

HPLC of Biological Macromolecules

Second Edition, Revised and Expanded

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Preface

Because its varied and complementary array of techniques has enabled many proteins to be isolated and characterized, chromatography has played a key role in the evolution of modern biochemistry. During the past two decades, high-performance liquid chromatography (HPLC) has emerged as a dominant technique in this field. Although HPLC is predominantly a major refinement of classical chromatographic techniques, its hundredfold reductions of analysis times and improved resolution and recoveries yield the results demanded by the scientists of today. The one mode that is unique to HPLC and does not have a classical predecessor—reversed-phase chromatography—has proven to be an invaluable tool that has revolutionized the study of both peptides and proteins.

The first edition of this book was written as a practical guide for scientists who are required to fractionate proteins, peptides, and polynucleotides. This edition includes many of the same topics, updated for discoveries of the past ten years. It also includes sections on new areas that have emerged from the field, such as the use of specialized detection systems. Many of the authors of these chapters are the pioneers who first attempted to use HPLC in the investigation of biomolecules in their areas of interest. If they as a group had not dared to try this technique in the 1970s, it would not be the fundamental method of protein and peptide characterization it is today.

Part One presents the fundamental concepts of HPLC, generally in the context of biological macromolecules. Both theoretical and practical aspects are presented to aid in understanding the material. Part Two integrates the concepts of

Part One into practical applications of fractionating specific groups of biological macromolecules. The general format of these chapters is a brief discussion of the chemical and biological properties of the biomolecule family, followed by detailed HPLC methods that have been used successfully for their analysis. Part Three addresses detection methods that are especially suited for qualitatively and quantitatively analyzing the complex mixtures and minute quantities encountered in biochemistry.

It is hoped that this compilation of subjects will prove invaluable for scientists who need to quickly evaluate and implement methods for the analysis of biological macromolecules.

*Karen M. Gooding
Fred E. Regnier*

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Silica as a Support in HPLC of Biomacromolecules

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INTRODUCTION

The use of silica as a support for biopolymer separation by high-performance liquid chromatography (HPLC) notably began around 1980. The traditional coarse soft gels such as cellulose, dextran, agarose, polyacrylamide, and other synthetic hydrophilic polymers [1] did not give the same column performance in terms of resolution and speed as the microparticulate silica packings employed for the HPLC of low-molecular-weight compounds. The impetus for using silica in the separation of biopolymers came mainly from two achievements: Macroporous silicas of an average pore diameter larger than 50 nm were synthesized and could be applied as supports, and the rich knowledge of bonding chemistry accumulated in the 1970s could be easily adapted to the manufacture of bonded silicas for biopolymer separation [2].

As compared with soft gels, porous silicas have the advantage of high mechanical stability. Furthermore, their specific surface area and pore size can be tailored to match the mass transfer kinetics and the retention of analytes in chromatographic separation techniques. High selectivities can be achieved by binding ligands with high molecular recognition abilities. Yet, even at optimum

surface modification, the silica matrix remains accessible, which gives rise to undesired nonspecific interactions that impair resolution and biorecovery. Finally, the limited chemical stability at a pH above 8 poses severe constraints on the application of bonded silicas at higher pH values.

CRITERIA FOR SILICA PACKINGS IN RELATION TO THE SEPARATION OF BIOPOLYMERS BY HPLC

The principal motives of a chromatographer attempting the separation of biopolymers by HPLC are high resolution and high biorecovery from the columns employed. In the case of preparative isolation, a high loading capacity without loss in selectivity is desired. Furthermore, it is essential that both high resolution under analytical conditions and high throughput under preparative conditions are achieved in an adequate period of time. The major difference in running a biopolymer separation compared with running a mixture of low-molecular-weight compounds is that biopolymers require the operation of columns under gradient elution conditions, providing a larger peak capacity than isocratic elution [3]. In order to optimize resolution and throughput, respectively, multicolumn operation is the method of choice, either in tandem or by applying a column switching technique. In the isolation of biopolymers, the main concerns are selectivity and loadability. The latter is defined as the maximum sample input corresponding to defined requirements with respect to resolution, peak shape, purity, and bioactivity. The aim is to isolate the product at a high mass recovery and biological activity.

Given these considerations, it is apparent that a number of requirements can be established with regard to the design and properties of supports used as column packings in biopolymer separation by HPLC. Supports should have rigid particles that are highly uniform in shape and size to guarantee a stable column bed with optimum hydrodynamic properties. This means a regular flow pattern under the operating conditions, e.g., by varying the flow rate and the mobile phase composition. This holds for highly efficient analytical columns packed with 3- to 5- μm particles and even more so for preparative columns filled with larger particles. In order to match the column selectivity in the various HPLC modes and to mimic the structure of biopolymers for specific chromatographic recognition, the surface of the support needs to be modified and tailored by means of a sophisticated and reproducible bonding chemistry. In general, any modification serves three major purposes: to eliminate to a large extent undesired matrix effects originating from the parent support material, to produce a chemically stable bond to the ligand, and to bind ligands for selective protein-surface interactions.

Because the high molecular weight of biopolymeric solutes, diffusion coefficients are one to two orders of magnitude lower than for small low-

molecular-weight compounds. Restricted diffusion can occur in the pores filled with the stagnant mobile phase and is related to the solute size relative to the pore size of the support. Furthermore, the kinetics of adsorption and desorption of such large molecules are inherently slow. Also, the kinetics due to secondary equilibria, i.e., solvation or dissociation, are complicated and conformational changes must be taken into account. In order to minimize these drawbacks, rapid diffusion must be achieved by designing supports with sufficiently large pores and small particles to provide maximum access of the stationary phase surface. It becomes evident from this discussion that both the physical and the chemical properties of the support exert a major impact on the performance of a chromatographic phase system applied to biopolymer separation.

STRUCTURE OF SILICA

Bulk Structure of Silica

Silica (silicon dioxide) is an inorganic polymer that consists of a three-dimensional framework of linked SiO_4 tetrahedra or octahedra. The terminating silicon atoms are coordinated to hydroxyl groups. Silica occurs in amorphous and in crystalline, ordered modifications. About 35 crystalline modifications are known that differ in the Si—O bond length, in the Si—O—Si bonding angle, in the Si—O bond topology, in coordination, and in their framework density, (d_f) [4]. Here d_f is defined as the number of SiO_2 units per $1000 \text{ \AA}^3$ of unit volume of the bulk phase. The framework density values range between 17 and 43. The latter is related to the high-density silica modifications such as stishovite; the lower value represents a low-density silica such as silicalite-I, which is the alumina-free end member of MFI-type zeolites [5]. The bulk structure of silica has been characterized by x-ray diffraction, solid-state nuclear magnetic resonance (NMR) spectroscopy, small-angle x-ray scattering spectroscopy, infrared (IR) spectroscopy and other techniques [6].

Porous silicas employed as packings in HPLC are amorphous; i.e., they do not possess a long-range order of their bulk structure. Most information on the bulk and surface structure of silica and its bonded derivatives can be drawn from ^{29}Si and ^{13}C solid-state NMR spectroscopy [7].

Pore Structure of Silica

The pore structure of a solid is generally characterized by its porosity, the pore diameter, the pore shape, the pore connectivity, the pore size distribution, the specific pore volume, and the specific surface area [8]. These parameters are obtained from sorption measurements with nitrogen, intrusion techniques, radiation scattering, pycnometry, thermoporometry, calorimetric measurements, xenon

NMR spectroscopy, and other techniques. The calculation methods are based on certain assumptions regarding the pore shape and the mechanisms of pore filling.

The term porosity refers to an open porosity accessible to molecules in the gas phase or liquid phase. With respect to silicas, the framework density is used to discriminate between porous and nonporous solids. Silicas having a framework density of (d_f) smaller than 21 SiO₂ units per 1000 Å³ are termed porosils according to Liebau [4]. For instance, clathrasils, a subgroup of porosils, possess pore openings smaller than 0.3 nm and hence allow access to the pore system only for small molecules such as helium and hydrogen. According to the IUPAC classification [9], the porous materials are divided into microporous, mesoporous, and macroporous according to the value of the pore diameter (P_d):

Microporous solids: pore diameter	$P_d < 2 \text{ nm}$
Mesoporous solids: pore diameter	$2 < P_d < 50 \text{ nm}$
Macroporous solids: pore diameter	$P_d > 50 \text{ nm}$

Silica supports in HPLC have mesopores and macropores. The presence of micropores leads to slow desorption kinetics and a lower loading capacity than found in supports with mesopores. The pore system can be built up by a unidimensional, two-dimensional, and network system of interconnected and intersecting pores, respectively MCM-41, as a member of the family of M41S materials, for instance, exhibits a unidimensional pore system of hexagonally arranged channels with a pore diameter ranging from 2 to 6 nm (Fig. 1) [10]. As the pore walls of MCM-41 are made of amorphous silica, the material is not considered to be a crystalline solid in its strict physical meaning but is rather termed an ordered mesoporous silica [11].

As previously mentioned, silicas applied as packing materials in HPLC are basically amorphous materials. Depending on the type of posttreatment, one can generate a minor degree of ordering or crystallinity of the bulk and the surface structure. The majority of silica supports in HPLC are porous materials having a pore diameter between 6 and 50 nm or larger and exhibiting an open, highly interconnected pore system that can be visualized by a high-resolution transmission electron micrograph of a cross section of a mesoporous silica particle (Fig. 2). Nonporous silicas as micron-size beads of 1 to 2 μm particle diameter have also been developed particularly for biopolymer separations in HPLC [12] (Fig. 3). Monolithic silicas have been successfully synthesized with a bimodal pore system composed of about 2-μm size macropores and 10-nm size mesopores (Fig. 4) [13].

Surface Structure of Silica

Native silica is a hydrophilic adsorbent that adsorbs about 5 to 10 (w/w) physisorbed water when exposed to humid air. The silica surface bears hydroxyl

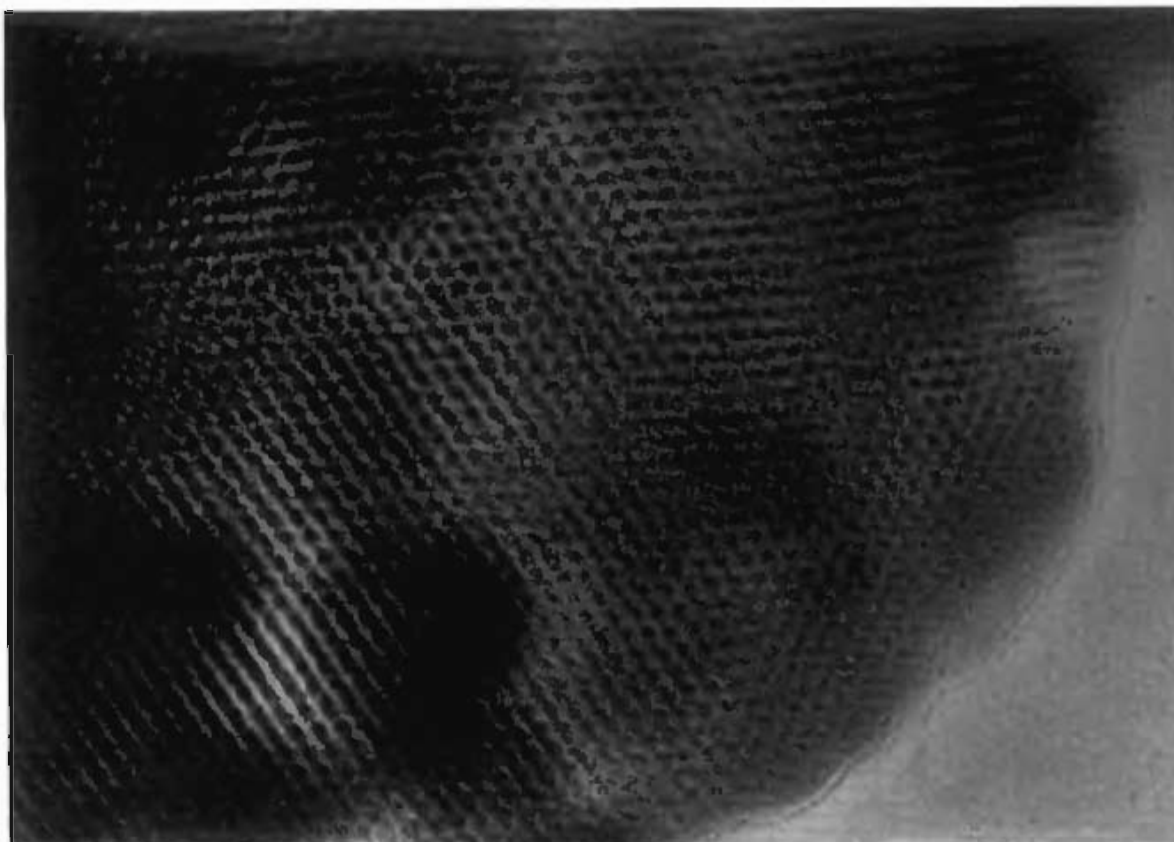


FIGURE 1 Electron transmission micrograph of a MCM-41 sample with an average pore diameter of about 4 nm (magnification 250,000).

groups, which can be divided into three types according to their coordination to surface silicon atoms: free or isolated, geminal, and vicinal (hydrogen-bonded) groups. They can be identified by IR spectroscopy, ^{29}Si CP MAS NMR spectroscopy, and other physicochemical methods [14–16]. In the fully hydroxylated state, the surface concentration of hydroxyl groups, α_{OH} , amounts to about 8 to 9 $\mu\text{mol}/\text{m}^2$ [14,17]. Physisorbed water is usually removed from the silica surface by subjecting the silica to a heat treatment under vacuum at about 150°C. With increasing calcination temperature, the surface hydroxyl groups condense to siloxane groups, whereby water is evolved. A drastic decrease in α_{OH} is observed in the range between 200 and 400°C. Above 600°C, mainly free or isolated hydroxyl groups condense. Rehydroxylation of the silica surface by exposing the dehydroxylated surface to water vapor is reversible up to a calcination temperature of about 600°C. Above 600°C rehydroxylation becomes a slow process or even remains incomplete [18].

Exposing porous silica to water or water vapor at an elevated temperature causes changes in the pore structure of the silica by hydrothermal treatment. As a result, the specific surface area diminishes, and the average pore diameter increases; however, the specific pore volume remains constant. The magnitude

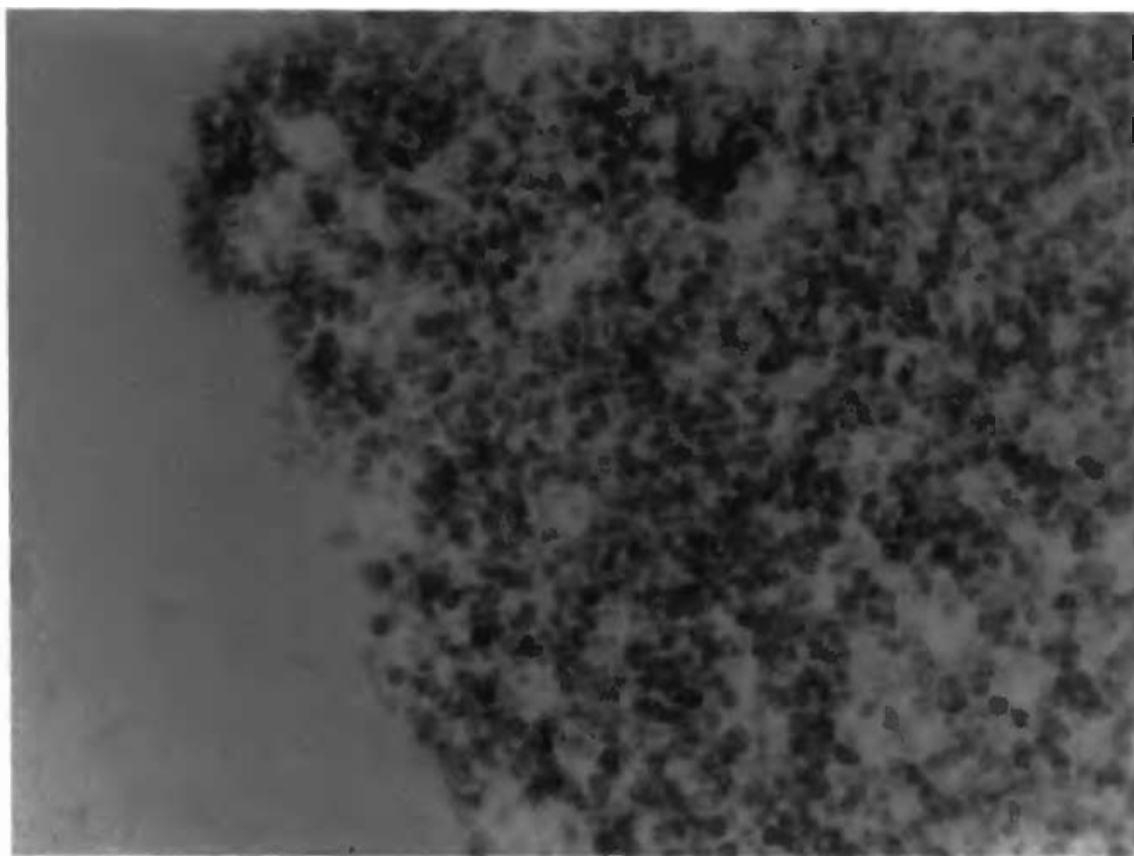


FIGURE 2 Electron transmission micrograph of a porous silica particle indicating the corpuscular structure of the silica skeleton (magnification 250,000).

of the effect is dependent on the temperature and on the duration of hydrothermal treatment [19].

The surface hydroxyl groups are weak Brønsted acids with a pK_a value of about 7 [14,15]. However, depending on the method of determination, the pK_a value has been reported to be between 2 and 10 [15]. Another reason for the wide variation of pK_a values might be from metal impurities in the silica. Silicas made from water glass or from colloidal silica often contain significant amounts of sodium, aluminum, titanium, etc. In the presence of aluminum strong Brønsted acid sites of type $\text{Si}-\text{OH}-\text{Al}$ can be formed, as in crystalline MFI-type zeolites [20]. Also, acidic impurities might be present that are left from aftertreatment or from particle shaping processes.

The pH of zero charge of the silica particles, pH_{zc} , is related to the pK_a value(s) according to

$$\text{pH}_{\text{zc}} = 0.5(pK_1 + pK_2)$$

The pH of zero charge of silica varies between 1.5 and 3 [21]. Above the pH_{zc} , silica acts as a weak cation exchanger and below it as an anion exchanger. The

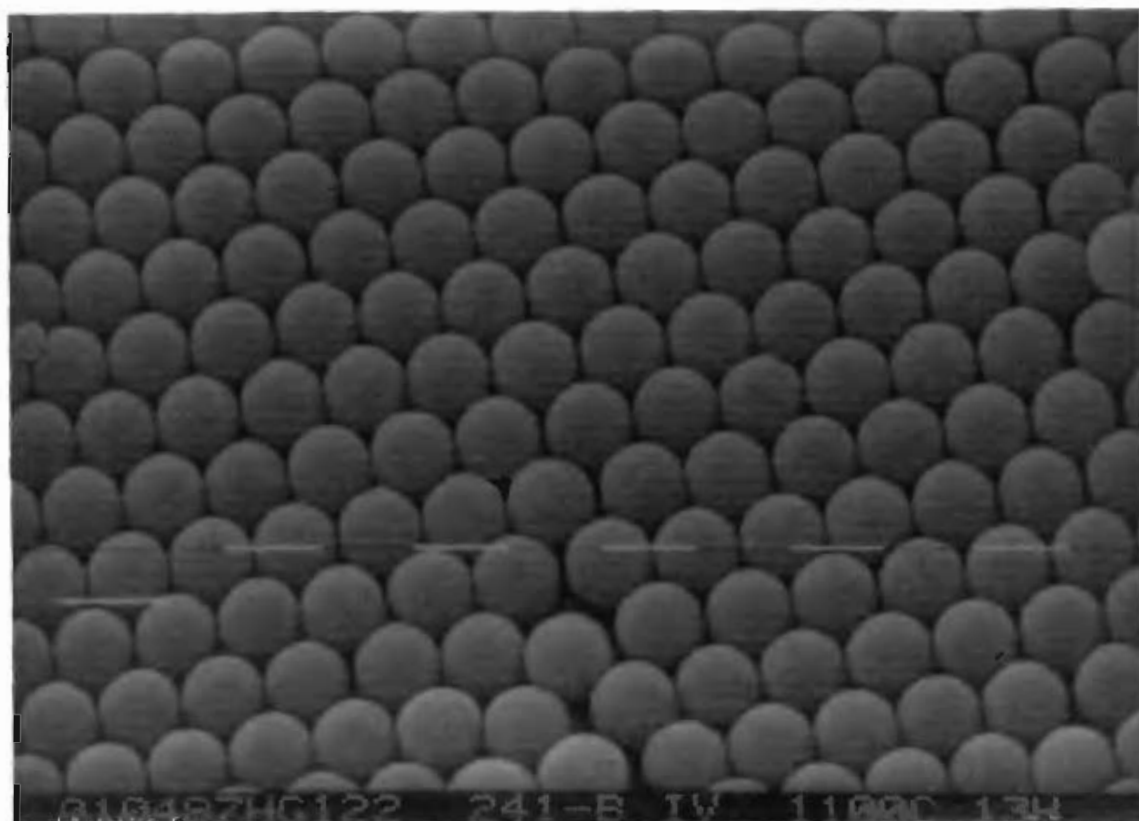
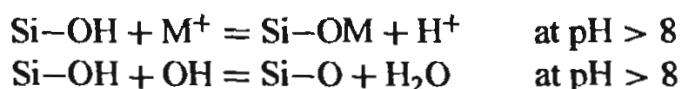


FIGURE 3 Electron scanning micrograph of nonporous silica particles with an average particle diameter of 1.5 μm (1 bar corresponds to 1 μm).

following ion-exchange processes have been postulated by Allen and Matijevic [22]:



On increasing the pH of a silica suspension from 3 to 8, the surface becomes increasingly negatively charged. This is evidenced by the dependence of the electroosmotic velocity as a function of pH in packed silica capillaries in electrochromatography [23]. Whereas deprotonation of the surface hydroxyl groups is a fast process, the protonation is rather slow; i.e., a pronounced hysteresis is observed in the titration curve of silica with sodium hydroxide and hydrochloric acid, respectively. Above pH 8, silica starts to dissolve as soluble silicates. Solubility increases exponentially above pH 10 [24].

Before use and chemical modification, porous silicas are subjected to an activation process that consists of either a thermal treatment or a treatment with a concentrated solution of an acid (except hydrofluoric acid) to decrease the surface heterogeneity and to remove inorganic impurities from the surface [25].

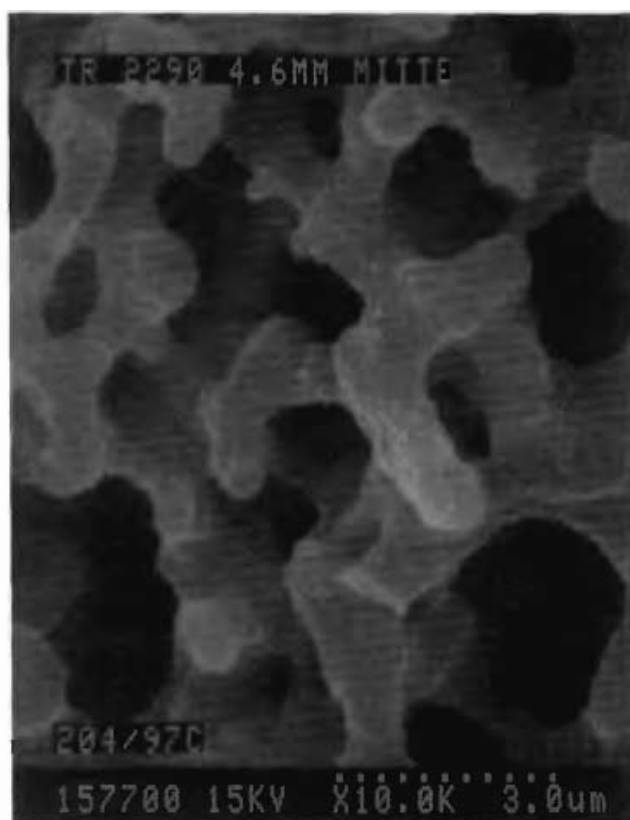


FIGURE 4 Electron scanning micrograph of a Silica Rod piece indicating the coherent macropore system (magnification 160,000). (Courtesy of Dr. K. Cabrera, Merck KGaA, Darmstadt, Germany.)

DECISIVE PROPERTIES OF SILICAS AND BONDED SILICAS AS PACKINGS FOR THE SEPARATION OF BIOMACROMOLECULES

From the foregoing discussion on the bulk structure, the pore structure, and the surface chemistry of silica, one can derive some important conclusions and practical guidelines that are highly relevant for the separation of biopolymers by HPLC.

Chemical Purity

During the last decade, a new family of high-purity silicas has replaced the traditional ones, which were mainly synthesized from water glass solutions and acids. The latter contained large amounts of metal impurities such as sodium, magnesium, calcium, iron, titanium, and aluminum [26]. Typical values are sodium 1000 ppm, aluminum 200 ppm, and iron 150 ppm. The high-purity silicas are mainly synthesized from tetraethoxysilane, highly pure colloidal silicas, or

silicas that were acid washed after synthesis. The metal content of these silicas is reported to be lower than 20 ppm. Metal impurities in the bulk and at the surface of silica give rise to a chemical surface heterogeneity, which leads to irreversible adsorption of proteins or tailing of the peaks for any bonded silica packing employed for biopolymer separations by HPLC.

Specific Surface Area, Porosity, and Average Pore Diameter

It was recognized in the early 1980s that the packings for biopolymer separations needed larger pores than those used for the separation of low-molecular-weight compounds. To avoid restricted diffusion of the biopolymeric solutes into the pores of the silica packing during the separation process and to provide full access of the bonded ligands at the pore walls, the average pore diameter (P_d , should be about 10 times larger than the calculated Stokes diameter of the biopolymer. As a rule, silica packings with a P_d of about 15 nm should be optimum for biopolymers up to 20 kDa. Larger proteins, so-called macroporous silicas, require a pore diameter in excess of 50 nm. It should be emphasized that bonding of ligands or coating the inner surface of silica causes a diminution of the average pore diameter of the parent silica. The pore narrowing effect is more drastic for mesoporous than for macroporous silicas with $P_d > 50$ nm.

The effect of pore size of the packing on the mass transfer kinetics of biopolymeric solutes in HPLC column separations has been a subject of intensive studies (as an example see Ref. 27). To optimize the mass transport properties of the supports, the focus was directed to packings with a bimodal pore size distribution having mesopores (diffusional pores) and macropores (transport pores). This has led to a new design of porous packings suitable for fast biopolymer separations at a preparative scale that exhibit a high dynamic capacity for biopolymers [28,29].

The larger the average pore diameter, the lower the specific surface area and hence the chromatographic retention. A 50-nm pore size silica exhibits a specific surface area of about 50 m²/g, about five times lower than that of a 15-nm pore size material. However, due to the multisite interactions of a biopolymer with a bonded silica in HPLC modes such as reversed phase or ion exchanger, the value of the specific surface area is not a critical parameter for the resolution of biopolymers. It has been demonstrated that even the low, entirely external specific surface area of about 1 to 2 m²/g of 1- to 2- μ m nonporous silica particles is sufficient to retain and to resolve biopolymeric analytes [12].

Acidity and Ion-Exchange Properties

Silicas with an appropriate pore size are chemically modified to serve as reversed-phase, hydrophobic interaction, ion-exchange, and affinity packings for biopoly-

mer separations [30]. Even after the chemical modification has been performed and novel ligands have been introduced, the original surface of the silica remains partly accessible to the analytes. Due to the negative charge of the silica surface at pH above 3, ion exclusion phenomena can take place with negatively charged biopolymeric solutes. The analytes then are eluted either at the dead volume of the column or ahead of it. The access of the matrix to the analytes often gives rise to nonspecific interactions that notably affect retention and selectivity and lead to peak tailing.

Bonded silicas are fairly stable in an acidic pH range of 1 to 2. Such conditions are usually applied in the resolution of peptides and proteins by reversed-phase HPLC. Separation of biopolymers is performed in the pH range between 5 and 8 with buffers in ion-exchange and hydrophobic interaction chromatography. The type and concentration of buffer have a remarkable effect on the stability of the bonded silica. For instance, phosphate buffers at pH 7 are not recommended, particularly when separations are performed at elevated temperatures. The lifetime of the column in phosphate will be much shorter than in Tris buffer [31]. Buffers with a pH above 8 should not be applied due to the dissolution of the silica matrix.

PACKED MICROPARTICULATE AND MONOLITHIC AND SILICA COLUMNS

The modern silica packings are spherical in shape and have average particle sizes between 5 to 10 μm . The value of the ratio of dp_{90} to dp_{10} where dp_{90} (dp_{10}) is the average particle diameter at 90% (10%) of the cumulative particle size distribution, should be about between 1.5 and 2. This width of the particle size distribution is required for two reasons: fine particles, in particular those below 1 μm , generate a high column back pressure, and large particles impair the column performance. To manufacture a column with microparticles is a tedious multistep process. First, the packing has to be subjected to a sizing, which is usually done before chemical surface modification. Second, the packing must be suspended in a liquid and the column filled by applying a high flowrate and a high pressure. After conditioning, appropriate tests have to be performed to assess the efficiency and the selectivity of the column.

In the manufacture of nonporous 1- to 2- μm particles, spherical silica particles are formed at an adjustable particle size with a narrow particle size distribution. Thus, no sizing is required. However, due to the small particle size, column back pressures on the order of 150 to 200 bar are generated even for columns 30 mm in length [12].

In order to overcome the disadvantages of particles in the manufacture of HPLC columns, so-called monolithic columns have been prepared [13]. The procedure is based on advanced sol-gel chemistry using hydrophilic polymers as aiding agents. A silica hydrogel is prepared with an embedded polymer, forming an independently coherent system. After drying and calcination, where the polymer is burned out, a highly interconnected macropore system is obtained with porous silica as a skeleton. The silica itself is also porous, containing mesopores. Both pore sizes can be independently varied. This concept, developed by Nakanishi and introduced by Tanaka into HPLC, allows very fast separations with very low column back pressures and high column efficiencies [32]. Such columns were introduced as analytical reversed-phase columns by Merck KGaA, Darmstadt, Germany as ChromolithTM columns. Monolithic columns have also been made from synthetic polymers and cross-linked agarose [33–35].

SURVEY OF COMMERCIAL HPLC SILICAS

Some of the procedures for manufacturing spherical silica beads are shown in Figure 5. Shaping is achieved by gelling a silica under certain conditions in a water-immiscible liquid, by agglutination of a silica sol in the presence of a hydrophilic polymer, and by spray drying of a silica sol. Another method is to use tetraethoxysilane as a silica source and to perform hydrolysis and condensation in an emulsion/dispersion by vigorous stirring.

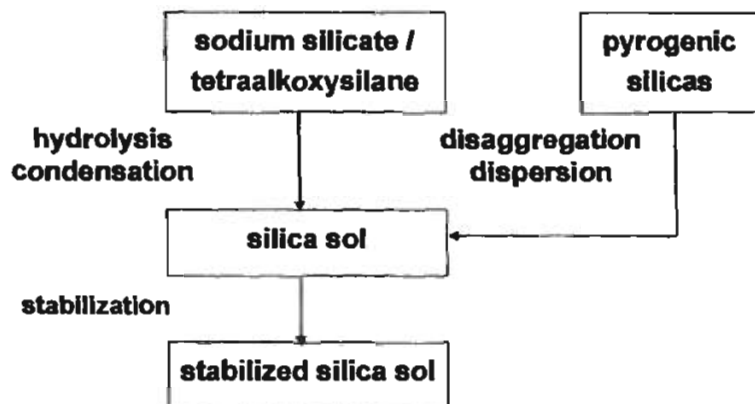
In conclusion, there are several ways to manufacture porous silicas for HPLC. It is characteristic that the shaping process be implemented in the manufacturing process. In most cases, the silicas are subjected to an after-treatment to make the surface homogeneous, to remove the impurities, to activate the surface prior to bonding, and to finish up the product for better mechanical stability. A number of companies around the world that have specialized in the manufacture of silica HPLC packings are listed in Table 1.

CONCLUSION AND PERSPECTIVES

Summarizing all the information presented in this chapter, silica and its bonded derivatives have gained widespread application as packings in biopolymer separation. Thorough attempts were made to improve the quality of native silica during the past decade, which led to the family of high-purity grades. Although the development of high-resolution spectroscopic techniques such as solid-state NMR spectroscopy has given much better insight into the surface

Formation of porous silica beads

A. Preparation of silica sols



B. Aggregation of silica sols to silica beads

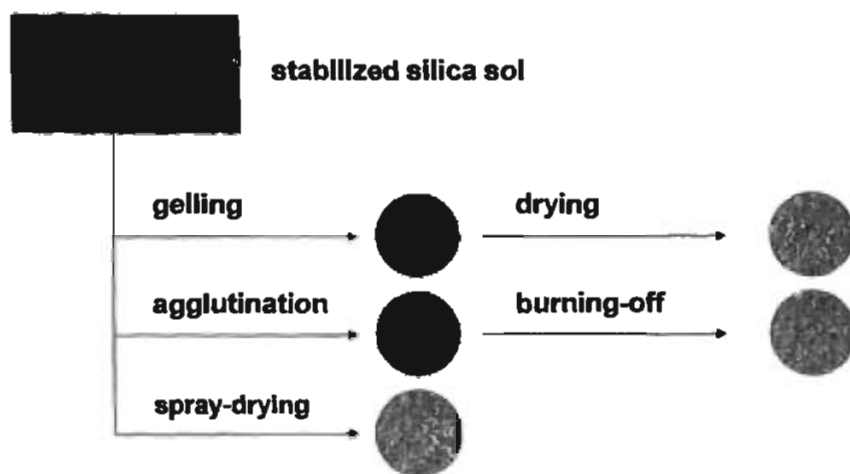


FIGURE 5 Scheme of the formation of silica beads.

chemistry of silica, many of the phenomena that occur are still not well understood. In particular, these are adsorption and ion-exchange properties of silica in aqueous and buffered solutions. I personally believe that with highly pure and tailored silicas, a renaissance in HPLC of biopolymers will be seen in the future.

TABLE 1 Suppliers of HPLC Grade Silicas (Alphabetical Order)

Bio-Rad Laboratories 2000 Alfred Nobel Dr. Hercules, CA 94547 Phone: (510) 741-1000	Macherey-Nagel GmbH & Co KG PO Box 101352 D-52313 Düren Germany Phone: +49 2421 969-0 Fax: +49 2421 969 199
Bischoff Chromatography Analysentechnik u.-geräte GmbH Böblinger Straße 23 71229 Leonberg Germany Phone: +49 7152-6064-0 Fax: +49 7152-6064-35	Mallinckrodt Baker PO Box 1 Rijsterborgherweg 20 Deventer NL7400 AA Netherlands Phone: +31 (570) 687500 Fax: +31 (570) 611342
Daiso Co. Ltd. 10-8 Edobori 1-Chome, Nishi-ku Osaka 550 Japan Phone: +81 (6) 443-5996 Fax: +81 (6) 445-5787	Merck KGaA Frankfurter Strasse 250 D-64271 Darmstadt 1 Germany Phone: +49 6151-72-4516 Fax: +49 6151-72-7495
Eka Chemclas AB Bohus S 445 80 Sweden Phone: +46 31 58 70 00 Fax: +46 31 58 77 27	MICRA Scientific, Inc. 1955 Techny Road, Suite 1 Northbrook, IL 60062 Phone: (847) 272-7877 Fax: (847) 272-7893
Hewlett-Packard Co. Newport Site 538 First State Boulevard Newport DE 19804 Phone: (302) 633-5887 Fax: (302) 633-5893	Phase Separations Inc. 20 Liberty Way Franklin, MA 02038 Phone: (800) 243-4398 Fax: (508) 482-4820
Hypersil Chadwick Rd., Astmoor Runcorn Cheshire WA7 1PR United Kingdom Phone: +44 (1928) 562633 Fax: +44 (1928) 581078 Fax: +44 (1244) 289500	

(continued)

TABLE 1 (continued)

Phase Separations Ltd. Deeside Industrial Park Deeside Flintshire CH5 2NU Wales Phone: +44 (1244) 288500	The Separations Group (Vydac) 17434 Mojave St. Hesperia, CA 92345 Phone: (760) 244-6107 Fax: (760) 244-1984
Phenomenex 2320 W 205th St. Torrance, CA 90501 Phone: (310) 212-0555 Fax: (310) 328-7768	Waters Corporation 34 Maple St. Milford, MA 01757 Phone: (508) 478-2000 Fax: (508) 872-1990
Supelco Supelco Park Bellefonte, PA 16823 Phone: (800) 247-6628 Fax: (800) 447-3044	Whatman Inc. 9 Bridewell Pl. Clifton, NJ 07014 Phone: (973) 773-5800 Fax: (973) 472-6949

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Organic Polymer Support Materials

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INTRODUCTION

In the early days of chromatography, the pioneering work of Tsvett [1], Lederer [2], and Martin [3] focused on separations using beds of natural porous inorganic materials as the separation media. Polymeric supports based on modified natural polysaccharides were introduced in the mid-1950s and soon became standard for the separation of biomacromolecules in low-pressure size exclusion and ion-exchange chromatographic mode [4–6]. High-performance liquid chromatography (HPLC) emerged about three decades ago as a result of advances in the preparation of microparticulate silica [7,8]. This new separation technique was first used for the separation of small molecules. The remarkable speed of separation that could be achieved by using HPLC encouraged the development of a broad spectrum of separation media. These media were based on different materials such as natural and synthetic polymers, glass and inorganic oxides. Biological molecules were first separated using the HPLC technique in the mid-1970s when macroporous silica became available. Further rapid development of the HPLC of biopolymers was observed after the introduction of hydrophilic organic resins [9,10].

The increasingly firm requirements of regulatory agencies to monitor and control biotechnology processes and the desire of the industry to reduce overall process time and cost are the most important driving forces in the development of techniques that allow rapid analysis and/or efficient isolation and separation of biomacromolecules. High resolving power makes HPLC the hottest candidate to become a completely routine tool for both analytical and process scale separations in the very near future.

It has been correctly pointed out that the most significant advances in chromatography have always followed the appearance of enhanced support matrices [11]. Therefore, much effort has been devoted to research and development on novel, highly efficient, and selective separation media. For example, bonded silicas started the era of reversed-phase HPLC, and this technique is now the most widely used chromatographic mode [8]. Development of rigid hydrophobic porous polymers led to development of size exclusion chromatography (SEC) in nonaqueous media [12]. Later, with novel supports, aqueous high-performance SEC of water-soluble polymers and biopolymers also became possible. Polymeric stationary phases currently dominate some chromatographic modes such as ion-exchange, hydrophobic interaction, and affinity chromatography as a result of their stability over the entire pH range and their accessibility with very different surface chemistries. All of these chromatographic modes are of particular relevance to the subject of this book.

Hundreds of original papers in scientific journals that are published every year, together with many excellent books and reviews [for example, 7,8,10,13–22], cover various aspects of the support technology for the separation of biomacromolecules. In addition, a plethora of commercial literature on HPLC separation media is available from the individual manufacturers and vendors. Therefore, this overview will focus on the organic supports that have been developed and marketed recently rather than the polymeric separation media in their entirety.

TYPICAL SHAPES OF PARTICULATE SEPARATION MEDIA

In the early days of chromatographic packings, the simplest way to obtain small particles was to crush bulk materials. For example, large pieces of porous silica or dried dextran gels were crushed in ball mills and then fractionated according to size. Although they are easy to prepare, irregular particles packed poorly in columns and created large voids, thus robbing the column of its efficiency. The many sharp edges of the particles also fractured easily, causing clogging of the systems. Therefore, spherical beads that by default do not suffer from these significant flaws were soon developed.

In general, spherical particles are manufactured by suspension polymerisation, which was invented in Germany in 1912 [23]. In aqueous suspension

polymerization, a water-insoluble polymerization mixture containing a dissolved free radical initiator is stirred in a large volume of water containing a suspension stabilizers such as water-soluble polymers [cellulose derivatives, starch, poly(vinyl alcohol), poly(vinyl pyrrolidone)] or very fine inorganic powders to form small droplets of the dispersed organic phase in the continuous aqueous phase. These droplets adopt a strictly spherical shape to minimize their interfacial free energy, and their average size is roughly controlled by the rate of stirring and the amount and type of suspending stabilizer used. The free radical polymerization is initiated by increasing the temperature of the stirred mixture, and solid polymer beads are formed [24,25]. This technique is currently the method of choice for the preparation of separation media in a regular spherical shape for high-performance liquid chromatography.

MORPHOLOGY

Current separation media can be divided into two major categories: gels and macroporous materials. The former family is represented by polymers that typically do not exhibit any porosity unless swollen in water or another solvent. This solvation results in the separation of the polymer chains from one another to form "pores", which are solvent-filled voids. This type of porosity is temporary because of the reversible nature of solvation and exists only as long as the solvent remains within the polymer network. Lightly cross-linked synthetic polymer beads—often termed "swellable" or "microporous" gels—acquire porosity only upon swelling. Well-known examples of separation media without permanent porosity are natural polysaccharides such as dextran, guar, and agarose, and hydrogels such as acrylamide and hydroxyethyl methacrylate-based polymers. Once swollen, materials are usually soft and easily deform under pressure, which generally prohibits their use in tall packed beds and at the high flow velocities required for high-performance liquid chromatography columns.

Unlike the preceding materials that require solvent swelling to become porous, macroporous polymers are characterized by a permanent porous structure formed during their preparation that persists even in the dry state. Their internal structure consists of numerous interconnected cavities (pores) of different sizes, and their structural rigidity is secured through extensive cross-linking. Macroporous polymers emerged in the late 1950s as a result of the search for mechanically resistant ion-exchange resins with enhanced exchange kinetics [26]. In contrast to gels, columns packed with these resins generally exhibit good flow characteristics. Macroporous beads are widely used not only for the preparation of chromatographic media but also as catalysts, adsorbents, supports, carriers, and ion-exchange resins. All of these applications take advantage of their rigid porous structure that remains unaffected by changes in their environments. Although macroporous materials swell much less than microporous gels in any

solvent regardless of its polarity, mass transport in and out of the pores is faster than in *unswollen* gels because they contain permanent pores available even in the dry state. Molecules can diffuse freely through these pores, which constitute a labyrinth of tortuous interconnected cavities of different sizes.

Because none of these morphologies is perfect for the preparation of chromatographic separation media, numerous hybrid materials have also been developed in an attempt to preserve advantages of both gels and macroporous materials while trying to avoid their drawbacks. In addition, nonporous micropellicular beads are available for the rapid separation of biopolymers.

“Rigid” Gels

Gel-type separation media prepared from natural polysaccharides were introduced to chromatography in the mid-1950s [4]. The original supports were modified cellulose powder [4,27] and soft gels of cross-linked dextran [5] and agarose [6]. Beads based on other polysaccharides such as chitosan, starch, and guaran were added to this group over time. Generally, these materials are not suitable for high-performance separation techniques because the back pressure in columns steeply increases at higher flow rates as a result of compressibility of the bed. However, they are readily available from renewable sources and provide both excellent hydrophilicity and chemical stability. Therefore, several attempts were made to improve the properties such as shape and mechanical strength of polysaccharide-based gel supports, thus making them useful even at higher flow rates. These efforts were most successful with cellulose- and agarose-based media.

Cellulose Supports

Cellulose is a polymer formed from β -1,4-linked D-pyranose repeat units. Cellulose particles consist of chains in ordered, crystalline domains interspersed with amorphous regions. High crystallinity resulting from a regular linear structure and extensive hydrogen-bonding between the polymer chains make cellulose insoluble in standard solvents. In contrast, functionalized cellulose is more soluble because the newly introduced groups remove the chains from each other and break the hydrogen bonds. For example, cellulose ion exchangers swell in water and may even dissolve completely, depending on their degree of functionalization.

Originally, cellulose was used in the shape of irregular fibrous particles (microcrystalline cellulose), which are not suitable for chromatographic applications requiring high flow rates. This led to the development of porous cellulose beads. An extensive review of this topic was published by Stamberg [28]. In a typical procedure, a solution of cellulose is dispersed in an immiscible liquid to small droplets, and these are solidified, regenerating the original cellulose.

Even highly porous cellulose beads, which contain only 2 vol.% solid and as much as 98% of pores typically filled with water, are more rigid than their dextran- and agarose-based counterparts. Therefore, they do not easily deform under pressure and can be packed into high beds that exhibit essentially lower flow resistance compared with columns packed with hydrogels. The sizes of cellulose beads available on the market vary in a broad range from 25 μm up to a few millimeters. Obviously, even the smallest bead size is too large and disqualifies these cellulose beads from analytical HPLC separations; however, they are well suited for large preparative scale systems. Low-solid-content cellulose separation media with a widely opened porous structure allows rapid mass transfer and good separations, even in columns packed with rather large beads at very high flow velocities [29].

Although these supports can be used directly for size exclusion chromatography (SEC) and desalination of protein solutions the major applications are expected in ion-exchange and affinity chromatography. To achieve this, the original matrix has been chemically modified [18,28-30]. Cellulose-based separation media for SEC and ion-exchange chromatography are commercially available under the trade names Sephacel (Amersham Pharmacia Biotech, Uppsala, Sweden), Perloza (SCHZ, Lovosice, Czech Republic), Spherilose (ISCO, Lincoln, NE), and Macrobead Hydrogel (LigoChem, Fairfield, NJ).

Agarose Supports

Agarose is a linear alternating copolymer of galactopyranose and anhydrogalactopyranose produced by seaweed. This polysaccharide dissolves in water only at a temperature higher than 50°C; a swollen gel forms upon cooling to a lower temperature. Stirring a hot aqueous agarose solution in an apolar organic solvent, followed by cooling, leads to regular beads consisting of spontaneously aggregated polymer chains arranged in an open three-dimensional network structure [6]. The pore size depends on the concentration of agarose in the original solution and can be controlled during the preparation of the beads. As a rule, the higher the concentration, the smaller the pores. For example, an average pore size of 30 nm is typical for a swollen 4% agarose gel prepared from a 4% agarose solution. This pore size allows size exclusion fractionation range of 30,000–6,000,000 as determined for dextran standards [16].

Cross-linking of the polysaccharide chains using bifunctional compounds such as epichlorohydrin, divinylsulfone, and bis-epoxides during the solidification dramatically increases the mechanical stability of the beads and allows the manufacture of beads as small as 10 μm . Although cross-linking does not significantly change the morphology of the beads, the number of hydroxyl groups is reduced to about 50% of the original value. Despite this, the number of hydroxyl groups remains large and the cross-linked agarose materials remain

very hydrophilic. These beads are an intermediate for the production of other separation media.

Agarose beads cross-linked using a two step procedure, which involves a reaction with butanediol diglycidyl ether during the preparation of beads followed by an additional modification with epichlorohydrin in an aqueous hydroxide solution, are marketed under the trade name Superose (Amersham Pharmacia Biotech). Rigidity, together with a small particle size, makes them suitable for SEC of proteins and nucleic acids at linear flow velocities of up to 185 cm/hr [22,31]. Highly cross-linked 6% agarose beads (Sephacrose Fast Flow) are also used for the manufacture of high-capacity ion exchangers and activated matrices for the preparation of affinity chromatography media.

Another family of new ion-exchange and affinity separation media designed for large scale purifications called Streamline (Amersham Pharmacia Biotech) is characterized by an average particle density of about 1.2 g/mL, which is significantly higher than that of plain beads. These beads consist of 6% cross-linked agarose with an embedded crystalline quartz core. In contrast to processes typical of packed columns, these beads are fluidized to an expanded bed in a column by an upward flow of the mobile phase. They tolerate easily the crude feedstock that can contain cells, cell debris, and other solids which would be lethal for a packed column, because these contaminants pass freely through the expanded bed. After the beads are saturated with the target compounds such as proteins and peptides and the solid impurities are washed out, the flow of the liquid is reversed and the beads sediment and form a typical chromatographic bed from which the proteins are eluted. This procedure considerably simplifies protein recovery from crude streams because it requires only a single-pass operation without the need for prior clarification.

Although advanced polysaccharide supports currently have high binding capacities and more favorable flow properties, their size and mechanical properties still do not meet the requirements of rapid analytical HPLC separations. These remain the domain of rigid porous beads based on both inorganic and organic polymer materials.

Macroporous Polymers

In order to obtain a rigid material that exhibits macroporosity, the polymerization mixture for its preparation must contain not only the monovinyl monomer but also a substantial amount of a cross-linking divinyl monomer and a porogen. The porogen does not react during the polymerization process but remains within the newly formed beads, where it is surrounded by polymerized material in areas that will ultimately become the pores after the porogen is finally removed during work-up. In most cases, the porogen is a simple organic solvent with a sufficiently high boiling point. As a result of both cross-linking and solubility changes

associated with increased chain length, polymer molecules formed during the polymerization process precipitate from the surrounding medium of porogen and remaining monomers. This phase separation occurs at an early stage of the polymerization, leading to the formation of microscopic globular entities that continue growing but do not coalesce due to cross-linking. As the polymerization proceeds, these so-called microglobes come into contact with each other and associate to form clusters consisting of both interconnected globules and voids or pores (Fig. 1). Typically, the pore volume within a macroporous material correlates well with the volume of porogen used [26,32–34].

Macroporous polymer beads are rigid and, therefore, well suited for HPLC. A well-defined pore size distribution is also important for the design of macroporous chromatographic separation media. Small pores and large surface areas are essential for the HPLC of small molecules, whereas the separation of large molecules such as proteins and nucleic acids requires significantly larger pores. As a rule, the larger the pores, the lower the overall surface area. In fact, development of some novel chromatographic techniques (perfusion chromatography) and media (continuous monoliths) (see later) were based on the availability of porous materials with extremely large pores.

Although the number of variables characteristic of a system for the preparation of macroporous materials is large, only a few are useful for the control of porous properties. These are the percentage of both the cross-linking monomer and the porogen in the polymerization mixture, the composition of the porogen, and the reaction temperature. Changes in the first two variables also affect both the composition of the polymer and its mechanical properties. In contrast, temperature and type of porogen are useful for the preparation of porous polymers with a fixed chemical composition that differ only in their porous properties.

Macroporous Separation Media

Specifics of the macroporous polymer stationary phases for HPLC have been thoroughly discussed in several review articles [34–39]. Macroporous styrene-divinylbenzene resins were first used for SEC in the mid-1960s [12]. Because they can be used in organic solvents, they significantly facilitated the determination of molecular weight distributions of organic polymers. In addition, their hydrophobicity makes them suitable for separations in the reversed-phase mode [10,34,40,41].

Lee [42] correlated porous properties with the chromatographic behavior of macroporous poly(styrene-divinylbenzene) beads PRP-1 and PRP-3 (Hamilton Co., Reno, NV) with specific surface areas of 100 and 400 m²/g, respectively, and observed less than a 5% increase in retention for PRP-3. Obviously, high surface area alone appears not to be the most important feature affecting the separation. In order to achieve both high selectivity and capacity, the polymer

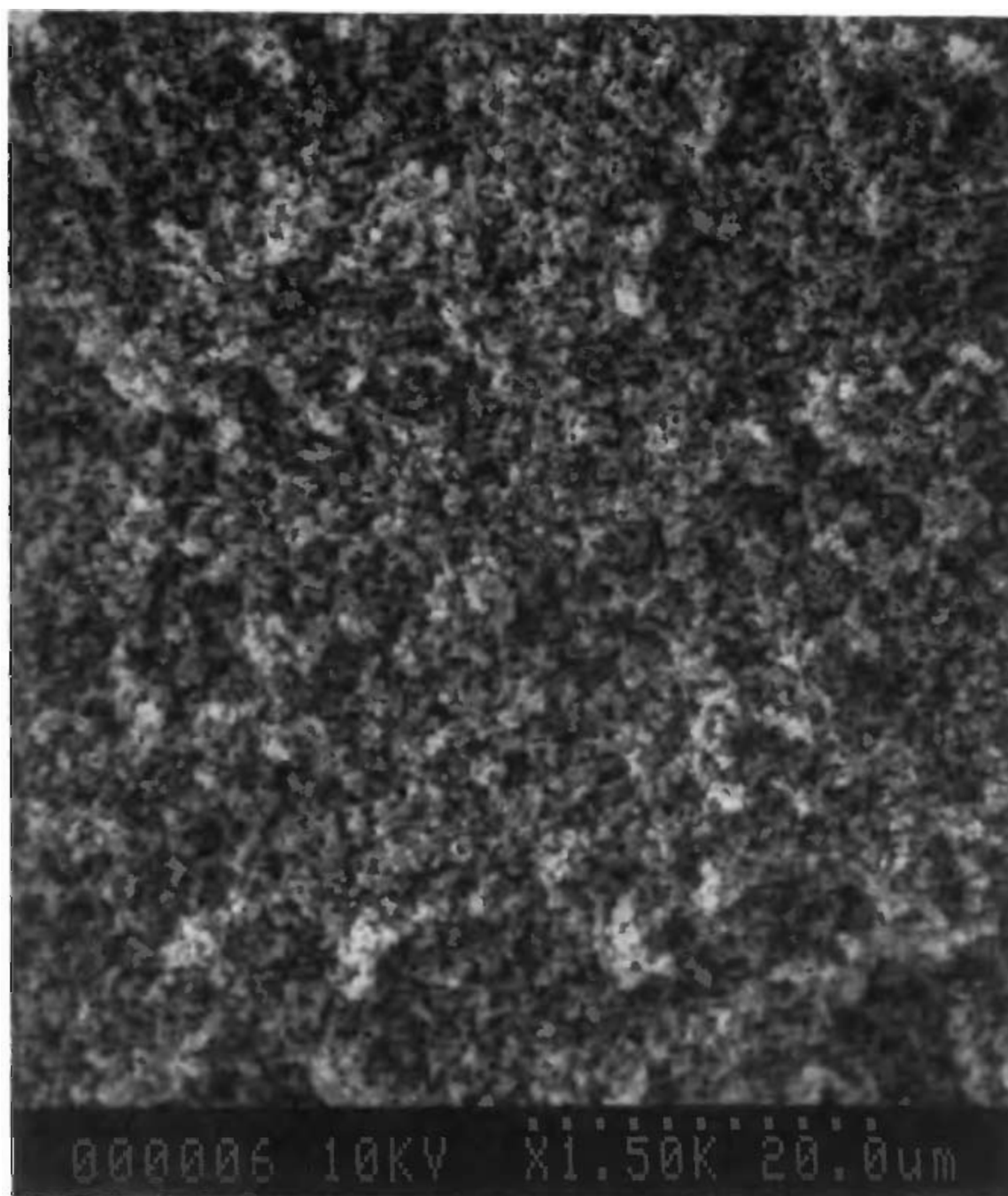


FIGURE 1 Scanning electron micrograph of inner part of a macroporous polymer.

support must have a sufficiently large number of pores with a size capable of accommodating the proteins. Polymer Laboratories (Amherst, MA) developed a series of micrometer-sized macroporous poly(styrene-divinylbenzene) beads PLRP-S with pore diameters of 100, 300, 1000, and 4000 Å. Beads with 100 Å pores are not suitable for the separation of large molecules, but the others exclude only proteins with molecular weight larger than 3×10^5 , 10^6 , and 10^7 , respec-

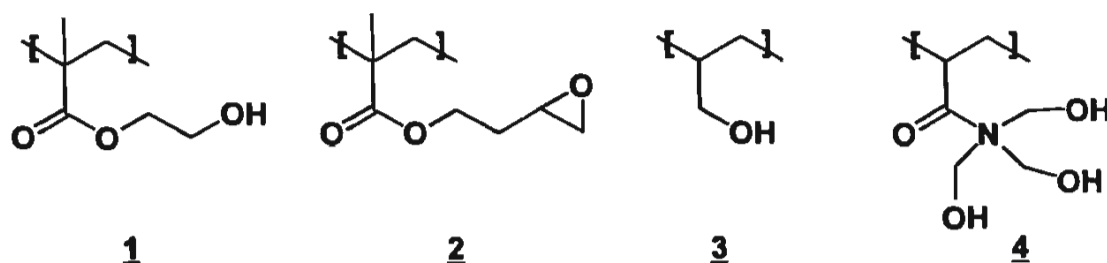
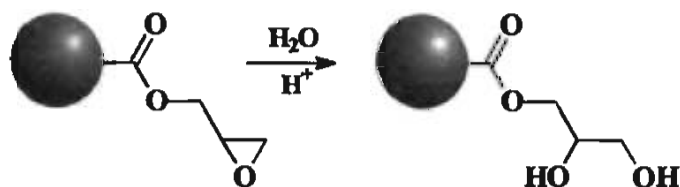


FIGURE 2 Examples of beads with typical hydrophilic functionalities.

tively. Those pores are adequate for the separation of very large biomolecules. The retention of proteins in PLRP-S columns was reported to be similar to that of columns packed with C_{18} silica [36].

The quest for more hydrophilic macroporous beads suitable for aqueous mobile phases resulted in several highly cross-linked resins prepared by a copolymerisation of 2-hydroxyethyl methacrylate **1**, glycidyl methacrylate **2**, vinyl alcohol **3** (actually, hydrolyzed vinyl acetate), and *N*-tris(hydroxymethyl) methylacrylamide **4** monomers shown in Fig. 2. Obviously, the epoxide rings of poly(glycidyl methacrylate) have to be hydrolyzed first to obtain the hydrophilic diol functionalities (Scheme 1). These materials were commercialized under trade names such as Biosphere (Labio, Prague, Czech Republic), Fractogel (EM Separation Technology, Gibbstown, NJ), OHpak (Showa Denko K.K., Tokyo, Japan), PL-aquagel-OH (Polymer Laboratories, Amherst, MA), Suprema (Polymer Standard Service, Mainz, Germany), Trisacryl (Biosepra, Marlborough, MA), and TSK gel PW (Tosoh Biosep, Montgomeryville, PA). A broad range of particle sizes is available extending from 5 μm for analytical columns to 30–60 μm (230–400 mesh) for the preparative scale separations. These beads often have well-controlled porous properties and therefore also analyte size selectivities defined as SEC exclusion limits. As a result of advanced polymerization processes, modern SEC media yield columns with high efficiency. For example, Tosoh Biosep guarantees more than 45,000 theoretical plates/m for columns packed with 6- μm polymer beads such as TSKgel G3000PW_{XL}. Many of these beads also serve as intermediates for the preparation of functionalized separation media.



Scheme 1

Separation Media with Surface-Modified Pores

There are two major reasons for modification of pore surfaces in some polymeric resins: (1) hydrophilization of hydrophobic surfaces and (2) an increase in loading capacity and kinetics of ion-exchange resins. The former is typical of styrene-divinylbenzene copolymers that are too hydrophobic to be used directly for the separation of biopolymers in some modes. In this case, hydrophilic polymers such as dextran, poly(oxyethylene), poly(ethylene imine), and poly(vinyl alcohol) are adsorbed and cross-linked on [35,43] or covalently linked to the pore surface to form a thin "biopolymer friendly" barrier on the hydrophobic surface. Regnier [44] was one of the first to develop a covalently attached hydrophilic coating that substantially decreased the nonspecific irreversible adsorption of proteins.

In typical macroporous supports, only the functionalities that are exposed at the "rigid" pore surface (Fig. 3a) can interact with analytes. This not only limits the number of functional groups but also restrains their motion and prevents them from adopting the optimal spatial configuration required for the most efficient interaction. Attachment of a linear polymer containing segments of opposite polarity (hydrophilic and hydrophobic) affords a "fimbriated" stationary phase [45] with loops and trains similar to those shown in Fig. 3b. This arrangement increases the mobility of functional groups and, indeed, improves the chromatographic properties. Another process that provides the surface with loose chains is the preparation of functional polymers in situ within the pores by graft polymerization. The resulting "brushlike" structure is schematically depicted in Fig. 3c. Müller [46] used free radicals generated from hydroxyl groups of porous Fractogel TSK (EM Separations Technology) beads using cerium (IV) ions and grafted functional monomers shown in Fig. 4 such as dimethylaminoethyl acrylamide 5, diethylaminoethyl acrylamide 6, trimethylaminoethylacrylamide 7, 2-acrylamido-2-methyl-1-propanesulfonic acid 8, and acrylic acid 9. This technique allows the control of the density of functionalities by simple dilution of

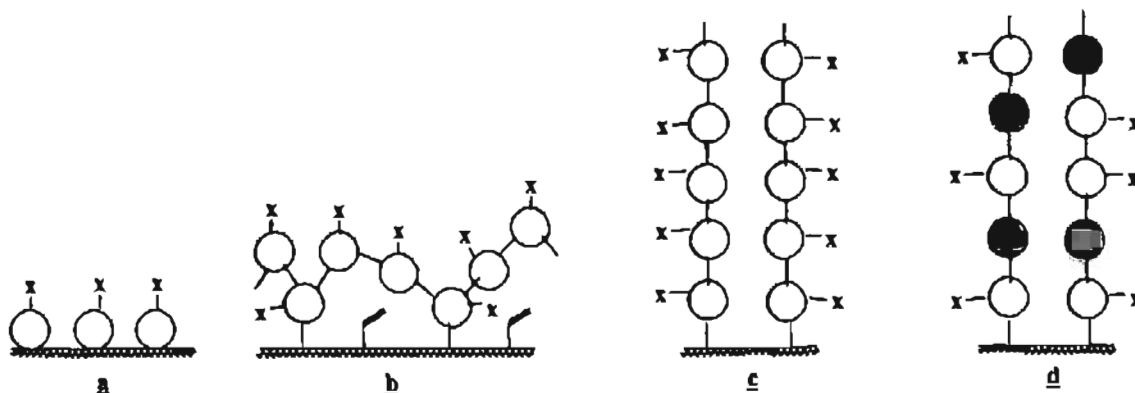


FIGURE 3 Examples of architectures of surface-modified pores.

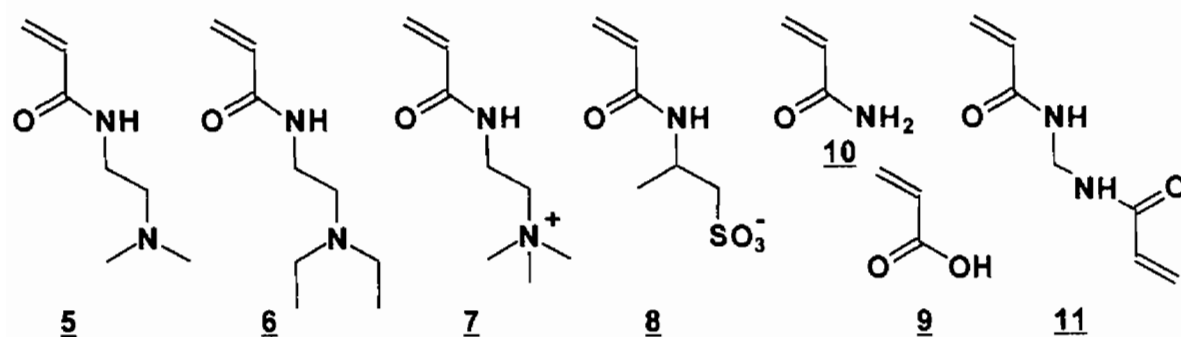


FIGURE 4 Examples of common acrylamide-based monomers.

the functional monomers with an inert monomer such as acrylamide (**10** in Fig. 4) and obtaining structures similar to that shown in Fig. 3d. Grafted ion exchangers for protein separations featuring both higher dynamic binding capacity and faster kinetics compared with “classical” materials are available under the trade name EMD Tentacle Ion Exchangers (EM Separations Technology).

Composite Packings

The major advantage of soft gel separation media is the accessibility and mobility of the functionalized chains constituting the matrix. On the other hand, rigid macroporous materials are characterized by much higher mechanical strength. A combination of benefits of both categories of materials led to the development of hybrid (composite) packings consisting of a rigid macroporous polystyrene-silica composite scaffold that contains pores filled with polymerized acrylamide monomers like **7** and **8** shown in Fig. 4 slightly cross-linked with methylenebisacrylamide (**11** in Fig. 4) [47]. Both strong acid (S-HyperD) and strong base (Q-HyperD) media are commercially available from Biosepra. These separation media exhibit very high dynamic loading capacities of about 150 mg BSA/mL and faster mass transfer kinetics compared with typical macroporous beads [47]. Although the mechanism of mass transfer within the gel phase in the pores is not yet completely explained, some preliminary results indicate that the mass transport is not controlled by diffusion [48].

“Gigaporous” Materials

The transport of molecules within the pores of a standard macroporous material is controlled by diffusion. Small entities such as gases, small organic molecules, and ions move relatively quickly, whereas the transfer of large molecules such as proteins, nucleic acids, and synthetic polymers is considerably slower because their diffusion coefficients are several orders of magnitude smaller than those of low-molecular-weight compounds. This effect is detrimental to processes in which the speed of the mass transfer limits the overall rate, as is the case in chromatography.

Consider what happens when a liquid is forced through a tube packed with standard macroporous particles. The liquid flows readily through the interstitial voids between the particles where resistance to its flow is smallest. In contrast, the liquid present in the pores does not move and remains stagnant. If a small amount of a substance or a mixture of compounds is injected into the stream of flowing liquid (the mobile phase), these compounds will also be carried through the voids. However, because of the concentration gradient between the solution in the voids and the stagnant liquid within the pores, diffusion enables transport of these compounds into the pores until their concentration is equal in both stream and pores. Once the concentration “pulse” has passed by the bead, the amount of compound in the main stream decreases steeply and the concentration gradient is reversed. The compound then diffuses back from the pores into the surrounding liquid and eventually only the original stagnant phase remains within the pores.

Because the diffusion rate for small molecules is quite high and the equilibrium concentration within the pores is reached quickly, their concentration in the pores is almost identical to that in the pulse. The situation is quite different in the case of macromolecules. The slow diffusional mass transfer of macromolecules in macroporous media result in severe peak broadening. The efficiency of the whole separation system deteriorates rapidly as the flow rate increases. As a result, longer columns or slower flow rates must be used to achieve the desired separation [8].

A considerable improvement in catalytic activity was observed when heterogeneous catalysts were used with large pores allowing convection of reactants through the particles [49]. In contrast to diffusion, in which the concentration gradient is the driving force, convection driven by flow dramatically accelerates the mass transfer. However, most pores found in typical macroporous polymers are much too small (< 100 nm) to allow convection. According to the Hagen-Poiseuille equation, the pressure needed to force a liquid through a straight tube increases exponentially as the tube cross-section decreases. In the case of macroporous beads, the pressure needed to achieve convection through typical pores with a diameter of 100 nm or less would be too high to be realistic with today's equipment. Therefore, Regnier [50] used polymer beads with pores as large as 600 to 800 nm (Poros, Applied Biosystems, Foster City, CA) and achieved some flow through these pores. He also coined the term *perfusion chromatography* for this technique [11,51]. The extent of convection depends on the overall pressure drop along the column, which in turn is a function of flow rate. The higher the flow rate, the more mobile phase flows through the pores. This manifests itself in very good separations of biomacromolecules even at flow rates that were deemed inconceivable in the past without considerable loss of column efficiency [52,53]. Perfusion chromatography is easily scalable and the high flow rates translate into high-throughput processes. The perfusion approach has also been extended to diagnostics and catalysis using immobilized enzymes.

Nonporous Beads

The use of nonporous microparticles which are functionalized only at their external surface (micropellicular beads), is the ultimate solution to the problem of diffusion within the pores. Although silica-based micropellicular materials for the separation of biopolymers were introduced in the mid-1960s by Horváth et al. [54], the real advantages for rapid separation of large molecules were demonstrated only after very small beads became available almost two decades later [55]. Both theoretical calculations and experimental data document that rapid mass transfer between the mobile and stationary phases and the absence of intraparticle diffusion allow the separations of biomacromolecules to be finished within a few seconds [55]. In addition, a higher working temperature that may easily exceed 100°C further accelerates mass transfer and increases column efficiency [56,57].

Hamilton Co. developed nonporous 5- μm poly(styrene-divinylbenzene) beads PRP- α in the late 1980s. However, their chromatographic properties were inferior to those found for porous supports [42]. This is most likely due to their surface chemistry and relatively large size that translates into very small surface area. Typical macroporous polymers exhibit surface areas in the range from tens to hundreds of square meters per gram. To achieve such surface areas with nonporous beads, extremely small particles would have to be used, but such small particles are not practical because the high pressure needed to pump the mobile phase through such a column would require very slow flow rates. Nonporous 2 to 3- μm beads provide a reasonable compromise between back pressure and capacity. Therefore, Tosoh Biosep markets a series of nonporous (NPR) 2.5- μm hydrophilic methacrylate polymer resins packed in 35-mm-long, 4.6 mm inside diameter (ID) columns for the separation of biopolymers in ion-exchange, reversed-phase, and hydrophobic interaction chromatography (Table 1). However, even this relatively short column exhibits a back pressure of about 10 MPa (1450 psi) at a flow rate of 1 mL/min [58]. Because some chromatographic modes require a direct interaction with surface functionalities whose number depends on the extent of surface area, the absence of internal porosity translates into low loading capacity. Therefore, the nonporous supports are preferred only for high speed separations of peptides, proteins, and nucleic acids on an *analytical* scale.

SURFACE CHEMISTRIES

A large number of surface chemistries have been developed for HPLC separation media useful in numerous separation modes. For example, hydrophilic functionalities are advantageous for size exclusion separation in the aqueous phase, hydrophobic chemistry is essential for reversed-phase and hydrophobic interac-

TABLE 1 Properties of Commercial 35 × 4.6 mm ID Columns Packed with 2.5 μm Nonporous TSK-GEL Beads

Packing	Chromatographic mode	Surface chemistry	Capacity ^a (mmol/mL)	Minimum number of theoretical plates/column ^c
SP-NPR	Cation exchange	$-(\text{CH}_2)_3\text{SO}_3^-$	0.1	1300
DEAE-NPR	Anion exchange	$-(\text{CH}_2)_2\text{N}-(\text{CH}_2\text{CH}_3)_2$	0.15	1300
DNA-NPR	Anion exchange	$-(\text{CH}_2)_2\text{N}-(\text{CH}_2\text{CH}_3)_2$	0.15	7000 ^b
Butyl-NPR	Hydrophobic interaction	$-(\text{CH}_2)_3\text{CH}_3$	n.a.	n.a.
Octadecyl-NPR	Reversed phase	$-(\text{CH}_2)_{17}\text{CH}_3$	n.a.	1000

^aIon-exchange capacity according to Ref. 31.^bColumn length 70 mm.^cAccording to Tosoh Biosep catalog.

tion chromatography, charged functionalities serve the needs of ion-exchange chromatography, and reactive functionalities are required for the binding of ligands for affinity chromatography.

Hydrophilic Beads

Although acrylamide-based polymers are rather hydrophilic, they are soft and often swell excessively in water. Therefore, these stationary phases are currently not well suited for HPLC. In addition, the high solubility of acrylamides in water makes them less practical for polymerization in aqueous suspensions. In contrast, acrylates and methacrylates containing hydroxyl groups in lateral chains can be used for the preparation of beads by means of typical suspension polymerization. This is why the majority of commercially available rigid hydrophilic beads suitable for HPLC are manufactured from these monomers. Some of these typical chemistries were already shown in Fig. 2.

Hydrophobic Beads

Reversed-Phase Chromatography

A highly hydrophobic surface chemistry is typical of supports such as octadecyl silica (ODS) and poly(styrene-divinylbenzene) which require a mobile phase containing organic solvents (acetonitrile, methanol, tetrahydrofuran) for elution of adsorbed compounds from them. Excellent separations of proteins were demonstrated with commercial macroporous styrene-divinylbenzene beads PLRP-S [35,42]. In contrast, the Tosoh Biosep approach starts with hydrophilic TSKgel-PW beads and linear octadecyl alkyl chains are attached to the surface hydroxyl groups through an ether linkage to make the surface hydrophobic. This modification affords resin with chromatographic properties similar to those of typical ODS. Compared with styrene-divinylbenzene media, the separations using octadecylated polymer resins are achieved in a shorter period of time and are less pH dependent. In addition, Tosoh Biosep markets the TSKgel Phenyl-5 PW RP support which is also prepared by chemical bonding and characterized by a high density of phenyl groups. The rather high degree of functionalization differentiates this support from that used for hydrophobic interaction chromatography.

Hydrophobic Interaction Chromatography

A lower density of hydrophobic ligands interspersed within a highly hydrophilic surface allowing elution with entirely aqueous eluents is typical of supports for hydrophobic interaction chromatography (HIC). Because no organic solvents are involved, HIC is a very gentle separation method for proteins and preserves their

activity completely. The concept of HIC relies on the interaction of hydrophobic patches of proteins with hydrophobic ligands located on the separation medium using an aqueous salt solution as a mobile phase that promotes these interactions. Elution is achieved by a decreasing gradient of salt in the mobile phase. Currently, several types of stationary phases are offered by industry for this chromatographic mode. They include functionalities such as phenyl, alkyl (methyl, isopropyl, butyl, *tert*-butyl), and alkylether. HIC separation media are manufactured from hydrophilic beads as already described; the hydrophobic groups are typically attached to the matrix through an epichlorohydrin-mediated ether linkage which is very stable. The choice of a specific chemistry depends on the characteristics of the separated proteins. A large variety of separation media for HIC based both on soft and rigid matrices is commercially available.

Ion-Exchange Resins

Ion-exchange chromatography (IEC) is one of the most popular methods for the separation of charged molecules such as peptides, proteins, and nucleic acids. This separation mode benefits from more than 50 years of a tradition of ion exchangers that are well established commodity products mostly used for water treatment in very large demineralization units of power plants. Typical advantages of IEC are high capacity, high resolving power, the use of aqueous salt solutions for elution, and straightforward scale-up. The mechanism that is at the heart of IEC is a reversible interaction of charges located on separated molecules with opposite charged groups attached to the matrix surface. The strength of this interaction depends on the net charge of the analyte molecule, the strength of the ion-exchange functionality (pK_a), and the ionic strength and pH of the mobile phase. In contrast to HIC, elution is achieved in an increasing gradient of salt concentration.

Except for weak cation-exchange resins which can be prepared directly by the copolymerization of acrylic acid, all other common ion-exchangers are produced by chemical modification of existing beads. Here again, hydrophilic beads are frequently the starting material for the preparation. Examples of typical reactions that lead to the common ion-exchange functionalities are shown in Fig. 5. Some of these reactions, such as those with chloroacetic acid, 1,3-propanesultone, and 2-dimethylaminoethyl chloride which yield CM (carboxymethyl), SP (sulfopropyl), and DEAE (diethylaminoethyl) resins, respectively, are used for the functionalization of both polysaccharide and synthetic polymer beads. Some other ion exchangers are made from beads activated with epichlorohydrin (epoxy beads) that reacted with diethylamine and trimethylamine to afford slightly different types of DEAE and Q (quaternary amine) resins respectively. Typical characteristics of industrial ion-exchange resins are their total ion-exchange capacity for small ions such as Cl^- or Na^+ . Although these data are often

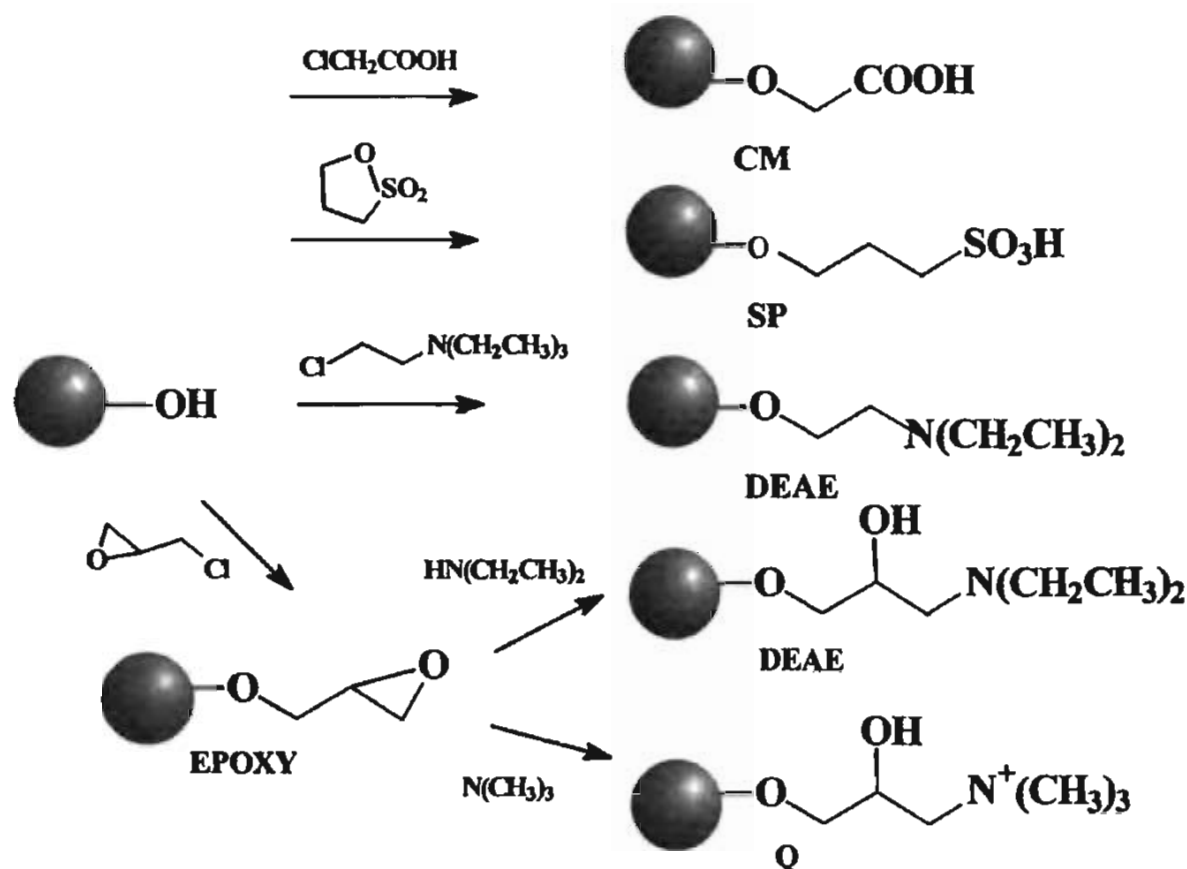


FIGURE 5 Preparation of ion-exchange resins from hydrophilic supports. Derivatives: CM, carboxymethyl; SP, sulfopropyl; DEAE, diethylaminoethyl; Q, quaternary amine.

available for chromatographic media and lie within a narrow range of 0.1–0.3 mmol/mL, they do not reveal much about the sorption capacity for large molecules which is actually the most important operational value in chromatography. Table 2 lists data published by individual manufacturers for selected anion-exchange resins and their sorption capacities for serum albumin which vary in a broad range of 40–150 mg/mL. Because the catalogs do not describe in detail the experimental conditions used, it is unlikely that all the data were obtained under identical operational conditions such as flow velocity, sample size, and steepness of gradient. A number of other variables such as the chemical structure of the beads, their porous properties, particle size, and the method used for the functionalization also affect the chromatographic parameters of a resin. Therefore, the data shown in Table 2 are intended only to illustrate the limits of the materials rather than to be used for direct comparison.

Media for Bioaffinity Chromatography

Affinity separations are the most selective among all of the chromatographic methods [18]. In fact, what is called *bioaffinity chromatography* is a simple binary

TABLE 2 Examples of Commercial Anion-Exchange Resins for Ion-Exchange Chromatography of Proteins

Packing	Vendor	Particle size (μm)	Capacity for BSA (mg/mL) ^a	
			Q	DEAE
Bio-Q	Cohesive Biotechnologies	20	110	n.a.
Fractogel EMD	EM Technology	25–40	100	100
Hyper-D	Biosepra	35	150	n.a.
Macro-Prep	BioRad Laboratories	50	40 ^b	35
Mono Q	Amersham Pharmacia Biotech	10	65 ^c	n.a.
Poros II	Applied Biosystems	50	55 ^d	55
Sepharose Fast Flow	Amersham Pharmacia Biotech	45–165	120 ^c	110 ^c
Source 30Q	Amersham Pharmacia Biotech	30 ^e	40	n.a.
Q-Sepharose XL	Amersham Pharmacia Biotech	45–165	130	n.a.
Toyopearl Super Q	Tosoh Biosep	40–90	143	n.a.
TSKgel	Tosoh Biosep	10	100	43

^aThe dynamic binding capacities for bovine serum albumin (BSA) are taken from catalogs. They may not be directly comparable as they are reported for different flow velocities and both analytical and preparative scale particle sizes.

^bMacro-Prep High Q.

^cCapacity for human serum albumin.

^dFor HQ derivative.

^eMonosized beads.

separation rather than a typical chromatographic method: the compound of interest is selectively “fished out” from a mixture by the sorbent in the adsorption step and then released in the following elution step. In contrast to the “true” chromatographic modes in which selectivity is at least partly controlled by the composition of the mobile phase, selectivity in affinity techniques results entirely from highly specific interactions between the bound ligand and the compound in solution. A large diversity of biomacromolecules requiring numerous specific sorbents creates difficulty for the manufacturers. This commercial problem has two possible solutions: (1) manufacture a limited number of resins with ligands featuring group selectivity or (2) marketing of activated supports for the preparation of customized affinity separation media by the end users [59].

Media with Group-Selective Ligands

Low-molecular-weight ligands for affinity chromatography include those shown in Fig. 6: benzamidine with selectivity toward serin proteases [60], boronic acid for carbohydrates and glycoproteins [61], and iminodiacetic acid, which offers chelation with transition metal ions (Cu, Zn, Ni) and exhibits selectivity for proteins and peptides containing histidine residues [62].

Midsized molecules of reactive dyes such as Procion Red HE3B or Cibachrom Blue F3GA (Fig. 7) are another example of group-selective ligands which interact specifically with dehydrogenases, kinases, interferons, lipoproteins, and albumins [18]. These dyes were invented long ago for the coloring of cotton fabrics composed of pure cellulose. The chloro-*s*-triazine building block reacts readily with hydroxyl groups and, therefore, these dyes are also well suited for modification of typical hydrophilic supports.

Finally, there is a group of high-molecular-weight ligands including protein A and protein G, both with high affinity against immunoglobulins, lectins for separation of sugar-containing molecules, DNA for DNA binding proteins, heparin for serum coagulation factors, and some others. Their exceptional selectivity makes them a very useful tool in modern biotechnology [18]; however, their price is much higher than that for low-molecular-weight ligands and dyes.

Beads with Reactive Functionalities

Reactive beads are finding numerous applications as supports for catalysts, in combinatorial chemistry, and in chromatography. Various modifications of some reactive polymer matrices were described in previous sections. For example, epoxide beads were used for the preparation of separation media for anion-exchange chromatography. However, the small number of typical ion-exchange functionalities require only a narrow range of modification reactions. Therefore, the preparation of ion-exchange resins does not require any excessive chemical creativity. In contrast, media for bioaffinity chromatography are often “tailor made” for the separation of each individual compound or small family of compounds and require materials carrying a large variety of surface chemistries. It would go far beyond the scope of this brief chapter to show all published reactive chemistries that have been used for the preparation of bioaffinity

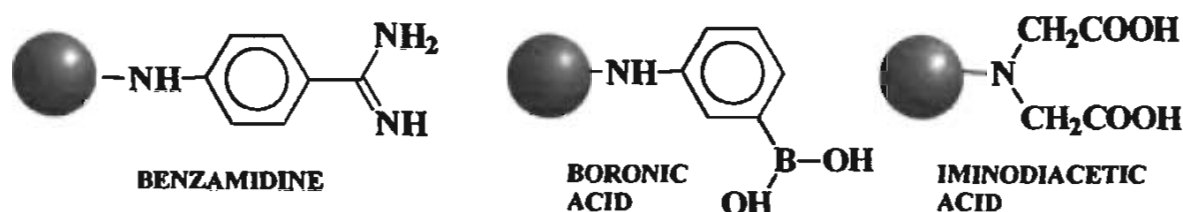


FIGURE 6 Examples of low-molecular-weight-affinants.

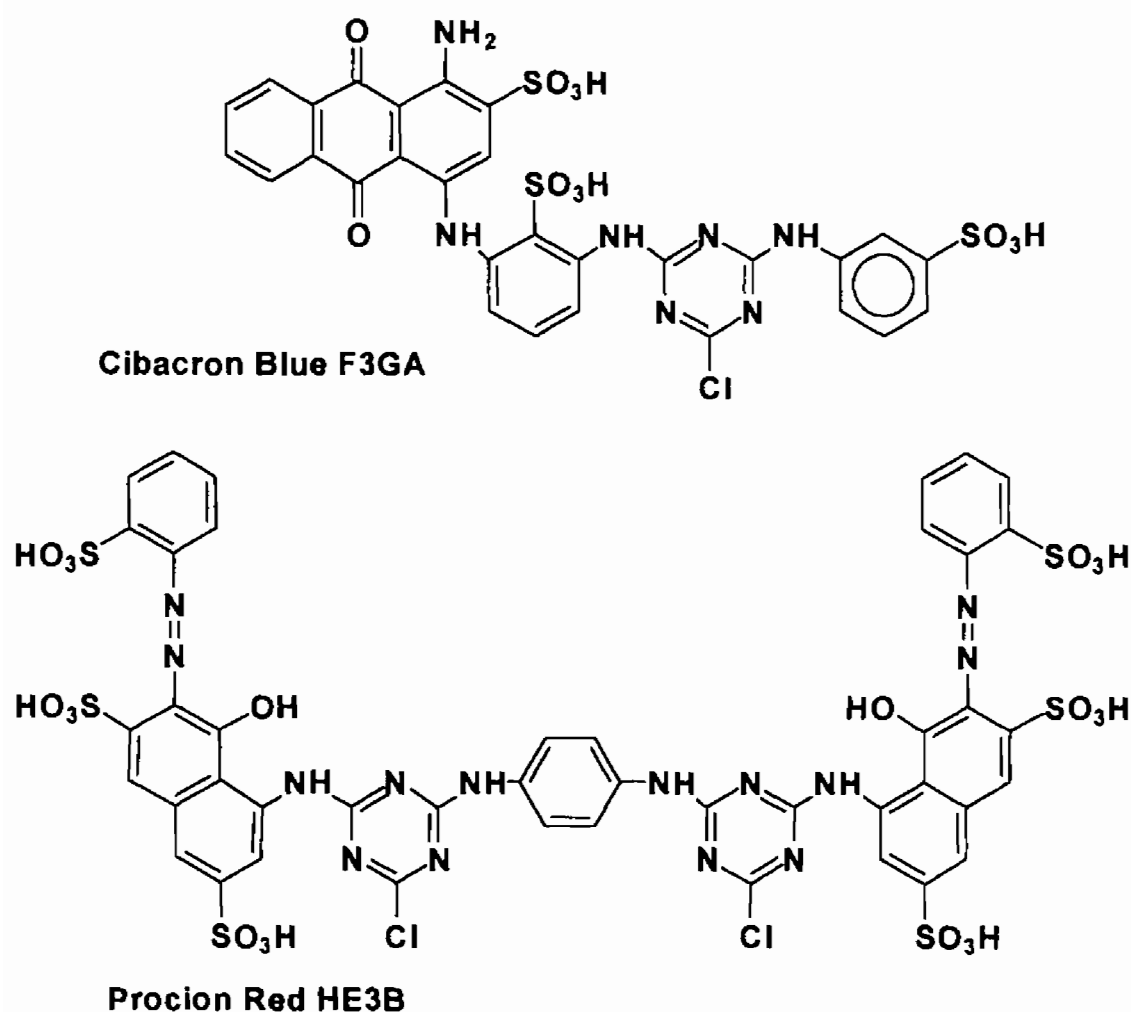


FIGURE 7 Reactive dyes for the preparation of supports for bioaffinity chromatography of proteins.

separation media, but Ref. 59 is highly recommended as an excellent guide that shows the majority of chemistries characteristic of current affinity supports.

Two basic methods are available for the preparation of reactive polymers: (1) activation of existing beads and (2) polymerization of reactive monomers. The first technique is typical of all natural polysaccharide beads and many synthetic polymers that contain functionalities such as hydroxyl, amine, carboxylic acid, and thiol groups. The most famous activated matrix, classical cyanobromide-activated agarose, was introduced in 1967 [63]. Many other activation procedures such as reaction with bisepoxides, *N*-hydroxysuccinimide, cyanuric chloride, and divinyl sulfone lead to materials that are shared by scientists preparing affinity media and immobilizing enzymes [59]. Although activated polysaccharide beads are still commercially available from Amersham Pharmacia Biotech and BioRad Laboratories, the use of activated supports based on rigid hydrophilic beads is growing. However, even these new supports are often provided with the “old”,

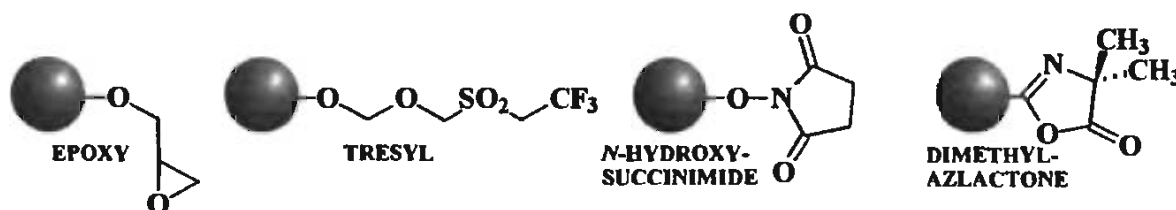


FIGURE 8 Examples of reactive supports for the preparation of bioaffinity separation media.

well-established reactive chemistries such as epoxide, tresyl, and *N*-hydroxysuccinimide ester groups illustrated in Fig. 8.

The polymerization approach is exclusive for synthetic polymers for which several reactive monomers shown in Fig. 9 such as glycidyl methacrylate **12**; 2-vinyl-4,4-dimethylazlactone **13**; and methacrolein **14** are available. However, even here the number of truly new chemistries is very limited. For example, 3M company (St. Paul, MN) introduced porous poly(vinyl dimethyl azlactone) beads in the early 1990s [64] that are now marketed by Pierce (Rockford, IL) under the trade name UltraLink Biosupport. The azlactone ring reacts under mild conditions with amine, hydroxyl, and thiol nucleophiles (ligands) as shown in Fig. 10 and binds covalently up to 35 mg/mL of proteins. EM Separations Technology extended their original concept of EMD “tentacle” ion exchangers to supports for affinity chromatography with grafted chains of poly(vinyl dimethyl azlactone) and poly(glycidyl methacrylate). The former is best suited for the immobilization of macromolecular ligands such as protein A or antibodies for immunoaffinity

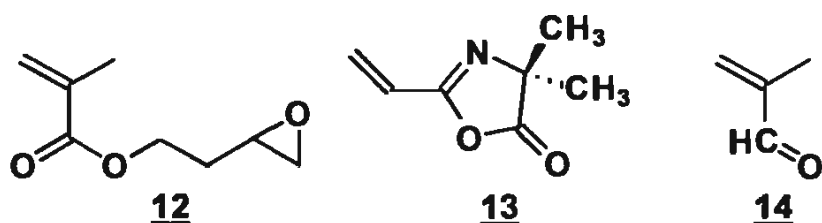


FIGURE 9 Reactive functional monomers useful for the preparation of supports for affinity chromatography.

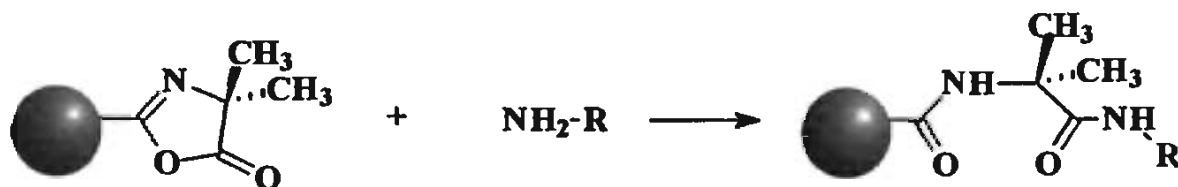


FIGURE 10 Immobilization of a ligand with amine groups on azlactone support.

chromatography. The latter couples readily with low-molecular-weight ligands and is an intermediate for the preparation of an iminodiacetic acid matrix for immobilized metal ion affinity chromatography (IMAC) and sulfur-containing functionalities for thiophilic chromatography.

Development of New Ligands

The quest for new, more selective, and perhaps also cheaper ligands for isolation of numerous new compounds produced using biotechnology processes goes beyond the current limits of the natural affinants. Today, discovery of new powerful ligands may involve methods of combinatorial chemistry that allows the creation of large libraries of compounds in a very short period of time [65]. For example, the total number of different hexamer peptides that can be prepared from 20 natural amino acids exceeds 65 million. The large diversity of compounds that result from combinatorial synthesis fits well in the discovery process of new affinants. The compound with the highest affinity against the target can be found through rapid automated screening. The next step is deconvolution of the affinant structure (if necessary) followed by its preparation on a larger scale and immobilization onto a support [66]. Several leading companies such as Amersham Pharmacia Biotech, Biotage of Dyax Corp. (Cambridge, MA), and Applied Biosystems are using affinants selected from large libraries for the development of new separation media [67–73].

UNIFORMLY SIZED BEADS

Although stirring provides some control over the average size of droplets in the initial stages of a “classical” suspension polymerization, the beads that are obtained have a broad distribution of sizes. Because these beads are packed in columns, this size polydispersity leads to a nonideal three-dimensional array of particles and which affect efficiency and flow through the column. As a direct consequence, most commercially available beads for high-end applications such as HPLC are size fractionated. In order to optimize the performance of beads and to eliminate the considerable waste of material that results from fractionation, processes have been developed for the production of uniformly sized spherical particles. The major advantages of columns packed with monodisperse beads are lower back pressure, lower void volume, and higher efficiency compared with polydisperse packings.

Simple techniques such as emulsion and dispersion polymerization which provide monodisperse beads are not suitable for the preparation of macroporous media. Alternative approaches based on the dropwise introduction of a polymerization mixture containing a thermally or photochemically triggered initiator into a heated immiscible liquid in which the polymerization proceeds under conditions that avoid coalescence have met with some success. The droplets

are produced by pushing the monomer phase through the thin orifices of syringe needles [74], porous glass membranes [75], or various capillaries [76]. Although these simple techniques yield beads with relatively narrow particle size distributions, they are not completely monodisperse.

Small, nonporous, uniformly sized spherical particles of linear polymers are readily obtained by modified emulsion polymerisation. These small nondisperse spheres are utilized as “shape templates” in another concept for the preparation of large macroporous beads. Such a process increases their size manyfold in a uniform fashion, but also introduces pores. A stabilized aqueous dispersion of micrometer-size uniform particles is swollen to the target size of the final macroporous product with a combination of solvents, monomers, and cross-linking agents. The swollen particles are then polymerized. Although the shape and size uniformity of the original templates is preserved, their size has been increased considerably and the presence of a solvent during polymerization leads to the desired macroporous structure. In practice, the equilibrium between interfacial energy and swelling forces considerably limits the extent of swelling of the original template beads, but the multistage swelling processes pioneered by Vanderhoff [77] and Ugelstad [78] and their coworkers enable the preparation of larger monodisperse beads. The size of the final beads is determined no longer by the stirring conditions but by the swelling of the shape-template particles [39].

Obviously, the large-scale preparation of porous monosized beads is more sophisticated than a standard suspension polymerisation technique. Therefore, only a few companies offer truly monodisperse organic polymer particles in their catalogs. Amersham Pharmacia Biotech was the first in marketing MonoBeads for fast ion-exchange chromatography of proteins. The preparation of these uniformly sized beads is based on technology developed by Ugelstad et al. [78]. Uniform 10- μm poly(styrene-divinylbenzene) beads are coated with a layer of hydrophilic polyether [poly(oxyethylene)] and modified with sulfonic acid groups (MonoS) or quaternary ammonium functionalities (MonoQ). MonoBeads were recently complemented with uniform 3- μm Q and S MiniBeads, and 15- and 30- μm beads called Source. In contrast to Mono- and MiniBeads that are sold only in prepacked columns, Source materials are marketed in bulk and additional chemistries such as those for hydrophobic interaction and affinity techniques are also available. Polymer Laboratories developed their own 8-, 9-, and 10- μm monodisperse poly(styrene-divinylbenzene) beads PL Hi-Plex for HPLC of carbohydrates, organic acids, alcohols, and synthetic polymers.

MEDIA WITH REDUCED DISCONTINUITY

The lowest theoretical interparticular volume of perfectly packed uniformly sized spherical beads is calculated to be about 27% of the total column volume. In

practice, even the best-packed columns contain much larger void volumes. Separation in a medium that is in fact a single piece and does not contain interparticular voids, which normally contribute to peak broadening, has already been treated theoretically [53], but experimental work remained scarce as a result of the lack of better alternatives to beads.

The problem of interparticular volume does not exist in systems in which a membrane is used as the separation medium. Both theoretical calculations and experimental results [79] clearly document that membrane systems can be operated in a “dead-end”, or filtration mode at much higher flow rates than packed beds because all the substrate solution flows through the support and the mass transfer is much faster as a result of this convective flow. Obviously, a reasonable compromise between the membranes with their fast mass transfer and beads with high capacity may result in separation media with enhanced properties.

Stacked Thin Sheets

The simplest available porous “membrane” is filter paper made of pure cellulose. Numerous modification methods are well known for this material because modified microcrystalline cellulose powders have been utilized in chromatography of biopolymers for a long period of time. This may be the most important driving force for the use of modified cellulose sheets in various separation devices. Because the binding capacity of a single sheet would be too small, several sheets are stacked in a specifically designed cartridge. For example, MemSep cartridge originally produced by Millipore (Bedford, MA) contained 40 stacked membranes. In contrast Sartobind membrane adsorbers (Sartorius, Edgewood, NY) consist of only three or four sheets. The pore size of these membranes is an important variable that effects the separation [80]. As a result of their large width and low height, the cartridges allow high volumetric throughputs at low back pressure. The residence time within the cartridge is generally short and reduces the danger of denaturation of biopolymers during the separation process. Numerous analytical and preparative separations of proteins in various chromatographic modes were performed in membrane cartridges with very good results [81–83].

Theory predicts a dramatic increase in efficiency, perhaps by a factor of 10, for adsorptive membrane devices compared with that of beds packed with particles [83]. However, the nonuniform flow and dead volumes typical of many current cartridges [84] do not allow one to utilize fully the intrinsic separation ability of the membranes. Therefore, their design should be improved to compete successfully with packed columns.

Porous Polymer Disks

The problem of distorted flow and voids between the membranes in the stack does not exist in cartridges that accommodate only a single thicker disk of porous material. In addition, Belenkii's theory of critical distance in adsorption chromatography leads to a conclusion that even a very short layer of a separation medium such as the disk can be sufficient for an efficient separation of large molecules [85]. These provisions led to development of rigid disks for rapid chromatography of biopolymers by Tennikova and Svec in the late 1980s [86].

Flat sheets of porous material are easily prepared in a single step by free radical polymerization within a closed flat "sandwich" mold. Disks are cut with a blade from these sheets and placed into special cartridges. In a different implementation, a monolithic cylinder is first prepared and then "sliced" to the disks [87]. If needed, the porous material can be provided with special functionalities by chemical modification. Typically, a solution of a reagent is soaked into the molded material and the modification is performed in a flask [87,88]. Josic and Strancar studied numerous applications of rigid macroporous disks [89] and found that a well-designed cartridge together with optimized disks dramatically improved performance and allowed rapid chromatography of proteins. Separations of protein mixtures in different chromatographic modes such as ion exchange and hydrophobic interaction were easily achieved in a few seconds [90,91]. The macroporous disk technology was recently commercialized by BIA Separations (Ljubljana, Slovenia). This technology has been described in detail in a series of excellent review articles [92,93].

Disks of regenerated cellulose provided with ion-exchange functionalities and placed in disposable cartridges (Seprisorb) are marketed by Sepragen Corp. (Hayward, CA). These devices are characterized by very large through pores that allow direct purification of dirty feedstocks such as homogenized yeast cells at high flow rates [94].

Rolled Sheet

Although the flat membranes and disks afford good separations, their aspect ratio requires specifically designed cartridges with sophisticated distributors of the mobile phase all across the surface of the disk. In contrast, tubular devices such as columns do not need these distributors because the top part of the bed itself serves this purpose and their construction is much simpler. This led Ladish to the fabrication of stationary phases from rolled flat materials—woven textile fabric [95]. The cylindrical roll is then inserted into typical HPLC column. The number of chemistries is limited only by the variety of fibers. Aramid columns behave as a hydrophobic interaction phase [95] and cotton fabrics transformed to an ion-exchange derivative can be used in IEC [96]. Ladish et al. [97] published a

detailed account of rolled separation media. Rolled columns are very stable and have much better flow properties than columns packed with 1-mm-long fibers [98]. As expected, the efficiency (plate height) does not change with flow velocity because the mass transfer is substantially enhanced by convection of the mobile phase through the stationary phase.

Kennedy and Paterson [99] used a similar approach and rolled a “rod” from a paper-like cellulose sheet. After cyanogen bromide or 1,1'-carbonyldiimidazole activation, protein A and protein G were immobilized onto the cellulose and the column was successfully used for the affinity separation of immunoglobulin G (IgG).

Compressed Gels

Slightly cross-linked polyacrylamide beads have been known for a few decades as an excellent packing for the low-pressure liquid chromatography of biomacromolecules. The general perception is that their swelling and softness prevent them from use in tall chromatographic columns. However, an “old” study of the resolving power of another soft gel bead—agarose—revealed that compressed beds of these particles had better efficiency than the original columns [100]. Hjertén et al. [101] transformed this experience into the preparation of highly cross-linked (30–60%) methylenebisacrylamide copolymers with functional monomers that were eventually compressed to a fraction of their original volume and used for chromatographic separations. In a typical example, 0.36 g of monomers is dissolved in aqueous buffer and polymerized in a 35-cm-long tube. The insoluble material is then compressed by pumping buffer solution followed by mechanical compaction to the final height of 3.5 cm, i.e., to one tenth of the original volume. This plug has a porosity of about 66%; however, this porosity mainly represents large pores while the highly cross-linked and compressed nodules are *nonporous* for proteins [102]. Therefore, separations of large molecules are rather fast. This technology was later extended to the preparation of microcolumns and microchips [103]. Materials for chromatographic separations in different modes are obtained either directly by copolymerizations that involve different charged or hydrophobic monomers or using in situ modifications [104]. Hjertén [105] nicely presents his technology and its historical development in a review.

BioRad Laboratories commercialized these compressed media under the trade name UNO. Both quaternary ammonium and sulfonic acid columns for IEC are claimed to have good stability and a high dynamic binding capacity that does not change with an increase in flow velocity. For example UNO-S binds 42 mg of bovine IgG per 1 mL of column volume.

“Molded” Monolithic Columns

A new procedure in which rigid macroporous materials of any desired shape are prepared in their final size by an in situ polymerization was developed in the early 1990s [106]. Separation media are thus not packed but “molded” within the confines of the tube of a chromatographic column. In this procedure, the tube (sealed at one end) is filled with the polymerization mixture; then it is sealed at the other end and heated in a water bath. Once the polymerization is complete, the seals are removed and the column is provided with fittings, attached to the HPLC system, and washed. Obviously, an ideal monolith should contain both large pores to achieve flow of the mobile phase through the column at a reasonable back pressure (convection) and a connected network of shorter and smaller pores for diffusion. Indeed, an extensive study of the effects of individual variables of bulk polymerization with particular attention paid to temperature (polymerization kinetics) and porogen composition (phase separation process) on the porous properties of products and the optimization of the polymerization conditions used to prepare monoliths led to highly porous materials with a bimodal pore size distribution that is completely different from that found for macroporous beads prepared from an identical polymerization mixture (Fig. 11) [107]. Typically, these monoliths contain not only small diffusive pores but also a large number of flow-through pores with diameters in the range of 700 to 2000 nm [107,108]. This

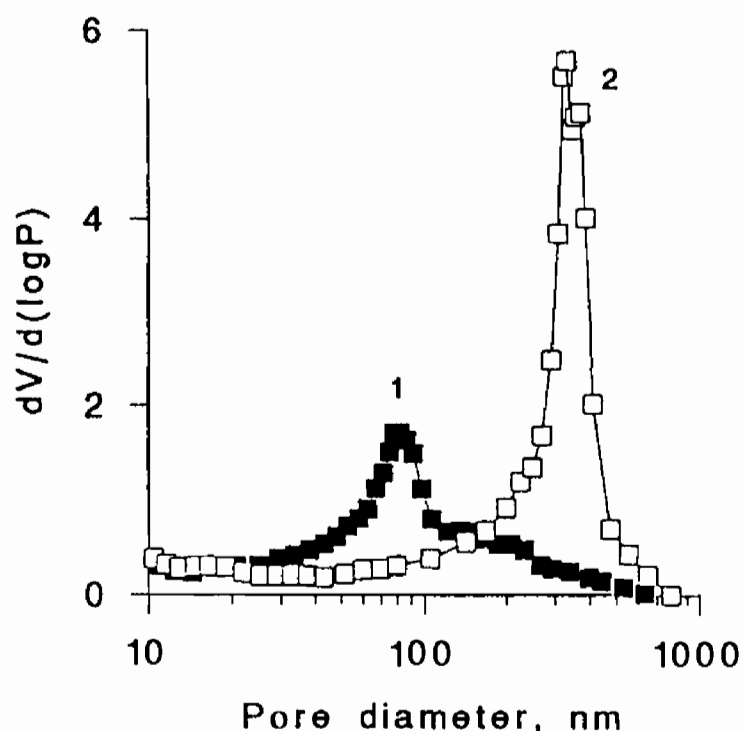


FIGURE 11 Differential pore size distribution curves of the poly(glycidyl methacrylate-co-ethylene dimethacrylate) beads (1) and monolith (2) prepared at a temperature of 70 °C.

structure accelerates mass transfer of the sample within the porous material by convection, resulting in much faster chromatographic runs without sacrificing the separation power of the monolithic column. Various types of monolithic columns and materials were described in detail in recent reviews [109,110].

Poly(styrene-divinylbenzene) monoliths are well suited for the rapid separation of proteins in the reversed-phase mode. For example, four proteins were separated in 24 sec at a flow rate of 5 mL/min in a 10×0.8 cm ID molded column [39]. In fact, this column easily tolerates even a flow rate of 25 mL/min. Rigid reactive poly(glycidyl methacrylate-ethylene dimethacrylate) monoliths can be easily provided with special functionalities such as ion-exchange or hydrophobic groups by chemical modification. Typically, the reagent is driven at a slow flow rate through the column kept at the desired reaction temperature. The experimental work done so far confirms the great potential of these molded continuous supports. Figure 12 shows the rapid separation of five proteins that is accomplished in about 15 sec [111]. Besides reversed-phase, hydrophobic interaction, ion-exchange, and precipitation chromatography, these materials have also been used for capillary electrochromatography and are characterized by very high

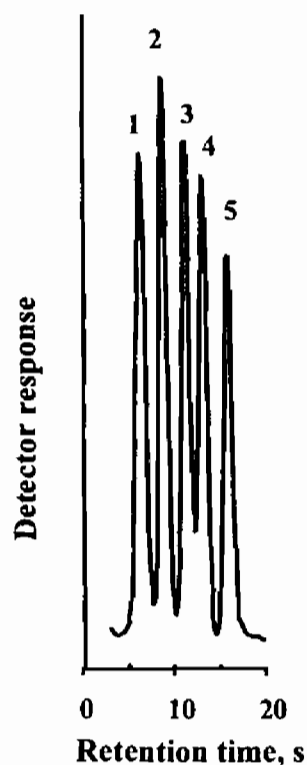


FIGURE 12 Rapid reversed-phase separation of proteins at a flow rate of 10 mL/min. Monolithic column 50×4.6 mm ID, mobile phase gradient 42 to 90% acetonitrile in water with 0.15% trifluoroacetic acid in 0.35 min. Peaks: ribonuclease (1), cytochrome *c* (2), bovine serum albumin (3), carbonic anhydrase (4), chicken egg albumin (5). Detection: ultraviolet 280 nm. (From Ref. 111.)

efficiency [112,113]. The approach has also been exploited for the preparation of imprinted molded materials for molecular recognition. Among numerous advantages of the monoliths are their ease of preparation and ruggedness, the versatility of their chemistries, and their excellent efficiency in the separation of biological and synthetic molecules.

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Size Exclusion Chromatography

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INTRODUCTION

Separation of molecules by size is an important technique in both the life sciences and polymer chemistry. Unfortunately, nomenclature associated with this technique developed independently in these disciplines. In the pioneering work of Porath and Flodin [1], separation of proteins by size in a porous gel medium was designated gel filtration chromatography (GFC), which became acceptable in the biological fields. Moore [2], on the other hand, referred to size separation of synthetic polymers in beds of polystyrene-divinylbenzene particles as gel permeation chromatography (GPC). The utility of Moore's work in synthetic polymer fractionation led to general acceptance of his definition in the polymer industry. This confusing difference in nomenclature has, unfortunately, fostered the growth of two sets of scientific literature pertaining to the same technique. Adequate discussion of state-of-the-art separation of molecules by size requires an examination of literature from both fields.

For the sake of continuity and clarity, a uniform nomenclature should be used. Neither gel filtration nor gel permeation can be defended as a technically accurate description of this technique. "Filtration" is not mechanistically correct, and reference to the support matrices as "gels" is equally inadequate. A more accurate term for the separation of molecules by size is size exclusion chromatography (SEC). The logic for this term will become apparent as this chapter is developed and the term will be used exclusively in this book to describe separation of molecules by hydrodynamic volume.

RETENTION

Mechanism

Two approaches may be taken to describe retention in SEC systems. The first is nonmechanistic in which the only assumptions are:

1. That there is a stagnant phase of liquid of volume (V_i) in the column
2. That there is differential permeability of solutes into this stationary phase on the basis of molecular size.

Because this approach is the simplest, most widely used, and has been the most successful in dealing with SEC systems, it will be used in this discussion. A second approach is an exact mechanistic description based on solute dimensions relative to pore size and pore geometry. Such explanations are primarily theoretical because supports of rigorously defined pore size and pore geometry are not available to test mathematical models. Mechanistic models of SEC have been developed by Ackers [3], Casassa [4], Giddings et al. [5], Kubin [6], and others.

Solute retention in terms of retention volume (V_R) in a chromatographic system may be explained in terms of three variables: the distribution coefficient (K_D), mobile phase volume (V_M), and stationary phase volume (V_S). In SEC, stationary phase volume is usually considered to be the volume of liquid in the support pores (V_i). The void volume is equal to the elution volume of a peak (V_o) which cannot penetrate the pores and must be the volume of liquid in the interstitial space outside the pores. The mobile phase volume is the sum of the two. The distribution coefficient in SEC specifies the fraction of internal volume (V_i) available to a molecule and, therefore, should not exceed unity. SEC distribution coefficients are frequently identified as K_D . Using these defined parameters, the retention volume (V_R) can be defined:

$$V_R = V_o + K_D V_i \quad (1)$$

A pictorial representation of these parameters is shown in Fig. 1. Large molecules that do not penetrate the pores of the support matrix elute in the

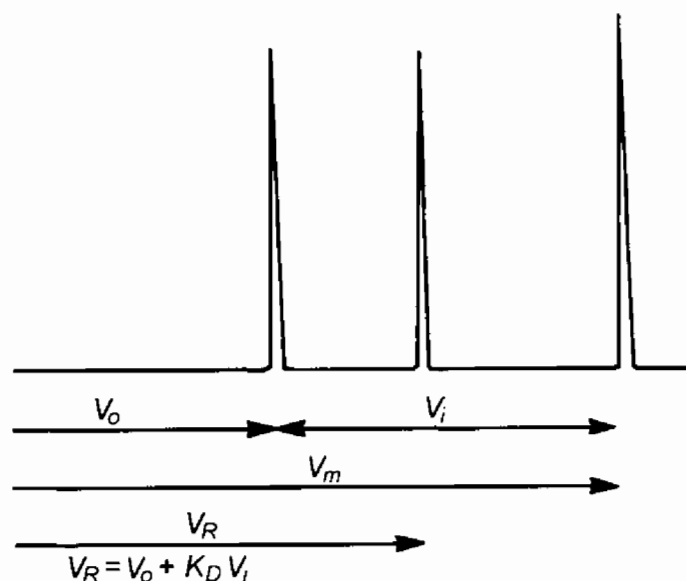


FIGURE 1 Pictorial representation of the terms used in size exclusion chromatography.

column void volume (V_o), while small molecules that totally penetrate the support pores elute in the mobile phase volume (V_M). This volume, which is equal to the sum of the void and internal volumes ($V_i + V_o$), is also referred to as the totally included or permeation volume. It can be seen in Fig. 1 that the elution characteristics of a substance may be expressed in terms of either elution volume (V_R) or distribution coefficient (K_D). Because V_R is dependent on column dimensions, K_D is a more universal and desirable expression for the SEC behavior of a substance.

A typical profile from a high-performance size exclusion chromatography (HPSEC) column is shown in Fig. 2. With ideal SEC systems, plots of the log of molecular weight against K_D produce calibration curves like those shown in Fig. 3. SEC supports of a single pore size with a narrow pore distribution should produce curves that are linear over a wide range of K_D values, usually 0.1–0.8. In order to achieve a linear calibration curve, it is essential that there be no interaction between the column support material and the solute. Because solute-support interactions have been observed in every support material that has ever been introduced for SEC, the nature and characterization of these retentive phenomena will be discussed in detail later in the chapter.

It is naive to correlate K_D directly with molecular weight because it is apparent that the relationship actually exists between the retention volume of a molecule and its hydrodynamic size rather than its molecular weight. The radius of a molecule in solution, relative to its molecular weight, is influenced by its degree of hydration, its molecular asymmetry, and, occasionally, its ionic and/or hydrophobic character.

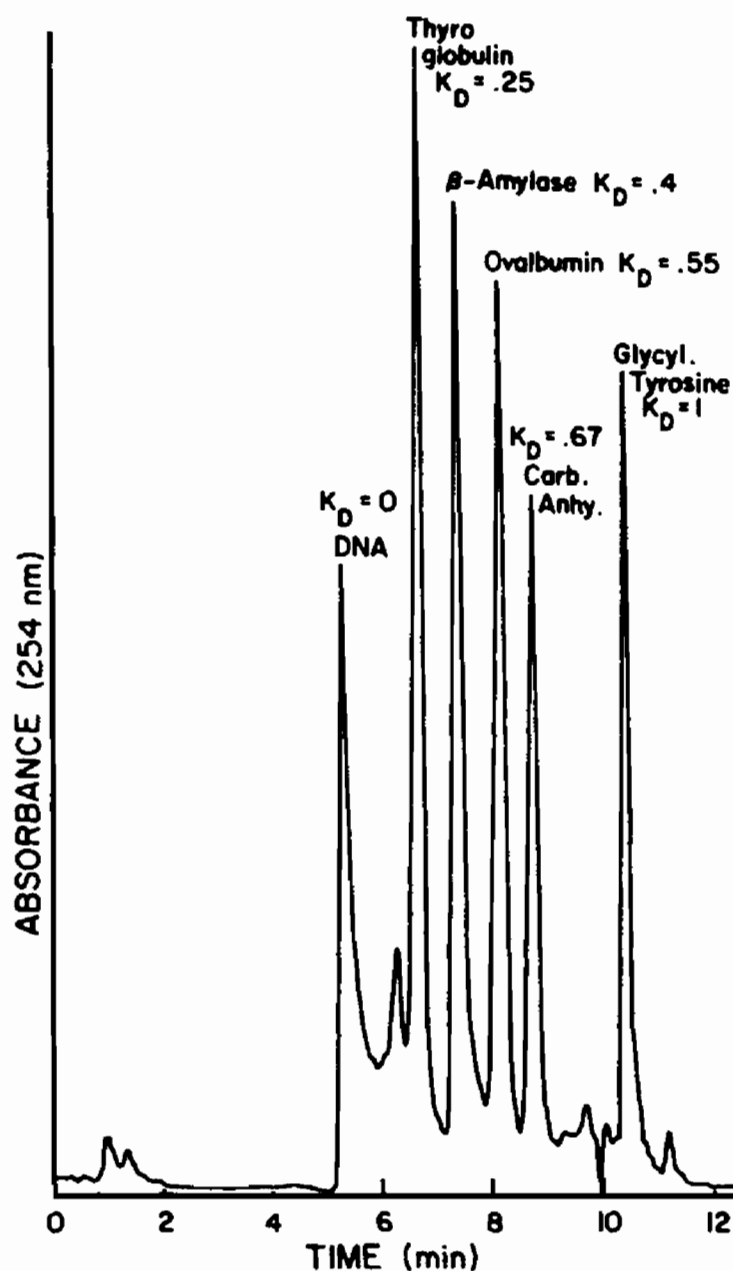


FIGURE 2 Typical profile obtained from high-performance size exclusion chromatography. Column: SynChropak GPC 300, 300 × 7.8 mm ID Mobile phase: 0.1 M KH_2PO_4 , pH 7. Flow rate: 1.0 mL/min.

Hydration

The polar nature of proteins causes them to bind multiple molecules of water, the number of which depends on solution pH and ionic strength. For proteins, the degree of hydration commonly varies from 0.3 to more than 1.0. Cantor and Schimmel [7] calculated that a monolayer of water bound to the surface of a 30,000-Da spherical protein would cause a 13.6% increase in the hydrated molecular radius. It is apparent that the relative degree of hydration will cause

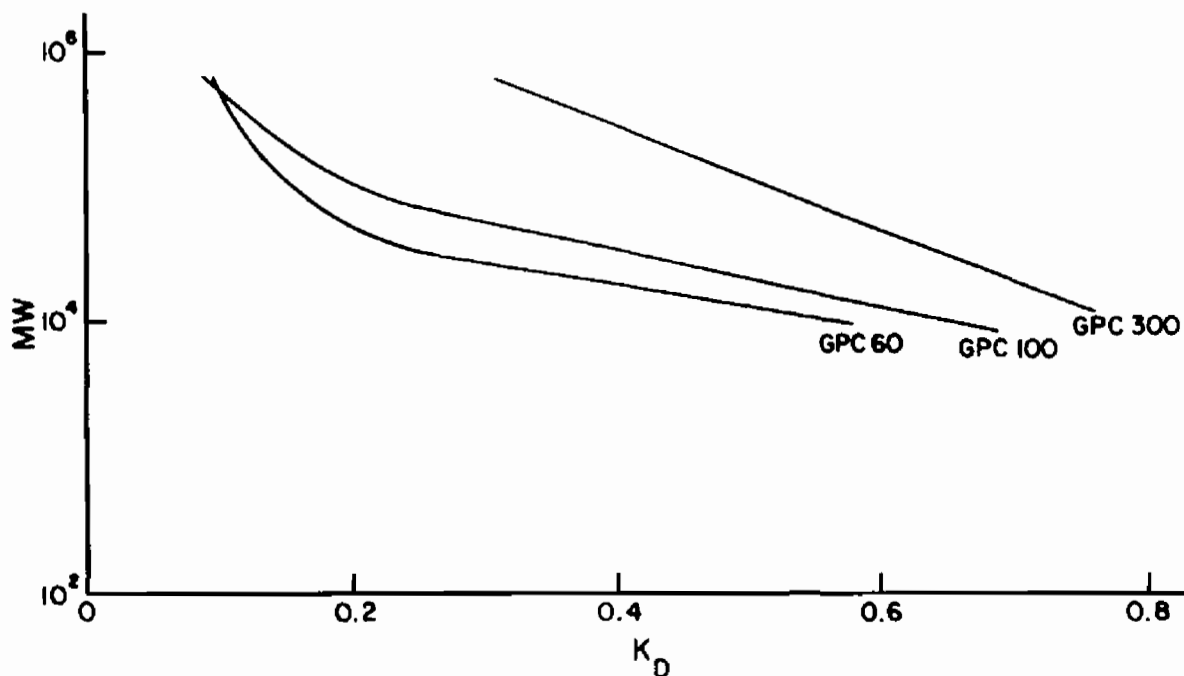


FIGURE 3 Calibration curves for proteins on size exclusion supports having 60-, 100- and 300-Å pore diameters.

the radius to be larger than it would be in the nonhydrated or dry state. As the degree of hydration increases, K_D becomes smaller, and a molecule appears to have a larger molecular weight. For some macromolecules, solution pH and ionic strength can have a major influence on molecular dimensions. In the case of carboxymethylcellulose, changing the mobile phase from 0.23 M phosphate buffer (pH 7.0) to distilled water caused the K_D to decrease threefold [8].

The ability of proteins to adsorb a variety of small molecules onto their surface and still retain most or all of their original conformation should also be considered when using complex mobile phases. It is possible that low concentrations of additives, such as detergents or organic solvents, can be adsorbed in sufficient quantities to change the volume of a protein. Figure 4 illustrates this effect on the calibration curves of a series of proteins when 0.1% sodium dodecyl sulfate (SDS) is added to the mobile phase.

Molecular Shape

The second major variable determining the hydrodynamic volume of a molecule is its shape. The radius of gyration of a spherical polymer is less than that of a linear polymer of the same molecular weight. It can be seen in Fig. 5 that linear sulfonated polystyrenes (SPSs) do not penetrate the pores as much as globular proteins of the same molecular weight. Table 1 lists the radii of gyration for molecules with varying asymmetry. As the shape of the 500,000-Da model is

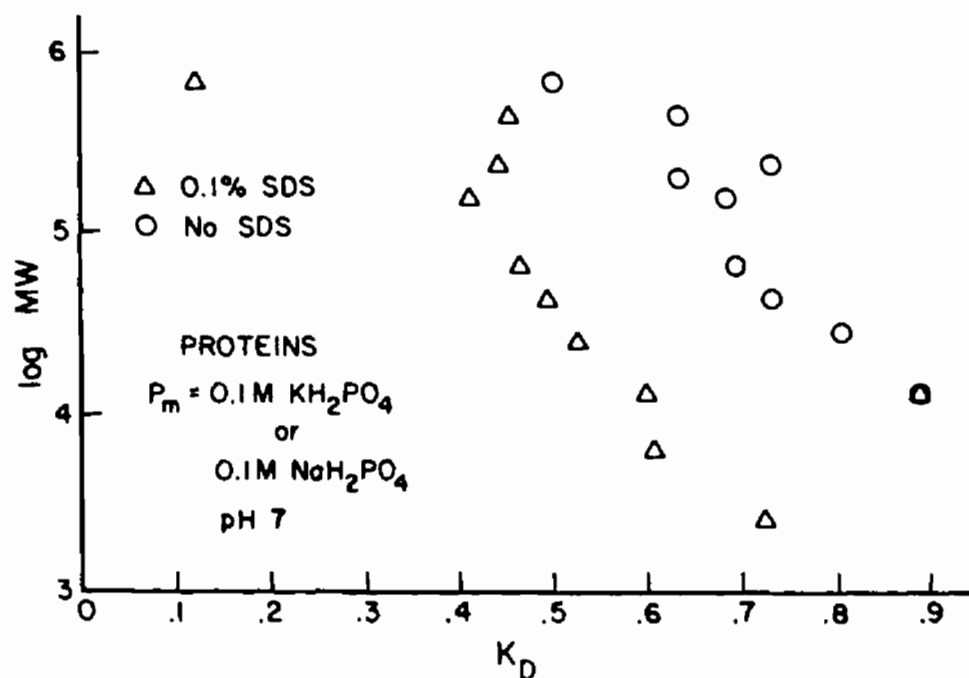


FIGURE 4 Displacement of protein calibration curve in the presence of 0.1% SDS in the mobile phase. Column: SynChropak GPC 100, 250×4.6 mm D. Flow rate: 0.5 mL/min. Standard proteins.

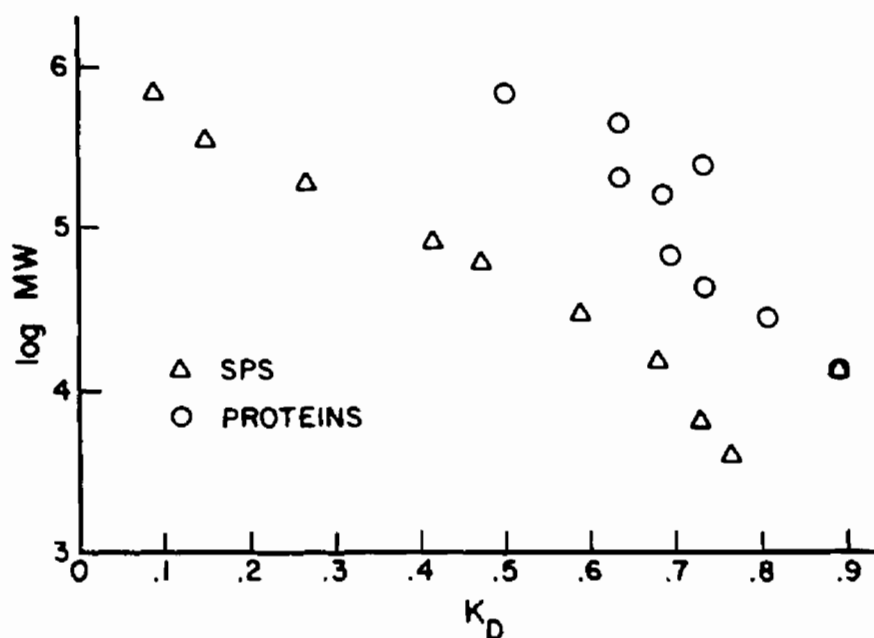


FIGURE 5 Effect of solute shape on calibration curves. Column: SynChropak GPC 500, 250×4.6 mm ID. Flow rate: 0.5 mL/min. (Δ) Sulfonated polystyrenes in 0.1 M sodium sulfate mobile phase. ($\circ$) Standard proteins in 0.1 M potassium phosphate, pH 7.

TABLE 1 Radius of Gyration (R_G) of Some Molecules

	Molecular weight	Calculated R_G (Å)	Experimentally determined R_G (Å)
Solid sphere (model molecule)	500,000	45	
Flexible coil (model molecule)			
Long rod (model molecule of 25 Å diameter)	500,000	410	
Long rod (model molecule of 15 Å diameter)	500,000	1150	
Serum albumin	66,000	21 ^a	29.8
Catalase	225,000	31 ^a	39.8
Myosin	493,000	41 ^a	468.0
DNA	4×10^6	74 ^a	1170.0
Bushy stunt virus	10.6×10^6	113 ^a	120.0
Tobacco mosaic virus	39×10^6	175 ^a	924.0

^aCalculated as the radius of an unsolvated sphere.

Source: Data from Ref. 57.

varied from a sphere to a rod of 15 Å diameter, the radius of gyration increases 26-fold. The very large differences between the calculated radius values for a sphere and the experimentally determined radii for myosin and tobacco mosaic virus are due to the substantial asymmetry in these molecules.

Application of this knowledge about hydrodynamic volume to size exclusion separations of proteins is complicated by several factors. With the exception of Ackers' work [3], most of the experimental examinations of pore models and molecular shape contributions to retention have been carried out with synthetic polymers in organic mobile phases. In addition, molecular dimensions of synthetic polymers are given in terms of effective radius or radius of gyration, whereas protein dimensions are most often expressed in terms of the Stokes radius. This radius can be readily obtained from centrifugation studies, but the calculations are so different from those used to calculate radius of gyration that no simple relationship can be derived. A final consideration is that proteins are not part of a homologous series and have no universally common shape factors. Potschka [9] proposed an alternative calibration method that is based on the viscosity radius, which is the molecular volume times a shape function defined by the intrinsic viscosity. When this procedure was used, there was less deviance from the calibration curve for proteins with nonglobular tertiary structures.

Expressing theoretical and experimentally observed relationships in some quantitative form that applies to all biopolymers has not been totally successful. Because retention in SEC is a function of molecular volume, degree of molecular

hydration, and shape as well as generally unknown column pore geometry characteristics, little can realistically be said about the molecular weight and shape of an unknown molecule. Although SEC can be very useful in molecular weight estimations for homopolymers such as sulfonated polystyrenes or dextrans, it must be recognized that for heteropolymers such as proteins, this convenient technique can produce large errors when used for the estimation of molecular weight of an unknown, especially in the absence of additional information such as that derived from centrifugation, viscosity, electrophoresis, and/or light-scattering studies.

RESOLUTION

General

The principal goal of any chromatographic analysis is the resolution of sample components. It is therefore critical that one have quantitative techniques to measure resolution as well as an understanding of the factors that contribute to it. One of the simplest and most widely used expressions for the calculation of resolution (R_s) is the expression:

$$R_s = \frac{2(V_{R2} - V_{R1})}{w_2 + w_1} \quad (2)$$

where V_{R2} and V_{R1} are the respective elution volumes of two chromatographic peaks and w represents the width of each peak in volume. This expression requires no knowledge of the nature of the solutes or the chromatographic system. It is the broad nature of this equation that makes it less useful in SEC systems. In SEC, it is more desirable to express resolution in terms of molecular size or molecular weight. One such expression of resolution is the equation of Bly [10]:

$$R_{SP} = R_s \left[\frac{1}{\log(M_1/M_2)} \right] \quad (3)$$

in which R_{SP} is defined as the specific resolution factor and M_1/M_2 is the molecular weight ratio of two standards. A column has an R_{SP} of one when solutes a decade apart in molecular weight are resolved with a resolution of one. In view of the fact that plate height varies with K_D , general R_{SP} values for a column may not be able to predict accurately the resolution of close-lying protein solute pairs at different K_D values from 0.2 to 0.8.

Resolving Power

The most desirable column for SEC is that which produces the minimum molecular weight difference between adjacent chromatographic peaks. This

minimum molecular weight difference, expressed as the ratio M_1/M_2 , may be referred to as the resolving power of the column [11]. The minimum molecular weight ratio (R_M), where $R_M = M_1/M_2$, can be defined as a special case of Eq. (3). If two chromatographic peaks are immediately adjacent, their resolution will be unity and the ratio of their molecular weights defined as R_M . Under these circumstances, Eq. (3) reduces to

$$R_{SP} = \frac{1}{\log R_M} \quad (4)$$

If it is assumed that with unit resolution, two adjacent solute peaks have roughly the same peak width (w) and the peak maxima are separated by ΔV_R , substitution into Eq. (1) gives an expression that includes the difference in the distribution coefficients of these two solutes,

$$V_{R2} - V_{R1} = V_i \Delta K_D \quad (5)$$

With these assumptions, the basic plate height equation yields:

$$\Delta V_R = w = \frac{4V_{R1}}{N^{1/2}} \quad (6)$$

A combination of Eqs. (5) and (6) yields a relationship between K_D and the basic properties of the column, theoretical plates (N) and internal volume (V_i):

$$\Delta K_D = \frac{4V_{R1}}{V_i N^{1/2}} \quad (7)$$

In order to define the resolving power of a column in terms of molecular weight, there must be an equation that relates ΔK_D and R_M . This can be calculated by an equation defining the linear portion of the SEC calibration curve:

$$\log M = b - mK_D \quad (8)$$

The slope of this line would be:

$$m = \frac{\log M_2 - \log M_1}{K_{D2} - K_{D1}} \quad (9)$$

Because $R_M = \log M_1 - \log M_2$, it may be shown that

$$m \Delta K_D = \log R_M \quad (10)$$

Combining Eqs. (7) and (10) yields a basic resolution equation for SEC systems:

$$\log R_M = \frac{4mV_{R1}}{V_i N^{1/2}} \quad (11)$$

This simple expression relates the resolution of a column to the molecular weight ratio of adjacent peaks and the basic parameters of the column, N and V_i . This

equation may be converted into terms of column length (L) and porosity (ϕ), where $N = L/H$ and $\phi = V_i/V_o$ to show their effects on R_M :

$$\log R_M = \left(\frac{4m[K_D(\phi - 1) + 1]}{\phi} \right) \left(\frac{H}{L} \right)^{1/2} \quad (12)$$

Peak Capacity

Resolution may also be expressed in terms of the maximum number of peaks that may be fit into an SEC chromatogram with baseline resolution of components. This peak capacity (PC) concept was proposed by Giddings [12] when he showed that

$$PC = 1 + \frac{\sqrt{N}}{4} \ln(\phi + 1) \quad (13)$$

Table 2 list peak capacities for several values of porosity and theoretical plates. Generally, silica-based supports possess pore volumes of about 1 and only gel columns, which yield lower efficiencies, have very high pore volumes.

Increasing the number of theoretical plates by coupling columns or by recycling can improve the peak capacity, but it is also expensive and slow. Peak capacities of SEC columns will usually be three to five times lower than those in other modes of liquid chromatography.

The preceding equations identify three independent factors that contribute to SEC resolution: (1) the number of theoretical plates (N); (2) the difference in the distribution coefficients of adjacent peaks (ΔK_D), which is related to calibration curve slope (m); and (3) the phase ratio or porosity (V_i/V_o or ϕ). All of the aforementioned retention models are based on the fact that the ratio of molecular radius to pore radius determines retention. If the support in a column has a wide pore distribution, with a variety of pore diameters, the difference between the molecular weights separated with a resolution of one will increase,

TABLE 2 Peak Capacities
Based on Pore Volumes and
Efficiency

Porosity	Theoretical plates	
	12,000	3000
1.0	19	10
1.5	26	13
3.0		19

resulting in an increase in the slope and, therefore, in the R_M . The net effect is that the fractionation range is increased at the expense of resolution.

Porosity

When a column is well packed with uniformly sized spherical porous particles, the interstitial volume, or volume between the particles, is 37–39% of the total volume of the column. Interstitial volumes lower than this are unusual and result from the presence of fines that partially fill the void spaces, physical distortion [13], fragmentation of the support during packing, or the presence of an exterior organic layer on the support that swells in the mobile phase. Void volumes that are as low as 34% of the column volume have been observed by Knox and McLennan [14]. When discussing the phase ratio or porosity (V_i/V_o) of an SEC column, it will be assumed that the void volume (V_o) is a constant for all spherical supports ($V_o = 0.38V_C$) where V_C is the volume of the column. Consequently, differences in V_i/V_o observed when comparing supports are a result of variations in the internal or pore volume (V_i). The remaining volume of a column is that occupied by the support itself (V_S), which is usually 15–40% of the column volume. If it is assumed that V_o is a relatively constant 38% of V_C , V_i/V_o will range from 0.5 to a limit of approximately 1.5. The contribution of V_i/V_o to R_M for different values of theoretical plates (N) and calibration curve slope (m) is seen in Fig. 6.

It would appear that a sound strategy for increasing resolution in SEC would be to find supports with very high pore volumes. There are, however, physical limits on the pore volume of an HPSEC support. The volume of a column (V_C) is composed of interstitial space (V_o), pore volume (V_i), and the volume of the support itself (V_S). Table 3 shows that in a column having $V_o = 38\%V_C$, the fractional amount of solid material in the support decreases as the V_i/V_o increases, to a limit of 1.63 where $V_S = 0\%V_C$.

Support particles must be composed of at least 10% solid material to give the permeation matrix mechanical stability. It may be calculated from Eq. (12) and Table 3 that in going from $\phi = 1.3$ with 13% of the support particle being solids to $\phi = 1.5$ with 5% solids, R_M will increase in only 4%. Weighing the gain in resolution against the probable increase in support fragility, it would be unwise to try to increase support pore volume above 85% of the volume of a support particle.

Packing density is also quite important in SEC resolution. It was noted before that with spherical particles, approximately 38% of the total column volume is interstitial space (V_o). In the case of irregularly shaped rigid particles, the void volume may constitute up to 47% of the column volume. Occasionally, a column has a void volume as low as 32%, which can be the result of particle deformation into the interstitial space [13], swelling of the particles into the

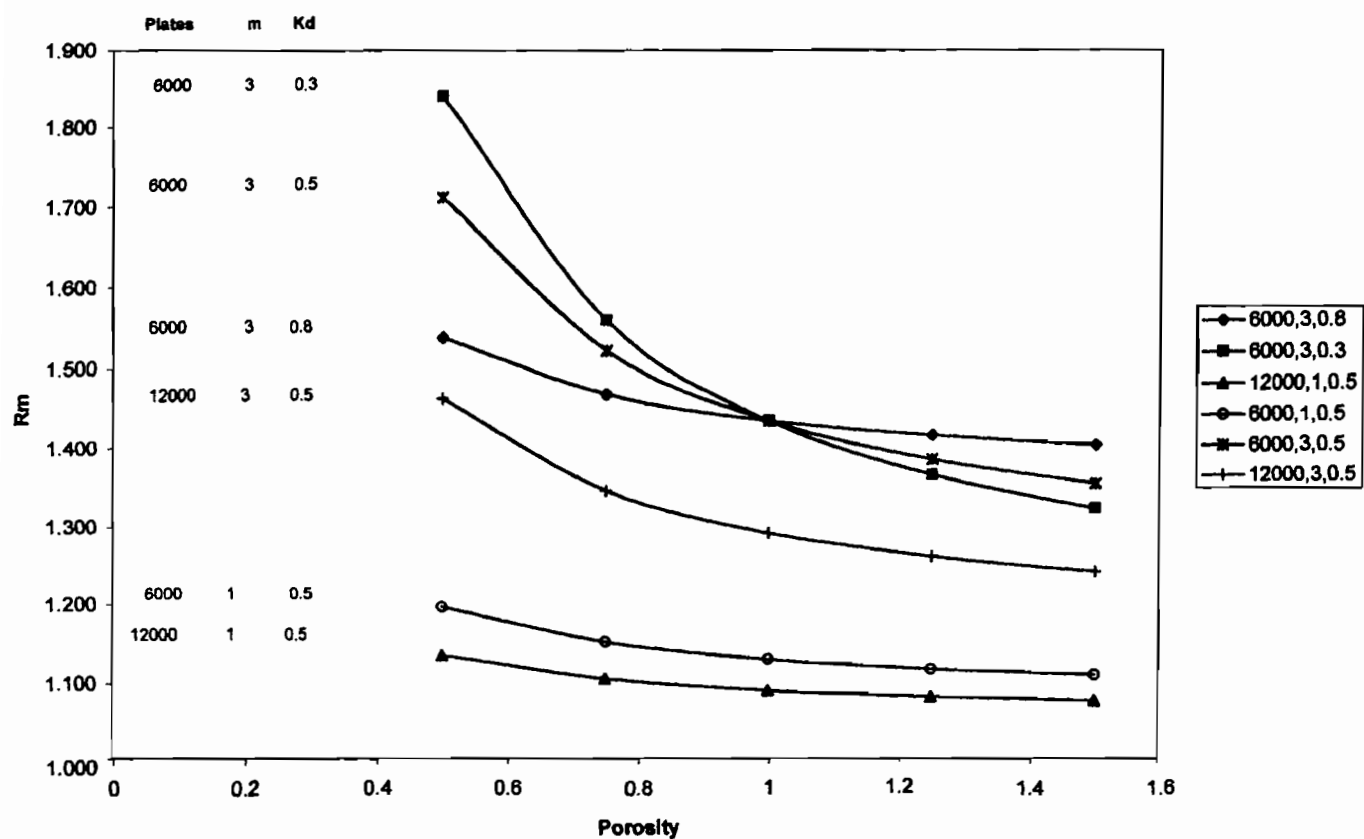


FIGURE 6 Effect of porosity on minimum molecular weight using Eq. (12).

TABLE 3 Volume Fractions of HPSEC Supports

Φ	% V_o	% V_i	% V_s
0.5	38	19	43
0.6	38	23	39
0.7	38	27	35
0.8	38	30	32
0.9	38	34	28
1	38	38	24
1.1	38	42	20
1.2	38	46	16
1.3	38	49	13
1.4	38	53	9
1.5	38	57	5

$$V_R = V_o + V_i + V_s.$$

interparticle voids, or extremely efficient packing. The influence of V_o on resolution may be examined theoretically by assuming a ratio of pore volume to solids (V_i/V_s) of 3, a column with 12,000 plates for a protein, and a solute K_D of 0.5. With these values, it can be calculated that spherical supports inherently have 19% more resolution than irregular particles of identical V_i/V_s ratio, specific pore volume, specific surface area, and pore diameter. This advantage is due to the higher packing density. In similar fashion, deforming or swelling the particles into the interstitial space to decrease V_o from 38 to 32% of V_c will increase resolution by about 13%.

Band Spreading

By definition, plate height (H) may be viewed as the rate of change in chromatographic peak variance (in units of length) relative to the distance migrated. This simply recognizes that band spreading is a fundamental property of flow through porous media and is not connected to any retention model. Based primarily on the work of Giddings [15], various factors that contribute to band spreading have been identified. In the case of SEC:

$$H_{SEC} = \frac{C_{SM}d_p^2u}{D_M} + K_D \quad (14)$$

The major cause of band spreading in the SEC of proteins is mass transfer (C_{SM}) of solutes between the mobile phase and liquid in the support pores [7,15,16]. Band spreading is also dependent on the particle diameter of the support (d_p), the

mobile phase velocity (u), the diffusion coefficient of the solute (D_M), and K_D . To understand the band-spreading process more fully, a systematic evaluation of the variables will be undertaken next.

Mobile Phase Velocity

It is apparent that as mobile phase velocity (u) is increased, macromolecules will experience mass transfer limitations, producing increased plate height and decreased resolving power of the column. This phenomenon has been examined experimentally in several cases with proteins [11,17]. The influence of mobile phase velocity on band spreading for a series of proteins is seen in Fig. 7. As expected, the effect of velocity increases with the molecular weight of the solute.

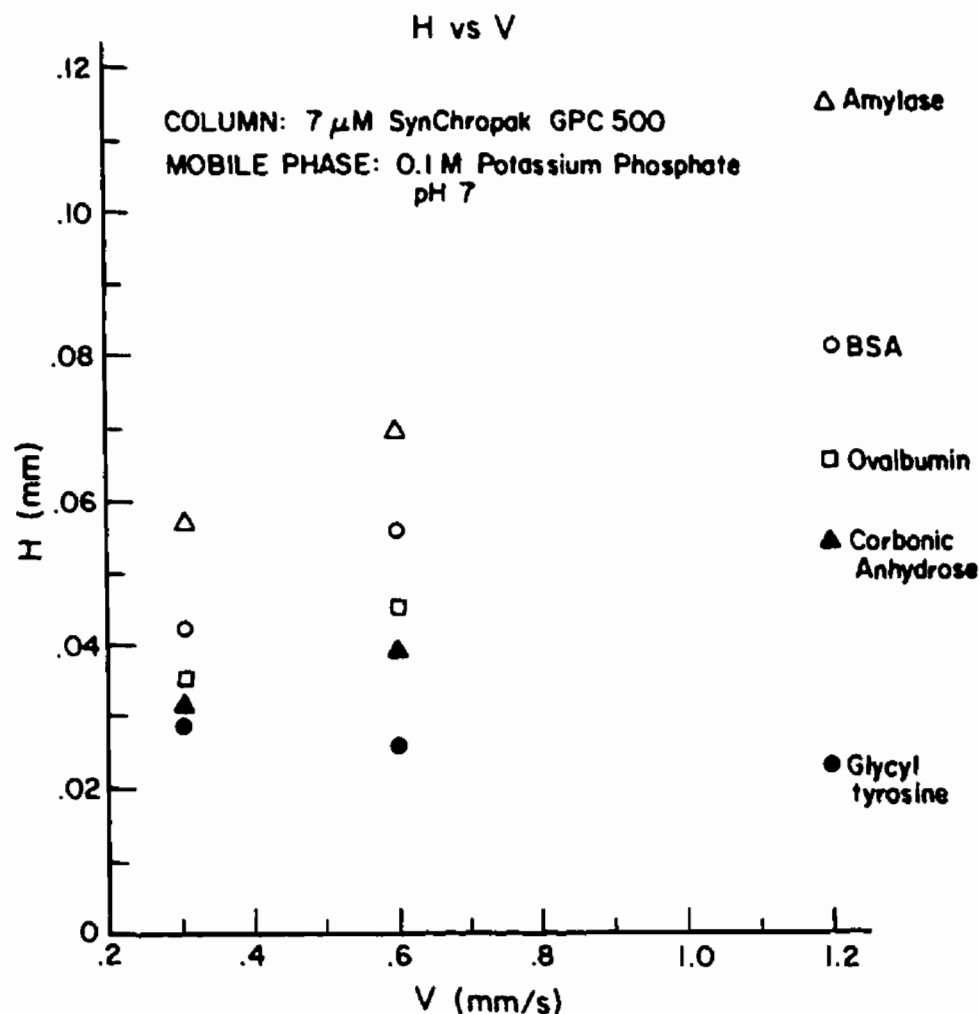


FIGURE 7 Effect of mobile phase velocity on band spreading for solutes with different molecular weights.

The relationship will be examined subsequently in the discussion of diffusion coefficient.

Obstructive Factor

Band spreading is also related to an obstructive factor that is not a constant in a column bed. Both Kubin [6] and Pfannkoch et al. [11] have shown that plate height varies as a function of K_D . It appears that as its molecular size approaches the pore dimensions, a solute experiences diffusion limitations, decreasing its effective diffusion coefficient. This influence of restricted molecular movement on plate height (H) can be readily observed in SEC profiles. The first peak to elute after the void volume marker is frequently the broadest peak in the chromatogram. If one were able to obtain columns with different pore diameters but similar plate counts, pore volumes, and calibration curve slopes, it would be best to select a column on which K_D would be 0.2 or greater for the solutes to minimize the effect of the obstructive factor.

Particle Diameter

As expected, plate height decreases with the particle diameter (d_p). There are, however, practical limitations on how small particles may be to continue to function well in HPSEC columns. These limitations are partially based on the ability to pack very small particles well and on current HPLC hardware technology. In addition, the maximum size of the pores is limited by the diameters of the particles; e.g., 1000 Å pores (0.1 µm) could be found only in particles with diameters large enough to accommodate them.

Diffusion Coefficient

The contributions to plate height of particle diameter, obstructive factor, and linear velocity are either inherent in a column or arise from the way it is operated. There is another set of variables, however, that arise from the sample itself. The contribution of these variables to band broadening is through the diffusion coefficient (D_M). The Einstein equation:

$$D_o = \frac{7.4 \times 10^{-8} T(XM)^{1/2}}{\eta(V_b)0.6} \quad (15)$$

shows that at high dilution, the solute diffusion coefficient (D_o) is a function of temperature (T), the association constant (X) and molecular weight (M) of the solvent, the viscosity (η) of the solution, and the molar volume (V_b) of the solute. Substitution of this equation into Eq. (14) shows that plate height (H) will

decrease as the operational temperature is increased, It is therefore desirable to run SEC columns at room temperature rather than at 4°C whenever possible. This is generally feasible because separation times in HPSEC are short, and thus protein stability is a minimal problem. It was noted earlier that D_0 was the diffusion coefficient observed at high dilution, a condition that is not often found in chromatography columns. Because the diffusion coefficient of a solute may be influenced by solution viscosity (η), its own concentration, or the concentration of other molecular species, the diffusion coefficient in the actual environment of a column is represented as D_M . The inverse relationship of the diffusion coefficient to the molar volume of a solute (V_b) implies that as molecules become more asymmetrical, their diffusion coefficients will become smaller, producing greater bandwidths. Viscosity also increases with molecular asymmetry and it is inversely proportional to concentration.

When all of the variables that contribute to the diffusion coefficient are examined in unison, it is seen that temperature, molecular shape, solute concentration, molecular weight, molecular hydration, intrinsic viscosity of the sample, and mobile phase viscosity all play a role in the ability of a solute to diffuse readily into the pores of a support.

SUPPORTS

General

In view of the ionic and hydrophobic character of proteins, it is important that a support matrix for SEC be neutral and hydrophilic to prevent non-size exclusion partitioning between the support material and the proteins. Classically, SEC separations of proteins have been achieved on gel supports composed of cross-linked polysaccharides or polyacrylamides [1,18,19]. The hydrophilic nature of the classical gel supports causes them to imbibe large quantities of water and to swell to many times their dry volume. Through this swelling process, the pore structure is also expanded and the supports become macroporous. Any manipulation of the support, either chemical or mechanical, that alters support particle shape and volume will also change pore volume and particle porosity, affecting SEC due to the relationship between particle volume, pore volume, and pore diameter. Classical hydrophilic gel matrices have very poor mechanical stability under pressure and cannot be effectively used for HPSEC, although more rigid agarose supports with smaller particle diameters have been developed by Hjerten et al. [13] and others.

Based on the observations in this chapter about resolution and band spreading in SEC and on the experiences of protein chemists over the past decades with protein-matrix interactions, the “ideal” HPSEC support should be:

1. Rigid and capable of withstanding pressures of at least 100 atm
2. Macroporous to allow the penetration of macromolecules
3. Totally hydrophilic to prevent solvophobic partitioning
4. Nonionic to prevent ion-exchange partitioning
5. Microparticulate and spherical to minimize mass transfer limitations with macromolecules and to increase resolution
6. Of uniform pore diameter and geometry
7. Of high pore volume
8. Easy to pack
9. Chemically stable over a broad pH range during elution with thousands of column volumes of mobile phase
10. Nonbiodegradable
11. Inexpensive

Although there is no single support that can meet all of these criteria, most commercially available supports for HPSEC try to encompass many of these requirements. There are two major types of HPSEC supports: surface-modified, controlled-porosity inorganic materials and rigid controlled-porosity organic packings. Both categories were initially developed in the early to mid-1970s. In 1974, Regnier designed a glycerylpropylsilane bonded phase on 37 to 74- μm controlled-porosity glass that could be used for SEC of proteins [20,21]. The origin of rigid organic SEC supports for protein analysis is slightly more difficult to trace. Soft gels composed of agarose, dextran, or other carbohydrate matrices have been the state of the art for protein purification since the 1960s [1,18,19]. In 1973, Coupek and his coworkers [22] reported a macroreticular, hydrophilic glycomethacrylate gel that they named "Spheron." They applied Spheron to the separation of proteins by size [23], but Strop and coworkers [24] later showed that hydrophobic interactions with the methacrylate matrix were principally responsible for the separation. Hjerten et al. [13] developed agarose gels that allow SEC under moderate pressures.

Inorganic Packings

The rationale used in developing inorganic bonded phase supports for SEC is based on the following facts:

1. Inorganic supports that range in pore diameter from 20 to 10,000 Å are available.
2. Porous inorganic materials can withstand pressures greater than 300 atm.
3. Silylation reactions at the surface of inorganic supports sequester hydroxyl groups on the support and render them neutral.

4. Using a variety of organic reactions, organosilanes may be used in surface silylation reactions to attach hydrophilic groups to the support.

Through a variety of different techniques, it is possible to obtain inorganic supports composed of silica, glass, alumina, titania, and ceramics in many pore sizes. Although these materials are macroporous, they are of limited value in SEC when used without modification because their ionic character causes them to behave as weak ion exchangers. In addition, many inorganic supports are unsuitable for HPSEC because of excessive variation in pore distribution, low pore volume, or incompatible surface chemistry.

In an ideal SEC system, there is no interaction between solutes and the support matrix, yielding retention strictly on the basis of hydrodynamic volume. Because native inorganic supports usually adsorb proteins and peptides, it is necessary to understand the chemistry of the surface before it can be controlled effectively. Most inorganic SEC supports such as controlled-porosity glass and silica are essentially pure silica that may contain low levels of metal impurities. The most important surface characteristic of siliceous supports is that they behave as weak acids and therefore are cation exchangers. This behavior is usually attributed to the ionization of surface silanol groups. The structure of silica surfaces is complex and they have no single or sharp pK_a due to the ionization of surface silanols. Rather, ionization begins at pH 2 and continues until at least pH 9. An in-depth discussion of inorganic supports can be found in Chapter 1.

Achievement of SEC separations on native inorganic supports requires either surface passivation or the analysis of solutes that do not interact with silanols. Unfortunately, this restriction eliminates most biological molecules. Proteins with high pI values are especially vulnerable to adsorption. Surface passivation by mobile phase manipulation using high-ionic-strength buffers, low pH, or the addition of sodium dodecyl sulfate, polyethylene oxide, or polyethyleneimines has been generally unsuccessful in solving the problem of nonspecific adsorption of proteins to inorganic surfaces. The poor protein recoveries and destruction of enzyme activity observed with uncoated inorganic supports produced an initial reluctance in biochemists to using deactivated silica-based supports. The advances in surface modification of improved silica supports in the past two decades have resulted in some excellent HPSEC columns.

Because the surface silanols on native silica are responsible for the adsorption and denaturation of proteins and enzymes, these silanols must be substantially eliminated. The most effective way of achieving this is by chemical modification through reaction of a hydrophilic neutral molecule with the surface silanols. The first successful surface-modified inorganic SEC support for proteins was made by Regnier when he bonded glycerylpropylsilane to controlled-porosity glass using the commercially available γ -glycidoxypropyltrimethoxysilane [20,21]. Without filling the pores of the support, this neutral, hydrophilic

silane coating substantially decreased the nonspecific adsorption of proteins to the silaceous surface. The support materials were capable of withstanding pressures greater than 300 atm and allowed the separation of proteins by SEC in less than 30 min. The Glycophase G supports manufactured by Corning Glass Works in 1974 were the result of this research [25]. In 1978, SynChrom introduced a similar product, SynChropak GPC, on controlled-porosity silica [26]. In 1978, Toyo Soda presented the TSK SW series of supports, which are also based on silica and deactivated by a proprietary process [27]. The ZORBAX GF series of supports also possess a diol bonded phase on silica that has been stabilized with a zirconium salt before application of the bonded phase, substantially extending the column lifetime [28]. Although 100% coverage cannot be achieved with the silane reactions, modifications to the methodology have resulted in HPSEC supports with minimal exposed silanol groups and high enzymatic recoveries. Commercial HPSEC supports that are classified as "diol" are generally synthesized by a modification of this technique.

Engelhardt and Mather [29] synthesized a similar HPSEC support by reacting silica with amides that had been synthesized from γ -aminopropyltriethoxysilane. The resultant supports exhibited good SEC properties and protein recoveries. Because the organosilane reactions cited before are reversible, the coatings can slowly leach with use, especially under acidic conditions. It was observed that when a column of similarly derivatized silica was placed before an analytical column, the bonded phase would actually transfer and extend the lifetime [30].

One method of extending the column lifetime of SEC supports is to use polymeric bonded phases. Regnier and Chang synthesized a polymeric layer from a glycerylpropyl bonded phase cross-linked with a multifunctional oxirane using boron trifluoride [31]. Komiya et al. [32] of Toyo Soda also described the synthesis of a polyglycerol coating through a similar reaction. Another hydrophilic polymeric coating was made by Gupta and Regnier [33] in a process in which they coupled a polyamine to the silica surface through an organosilane coupling agent. The positive charge on the coating was neutralized by exhaustive acetylation. The remaining silanols and tertiary amines did not cause ionic partitioning during operation under HPSEC conditions. In a further refinement by Berek and coworkers [34], dextran was grafted to silica that had been modified with γ -glycidoxypropyltrimethoxysilane. The resultant support, which had a slight positive charge, showed minimal hydrophobic or ionic characteristics.

Organic Packings

Families of chromatography packings often have general limitations. For inorganic supports, the inability to operate above pH 8, the leaching of organic bonded phases, and residual surface silanols are general problems that dominate

synthetic and operational procedures. The totally organic supports have a different set of restrictions. Although it is relatively easy to obtain organic matrices that are neutral and hydrophilic, most materials with these properties also have poor mechanical strength. Rigidity may be improved by increasing the degree of cross-linking, but this also adds hydrophobicity and decreases the pore volume. Agarose matrices such as highly cross-linked Superose have been used as HPSEC supports with success [13].

Coupek and coworkers [22] prepared macroreticular, hydrophilic glyco-methacrylate gels by copolymerization of hydroxyethyl methacrylate and ethylene dimethacrylate. These materials are capable of withstanding pressures in excess of 100 atm and can be eluted with aqueous and organic solvents. Because of the relatively high content of aliphatic residues in these packings, some proteins were adsorbed hydrophobically [23,24]. Another set of rigid organic supports for SEC of proteins are the TSK PW gels that Fukano et al. [27] of Toyo Soda described in 1978. The semirigid agarose-based Superose has more recently been developed for HPSEC using low to moderate flow rates [13]. Amersham-Pharmacia has also developed Superdex, which has peptide applications [35]. Shodex and Toyo Soda manufacture polymeric HPSEC supports of proprietary designs. Many polymeric supports are more hydrophobic than those that are silica based; however, they are also more resistant to basic conditions. An in-depth discussion of organic supports may be found in Chapter 2.

METHODOLOGY

General Observations

Based on the number of papers reporting the use of SEC, it may be assumed that it is the most widely used chromatographic technique in biochemistry. There are several factors that make this fact surprising:

1. SEC is a low-resolution technique, often yielding only six to eight peaks and requiring at least a 1.5 to 2-fold difference in molecular weight for resolution.
2. Due to its basis on hydrodynamic volume, SEC is of questionable utility in molecular weight estimation of new and uncharacterized biopolymers.
3. Nonideal SEC behavior is common with proteins and difficult to recognize.
4. Sample capacity in SEC is low.

The explanation for this apparent paradox is simple: no other type of chromatography is as simple and allows as broad a range of mobile phases as SEC. Frequently, mobile phases may be altered to fit the unique requirements of a

protein without influencing the chromatographic characteristics. The utility of SEC in changing the buffer environment of a protein or in desalting is well known. In addition, results may often be obtained without complete understanding of either the physical characteristics of a particular protein or even the chromatographic methods. No other chromatographic technique for separating proteins has the versatility and speed of SEC. Given these attributes, the inaccuracy of the method for molecular weight determinations is an acceptable limitation. Most of the applications chapters in this book describe specific methodologies for separations of various types of biopolymers by SEC.

Mobile Phase

In SEC, the mobile phase has several important functions. Most important, it must totally solubilize all of the sample components or they will not elute purely by size. Secondly, the mobile phase must mask any hydrophobic or ionic characteristics of the support or the solute. For proteins, this generally requires a buffer at a pH near neutrality with salt concentration of 0.05–0.2 M; addition of a small percentage of organic solvent may also be necessary [36]. The specific composition of the ideal mobile phase varies with both the column and the analytes. The mobile phase may also affect the size or hydrodynamic volume of the solutes. Ionic polymers such as sodium poly(styrene)sulfonate (SDS) compounds expand at low ionic strength and contract at high [37]. In denaturants, proteins can expand into linear configurations such that proteins were denatured in 6 M guanidinium hydrochloride in a uniform manner, producing a linear calibration curve offset from that of the proteins in their native state. On the other hand, 0.1% sodium dodecyl sulfate (SDS) did not yield consistent effects on hydrodynamic volume, especially for proteins smaller than 10,000 Da [38].

Peptide mixtures are very susceptible to interaction with SEC supports and their general heterogeneity makes it difficult to optimize the mobile phase [35,39]. Each class of peptide (acidic, basic, hydrophobic, and neutral) was observed to react differently to changes in pH, ionic strength, or addition of organic solvent when they were run on a Superdex Peptide column [35]. It was determined that acidic peptides were best run at high pH and required addition of 30% acetonitrile at low pH, whereas basic peptides had the best peak shapes at low pH.

In a study of antibody analysis by SEC, it was observed that the optimum pH and ionic strength for good peak shape and noninteraction differed between antibodies [40]. This again emphasized the necessity of mobile phase optimization for attainment of ideal SEC.

Another effect of mobile phase is the general one of degradation of the column. For silica-based supports, high pH is generally harmful. In addition, certain salts, notably phosphate, have been shown to hasten column degradation

[41,42]. For the analysis of proteins modified with polyethylene glycol (PEG), it was seen that the addition of 10% ethanol to a sodium chloride/phosphate mobile phase improved column performance and longevity [42].

Column Evaluation

General

A discussion of column evaluation may seem inappropriate to biochemists who have traditionally packed gel SEC columns themselves and run them without testing. Such columns would eventually be unpacked when the performance degraded, and the packing material would be cleaned or discarded. The quality of the column was not significant in relationship to the time necessary to evaluate it. HPSEC columns are usually more expensive than their classical counterparts, and consequently, users are concerned about quality assurance, column longevity, sample recovery, and extension of column lifetime. Because there are major differences in columns from different manufacturers, users must have a qualitative basis for column comparison. Columns should also be evaluated periodically during their use so that loss of efficiency can be monitored and columns cleaned, when necessary, to restore resolution.

Three types of column evaluations should be implemented:

1. In-depth evaluation of a column that is totally new to the laboratory
2. Quality control testing on a new lot of a previously used column type
3. Routine efficiency testing to determine whether any column or instrument components need maintenance or replacement

New Columns

The void volume (V_o) pore volume (V_i), elution volume (V_R), and slope of the molecular weight calibration curve (m) should remain constant in a column that does not develop mobile phase channeling or bonded phase degradation. To give results identical to those obtained with a previous column, a replacement column must have identical values of these characteristic constants. Because it is often impossible to obtain identical columns, especially over a long period of time, it is always best to determine these parameters for every new column and to make operational condition adjustments when necessary.

In the course of preparing the SEC calibration curve, It is necessary to establish the V_o , V_i , and V_R for a series of compounds. It is important to choose solutes that are totally excluded for the V_o and ones that are totally included and have no interaction with the support for the total volume (V_M). DNA is a good molecule for determination of exclusion limits in aqueous systems near neutral pH. Blue dextran may be suitable, but it should be used with caution because it may bind irreversibly to certain columns. A hydrophilic small molecule such as

glycyltyrosine can be used to quantitate the total volume ($V_o + V_i$). If the resolution of a new column is not identical to that of an old column, it is possible to isolate the problem by comparing values of calibration curve slope, V_o , V_i , and theoretical plates for one or more compounds. A larger absolute value for the slope indicates an increase in pore distribution. Loss of pore volume (V_i) could be caused by an increased bonded phase layer on the support or by a difference in the support matrix itself. Lower plates reflect the quality of the packing of the column or a larger particle diameter.

Daily Testing

It is strongly recommended that a standard test sample of proteins or molecules similar to the analytes be run daily on a routinely operating HPSEC column. Even a simple visual comparison enables the analyst to monitor the resolution of the column, any decline in the sensitivity of the instrument, development of baseline noise, problems with an inlet valve, or the deterioration of the test mixture.

Daily performance testing may be implemented in one or a combination of four techniques:

1. Theoretical plate determination for a small molecule
2. Theoretical plate determination for a macromolecule
3. Determination of the resolution between two macromolecules
4. Determination of the minimum molecular weight resolution (R_M)

Pfannkoch et al. [11] examined the utility of a number of small molecules as test probes for HPSEC using the requirement that a molecule must have minimal interaction with the support. The dipeptide glycyltyrosine functioned adequately when aqueous mobile phases with neutral pH were used. An inexpensive and stable protein that is available in high purity, such as ovalbumin, is generally well suited for macromolecular plate height determinations. It should be noted that plate height is also a function of operational parameters such as mobile phase velocity, viscosity, and temperature; therefore, care must be exercised to perform evaluations under identical conditions. The simple evaluations described earlier will not yield any information about pore volume or slope of the calibration curve.

A useful technique for routinely evaluating resolution is to use a mixture of two proteins that vary two- to threefold in molecular weight. Determination of the elution volumes or retention times and peak widths will allow calculation of resolution by means of the standard equation:

$$R_S = \frac{2(t_{R2} - t_{R1})}{w_2 + w_1} \quad (16)$$

Any two proteins may be used, but it is best if they both fall in the linear portion of the calibration curve and have K_D values of 0.2–0.8. The resolution declines

sharply outside this region because of the nonlinearity of the calibration curve near the extremes.

Minimum molecular weight resolution is slightly more difficult to calculate than resolution and it may be of no greater value in daily testing. Nonetheless, it should be standard practice to determine the void volume, pore volume, and calibration curve for any size exclusion column that is used in the laboratory. Thereafter, the slope (m) can be calculated and the minimum molecular weight resolution easily computed.

Many laboratories have found that the plate height (H) of an HPSEC column can be strongly dependent on the instrument on which it is operating. Extra column effects from the valve, detector, or connecting tubing can easily cause a 50% decline in plates. The problem is especially acute when using 250×4.6 mm ID or microbore columns where elution volumes are small. One must be particularly sensitive to instrumental degradation of resolution in HPSEC because the technique is more susceptible to dead volume problems than modes of chromatography that involve binding to the supports.

Loading Characteristics

In SEC, peak widths are related to solute diffusion coefficients; therefore, band broadening will occur if mass or volumetric changes affect the viscosity or diffusion characteristics [43]. Figure 8 illustrates the effect of both volume and mass on the resolution of BSA and myoglobin using a 305×7.5 mm ID column [43]. It can be seen that the column tolerated volumes of 150–200 μL and masses of 700 μg before resolution began to deteriorate substantially. This volume is approximately 2% of the column volume. For smaller columns (250×4.6 mm ID), the maximum volume and maximum mass will be lower in proportion to the column volume.

NONIDEAL SEC

According to Eq. (1), it would be expected that all small molecules not associating with the surface of an SEC packing would have a K_D of 1. Any K_D values greater than 1 would indicate non-size exclusion partitioning. When a low-ionic-strength mobile phase is used, it would be expected that ionic interactions between the support and the solute would be enhanced and hydrophobic interactions would be minimized. On the other hand, high-ionic-strength mobile phases would strengthen hydrophobic interactions and minimize ionic partitioning. When Pfannkoch et al. [11] made an extensive study of such partitioning on HPSEC columns from several manufacturers, they found that cationic effects could be tested by arginine or lysine and anionic effects by glutamic, oxalic, or

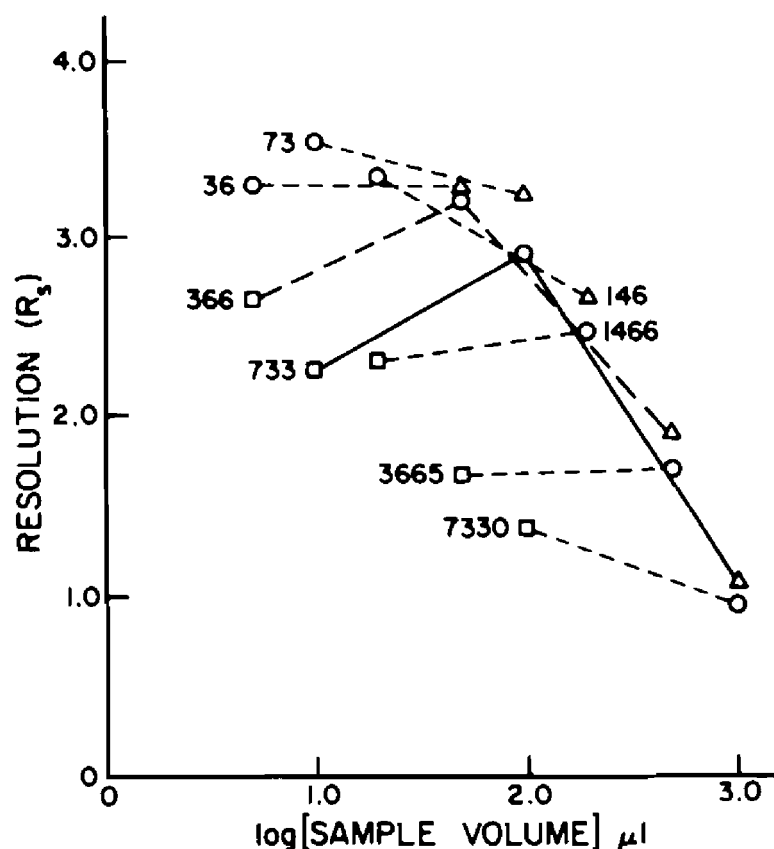


FIGURE 8 Effect of sample size on resolution of the bovine serum albumin–myoglobin pair. Sample concentration: (Δ) 0.73 mg/mL; ($\circ$) 7.33 mg/mL; ($\square$) 73.3 mg/mL. Column: TSK G3000SW. Flow rate: 0.5 mL/min. (From Ref. 43.)

citric acid. Retention of water-soluble neutral molecules, such as benzyl alcohol or phenyl ethanol, predicted hydrophobic partitioning.

Whenever the K_D of a cationic molecule is greater than 1, an HPSEC support is acting as a weak cation exchanger. Table 4 shows this phenomenon for arginine on several columns [11]. Ion exclusion, a process in which negatively charged solutes are repulsed or excluded by negative silanols or functional groups within the pores of a support, is indicated when the K_D of a small anionic molecule is less than 1. Table 5 illustrates this effect for citrate [11]. Hydrophobic interactions, which are enhanced by high salt concentrations, are illustrated in Table 6 for phenylethanol [11]. In this case, phenylethanol interacted with all the supports to some extent and never eluted at or before the total volume (V_M). Glycyltyrosine showed little deviation under most conditions on many of the columns and was thus chosen as a good probe for V_M in SEC. It can be seen that each brand of column used in this study had different problems related to nonideal SEC behavior. None was or is perfect. All of the columns exhibited some anionic characteristics that were manifested by ion exclusion of anionic molecules or adsorption of cationic solutes. All of the columns also exhibited hydrophobic partitioning under certain conditions. These facts show that it is

TABLE 4 K_D of Arginine as a Function of Mobile Phase Ionic Strength

Column	Mobile phase ionic strength ^a					
	0.026	0.12	0.24	0.60	1.20	2.40
TSK SW 3000	1.30	1.05	1.02	1.00	—	0.98
SynChropak GPC 100	1.35	1.06	1.01	—	—	0.98
LiChrosorb Diol	1.53	1.15	1.05	0.99	—	1.07
TSK SW 2000	1.57	1.06	1.02	0.99	—	0.98
Waters I-125	1.70	1.23	1.16	1.08	—	1.05
Waters Bondagel	1.75	1.11	1.06	1.02	—	1.00
Shodex OH pak B-804	2.06	1.16	1.07	1.02	1.02	—

^aThe mobile phase consisted of pH 7.05 phosphate buffer of the ionic strengths indicated above.

Source: Ref. 11.

imperative to choose mobile phase conditions that are best suited to one's specific analysis and minimize interactions between the support and solute. It can be seen that certain guidelines are generally true for choosing mobile phases that minimize non-SEC effects. Mobile phases with ionic strengths over 0.6 M often enhance hydrophobic interactions and those below 0.1 M frequently encourage ionic effects.

The nonideal effects seen before for small molecules are much greater for proteins or other biopolymers that have multiple ionic and hydrophobic moieties

TABLE 5 K_D of Citrate as a Function of Mobile Phase Ionic Strength

Column	Mobile phase ionic strength ^a			
	0.026	0.12	0.24	2.40
Shodex OH pak B-804	0.83	0.88	0.91	0.94
TSK SW 3000	0.66	0.89	0.92	0.94
LiChrosorb Diol	0.54	0.81	0.95	0.99
SynChropak GPC 100	0.46	0.76	0.89	0.91
TSK SW 2000	0.43	0.75	0.84	0.88
Waters I-125	0.39	0.72	0.79	0.88
Waters Bondagel	0.39	0.73	0.80	0.88

^aThe mobile phase consisted of pH 7.05 phosphate buffer of the ionic strengths indicated above.

Source: Ref. 11.

TABLE 6 K_D of Phenylethanol as a Function of Mobile Phase Ionic Strength

Column	Mobile phase ionic strength ^a					
	0.026	0.12	0.24	0.60	1.20	2.40
SynChropak GPC 100	1.44	1.49	1.53	1.63	1.81	2.33
TSK SW 3000	1.47	1.50	1.53	1.61	1.81	2.35
Waters I-125	1.83	1.88	1.88	2.03	2.29	3.03
TSK SW 2000	1.95	2.02	2.10	2.30	2.71	4.01
LiChrosorb Diol	2.49	2.56	2.64	2.93	3.52	5.31
Waters Bondagel	5.32	5.19	5.37	5.97	7.44	11.47
Shodex OH pak B-804	6.36	6.65	6.96	8.47	10.96	—

^aThe mobile phase consisted of pH 7.05 phosphate buffer of the ionic strengths indicated above.

Source: Ref. 11.

in their structures. Mathes and Engelhardt [44] reported that at low ionic strength some proteins will be ion excluded whereas others will be adsorbed to SEC supports. Mathes and Engelhardt [44], Becker and Unger [45], Karger et al. [46], and Pfannkoch et al. [11] all demonstrated that there are electrostatic interactions between proteins and silica-based packings, that manipulation of ionic strength and pH can control ionic effects, and that mixtures can be resolved by exploiting these non-size exclusion effects. Peptides are especially liable to hydrophobic partitioning and mobile phases are always being sought to eliminate interactions. Mobile phases containing trifluoroacetic acid, with or without sodium chloride, have been used on HPSEC columns to differentiate peptides by molecular size and charge [47,48].

The slight hydrophobic character of organic size exclusion bonded phases may be used in a reversed-phase mode to retain proteins [44]. In a study where the concentration of trifluoroacetic acid (TFA) in the isocratic analysis of a peptide mixture on an SEC column was varied, the retention and the peak shapes of the components were affected differentially, yielding good resolution that was not by size [49].

It should be recognized that it is hard to know whether a column is operating exclusively in the SEC mode. The studies outlined previously can help one ascertain whether interactions are occurring. In addition, Becker and Unger [45] noted that SEC is a temperature-independent entropic process, whereas surface interactions are temperature-dependent enthalpic processes. Therefore, changes in retention that result from changes in temperature would indicate non-size exclusion, partitioning mechanisms.

APPLICATIONS TO PROTEIN ANALYSIS

SEC has proved to be a useful technique in the observation and control of protein folding and aggregation. Its mild conditions and short analysis times, along with compatibility with many different mobile phases, maximize its utility. Specific detection methods, such as second-derivative ultraviolet (UV) absorption spectroscopy [50] or laser light scattering [51] with low angle or multiangle methods, can identify aggregates or some conformational changes.

When the effect of the concentration of guanidinium hydrochloride (GuHCl) on protein refolding was observed for several growth hormones and prolactin using SEC, it was found that human growth hormone exhibited a refolding transition at a higher concentration of GuHCl than the other proteins [52]. In this type of study, the presence of aggregates and the extent of refolding can also be monitored.

The problems of inclusion bodies formed by recombinant proteins can also be addressed using SEC. SEC was used to perform buffer exchange from a solubilizing to a refolding solution [53]. During the process, unfolded lysozyme was gradually refolded as it traversed the column and its mobile phase environment changed to the refolding solution. This gradual solvent exchange process minimized protein aggregation.

Insoluble peptides produced from tryptic or cyanogen bromide-digested proteins were effectively analyzed by SEC with SDS in the mobile phase to separate families of peptides that could subsequently be analyzed by gel electrophoresis or another technique [54]. Complexes between heat shock proteins and unfolded substrate proteins have also been monitored successfully using SEC [55]. The effect of ATP and ADP on the complexation could be readily ascertained with this technique.

CONCLUSIONS

It is apparent from current literature and the preceding discussion that certain conclusions can be drawn about commercially available HPSEC supports:

1. All of the commercial HPSEC columns are weak cation exchangers.
2. All of the commercial HPSEC columns show some hydrophobicity.
3. The optimum mobile phase ionic strength for analysis of most solutes is 0.1–0.6 M.
4. Operating columns outside the 0.1–0.6 M range often results in nonideal SEC.
5. SEC resolution may be broken down into the contributing components of column plate count (N), pore volume (V_i), and calibration curve slope (m).

TROUBLESHOOTING

HPSEC columns allow the use of a wide variety of mobile phases and will tolerate most of the buffers and additives that have been used in classical columns. The major limitation is that silica-based columns should not be operated outside the pH range of 2–8. At basic pH values, silica slowly dissolves and the packing is destroyed. This is not frequently a restriction because the columns can be run with mobile phases containing additives such as 0.1% trifluoroacetic acid [47,48] or organic solvents to help solubilize samples or impurities that may have adsorbed on the column.

Column failure is often a function of column usage. All columns will eventually fail, but their longevity can be enhanced by protective measures such as the use of guard columns, filtration of samples and mobile phases [42,56], and routine cleaning measures according to the manufacturer's instructions. Because bacteria grow in most of the buffers used in HPSEC, columns should be stored for prolonged periods in something not conducive to bacterial growth, such as 10% isopropanol or 0.1% sodium azide in water. To prevent precipitation of proteins or salts on the columns, it is often best to avoid using 100% organic solvents after usage in buffers. If organic solvents are to be used after salts, the columns should first be washed extensively with water (at least 10 column volumes).

Blockage of column frits or channeling in a column bed will cause a loss of resolution. Any packing void will be a source of dead volume and thus of band spreading; this can be checked by carefully removing the front frit from the column. A void is indicative of the imminent end of the column's usefulness, especially in SEC, which is inherently dependent on peak widths and column efficiency.

TERMS

K_D	solute distribution coefficient
V_i	internal volume (within the pores); interstitial volume
V_o	void volume; excluded volume (outside the matrix)
V_M	total mobile phase volume in a column; dead volume
V_R	retention volume

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Ion-Exchange Chromatography

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INTRODUCTION

Ion-exchange chromatography has been used in the separation of ionic species for more than half a century. Early separations were achieved with either sulfonated or quaternized polystyrenedivinylbenzene (PSDVB). Unfortunately, these materials are often unsuitable for protein separations because the strong nonspecific adsorption between the hydrophobic sorbents and proteins necessitates the use of organic solvent or extremes in pH to achieve elution. Such conditions are sufficiently harsh to denature protein three-dimensional structure and, consequently, alter biological activity.

In 1958, Sober and Peterson [1] described the use of diethylaminoethyl (DEAE)-derivatized cellulose in the anion-exchange separation of proteins. This hydrophilic matrix overcame many of the problems of the PSDVB-based materials. Shortly thereafter, carboxymethyl-derivatized cellulose was introduced for the cation-exchange chromatography of proteins. Separations achieved using

cellulose-based ion-exchange sorbents had high protein recovery, good loading capacity, and outstanding chemical stability in the pH range 2–12. With the addition of dextran and agarose support matrices in the early 1960s, a wide variety of carbohydrate-based ion-exchange materials became available for protein separations. These materials have played a major role in the evolution of both separation science and modern biochemistry because most fully characterized proteins have been purified at some stage using ion-exchange chromatography.

Soft gel materials, which include most carbohydrate matrices, have the primary limitation of poor mechanical strength. This precludes their use at high mobile phase velocity and in small particle diameters, both of which substantially increase the performance of liquid chromatography columns. The significant advances in the 1970s of chromatographic separations in terms of speed and resolution required the development of more pressure-stable ion-exchange packing materials.

The first pressure-stable, high-resolution ion-exchange packing materials for proteins were described in 1976 [2]. Through the use of macroporous, controlled-porosity glass or silica of 5–10 μm particle diameter with a polymeric surface layer containing ion-exchanging functional groups, it was possible to separate proteins in less than 10 min with resolution superior to that of gel-type packing materials. In recent years, a variety of high-performance ion-exchange materials based on both silica and organic polymer supports have become available. With the broad availability of commercial columns, high-performance ion-exchange chromatography has become an analytical and preparative technique of major importance in both the biochemistry and biotechnology communities.

RETENTION

Ion-exchange (or electrostatic interaction) chromatography would seem to be a simple process in which interactions between charged functional groups on a solute and those on a sorbent are the basis for adsorption. Furthermore, the strength of electrostatic interactions should be mediated by either the ionic strength or the pH of the mobile phase. Although this simple model is applicable in a broad sense, it is now known that unique features of both the ion-exchange sorbent and the solute play strong roles in the electrostatic interaction retention mechanism.

Sorbent Contributions

One of the more perplexing features of ion-exchange chromatography is that two sorbents of the same charge can behave very differently. This points out the

substantial contribution that the physical properties of the sorbents—both the stationary phase and the matrix—can make in the ion-exchange process.

Stationary Phase

Functional Groups

Stationary phases for ion-exchange chromatography are generally referred to as either weak or strong. This unfortunate nomenclature gives the impression that weak ion-exchange sorbents adsorb proteins more weakly than strong ion-exchange materials. Actually, the terms weak and strong refer to the pK_a of the stationary phase groups, similar to the designations for acids and bases. Functional groups on strong ion-exchange materials are totally ionized across the complete operational pH range used in ion-exchange chromatography, whereas the stationary phase ligands on weak ion-exchange sorbents vary in ionization within the range. For example, sulfonic acids and quaternary amines are considered to be strong ion-exchange groups because their ionization does not vary appreciably from pH 1 to 14. In contrast, carboxyl groups and tertiary amines are said to be weak ion-exchanging functional groups because their ionization goes from zero to 100% across the whole pH range, as shown by titration curves. Functional groups used in ion-exchange chromatography of proteins and their operating ranges are shown in Table 1. It can be seen that strong ion-exchange sorbents should have a broader operating range than weak ion-exchange materials.

TABLE 1 Some Common Functional Groups Used in Ion Exchange Chromatography

Type of exchanger	Functional group	Operating	
		Type	Range
Strong anion	$\begin{array}{c} \backslash \\ -N^+ - CH_3 \\ / \end{array}$	Quaternary amine	1–11
Weak anion	$-NH_2$	Primary amine	1–8
	$-NH$	Secondary amine	1–7
	$\begin{array}{c} \backslash \\ -N \\ / \end{array}$	Tertiary amine	1–6
Strong cation	$-SO_3^-$	Sulfonic acid	1–14
Weak cation	$-COO^-$	Carboxylic acid	6–14

Charge Density

Stationary phase density is another variable that influences retention. Charge density may be varied in either of two ways. In the case of weak ion-exchange sorbents, charge density decreases as the mobile phase pH approaches the pK_a of the charged functional groups. Some materials, such as the polyethyleneimine (PEI) adsorbed sorbents, have a broad pK_a , with the charge density varying in almost a linear fashion over four pH units. The positive feature of using weak ion-exchange sorbents is that mobile phase pH can be used to desorb very strongly adsorbed proteins by decreasing the surface charge of the sorbent. The negative aspect of these sorbents is that optimization of separations becomes more difficult because the charge density is not constant.

Charge density can also be varied synthetically. The distance between ligands on high-charge-density sorbents can be as close as 2 Å. Spacing on low-charge-density materials can be 10–15 Å. In general, high-charge-density sorbents have higher loading capacity, adsorb proteins and nucleic acids more strongly, and are often of superior resolution. Their most negative characteristics are that protein recovery can be lower and fouling greater than on sorbents with lower ligand density. Low-charge-density materials are primarily used in preparative chromatography of very crude mixtures where it is desirable that proteins and debris be easily removed from the column.

It should also be noted that charge density can play a role in selectivity. Even though high-charge-density materials seem to be best in most cases, some solute pairs are resolved much better on low-charge-density columns. For this reason, it is recommended that sorbents with high charge density be used in analytical separations where one must deal with a large number of different compounds and the time required precludes optimization of separation conditions. Preparative and process scale applications, in contrast, involve repeated separations of the same sample and hence it is worthwhile to explore multiple alternatives toward stationary phase optimization.

Matrix Effects

Stationary phase groups must be supported on some type of matrix. Ideally, this matrix should be inert, i.e., not interact with the solutes being separated. In practice, this is seldom the case. Residual silanols, for example, are always present on silica-based supports and must be totally covered or masked by the bonded phase. Above pH 5, silanol groups ionize sufficiently to act as weak cation-exchange ligands. This cation-exchange behavior can either cause the sorbent to act as a mixed-mode exchanger in anion exchange or moderate the properties of the stationary phase. In the latter case, the charge density of a silica-

based quaternary amine sorbent may vary as a function of pH because the silanols, which have a broad titration curve ranging from pH 5 to 8, can ion-pair with amine groups on the stationary phase. Such ion-paired amines are unavailable for adsorption with solute molecules, thus decreasing charge density for these anion-exchange sorbents.

The organic matrix of resins can participate in two types of nonspecific interactions associated with protein retention. Small numbers of carboxyl groups containing monomers can be incorporated into the resin matrix during synthesis, giving the support weak cation-exchanging properties. The influence of these anionic functional groups in the matrix is similar to the case of silanols already described. This problem is most easily eliminated during synthesis by using monomers of higher purity.

Resin-based ion-exchange sorbents generally have some hydrophobicity associated with the organic matrix. Because the backbones in polyvinyl alcohol, polyacrylate, polymethacrylate, and polystyrene-divinylbenzene polymers are hydrocarbons, there is ample opportunity for nonspecific hydrophobic interactions between proteins and the support matrix. Hydrophobic interactions are most likely to occur along the hydrocarbon backbone of the polymer where there are no hydrophilic functional groups, such as at points of cross-linking or where the hydrocarbon skeleton was not completely derivatized. Hydrophobic interactions on ion-exchange matrices are promoted by mobile phase conditions of high salt concentration. Therefore, in gradient elution separations, protein retention affected by hydrophobic interactions generally occurs at the end of the gradient. Good markers for measuring nonspecific adsorption due to hydrophobic interaction in cation-exchange chromatography are cytochrome *c* and lysozyme. In a purely cation-exchange separation, these two proteins elute immediately adjacent to each other. Their resolution increases with increasing matrix hydrophobicity. Ovalbumin and soybean trypsin inhibitor are similarly good hydrophobicity markers for anion-exchange columns. Ovalbumin is hydrophilic and elutes first. As matrix hydrophobicity increases, the separation between these two substances increases.

Hydrophobicity in an ion-exchange column can have both positive and negative attributes. An attractive feature is that it can change the selectivity and increase the resolution between some species. The negative aspect of columns that are both charged and hydrophobic is that they often show lower protein recovery. An extreme case is the use of sulfonated polystyrene-divinylbenzene columns on which recovery of proteins is often very poor.

Minimization of solute-sorbent hydrophobic interactions can be attained using organic modifiers such as ethylene glycol, propanol, and acetonitrile in the mobile phase buffers. Even though ethylene glycol is the most viscous, causing the most band spreading of the three solvents, it is the most desirable because it is the least likely to initiate protein denaturation.

Solute Contributions

Both electrophoresis and ion-exchange chromatography separate biological macromolecules on the basis of charge. However, comparison of ion-exchange chromatograms and electropherograms of the same mixture shows that they do not separate charged macromolecular species in the same way. The following discussion will develop the basis for discrimination between charged macromolecules on ion-exchange sorbents and shed some light on how ion-exchange chromatography and electrophoresis differ.

Titration Curves of Proteins

Proteins and nucleic acids contain large numbers of charged functional groups, which, due to their chemical nature and location in a macromolecule, may have pK_a values ranging from 2 to 11. For example, the pK_a of a histidine residue in a protein may vary by up to 1 pH unit in either direction depending on its local environment. The same can be true of carboxyl and amino groups. An extreme case has been observed wherein the pK_a of lysine was shown to be as low as 5.9 [3]. Because the pK_a values of functional groups in a macromolecule are sensitive to the surrounding structure and other neighboring amino acids, macromolecules with 30–40 charged groups derived from three to five different ionic species have very complex titration curves. Two molecules can have identical charge characteristics at one pH but be different at another pH due to perturbations in the ionization of charged functional groups [4]. In the case of hemoglobin, it has been found that substitution of one neutral amino acid for another can change the titration curve [5]. The experimental challenge in both electrophoresis and ion-exchange chromatography is to find the pH at which polyelectrolytes are different.

Isoelectric Points

Because the charged functional groups in proteins are composed of both weak acids and weak bases, proteins have an isoelectric point (pI) where the net charge of the protein is zero. Logic would suggest that separations on an anion-exchange column should be carried out above the pI, where the protein has a net negative charge, and on cation-exchange columns below the pI, where the protein has a net positive charge. This concept has been found to be true experimentally for electrophoresis in terms of the direction of migration. Electrophoretic properties are easily explained in terms of a “net charge” model whose central premise is that electrophoretic mobility is directly proportional to the number of excess charges (either positive or negative) that exist after all the charges have been

summed. Therefore, in an electrophoretic separation, a protein with 12 negatively charged and 14 positively charged amino acid side chains has a net charge of 2^+ and migrates toward the cathode.

Similar logic in the application of this model to ion-exchange chromatography would suggest that chromatographic retention should increase in direct proportion to the net charge. A graphic representation of this model as it applies to ion-exchange chromatography is seen in Fig. 1. Experimentally, this model fails in ion-exchange chromatography, as shown in Fig. 2, using a series of retention maps in which chromatographic retention is plotted as a function of pH on both anion- and cation-exchange columns. Although the net charge retention model implies that proteins should not be retained on ion-exchange columns at their pI, this is not the case with many proteins. It is clearly seen that many proteins can be retained on ion-exchange columns even when their net charge is zero, a behavior totally different from what would be seen in electrophoresis. Furthermore, there seems to be little relationship between net charge and chromatographic retention. Chromatographic retention of β -lactoglobulin (β -LAC) (Fig. 3) levels off within two pH units of the pI, whereas electrophoretic mobility increases in direct proportion to charge.

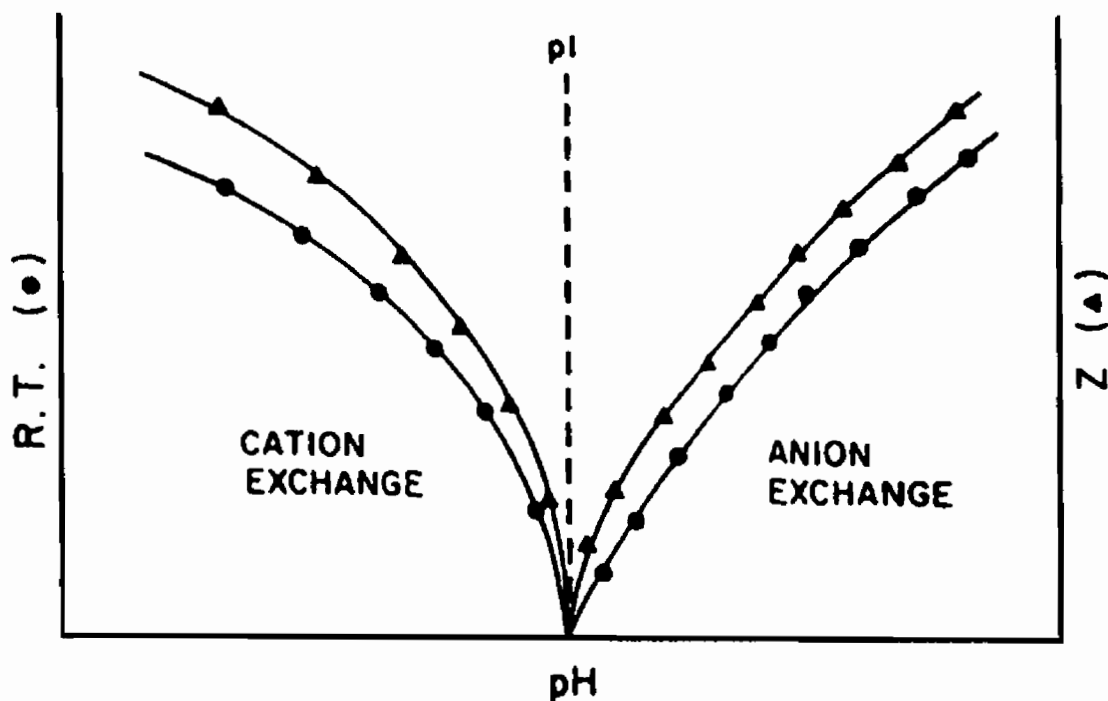


FIGURE 1 Hypothetical relationship between protein net charge and chromatographic retention. Net charge Z is depicted by a titration curve, and pH-dependent chromatographic behavior is represented by a retention map (retention time vs. pH).

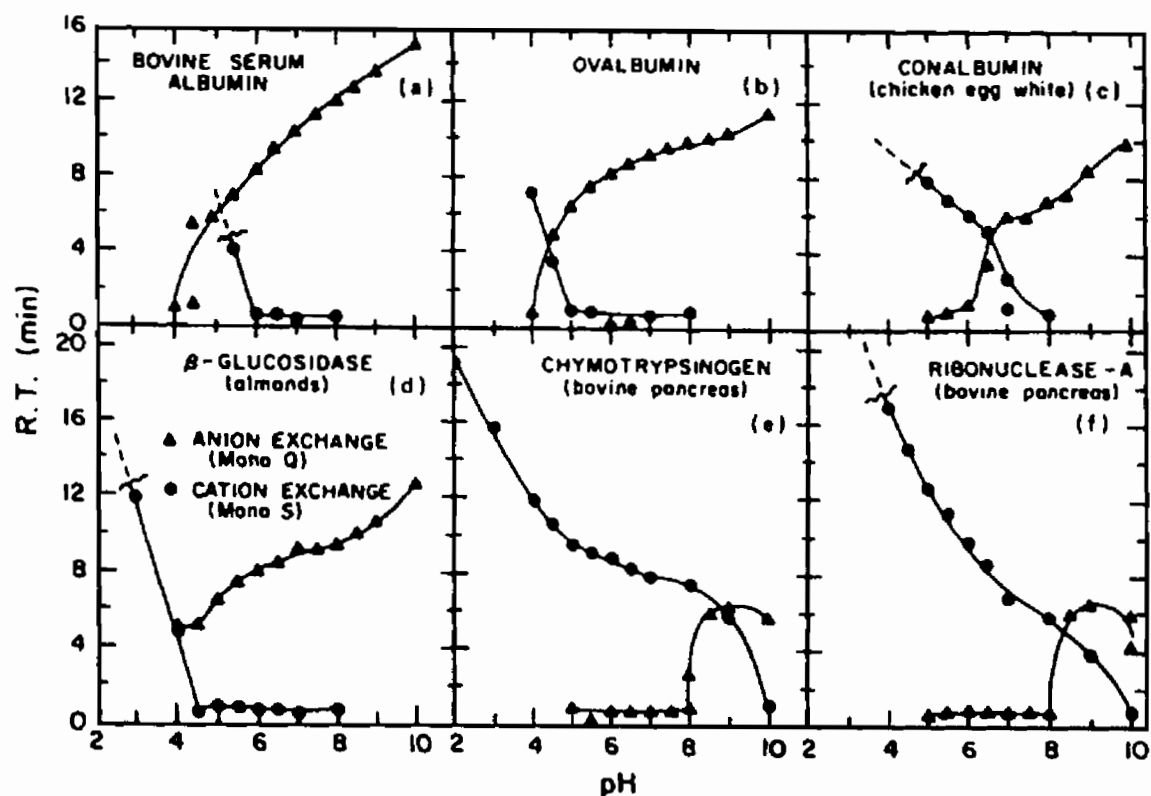


FIGURE 2 Retention maps for six proteins that were retained on at least one of the strong ion-exchange columns at their respective pH values. Proteins and their respective pI values are as follows: BSA, 5.07; OVA, 4.7; CON, 6.3; β -GLU, 7.3; CHY, 9.2; RIB, 8.7.

Three-Dimensional Structure

Many proteins and polynucleotides are of rather rigid three-dimensional structure that is maintained even when adsorbed to surfaces. This has been demonstrated most dramatically in the case of immobilized enzymes and antibodies, where unimpaired biological activity is retained after adsorption by any of a variety of techniques. This is significant because it means that under physiological conditions: (1) the three-dimensional structure of most biological macromolecules will be retained during the adsorption process required for chromatographic separations, and (2) only a portion of the protein or polynucleotide surface can be in contact with the sorbent surface at any one time. In the case of ion-exchange chromatography, this means that not all of the charged groups in a molecule can participate simultaneously in the retention process.

Cooperativity

Large molecules, such as proteins and polynucleotides, have many charged groups that interact with an ion-exchange sorbent at many sites. Multiple-site

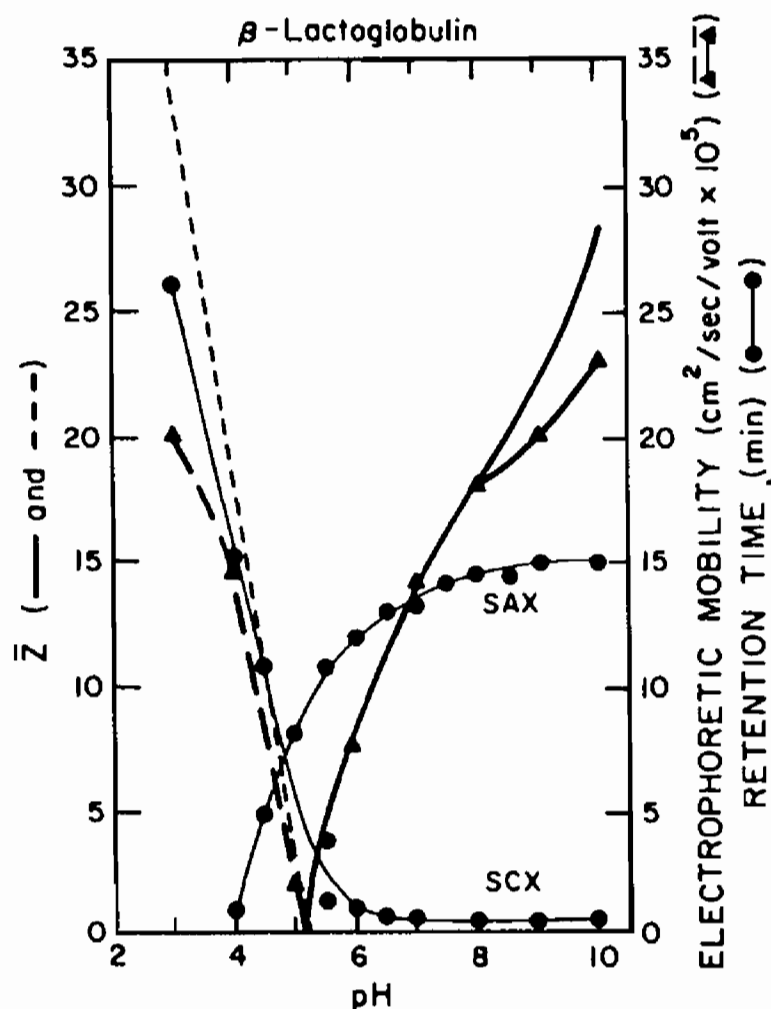


FIGURE 3 Chromatographic retention compared with titration and electrophoretic mobility curves for β -lactoglobulin, $pI = 5.1$. SAX refers to the Pharmacia Mono Q column and SCX to the Pharmacia Mono S column.

adsorption of macromolecules results in unique retention behavior not found in small solutes. The most significant property is that small changes in mobile phase composition can produce large differences in retention behavior. For example, a molecule adsorbed to a column at 20 sites might be infinitely retained on an ion-exchange column at 0.40 M salt but elute at 0.41 M salt. This unusual behavior is the result of cooperativity. A cooperative process in chromatography refers to cases in which binding at one site makes binding at a second site easier or more likely. When surface charged groups are in close proximity in a molecule, the binding of the molecule to the sorbent at one site increases the probability of binding for other nearby charged groups on the protein surface. Similar effects are seen in helix formation with polypeptides and polynucleotides. The relationship of cooperativity to chromatographic retention will be further discussed later under "Retention Model."

Charge Distribution

The two characteristics of proteins that set them apart from synthetic polymers are their defined three-dimensional structures and the asymmetric spatial distribution of amino acids. Asymmetric distribution of amino acids is fundamental to organizing molecular structure and intermolecular interactions. For example, the high concentration of hydrophobic amino acids on one side of an amphiphilic helix of a protein is important for protein folding or association of proteins with membranes and receptors. Some proteins have an analogous high concentration of charge on one side that facilitates binding or docking to a specific surface or receptor. Another type of amino acid distribution in proteins is one in which a high concentration of hydrophilic residues or carbohydrate groups in a particular part of the molecule plays a role in intermolecular interaction. Thus, asymmetry with respect to amino acid type (hydrophobicity, charge, or hydrophilicity) is important in natural systems where molecules interact with surfaces because of the orientation of the molecular species during intermolecular docking.

There is reason to believe that the same is true in chromatography. For example, it has been shown with amphiphilic helices that the hydrophobic side of the helix dominates chromatographic behavior in hydrophobic interaction chromatography (HIC) [6]. In a similar manner, the more hydrophobic side of lysozyme determines chromatographic behavior in HIC. Chromatographic retention in metal interaction chromatography (MIC) is dominated by portions of the extreme surface of a protein that are rich in histidine, cysteine, or tryptophan. Very specific binding sites are also common with immunosorbents in affinity chromatography. Portions of the external surface of a macromolecule that interact with the surface of a chromatographic column and determine chromatographic behavior have come to be known as the "chromatographic contact region." This concept of the chromatographic contact region is also referred to as the "footprint" model of retention.

Asymmetric spatial distribution of charged groups within a protein, or charged "patches" as they are sometimes called, plays an important role in ion-exchange retention behavior. This is most easily seen at the pI of a protein. When positive and negative charge is uniformly distributed on the protein surface, little retention on ion-exchange columns at the pI of the protein is observed. The case where one type of charge (either positive or negative) is uniformly distributed, while the opposite charge is asymmetrically distributed, is exemplified by β -lactoglobulin (β -LAC, seen in Fig. 3). Because the positively charged surface amino acids are uniformly distributed in β -LAC, it is not retained on a cation-exchange column at pH values above its pI. In contrast, the negatively charged residues in β -LAC are asymmetrically distributed, producing retention of the protein on anion-exchange columns one pH unit below its pI. The other case of charge distribution is that where both positively and negatively charged residues

are located asymmetrically on the surface of the molecule. These proteins would be likely to be retained at their pI on both anion- and cation-exchange columns.

Hydrophobic Group Contributions

It was noted earlier that ion-exchange sorbents can possess some hydrophobic characteristics, providing the opportunity for hydrophobic interactions with the solute. Hydrophobic residues of proteins can likewise produce interaction. Although most of the hydrophobic amino acids are buried within the interior of the three-dimensional structure, significant numbers also occur at the surface of a protein. These groups will interact with a weakly hydrophobic ion-exchange sorbent at high salt concentrations and subsequently increase protein retention. Hydrophobic effects are easily recognized in ion-exchange chromatography through the use of different salts and organic solvents. Kosmotropic salts, such as ammonium sulfate, have high molal salting-out coefficients and induce hydrophobic effects. In contrast, chaotropes such as sodium chloride are relatively poor at driving hydrophobic interactions. Increased retention in applications when ammonium sulfate is used as the displacing salt in ion-exchange strongly implies that nonspecific hydrophobic interactions are taking place. Organic solvents such as ethylene glycol and propanol reduce hydrophobic effects and decrease retention when hydrophobic interaction is a strong contributor to chromatographic retention. However, it should be noted that organic solvents can also change the integrity of protein structure, thus causing changes in retention that have nothing to do with diminishing hydrophobic effects. Hydrophobic interaction with the sorbent matrix can also play a significant role in the separation of polynucleotides [7].

Retention Model

Theory

The importance of three-dimensional structure to chromatographic behavior is reflected in the nonmechanistic model, the stoichiometric displacement model (SDM). The central hypothesis of the SDM is that the displacement of a solute from a surface is accompanied by the adsorption of a stoichiometric amount of displacing agent. The process may be described by the equilibrium expression:



where P_0 refers to the analyte in solution, capable of being adsorbed on a surface at multiple sites, D_b refers to adsorbed displacing agent, P_b is the analyte bound to the stationary phase, D_0 is the free displacing agent, and Z is the number of interaction sites for a particular analyte and stationary phase in a given system.

Simple transformation of the equilibrium expression provides the formation constant (K_f):

$$K_f = \frac{[P_b][D_0]^Z}{[P_0][D_b]^Z} \quad (2)$$

The formation constant (also known as a selectivity coefficient) should not be confused with the thermodynamic equilibrium constant in which the activities of the species are used.

Relating the formation constant to chromatographic retention requires that it be expressed in terms of a partition or distribution coefficient (K_1). The partition coefficient is defined as the ratio of solute concentration in the stationary phase to that in the mobile phase

$$K_1 = \frac{C_s}{C_M} = \frac{[P_b]}{[P_0]} \quad (3)$$

Combining Eqs. (2) and (3) produces the expression:

$$K_1 = \frac{K_f[D_b]^Z}{[D_0]^Z} \quad (4)$$

Although the partitioning process in liquid chromatography is defined in terms of K_1 , solute retention on an ion-exchange column is usually expressed in terms of the capacity factor k :

$$k = K_1 \frac{V_S}{V_M} \quad (5)$$

where V_S and V_M are constants for a given solute-support combination and correspond to the available stationary phase and mobile phase volume, respectively. The term V_S/V_M is also referred to as the phase ratio. It should also be noted that when a column is being operated in the analytical mode and protein loading is low, D_b is a constant. Hence, solute retention (expressed by the capacity factor k) has been related to the displacing salt concentration $[D_0]$ by:

$$k = \frac{I}{[D_0]^Z} \quad (6)$$

where I is a constant incorporating the equilibrium formation constant, the phase ratio, and the bound solute concentration.

It has been shown in anion-exchange chromatography of oligonucleotides that Z values reflect the stoichiometry of ionic displacement of solute from the sorbent surface. The relationship between three-dimensional structure and Z number was examined using oligonucleotides of varying length as models [7]. With oligonucleotides of less than 10 bases, the Z number reflected the total

number of apionic positions on the molecule, whereas oligonucleotides larger than 10 bases, including tRNA (>70 bases), have a fractional ratio of Z number/total surface charge. Other applications of the SDM to the relationship between three-dimensional structure and protein retention have revealed that only a fraction of the total surface charge on a protein interacts with the sorbent matrix at any one time. An unrelated retention study of the quaternary structure of lactate dehydrogenase isoenzymes showed that Z number values varied depending on the subunit composition [8]. That study also led to the conclusion that in a multimeric protein there may be equivalent areas on the molecular surface that participate in the adsorption process. Consequently, three-dimensional structure plays a vital role in the chromatographic behavior of proteins.

Structural Changes

Because three-dimensional structure sterically defines which amino acids may interact with a chromatographic column, conformational changes associated with the protein surface can affect the affinity between solute and sorbent. Depending on the severity of the transition and the area affected, it is possible to eliminate protein adsorption completely, although in most cases only partial restriction of electrostatic interaction results. On the other hand, it is also conceivable that retention can be enhanced as a result of changes in the protein structure. A systematic examination of the effects of small changes, with strict control over the location and type of structural manipulations incorporated onto the protein surface, is necessary to explore the retention process further.

With the advent of site-directed mutagenesis, it is now possible to change any amino acid in a protein. Proper application of this technique permits the construction of similar structural variants differing by as little as one amino acid without any global shifts in the three-dimensional structure. Chromatographic analysis of such engineered proteins can expand our understanding of biomolecular retention processes. Provided the protein crystallographic structure and the chromatographic contact area have been determined, amino acid contributions to protein retention can be quantitatively examined using the SDM. Although only the surface residues within the footprint region can directly interact with the stationary phase of the sorbent, neighboring amino acids can influence the surrounding microenvironment, indirectly affecting retention. In this way, minute changes incorporated into the primary structure of proteins with similar three-dimensional structure can contribute to the overall chromatographic characteristics of the molecule.

A study investigating the chromatographic behavior of genetically engineered subtilisin reported the separation of various single-residue substitution variants from each other on a cation-exchange column [9]. The sensitivity of cation-exchange chromatography was demonstrated with the resolution of two variants differing by only a methylene group in this 27,500 Da protein. It was

concluded that both neutral and charged amino acids, located in the microenvironment of the chromatographic contact area, contributed to protein retention in this separation mode. Thus, small changes in the primary structure that did not alter the global shape of the molecule still significantly affected the retention process. Future studies using site-directed mutagenesis will perhaps yield additional information on the importance of surface microenvironments and their roles in retention mechanisms.

RESOLUTION

It is important to identify the variables that control resolution in ion-exchange chromatography so that they can be optimized. The degree of separation between a solute pair in any chromatographic system can be expressed by the equation:

$$R_s = 1 \left(\frac{V_2 - V_1}{w_1 + w_2} \right) \quad (7)$$

where R_s is resolution, V_2 is the elution volume of the more strongly retained solute (S_2), V_1 is the elution volume of the less strongly retained solute (S_1), w_1 is the peak width of S_1 , and w_2 is the peak width of S_2 . It is seen from this equation that resolution is determined by two variables: the sum of the peak widths ($w_1 + w_2$) and the relative difference in elution volume between the two solutes ($V_2 - V_1$). The first of these variables, ($w_1 + w_2$), is a function of band spreading and the second, ($V_2 - V_1$), is determined by the selectivity of the system. Because band spreading is a general feature of all chromatographic systems and is not unique to ion exchange, it will not be discussed further here. In contrast, variables that determine selectivity in ion-exchange chromatography will be identified and discussed.

Selectivity

Separation science is entering an era for which separation systems of more than 10^6 theoretical plates will be common. This breathtaking prospect conveys the impression that with enough theoretical plates, it will be possible to separate any mixture. Obviously, this will be true only if solute retention is at least slightly different or if some way can be found to make the interaction between distinct solutes and the sorbent unique. Adjusting the selectivity, which is essential in the fractionation of any mixture, can be accomplished in numerous ways in ion-exchange chromatography. The great functional group diversity in polypeptides allows their selectivity to be manipulated more easily than that of polynucleotides.

Solute Contributions

Modification of charge is obviously one of the more direct methods of changing selectivity in ion-exchange chromatography. It has already been noted that even minute differences in protein structure are manifested in the titration curves. Unfortunately, because the structure of proteins is so complex, it is not possible to predict the pH at which these charge differences occur and it must be determined experimentally. The most unequivocal way to find the optimum pH for the separation of a solute pair is by retention mapping, as shown in Figs. 2 and 3. A pH retention map is constructed by plotting gradient elution retention time versus mobile phase pH with the same displacing salt. With the advent of mobile phase switching devices in modern LC instrumentation, it is now possible to automate the experimental component of data acquisition for the construction of retention maps. The chromatographic retention map is similar in form to the isoelectric titration curve used in electrophoresis for determining the point at which the charges of a solute pair are different. The maps are, however, far from equivalent because of the large contribution that charge asymmetry makes in ion-exchange chromatography.

Mobile Phase Contributions

Salt Effects

It has already been noted that proteins and ion-exchange sorbents vary widely in surface hydrophobicity. These differences are easily exploited with simple changes in mobile phase composition. Solute displacement from an ion-exchange column is usually achieved by increasing the ionic strength of the salt in the mobile phase buffer. The molal salting-out coefficient and concentration of the displacing salt determine the extent of nonspecific hydrophobic interactions between the sorbent and solute species. For example, most proteins and polynucleotides are displaced from an ion-exchange column at less than 1 molar salt. Hydrophobic interactions induced by sodium chloride are very weak at these concentrations. In contrast, ammonium and sodium sulfate strongly induce hydrophobic interactions. When sodium or ammonium sulfate is substituted for sodium chloride in ion-exchange chromatography, there is often a large change in selectivity with some solute pairs. This is particularly true at the end of the gradient where mobile phase ionic strength is highest. Separations carried out on the same column using different salts provide an alternative fractionation of a sample mixture due to changes in selectivity.

Displacing salts apparently contribute to selectivity in other ways that are not well understood. Studies of displacing agents in both cation- and anion-exchange chromatography have shown that the type of salt can have a large

impact on selectivity [10–14]. Unfortunately, this must be determined experimentally and cannot be predicted a priori. Figure 4 illustrates the effect of salt substitution on the retention of various proteins by ion-exchange chromatography. It is interesting to note that both ions of the salt affect the retention—not only the counterion to the functional group on the stationary phase. This is most likely due to the association of the other ions with the solute; these ions must also be

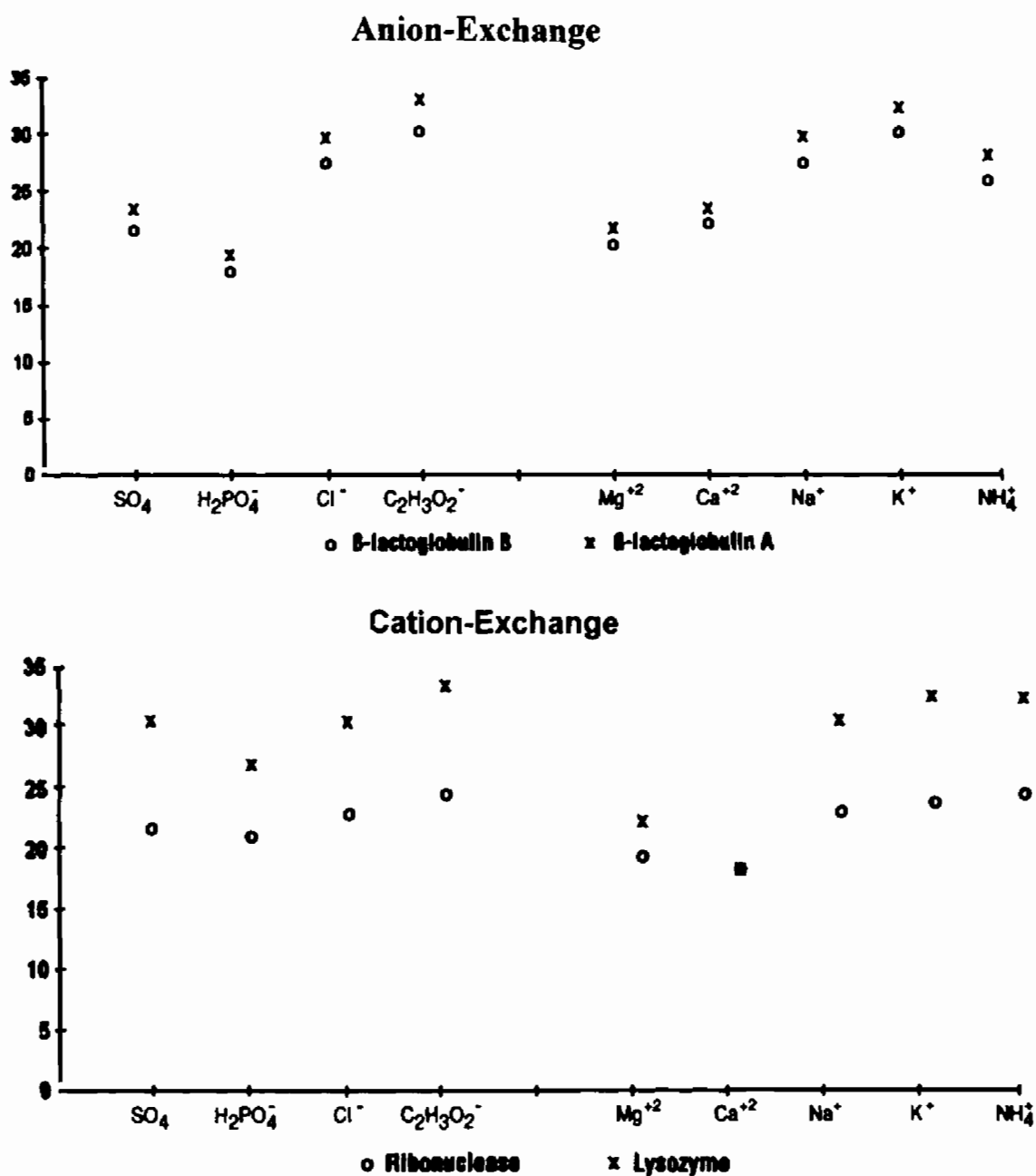


FIGURE 4 Effect of salt on retention in IEC. AEX; SynChropak AX300; CEX; SynChropak CM300; 30 min gradient (0–1 N) of sodium or chloride salts in 0.02 M Tris, pH 7. (From Ref. 14.)

exchanged during binding. The relative size of ions and their strength of binding to the solutes or functional groups ultimately determine retention.

pH Effects

Gradients of pH, although less frequently employed than salt concentration, can also produce excellent resolution in ion-exchange chromatography. More frequently, pH gradients are combined with salt gradients to effect separation.

Sorbent Contributions

Variables such as pore diameter, loading capacity, and type of stationary phase also contribute to selectivity and resolution in chromatographic separations. The stationary phase contributions of weak and strong ion exchangers to selectivity have been described in a previous section. Pore diameter is an indirect determinant of the mass capacity. Because more than 95% of the surface area of a porous sorbent is within the pore matrix, pores must be sufficiently large to give macromolecules access to the interior. A pore diameter of 300–500 Å is optimum for proteins of 30–100 kDa, while materials of 800–1000 Å pore diameter function well for proteins that exceed 100 kDa. Table 2 illustrates the effect of pore diameter on loading capacity for macromolecules.

Because the effect on resolution attributed to band spreading is innately important in chromatography, the loading capacity of a column directly influences the efficiency of separations performed on ion-exchange sorbents. As the concentration of protein injected overloads the column, the plate number decreases due to band broadening, reducing the resolution between separated species. Column efficiency can be increased by using microparticulate supports

TABLE 2 Ion-Exchange Capacity Relative to Pore Diameter^a

Pore (nm)	Surface area (m ² /g)	Ion-exchange capacity		
		Picric acid (μmol/g)	Ovalbumin (mg/g)	BSA (mg/g)
10	250	1415 ^b	59 ^b	64 ^c
30	100	656 ^b	98 ^b	130 ^c
50	50	308 ^b	76 ^b	59 ^c
100	20	129 ^b	26 ^b	57 ^c

^aPEI bonded phase.

^bFrom Ref. 15.

^cFrom Ref. 16.

with the appropriate porosity (as described earlier) to maximize the mass capacity for protein samples.

CONCLUSIONS

Ion-exchange chromatography is a powerful separation technique for proteins because it is very selective and has high loading capacity. Selectivity can be easily varied by adjusting the mobile phase composition—either the salt or the pH. The asymmetric distribution of surface amino acids on proteins is a major determinant of the excellent selectivity of ion-exchange chromatography.

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Reversed-Phase and Hydrophobic Interaction Chromatography of Proteins and Peptides

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INTRODUCTION

Background to Chapter

Reversed-phase chromatography (RPC) and hydrophobic interaction chromatography (HIC) are two important separation technologies, arguably now the most important, for the analysis and purification of proteins and peptides. Central to these methods, hydrophobic ligands are either chemically attached to the surface of hydrophilic support matrices or alternatively form part of the backbone of macroreticular polymeric resins, as occurs, for example, with different types of polystyrene-divinylbenzene (PS-DVB) microparticulate or monolithic chromatographic sorbents. A large range of support materials, suitable for high-performance separations as RP-HPLC and HP-HIC analytical and laboratory-scale procedures, as well as lower performance gels more suited to larger scale preparative applications, can be used. Retention is achieved through discrete interactions between these nonpolar ligands and hydrophobic patches accessible on the surface of a protein or peptide. This feature sets RPC and HIC apart from

the other chromatographic separation procedures that employ surface interactive phenomena to resolve mixtures of peptides or proteins.

In this chapter, a perspective is presented on the experimental opportunities that RPC and HIC offer for the isolation, analysis, and physicochemical characterization of proteins and peptides. Following an introductory section on the historical background that has led to the developments in RPC and HIC techniques as practiced today, the principles and theory of RPC and HIC will be discussed, with an emphasis on the importance of protein or peptide hierarchical structure in their interactions with various types of nonpolar chromatographic surfaces. These sections will then be followed by a discussion of the influence of operating parameters such as the effects of different surface chemistries and morphologies of the sorbent packing materials; the influences of different concentrations of organic solvents, salts, or other mobile phase additives; the impact of pH; and the role of temperature. The impact of conformational changes and related phenomena will then be explored from the perspective of their thermodynamic implications and effects on resolution. Some practical aspects of RPC and HIC methods will next be described, followed finally by a survey of selected applications in order to highlight the wide scope, versatility, and importance of RPC and HIC procedures in modern biology and biotechnology. In this context, RPC and HIC methods must be considered some of the more powerful experimental procedures now available. Because of the continuous, major advances in this field that have occurred over the past decade, RPC and HIC procedures can be expected to become even more dominant as exquisite laboratory and process separation technologies *par excellence* for the analysis, quantitation, purification, and manufacture of peptides and proteins over the early decades of the millennium.

In the case of RPC, elution is usually achieved with a defined (isocratic) and/or increasing (gradient) concentration of an organic solvent that forms part of a water–organic solvent mobile phase, often of low pH. In HIC, elution is effected with a step decrease or a gradient decrease in the concentration of a salt, frequently kosmotropic (water-structuring or antichaotropic) in its properties, and usually at or near neutral pH. Thus, the same fundamental principle is exploited when an RPC or HIC separation is carried out with a protein or peptide, namely the surface tension of the surrounding solvent is manipulated, enabling retention to be increased or decreased with a defined sorbent through the simple expediency of changes in the concentration of an organic solvent modifier or kosmotropic salt.

Illustrative of the use of conditions of an ascending organic solvent gradient in RPC and a descending salt gradient in HIC, thus achieving these surface tension changes, are the separations shown in Figs. 1 and 2 for the resolution of the *Thermus thermophilus* (*Tth*) 50S ribosomal proteins [1] and several rabbit reticulocyte hexokinase isoforms [2], respectively. In order to achieve the

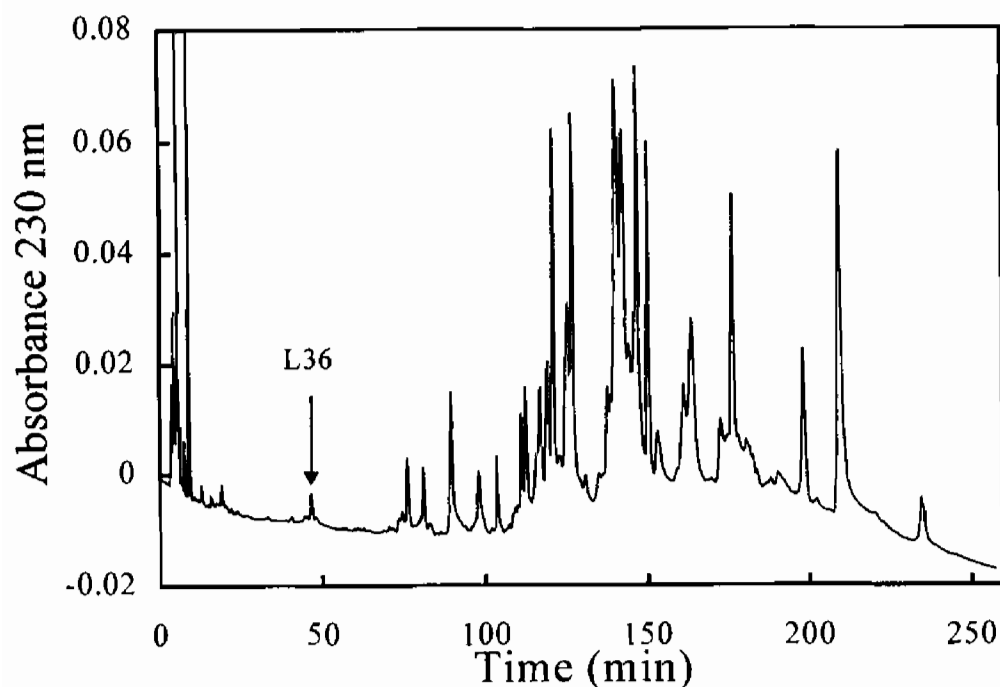


FIGURE 1 Separation of the ribosomal proteins of the 50S subunit of *Thermus thermophilus* by reversed-phase HPLC at a flow rate of 0.7 ml/min. The 50S ribosomal proteins were separated with a thermostatically controlled LiChrospher C-18 column (10 μ m, 300 Å, 250 $\times$ 4 mm ID). An optimized linear gradient of acetonitrile-water of 257.6 min duration, derived using the DryLab G/*plus* software, was employed using as eluent A 500 mM NaH₂PO₄, 500 mM H₃PO₄, pH 2.1, and eluent B 200 mM H₃PO₄, pH 2.1-acetonitrile (25:75) (v/v). The experiments were carried out at 20 $\pm$ 0.1°C and detection was at 215 nm. The collected fractions were desalted by a second RP-HPLC procedure using a Nucleosil C-4 column (10 μ m, 300 Å, 30 $\times$ 4 mm ID) with a linear gradient of 0.1% TFA in water to 0.09% TFA in 60% (v/v) acetonitrile-water over 60 min at a flow rate of 1.0 ml/min and detection at 215 nm. The location of the ribosomal zinc finger *TthL36* protein is indicated by the arrow. Amino acid sequencing of the isolated ribosomal proteins was achieved using an ABI 473A system with standard procedures for the quantification of the derived PTH-amino acid derivatives by RP-HPLC. (Data from Ref. 1.)

resolution of the *Tth* ribosomal proteins shown in Fig. 1, an optimized low pH, water-acetonitrile gradient from 500 mM NaH₂PO₄, 500 mM H₃PO₄, pH 2.1 to 200 mM H₃PO₄, pH 2.1-acetonitrile (25:75) with a non-end-capped LiChrospher C-18 sorbent was employed, permitting the isolation for the first time of many of these proteins, including the zinc finger motif protein *TthL36*. In Fig. 2, the complete isoenzyme elution profile for the rabbit reticulocyte hexokinase isoforms obtained with a Toyopearl 650 S sorbent and a decreasing ammonium sulfate gradient is compared with the corresponding profile for the individual isoforms previously isolated using a weak ion-exchange sorbent (Toyopearl DEAE 650 S) derived from the same support material. As evident from Fig. 2,

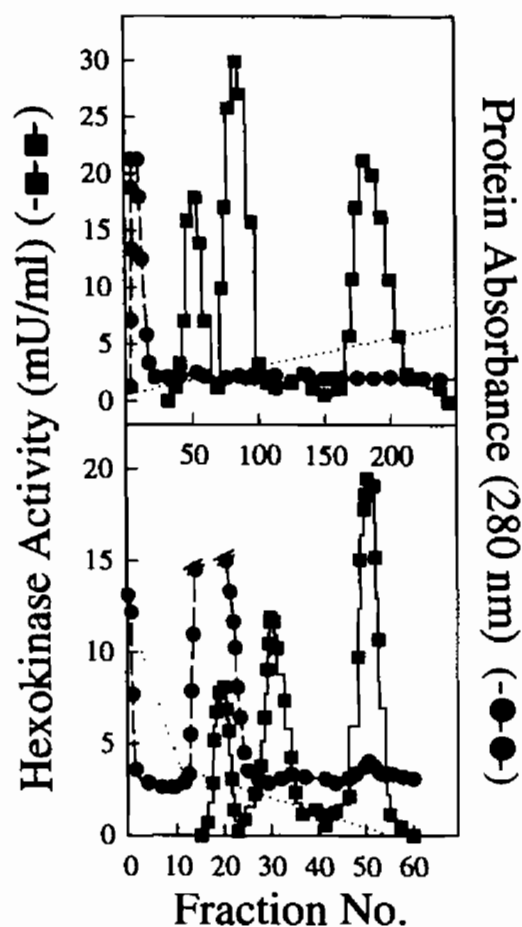


FIGURE 2 Separation of multiple forms of hexokinase from rabbit reticulocytes. (A) Fractionation of a hemolysate sample with a protein concentration of 50 mg/mL on a column of Toyopearl DEAE 6505 equilibrated in 5 mM sodium potassium phosphate buffer (pH 8.1) containing 3 mM KF, 3 mM β -mercaptoethanol, 1 mM dithiothreitol, and 5 mM glucose and operated at 0.3 mL/min. The elution of the hexokinase isozymes was achieved using a linear gradient from 40 to 200 mM KCl in the same buffer. (B) Fractionation of sample of hexokinase (190 mg/mL) from rabbit reticulocytes on a column of Toyopearl Phenyl 650S. The column was equilibrated in 5 mM sodium potassium phosphate buffer (pH 8.1) containing 3 mM KF, 3 mM β -mercaptoethanol, 1 mM dithiothreitol, 5 mM glucose, and 30% (v/v) ammonium sulfate, operated at 0.5 mL/min. Elution was achieved using a descending linear gradient of ammonium sulfate to effect the resolution of several rabbit reticulocyte hexokinase isoforms. (Data from Ref. 2.)

the hexokinase isoform 1a behaves as the least hydrophobic, followed by the isoform 1b and then the mitochondrial-associated hexokinase 1c. Results similar to those shown in Figs. 1 and 2 can readily be achieved with most biological extracts containing peptides or proteins using similar RPC or HIC procedures. Moreover, resolution of synthetic peptides prepared by solid- or solution-phase chemical methods is now routine by analytical or preparative RPC methods.

Illustrative of this wide applicability, a recent (May 2000) CD-ROM database search of relevant scientific journals, pertinent to the analysis and isolation of peptides and proteins, revealed that over 3650 publications arise each year as part of the scientific literature on the use of RPC and HIC in various fields of biology and biotechnology; the food, environmental, and pharmaceutical industries; and from biomedical and life science research endeavours.

Currently, the analytical separation and purification of peptides is largely the province of RPC techniques, and HIC has been found to be particularly useful for the purification of globular proteins while maintaining their biological activity. A concern frequently expressed by less experienced investigators about the use of RPC is that the technique is too harsh on proteins and thus is unsuited for large-scale preparative applications. Yet, numerous exceptions can be found in the literature [3,4] whereby bioactive, middle molecular weight proteins and large polypeptides have been successfully purified at laboratory scales by RPC procedures. Therein lies the challenge for investigators to better understand the origin of the selectivity (thermodynamic) and zone broadening (dynamic) behavior manifested by proteins and peptides in RPC and HIC environments. As both techniques provide orthogonal alternatives to the other methods of bio-polymer separation, they can be rightly considered as complementary and essential components of the experimental arsenal that should be used in high-resolution separations of complex mixtures of proteins and peptides.

Of equal importance, the use of RPC and HIC techniques provides a very powerful avenue to explore the molecular basis of the hydrophobic effect *per se* that these biomacromolecules exhibit. Since the time of the initial attempts, commencing over 50 years ago, to exploit the hydrophobic effect as part of robust separation procedures, RPC and HIC have thus come to assume a dominant position for the isolation and analysis of many proteins and now represent the techniques *par excellence* for the purification and analysis of polypeptides prepared by solid- or solution-phase synthetic procedures. Equally, these techniques provide an opportunity to explore the role of the hydrophobic effect in the stabilization and folding of proteins and polypeptides, the molecular forces that are involved in these processes, the thermodynamics of their interaction with relatively well-defined nonpolar surfaces, and the biophysics of peptide or protein nonpolar interactions in general.

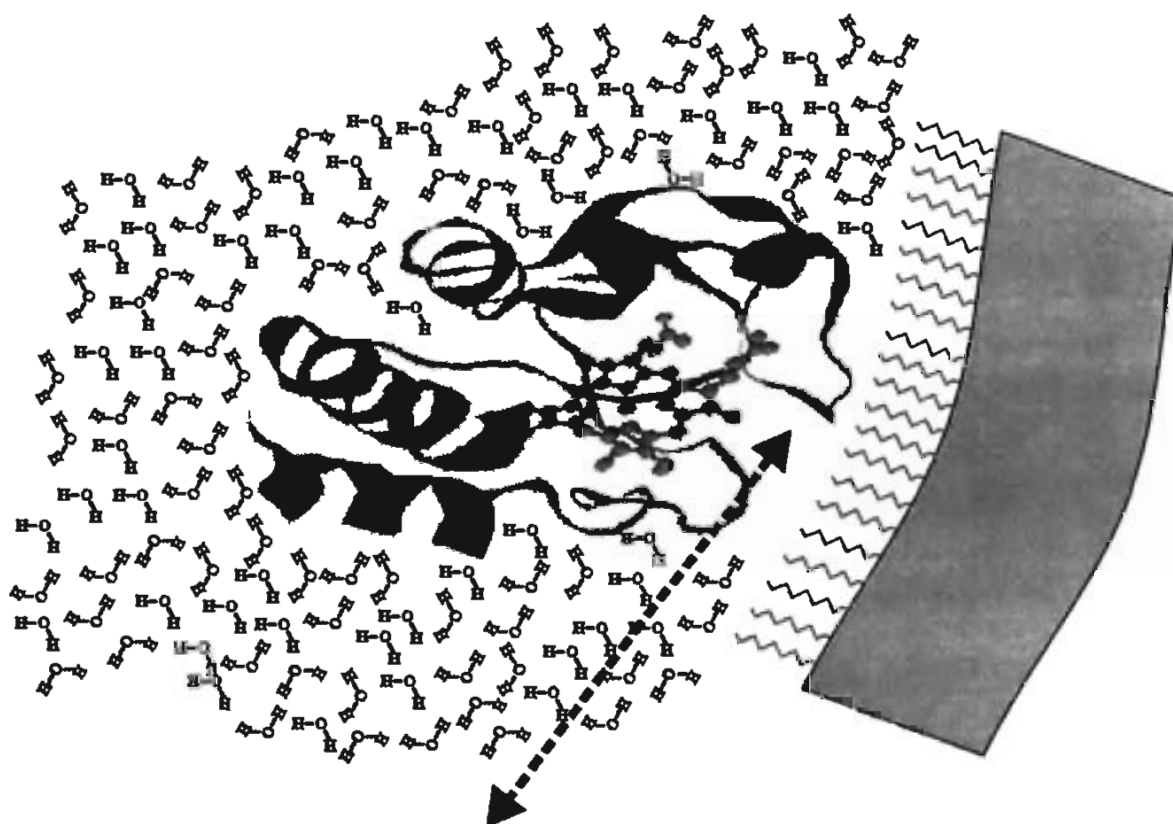
Historical Origins of RPC and HIC

In science, confluence of ideas often occurs near the same dates. Such was the case leading to the initial observations on the use of RPC and HIC techniques. When introduced in 1950 by Howard and Martin [5], the chromatographic approach of RPC represented a novel concept because the polarities of the chromatographic phases were “reversed” with respect to those used in other

chromatographic systems in vogue at that time, i.e., encompassed a nonpolar stationary phase and a polar mobile phase. Similarly, when HIC was described in 1948 by Tiselius [6,7] as "salting-out chromatography," a more polar eluent was used in conjunction with a less polar paper chromatographic sorbent for the separation of dyes in the presence of relatively high concentrations of salts, e.g., sulfate and phosphate salts. In the subsequent five decades, these initial reports have been expanded upon by a large number of investigations directed to shed light on the physicochemical basis of the retention mechanism in RPC and HIC. Significant advances in concept and practice have been made essentially in a cyclical fashion every 10 years or so as new analytical and instrumental technologies became available to aid the understanding of the retention, kinetic, and isothermal adsorption behavior of proteins and peptides, as well as the influence of various solvents and salts on the stability of these biomacromolecules, under RPC and HIC conditions [8].

From a historical perspective, HIC developments based on soft gel materials prospered more during the years up to the late 1970s than RPC, until suitable RPC support materials for peptide separation became generally available based on the development of stable pellicular and porous silicas. Retention behavior typical of hydrophobic interaction effects was noted in the size exclusion chromatography (SEC) of amino acids and proteins with agarose-based gels as early as 1960 by Gelotte [9] and Porath [10]. Similarly, studies with small molecules, such as *n*-alcohols, demonstrated that their adsorption to SEC gels increased with the carbon number of the alcohol [11]. Such behavior is consistent with the binding being mediated by the intrinsic hydrophobicity of the polysaccharide backbone and/or the nonpolar groups introduced into the sorbent as part of the cross-linkage chemistry. Illustrative of these types of interactions is the schematic representation of the hydrophobic interaction of a globular protein with an amphiphilic sorbent, such as a highly cross-linked agarose or a silica-based material that had been chemically modified with *n*-alkyl chains, as shown in Fig. 3. As evident from this Fig. 3, the protein will exist in a highly solvated cavity surrounded by water via hydrogen-bonding effects and other dipole interactions, but on approaching the nonpolar surface, incremental desolvation will occur from regions of the protein of highest hydrophobicity, permitting the generation of a preferred contact region for intercalation with the nonpolar ligates through partition and/or adsorption effects. The free energy changes for a favorable interaction, given by $\Delta G_{\text{assoc},i}^{\circ}$, will be negative. Depending on the magnitude of the $\Delta G_{\text{assoc},i}^{\circ}$ for this interaction, molecular forces can be generated that may exceed the corresponding $\Delta G_{\text{fold},i}^{\circ}$ term associated with the (favorable) folding of the protein, and the protein may partially or completely denature.

Observations on the participation of hydrophobic interactions parallel to those typically observed with low- and high-performance size exclusion chromatographic materials can also be found with biospecific affinity chromato-



Preferred Hydrophobic Contact Sites

FIGURE 3 Illustrative of these types of interactions is the schematic representation of the hydrophobic interaction of a globular protein solvated in water with an amphiphilic sorbent. The arrow identifies the preferred (hypothetical) hydrophobic contact region of the globular protein, P_1 . When binding occurs, a reduction in the cavity volume, $V_{cav,i}$, of the interacting protein–nonpolar ligate system occurs. Under such conditions, the change in the overall free energy, $\Delta G_{assoc,i}^\circ$, for the RPC or HIC chromatographic process is given by $\Delta G_{assoc,i}^\circ = \Delta G_{g/p,i}^\circ + \Delta G_{cav,i}^\circ + \Delta G_{int,i}^\circ - RT \log(RT/PV)$, where $\Delta G_{g/p,i}^\circ$, $\Delta G_{cav,i}^\circ$, and $\Delta G_{int,i}^\circ$ are the free energy changes for the association process in the hypothetical gas phase, the formation of the appropriate cavity, and the interaction between the protein species and the surrounding molecules, respectively. R is the gas constant, and T , P , and V are the standard temperature, pressure, and volume terms.

graphic sorbents, where n -alkyl spacer groups (such as the n -hexyl moiety of caproic acid) are frequently interposed between the (bio)specific affinity ligand and the agarose gel to increase steric accessibility of the affinity ligand to the protein. Such hydrophobic interaction effects between a nonpolar spacer group and proteins were exploited [12] as the basis for the isolation of several proteins and enzymes, such as the separation of α -chymotrypsin from α -chymotrypsinogen [13–15]. However, these types of interactions with proteins and peptides were considered at the time to be generally problematic, generating nonspecific

effects that could not be rationally controlled or explained. Although RP-HPLC, the most important branch of high-performance liquid chromatography (HPLC), was finding increasing application for the separation of low-molecular-weight molecules by the commencement of the 1980s, analogous “nonspecific” effects were frequently observed when proteins or large polypeptides were chromatographed with nonoptimized mobile phase systems on the different types of developmental or commercial *n*-alkylsilicas available at that time. Thus, acceptance of an important role for RPC techniques in peptide and protein chemistry was still treated in the late 1970s and early 1980s with some scepticism. A second major reason for the hesitancy to adopt RPC in protein chemistry can be traced to the (misguided) belief that the RPC conditions always lead to denaturation of proteins, with the denaturation occurring as a direct consequence of the contact of protein molecules with water–organic solvent mixtures and/or the strongly hydrophobic RPC surfaces. With the emergence of biotechnology and genetic engineering, as well as the wider availability of greatly improved types of solid-phase peptide synthesis (SPPS) technologies, new separation tools were, however, urgently required to permit the isolation and analysis of an ever-expanding range of peptides and proteins or their posttranslational variants. This pressure, coupled with a number of significant conceptual and experimental advances, started the revolution that has today become known as *molecular chromatography* in which RPC and HIC techniques have become major players.

Because these biopolymers have high molecular weights and complex tertiary structures, their diffusional and mass transfer properties in solution or at liquid-solid interfaces differ considerably from those of small molecules, while profoundly different dynamic processes of interaction can occur. Furthermore, polypeptides and proteins can undergo conformational changes either at the liquid-solid interfaces of a stationary phase and/or in the mobile phase. Thus, adsorption phenomena with proteins or polypeptides at hydrophobic surfaces normally involve a “dwell”- or “residency”-induced conformational change [16–19] in which these molecules can further unfold at the solid-liquid interface. Figure 4 shows one such (simplified) pathway involving only two unfolded states, P_{UM} and P_{US} , that can occur when a globular protein interacts with immobilized nonpolar ligands in the presence of an aquo-organic solvent or chaotropic mobile phase system. Denaturation effects associated with these unfolding/conformational changes can thus complicate an evaluation of the retention processes in RPC and HIC of proteins. Although thermodynamic studies have elucidated some aspects of the behavior observed with protein adsorption from water–organic solvent mixtures to solvated nonpolar ligates [17,20], most of our understanding of the retention processes that involved RPC and HIC separations has started with a more empirical examination of the properties of the stationary phase ligates or the mobile phase composition.

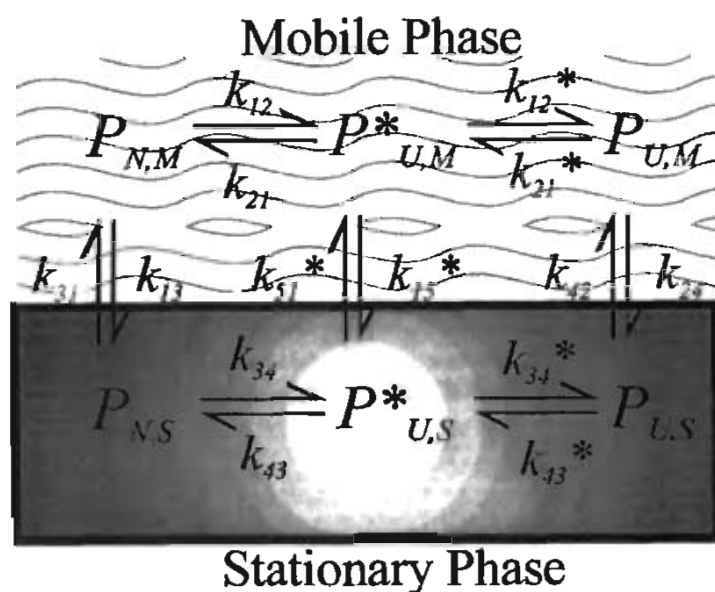


FIGURE 4 Schematic representation of a retention cycle pathway for the conformational interconversion of a globular protein or polypeptide, $P_{N,M}$, in free solution in the mobile phase and in two unfolded states, $P_{U,M}$ and $P_{U,S}$, which occur in the presence of a liquid-solid interface involving immobilized nonpolar ligands of an RPC or HIC sorbent in the presence of an aquo-organic solvent, a kosmotropic or a chaotropic mobile phase system. If the globular protein or polypeptide undergoes a two-stage interconversion in the mobile phase and at the surface, the corresponding distribution process between the two chromatographic phases will involve the unfolded intermediates, $P_{U,M}^*$ and $P_{U,S}^*$. Also shown are the corresponding rate constants, k_{ij} , for these interconversions. The subscripts refer to the native and unfolded states, N and U, respectively, and the mobile and stationary phase, M and S, respectively.

Over the last 15 years, a great deal of experience has been accumulated related to the characterization of RPC and HIC chromatographic systems, their method development, and understanding the physicochemical basis of the retention and kinetic phenomena that proteins and peptides manifest in these systems. In common with other modes of interactive chromatography, retention in RPC and HIC is due to the interaction of certain amino acid side chain and main chain functional groups accessible at the molecular surface of a peptide or protein with the stationary-phase ligates. However, in contrast to low-molecular-weight compounds, in RPC and to a lesser extent in HIC globular proteins can unfold to a greater or smaller amount as they traverse the column. Under these conditions, the interior segments of the proteins, which are usually richer in hydrophobic residues, become exposed and reinforce the interaction with the nonpolar chromatographic surface. In RPC and HIC, proteins can unfold to a greater or

lesser extent depending on the hydrophobic characteristics of the sorbent surface and the properties of the mobile phase in terms of its intrinsic polarity, surface tension, temperature, and pH. The presence of different types of organic solvents; kosmotropic, neutral, or chaotropic salts; or other types of mobile phase additives such as ion-pairing agents or detergents has a dual role in the retention and conformational equilibrium of the system. When unfolding processes do occur, different selectivities will be observed for the “native” compared with the “unfolded” state of the protein or polypeptide. As a result, these selectivity differences will lead to elution profiles in RPC and HIC that are fundamentally different from those obtained by other chromatographic or electrophoretic procedures.

The participation of an immobilized nonpolar ligand, such as an *n*-alkyl group chemically attached to porous silica and other ceramic materials, in the interaction with a protein or peptide represents one common theme between RPC and HIC. In the case of macroreticular polystyrene-divinylbenzene, alkylated polyhydroxymethylacrylate, cross-linked agaroses, or other types of derivatized polymeric sorbents, the presence of a nonpolar surface cluster can be achieved via a variety of different polymer- and subsequent surface-modification chemistries. Thus, Shaltiel and coworkers [21,22] have immobilized different *n*-alkyl groups onto cyanogen bromide-activated agarose gels for use in protein isolation. In this approach, a series of low-density *n*-alkylated matrices of increasing carbon number were produced, with the isourea linkage between the agarose and the *n*-alkyl moiety providing a reasonable level of chemical stability. To describe this technique, Shaltiel and Erel [22] coined the term hydrophobic chromatography. For a given mobile phase condition, adsorption increased with the *n*-alkyl chain length. In this manner, the shortest chain length necessary to adsorb a particular protein could be determined. Due to the assumed weak nature of the interaction, induced structural changes in the protein of interest were considered to be minimal. Moreover, mild elution conditions could be selected using weakly chaotropic buffers (e.g., imidazole citrate, sodium glycinate at low pH) that were thought to generate only small, reversible structural changes in the protein. Based on this strategy, several key enzymes involved in the glycolysis/gluconeogenesis pathways were purified [22–24] from crude mixtures by this procedure.

Somewhat analogous procedures have been used by other investigators [24–28] with silica-based support materials to immobilize *n*-alkyl groups of different chain length, permitting the impact of *n*-alkyl chain length on the RPC and HIC behavior of peptides and proteins to be studied. Arising from these investigations, a consensus view has emerged with RPC sorbents that *n*-alkyl chain lengths between C₃ and C₈ were preferred for middle to higher molecular weight proteins, whilst the C₈ to C₁₈ chain lengths offered additional selectivity options with peptides. Thus, a perceived problem (i.e., the participation of non-

specific interactions) in classical affinity chromatography and soft gel SEC was turned to advantage, permitting the possibly denaturing conditions of RPC conditions to be more fully exploited in the analytical and preparative separations of peptides and some proteins, and the influence of different salts to be exploited in protein purification by HIC, in both the low- and high-performance modes.

With regard to subsequent developments of HIC procedures with proteins, additional studies [29–37] have confirmed that retention can be enhanced with longer chain length *n*-alkyl moieties on the support surface [29,31–34] and higher concentrations of kosmotropic salts [30–32] in the mobile phase. To emphasise the nonspecific and noncovalent nature of the binding of proteins to such HIC stationary phase, Hofstee [35] introduced the term “hydrophobic (or nonionic) adsorption chromatography,” whereas other investigators using uncharged hydrophobic sorbents [30–32] considered that the separation mode was better described under the rubric of “hydrophobic affinity chromatography.” The term “hydrophobic interaction chromatography” introduced by Hjerten and colleagues [36,37] remains the more popular description, consistent with a variety of experimental studies on the adsorption behavior of proteins to nonpolar surfaces, including the work by Morris [38,39], Jennissen [40–43], Andrade [44,45], or Hearn [46,47] and their colleagues on the different operating parameters that influence retention and the molecular basis of the separation mechanism. In common with RPC sorbents, ligand density in HIC separations has a profound effect on protein adsorption, resulting in the binding processes occurring through multiple sites. Building upon these studies, a variety of reports have documented the power of HIC as a tool for the purification of enzymes and other proteins [2,15,17,22,23,48–51]. Since that time, HIC using soft gel matrices and high-performance sorbents has been considerably refined and widely used as a generic tool for the purification of many proteins and even plasmids [52], DNA [53], cells [54,55], and virus-like particles [56].

Once issues about the role of secondary chemical equilibrium processes in the RPC of peptides and proteins had been resolved in the early 1980s [57,58], the evolution of stable, efficient RPC systems followed a somewhat similar path to the HIC experience. Various investigations examined the influence of chain length, density, and distribution of the immobilized *n*-alkyl moieties. What sets RPC apart from HIC was that almost immediately, once reproducible procedures for the manufacture of macroporous materials became available, high-performance, microparticulate sorbents were employed, rather than the soft organic or naturally occurring types of polymer gels. Although some aspects of protein adsorption from hydro-organic solvent mixtures to nonpolar stationary phases emerged from work carried out with classical “reversed-phase” paper and column partition chromatographic systems, most of the significant work related to development of an understanding of the chromatographic processes by which

peptides and proteins migrate in RPC systems was inferred from information acquired with low-molecular-weight (pharmaceutical) molecules using *n*-alkylsilicas. For this reason, some key features of the use of macroporous *n*-alkylsilica and other types of hydrophobic sorbent materials will be summarized.

General Characteristics of RPC and HIC Sorbents

Both RPC and HIC employ stationary phases with hydrophobic surfaces. In most RPC cases, these stationary phases consist of a microparticulate rigid porous support, usually silica, with covalently bound hydrocarbonaceous ligates at the surface. In contrast to many of the soft, flexible gel materials used in HIC, where particle sizes are typically in the range 15–65 μm , for analytical and process scale RPC separations rigid particles in the range of average particle diameters (d_p of 1.5–25 μm are usually employed as high-efficiency packings, coated with relatively stable hydrophobic stationary phases. This characteristic of the stationary phase defines the chromatographic system and hence can be considered the heart of the separation method. The average particle diameter (d_p) of an RPC or HIC sorbent will consequently directly affect the resolution of a peptide or protein separation. For most analytical peptide separations by RP-HPLC, for example, *n*-alkylsilicas in the range of $d_p = 1.5$ to 5 μm are now currently employed, with significant differences in efficiencies, separation speed, and resolution evident with the small particles. The penalty here is reduced sample capacity. Numerous illustrations of the very high resolution that can be achieved by such RP-HPLC procedures with *n*-alkylsilicas encompassing this particle size range can be found in the scientific literature for the separations of tryptic maps of proteins. The RP-HPLC separation of the tryptic peptides, labeled *in situ* with the fluorescent reagent 5-*N*-[(iodoacetamidoethyl)amino]-naphthalene-1-sulfonic acid (5-I-AEDANS), of bovine thyrotropin β -subunit [59], achieved under low pH, linear gradient acetonitrile-water elution conditions with an *n*-octadecylsilica of average $d_p = 5 \mu\text{m}$, shown in Fig. 5, is representative of the excellent resolution that can be achieved for peptides in such applications with high-performance microparticulate sorbents.

Once a particular sorbent has been selected, the choice of the mobile phase dictates the relative affinity and selective changes that can be achieved. Depending on the immobilization chemistry, as well as the type of the hydrocarbonaceous ligand and support matrix, a great many types of nonpolar stationary phases with different surface characteristics have been prepared over the past decades, all of which can loosely be encompassed within the category of RPC sorbents. Thus, the shape and pore morphology of the support material, the chemical nature of the immobilized ligand, and the chemistry employed in the bonding reaction all affect the performance of an RPC packing material [60–62]. Linear and branched *n*-alkyl groups of up to 20 carbon atoms have been bound [25,63] to silica surfaces

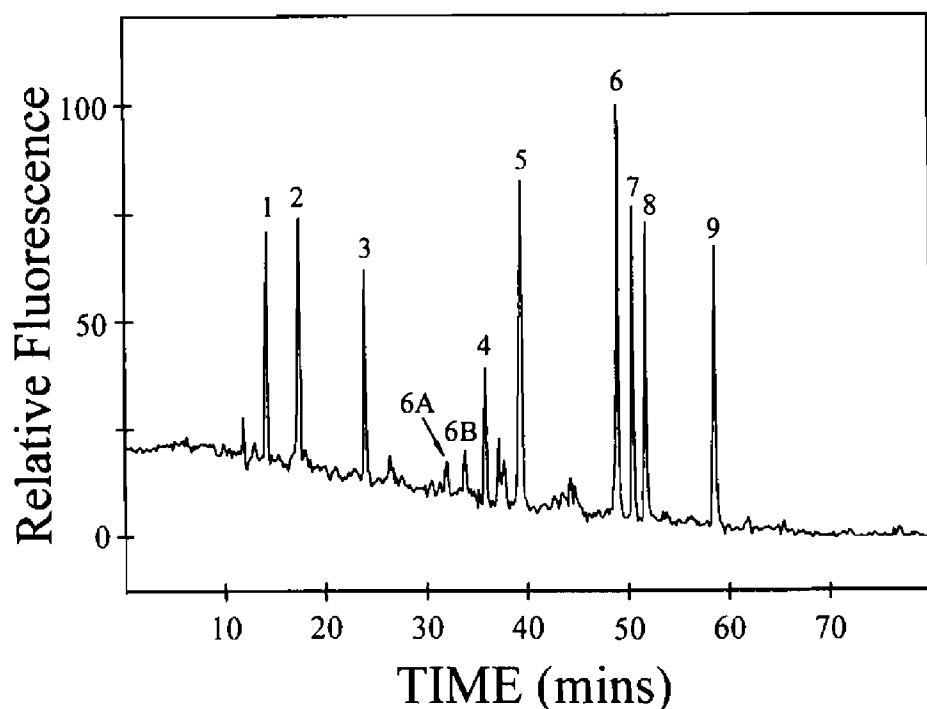


FIGURE 5 Representative RP-HPLC separation of the tryptic/chymotryptic peptides of bovine thyrotropin β -subunit, bTSH β , labeled in situ with the fluorescent reagent 5-*N*-[(iodoacetamidoethyl)amino]-naphthalene-7-sulfonic acid (5-I-AEDANS). The separation of these peptides was achieved under low pH, linear gradient acetonitrile-water elution conditions with an *n*-octadecylsilica of average $d_p = 5 \mu\text{m}$. In this experiment, bovine TSH β was reduced with a 1000-fold molar excess of dithiothreitol (DTT) at pH 8.2 for 3 hr on ice, then alkylated by the addition of 5-I-AEDANS at a five-fold molar excess over the DTT present. The remaining disulfide bonds were then fully reduced and the fluorescently labeled bTSH β -subunit preparation digested first with trypsin followed by chymotrypsin. The fluorescently labeled peptides (1–9) were then separated by RP-HPLC on a Bakerbond C₁₈ column preequilibrated in 0.1% (v/v) phosphoric acid. Elution was achieved with a linear gradient of 0–50% (v/v) acetonitrile in 0.1% (v/v) phosphoric acid over 120 min. All fluorescently labeled peptides, except for the tryptic/chymotryptic glycopeptide, bTSH β [Y¹⁹€LTINTTVEAGY³⁰], where the symbol € corresponds to AEDANS-labeled Cys and the symbol ¶ to the N-linked glycosylation site, were eluted during the first 80 min and could be subsequently characterized. Fluorescent peptides were monitored using a fluorescence detector fitted with a 340-nm excitation filter and 500 nm emission filter at gain 128. The major peptide peaks (1 to 9) were subsequently identified by automated gas phase Edman amino acid sequence analysis, permitting the disulfide connectivities and their relative stabilities of the bTSH β subunit to be ascertained. The peaks 6A and 6b corresponded to further enzymatic digestion of peak 6, namely fragmentation of the peptide C⁸⁸NTDYSDCIHEAIK¹⁰¹. (Data from Ref. 59.)

for RPC separations of peptides and proteins. Figure 6 illustrates the influence of *n*-alkyl chain length from C₁ to C₂₀ on the retention of paracelsin-related peptides separated [25], under the same elution conditions. Paracelsin A–D are 20-meric, amphipathic α -helical peptides, involving alanine (Ala) to aminoisobutyric acid (Aib) and/or valine (Val) to leucine (Leu) substitutions at positions 6 and 9 in their amino acid sequences, respectively. The observed changes in k' as a function of *n*-alkyl chain length clearly reflect differences in the hydrophobic contact areas that these closely related peptides generate on binding to the nonpolar ligands. Edmundsen wheel projections of paracelsin A–D reveal that the Ala $\rightarrow$ Aib and

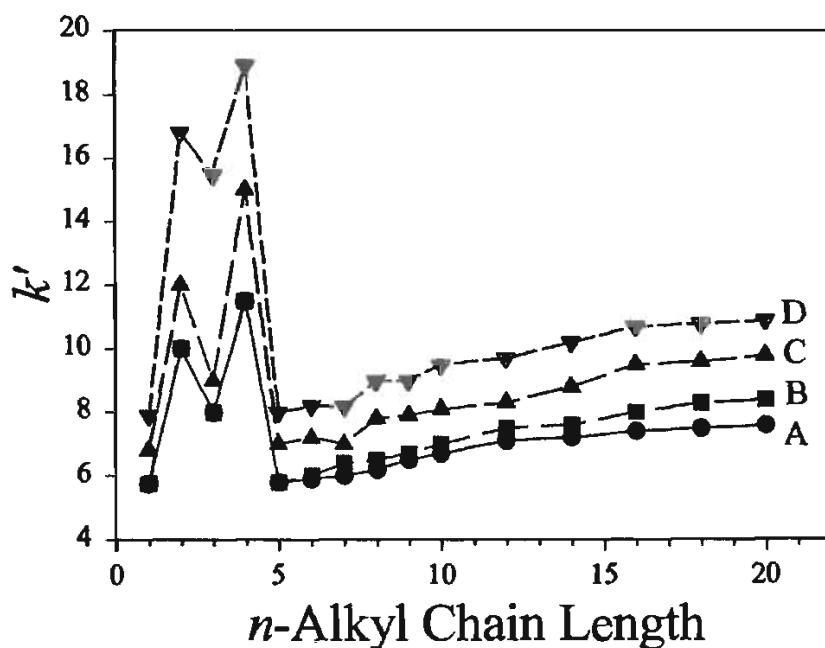


FIGURE 6 Capacity factors, k' , of the paracelsin A–D as a function of the *n*-alkyl chain length of reversed-phase silica sorbents of constant ligand density in water-methanol-acetonitrile (22:39:39, v/v/v). The amino acid sequences of the paracelsin A:D peptides are as follows:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-PheOH	(A)																			
Ac-Aib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-PheOH	(B)																			
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-PheOH	(C)																			
Ac-Aib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-PheOH	(D)																			

The progressive increase in the k' values of the different paracelsin peptides as the *n*-alkyl chain length was increased is consistent with the expected behavior for hydrophobic effects dominating the interaction, while the significant changes in the k' values from C₁ to C₅ are consistent with the transition from the rigid picket fence type of nonpolar sorbent surface typical of C₄ phases to a more relaxed, collapsed phase frequently found for immobilized C₈ to C₁₈ chains. (Data adapted from Ref. 25.)

Val → Leu replacements are in close proximity on the same face when these peptides fold into their α -helical conformations.

Moreover, better mass balances and higher recovery of activity have been found with proteins when RPC stationary phases containing relatively short *n*-alkyl chains [64–68] are employed with either nonporous or porous silicas, with the *n*-propyl, *n*-butyl-, or *n*-octylsilica phases currently the most widely used in protein RPC. However, when short linear *n*-alkyl chains (i.e., $< C_4$) are used, the RPC stationary phases can tend to be less stable than those obtained with longer nonpolar ligates, while the C_8 – C_{18} chains at an equivalent ligand density tend to shield the underlying silica surface more effectively from hydrolysis by the aqueous mobile phase [69,70]. For this reason, shielded phases, such as the bisisobutyl-*n*-alkylsilicas, have emerged [71] as a preferred option for the RPC of peptides and some proteins.

Diphenyl-, cyanopropyl-, and acylated aminopropyl ligates have also been employed as alternatives to *n*-alkyl groups in peptide and protein RPC [4,60–62,72]. Due to their aromatic $\pi \rightarrow \pi$ bonding characteristics, diphenyl groups provide different selectivity from the more conventional *n*-alkyl systems and tend to be less retentive. Cyanopropyl groups are also less hydrophobic and hence also tend to be less retentive with peptides and proteins. As a consequence, peptides and proteins can be eluted from diphenyl- and cyanopropyl-bonded silicas under milder RPC elution conditions, resulting in some cases in better recoveries than can be achieved with the more nonpolar hydrocarbonaceous phases. However, selectivity differences may not be as significant as found with *n*-alkylsilica sorbents, and the column stability can be lower with diphenyl-, cyanopropyl- and acylated aminopropyl phases immobilized onto silica. Analogous situations prevail with the so-called phenyl and cyanopropyl-bonded HIC sorbents, which also behave in a less hydrophobic manner with proteins in the presence of decreasing gradients of kosmotropic salts. The so-called ether-type phases and other mildly hydrophobic stationary phases developed for use in high-performance HIC [12,17,24,73–76] can also be employed in the RPC mode for the separation of very hydrophobic proteins with water–organic solvent eluents [77].

The selectivity of RPC sorbents is affected by the bonding chemistry in the case of silica- or ceramic-based sorbents or the type of monomer units in the production of polymeric RPC sorbents. Treatment of silica or other inorganic oxide particles is usually carried out with mono-, di-, or trifunctional organosilane reagents [60–71,78,79] under anhydrous conditions in the presence of a small amount of catalyst. Alternatively, triethoxy-*n*-alkylsilanes can be used under either anhydrous or aqueous modification conditions. Monofunctional chlorosilane reagents, including the sterically shielded phase reagents [80], generally yield more efficient sorbents. Higher surface coverages may be attained by using di- and trifunctional silanes [61–71,81] at the cost of reduced mass transport kinetics. In some cases with sorbents derived from surface modification with

trifunctional silanes, such as triethoxy-*n*-alkylsilane, greater sorbent stability at low pH can be obtained compared with the use of monofunctionalized *n*-alkylsilane, although typically resistance to hydrolytic degradation with buffers containing inorganic anions or cations is more impaired [82] at higher pH values such as pH 9–10. As an alternative, stable high-coverage nonpolar stationary phases can be prepared by cross-linking a polymer or oligomer at the support surface, via either free radical [83] or condensation [84] polymerisation chemistry. In this case, the underlying silica is partly protected from hydrolytic degradation due to the presence of the hydrophobic polymer film coating that effectively shields the support material. Similar procedures have been employed [85–87] to chemically modify the surface of a wide variety of other support materials, such as porous zirconia, titania, or alumina, to impart further resistance to degradation when alkaline mobile phase conditions are employed. Porous polystyrene-divinylbenzene sorbents, because of their intrinsic hydrophobicities, also fulfill useful roles in the analytical RPC of peptides and proteins. Although these types of particles are resistant to alkaline pHs, most applications still favor low pH elution conditions to achieve resolution of peptides and proteins. With the macroporous polystyrene-divinylbenzene sorbents, such as the perfusive Poros RPC materials [88,89], high superficial velocities can be employed without sacrificing resolution. The advent of monolithic hydrophobic sorbents has introduced [90–93] a further opportunity to modulate selectivity and resolution with peptides and proteins under conditions of high superficial velocities and mobile phase compositions that encompass high pH values.

Although a large number of protein purifications have been achieved with soft gel HIC systems, these sorbents require the use of low flow rates and hence involve long separation times. For this reason, high-performance HIC (HP-HIC) sorbents have been developed based on the same considerations as employed for the generation of RP-HPLC sorbents. Thus, pressure-stable, microparticulate HP-HIC packings, derived from silica materials with average d_p values in the range 5–10 μm are now available. HP-HIC sorbents based on 1.5- μm nonporous silica are also finding application at relatively high flow rates with excellent chromatographic peak efficiencies [94]. The evolution of HP-HIC sorbents has followed a similar path of development and with selection criteria used for the choice of ligand chemistries similar to those used for the preparation of the corresponding soft gel HIC systems or for that matter RP-HPLC sorbents. Thus, initial studies with high-performance size exclusion chromatographic gels (HP-SEC) documented that the elution volume of some proteins exceeded the total internal volume of the column; i.e., the protein was retained by the surface of the stationary phase at low and/or high ionic strength [95–99]. Typical of this behavior with silica-based HPSEC sorbents are the plots [98] shown in Fig. 7 of the dependence of the size exclusion distribution coefficient, K_D , for the small globular protein, hen egg white lysozyme, versus the ionic strength, I , of the mobile phase for several

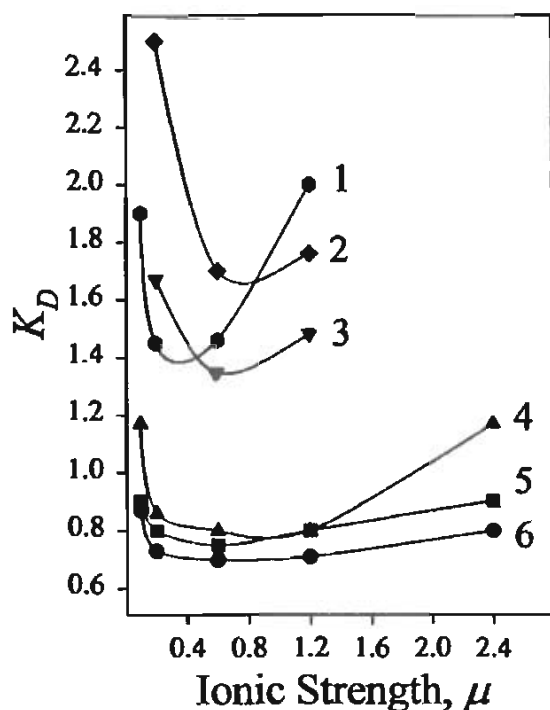


FIGURE 7 Dependences of the size exclusion distribution coefficient, K_D , for the small globular protein hen egg white lysozyme versus the ionic strength, I , of the mobile phase for several notional size exclusion sorbents of different average pore diameter, particle size, and surface chemistry characteristics. The sorbents employed in these investigations were 1, Synchronapak GPC 100; 2, Waters I-125; 3, Shodex OH Pak B-804; 4, Lichrosorb Diol; 5, Tosoh TSK SW 3000; and 6, Tosoh TSK SW 2000. The interplay of hydrophobic interaction and electrostatic phenomena, superimposed upon the size exclusion effect due to the differences in the pore sizes of the support materials, is particularly evident with these sorbents at high- and low-ionic-strength conditions. (Data adapted from Ref. 98.)

sorbents of different average pore diameter, particle size, and surface chemistry characteristics. The participation of hydrophobic interaction and electrostatic effects is particularly evident for HEWL with the sorbents shown in Fig. 7 for the high- and low-ionic-strength elution conditions, respectively.

Elution dependences of this type are commonly experienced with silica-based HP-SEC sorbents and other highly cross-linked polymer-based gels. Characteristic U-shape dependences arise with such sorbent systems due to the interplay of contributions at low ionic strength of electrostatic interactions between the protein and the stationary phase, size exclusion at intermediate ionic strength, and hydrophobic interactions at high ionic strength. This retention behavior, attributable to the combined influences of the surface chemistry of the silica support material and the procedures used to “passivate” the silica surface *via* chemical modifications with, for example, γ -glycidoxylpropyltrimethoxysilanes (Glyco phases) is, in fact, characteristic of all interactive modes of chromatography with peptides and proteins. The important question is which

of the separation modes—electrostatic, size exclusion, or hydrophobic interaction—dominates the retention mechanism under a particular set of experimental conditions. As was the case in the early days of soft HIC gels, the use of high-ionic-strength conditions for enhancement of the interaction of proteins or peptides with weakly hydrophobic surfaces was initially viewed as detrimental rather than of great potential.

Fortunately, conjoint with these HP-HIC developments, RP-HPLC procedures were emerging in the early 1980s as very powerful tools for peptide separations [58,72,100,101] and were under active development at that time for the analysis and purification of proteins. A surprising observation that emerged from these initial comparisons between RP-HPLC and HP-HIC was that the elution order of proteins in RPC was significantly different from that found in HIC. Not as surprising was the observation that RPC often yielded lower recoveries of biological activity than HIC for the same proteins. Illustrative of these effects are the results described by Fausnaugh et al. [15] on the elution order for 12 commonly available proteins separated on columns of similar dimensions packed with HIC and RPC sorbents of the same particle ancestry and characteristics. Thus, with the HIC sorbent, the elution order was cytochrome *c* < myoglobin < ribonuclease < conalbumin < ovalbumin < lysozyme < β -glucosidase < chymotrypsin < α -chymotrypsinogen < lactoperoxidase < BSA < ferritin, while with the RPC sorbent the elution behavior followed the order β -glucosidase < ribonuclease < cytochrome *c* < α -chymotrypsin < lysozyme < myoglobin < ferritin < α -chymotrypsinogen < BSA < conalbumin < ovalbumin < lactoperoxidase. Moreover, the selectivity factors, α , between adjacent protein peaks were different under these HIC and RPC elution conditions.

Such experimental comparisons between HIC and RPC with peptides and proteins are of particular interest because they provide discriminatory information about the specific processes involved in the interaction despite the retention mechanism *per se* being based on the same fundamental principle. Because the tertiary structure of proteins may be significantly different under RPC conditions from that occurring in HIC, the two methods are, in effect, often separating species of different molecular structure. The relative accessibility of the nonpolar amino acid residues in proteins and peptides will to a large measure dictate the extent of retention in both RPC and HIC. The exposure or greater accessibility of previously buried hydrophobic amino acid side chains in globular proteins or, alternatively, peptides with well-developed secondary structures will have a significant effect on the relative binding affinities to hydrocarbonaceous bonded phase surfaces. Detailed studies [18,102–109] have shown that a major contributor underpinning this difference between RPC and HIC retention with proteins and polypeptides is the extent of changes in conformation mediated by differences in ligand composition and/or density at the surface of the sorbent or the mobile phase composition.

Comparative examination of these outcomes with the early types of HIC sorbents suggested that rigid, microparticulate macroporous stationary phases, analogous to those increasingly being used in RP-HPLC, could be employed for the high-performance separation of proteins without denaturation. In this case, the HIC support surface required chemical modification with ligands that were significantly weaker in terms of their intrinsic hydrophobicities; i.e., modification with the so-called ether-type ligands and their siblings resulted in “biocompatible” mildly hydrophobic immobilized coatings [73,76,110,111]. With such HP-HIC sorbent available, the implications of the earlier results obtained for protein separations with RP-HPLC and HP-SEC systems became clear: kosmotropic salts could be used in the mobile phase to affect a change in the water structure and surface tension; thereby the separation of proteins could be based on weak hydrophobic interaction, thus permitting recovery of the proteins after separation without denaturation.

RETENTION MODELS IN RPC AND HIC

General Characteristics of Peptides and Proteins

In all RPC and HIC separations, the fundamental structural properties of the amino acid entities themselves, which form the building blocks of peptides and proteins, determine the overall selectivity that can be achieved with a defined chromatographic system. What sets peptides and proteins apart from low-molecular-weight organic compounds is their structural hierarchy. Thus, the chemical organization dictated by the amino acid sequence and the various levels of the folded (i.e., the secondary, tertiary, and quaternary) structure determine the overall topographical features of these molecules and how they will interact with the solvent or nonpolar ligates. Superimposed upon the molecular features of these biomolecules are the chemical and physical attributes of the chromatographic system *per se*. These characteristics of a RPC and HIC system will directly affect the molecular properties of peptides or proteins, whether or not they have been modified chemically or posttranslationally with carbohydrate moieties, lipids, or other structural entities.

Depending on the nature of the amino acid sequence, unique regions within a peptide or protein can adopt preferred secondary structures such as α -helical, β -sheet, or β -turn motifs. These motifs can then consolidate and close pack to generate transient tertiary structures (molten globule intermediates) that undergo final reorganization and disulfide bond formation to generate the tertiary structures of a correctly folded protein or polypeptide. The propensity for these structures to form in solution or at a solid-liquid interface is dictated by the presence of nucleation sites for folding within the peptide or protein, by the properties of the amino acids themselves, and by the characteristic features of the

surrounding solvent. Although the fundamental properties of amino acids in terms of their mass, volume, accessible surface area, partial specific volume, pK_a , relative occurrence, and hydrophobicity have relevance in this context (Table 1), it is the magnitude of the overall free energy changes associated with the folding and retention processes that determines what conformation a peptide or protein will adopt under a defined set of chromatographic conditions.

Typically, a protein in its native conformations has a small, favorable free energy for folding, i.e., a small negative $\Delta G_{\text{fold},i}^\circ$ value. For monomeric globular proteins, $\Delta G_{\text{fold},i}^\circ$ is typically in the range of -5 to -15 kcal/mol. The change in the free energy, $\Delta G_{\text{assoc},i}^\circ$, associated with the interaction of a peptide or protein with the immobilized nonpolar ligands of an RPC or HIC sorbent is frequently of similar magnitude. The contributions from the various enthalpic and entropic terms associated with solvation, hydrogen bond formation, other nonbonding organizational processes and changes in order of the system that occur in solution or following interaction at the liquid-solid interface of a chromatographic sorbent can have large positive or negative values depending on the process. Overall, however, the result is a compensatory effect with the summated values leading to the observed similar values of $\Delta G_{\text{fold},i}^\circ$ and $\Delta G_{\text{assoc},i}^\circ$. The participation of these thermodynamic and extrathermodynamic processes sets the stage in the bulk solution for different sequence regions within an unfolded polypeptide or protein to progressively nucleate, form secondary structures, and finally to adopt, if the conditions are favorable, a native three-dimensional structure. Along the pathway to this energetically favored folded state, the protein or peptide can sample in solution a relatively large proportion of its conformational space. Similar considerations apply, under some chromatographic conditions, to the transitions that a folded or partially folded protein or polypeptide can undergo, leading progressively to the emergence of the fully unfolded (random coil) state. Both RPC and HIC procedures offer considerable potential when carried out in these biophysical modes to reveal important information on these changes in Gibbs free energy and the enthalpy or entropy changes associated with the unfolding processes. The same methods can be used to examine the reverse of this process, namely the unfolding trajectories. It is thus essential to assess constantly when undertaking an RPC or HIC separation of peptides and proteins how the choice of the chromatographic conditions and the experimental adjustments, such as flow rate and temperature, might affect the conformational status of these biomolecules. The question is, by how much?

It is well known that conformational stability, and frequently the solubility, of peptides and proteins can be manipulated by exposure to different salts, organic solvents, organic polymers, and pH changes particularly near the isoelectric point or alternatively to aqueous-aqueous or aqueous-nonaqueous two- or multiphase liquid-liquid systems. In all of these cases, advantage is taken of the changes in the bulk hydration and molecular cavity properties of the

TABLE 1 Fundamental Properties of Amino Acid Residues in Terms of Occurrence in Proteins, Molecular Mass, Molecule Volume, Accessible Surface Area, Partial Specific Volume, pK_a of Ionizing Side Chains, and Relative Hydrophobicity

Residue	One-letter abbreviation	Occurrence in proteins [8,573,574] (%)	Mass (Daltons)	Volume (A^3) [575]	Accessible surface area (A) [576]	Partial specific volume (mL/g) [575]	pK_a of ionising side chain [577]	Relative hydrophobicity [212]
Ala	A	9	71.08	88.6	115	0.748		0.06
Arg	R	4.7	156.2	173.4	225	0.666	~12	-0.85
Asn	N	4.4	114.11	117.7	160	0.619		0.25
Asp	D	5.5	115.09	111.1	150	0.579	4.5	-0.20
Cys	C	2.8	103.14	108.5	135	0.631	9.1 to 9.5	0.49
Gln	Q	3.9	128.14	143.9	180	0.674		0.31
Glu	E	6.2	129.12	138.4	190	0.643	4.6	-0.10
Gly	G	7.5	57.06	60.1	75	0.632		0.21
His	H	2.1	137.15	153.2	195	0.67	6.2	-2.24
Ile	I	4.6	113.17	166.7	175	0.884		3.48
Leu	L	7.5	113.17	166.7	170	0.884		3.50
Lys	K	7	128.18	168.6	200	0.789	10.4	-1.62
Met	M	1.7	131.21	162.9	185	0.745		0.21
Phe	F	3.5	147.18	189.9	210	0.774		4.80
Pro	P	4.6	97.12	122.7	145	0.758		0.71
Ser	S	7.1	87.08	89	115	0.613		-0.62
Thr	T	6	101.11	116.1	140	0.689		0.65
Trp	W	1.1	186.21	227.8	255	0.734		2.29
Tyr	Y	3.5	163.18	193.6	230	0.712	9.7	1.89
Val	V	6.9	99.14	140	155	0.847		1.59
		α -Amino	6.8 to 7.9		α -Carboxyl	3.5 to 4.3		

Source: Ref. 6.

peptides and proteins when they are placed in solution. Depending on the solution conditions, all peptides and proteins are capable of forming finite and/or infinite intermolecular networks of solvated monomeric structures, which can lead to self-associated (isodesmic) species and finally to aggregates. Whether the peptide or protein exists as a monomer or as an aggregate will thus depend on its concentration and its solubility parameter under a particular set of solvent, pH, and ionic strength conditions. Participation of these processes thus clearly involves solution chemical equilibrium. Hence, the potential exists to modulate rationally separation selectivities and zone-broadening processes of peptides and proteins in both RPC and H[C chromatographic systems by taking advantage of their molecular characteristics at the same time as examining the influence of the experimental conditions on the conformational status.

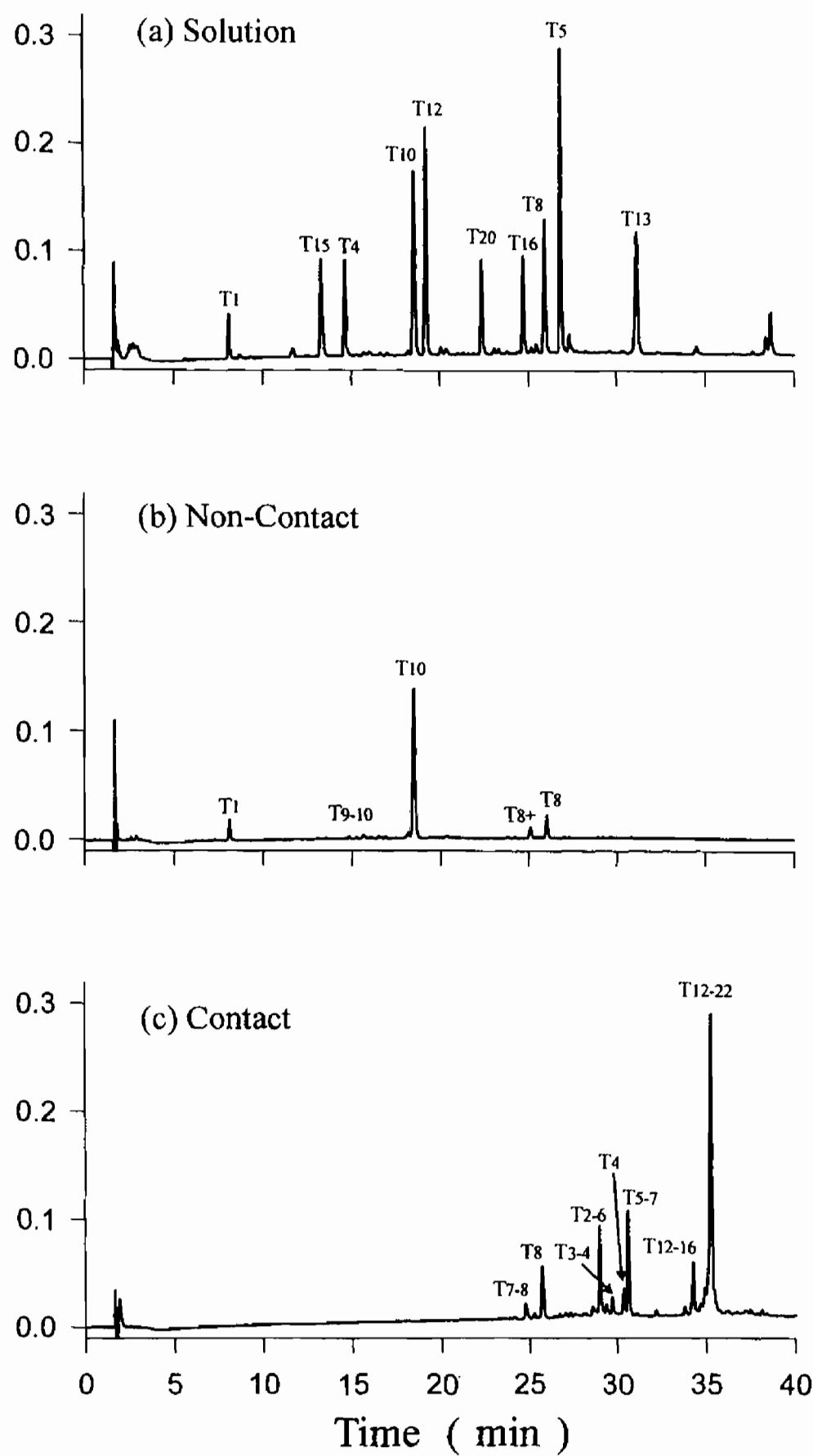
Structure, Conformation, and Spectroscopic Tools

In order to elucidate the molecular forces involved in these processes, a comprehensive biophysical experimental strategy is required. The current generation of instrumentation—particularly high-field ^1H nuclear magnetic resonance (NMR) or solid-state heteronuclear NMR [112–114], mass spectrometry in its different modes (e.g., electrospray ionization mass spectrometry (ESI-MS) or matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF) [115–118], circular dichroism–optical rotary dispersion spectroscopy (CD-ORD) [119,120], derivative ultraviolet-visible spectroscopy (UV-Vis) [121,122], time-resolved polarization fluorescence [123,124], diffuse reflectance Fourier transform infrared spectroscopy (DRIFT) [125,126], laser-activated light scattering (LALLS) [127,128], scanning differential micro-calorimetry [129–130], and Raman and other spectroscopic techniques [131,132]—provides a basis to explore these physicochemical aspects at the molecular level. Until recently, the interaction of solvent ions, and free or immobilized ligands with peptides and proteins has, however, been largely explored only by methods that distinguish bulk differences in the physical characteristics of these biosolutes when in the bulk mobile phase or after desorption. In the latter case, assumptions have frequently been made that interconversion between different conformational or association states following desorption is associated with slow kinetics. Precise information on the microenvironmental changes that occur in the physical state or chemical properties of the peptide or protein solute in contact with the chromatographic surface has usually not been obtained to substantiate these claims, with the consequence that some caution [133] should be adopted to avoid the overinterpretation of experimental results, which has arisen with some recent studies [134] due to lack of sufficient experimental data.

The availability of orthogonal spectroscopic procedures and more powerful computational modeling methods have started to provide a basis to explore in

greater detail the precise mechanisms of interaction and the forces involved in these molecular recognition events when peptides and proteins are docked with solvated immobilized nonpolar ligands. For example, with the availability of powerful instrumental techniques such as surface-activated ionisation mass spectrometry (SAI-MS), $^1\text{H} \rightarrow ^2\text{H}$ amide exchange ESI-MS [118,135], or heteronuclear two and three-dimensional NMR [136,137], coupled with experimental approaches based on molecular footprinting procedures [59,138–140], the possibility now exists to define precisely the molecular features of a peptide or protein when it docks to a chromatographic surface. Findings [138,139,141] based on this molecular footprinting–spectroscopic approach have delineated how different cytochrome *c*'s and growth hormones dock to *n*-butyl ligate surfaces. For example, proteolytic digestion of proteins bound to RPC or HIC sorbents can be employed to delineate putative binding sites. In these cases, the experimental design involves the analytical RP-HPLC separation of tryptic (or other enzymatically generated) peptides generated from the complete digestion of the protein in the free (bulk solution) and bound states. Figure 8 illustrates one such case, wherein the putative binding site for equine cytochrome *c* on ligate capture by a *n*-butylsilica sorbent has been elucidated. Other examples, based on the use of similar techniques coupled with molecular modeling, including molecular dynamics procedures, have led [108,109,142] to new insight into the manner in which bioactive peptides can be organized at the surface of RPC and HIC sorbents.

Gel filtration–size exclusion chromatographic (SEC) methods and other noninteractive separation techniques largely reflect a bulk property of a peptide or protein in terms of its average molecular weight or average Stokes hydrodynamic radius. Such bulk properties are often taken to imply either a fixed physical state or a state of slowly interconverting conformers for a biopolymer. In reality, all peptides and proteins undergo continuous, dynamic changes in their shape, self-association, and mobility in response to variations in the properties of the surrounding liquid environment. In all cases, these environmental changes affect the chemical potential of these biopolymers and involve reversible solution equilibrium processes. When such phenomena are rapid (i.e., with half-lives in the range of nanoseconds to milliseconds), they have a minimal influence on RPC or HIC selectivities or peak shapes—to all intents and purposes, the nonpolar ligates are sampling the same set of rapidly averaging molecules. However, when these phenomena have long relaxation times (i.e., of the order of seconds or even minutes to hours), major variations in selectivity can arise as part of the RPC or HIC separation. In some cases, these effects can have catastrophic consequences, i.e., lead to dissociation of multimeric proteins, formation of long-lived, inactive conformational intermediates, and denaturation or precipitation, and are associated with very complex adsorption isotherms. Illustrative of the interplay of such effects is the dissociation of the heme moiety from sperm whale myoglobin



under low-pH elution conditions (Fig. 9) in the RPC system where the *apo*-protein is generated as the major chromatographic protein species, which still retains a considerable proportion of the native secondary and tertiary structure. In this case, the use of diode array (DAD) spectrometry provided a very facile procedure to characterize the dissociation of the prosthetic group as the *apo*-

FIGURE 8 RP-HPLC separation of (a) solution tryptic fragments, (b) noncontact fragments, and (c) contact fragments of horse heart cytochrome *c*. The fragments in (b) and (c) were generated by tryptic digestion of horse heart cytochrome *c* adsorbed to a C₁₈ sorbent; the peptides for the experiment shown in (a) were generated by bulk tryptic digestion of a sample of horse heart cytochrome *c* in bulk solution. These analyses of the tryptic fragments were performed with Zorbax 300 SB-C8 reversed-phase columns with dimensions of 4.6 mm × 15 cm with particles 5 μm in diameter and 300 Å average pore size. Linear gradient elution was performed from 0.1% (v/v) TFA in deionized water (buffer A) to 0.09% (v/v) TFA with 50% (v/v) aqueous acetonitrile (buffer B) with a gradient time of 50 min at a flow rate of 1 mL/min. The sorbent used for the in situ digestion experiments was a Vydac C₁₈ silica (5 μm particle size and 30 nm pore diameter) (2 mg per mg of horse heart cytochrome *c*) washed with 1 mL methanol, 3 times with 1 mL 35% (v/v) acetonitrile-water, and then suspended for 2 hr in 1 mL 0.1% TFA containing horse heart cytochrome *c* under continuous agitation conditions to allow maximal binding. After centrifugation, the pellet was washed four times with the cleavage buffer (50 mM ammonium bicarbonate, 2 mM calcium chloride, 1 mL) to remove all unbound horse heart cytochrome *c*. The sorbent with bound horse heart cytochrome *c* was then incubated with TPCK-treated trypsin in the cleavage buffer, using a protein/trypsin ratio of 10 : 1. The enzymatic proteolysis was stopped after 18 hr with two drops of 2 M HCl. After centrifugation of the sorbent, an aliquot of the collected supernatant containing tryptic peptides generated from regions of horse heart cytochrome *c* that did not form part of the contact region or alternatively did not rebind to the C₁₈ sorbent [the noncontact fragments (b)] following proteolysis of the protein in the presence of the cleavage buffer was analyzed by RP-HPLC under the same chromatographic conditions as used for the tryptic digest of horse heart cytochrome *c* generated in the absence of sorbent material, with the same protein/protease ratio and the same cleavage conditions [the free solution digest (a)]. The remaining tryptic peptides, which were bound to the sorbent [the contact fragments (c)] were eluted from the sorbent with 75% acetonitrile/0.1% TFA. After centrifugation, an aliquot of the combined supernatants of these "contact" fragment peptides was analysed by RP-HPLC. Amino acid composition analysis, N-terminal sequence analysis, electrospray mass spectrometry, and capillary electrophoresis of the tryptic peptides confirmed the structures of the individual peptides (T1–T16) and thus the regions of horse heart cytochrome *c* protected from proteolysis when the protein is bound to the hydrophobic sorbent. (Data from Ref. 140.)

myoglobin species was formed. Similarly, on-line electrospray ionization mass spectrometry (ESI-MS) provides a powerful tool to characterize similar dissociation events induced by the chromatographic conditions. Knowledge of these characteristics of a peptide or protein on interaction with components in the mobile phase or those present on the stationary phase surface and optimization of their consequences, are particularly pertinent to the development of robust separations strategies for RPC or HIC applications.

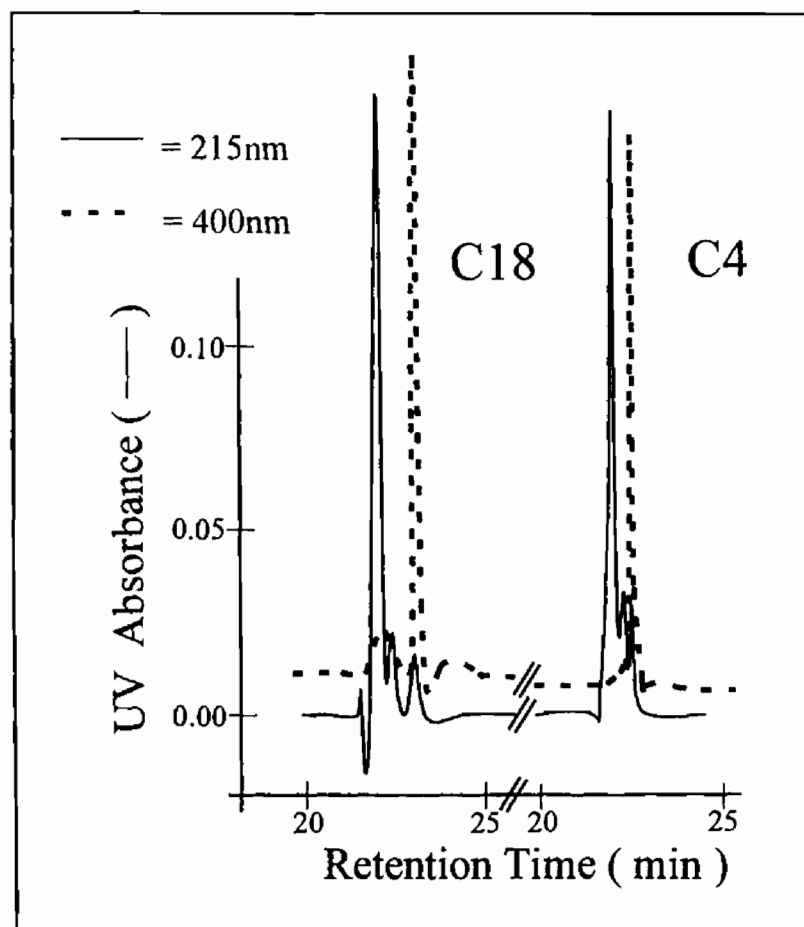


FIGURE 9 Elution profiles for sperm whale myoglobin (SWMYO) chromatographed on Bakerbond C₁₈ and C₄ sorbents, monitored at 215 and 400 nm. The mobile phase consisted of a linear 60 min gradient of 0.1% trifluoroacetic acid-H₂O to 90% acetonitrile-H₂O containing 0.1% trifluoroacetic acid (buffer B), at a flow rate of 1 mL/min. In this study, the SPMYO sample was chromatographed on the C₁₈ and C₄ sorbents (nominal 5 μ m particle size and 30 nm average pore diameter) packed into columns of dimensions of 250 $\times$ 4.6 mm ID. at 25°C. On-line photodiode array detection (DAD) was achieved using an LKB 21440 Rapid Spectral Detector, and the spectra were analyzed using the Wavescan software. The later eluting peak, with absorption at 400 nm, corresponds to the heme moiety. (Data from Ref. 106.)

Hydrophobic Effects and Solvophobic Considerations

The interaction between a solvated peptide or protein and a chemically modified RPC and HIC stationary phase in a fully or partially aqueous solvent environment can be discussed in terms of the interplay of weak physical forces. The main types of physical interactions that are involved in order of relevance and dominance for the establishment of the selective recognition and binding between a peptide or protein and RPC and HIC ligates are (1) hydrophobic interactions and related phenomena mediated by polarized electron donor or electron acceptor processes, (2) Lifshitz-London forces and van der Waals and associated weak dipolar interactions, (3) $\pi \rightarrow \pi$ and $\pi \rightarrow$ dipole interactions, (4) hydrogen bond interactions, (5) electrostatic interactions, (6) metal ion coordination interactions, and (7) secondary macromolecular interactions involving force field effects.

Figure 10 illustrates the respective contributions to the changes in the energy of interaction that occur due to these effects. Close-packing interactions mediated by van der Waals effects between nonpolar amino acid side chains have been shown [143] to play an important role in the folding of peptides and proteins. However, the weak van der Waals interactions make only a small contribution to the negative change in enthalpy that arises when a peptide or protein is placed into water. As the nonpolar ligands more closely approximate the sequestered environment that exists for the nonpolar side chains in a folded protein, van der Waals interactions probably make a greater contribution to the overall free energy change when the biosolute is in the bound state. The London-van der Waals forces are influenced by the size and geometry of the molecules involved in the interaction(s) and by the ease of polarization of their electron clouds. These momentary shifts in the symmetry of the electron clouds of a peptide or protein molecule when approaching a nonpolar surface will thus be conditional on the solvent environment and composition, the type of nonpolar ligate (i.e., *n*-alkyl versus phenyl ligands), the temperature, and the chemical nature of the support material that forms part of the sorbent. Of all of the forces, the contributions from hydrogen-bonding effects probably represent the greatest impact for folded polypeptides and proteins. Although the differences in the hydrogen bond energies for a folded and unfolded polypeptide or protein may overall be small, it should be recalled that hydrogen bond energies are typically about 20–40 kJ mol⁻¹, and thus only a few hydrogen bonds may have to be disrupted for fragile proteins to unfold irreversibly.

According to solvophobic considerations, when a peptide or protein is dissolved in a solvent, a cavity is formed with the molecular dimensions of the solute. If water is the solvent, then a negative change in enthalpy will arise because overall there will be a net reduction in the number of hydrogen bonds in the system. Concomitantly, there will be an increase in the order of the water molecules near the cavity containing the peptide or protein, resulting in a negative

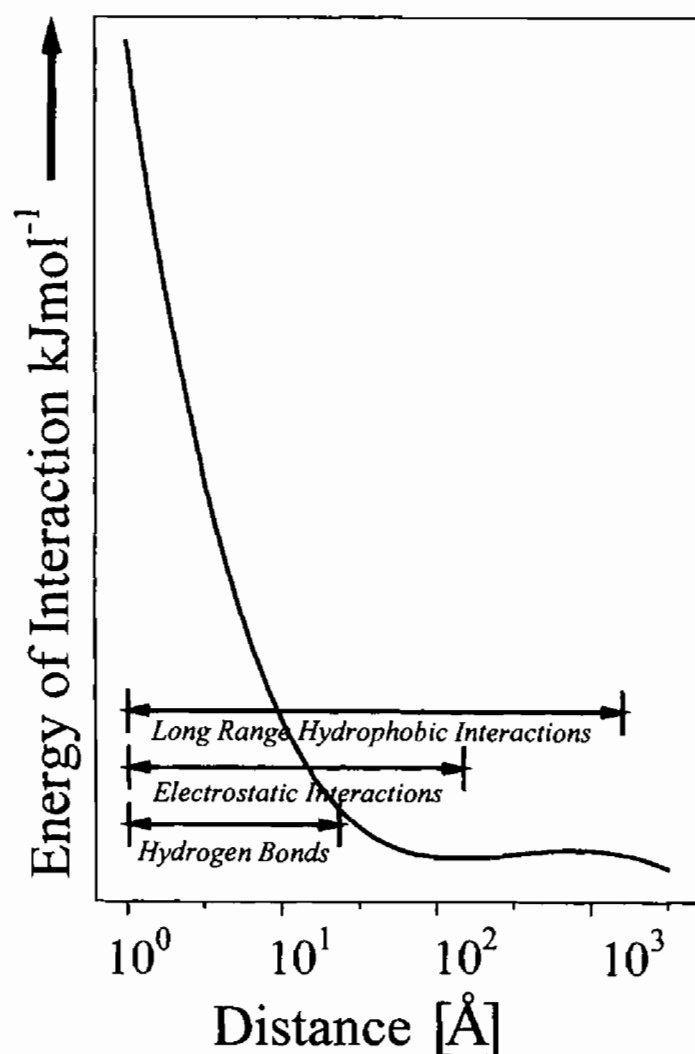


FIGURE 10 Plot of the energy of interaction (in kJ mol^{-1}) versus the distance between the interacting species during the very early stages of an adsorption process. When the distance is relatively large in atomic units, the respectively orientated primary interactions are based on contributions from the long-range Lifshitz–London–van der Waals forces and/or hydrophobic effects. As the approach distance decreases, the energy term exhibits a greater electrostatic component, with a dependence of $1/r^2$. On further closure of the approach distance, hydrogen bond effects become increasingly significant, with a distance dependence of $1/r^3$. On closer association the short-range London forces–van der Waals attractions mediated by fluctuating electrical charges and dipoles of the polypeptide or protein will occur, followed by strong repulsion and large increases in the energy of interaction at very small approach distances.

change in entropy. When an interaction occurs with the immobilized hydrophobic ligands of the RPC or HIC sorbent, an increase in entropy will arise as the solvent molecules leave the more structured environment of the partially solvated solute for the unstructured environment of the bulk mobile phase. The outcome is a

positive change in enthalpy, but because this will typically be smaller than the change in entropy, enthalpic changes do not normally control the spontaneous association, at least within the usual range of temperatures employed in RPC or HLC separations of peptides or proteins; i.e., the process is entropy driven. Although no comprehensive, de novo molecular dynamic or biophysical treatment of the binding mechanism of peptides and proteins to nonpolar ligates has been yet established, various avenues of investigation have indicated that the binding processes can be modeled as a multistage reaction. Thus, for phosphor-ylase *b*, the rate-limiting step appears [43] to be a slow conformational change and/or structural reorganization of the protein in association with the nonpolar ligands rather than the mass transfer of the protein to the lipophilic sorbent. Similarly, with subtilisin muteins [144], avian lysozymes [145] as well as from data acquired with other homologous proteins [146], it is apparent that both the distribution of nonpolar amino acid residues and the effective hydrophobic surface area of a globular protein influence the affinity of the interaction with HIC sorbents, and similar effects are evident with the corresponding RPC sorbent materials.

Both entropic and enthalpic processes thus contribute to retention via the hydrophobic effect, with the influence of enthalpic and entropic processes varying considerably with temperature. As a consequence, the hydrophobic effect changes from an entirely entropically driven process at temperatures near 25°C to an entirely enthalpically driven process at about 113°C, assuming that the change in the heat capacity term ($\Delta C_{p,i}^\circ$) for the different states of the peptide or protein remains constant at higher temperatures. Retention in RPC and HIC can be considered to be primarily due to a solvent-mediated hydrophobic effect and thus from a conceptual perspective involves the noncovalent association of non-polar moieties present within the protein or peptide with the nonpolar ligates, both of which have been solvated to differing extents in an aqueous or semiaqueous environment. Since it was first described by Kauzrnan [147], the hydrophobic effect has been recognized to play a very important role in the all areas of biomolecular recognition and interactions, in protein architecture, and in the establishment of integratrd biological systems [148].

The retention process in RPC and HIC can be described from a number of theoretical perspectives, either in terms of rigorously consistent treatments based on the thermodynamics of interaction or from more empirical considerations. Of the approaches that have gained currency, the solvophobic theory [149,150], the “close-contact” model [151–153] with the interactions based on van der Waals and other short-range forces, and the stoichiometric solvent displacement model [154,155] have attracted the most interest. All theories assume that “near-equilibrium” conditions are achieved in the interactions of proteins or peptides with the nonpolar ligates. In most models, the factors affecting the magnitude of the changes in retention have been assumed to be either independent or,

alternatively, first order with regard to each other. Proteins and peptides are also often assumed to bind to RPV or HIC sorbents in such a way that close contact is established between the hydrophobic ligates of the sorbent and the hydrophobic moiety accessible at the surface of these biomolecules. In some models, no distinction is made between an adsorption event, with the retention thus dependent on surface area relationships, and a partition event, whereby the bioanalyte penetrates into the immobilized ligands and the retention is dependent on partition coefficient relationships. Existing theories, of which the solvophobic approach is the most comprehensive, attempt to explain the changes in retention in terms of changes in the strength or affinity of the binding, as measured by the logarithmic retention factor, $\log k'$. In the adsorption model, $\log k'$ is proportional to the molecular contact area, $\Delta A_{\text{contact}}$, whereas in the partition model, $\log k'$ has a dependence on $\log P$, where P is the partition coefficient.

Over the last 20 years, the concept that the magnitude of the hydrophobic effect can be equated with the free energy of transfer of hydrophobic species from a nonpolar phase to water thus has gained considerable experimental and theoretical support. Both solubility determinations with amino acids and peptides and RPC measurements indicate [156] that the hydrophobic effect is strongest when the entropic component dominates the process—observations that are discordant with classical thermodynamic interpretations. Although the involvement of hydrophobic effects in RPC and HIC can be interpreted [152,153] in terms of van der Waals interactions between nonpolar groups on a peptide or protein, with the solvation of these groups by water leading to a favorable van der Waals effect (i.e., the close-contact model), other contributions from the hydrogen bonds involving interactions with the bulk water and solvent displaced from the peptide or protein when it docks with the nonpolar ligate may negate this type of van der Waals interaction. To overcome this limitation, alternative explanations of the role of the hydrophobic effect in RPC and HIC interactions can be proposed [157–159], based on the flickering cluster model, the random network model, or the continuum model for liquid water.

According to these adapted theoretical treatments, the hydrophobic effect can be equated with the change in order/disorder of the water surrounding a cavity, which has molecular dimensions corresponding to that of the peptide or protein in solution. These models satisfactorily describe the entropy component at low temperatures, based on the formation of clathrate-like cages of highly structured water surrounding the nonpolar moieties of the peptide or protein. Common linkages exist between these models for a peptide or protein in bulk aqueous solution and the solvophobic model for RPC and HIC. Thus, in the bulk mobile phase, as the temperature increases, the hydrophobic effect becomes more enthalpic in character, with the hydrogen bonds, dipole interactions, and van der Waals close contacts associated with the higher order structures of the peptide or protein becoming disrupted, while the surrounding highly ordered, structured

water collapses, permitting the cavity to expand and the peptide or protein to commence to unfold. Similar effects can be invoked for peptides or proteins when bound to the nonpolar ligates of the RPC or HIC stationary phase. In such cases, the magnitude of the hydrophobic effect decays exponentially up to a distance of about 1000 Å. Moreover, these models indicate that the hydrophobic effect provides a significant driving force for even a polar polypeptide or highly charged protein to be excluded from an aqueous environment and to interact with nonpolar ligates, such as those present on RPC or HIC sorbents.

Of the available models, the solvophobic model provides a rigorous theoretical framework to evaluate hydrophobic effects in the RPC and HIC of peptides and proteins. In order to place a polypeptide or protein into a solvent, a cavity of the same molecular dimensions must first be created. The energy or work required to create this cavity is related to the cohesive energy density or the surface tension of the solvent. If each accessible unit of a peptide or protein, for example, an amino acid side chain, is conceptually treated as an individual microcavity, sensed by a molecular probe of the dimensions of a water molecule, then fusion of these microcavities into a combined cavity corresponding in molecular dimensions of the peptide or protein will effectively reduce the total surface area and hence the free energy of the system. A driving force thus exists to sequester these nonpolar moieties away from a highly polar (water) solvent environment. In RPC and HIC, this involves energetically favorable interactions between the biosolute and the nonpolar ligates. The surface area dependence of the free energy of transfer can then be employed to describe at the microscopic level the magnitude of the hydrophobic effect(s), while at the macroscopic level, the magnitude of the hydrophobic effect can be assessed from the change in surface tension of the solvent that is required to effect desorption from a nonpolar surface. The duality of these microscopic and macroscopic phenomena permits a rigorous theoretical framework for hydrophobic processes to be elaborated in terms of both linear free energy concepts and the molecular parameters associated with the interaction of peptides and proteins with nonpolar surfaces.

Relationships Between Retention, the Equilibrium Association Constant, and Mobile Phase Parameters

The effect of the organic modifier in RPC or a kosmotropic salt in HIC is such that the retention decreases, to a good first approximation, exponentially with increasing organic modifier concentration in RPC or exponentially with decreasing kosmotropic salt concentration in HIC. Thus, the plots of $\log k'$ against the mole fraction, ψ [or volume percentage (v/v)], of an organic modifier in RPC or the corresponding plots of $\log k'$ against the mole fraction, ζ , of a kosmotropic salt in HIC are quasi-linear [133,160–164]. Illustrative of such dependences are the plots [161] of $\log k'$ versus the mole fraction, ψ for the polypeptide bombesin

separated on an *n*-butylsilica sorbent with low-pH water-acetonitrile and water-methanol mobile phases shown in Fig. 11. Acetonitrile-water and methanol-water systems represents the two most frequently used binary solvent systems in the RP-HPLC of polypeptides, although many other solvent combinations have been employed over the years. Acetonitrile-water and methanol-water generate solvent mixtures of different molecular organization [133,161,165–167]. The solution chemistry characteristics of methanol-water mixtures are predominantly controlled by competitive hydrogen bonding [168]. Acetonitrile, on the other hand, poorly forms hydrogen bonds [169] and is organised in aggregates or loosely defined clusters in aqueous solution [170]. Even when mobile phases of comparable relative elutropicities are employed, i.e., under conditions of equivalent surface tension, these differences in the molecular organization of acetonitrile-

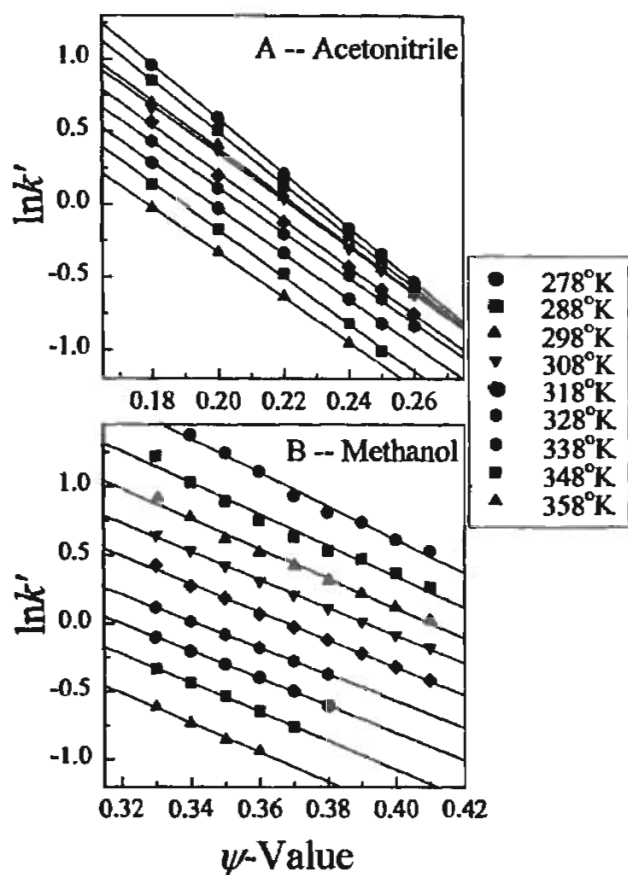


FIGURE 11 Plots of the logarithmic capacity factor, $\ln k'$, versus the volume fraction of the organic solvent, ψ , for the 14-mer peptide, bombesin, using acetonitrile-water (A) or methanol-water (B) mixtures containing 0.1% TFA and a Bakerbond wide-pore *n*-butylsilica of 5 μm nominal particle size and 30 nm average pore size at temperatures $T = 278\text{--}358\text{ K}$. The plots show the experimental data and the lines of best fit according to the quadratic form of the relationship given as Eq. (5). (Data from Ref. 161.)

trile-water and methanol-water mixtures have a fundamental influence on the selectivity. Similarly, effects are also evident when other organic solvents are employed in combination with water in RPC, e.g., with ethanol, 1- or 2-propanol, tetrahydrofuran, or dioxane, as well as with different kosmotropic salt combinations in HIC. These differences in the molecular organization of the mobile phase give rise to distinctive features in the surface tension, γ , versus volume fraction, ψ , of the organic solvent in the case of RPC and the surface tension, γ , versus salt concentration, $[C]$, in HIC as demonstrated in Fig. 12. As clearly evident from Fig. 12, a reverse trend occurs in terms of the respective surface tension dependences as the organic solvent or salt concentrations are increased in RPC or HIC, and this behavior is translated as capacity factor, k' , values that are higher in the absence of an organic solvent in RPC but also higher in the presence of a high salt concentration in HIC separations.

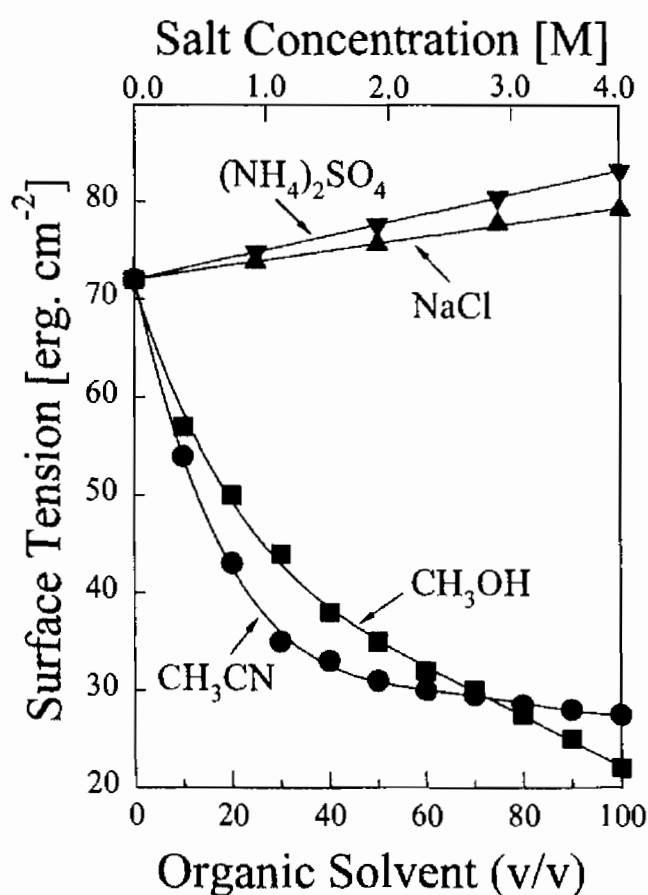


FIGURE 12 Differences in the molecular organization of the mobile phase revealed from the distinctive features of the dependences of the surface tension, γ , on the volume fraction, ψ , of the organic solvent in the case of RPC mobile phase systems and the surface tension, γ , versus salt concentration, $[C]$, in the case of HIC mobile phase systems. (Data adapted from Ref. 579.)

Illustrative of these differences between RPC and HIC behavior with peptides and proteins, mediated in part by the mobile phase composition, are the plots of $\log k'$ versus the mole fraction, ψ , of the "displacing" species with acetonitrile-water mixtures containing 0.1% TFA for horse heart and other cytochrome *c*'s separated [171] on *n*-butylsilica, shown in Fig. 13. Similarly, the use of water-ammonium sulfate, pH 6.5, mobile phases of different ammonium sulfate concentrations, ζ , for the separation [104] of horse heart cytochrome *c* and horse muscle myoglobin on different Tosoppearl sorbents is shown in Fig. 14. As evident from Figs. 13 and 14, the slope of the $\log k'$ versus ψ plots for the cytochrome *c*'s in the RPC mode and the $\log k'$ versus ζ plots for horse heart cytochrome *c* and horse muscle myoglobin in the HIC mode are similarly negative, as the mole fraction of the displacing species is increased. However, a fundamental difference exists between these plots, namely that in the RPC mode the organic solvent concentration is increasing, while in the HIC mode the concentration of the kosmotropic salt is decreasing as the concentration of the displacing species, water, is proportionally increasing. However, in both cases the slope is roughly proportional to the size of the elute molecule and the contact area established at the ligate surface. On the other hand, if the retention dependence in HIC separations is plotted as a function of the concentration of the kosmotropic (chaotropic) salt in the mobile phase, then slopes of positive value are typically observed, as evident from the plots of the logarithm of the elution volume ($\log V_e$) versus ammonium sulfate concentration for several proteins shown in Fig. 15. Irrespective of whether the retention dependences are presented as $\log k'$ versus ψ in RPC separations, or $\log k'$ versus ζ and $\log k'$ versus [Salt] in HIC separations, the underlying mechanisms of interaction and desorption are similar. Fundamental differences thus exist between the chromatographic behavior of small and large molecules in RPC and HIC systems, namely with higher molecular weight polypeptides and proteins, $\log k'$ values are much more sensitive to small changes in modifier concentration with a particular RPC or HIC sorbent. As a consequence with many biomolecules, the "window" of ψ or ζ values over which isocratic desorption can be achieved is very small; see the differences in retention for horse heart myoglobin with different HIC sorbents in Fig. 14.

Because electrostatic, size exclusion, and other interactive effects can also be manifested over different ranges of mobile phase conditions with a particular type of sorbent, then these $\log k'$ versus ψ or $\log k'$ versus ζ plots can take on the characteristic U-shaped dependences [3,72] illustrated in Fig. 16 for several peptides chromatographed with a RPC system. This behavior with biomacromolecules such as peptides and proteins usually necessitates the use of gradient elution conditions for the separation of protein mixtures, unless closely related or homologous proteins are being separated. Moreover, this behaviour indicates that special care must be taken to avoid that self-association or

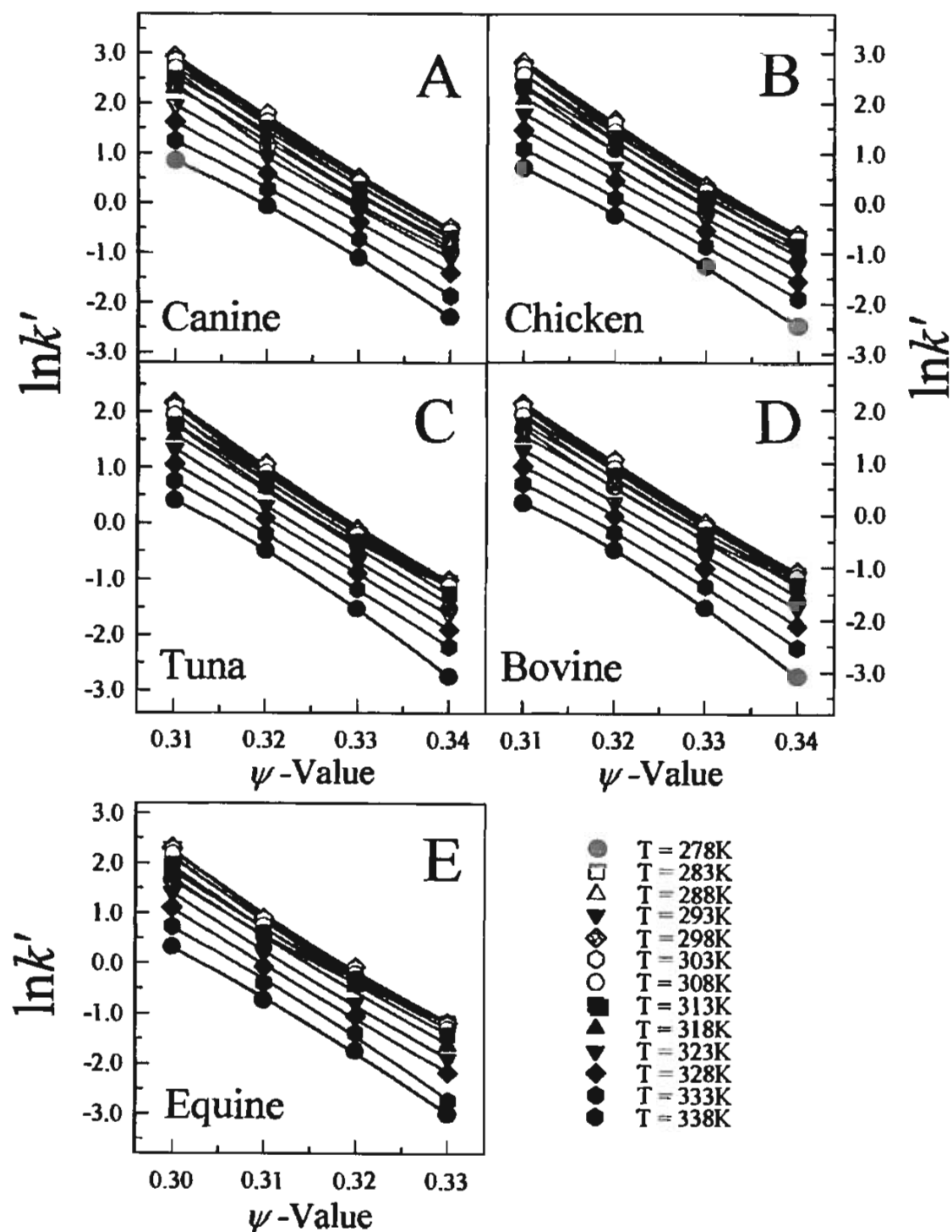


FIGURE 13 Plots of $\log k'$ versus the mole fraction, ψ of the "displacing" species with acetonitrile-water, 0.1% TFA, mobile phases of different acetonitrile concentrations for cytochrome c from different species, namely (A) horse, (B) canine, (C) tuna; (D) bovine, and (E) chicken, using a Zorbax 300 SB-C8 reversed-phase column with dimensions of 4.6 mm $\times$ 15 cm with particles 5 μ m in diameter and 300 Å average pore size. (Data from Ref. 171 and A. Jong, R. I. Boysen, and M. T. W. Hearn, unpublished observations.)

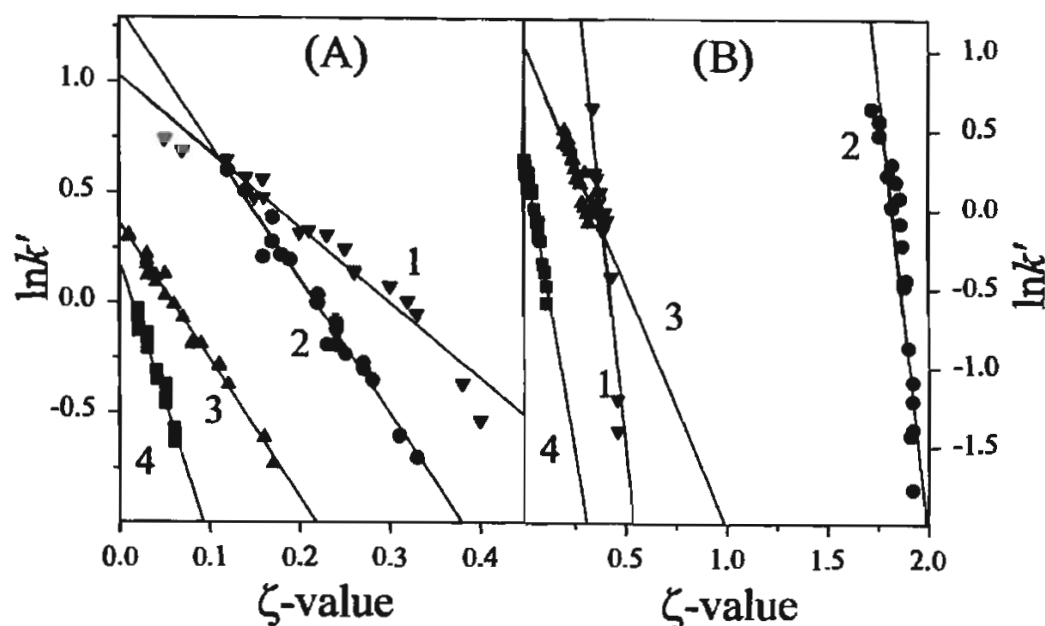


FIGURE 14 Plots of $\log k'$ versus the mole fraction, ζ , of the "displacing" species with water—ammonium sulfate, 50 mM potassium phosphate, pH 7.0, mobile phases of different ammonium sulfate concentrations for horse heart cytochrome *c* (A) and horse heart myoglobin (B) separated on hydrophobic interaction sorbents of different ligand density and ligand type. The sorbents employed in these studies were 1, Toyopearl Butyl 650 BUM 72W; 2, Tosopearl Ethyl MDP ETVO7; 3, Tosopearl Phenyl MDP PHW01; and 4, Tosopearl Butyl MDP BUW01. With more favorable elution of these proteins occurring as the ammonium sulfate concentration decreases, a corresponding increase in the concentration of "structured" water of the system occurs, with the change in the mole fraction, ζ , of the displacing species effectively corresponding to an increase in the water content. (Data from Ref. 104 and Y. Q. Wang, R. I. Boysen, and M. T. W. Hearn, unpublished observations.)

isodesmic aggregation effects [172], gross conformational changes [173], or reduced solubility effects, [174] do not arise and the Gorgon's knot of nonreproducible separations generated.

With globular proteins, such as the preceding example of cytochrome *c* or myoglobin, the polypeptide chain is typically folded to ensure that most of the more polar amino acid side chains (e.g., Arg, Asn, Asp, Gln, Glu, His, Lys, Ser, Thr) are exposed to the aqueous solvent, while the majority of the hydrophobic amino acid side chains (e.g., Ala, Ile, Leu, Phe, Trp, Tyr, Val) are buried within the interior of the molecule. Similarly, many polypeptides of sequence length over ~ 18 amino acid residues attempt to assume a preferred secondary structure in solution. This nascent folding process can lead, depending on the amino acid sequence, to transient or long-lived secondary structures with all or most of the nonpolar residues on the same topological face; i.e., the polypeptide assumes an

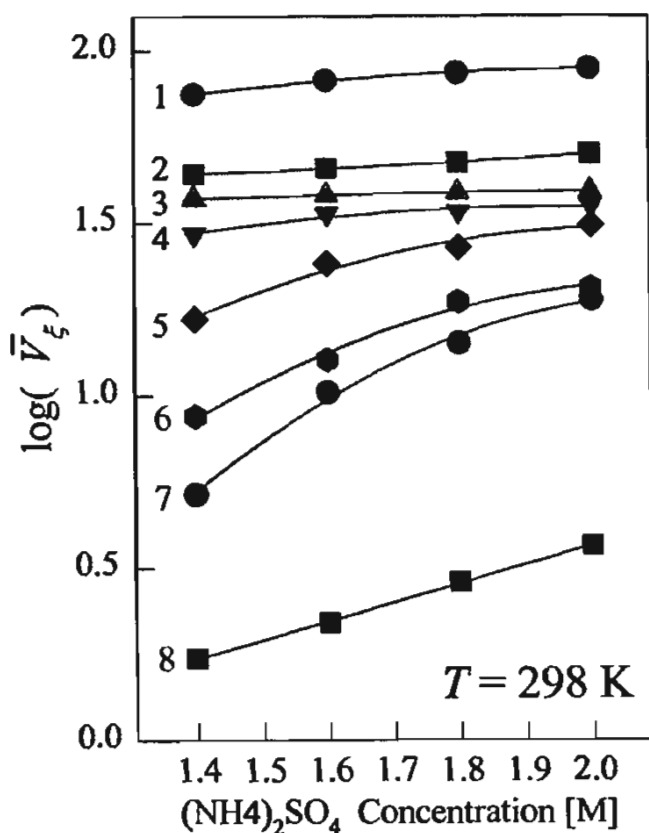


FIGURE 15 Plot of the logarithm of the retention volume ($\log V_r$) versus the concentration of the displacing salt, ammonium sulfate, in the HP-HIC mode separation at 278 K of the polypeptides and proteins: 1, insulin; 2, insulin B chain; 3, bovine trypsin inhibitor; 4, bovine trypsinogen; 5, insulin A chain; 6, ribonuclease A; 7, sperm whale myoglobin; 8, horse heart cytochrome c. As evident from these plots, the lower molecular weight polypeptide species show smaller changes in retention behavior as the concentration of the kosmotropic salt is varied. (Data from Ref. 207.)

amphipathic structure. When this organization array occurs, then these nonpolar regions can preferentially either bind more strongly to the nonpolar ligates or alternatively attempt to self-assemble into dimers, oligomers, etc. In the extreme case, a self-association event can lead to fibril-like structures, with the polypeptide molecules loosely or strongly bound together as an aggregate. Fortunately for the application of RPC or HIC techniques, complete burial of all the hydrophobic groups in a protein or a polypeptide is not possible. These exposed hydrophobic groups form accessible patches on the surface of the molecule [175], which can interact with a hydrophobic matrix as shown in Fig. 3 as a schematic representation. In a number of cases the biological importance of these nonpolar patches have been documented [176]. For example, hydrophobic patches have been found to occur in or near crevices or pockets of many multidomain proteins [177,178],

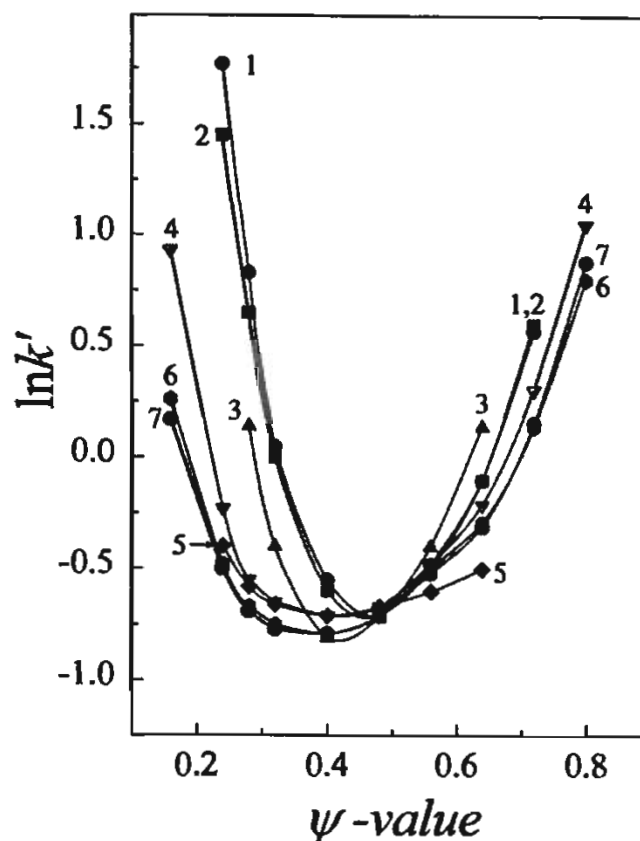


FIGURE 16 Plots of $\ln k'_i$ versus ψ for several polypeptide hormones. The experimental conditions used in this study were: column, μ Bondapak C₁₈ (4.0 $\times$ 300 mm, d_p 10 μ m); flow rate 2.0 mL/min; mobile phase, 15 mM triethylamine–4 mM H₂SO₄–15 mM H₃PO₄, with the volume fraction, ψ , of acetonitrile adjusted from 0.0 to 0.8. The pH value for the 100% water experiment was pH 2.2. The key to the polypeptide structures is: 1, human β -Leu⁵ endorphin; 2, human β -Met⁵-endorphin; 3 porcine ACTH[1–39]; 4, angiotensin I; 5, oxytocin; 6, angiotensin II; 7, angiotensin III. (Data adapted from Refs. 162 and 580.)

giving rise to greater stabilization of the domains and an ability to correctly assemble the catalytic site. Bending of the two-domain structure of lysozyme [119,179] is believed, for example, to arise by solvent exchange around this type of hydrophobic patch.

Other examples documenting the direct interaction between hydrophobic regions accessible on the surface of a protein and the corresponding nonpolar ligates on the stationary phase of a RPC or HIC sorbent have been described in the scientific literature. For example, as noted earlier, the hydrophobic contact site of equine cytochrome *c* and porcine growth hormone in the presence of *n*-butyl ligands has been delineated [140,141] using a combination of footprint enzymatic procedures and electrospray mass spectrometry. A less direct approach is based

on the observation that specific additives, when present in the mobile phase, can selectively bind to the protein or polypeptide and thus cause significant changes in retention. This concept can then be employed to address vexing questions about the nature of the hydrophobic contact sites. Illustrative of this indirect approach are the results reported for calmodulin and troponin C, two calcium-binding proteins, where the hydrophobic binding regions expressed on the surface of these proteins have been implicated based on the use of limited ex columnae proteolytic and other methods [180].

Central to these studies was the observation that these proteins were able to interact strongly with phenyl-Sepharose only when 1 mM Ca^{2+} was present in the mobile phase. This finding suggests that the Ca^{2+} ions were responsible for the exposure of specific hydrophobic regions of these proteins. In these cases, the Ca^{2+} ion has apparently induced destabilizing conformational changes on binding to these proteins, exposing additional hydrophobic regions that were then able to interact with the HIC sorbent. Interestingly, the opposite effect was observed for another calcium-binding protein, α -lactalbumin, when Ca^{2+} ions were present [181]. This protein can be adsorbed to phenyl-Sepharose in the presence of EDTA but can be eluted by the addition of Ca^{2+} to the mobile phase. In this case, the presence of Ca^{2+} ions apparently induces a conformational change in α -lactalbumin resulting in the formation of a less hydrophobic species and elution then occurs. Spectroscopic studies have indicated that a tryptophan residue, exposed on the surface of Ca^{2+} -free α -lactalbumin, becomes more buried when Ca^{2+} ions are bound [183]. Collectively, these and related studies [8,16,40–47,77,101,104] have demonstrated that both RPC and HIC are powerful tools to monitor the effects of mobile phase additives, temperature, and pH on protein conformation. Moreover, various examples that have appeared in the scientific literature over the past 10 years or so related to the retention behavior of peptides and proteins [183–185] have given further credibility to the concept that retention in RPC and HIC is based on mutual interaction between hydrophobic regions accessible on the surface of the protein or peptide and nonpolar ligates on the surface of the stationary phase.

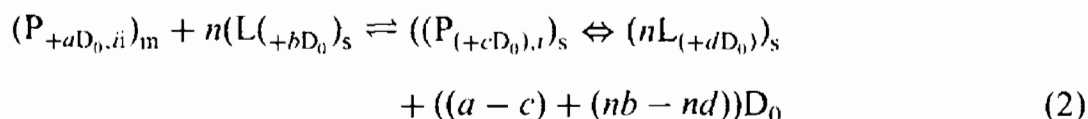
The Nature of the $\log k'$ versus ζ Dependences

In common with all interactive chromatographic systems, the relative retention can be expressed in terms of the equilibrium association constant, K_{assoc} , the concentrations (in moles/liter) of the biosolute present in the stationary and mobile phases, i.e., $[X]_s$ and $[X]_m$, and the volumes of the stationary and mobile phases, V_s and V_m , respectively, within the column of length, L , and internal diameter, d_c . Since the ratio $[X]_s/[X]_m$ is K_{assoc} while the ratio V_s/V_m defines the

phase ratio, Φ , of the chromatographic system, the capacity factor, k' for species P_i , can be expressed in the form

$$k'_i = \Phi[X_i]_s/[X_i]_m = \phi K_{\text{assoc},i} \quad (1)$$

An extensive literature has been developed over the past two decades related to the preferential interaction of different solvent environments with proteins or peptides in the bulk solution state [186–191] and these concepts can be incorporated into descriptions of similar effects in RPC and HIC. Thus, the dependence of the equilibrium association constant, $K_{\text{assoc},i}$ and the concentration of the solvent in RPC or salt in HIC during the adsorption process can be empirically described [154,192,193] in terms of a nonmechanistic, stoichiometric solvent displacement model or a preferential hydration model, whereby the mass distribution of a protein or peptide, P_i , with n nonpolar clustered ligands, each of which is solvated with the solvent molecules, D_0 , is given by



where $(P_{+aD_0,i})_m$ is the protein in bulk (mobile phase) solution solvated with aD_0 solvent molecules; $(P_{+cD_0},i)_s$ is the protein adsorbed to the stationary phase surface with cD_0 solvent molecules; $(L_{+bD_0})_m$ is the non-polar ligand in the free state solvated with dD_0 solvent molecules; $(L_{+dS})_s$ is the nonpolar ligand in the protein bound state solvated with dD_0 solvent molecules; $[(a-c) + n(b-d)]$ represents the number of solvent molecules that are released from the protein and the ligands on adsorption of the protein; $[(a-c)]$ represents the number of solvent molecules required to solvate the contact area of the peptide or protein in contact with the nonpolar ligate surface; and the subscripts m and s refer to the mobile and the stationary phase, respectively. When “near-equilibrium” chromatographic conditions prevail for the distribution process, i.e., when T , P , V , Φ , and the flow rate, F , are constant, and the adsorption process approximates a linear (Langmuirean) isotherm, then this dependence of $K_{\text{assoc},i}$ and hence the logarithm of the corresponding capacity factor $\log k'_i$ on the solvent or salt concentration in the mobile phase $[\mathfrak{I}]_m$ can be written in terms of the familiar Taylor series for both RPC and HIC separations, as follows:

$$\log k'_i = \log k'_{i,0} + S_s(\log[1/\mathfrak{I}]) + S'_s(\log[1/\mathfrak{I}])^2 + S''_s((\log[1/\mathfrak{I}])^3 + \dots \quad (3)$$

$$\log k'_i = \alpha + \beta(\log[1/\mathfrak{I}]) + \gamma(\log[1/\mathfrak{I}])^2 + \delta(\log[1/\mathfrak{I}])^3 + \dots \quad (4)$$

These representations also form the basis of the empirical stoichiometric model discussed in the following. In both RPC and HIC, the change in the equilibrium association constant K_{assoc} , can be readily derived from knowledge of

the change in the retention behavior (usually expressed as the logarithm of the corresponding logarithm of the capacity factor, $\log k'_i$) following an increase in concentration, $[\text{Solvent}]_m$, or volume fraction, ψ_{solvent} , of an organic solvent, or a decrease in the concentration of a salt species, $[\text{Salt}]_m$, in the mobile phase. The respective $\log k'_i$ values can, in turn, be empirically evaluated from the following expressions:

$$\log k'_i = \log k'_{i,0} - S\psi_{\text{solvent}} + S'(\psi_{\text{solvent}})^2 - S''(\psi_{\text{solvent}})^3 + \dots \quad (5)$$

$$\begin{aligned} \log k'_i = \log k'_{i,0} + S_c \log(1/[\text{Solvent}]_m) + S'_c \log(1/[\text{Solvent}]_m)^2 \\ + S''_c \log(1/[\text{Solvent}]_m)^3 + \dots \end{aligned} \quad (6)$$

$$\begin{aligned} \log k'_i = \log k'_{i,0} + H_c \log(1/[\text{Salt}]_m) + H'_c \log(1/[\text{Salt}]_m)^2 \\ + H''_c \log(1/[\text{Salt}]_m)^3 + \dots \end{aligned} \quad (7)$$

The coefficients $S, S', \dots, S_c, S'_c, S''_c, \dots$ and $H_c, H'_c, H''_c, \dots$, etc. correspond to biosolute-specific parameters associated with the reversed phase or hydrophobic binding processes that, in principle, occur as part of the ligand-ligand interaction. In fact, $k'_{i,0}$ is the capacity factor when $\psi_{\text{solvent}} \rightarrow 0$, the coefficients $S, S', S'' \dots$ are the tangents of the plot of $\log k'_i$, versus ψ_{solvent} , the coefficients $S_c, S'_c, S''_c \dots$ are the tangents of the plot of $\log k'_i$ versus $\log(1/[\text{Solvent}]_m)$, and the coefficients $H_c, H'_c, H''_c \dots$ are the tangents of the plot of $\log k'_i$ versus $\log(1/[\text{Salt}]_m)$ at defined values of ψ_{solvent} , $[\text{Solvent}]_m$ or $[\text{Salt}]_m$, respectively. When "ideal" linear dependences prevail between $\log k'_i$ and ψ_{solvent} , $\log(1/[\text{Solvent}]_m)$, or $\log(1/[\text{Salt}]_m)$ in RPC or HIC separations with peptides or proteins, then these empirical dependences reduce to the familiar form given by Eqs. (8) to (10).

$$\log k'_i = \log k'_{i,0} - S\psi_{\text{solvent}} \quad (8)$$

$$\log k'_i = \log k'_{i,0} + S_c \log(1/[\text{Solvent}]_m) \quad (9)$$

$$\log k'_i = \log k'_{i,0} + H_c \log(1/[\text{Salt}]_m) \quad (10)$$

Under such linear dependence conditions, these coefficients can be derived from the slope of the plots of the logarithmic capacity factor, $\log k'_i$, for a particular polypeptide or protein *versus* the logarithm of reciprocal of the concentration of the organic solvent modifier in the case of RPC separations, or $\log k'_i$ *versus* the logarithm of reciprocal of the concentration of displacing ion in the case of HIC separations. Depending on the magnitude of the S, S_c , and H_c parameters and the corresponding $k'_{i,0}$ terms, a variety of retention time *versus* mobile phase elutropic strength scenarios can thus be derived for the interaction of any biomacromolecule with immobilized nonpolar ligates in an RPC or HIC separation mode.

Figure 17 represents typical examples of the limiting cases of such $\log k'_i$ *versus* ψ_{solvent} or $\log k'_i$ *versus* $\log(1/[\text{Salt}])$ retention dependences [see Eqs. (5)–(10)]. Case (a) represents a typical scenario for the behavior of polypeptides

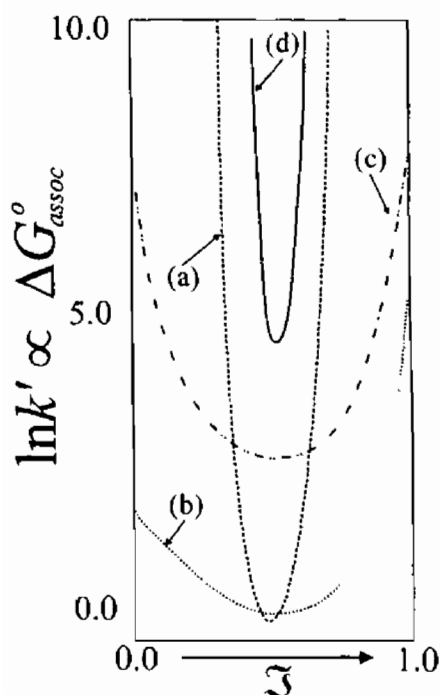


FIGURE 17 Schematic illustrating four limiting cases of the retention dependences manifested between $\ln k'_i$ (proportional to the change in free energy $\Delta G'_{\text{assoc},i}$) and the solvent or salt concentration in the mobile phase, $[\mathfrak{Z}]_m$, often expressed in terms of the mole fraction of the displacing species, ψ or ζ (depending on whether the process involves a reversed-phase or hydrophobic interaction sorbent) over the mole fraction range of 0 to 1.0 [see Eqs. (6)–(11)]. As the contact area and the retention factor, k'_i , of the polypeptide or protein increase, the slope of the plot of $\ln k'_i$ versus $[\mathfrak{Z}]_m$ increases, resulting in a narrowing of the elution window over which the biosolute can be desorbed from the HPLC sorbent. Cases (a) and (b) are typically observed in RP-HPLC or HP-HIC (as well as in HPIEX and some other interactive modes of HPLC) with globular proteins and small peptides; cases (c) and (d) are more representative of the behavior evident with hydrophobic polypeptides, membrane proteins, and other nonglobular proteins. As evident from these plots, the shape of these $\log k'_i$ versus $[\mathfrak{Z}]_m$ plots can take on the characteristic U-shaped dependences typical of multimodal interactions such as those found with silica-based or polymer-based sorbents. This dependence can be expressed in terms of Eqs. (3)–(8).

and globular proteins in the presence of RPC or HIC sorbents [16,20, 47,94,104,133,183,194–198]. There is a steep dependence of $\log k'_i$ on the change in concentration of the displacing species $\mathfrak{Z}$, leading to a minimal value at a specific value of $[\mathfrak{Z}]$, followed by an increase in $\log k'_i$ as $[\mathfrak{Z}]$ is changed further. With RPC methods the volume (or mole) fraction of the displacing species increased from a low value to a higher value, whereas in HIC procedures the reverse occurs with the value of the volume (or mole) fraction of the displacing species decreased to achieve elution. Case (b) is typified by shallow

$\log k'_i$ versus ζ (or $\log 1/[C]$) dependences with small values of $\log k'_i$ as ξ or $\log 1/[C] \rightarrow 0$. Case (b) thus represents a commonly observed situation with small peptides separated under RP-HPLC, where relatively small free energy changes arise over the range of elution strength employed [94,103,104,199–201]. Shallow dependences in terms of the $\log k'_i$ versus ζ (or $\log 1/[C]$) dependences can also be observed under some conditions with more retentive biosolutes, but in this case much larger values of $\log k'_i$ are the norm. Such a situation is illustrated by case (c), which is representative of the behavior of middle molecular weight proteins and very hydrophobic polypeptides under some RPC conditions. Some examples of polypeptide displacement RP-HPLC [202] also fall within the boundaries of case (c). Because the $k'_{0,i}$, $k'_{h,i}$, ... terms are large with case (c) biosolutes, small changes in a secondary mobile phase component or condition, e.g., pH or salt type, can lead to significant secondary retention effects. When such behavior is evident, the limiting chromatographic conditions are frequently chosen from practical considerations so that the minimum of the plot of $\log k'_i$ versus ζ (or $\log 1/[C]$) corresponds to k'_i values equal to unity. Typically, this criterion is easier to achieve in HPIEX than RP-HPLC or HP-HIC separations, although sufficient empirical information is now at hand to enable sensible choices to be made for the latter HPLC modes. In situations associated with the purification of large globular multisubunit proteins or hydrophobic membrane proteins, retention behavior typified by cases (a)–(c) with smaller globular proteins and polypeptides in the RPC or HIC modes are rarely seen. With more hydrophobic proteins, retention dependences approaching case (d) are often observed, resulting in a narrow desorption window, with evidence of significant secondary high-affinity sorption effects and associated poor mass balance and recovery of bioactivity.

Alternatively, changes in the magnitude of $\log k'_i$ with solvent or salt concentration in peptide- or protein-ligand interactions involving hydrophobic effects can be related in solvophobic terms to the chemical potential for the process, $\Delta\mu^\circ$, or to the surface tension, γ , of the solution (solvent) through the expressions:

$$\log k'_i = \log \Phi + \frac{\Delta\mu^\circ}{RT} = \frac{Z_{\text{mol}}}{RT} \left[W_{\text{sc}} - W_{\text{sa}} + \frac{W_{\text{aa}}}{2} - \frac{W_{\text{cc}}}{2} \right] \quad (11)$$

or

$$\log k'_i = \log k'_0 - \gamma \frac{[N\Delta A_{\text{hydr}} + 4.836N^{1/3}(\kappa^e - 1)V^{2/3}]}{2.3033RT} \quad (12)$$

where Z_{mol} is the number of the nearest neighboring (solvent) molecules that surround the polypeptide or protein in solution; W_{sc} , W_{sa} , W_{aa} , and W_{cc} are the reversible work required to achieve adsorption/partition of the biosolute into a liquid film of the desired composition; R is the gas constant; N is Avogadro's

number; V is the partial molar volume; ΔA_{hydr} is the hydrophobic contact surface area; and κ^e is defined as the ratio of the energy required to form a cavity with surface area equal to the surface area of the solute and the energy required to extend the planar surface of the liquid by the same amount. Equation (11) assumes that the interaction can be described in terms of a partition model, while Eq. (12) accounts only for the solvent cavity formation/deformation in the mobile phase and not in the stationary phase; i.e., for a specific sorbent, the retention is assumed to be independent of the structural organization of the nonpolar ligates on the stationary phase surface. As documented elsewhere [4,70,108,109,203], with nonpolar n -alkylsilica sorbents of different ligand densities, this assumption is sustainable only when the ligand density is $\geq 2.7 \mu\text{mol}/\text{m}^2$. Thus, when well-bonded, high-coverage n -alkylsilicas are employed, solvophobic predictions have proved to be remarkably precise with polypeptides that lack secondary structure; i.e., linear relationships have been observed between $\log k'_i$ and γ . Illustrative examples of these dependences are the plots of the experimental $\log k'_i$ (proportional to $\Delta G_{\text{assoc},i}^\circ$) versus the mole fraction of the organic solvent, ψ , and $\log k'_i$ versus the mobile phase surface tension, γ , for several small peptides and other hormonal polypeptides analysed by RPC procedures [204] using n -butyl silica sorbents (Figs. 18 and 19). Figure 20 shows the dependence of $\log k'_i$ versus the

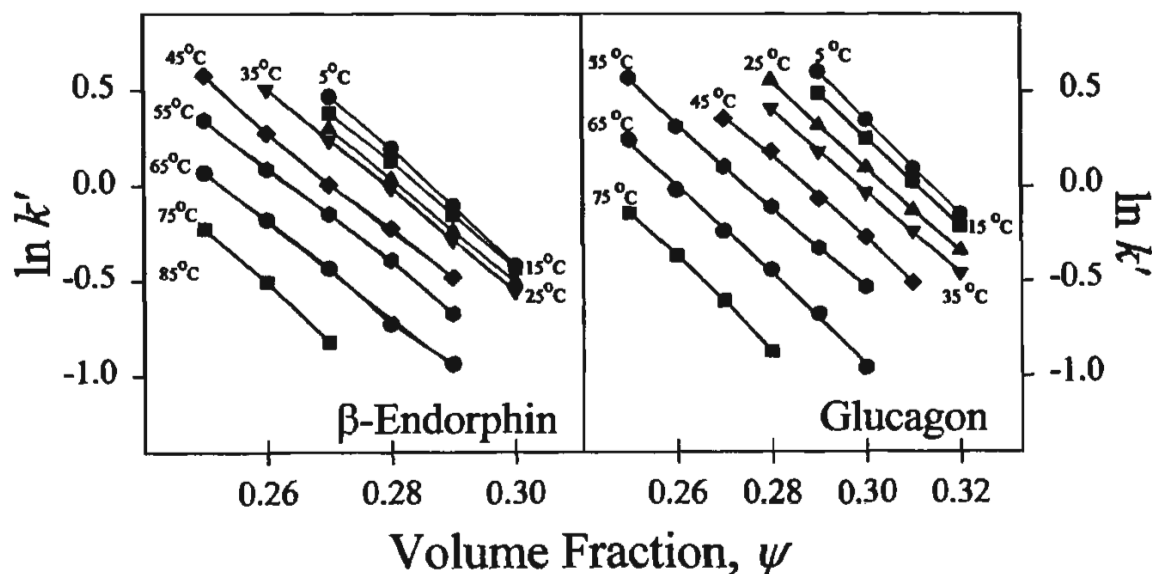


FIGURE 18 Retention dependences for the polypeptide hormones β -endorphin (a) and glucagon (b) as plots of the experimentally determined $\ln k'_i$ values (proportional to $\Delta G_{\text{assoc},i}^\circ$) versus the volume fraction of organic solvent, ψ , measured with an n -butylsilica reversed-phase sorbent at temperatures from 5 to 85°C under isocratic elution conditions, at pH 2.3 and at a flow rate of 1 mL/min, encompassing the range of acetonitrile concentrations $0.25 < \psi < 0.30$. (Data adapted from Refs. 133 and 307.)

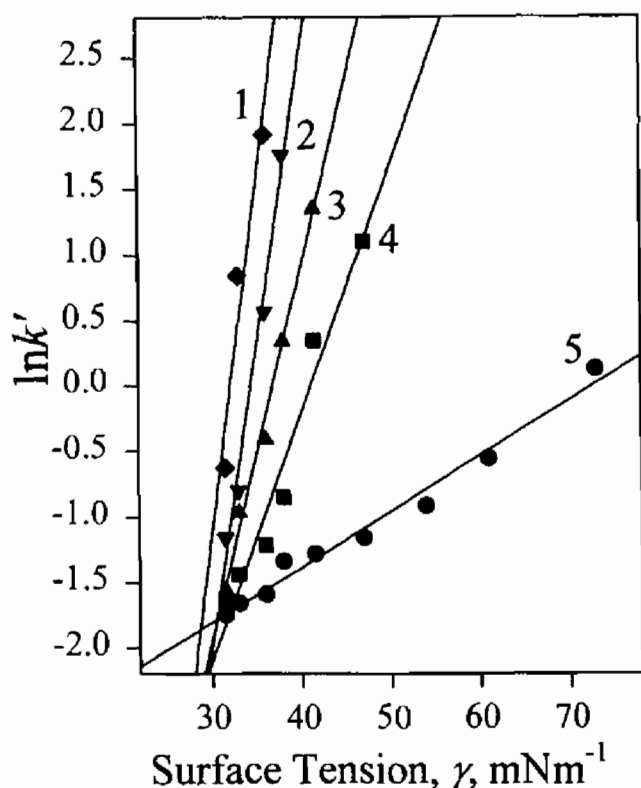


FIGURE 19 Illustrative examples of the dependence of the experimental $\ln k'_i$ (proportional to $\Delta G_{\text{assoc},i}^\circ$) on the surface tension, γ , for the polypeptides analyzed by RPC procedures using *n*-alkylsilica sorbents are these above plots of $\ln k'_i$ versus γ for several peptide homologues analyzed by RP-HPLC procedures using methanol as the organic solvent modifier. The experimental conditions were: column, μ Bondapak C_{18} (4.0×300 mm, $d_p = 10$ μ m); flow rate 2.0 mL/min; mobile phase, water–20 mM H_3PO_4 , with the surface tension adjusted from 78 to 32 mNm^{-1} by varying the volume fraction, ψ , of methanol from 0.0 to 0.56. The pH value for the 100% water experiment was pH 2.2. Key to the peptide structure: 1, pentaphenylalanine (F5); 2, tetraphenylalanine (F4); 3 triphenylalanine (F3); 4, diphenylalanine (F2); 5, phenylalanine (F1). Also shown are the linear regression lines for each plot, where the corresponding correlation coefficients were obtained: $r_{F5}^2 = 0.982$; $r_{F4}^2 = 0.998$; $r_{F3}^2 = 0.991$; $r_{F2}^2 = 0.988$; $r_{F1}^2 = 0.967$. (Data from Ref. 204.)

mobile phase surface tension, γ , for hen egg white lysozyme, hen egg ovalbumin, and concanavalin A chromatographed on a TSK Phenyl-5PW sorbent in the presence of different concentrations of several kosmotropic salts. As particularly apparent from these $\log k'_i$ versus γ plots, the unifying principle that retention decreases as the surface tension of the mobile phase also decreases is followed in both the RPC and HIC modes.

As discussed elsewhere in this chapter, the variations that occur in the $\log k'_i$ dependences on ψ_{solvent} , $\log(1/[\text{Solvent}]_m)$, $\log(1/[\text{Salt}]_m)$, or γ as the temperature, T , of the system is systematically changed provide fundamental information

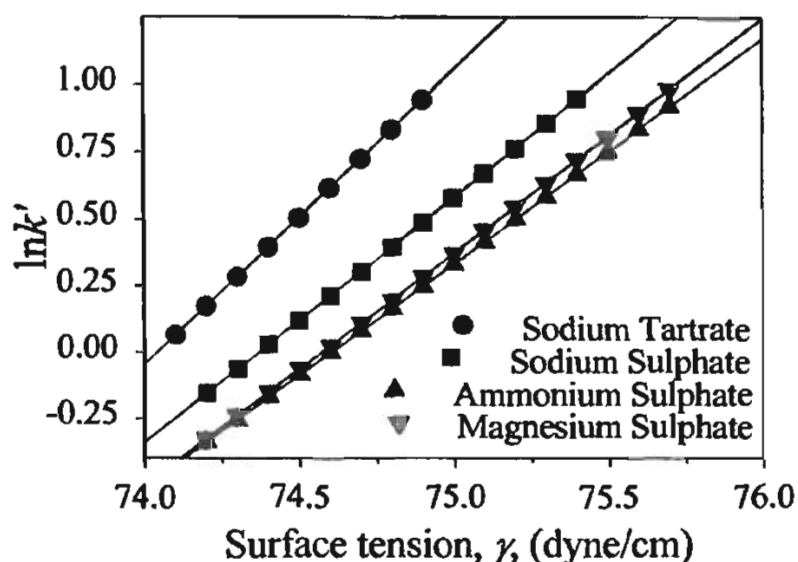


FIGURE 20 Influence of salt type on the dependence between $\ln k'_i$ and surface tension, γ , for the protein hen egg white lysozyme (HEWL). These experimental data were acquired using a TSK Phenyl-PW sorbent equilibrated in 10 mM potassium phosphate buffer at pH 7.0 and increasing concentrations of the designated salt. (Data from Ref. 144.)

on the thermodynamic properties and physicochemical characteristics of the interaction of proteins and peptides with nonpolar surfaces such as used in RPC or HIC. Appropriate utilization of these dependences, with careful assembly of the experimental data, provides an avenue to explore the biophysical behavior of polypeptides and proteins in nonpolar environments and to give insight into the relationships that exist between conformation, interactive behavior and function with these biomacromolecules.

Basic Thermodynamic Considerations

As far as the thermodynamics of the interaction of a protein or peptide species i with the nonpolar stationary phase of an RPC or HIC sorbent is concerned, retention is associated with a decrease in the Gibbs standard free energy ($\Delta G_{\text{assoc},i}^\circ$). Because the equilibrium distribution coefficient, $K_{\text{assoc},i}$, is related to the overall energy change in Gibbs free energy, i.e.,

$$\Delta G_{\text{assoc},i}^\circ = -RT \log K_{\text{assoc},i} \quad (13)$$

the change in $\Delta G_{\text{assoc},i}^\circ$ and its relationship to the relative retention for an RPC or HIC separation process carried out at constant pressure, P , and constant molar volume, V , can be expressed in terms of the well-known, fundamental thermodynamic dependence:

$$\log k'_i = \log \Phi - \frac{\Delta G_{\text{assoc},i}^\circ}{RT} \quad (14)$$

where R is the gas constant and the T the absolute temperature (in kelvins). The use of the capacity factor, k'_i , concept thus allows a particular RPC or HIC process, operated at a constant flow rate and temperature, to be defined in both empirical and thermodynamic terms. Changes in the thermodynamic parameters of the ligand-ligand interaction can thus be depicted in terms of criteria specified by the Gibbs-Helmholtz relationship, namely:

$$\Delta G_{\text{assoc},i}^{\circ} = \Delta H_{\text{assoc},i}^{\circ} - T\Delta S_{\text{assoc},i}^{\circ} \quad (15)$$

Hence, the logarithm of the capacity factor, $\log k'_i$, can be related from fundamental thermodynamic considerations to the temperature, T , through the expression:

$$\log k'_i = -\Delta H_{\text{assoc},i}^{\circ}/RT + \Delta S_{\text{assoc},i}^{\circ}/R + \log \Phi \quad (16)$$

where $\Delta H_{\text{assoc},i}^{\circ}$ and $\Delta S_{\text{assoc},i}^{\circ}$ are the changes in enthalpy and entropy associated with the interaction and $\log \Phi$ is the phase ratio of the system, i.e., the logarithm of the ratio of the stationary phase volume, V_s , to the mobile phase volume, V_m , within a column for a defined sorbent and mobile phase composition. Because the total free energy change incorporates the summation of all contributory free energy terms,

$$\Delta G_{\text{assoc},i}^{\circ} = \Delta G_{\text{es},i}^{\circ} + \Delta G_{\text{hydr},i}^{\circ} + \Delta G_{\text{vdw},i}^{\circ} + \Delta G_{\text{sol},i}^{\circ} + \Delta G_{\text{aggr},i}^{\circ} + \Delta G_{\text{red},i}^{\circ} \quad (17)$$

where $\Delta G_{\text{es},i}^{\circ}$, $\Delta G_{\text{hydr},i}^{\circ}(= \Delta G_{\text{cav},i}^{\circ})$, $\Delta G_{\text{vdw},i}^{\circ}$, $\Delta G_{\text{sol},i}^{\circ}$, $\Delta G_{\text{aggr},i}^{\circ}$, and $\Delta G_{\text{red},i}^{\circ}$ are the free energy differences associated with electrostatic effects (subscript es, i), hydrophobic processes including the formation of a solvent cavity of dimensions of the biosolute (subscript hydr, i or cav, i), van der Waals interactions (subscript vdw, i), solvational effects (subscript sol, i), self-association or heterogeneous association of the biosolute in the presence and absence of solvent (subscript aggr, i), and the reduction in free energy due to nonideal effects (subscript red, i), respectively, the fundamental relationship between $\Delta G_{\text{assoc},i}^{\circ}$ and $\log k'_i$ thus provides the basis for understanding the composite interplay between size exclusion phenomena, solvophobic, coulombic, and the other interaction processes that are evident as dominant features of all current HPLC stationary phases. Depending on the magnitudes of these individual Gibbs free energy–capacity factor dependences, the retention behavior of any biosolute with an interactive ligand system can be formalized in terms of the incremental contributions to the overall retention process and thus to the isocratic retention factor, k'_i , through the relationships given as Eqs. (16) and (17).

In both RPC and HIC, immediately prior to the interaction between the nonpolar ligates and the protein or peptide molecule(s), the nonpolar ligates and some of the nonpolar regions accessible at the surface of the biosolutes will still have structured water molecules associated with their nonpolar surfaces [205]. When these two surfaces are brought into close contact, solvent exclusion events

occur, releasing structured water molecules that in turn increase the entropy of the bulk mobile phase. This process of solvent exclusion can be viewed as the driving force for adsorption. Because van der Waals forces are expected to be a major contributor and the contact area of the protein or peptide with the nonpolar ligate surface is small, $\Delta H_{\text{assoc},i}^\circ$ may also be small (and either negative or positive) and $\Delta S_{\text{assoc},i}^\circ$ may be large. In this case, $\Delta G_{\text{assoc},i}^\circ$ is controlled by a large positive entropy change. Such an entropy-driven process would be characterized by greater retention at higher temperatures, as expected from Eq. (16). Indeed, in contrast to ion-exchange and affinity chromatography, higher column temperatures in well-controlled RPC and HIC separations in general lead to longer retention. One of the advantages of RPC and HIC, then, is that column operation at subambient temperatures will tend to result in a weaker interaction between the peptide or protein and the stationary phase, relative to similar experiments carried out at ambient or more elevated temperature. Use of lower temperatures will often translate into a stabilizing influence for globular proteins, but it should not be forgotten that low temperatures could also lead to the “cold” denaturation effect [186–189,206] with some proteins. The ability to operate at subambient temperature, however, and to minimize the chances of unfolding conformationally labile species represents valuable attributes of RPC and HIC techniques.

As is evident from the preceding discussion, the mobile and stationary phases as well as the protein structure all play critical roles in determining the magnitude of retention in RPC and HIC. Solvophobic theory [16,72,78,79,149,150,160,204,207] can be employed as a theoretical framework to describe quantitatively the effect of organic solvents on peptide or protein retention in RPC or salts in HIC. According to Eqs. (11) to (17), the change in the interfacial free energy upon adsorption of a peptide or protein to a solvated *n*-alkyl ligand immobilized onto the surface of a support material can be related to the change in the solvational state of the peptide or protein, the nonpolar stationary phase ligands, and the complex formed when the biosolute and the ligates dock. This process can be treated in terms of two distinct steps, the first of which involves the formation of a cavity of the size and shape that can accommodate the peptide or protein and the second involves the introduction of the peptide or protein into this cavity and its subsequent interaction with the surrounding solvent environment.

Surface Tension Considerations and the Solvophobic Model

According to Eq. (12), the retention of a peptide or protein, *P*, in an RPC or HIC system can be related to the surface tension of the mobile phase, γ , the magnitude of the cavity factor, κ_i^c , and the hydrophobic contact area, $\Delta A_{\text{hydr},i}$, which is established between the biosolute and the stationary phase surface. To a first approximation, $\Delta A_{\text{hydr},i}$ is proportional to the total surface area of the molecule,

$A_{\text{total},i}$, and thus can be related to the total molecular volume, $V_{\text{total},i}$, as calculated from the individual amino acid residues according to the procedures of Privalov and colleagues [208]. In turn, the total surface area, $A_{\text{total},i}$, can be calculated using the individual amino acid surface area values as determined by Rose and colleagues [209]. Similarly, the total van der Waals surface area, $A_{\text{vdw},i}$, and the solvent-accessible surface area, $A_{\text{acc},i}$, can be derived using the Connolly algorithm based on the partitioning of the total surface area into the area accessible to a 1.4 Å solvent probe [210], while the hydrophobic surface area, $A_{\text{hydr},i}$, can be calculated again using the Connolly algorithm, including only the amino acid residues with a hydrophobicity coefficient value > 2 as determined, for example, from the Hearn hydrophobic coefficient sets [211,212]. Finally, the solvent-accessible hydrophobic surface area, $A_{\text{solhydr},i}$, can be derived again using the Connolly algorithm and the total hydrophobic surface area accessible to solvent probes of different size, i.e., from 1.4 Å (water) to say 3.6 Å (acetonitrile). Thus, when hydrophobic interactions prevail in RPC and HIC, the change in free energy for cavity formation for a peptide or protein in the mobile phase is related to the surface tension, γ , and surface area of the molecule, $A_{\text{total},i}$, according to

$$\Delta G_{\text{cav},\text{mp},i}^{\circ} = [NA_{\text{total},i} + 4.8N^{1/3}(\kappa_i^e - 1)V^{2/3}] \gamma \quad (18)$$

The surface tension of an aqueous solution containing an organic solvent or a salt is a function of the molal solvent or salt concentration, m , and can be given by

$$\gamma = \gamma^{\circ} + \sigma m \quad (19)$$

where γ° is the surface tension of pure water (72 dyne cm^{-1}), σ is molal surface tension increment, a constant characteristic of each solvent or salt, and m is the molality of the salt. Listed in Table 2 are the molal surface tension increments of a typical selection of salts in order of their decreasing σ values. This order of σ values parallels the Hofmeister salting-out series. Similar information on σ values for various water-soluble organic solvents can be extracted from surface tension versus mole fraction tables [213]. In the Hofmeister series, anions that promote water-structuring events and/or hydrophobic interactions are to the left of the rank order series, and anions that disrupt water structure and act as chaotropic ions are placed to the right of the series. In addition, cations reinforce the strength of the interaction. Thus, the Hofmeister series follow the orders:

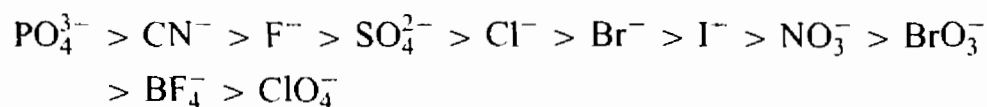
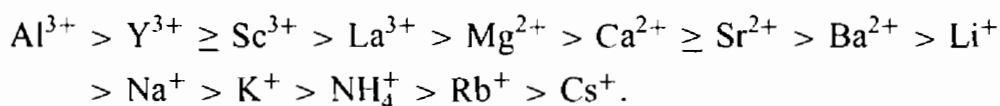


TABLE 2 Representative Examples of Salts Employed in HIC and Their Modal Surface Tension Increments, σ

Salt	Molal surface tension increment $\sigma \times 10^3 \text{ dyne g cm}^{-1} \text{ mol}^{-1}$
Calcium chloride	3.66
Magnesium chloride	3.16
Potassium chloride	3.12
Sodium sulfate	2.73
Potassium sulfate	2.58
Ammonium sulfate	2.16
Sodium dihydrogen phosphate	2.02
Potassium tartrate	1.96
Sodium chloride	1.64
Potassium perchlorate	1.40
Ammonium chloride	1.39
Sodium bromide	1.32
Sodium nitrate	1.06
Sodium perchlorate	0.55
Potassium thiocyanate	0.45

Source: Data from Refs. 144,160,186–188,207,217,308,309,579.

and



Assuming that the magnitude of the solvent or salt concentration has no effect on κ_i^c , $A_{\text{total},i}$ or V , then the free energy change associated with cavity formation in a mobile phase ($\Delta G_{\text{cav},\text{mp},i}$) of defined composition can be evaluated from

$$\Delta G_{\text{cav},\text{mp},i} = [NA_{\text{total},i} + 4.8N^{1/3}(\kappa_i^c - 1)V^{2/3}] \gamma_i^c \\ + [NA_{\text{total},i} + 4.8N^{1/3}(\kappa_i^c - 1)V^{2/3}] \sigma m \quad (20)$$

which can be expressed in a simplified form as

$$\Delta G_{\text{cav},\text{mp},i} = \Delta A_{\text{contact}} \sigma m + \text{constant}. \quad (21)$$

where $\Delta A_{\text{contact}} (= \Delta A_{\text{hydr},i})$ is the difference in surface area of the protein exposed to mobile phase in the bound and unbound states, i.e., equivalent to the molecular contact area upon binding. Thus, the greater the value of γ or $\Delta A_{\text{contact}}$, the greater will be the retention. When the contact area for protein-protein interaction exceeds the contact area for protein-ligand interaction and the respective free

energy change favors protein-protein interaction due to isodesmic self-association, then a complex, $(P)_n$, will form. Under these conditions, the solvophobic model reflects salting-out or precipitation phenomena [160,186–191], where the distribution occurs between a solvated state of the protein and a solid phase state rather than between a solvated monomer and a chromatographically bound state. Obviously, isodesmic self-association can also occur on the stationary phase surface, and these effects [214,215] can lead to bizarre retention processes and elution behavior that is highly condition dependent.

In a system of constant P and V , the net free energy change due to van der Waals interactions ($\Delta G_{\text{vdw},i}^\circ$) is assumed to be unaffected by the addition of exogenous salts or solvents in RPC or HIC and, therefore, is expected to follow the relationship

$$\Delta G_{\text{vdw},i}^\circ = \Delta G_{\text{vdw},\text{sp},i}^\circ - \Delta G_{\text{vdw},\text{mp},i}^\circ \quad (22)$$

The differential changes in the free energy associated with the changes in the salt or solvent concentration lead to the following expression

$$\ln(k'_i/k'_0) = -B_{\text{mp},i}m^{1/2}(1 + C_{\text{mp},i}m^{1/2}) - D_{\text{mp},i}\mu m + \Delta A_{\text{total}}\sigma m + vm + \text{constant} \quad (23)$$

where k'_0 is the retention factor at zero salt or solvent concentration. In the case of HIC separations, when the electrostatic component becomes negligible at sufficiently high ionic strength, the $-B_{\text{mp},i}m^{0.5}(1 + C_{\text{mp},i}m^{0.5})$ term approaches a constant value and then the logarithmic retention factor becomes linear with regard to the salt molality, i.e.,

$$\log(k'_i/k'_0) = \lambda_{\text{salt}}m_{\text{salt}} \quad (24)$$

where λ is a parameter that measures the retentive strength of the salt and is similar to the salting-out constant. Analogous expressions can be derived for RPC separations, although in this case the involvement of electrostatic effects has to be modulated because the protein or peptide assumes a fully protonated state under the low-pH conditions or requires the inclusion of an ion-pairing reagent as an additive to the mobile phase to achieve a near-neutral state. When electrostatic/polar interactions can be excluded from the retention process with peptides or proteins, the free energy changes associated with the cavity formation and the Lifshitz–London–van der Waals interaction represents the dominant basis of RPC and HIC separations. Solvents that have large negative σ values give rise to significant decreases in the surface tension of the mobile phase and hence cause reduced retention. In contrast, salts with large positive σ values give rise to significant increases in the surface tension of water and hence cause enhanced adsorption. In this context, distinction must also be made between organic solvents that are strongly hydrogen bonding with water and those that prefer to

form solvent clusters in water. Salts that increase the surface tension of the solvent can be described as kosmotropic (structure inducing) because they promote the ordering of water molecules at interfaces. Salts of many organic acids and bases have negative values of σ and consequently have an opposite influence, i.e., chaotropic effects, on protein structure or the adsorption of proteins and peptides to nonpolar surfaces. It is for these reasons that sodium, potassium, or ammonium thiocyanates have been commonly used to dissociate high-affinity protein-ligand interactions in circumstances where strong hydrophobic effects may dominate the overall interaction mechanism. Knowledge of the effect of the molal surface tension increment thus provides a quantitative measure of the effect of salt type and concentration on HIC retention.

As apparent from Eqs. (11), (12), and (18), the free energy of cavity formation is proportional to the surface area of a protein or peptide. Therefore, retention differences between proteins or peptides will reflect the incremental changes in their surface areas as a consequence of adsorption to the stationary phase ligands. In the presence of an organic solvent in RPC, or when the salt concentration is sufficiently high in HIC, the retention relationship for a specific protein or peptide takes the simplified linear form [8,160,207]

$$\log k'_i = \log k'_0 - \lambda m \quad (25)$$

where λ is the slope of the plot of $\log k'_i$ versus solvent or salt molality, m , and $\log k'_0$, corresponds to a solute-specific chromatographic system parameter. Moreover, experience has shown that λ is strongly correlated to σ with the slopes of the plots for a specific protein or peptide in different solvent or salt systems tending to follow the relative ranking of solvents of the same family type (alcohols, etc.) in terms of their relative elutropieities or salts in terms of their place in the Hofmeister series. For many globular proteins and peptides in RPC or HIC these plots are not coincidental with the same solvent or salt combinations when the retention data are normalized (see surface tension differences between acetonitrile or methanol shown in Fig. 12). Just as different organic modifiers can be employed to generate different selectivities in RPC, retention, and hence resolution, in HIC can be independently modulated by the use of different salts. As the hydrophobicity of the stationary phase increases, the λ value for a particular protein or peptide often increases with a defined mobile phase composition. Such behavior would be in accord with the concept that a larger nonpolar contact area will be generated when a protein or peptide interacts with a more nonpolar ligand system [161]. Several studies have also shown that for topologically similar proteins or peptides, with (nearly) identical types of hydrophobic patches accessible on their surfaces, the λ value in HIC or the S_c , value in RPC remains essentially constant [20,171].

Clearly, in the HIC situation, anions or cations that bind in a specific manner to proteins, or perturb the tertiary or secondary structure and thus destabilize the conformation of the protein in solution or at the solid-liquid interface, will cause divergences from these solvophobic predictions. In particular, anion-cation combinations that cause salting-in effects or result in disturbances in the dipole of the amide backbone structure of the protein or peptide can be expected to cause significant divergences. Salts of Ca^{2+} and Mg^{2+} , because of their propensity to bind selectively to many proteins, frequently show aberrant retention behavior in HIC with significant deviations from linear dependences of retention on surface tension despite their large σ values [145,216]. Analogous circumstances of specific binding of solvents are not as common in protein RPC (except for some enzymes where the solvent acts as a substrate/pseudosubstrate by binding to an allosteric or active site pocket [217]). Salts that bind selectively to proteins frequently cause changes in conformation, mimicking the R- to T-state transitions observed with enzymes. Thus, with some proteins a decrease, and in other cases an increase, in hydrophobic contact area may ensue in HIC. This facet of HIC is particularly evident with Ca^{2+} - and Mg^{2+} -binding proteins, but clearly such behavior can be expected for any protein or peptide that binds specifically to a salt of relatively high molal surface tension increment values. In fact, the interaction of some of the so-called hard metal ion salts, such as Al^{3+} salts with, e.g., prion-related polypeptides [218] fall into this category.

In the RPC of proteins, it can be argued that helix-inducing or secondary structure-stabilizing solvents should be used in preference to other solvent combinations. These solvent conditions will tend to favor preservation of the “native” conformation of these biomolecules under specific RPC conditions. Similarly, in HIC preference should be given to the use of kosmotropic salts because these salts are excluded from the immediate vicinity of the protein and enhance the structure of the surrounding water. The thermodynamic outcome of this preferential hydration is that the free energy of the protein solution becomes higher, and retention is thus favored because a smaller surface area of the protein or peptide (and concomitantly also the non-polar ligates) will be exposed to the solvent.

Preferential Interaction Model

In the preferential interaction model for adsorption, the equilibrium association constant K_{assoc} for the interaction of a protein or peptide with an RPC or HIC sorbent can be related via Eq. (2) to the changes in the preferential interaction of the salt and water with the protein, P , the ligands, L , and the protein-ligand complex, P_L , as a function of salt concentration. The effect of preferential

hydration/solvation or preferential salt interaction with a protein on k' can be represented in terms of the following expression [192]:

$$\frac{d(\log k'_i)}{d(\log m_3)} = 3 \left[\Delta v_3 - \frac{m_3}{m_1} \Delta v_1 \right] = Z^* \quad (26)$$

where m_3 is the molal concentration of salt and m_1 is the molal concentration of water (55.5 mol/L), Δv_1 and Δv_3 refer to the number of moles of water and salt, respectively, displaced upon adsorption per mole of protein, and Z^* corresponds to the slope of the plot of $\log k'$ versus $\log m_3$. The ratio m_3/m_1 will thus vary with the salt concentration and is a weighting factor for the water term. Because Z^* provides a measure of the changes in both salt and water bound to a protein or peptide as a consequence of the chromatographic distribution process, it can be related through solvophobic considerations to λ through the relationships

$$\lambda = \frac{d(\log k'_i)}{dm_3} = \frac{Z^*}{m_3} \quad (27)$$

Again, similar expressions can be derived to account for the effect of organic solvent concentration on the $\log k'_i$ values of proteins or peptides in RPC. The solvophobic theory and the preferential interaction theory thus have a common linkage through the slopes of these $\log k'_i$ versus $\log[1/\zeta]$ plots. From these dependences, and the relationship of $\log k'$ to the free energy change, $\Delta G_{\text{assoc},i}$, a unified theoretical approach is also provided to the thermodynamic and extrathermodynamic relationships of the interaction of proteins or peptides with hydrocarbonaceous ligands in both RPC and HIC.

Stoichiometric Solvent Displacement Model

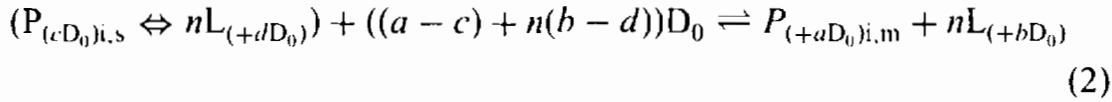
The stoichiometric solvent displacement model addresses an analogous set of considerations from an empirical perspective. According to this theoretical treatment, the slope of the plots of $\log k'_i$ versus the solvent or salt concentration can be employed as a measure of the contact area associated with the peptide or protein interaction. The retention and elution of a peptide or protein in RPC or HIC can then be treated as a series of microequilibria between the different components of the system, as represented by Eq. (2). Thus, the affinity of the desorbing agent (an organic solvent in RPC and water in HIC) for the free protein, P_i , in the mobile phase can be represented by:



and the affinity of the desorbing agent (an organic solvent in RPC and water in HIC) for the free nonpolar ligand, L , can be represented by



If n solvated nonpolar ligands simultaneously interact with the solvated peptide or protein, then the binary complex that forms can be represented by the equilibrium shown as Eq. (2). When the surface tension, i.e., the concentration of the organic solvent or salt, reaches a critical value, then the partially solvated protein, $P_{(+cD_0)i,s}$, bound to the stationary phase, will desorb and become resolvated to generate the species, $P_{(+aD_0)i,m}$. Thus,



and the change in retention can be represented by:

$$k' = \frac{[P_{(+cD_0)i,s}]}{[P_{(+aD_0)i,m}]} \Phi \quad (30)$$

According to the stoichiometric displacement model, the equilibrium constant for adsorption can be expressed in terms of Eq. (31), namely:

$$K_{\text{adsorb}} = \frac{[P_{(+cD_0)i,s}][D_0]^{((a-c)+n(b-d))}}{[P_{(+aD_0)i,m}][L_{(+bD_0)}]^n} \quad (31)$$

and thus

$$k' = \frac{([L_{(+bD_0)}]^n \Phi K_{\text{adsorb}})}{[D_0]^{((a-c)+(b-d))}} \quad (32)$$

By convention, the difference in number of solvent molecules associated with the adsorption of a peptide or protein to a nonpolar ligate in RPC has been represented by Z or S_c , and a corresponding term, H_c , can also be employed for HIC separations, where

$$Z = S_c = (a-c) + n(b-d) \quad (33)$$

and hence

$$\log k' = Z \log(1/[D_0]) + \log C \quad (34)$$

where

$$C = ([L_{(+bD_0)}]^n \Phi K_{\text{adsorb}}) \quad (35)$$

and Z (or S_c and H_c) is the slope of the plot of $\log k'$ versus the logarithm of $1/[D_0]$ assuming that a first-order dependence prevails. As noted previously [16,18,20,54,133], the Z (or S_c and H_c) and $\log C$ terms represent average values characteristic of the overall adsorption-desorption phenomena that a peptide or protein undergoes at the nonpolar surface of an RPC or HIC sorbent.

As illustrated in Fig. 21, linearity can be observed for these dependences of $\log k'$ on the logarithm of $1/[D_0]$ ($\log(1/[D_0]) = \mathfrak{Z}$) for several proteins and

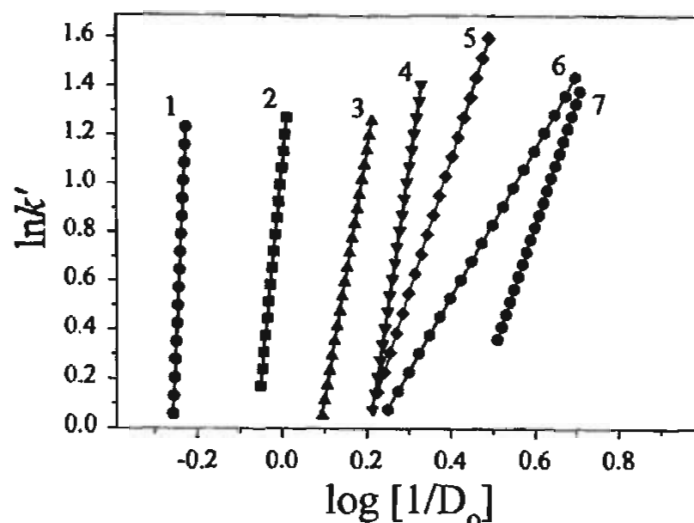


FIGURE 21 Dependences of $\ln k'$ on the reciprocal of the logarithm of the organic solvent concentration $\ln(1/D_0)$ (where $\ln(1/D_0) = \zeta$) for several proteins eluted from an *n*-octylsilica sorbent under RPC conditions using a 50% formic acid mobile phase and 2-propanol as the organic solvent modifier at a flow rate of 1.0 mL/min. The legend for the proteins: 1, hen egg ovalbumin; 2, bovine pancreatic trypsin inhibitor; 3, hen egg white lysozyme; 4, horse heart cytochrome c; 5, bovine insulin; 6, bovine glucagon; and 7, bovine pancreatic ribonuclease. (Data from Ref. 154.)

polypeptides eluted with a 50% formic acid mobile phase containing different concentrations of 2-propanol from *n*-alkylsilica under RPC conditions. Similarly, as shown in Fig. 22, the corresponding plots of $\log k'$ versus the logarithm of $1/[C]$ for several bird lysozymes also show linearity with a TSK Phenyl-5 PW sorbent under HIC conditions when analyzed by the stoichiometric displacement model. Clearly, the Z (or S_c and H_c) values so derived also incorporate the influence of other mobile phase additives, such as the presence of a particular acid or salt, the magnitude of the molal surface tension increment of the solvent or salt, and the type of buffer species present. In addition, the assumption is made that the peptide or protein structure remains constant throughout the chromatographic migration and that there is a constant number of ligands available for interaction over the complete range of mobile phase compositions required for elution. Consistent with the underpinning physicochemical relationships linking molecular structure and retention behavior, various studies have shown that the Z (or S_c and H_c) values are proportional to the molecular weight of peptides or proteins with a similar ratio of hydrophilic to hydrophobic amino acid residues. This empirical correlation, however, permits a more fundamental question related to the interaction of peptides and proteins with nonpolar ligands to be explored, namely what is the relationship between hydrophobic surface area or total surface

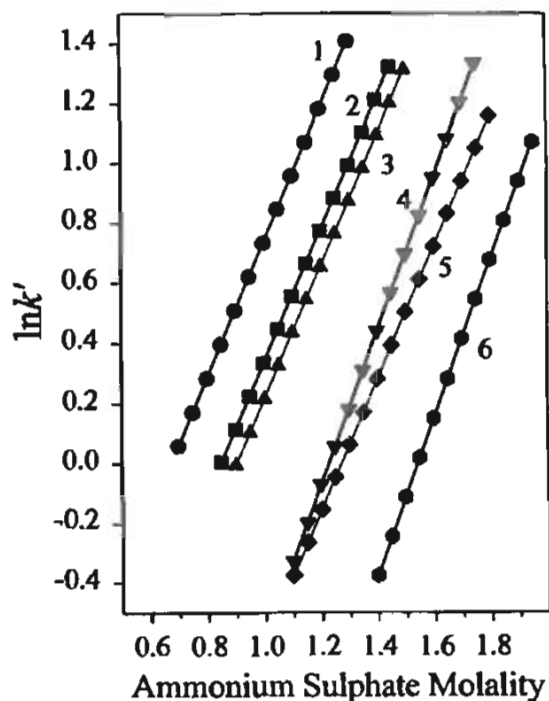


FIGURE 22 Plots of $\ln k'$ versus the salt molality for different bird lysozymes chromatographed under high-performance hydrophobic interaction chromatographic conditions using a TSK Phenyl-5PW sorbent with a mobile phase containing 10 mM potassium phosphate buffer, pH 7.0, and increasing concentrations of ammonium sulfate. The linearity observed with these plots has permitted analysis of the data by the stoichiometric displacement model. The similarity in the slopes of the plots indicates that similar hydrophobic contact areas are involved in the interaction with the nonpolar sorbent, with the value of the $\ln k_0$ intercept increasing as the lysozyme became more hydrophobic. The conclusion can be drawn that amino acid substitution per se with these seven lysozymes did not increase the size of the hydrophobic contact area but rather the affinity of the hydrophobic interaction. Protein code for the different lysozymes: 1, Japanese quail; 2, turkey; 3, hen egg white; 4, ring-necked pheasant; 5, Peking duck isoenzyme A; 6, Peking duck isoenzyme B; and 7, Peking duck isoenzyme C. (Data from Ref. 144.)

area of a peptide or protein in free solution and the contact area for these biomolecules when bound to a nonpolar surface?

van der Waals Effects and Protein Interactions

An alternative view has been advanced for the basis of the interaction of a protein or peptide with hydrophobic ligates, namely that the interaction is mediated via van de Waals and other close-range forces [152,153]. In this scenario, the driving force for retention in RPC and HIC is much more dependent on the interfacial characteristics of the interaction, which shows different distance dependences. Thus, in this model, the force of the interaction decays according to a distance

dependence of $1/r^4$ for uncharged atoms and $1/r^3$ for carboxylate oxygens, where r is the distance between the two interacting surfaces. In high-performance ion exchange chromatography (HPIEX), in comparison, the electrostatic interaction is mediated by formation of electrical double layers between the interacting surfaces with a dependence on $1/r^2$ and operates over considerably larger atomic distances. Similarly, in polar phase interactions, mediated primarily by hydrogen-bonding interactions between the protein or peptide and the polar sorbent surface, the distance dependence takes the form of a $1/r^3$ relationship but is also believed to be operative over relatively small atomic distances, 1.5–5 Å. This concept of a short-range interaction in RPC and HIC, and its relevance to the concept of interdigitation of the contact region of the protein or peptide with the hydrocarbonaceous surface, leads to a number of important outcomes. These outcomes and several other fundamental question related to the binding behavior of peptides and proteins with RPC and HIC sorbents are examined in greater detail in the next section of this chapter.

SORBENT AND MOBILE PHASE CONSIDERATIONS FOR RPC AND HIC SEPARATIONS

Sorbent Characteristics

Since the 1980s, RPC and HIC methods have been increasingly employed for the separation of peptides and proteins at both the analytical and the preparative scales. This popularity owns its origins to a number of key operational features. One of these is the sorbent characteristic. As discussed earlier, the choice of the reagents and the methods used for the chemical modification of a stationary phase surface is conditional on the molecular characteristics of the particle itself. For preparative RPC and HIC applications, a considerable amount of effort has been expended, particularly in recent years, on the development of more stable silica-based sorbents as well as sorbents derived from polymerization of organic monomeric materials. Many of these nonpolar stationary phase materials now have the desirable features of mechanical stability and good chemical or pH stability. In addition, related features inherent in high-performance macroporous, preparative sorbents (with d_p values in the range 25–50 μm), large pore diameters ($p_d > 500$ Å), lack of deformation with pressure drops up to 200 bar, or swelling in different mobile phase conditions can be achieved. Various types of styrene-divinylbenzene, polysaccharide-based materials, vinyl alcohol–polymethacrylate copolymers, perfluoro polymers, and other polymeric matrices have found their way into the application arsenal of RPC and HIC media for the separation of proteins and peptides. However, the chemically modified porous silicas have attracted the greatest attention for analytical RPC and HIC separations and for

some large-scale RPC applications, e.g., recombinant human insulin [219]. This versatility arises partly because of their ability to resist high pressure changes, thus allowing the chromatographic system to operate at higher linear flow velocities, but also because they provide a rigid scaffold for attachment of various types of nonpolar surface coatings.

These aspects have been particularly relevant to the laboratory scale activities important for high-performance applications where porous silica has been the most frequently used support material [60–62]. Naked silicas are now readily available in narrow ranges of average pore and particle diameters. Particles can be produced in spherical and irregular geometries, resistant to significant static or dynamic pressure changes, and with excellent mechanical stability. Moreover, the characteristics of the current generations of silica materials do not change in terms of their packing morphologies under conditions that are employed with other soft gel chromatographic sorbents and have good chemical stability at low pH values. With current manufacturing technologies, porous silica of defined particle sizes can be produced with good control over the distribution of pore diameter and tortuosity, although micropores may still contribute to loss of efficiency with some types of porous silica. However, this effect with currently available silica-based RPC sorbents appears to be smaller than that experienced with polymer-based RPC sorbents, where micropore effects make a significant contribution to loss of efficiency and peak dispersion [220]. Notably naked silicas contain reactive surface silanol groups that allow covalent attachment of a myriad of different types of RPC and HIC ligands by various manifestations of conventional siloxane [60] and disilazane chemistries [70]. The acidic nature of residual silanol groups accessible on the sorbent surface, even when the sorbent has been extensively endcapped, gives rise to the silanophilic component of the retention process. Depending on the nature of the protein or peptide, silanophilic contributions may represent an advantage or a constraint; i.e., highly basic peptides or proteins may require special attention [1,16,70,102,104,218,221,222], in terms of mobile phase selection, to ensure that adequate resolution and elution are achieved. The example cited previously for the separation of the *Thermus thermophilus* ribosomal proteins (Fig. 1) is illustrative of the approach that can be used with basic peptides and proteins when optimized mobile phase conditions are employed.

Because silica is rapidly dissolved in high-pH solution [60–62,223], this property represents a major disadvantage and has led to the search for other types of inorganic oxides, including alkaline earth metal ion-doped silicas [60–62,80,224], polymer-coating silicas [84,225–227], zirconias [85–87], aluminas [60,225], titanias [228,229], as well as graphite and other glassy carbon materials [230–232] as alternatives. Many of these alternatives have not yet gained significant popularity and typically have met with only limited success. Nevertheless, some of these new types of doped or clad-silicas and other inorganic

oxide stationary phases show improved chemical stability in alkaline mobile phases, i.e., up to pH 10. RPC sorbents derived from porous zirconia have been found [85] to be stable at pH 11 for considerable periods of time at elevated temperatures, conditions that would degrade conventional silica-based sorbents very rapidly. Illustrative of the excellent pH stability that can be achieved is the pH stability profile for an *n*-octadecylzirconia sorbent prepared *via* a novel plasma torch technology [233] and subsequent surface modification with *n*-alkyldimethylchlorosilanes, other derivatized silanes, and cross-linked polymeric coatings. The hydrolytic instability of silica-based RPC and HIC sorbents has often been claimed as the major impediment to their adoption as high-performance stationary phases in the process-scale purification of proteins or peptides by the pharmaceutical, biotechnology, and food industries. With most silica-based sorbents, cleaning in place (CIP) and column regeneration cannot be undertaken with sodium hydroxide solutions that have proved to be the effective means for the removal of foulants, cell lipids, and other strongly bound substances from the surface of soft gel RPC or HIC sorbents.

For the purification or analysis of peptides and proteins in the RPC or HIC modes, the macroreticular polymeric resins have shown considerable promise [88–93,234–236]. Resins made from styrene-divinylbenzene copolymers and other polymer combinations, e.g., methacrylates and polyvinyl-alcohol copolymers, are commercially available both with and without surface-bound *n*-alkyl ligates. Because styrene-divinylbenzene copolymer supports are already hydrophobic, no further derivatisation may be required for these materials to be used in the RPC mode of separation. The presence of unreacted vinyl or acidic groups in some commercial polymeric phases [237,238], however, means that these sorbents show mixed-mode effects with low-ionic-strength eluents. The major limitation for the use of polymeric support materials for the preparation of sorbents destined for use in RPC applications is that they tend to swell when in contact with organic solvents typically employed in RPC separations. Except for sorbents derived from highly cross-linked materials or those produced from specialized polymer systems, this swelling process can cause peak tailing due to a loss of the permeability of the sorbent. Many analytical HIC sorbents have been derived from Glymo-modified porous silicas. In contrast, most of the HIC sorbents available today for the preparative isolation of proteins are based on polymeric support materials, either from naturally occurring polysaccharide materials such as agarose or from various types of organic copolymers. Because these materials are used in aqueous environments with relatively high concentrations of salts, the swelling constraints evident with the use of the same or similar materials in RPC modes are not as troublesome. However, similar considerations about the cleaning regime must be taken into account, while at times the batch-to-batch reproducibility of polymer-based HIC sorbents has been claimed to be substantial.

In order to ensure that good performance is obtained from the RPC or HIC sorbent, attention must be given to the selection of the correct particle shape and size. After the chemical nature of the support, these physical attributes are key characteristics behind efficient, high-resolution separations of peptides or proteins by RPC or HIC procedures. Irregular and spherically shaped silica-based particles are available from a variety of manufacturing processes, typically as extensions of the sol-gel and spray-drying procedures, often followed by hydrothermal treatments. Many sources can be identified for the commercial availability of both RPC and HIC sorbents, and there are currently in excess of 500 different vendors [239]. The workhorses for most RPC applications with proteins and peptides have been the C₃, C₄, C₈, and C₁₈ *n*-alkylsilicas available as 3- to 10- μ m particles. The same sorbents are also popular for the analytical RPC of small organic molecules. Higher column efficiencies are typically achieved with spherical particles than irregular particles. Columns packed with spherical sorbents tend to have greater durability, partly because spherical sorbents are manufactured to better specifications and partly due to the close-packed morphology that can be achieved following the packing procedure. For preparative and process scale applications, of spherical and irregular particles with mean d_p values in the range of 15–20 and 40–50 μ m have had great ascendancy over the past decade, partly because of their higher productivities and lower unit operation costs [240,241]. Increasingly, particles of higher density such as surface-modified zirconia are finding their way into process applications [85–87,228,242], particularly for expanded bed capture in HIC modes, due to the rheological and viscosity characteristics of many process feedstocks.

Particle Size, Pore Diameter, and Ligand Density Effects

Because proteins and peptides have relatively large Stokes radii and hydrodynamic volumes compared with low-molecular-weight pharmaceutical substances and other organic molecules, the mean pore size and the pore size distribution of the support can have a significant influence on the separation efficiency and thus the resolution (through the dependence $R_s \propto \sqrt{N}$). In order to achieve adequate utilization of the surface area of the sorbent, and thus to achieve a reasonable level of capacity with the sorbent, the protein or peptide has to gain access to the internal regions of the porous particle. Increasing the porosity of the sorbent can achieve this outcome, but this gain is partly offset by the loss in specific surface area, and therefore column loading capacity, and by a decrease in mechanical stability. However, if it is recalled that on average <1% of the surface area of most typical porous and semiporous particles is associated with the external surface, the use of wide-pore materials achieves a significant gain in capacity arising from

the greater accessibility of the bulky proteins or peptides to RPC or HIC ligates within the pore chambers.

To achieve this requirement, the RPC and HIC sorbents will need to have pore diameters significantly larger than the mean diameters of the protein or peptide solutes being separated [243,244], ideally by a factor of at least 10. Consequently, pore diameters in excess of 300 Å are now the norm for many RPC and HIC sorbents in both analytical and preparative applications. However, wide-pore supports (particularly those with average pore diameters greater than 1000 Å) can show increased mechanical or pressure drop fragility, resulting in reduced column life. Gigaporous materials, i.e., sorbents with very large pores up to 10,000 Å, have also gained some popularity. With these macroporous materials, as well as with the advent of monolithic types of RPC sorbents [90–93], very high flow rates can be achieved without the sacrifice of equally high back pressures. The use of sorbents with gigantic pores or channels effectively acting as interconnecting tunnels results in convective or perfusive flow [88,89,245,246] of the mobile phase fluid along the main branches and diffusive flow in the side branches where pores of smaller mean diameter may exist [247]. Although RPC sorbents based on macroporous organic polymers, such as polystyrene, have been available for more than a decade and widely employed at high superficial fluid velocity, thus allowing very rapid analytical and semipreparative applications to be carried out, similar applications with the monolithic types of RPC sorbents at high flow rates have yet to gain popularity due to their lack of commercial availability.

An alternative approach to the issues of pore size, particle size, surface area accessibility, mass transfer kinetics, and mass loadability that has shown some promise involves the micropellicular RPC and HIC sorbent materials [220,248,249], which are based on microspherical, fluid-imperious particles with appropriate types of retentive coating. These types of particles also show improved peak efficiencies at high linear flow velocities because intraparticle diffusion resistances are absent, with the interior of the particle behaving as an essentially nonporous medium. One limitation with micropellicular RPC and HIC sorbents is their greatly reduced mass loadability. To circumvent this constraint, particles of smaller average diameter, d_p 1.5 to 3 μm , can be employed [25,63,67,94], but clearly in these cases the application area that is being targeted is the ultrafast, high-flow-rate analytical separations, often at elevated temperatures to decrease the viscosity of the mobile phase and permit low operating back pressure to be generated. Otherwise the consequences of the dependence of back pressure on particle diameter will become manifest, e.g., $\Delta P \propto 1/(d_p)^2$. Reducing the average d_p of an RPC or HIC sorbent is a simple way to increase column efficiency, and in this context the nonporous silicas, such as the Micra NPS-I or the polymeric TSKgel NPR reversed-phase sorbents, have gained some popularity. With such sorbents in well-packed (25–33 mm length) columns, peak

efficiencies of >300,000 plates/m can be achieved. For many analytical RPC and HIC separations of proteins or peptides, such minicolumns of the standard 4.5–5.0 mm inner diameter have been the norm for many years.

One outcome of using very small particle diameter micropellicular, porous or nonporous RPC (or HIC) sorbents is that microbore or capillary columns can be employed, although in these cases the issue of detector response and the flow turbulence caused by poorly designed end fittings, frits, and detector cells becomes critically important. Various solutions to this engineering problem have been proposed, including those based on modifications of conventional HPLC instrumentation, leading to an increase in detection sensitivity either by UV or by ESI-MS or MALDI-TOF MS techniques [116,117,250,251]. Increasingly, as the requirements for enhanced sensitivity of detection with even decreasing amounts of protein and peptide solutes become more demanding, the use of narrow-bore (1 or 2 mm ID), microbore (0.5–1 mm ID), and capillary (100–500 μm ID) columns has attracted greater attention [252–255]. The advantage of decreased solvent consumption arising from the lower flow rates employed with smaller diameter columns also becomes a consideration with these “miniaturized” systems. Compared with separation carried out on a conventional 4.6-mm internal diameter column, a 1-mm microbore column packed with the appropriate 3- μm RPC sorbent will typically show smaller peak volumes and a 5 to 10-fold increase in detection sensitivity when the same sample amount of, e.g., a tryptic digest of a protein is injected. The advantages of employing capillary columns packed with suitable RPC or HIC sorbents for the resolution of peptide or protein samples at the ultramicro analytical scale are obvious. Thus, as little as 2 pmol of a tryptic digest of horse skeletal muscle myoglobin can be readily separated [256] on a 180 mm ID capillary column, packed with a 5- μm d_p , 300 Å pore diameter C_{18} silica using a standard gradient system at a flow rate of 100 $\mu\text{L}/\text{min}$. Although the gain in sensitivity with microbore or capillary columns compared with conventional columns does not always correspond to the theoretical value, i.e., detection sensitivity gain = $(d_{c1})^2/(d_{c2})^2$ where $d_{c1,2}$ are the column diameters, typically >80% of the theoretical advantage in sensitivity can be achieved with conventional HPLC equipment. This enhancement in detectability is due to reduced dilution of the sample as it traverses the column, although the theoretical gain of ≈ 20 -fold in the preceding case is not realized due to the participation of other column and extracolumn zone-broadening effects. Such systems readily permit the effluent stream to be interfaced with an ESI-MS or gas-phase sequencer without additional handling steps.

Instrumentation is now available to deliver reliably flow rates in the range of 5–100 $\mu\text{L}/\text{min}$, thus permitting the general application of microbore and capillary columns packed with RPC and HIC sorbents to be employed. These developments have enabled exciting new modes of RPC to emerge, including

high-performance capillary electrochromatography (HP-CEC) of proteins and peptides [257,258]. Efficiencies of more than 250,000 theoretical plates per meter can be routinely obtained with such types of microbore or capillary columns, operated in the CEC mode, permitting very high sensitivity detection of the separated components, i.e., $\sim 100\text{--}200\text{ pg}$ of protein samples [259], to be achieved. Of particular interest to the further application of CEC procedures with nonpolar sorbents are the recent observations that the selectivity of peptides in CEC is fundamentally different from that manifested in the conventional RPC mode. As a consequence, peptide mixtures that have proved recalcitrant to RP-HPLC separation techniques have proved responsive to HP-CEC procedures [257,258,260]. Additional gains can be made with RPC separations carried out with capillary columns if the protein or peptide analytes can be detected by laser-induced fluorescence techniques [261,262]. With such microbore or capillary columns, by interfacing the effluent directly with a continuous-flow fast atom bombardment (FAB), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), or electrospray ionisation (ESI) mass spectrometer, peptides derived from solid-phase peptide synthesis or from the enzymatic digestion of proteins and separated by RPC under gradient elution conditions can be directly analyzed in terms of their mass, composition, and sequence within tens of seconds.

Surface Modification Considerations for RPC and HIC Sorbents

In the development of RPC sorbents based on silica support materials, chemically modified with an *n*-alkylsilane, several widely employed bonding strategies have been utilized [60–63,67–71,263–267]. One approach involves the reaction of an *n*-alkylsilane of the type RSiX_3 or R_2SiX_2 ($\text{X} = \text{chloro or ethoxy}$) with the silica, frequently preactivated by a hydrothermal treatment [60–63,67–71,85,268]. Silica modified with R_2SiX_2 silanes exhibits multifunctional surface characteristics. This strategy often leads to the generation of a polymer layer deposited at the silica surface, thus partially stabilizing the silica to hydrolysis. In addition, this strategy appears to partially obviate the participation of secondary binding processes by effectively masking certain undesirable properties of the residual acidic silanols present on the silica surface. In terms of reproducibility of the manufacturing process, these sorbent materials tend, however, to be harder to reproduce, with variation in the total organic coverage evident from batch to batch. Moreover, the thickness of the polymeric layer can lead to reduced mass transport, leading to impaired column efficiencies compared with the corresponding monolayer bonded phase materials.

The second popular approach is based on the reaction of an *n*-alkyldimethylchlorosilane or *n*-trialkylchlorosilane of the type RMe_2SiX or R_3SiX

(X = chloro). The advantages of this reaction are that it is reproducible and convenient. The resulting surface of the monolayer coverage exhibits excellent mass transfer characteristics that result in high column efficiencies for most peptides and proteins. The silanization reaction can be carried out under anhydrous or aqueous conditions, using a small amount of a basic catalyst such as lutidine. Moreover, when shielded *n*-alkyldiisopropylchlorosilanes or *n*-alkyldi-*tert*-butylchlorosilanes are employed, RPC sorbents with excellent pH and temperature profiles can be prepared. However, the very dense coverages, typical of surface-modified silicas when di- or trichlorosilanes are employed, may not occur with *n*-alkyldimethylchlorosilanes. In order to overcome the relatively low reactivity of monochlorosilanes, the reaction conditions of different chlorosilanes, organoaminosilanes, and the *n*-alkylenolate silanization, have been studied in great detail over the past 20 years. As a consequence, various approaches have evolved, including the use of more reactive chlorosilanes. Under comparable conditions, the reactivities of the chlorosilanes tend [63] to follow the relative order $C_8N(CH_3)_2 > C_8OCOCF_3 > C_8Cl \gg C_8OH = C_8OCH_3 \gg C_8OC_8$. In addition, di-*n*-alkyltetramethyldisilazane, such as di-*n*-butyltetramethyldisilazane, can be employed [70] in place of *n*-alkyldimethylchlorosilane, permitting proximal and geminal silanol groups to be more effectively modified due to the bifunctional nature of these reagents. With such disilazanes, under optimized conditions, ligand densities approaching the limited values can be reached, i.e., between 3.50 and 4.20 $\mu\text{mol}/\text{m}^2$, while typically with chlorosilanes ligand densities within the range of 2.5–3.2 $\mu\text{mol}/\text{m}^2$ are more usual. Considerably less information has been reported in the scientific literature about the methods used in the commercial production of polymeric RPC sorbents, partially due to the perceived commercial sensitivity about the actual characteristics of the polymer backbone but also due to the proprietary nature of the additive formulations used in the emulsion or free radical polymerization procedures to generate the porous network or to control particle diameter distribution with these cross-linked organic polymers/copolymers.

Procedures analogous to those employed to optimize the manufacture of RPC sorbents have also been developed for the synthesis of HIC materials. Currently, two major types of HIC materials can be identified: those based on inorganic oxide support materials and those made from organic copolymers. The ligand density of commercially available HIC sorbents typically falls within the range of 20–40 $\mu\text{mol}/\text{mL}$ sorbent as determined by solid-state NMR, gas chromatography, and, where feasible, elemental carbon analysis [36,269]. All silica-, zirconia-, or other metal oxide-based HIC sorbents can be operated at relatively high superficial velocities without particle deformation and are resistant to swelling and shrinkage under different mobile phase conditions. However, as noted with RPC sorbents, the use of monofunctional silane derivatives for the introduction of the nonpolar ligands either directly via ether-type dimethylchlor-

osilanes or via a γ -glycidoxypopyl modified metal oxide surface, such as a porous GLYMO-based silica support material, tends to result in stationary phases of lower stability than can be generated with trifunctional silanes [60–62,71,72], and as a consequence a subsequent cross-linkage step may be required to permit longer term ligand stability. As an alternative approach, passivated silica surfaces with dextran or dimethyl aminodextran coatings can be employed to generate a hydroxylated template onto which nonpolar ligands can be chemically linked or grafted. Likewise, with silica-based HP-HIC sorbents, the operational limit for use under alkaline pH conditions for extended periods of time without loss of performance is usually $\sim$ pH 7.5. When polymer-grafted coatings are employed as an intervening layer, thus protecting to some extent the silica support material, operation under alkaline pH conditions can be extended somewhat, with separations feasible for short periods of time up to pH 9.0. In fact, with HP-HIC sorbents, the knowledge gained from studies with the soft agarose gels has been directly adapted to silica-based (as well as polymer-based) support materials to allow enhanced chemical stability and performance.

To achieve this outcome, it has been necessary to develop generic strategies for the introduction of hydrophobic ligands of relatively short chain length ($n \leq 6$) and to immobilize these moieties at low densities either onto hydroxylated surfaces or en bloc as ether-like derivatives to allow the elution of enzymes and other proteins to be carried out without resorting to forceful elution conditions that may cause denaturation [50,51,270]. As a consequence, the preferred approach for the preparation of high-performance HIC sorbents is based on the concept of initially introducing an underlying bonded phase or coating that mimics the polar, highly hydroxylated characteristics of polysaccharide-based gels and then to introduce, in one or more synthetic bonding or coating steps, the relevant hydrophobic ligand groups, which can be selected to have a particular chain length or hydrophobicity. Thus, silica-based [110] and polymer-based [271] support materials developed for size exclusion applications can be derivatized with *n*-butyl- or phenyl-glycidoxyl ethers via an introduced hydrophilic layer to generate HP-HIC sorbents. Alternatively, weakly hydrophobic polymers, such as polyvinyl alcohols, oligoamino alcohols, and polyoxyethylenes of various molecular weights can be attached via multistep reactions to *n*-propyloxy-modified silicas via reactive functional groups to generate HIC sorbents of different hydrophobic characteristics [110,111]. Similar multistep procedures have been employed to introduce [272] *n*-alkylamines of different chain lengths into previously prepared polyaspartic acid-modified silicas, and polyamide-bonded silica support materials have been employed as a scaffold on which various nonpolar ligands [273] could be immobilized. In terms of the control over the derivatization chemistries, however, the preformation and subsequent immobilization of the complete hydrophobic ligand represents a more elegant approach. Based on this strategy, several different types of

HP-HIC sorbents have been described, including those derived from polyethersilanes containing *n*-alkyl groups of different ligand chain length and hydrophobicity [73] or from *N*-acetyl-amino-*n*-alkylsilanes [274].

Hydrophilic polymeric support materials provide alternatives to silica for the preparation of HIC sorbents. These materials are more stable to high-pH conditions used as part of the “clean in place” (CIP) regimes [46]. Representative examples of HIC sorbents are contained in Table 3, and many other in-house laboratory variants can be identified in the scientific literature. From the list of commercialized HIC sorbents found in Table 3, it is apparent that the majority of preparative HIC sorbents can be obtained in particle size ranges (typically 20–65 μm), pore size distributions (typically ≈ 600 –1000 Å), and surface chemistries suitable for large-scale applications with proteins and peptides. The TSK PS copolymer materials or the Pharmacia cross-linked Superose/agarose range of HIC sorbents are well-known examples in industry. Illustrative of the various uses of silica- and polymer-based HIC sorbents are the examples of avian lysozymes [144], human pituitary prolactin [275], recombinant subtilisin variants [145], and other globular proteins [276–278].

In common with analytical RPC sorbents, HIC sorbents destined for analytical and micropreparative separations typically involve small particle diameter materials ($d_p \leq 10 \mu\text{m}$) and large nominal pore size ($p_d \geq 300 \text{ Å}$). With the increased trend toward the use of microbore and capillary column formats for analytical applications, HIC sorbents based on 1.5- μm nonporous amide- or ether-bonded phase silicas represent attractive opportunities for very high speed separations of proteins, i.e., with retention times of the order of seconds rather than minutes. For example, Janzen et al. [94] described a high-speed HP-HIC application based on the use of nonporous 1.5- μm ether- and amide-bonded silicas with eight proteins resolved in less than 3 mm. Although preservation of biological activity is not a prerequisite for such high-speed HIC analytical applications, the choice of the ligand hydrophobicity must still be taken into account if adequate mass balances, and hence detectability, of labile proteins are to be achieved. As in RPC, in HIC applications the choice of the *n*-alkyl chain determines the sorbent hydrophobicity, which must be balanced against the lability of the proteins to be separated. Exemplifying this effect of sorbent hydrophobicity is the inability of α -lactalbumin to elute at 25°C from a TSK Phenyl PW phase whereas this protein could be readily eluted from a methylpolyether phase under similar chromatographic and salt gradient conditions [192,279]. Because the retention process is dictated by the affinity of the interaction between hydrophobic patches on the protein surface and the nonpolar ligates, sorbents of inappropriate hydrophobicity can lead to “irreversible” interactions as exemplified before with α -lactalbumin or with various other proteins [8,46,78,192–194], where denaturation effects lead to loss of protein recovery and greatly decreased detection sensitivity.

TABLE 3 Selected Commercially Available HP-HIC Sorbent Materials^a

Manufacturer's name and details	Particle/pore size	Phase material
Beckman		
Spherogel CAA-HIC	5 μm /300 Å	Methyl polyether/silica
BioChrom Labs		
Hydrocell C3 1000	15 μm /1000 Å	Allyl/polymer
Hydrocell C4 1000	15 μm /1000 Å	Butyl/polymer
Hydrocell C3 NP10	5 μm	Allyl/polymer
Hydrocell C4 NP10	5 μm	Butyl/polymer
Bio-Rad		
Bio-Gel MP7 HIC	7 μm /900 Å	Methyl/polymer
Bio-Gel Phenyl 5PW	10 μm /1000 Å	Phenyl/polymer
Methyl HIC	50 μm /1000 Å	Methyl/methacrylate
<i>t</i> -Butyl HIC	50 μm /1000 Å	<i>t</i> -Butyl/methacrylate
Interaction Inc		
Hydrophase HP-Butyl	10 μm	Butyl/polymer
J. T. Baker		
BakerBond HI-propyl	5 μm /300 Å	Propylsilica
BakerBond PREPSCALE HI-propyl	40 μm /275 Å	Propylsilica
Mitsubishi		
MCI Gel-HIC-Ethyl, Butyl, Phenyl	10 μm /1000 Å	Ethyl, butyl, phenyl/polymer
PE—Perspective Biosystems		
POROS PH	10 μm /8000 Å	PS/DVB beads coated with a cross-linked polyhydroxylated polymer with large transecting pores
POROS PE	10 μm /8000 Å	
POROS BU	10 μm /8000 Å	
POROS ET	10 μm /8000 Å	
Pharmacia		
Phenyl-Sepharose	34 μm	Phenyl/agarose
Butyl-Sepharose 4B	45–165 μm	Butyl/agarose
Octyl-Sepharose CL-4B	45–165 μm	Octyl/agarose
Phenyl-Sepharose CL-4B	45–165 μm	Phenyl/agarose
Butyl Sepharose 4 FF	45–165 μm	Phenyl/agarose
Phenyl Sepharose 4 FF	45–165 μm	Phenyl/agarose
Hi-Load Phenyl Sepharose	34 μm	Phenyl/agarose
Phenyl-Superose	10–13 μm	Phenyl groups cross-linked agarose
Alkyl-Superose	10–13 μm	Neopentyl/cross-linked agarose

TABLE 3 (continued)

Manufacturer's name and details	Particle/pore size	Phase material
Poly LC		
Polyethyl A	5 μm /330 Å	Polyalkylaspartamide/silica
Polypropyl A	5 μm /330 Å	Polyalkylaspartamide/silica
Shoda Denko		
Shodex HIC	35 μm /800 Å	Phenyl/polyhydroxy methacrylate
Supelco		
LC-HINT	5 μm	"Polar" diol bonded phase/silica
SynChrom		
SynChropak ^b Methyl, Propyl, Pentyl, Benzyl, Hydroxypropyl, SynChrorep ^c	6.5 μm /100 Å, 300 Å, 500 Å, 1000 Å 30 μm /300 Å	Polyamide coating with indicated ligand/silica
TOSOH ^d		
TSK Phenyl 5PW	10 μm /1000 Å	Phenyl groups/polymer base
TSK Ether 5PW	10 μm /1000 Å	Oligoethylene glycol groups/polymer base
TSK Butyl NPR	2.5 μm	Polymer
TSK Phenyl 5PW	20–40 μm /1000 Å	Phenyl groups/polymer base
TSK Ether 5PW	20–40 μm /1000 Å	Oligoethylene glycol groups/polymer base
TSK Ether 650	20–50 μm /1000 Å	Oligoethylene glycol groups/polymer base
TSK Butyl 650	40–90 μm /1000 Å	Butyl/polymer
TSK Phenyl 650	50–150 μm /1000 Å	Phenyl/polymer
YMC		
YMC-Pack-HIC	6.5 μm /300 Å	Propyl/cross-linked polyamide silica

^aThis list is intended not to be exhaustive but rather to represent a variety of products that are available from manufacturers with wide commercial distribution network.

^bAny combination of ligand type and pore size is available.

^cSynChrorep packings are available with any SynChropak ligand type.

^dTSK packings are distributed either through TOSOBIOSEP or TOSOH Corp.

In both RPC and HIC, theory predicts that retention should be greatest when the mobile phase pH is near the isoelectric point of the peptide or protein. Ionization changes of specific amino acids located within the chromatographic contact region can particularly affect the retention. As a consequence, pH changes can be indirectly used to influence significantly the magnitude of hydrophobic

interactions in RPC and HIC, with the opportunity, in principle, to carry out separations over a wide range of pH values [3,8,16,17,58,72,164,175,276,280]. Mobile phase pH conditions can thus often be chosen for HIC, as well as RPC, separations of peptides or proteins to obtain appropriate selectivities without necessarily compromising the stability of the solute to be purified.

Mixed Modality Sorbents

One of the consequences of the interplay of electrostatic, size exclusion, hydrogen-bonding, and hydrophobic interaction effects that occur between proteins or peptides with all types of chemically modified surfaces is that selectivity reversals and changes in resolution can be achieved with a single column by exploiting enhancement or suppression of a particular interaction via changes in pH, solvent composition, ionic strength, or the presence of different classes of additives [8,16,58,72,281]. Alternatively, during the chemical synthesis of the sorbent, different types of ligand characteristics can be purposely incorporated or “tailored” into the surface of the RPC or HIC sorbent, resulting in the generation of different retention modes and different selectivities. Recognizing the value of such mixed-mode selectivities, various manufacturers have introduced over the years a variety of RPC and HIC sorbents that take advantage of these effects [282–284]. One advantage of the mixed-mode approach lies in the potential for generating selectivity features that are different from those obtained with standard RPC or HIC surfaces. This leads to the opportunity for multiple utilization of the same sorbent, packed into a single column, in different (multimodal) modes, each of which manifests different selectivities according to the extent of variation in the mobile phase conditions. Thus, specific functional groups can be deliberately incorporated into a weakly hydrophobic HIC stationary phase to enhance retention of proteins at low salt concentration, where formerly they may have eluted with a standard HIC sorbent at the same ionic strength.

The option also exists with RPC and HIC sorbents to modulate selectivity for peptides and proteins through changes in the chemistry used at the end-capping stage of the synthesis of the sorbent. In fact, one of the advantages of the silanophilic characteristics present in all silica-based RPC sorbents is that selectivity can be adjusted at the different percentages of an organic solvent with a water–organic solvent mobile phase through the simple expedient of adding to the mobile phase a reasonable high concentration of a suitable salt. In so doing, two important properties of the system are adjusted. First, the surface tension of the mobile phase is changed, leading to a variation in selectivity in its own right. Second, electrostatic and hydrogen-bonding effects mediated by the geminal/vicinal type I or type II silanol groups are masked, again leading to a

change in selectivity and retention. Such a strategy was used for the separation of the 50S ribosomal proteins [1] shown in Fig. 1, where 300 mM KH_2PO_4 was added to the mobile phase with a weakly end-capped RPC sorbent (LiChroSpher 1000). Under these conditions, basic proteins, such as those present in the 50S ribosomal protein mixture from *Thermus thermophilus*, can be resolved under low-pH acetonitrile-water gradient elution conditions, where formerly this resolution could not be achieved with highly end-capped sorbents and the same eluents. The literature contains a large number of examples of the use of such multimodal RPC and HIC adaptations for the separation and/or purification of proteins and peptides, particularly when employed in the analytical or small-scale preparative HPLC mode. Some well-known HIC examples include the separation of monoclonal antibodies [285–288] of different subclass or isotype characteristics, various enzymes, e.g., cytochrome c_{553} [194], microsomal cytochrome P450 [289], calcium-independent phospholipase [290], proteinase inhibitor [291], therapeutic proteins [292–295], receptors [296], and other peptides [297] that have been isolated using these principles of multinodal resolution with a sorbent that is notionally of the RPC or HIC type.

Because sufficient knowledge is now at hand to direct the separation strategy, a further possibility of adapting standard RPC or HIC selectivities is to choose elution conditions that are known to amplify interactions based on the hydrophobic effect, but with sorbents of different types of ligate characteristics. Thus, with sorbents that are notionally ion exchange, or even metal ion affinity, it is possible with the judicious choice of elution conditions to obtain selectivities that manifest a significant component of the hydrophobic effect. For example, such a strategy has been followed with the use of several new classes of immobilized metal ion affinity sorbents based on the use of iminodiacetic acid (IDA) and other chelating ligands as their borderline (Cu^{2+} , Ni^{2+}) or hard (Fe^{3+} , Al^{3+}) metal ion complexes [298–302]. When descending gradients from high to low ionic strength are employed with such iminodiacetic acid or tacn-based IMAC sorbent systems commencing with relatively high ionic strength values, i.e., 4 M NaCl, these IMAC sorbents exhibit selectivities typically expected for an HIC resin and dramatically different from the selectivities observed when low-ionic-strength (≤ 0.2 M) buffers are employed. Obviously, similar approaches could be used with sorbents based on other types of ligand chemistries. Such behavior is indicative that more than one type of interaction force contributes to the overall retention. Moreover, with such systems the use of high concentrations of a kosmotropic salt will tend to stabilize a protein in its native conformation regardless of the characteristics of the sorbent surface. As a consequence, the possibility for higher recovery of biological activities may be an outcome when operating in this region of the separation window, rather than operating in another region where the selectivity of the sorbent is more “standard.” Advantage can thus be taken of these types of multimodal effects, and many thoughtful

investigators interested in achieving rapid, high-selectivity separations of complex feedstock mixtures of proteins do so today.

Certain types of thiophilic compounds, particular the aromatic sulfides, have been found to function extremely well as ligates in HIC under salt-promoted adsorption conditions. With such thiophilic ligates, charge transfer interactions may also occur, enhancing the effects mediated by the close contact of the hydrophobic binding site(s) on the protein with the hydrophobic surface of the sorbent. The incorporation of these structures into chromatographic sorbents has been described by Porath [303] and Wilcek et al. [304] under the rubric of salt-mediated chromatography. With these thiophilic sorbents, high concentrations of kosmotropic salts are required at the adsorption stage for the expulsion of proteins out of the bulk aqueous phase onto the surface of the matrix. Depending on the type and concentration of the salt and the feedstock compositions, selective adsorption of components from a mixture can be achieved. The participation of short-range interaction forces is thus essential to effect a tight association of the protein and the stationary phase. Such extensions of the HJIC concept have been applied to the fractionation of human serum. For example, ceruloplasmin can be selectively adsorbed onto *p*-methoxyphenylsulfide-agarose in the presence of 0.5 M potassium sulfate, and other serum proteins can be adsorbed with similarly functionalized thiophilic HIC sorbents [303].

Mobile Phase Characteristics in RPC and HIC

The ability of relatively inexperienced investigators to acquire readily the skills to manipulate the composition of the mobile phase and achieve within a short time the desired level of selectivity and resolution in RP-HPLC separations of peptide or protein mixtures is one reason why this technique has become so dominant in the life sciences over the last two decades. Analytical protein separations also can be generally expected to benefit from the flexibility to modify the mobile phase by using various additives. In RPC the mobile phase is more polar than the sorbent and is typically composed of a low-pH, buffered water-organic solvent mixture. As mentioned before, the profound dependence that applies between $\log k'$ and the volume fraction of the organic solvent modifier, ψ , in RPC systems with peptides and proteins necessitates in many cases that gradient elution protocols be applied with the ψ value progressively increased during the chromatographic experiment. Many types of gradient profiles can be generated, but from pragmatic considerations, linear gradient profiles are to be preferred. Under some RPC conditions, and similarly in HIC, a linear change in $\log k'$ per unit time can be observed when a linear gradient protocol of increasing organic solvent or decreasing salt concentration is employed. Such conditions satisfy the criteria of the linear solvent strength (LSS) model of gradient elution [16,58,72,305,306]. As a consequence, the influence of gradient time on the

chromatographic retention, peak efficiency, and capacity of the RPC or HIC separation can be predicted *de novo* under linear solvent strength criteria and the effect of column length and particle diameter rationalized. Approaches to optimize the resolution of peptides and proteins in RPC and HIC environments can thus be employed using computational algorithms of the retention behavior of peptides and proteins based on this and related simulation models.

The example shown in Fig. 1 for the 50S ribosomal proteins using RP-HPLC was based on such an optimization approach. Similarly, in Fig. 23 a further application of the LSS concepts is illustrated in the derivation of the $\log \bar{k}$ versus $\bar{\zeta}$ plots for several globular proteins [146] separated on an *n*-butyl TSK 650M sorbent under ammonium sulfate gradient conditions, where both the $\log \bar{k}$ and $\bar{\zeta}$ values were calculated with the *Pek-n-Eze* and *Hephaestus* software, which similarly treats gradient retention data in terms of the incremental changes in solvent strength across the peak zone and column. Good correlations have been found [15,16,106,161,164,305,307] in terms of the linearity of the $\log \bar{k}$ versus $\bar{\psi}$ and $\log \bar{k}$ versus $\bar{\zeta}$ plots over limited ranges of $\bar{\psi}$ or $\bar{\zeta}$ values, provided no

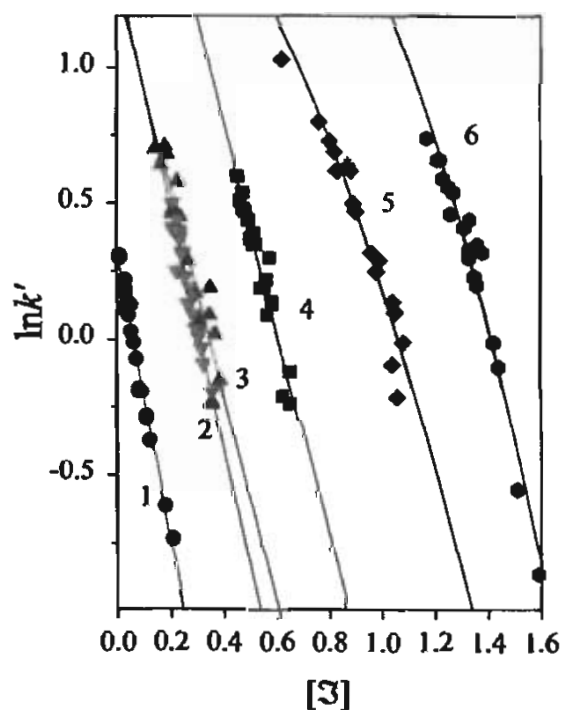


FIGURE 23 Application of the LSS concepts in the derivation of the $\log \bar{k}$ versus $\bar{\zeta}$ plots for several globular proteins [146] separated on an *n*-butyl TSK 650M sorbent using ammonium sulfate gradient conditions, where both the $\log \bar{k}$ and $\bar{\zeta}$ values were calculated with the *Pek-n-ese* and *Hephaestus* software. Code for the proteins is: 1, horse heart cytochrome c; 2, sheep muscle myoglobin; 3, horse muscle myoglobin; 4, ribonuclease A; 5, hen egg white lysozyme; and 6, α -lactalbumin. (M. T. W. Hearn, R. I. Boysen, and Y. Q. Wang, unpublished results.)

secondary effects, such as conformational changes, self-aggregation, specific binding to metal ions, and temperature-induced effects, significantly affect the chromatographic performance.

Because proteins can denature under some solvent and cosolvent conditions due to specific ion interaction or excluded water effects [308,309], it is important to employ mobile phase compositions that ensure adequate selectivity without inducing substantial changes in conformation, loss of cofactors or other weakly bound allosteric regulators, or protein-protein aggregation and precipitation. Consequently, the choice of the organic solvent, its concentration, the presence of ion-pairing reagents and other additives such as nonionic detergents, and the nature of the buffer species and the operational pH are all factors that must be taken into account to ensure successful RPC separations of peptides and proteins.

In RPC, peptide and protein separations are most commonly carried out at acidic pH, with ~15 mM trifluoroacetic acid (TFA), 15 mM phosphoric acid, 25–50 mM acetic acid [310], up to 60% formic acid [311,312], or 10 mM HCl [313,314] added to the mobile phase. The use of low-pH conditions has a desirable outcome with silica-based sorbents because under these conditions the silanol groups are not ionized and peak tailing with basic peptides can be partially suppressed. The volatile organic acids (trifluoroacetic, formic, or acetic acids) permit direct recovery of separated samples via lyophilization. In addition, these ionic modifiers are compatible with direct injection to a mass spectrometer for subsequent analysis of the separated peptides or proteins. Besides the issue of pH control, these acids also act as ion-pairing reagents with peptides and proteins, thus permitting improvement in the peak efficiencies and overall recoveries when they are present in low concentrations [58,72,100,102,106,161,162,204,315]. When RPC separations of peptides and proteins need to be carried out at higher pH values, 100 mM ammonium bicarbonate, 25–200 mM ammonium acetate, 50 mM triethylammonium phosphate, and 500 mM pyridine acetate have been favored, although in the latter case postcolumn derivatization and fluorescence detection are required. The availability of polymeric RPC sorbents makes feasible the exploration of higher pH ranges under conditions that are incompatible with the use of many silica-based RPC sorbents [207], although typically most investigators still favor elution using lower pH values, i.e., $\text{pH} \leq 4.0$, with polymer-based sorbents.

Experience has shown that the resolution by RPC methods of very closely related proteins, such as deamido forms, methionine *S*-oxide, or N-terminal methionyl variants, is best achieved if mobile phase conditions can be chosen whereby the native structures of the proteins are preserved in the RPC system. The choice of these conditions frequently dictates that higher pH conditions, i.e., pH 4.5–7.5, are selected in these cases. To address these requirements and to take advantage of the ionization status of the amino acid side chains, a very large number of different ion-pairing reagents (including those already indicated

before) have been developed [58,72,282] for use in the resolution of peptides and proteins by RPC procedures. Table 4 lists a selection of these reagents. Various studies have confirmed that these ion-pairing reagents modulate selectivity of peptides and proteins with RPC sorbents in a concentration-dependent manner. Typically, these ion-pairing reagents are employed in the range of 10–15 mM, although higher concentrations have been utilized in some applications.

Illustrative of the effects of these reagents on the retention and selectivity of peptides with high-performance *n*-alkylsilica sorbents are the plots of k' versus $[X]$, where X corresponds to the ion-pairing reagent *n*-hexylsulfonic acid, shown in Fig. 24. Because of the structural properties of many of these ion-pairing reagents, modulation of both the bulk mobile phase secondary chemical equilibria and equilibria associated with dynamic coating of the nonpolar sorbent can be achieved. Because some of these reagents permit RPC separations to be carried out at neutral and even alkaline pH values rather than acidic pH values, the use of these conditions offers a strategy to resolve peptides and proteins that may not be separable under low-pH conditions.

Thus, two different approaches can be used for the separation of microheterogeneous proteins. In the first approach, the microheterogeneity is associated with substitution, deletion, or minor posttranslational modifications of surface-accessible amino acid residues of the protein of interest, which exists in its native conformation. In this case, it may be prudent to use pH conditions that maximize the differences in charge state between the different microheterogeneous species and not use elution conditions that induce significant levels of unfolding. The correct choice of these conditions in terms of the solvent type, pH, and ion-pairing reagent thus becomes important. This selection and assessment of the origin of selectivity differences can often be guided using high-performance capillary electrophoresis (HPCZE) methods as a prelude. In the second approach, the microheterogeneity is due to major posttranslational modification or substitution of specific amino acid residue motifs that leads to significantly greater hydrophobicity. In this case, elution conditions that favor some unfolding may permit this change to be accentuated, thus permitting resolution of the microheterogeneous species. Such approaches have been employed to resolve growth hormone variants produced by recombinant engineering procedures [316] and have been generally applied to many other proteins, posttranslationally modified polypeptides, and also many diastereomeric or deletion peptides prepared by solid-phase peptide synthesis (SPPS).

Organic Solvent and Other Mobile Phase Properties

As already discussed, acetonitrile, methanol, ethanol, and 1- or 2-propanol have proved to be the most useful organic solvent modifiers for the RPC of peptides and proteins, although other organic solvents such as tetrahydrofuran, dioxane,

TABLE 4 Selection of Ionic and Neutral Species that at pH < 7.0 Modify the Retention Characteristics of Unprotected Peptides on Chemically Bonded Hydrocarbonaceous Stationary Phases^a

Anionic pairing ion	Reference ^b	Cationic pairing ion	Reference ^b
Ionic-pairing ion/dynamic liquid-liquid ion-exchange/mobile phase buffer species			
H ₂ PO ₄ ⁻ /HPO ₄ ²⁻	4,100–102,200,416,581	NH ₄ ⁺	4,100–102,582
Cl ⁻	1,199,310,583	CH ₃ NH ₃ ⁺	584
ClO ₄ ⁻	100–102,200,584	C ₃ H ₉ NH ₃ ⁺	585
SO ₄ ⁻	100–102	C ₁₂ H ₂₅ NH ₃ ⁺	586
CO ₃ ²⁻	100–102	HOCH ₂ CH ₂ NH ₃ ⁺	586
BO ₃ ³⁻	100–102	C ₆ H ₁₃ NH ₃ ⁺	586
HCO ₂ ⁻	587	(HOCH ₂ CH ₂) ₃ NH ⁺	586
Tartrate	588	(C ₂ H ₅) ₃ NH ⁺	589
Citrate	590	N-Methyl- morpholinium	591
CH ₃ CO ₂ ⁻	581,582,592	N-Methyl- piperidinium	100–102
CF ₃ CO ₂ ⁻	593,594	Piperazinium	100–102
C ₂ F ₅ CO ₂ ⁻	594	TEMED	100–102
C ₄ F ₇ CO ₂ ⁻	4,100–102	Triammonium propane	100–102
C ₆ F ₁₁ CO ₂ ⁻	4,100–102	(CH ₃) ₄ N ⁺	586
C ₄ H ₉ SO ₃ ⁻	4,100–102	(C ₂ H ₅) ₄ N ⁺	586
C ₅ H ₁₁ SO ₃ ⁻	4,100–102	(C ₃ H ₇) ₄ N ⁺	586
C ₆ H ₁₃ SO ₃ ⁻	4,100–102,584,586	(C ₄ H ₉) ₄ N ⁺	586
C ₇ H ₁₅ SO ₃ ⁻	4,100–102	Pyridinium	595
C ₈ H ₁₇ SO ₃ ⁻	4,100–102	Cationic metal chelates	4,100–102
C ₁₂ H ₂₅ SO ₃ ⁻	4,100–102	Group I and II inorganic cations	586
C ₁₂ H ₂₅ SO ₄ ⁻	4,100–102		
Camphor-10- sulfonate	580		
p-Toluenesulfonate	4,100–102		
Naphthalene- sulfonate	4,100–102		
Neutral polar-phase modifying reagents			
C ₁₁ H ₂₃ CH ₂ OH	4,100–102	(CH ₃) ₃ CCH ₂ OH	4,100–102
C ₇ H ₁₅ CH ₂ OH	4,100–102	(CH ₃ CH ₂ CH ₂) ₃ COH	4,100–102
C ₁₀ H ₂₁ CH ₂ CN	4,100–102		
C ₆ H ₅ CH ₂ OH	4,100–102		

^aThis selection of ionic and neutral species is generally compatible with UV detection below 235 nm or, in the case of the reagents with an aromatic nucleus, with fluorometric or UV (above 254 nm) detection. (The concentration of these compounds in the mobile phase is usually < 5 mM.)

^bFor a more complete compendium of applications, conditions, etc., see Refs. 58,72,101.

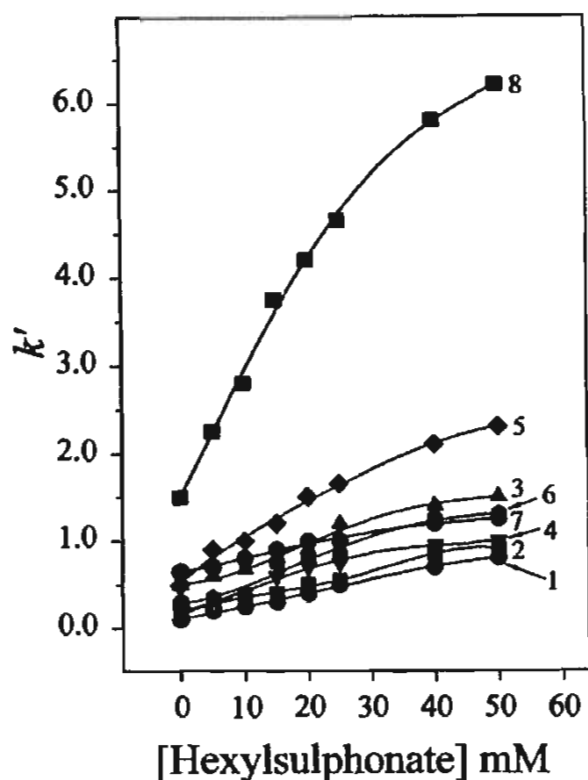


FIGURE 24 Illustrative of the effects of ion-pairing reagents on the retention and selectivity of peptides with high-performance *n*-alkylsilica sorbents are the plots of k' versus $[X]$, where X corresponds to the reagent *n*-hexylsulfonic acid. The experimental data were acquired using a μ -Bondapak C18 sorbent operated at a flow rate of 2.0 mL/min and a temperature of 20°C. The mobile phase composition was 50% methanol–50% water–50 mM NaH_2PO_4 with H_3PO_4 added to pH 3.0, containing various concentrations of the ion-pairing reagent. Peptide code: 1, Arg-Phe; 2, Arg-Phe-Ala; 3, Met-Arg-Phe; 4, Met-Arg-Phe-Ala; 5, Leu-Trp; 6, Leu-Trp-Met-Arg; 7, Leu-Trp-Met; 8, Leu-Trp-Met-Arg-Phe. (Data from Ref. 102.)

acetone, and *n*-butanol [317,318] have also been employed. Typically, binary water–organic solvent eluents are favored, partly because of the reproducibility in their preparation, but ternary organic solvent systems have also been used for a variety of applications. Several criteria can be used to select and discriminate the different organic solvents that can be employed in the RPC of peptides and proteins. First, the issues of purity and UV optical transparency of the solvent must be taken into account. As detection is commonly carried out by UV methods at 215 nm, very high purity solvents are required to ensure small baseline changes and good detectability of the separated peptides and proteins. Second, organic solvents that generate mobile phases of low viscosity are to be favored simply because the back-pressure of the chromatographic column will be lower. Third, the relative elutropicity of the solvent must be taken into account. As discussed earlier, the molecular architecture of acetonitrile–water mobile phases is fundamentally different from that found with, for example, methanol or the other alkyl

alcohols, and this difference will be reflected in an RPC separation through the dependences of $\log k'$ on ψ as well as the differences in the selectivities. Fourth, solvents that tend to stabilize the native conformation of large polypeptides and proteins are more likely to result in the recovery of the biosolute(s) in their active states. Such behavior has been confirmed with a large number of recombinant proteins, including human growth hormone variants separated by RPC methods.

When halide salts are employed in RPC or HIC separations of peptides or proteins with standard HPLC equipment made from type 316 stainless steel, it is essential that the equipment be properly flushed with neat water when not in operation to avoid corrosion by the residual halide ions, especially at low pH. Otherwise, the current generation of HPLC equipment has a high tolerance to most mobile phase conditions that can be contemplated for use in RPC or HIC applications with peptides and proteins. So-called biocompatible metal-free HPLC equipment has been marketed by several manufacturers, thus avoiding potential problems of equipment malfunction due to corrosion of stainless steel or the contamination of protein samples by low levels of leached heavy metal ions. Typically, with metal-free HPLC equipment, the wettable stainless components have been replaced by titanium, glass, or perfluoropolymeric components.

Care in the preparation of mobile phases cannot be overemphasised. Attention has to be given to the order of adding the different components of the mobile phase because volume expansion or contraction can arise due to exothermic or endothermic heats of mixing, thus generating an error in the value of the concentration or volume fraction of the modifier. For precise structure-retention measurements related to thermodynamic, partition coefficient, or other biophysical assessments, isocratic mobile phases should be used, prepared individually and not from binary mixtures generated with a preset gradient former. Slight increases in eluent strength can dramatically weaken the binding of peptides or proteins in RPC and HIC. Thus, mobile phase conditions for isocratic separations of peptides or proteins must be established with very high precision, with the organic modifier content of a mobile phase in an isocratic RPC separation typically specified to three significant figures. Care must also be taken in such RPC separations to avoid compositional changes caused 1) by evaporation of the organic modifier. Filtration of all mobile phases through 0.45- μm pore size membranes prior to use is essential, and appropriate degassing and purging of the mobile phase with a neutral gas is important prior to or during use with all high-pressure systems. In RPC, organic solvents with good optical transparency at 215 nm are to be preferred. As anticipated from their relative elutropicity and polarity, the higher alcohols, i.e., the propanols, are "stronger" solvents in RPC than the shorter chain alcohols or acetonitrile. With some proteins, the propanols also cause smaller perturbations to the secondary or tertiary structures. Illustrative of these effects are the changes in the CD and relative intrinsic fluorescence spectra of β -lactoglobulin, α -lactalbumin, cytochrome

c, and several other proteins as a function of 1-propanol concentration and pH as described by Sadler and colleagues. [319].

Obviously, with very hydrophobic globular or membrane proteins, specially tailored elution conditions must be employed to achieve successful RPC or HIC separations. In these cases, a common trend has been to use nonionic detergents as an additive to the mobile phase. The magnitude of the retention, the selectivity, and the adsorption kinetics can also be altered by including these additives in the mobile phase. The presence of detergents also affects the surface tension of the mobile phase. Because of their properties, the more nonpolar additives and ion-pairing reagents have the ability to modify the RPC sorbent surface into dynamic liquid-liquid phases [320]. Such dynamic coating effects frequently provide improved solubility for highly hydrophobic proteins and peptides in the mobile phase as well as providing enhanced resolution, but they have the downside that long reequilibration times are required due to the slow mass transfer kinetics (hence impaired peak efficiencies). Nevertheless, for the RPC separation of membrane proteins, nonionic detergents [321] can be used to keep the protein soluble and to minimize losses on the chromatographic surface. Adaptation of this dynamic coating concept with RPC systems provides a very interesting approach to study the behavior of amphipathic peptides in artificial biomembrane-like environments utilizing phospholipids [322,323]. Under such conditions, phospholipid densities approaching $2 \mu\text{mol}/\text{m}^2$ can be generated, which is close in value to that found for lipid vesicles. Various experimental results conform that the retention behavior of peptides with noncovalent, immobilized artificial biomembrane-like stationary phases correlates with the lipid-binding affinities of phospholipid vesicles. Similarly, HP-HIC approaches have been employed to simulate the binding behavior of polypeptides and proteins, such as cardiotoxin [324], that are believed to exert their biological effects by interaction with biological membranes. In these cases, correlations based on an index of accessible hydrophobic surface areas of these polypeptides proteins can be developed on the basis of the relative elution order and knowledge of the relative rank order of pharmacological toxicities.

Solvents, Salts, Detergents, and Surface Tension Effects

Whether an organic solvent, a nonionic detergent, or another type of additive is employed in the RPC of peptide or proteins, desorption is achieved through lowering of the surface tension of the mobile phase by increasing the volume fraction of the organic solvent modifier in the mobile phase. An analogous situation applies in the HIC of peptides and proteins, but in this case, a lower surface tension of the mobile phase is achieved by lowering the concentration of the added salt. In HIC, then, advantage is taken of the molal surface tension increment, σ , of a specific salt to affect the adsorption and desorption. Elution is

then achieved by decreasing the salt concentration, by decreasing the polarity of the mobile phase by the addition of up to 80% (v/v) ethylene glycol or 40% (v/v) 1-propanol, or by the use of a detergent, such as Brij 35, octyl-glucuronide, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonic acid (CHAPS), Nonidet P-40, or Tween 20. In many cases, elution can be affected simply with a decrease in the salt concentration, either via step elution or gradient elution methods, i.e., with an eluent of lower surface tension. In this case, the stronger solvent is water. When different salts are compared under the same elution conditions, the relative retention of a specific protein will follow the lyotropic ability of the salt, according to its place in the Hofmeister series. Retention of a particular protein will thus be proportional to the σ value of a salt provided it does not undergo specific binding effects with the protein. As also apparent from Table 2, the choice of the salt for use in HIC can be based on the same considerations that are applied when the salting out of a protein is undertaken as part of a solubility study.

Because retention of peptides or proteins in HIC is determined largely by the surface tension of the mobile phase, pH changes are not expected from the solvophobic theory to have a significant effect on the retention. However, pH changes can affect the net charge on the protein and may perturb the structure of the hydrophobic contact sites through titration of ion-pair bridges, affecting overall the ionization state of the protein surface. Changes in retention will thus become more evident when the pH of the mobile phase approximates the isoelectric point of the protein. When such circumstances arise, a second phenomenon due to the hydrophobic effect can arise, namely the preferential salting out of the protein, which may lead to precipitation [325]. For this reason, HIC separations of globular proteins should be carried out at pH values at least 1 pH unit away from the pI value of the protein(s) and preferably across a range of pH values [15,176,216,274,275,280,286,326], thus enabling the mobile phase pH to be chosen to obtain appropriate separation selectivity, while still ensuring that the stability and solubility of the protein do not become impaired.

In addition, the solubility of the salt is important if large values for the σ term in Eq. (19) are to be achieved. In this context, ammonium sulfate has generally been found to be the most useful salt for HIC as it has excellent solubility (~ 4 M) at low temperatures and has high UV transparency even at high concentrations. In addition, concentrated solutions of ammonium sulfate resist microbial growth. Despite its relatively low σ value, sodium chloride at high concentrations has also been employed in protein fractionation with HIC procedures, taking advantage of its low cost, high purity, and high solubility. From pragmatic considerations of purity and cost, sodium sulfate also represents a useful salt in HIC, despite its lower solubility limit (~ 1.5 M). Major concerns with several other salts with suitable σ values are their predisposition toward supporting microbial growth or the propensity to bind selectively to proteins.

Thus, sodium citrate, on the basis of its molal surface tension increment and high solubility, would appear to be a good choice as an HIC kosmotropic salt. However, citrate salts are well known for their ability to bind selectively to many proteins at low concentrations, a feature that can be exploited [189–191] in the stabilization of multimeric NAD-dependent enzymes.

Proteins with low water solubility, such as integral membrane proteins, are frequently strongly adsorbed to RPC sorbents with mobile phases of high organic solvent content or to HIC sorbents with mobile phases of low salt concentration. With such proteins, one or more hydrophobic domains are exposed to the nonpolar ligates in a manner reminiscent of the binding processes that occur with the lipid bilayer of a biological membrane. Various studies have documented that the chromatographic binding regions of such proteins are the same or near the membrane insertion site [322]. In these circumstances, desorption from an RPC or HIC sorbent usually requires the presence of a nonionic (e.g., Triton X-100), ionic (e.g., sodium dodecyl sulfate, cetyltrimethylammonium bromide), or zwitterionic (e.g., CHAPS) detergent [328,329] and/or an organic solvent of higher elutropicity and protein stabilization capabilities (e.g., ethylene glycol) in the mobile phase. Surfactants will alter the surface tension of solutions and from solvophobic considerations can be anticipated to influence the retention behavior of proteins in both RPC and HIC systems according to the linear dependence of $\log k'$ on γ . However, with simple globular proteins, no such correlation has been observed between the HIC retention behavior and the concentration of the detergent in the resulting mobile phase [330]. The conclusion that can be reached from this and related studies is that globular proteins in the presence of surfactants unfold beyond their “molten globule” intermediate states [331] and possibly reach conformational states near their random coil conformations, thus losing the ability to interact with the immobilized nonpolar ligands on the HIC (or RPC) sorbent in a topologically determined manner.

In contrast, integral membrane proteins appear to maintain a considerable amount of their native structure in the presence of detergents and will interact with the nonpolar HIC (or RPC) ligates in a manner analogous to that occurring in the lipid bilayer of a biological membrane. As a consequence, very hydrophobic or membrane proteins can be successfully separated on RPC and HIC sorbents provided attention is given to the choice of detergent, the pH, and the accompanying buffer system. The solubility characteristics of the kosmotropic salt also have to be factored into the selection of these mobile phase conditions to avoid salt precipitation. Knowledge gained about the properties of the membrane proteins from extraction experiments with detergents or organic solvents can often be useful in guiding the choice of the mobile phase conditions. In addition, polyhydric alcohols, such as glycerol, or polyethylene glycols have long been used to stabilize hydrophobic proteins and a similar benefit is observed in RPC and HIC separations with membrane or highly hydrophobic proteins. Illustrative

of the use of nonionic and zwitterionic detergents in the HIC resolution of membrane-associated proteins and enzymes without loss of activity [325,332,333] are the purifications of human skin gelatinase by HP-HIC in the presence of glycerol and Brij 35 [329], rat liver cytochrome P450 enzymes in the presence of 20% glycerol and cholate or octylethyleneglycol-dodecylether [289,332,333], the neuropeptide Y-2 receptor [335] in the presence of 1% octylglucoside, and the active human estrogen receptor with 10% ethylene glycol [336] with or without an initial gradient of decreasing ammonium sulfate concentration. Similarly, the purification of human erythrocyte acetylcholine esterase and other membrane proteins [77,235,337] can be achieved with wide-pore RPC sorbents, i.e., TSK-phenyl-RP or Vydac C₄, and aquo-organic solvent mobile phases containing octylglucoside or Brij 35.

In all modes of interactive chromatography, self-association of peptides and proteins can result in significant peak shape distortions, and RPC and HIC are no exception. Such behavior, often described in terms of isodemic interaction theory, is frequently a result of a specific molecular property of the peptide or protein, i.e., the propensity to form amphipathic helices or fibril-like structures or to self-associate, and can lead to multiple peak zones for an otherwise compositionally homogeneous sample. Participation of such self-association processes can be assessed [127,128,338] by on-line light scattering (LALLS) spectroscopic procedures through an examination of the peak shape response as a function of sample concentration under specified RPC or HIC conditions at a defined pH and temperature.

CONFORMATION BEHAVIOR AND THE EFFECT OF TEMPERATURE

General Thermodynamic Considerations

In the RPC and HIC of peptides and proteins, the “near-equilibrium” condition has generally been assumed for binding and desorption. Changes in the thermodynamic parameters of the ligand-ligate interaction can thus be depicted in terms of criteria specified by the Gibbs–Helmholtz relationship:

$$\Delta G_{\text{assoc},T}^{\circ} = \Delta H_{\text{assoc},T}^{\circ} - T\Delta S_{\text{assoc},T}^{\circ} \quad (36)$$

In most RPC and HIC investigations, a fixed temperature condition has been used. When the properties of the solute, the surrounding bulk solvent, and the interactive surface are invariant with regard to the temperature, T , the contributions from the corresponding changes in enthalpy, $\Delta H_{\text{assoc},T}^{\circ}$, and entropy, $\Delta S_{\text{assoc},T}^{\circ}$, of the system to the overall Gibbs free energy change, $\Delta G_{\text{assoc},T}^{\circ}$, associated with the solute-sorbent interaction, as well as the phase ratio of the system, Φ , will also be independent of temperature. When such conditions

prevail, the dependence of $\log k'_i$ on $1/T$ takes the form of the well-known linear van't Hoff plot. Such behavior has been observed experimentally with some small peptides [3,8,26,20,104,133,219,339–314], but with many polypeptides and proteins studied under RPC and HIC conditions, significant divergences from this ideal behavior have been found [8,16,20,133,146,161,171,223]. As noted earlier, the interaction of a peptide or protein, P_i , with the chemically defined ligate(s) of an RPC or HIC sorbent can be described in terms of the logarithm of the capacity factor, $\log k'_i$, and related from fundamental thermodynamic considerations to the temperature, T , through Eq. (16) as

$$\log k'_i = -\Delta H_{\text{assoc},i}^{\circ}/RT + \Delta S_{\text{assoc},i}^{\circ}/R + \log \Phi \quad (16)$$

For systems where the phase ratio, Φ , and the properties of the peptide or protein and the sorbent surface are invariant with temperature, the values of $\Delta H_{\text{assoc},i}^{\circ}$ and $\Delta S_{\text{assoc},i}^{\circ}$ associated with the interaction of P_i with the nonpolar ligates can be derived in the traditional manner by linear regression analysis of the $\log k'_i$ versus $1/T$ plot as the slope and intercept values, respectively. Moreover, with RPC and HIC sorbents, the extent of fit of the experimental data to the linearized form of the van't Hoff dependence can be employed to gain insight into whether first-order (linear) adsorption/desorption conditions prevail or whether additional factors are involved in the interaction of the polypeptide (or protein) with the nonpolar ligates, e.g., whether perturbation of secondary or tertiary structure of P_i occurs under the conditions of the interaction.

In the case of the RPC and HIC of peptides and proteins, three situations have been documented [16,20,146,171,342] for the interaction of these biomolecules with hydrocarbonaceous ligates, whereby the change in heat capacity, $\Delta C_{p,i}^{\circ}$, of the system is (1) zero and remains invariant with regard to T (the isothermic scenario), (2) not zero but is linearly dependent on T (the homothermic scenario); or (3) not zero and shows a nonlinear dependence on T (the heterothermic scenario). In the bulk state, the heat capacity, $C_{p,i}^{\circ}$, of a substance is usually defined as the quantity of heat necessary to raise the temperature of a unit mass of the substance by one kelvin. In the case of RPC or HIC, the change in heat capacity, $\Delta C_{p,i}^{\circ}$, for a peptide (or protein) interaction with a nonpolar ligate system as a function of T can be expressed according to the Kirchhoff relationships [343,344] as

$$\Delta C_{p,i}^{\circ} = T \partial S_{p,i}^{\circ} / \partial T \quad (37)$$

where

$$\Delta S_{\text{assoc},i}^{\circ} = \int \frac{\Delta C_{p,i}^{\circ}}{T} dT \quad (38)$$

Hence,

$$\Delta C_{p,i}^{\circ} = \partial H_{\text{assoc},i}^{\circ} / \partial T \quad (39)$$

and

$$\Delta H_{\text{assoc},i}^{\circ} = \int \Delta C_{p,i}^{\circ} dT \quad (40)$$

The change in the heat capacity can be represented as

$$\Delta C_{p,i}^{\circ} \partial T = \partial E + P \partial V = \mu_i \partial \mu_i \quad (41)$$

where $\partial \Delta E$ represents the incremental difference in the internal energy of the system. According to Eqs. (39)–(41), $\Delta C_{p,i}^{\circ}$ is also related to the change in the standard enthalpy and the standard pressure-volume product (∂PV) terms, while the term μ_i takes into account contributions from processes not defined by the criteria of the Stefan-Boltzmann-Maxwell law. If the interaction of P_i with the chromatographic ligates satisfies these criteria, i.e., no effects other than standard changes in P , V , and T are involved, then Eq. (41) becomes

$$\Delta C_{p,i}^{\circ} \partial T = \partial E + P \partial V \quad (42)$$

and hence

$$\Delta C_{p,i}^{\circ} \partial T = \partial H_{\text{assoc},i}^{\circ} - \partial(PV) + P \partial V \quad (43)$$

This relationship permits the change in heat capacity, $\Delta C_{p,i}^{\circ}$, associated with the interaction of a peptide or protein with nonpolar ligates to be evaluated from the change in enthalpy, $\Delta H_{\text{assoc},i}^{\circ}$, assuming that the change in pressure for the system is known, i.e.,

$$\Delta C_{p,i}^{\circ} \partial T = \partial H_{\text{assoc},i}^{\circ} - V \partial P \quad (44)$$

Evaluations of the interaction of a peptide or protein, P_i , with the hydrocarbonaceous ligates in terms of Eqs. (37)–(44), however, do not represent complete descriptions of the binding process because the condition(s) under which the thermal energy of the system is increased has not been specified. If the heat capacity of the system is increased under experimental conditions where the pressure, P , does not change significantly, then Eq. (43) can be simplified and the specific heat capacity can be redefined in terms of Eq. (45):

$$C_{p,i}^{\circ} = (\partial H_{\text{assoc},i}^{\circ} / \partial T)_P \quad (45)$$

In RPC and HIC, when the temperature of the system is changed, the viscosity, η , of the mobile phase will also change. This variation in viscosity will result in change in the pressure of the system, ΔP , according to the relationship

$$\Delta P = \frac{u\eta L}{\epsilon_p d_p^2} \quad (46)$$

where u is the linear flow velocity of the eluent, L is the length of the column, ϵ_p is the specific permeability of the sorbent, and d_p is the average particle diameter. Over the temperature range usually employed in most laboratories, 5–65°C, the ΔP variations for well-packed columns typically should not exceed more than 15–20% of the original pressure drop. Despite this change in ΔP as the temperature is increased, isocratic conditions at constant flow rate remain the preferred experimental option [20,146,171,219,223] for the measurement of thermodynamic parameters associated with the interaction of peptides and protein with the nonpolar ligates in RPC and HIC systems. Application of Eqs. (36)–(46) thus provides a useful and appropriate approximation of the ligand-ligate interaction with peptides and proteins in RPC and HIC, enabling evaluation of the relationship between k'_i , T , and the associated thermodynamic parameters $\Delta H_{\text{assoc},i}^\circ$, $\Delta S_{\text{assoc},i}^\circ$, and $\Delta C_{p,i}^\circ$ for a specific peptide or protein and a defined flow rate or mobile phase composition.

Relationship Between k'_i , $\Delta H_{\text{assoc},i}^\circ$, $\Delta S_{\text{assoc},i}^\circ$, $\Delta C_{p,i}^\circ$ and T

The equilibrium association constant, $K_{\text{assoc},i}$, for the interaction of P_i with the hydrophobic surface is a function of temperature, with the dependence taking the form:

$$K_{\text{assoc},i} = e^{-\Delta G_{\text{assoc},i}/RT} \quad (47)$$

where $\Delta G_{\text{assoc},i}^\circ$ is the Gibbs free energy change for the interaction. Moreover, the equilibrium association constant, $K_{\text{assoc},i}$, for the binding of the peptide or protein to the RPC or HIC ligate is related to the capacity factor, k'_i , through the relationship [see Eq. (1)]:

$$\log K_{\text{assoc},i} = \log k'_i + \log \Phi \quad (48)$$

From Eqs. (14)–(16) and (36), it can be shown that

$$\partial \log k'_i / \partial T = \Delta H_{\text{assoc},i}^\circ / RT^2 \quad (49)$$

In situations in which $\Delta H_{\text{assoc},i}^\circ$ and $\Delta S_{\text{assoc},i}^\circ$ are both invariant with temperature, i.e., under the conditions of the isothermic binding scenario,

where over a range of temperatures linear van't Hoff plots are observed, Eq. (49) can be given as the familiar expression:

$$\log k'_i = -\Delta H_{\text{assoc},i}^{\circ}/RT + I \quad (50)$$

with the term I incorporating contributions from both the entropic and the phase ratio terms in the form $I = \Delta S_{\text{assoc},i}^{\circ}/R + \log \Phi$. If the phase ratio, Φ , is assumed (or found) to be independent of temperature, then the plots of $\log k'_i$ versus $1/T$ will generate a straight line characteristic of linear van't Hoff plots. Thermodynamic data for a number of low-molecular-weight peptides have been interpreted in terms of this linear van't Hoff relationship, but in general nonlinear dependences are the norm. In these cases, $\Delta H_{\text{assoc},i}^{\circ}$ and $\Delta S_{\text{assoc},i}^{\circ}$ are dependent on temperature. When such temperature-dependent heat capacity conditions prevail, i.e., when $\Delta C_{p,i}^{\circ} \neq 0$ as observed for the homothermic and heterothermic binding scenarios [16,20], respectively, the dependence of $\log k'_i$ on T can be approximated by a polynomial expression as represented by Eq. (51):

$$\log k'_i = a + b(1/T) + c(1/T)^2 + d(1/T)^3 + \dots + \ln \Phi \quad (51)$$

and the change in enthalpy for these homothermic and heterothermic scenarios can be represented by

$$\Delta H_{\text{assoc},i}^{\circ} = -R\alpha(b + 2c(1/T) + 3d(1/T)^2 + \dots) \quad (52)$$

while the change in heat capacity can be represented as

$$\Delta C_{p,i}^{\circ} = \partial \Delta H_{\text{assoc},i}^{\circ} / \partial T \quad (53)$$

$$= m/(T)^2 + n/(T)^3 + \dots \quad (54)$$

where $m = 2Rc$ and $n = 6Rd$, etc.

With some peptides and proteins that exhibit in RPC and HIC nonlinear plots of $\log k'_i$ versus $1/T$, the value of $\Delta H_{\text{assoc},i}^{\circ} \rightarrow 0$ and $\log k'_i$ may thus reach a maximal value at a temperature value corresponding to the inflection temperature, T_H . In these circumstances, when $\log k'_i$ reaches a maximal value as T is varied and $\Delta C_{p,i}^{\circ}$ is constant for the process, the dependence of the $\Delta H_{\text{assoc},i}^{\circ}$ and $\Delta S_{\text{assoc},i}^{\circ}$ contributions on temperature can be expressed in terms of $\Delta C_{p,i}^{\circ}$ as

$$\Delta H_{\text{assoc},i}^{\circ} = \Delta H_{\text{assoc},i}^* + \Delta C_{p,i}^{\circ}(T - T_H^*) \quad (55)$$

and

$$\Delta S_{\text{assoc},i}^{\circ} = \Delta S_{\text{assoc},i}^* + \Delta C_{p,i}^{\circ} \log(T/T_S^*) \quad (56)$$

where the symbols T_H^* and T_S^* refer to the isoenthalpic and isentropic temperatures at which the plots of $\Delta H_{\text{assoc},i}^{\circ}$ versus T or $\Delta S_{\text{assoc},i}^{\circ}$ versus T , respectively, for the peptides (or proteins) intercept and attain a common value of $\Delta H_{\text{assoc},i}^*$ or $\Delta S_{\text{assoc},i}^*$, respectively, under the different experimental conditions.

When such ligand-ligate processes prevail in the RPC or HIC of peptides and proteins, then the dependence of $\log k'$ on T can also be approximated by the logarithmic expression [345]:

$$\log k'_i = \frac{\Delta C_{p,i}^\circ}{R} \left(\frac{T_H}{T} - \log \frac{T_S}{T} - 1 \right) + \log \Phi \quad (57)$$

From Eqs. (51)–(57), values of $\Delta H_{\text{assoc},i}^\circ$, $\Delta S_{\text{assoc},i}^\circ$, or $\Delta C_{p,i}^\circ$ corresponding to homothermic or heterothermic interaction processes for any peptide or protein can thus be evaluated from the nonlinear van't Hoff plots using nonlinear least-squares regression and associated curve-fitting procedures. The corresponding values of $\Delta G_{\text{assoc},i}^\circ$, the S term, $\log k'_0$, $\Delta A_{\text{contact}}$, $K_{\text{assoc},i}$, etc. can then be derived from the same data set using the appropriate relationships. Illustrative of this approach are the plots of $\log k'_i$ versus the volume fraction, ψ , of the organic solvent for the L- α -polypeptide, **1** H-DDALYDDKNWDRAPQRCYQ-OH and its retro-inverso isomer, **2**, H-QYYCRQPARDWNKDDYLADD-OH determined with an *n*-octylsilica sorbent over the range of $\psi = 0.17$ to $\psi = 0.21$ with water-acetonitrile mixtures and $\psi = 0.35$ to $\psi = 0.40$ with water-methanol mixtures, over the temperature range 278 to 338 K in 5 K increments shown in Fig. 25a–d, respectively. For these polypeptides, the trend expected for the hydrophobic effect dominating the interaction mechanism are clearly evident, with $\log k'_i$ decreasing as the ψ value was increased.

The corresponding three-dimensional grid plots for the S -values [derived according to Eq. (5)] of these two polypeptides as the temperature and ψ values were systematically varied are shown in Fig. 26a–d. In each case, the S values for polypeptides **1** and **2** were derived by regression analysis methods from the gradient of the experimental plots of $\log k'_i$ versus ψ at the specified ψ and T values with the regression coefficients ≥ 0.9985 . In turn, the S value of a peptide or protein in the presence of an RPC sorbent can be related [16,20,211,212] to extrathermodynamic parameters, such as the accessible molecular surface area, ΔA_{mol} , through the expression

$$S = a' \Delta A_{\text{hyd}} + b' \kappa^c + c' \quad (58)$$

where ΔA_{hyd} is the hydrophobic contact area (proportional to the accessible molecular surface area, ΔA_{acc}), κ^c is a solvophobic cavity factor related to the ratio in energy required for the formation of a cavity with surface area equal to ΔA_{acc} and the energy required to extend the planar surface of the liquid by the same area, and a' , b' , and c' are constants related to the molecular properties of the polypeptide.

Several features relevant to the general behavior of other peptides and proteins in (RPC environments are evident from Figs. 25 and 26. First, it can be noted from the comparison of the acetonitrile versus the methanol solvent

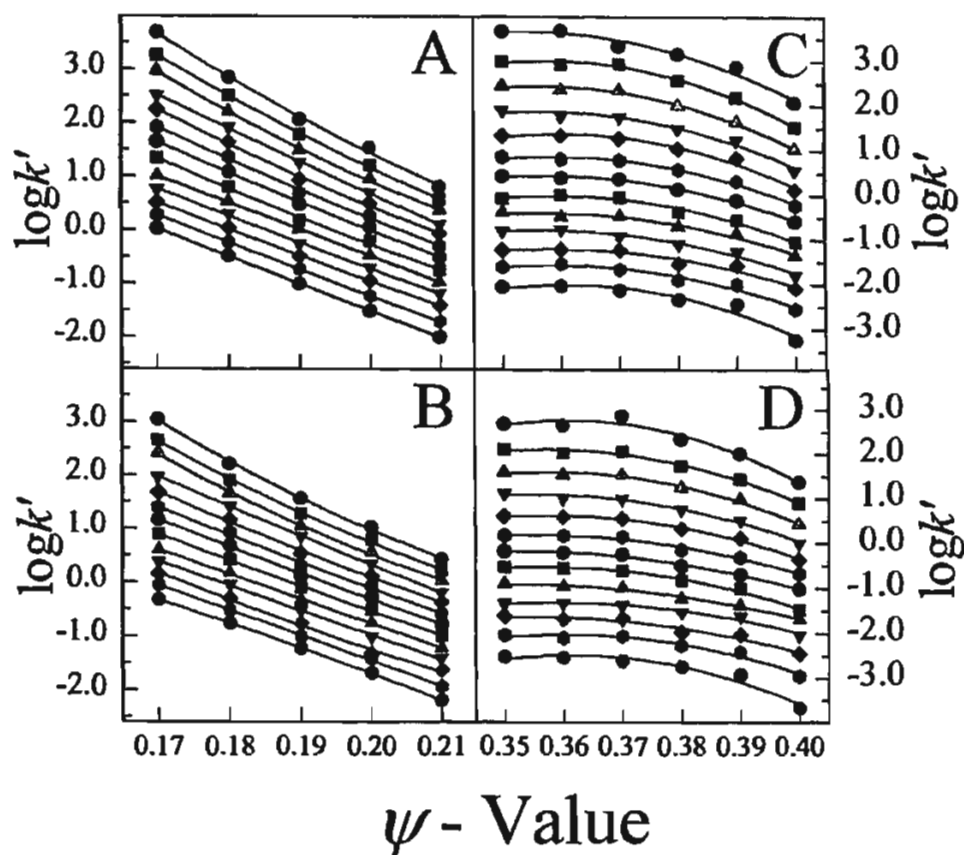


FIGURE 25 Plots of $\log k'_i$ versus the volume fraction, ψ , of the organic solvent for the L- α -polypeptide **1** (H-DDALYDDKNWDRAPQRCYYQ-OH) and its retro-inverso isomer **2** (H-QYYCRQPARDWNKDDYLADD-OH) determined over the range of $\psi = 0.17$ to $\psi = 0.21$ with water-acetonitrile mixtures (A and B) and from $\psi = 0.35$ to $\psi = 0.40$ with water-methanol mixtures (C and D) at different temperatures ranging from 278 to 338 K in 5 K increments, respectively. In each panel the data shown as the top $\log k'_i$ versus ψ plot (shown as the $\bullet-\bullet-$ values) correspond to $T = 278$ K and the bottom $\log k'_i$ versus ψ plot (shown as the $\bullet-\bullet-$ values) correspond to $T = 338$ K. (Data from Ref. 20.)

systems shown in Fig. 25a–d and Fig. 26–d that the choice of solvent composition results in significant changes in the shapes of these plots at a specific temperature. Second, the overall similarity of the $\log k'_i$ versus ψ data sets for polypeptides **1** and **2** with acetonitrile or alternatively with methanol confirm that these two 20-mers exhibit similar interactive behavior, consistent with their ability to assume similar conformational properties, and thus very similar surface topologies when in the same solvated ligate environment. Similar types of behavior have been observed for homologous proteins in RPC and HIC systems when tertiary structure was preserved.

Shown in Fig. 27 are the corresponding plots of $\log k'_i$ versus $1/T$ for the hormonal polypeptides β -endorphin and glucagon determined at different

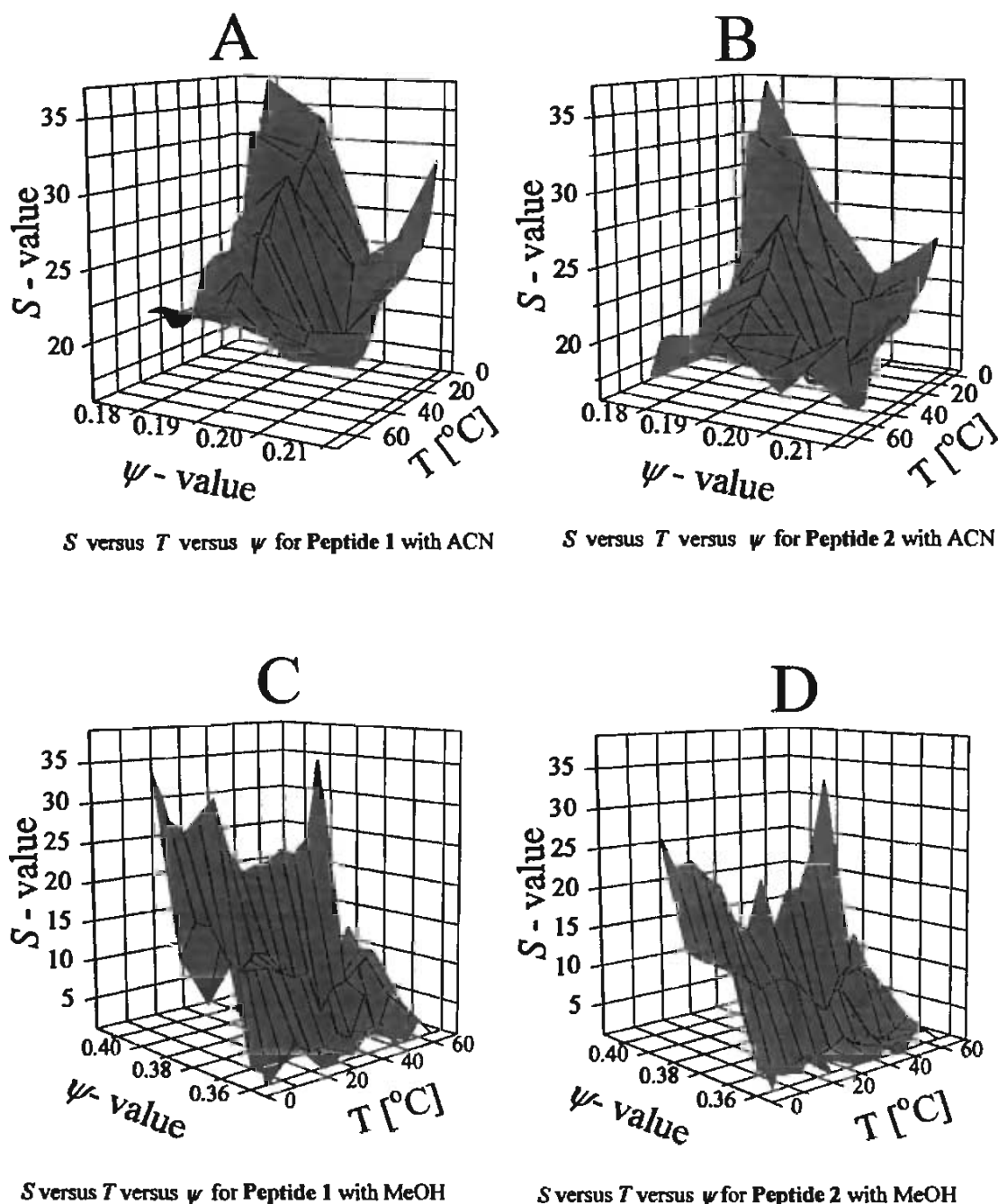


FIGURE 26 The corresponding three-dimensional grid plots for the S -values [derived according to Eq. (5)] for the L- α -polypeptide **1** (H-DDALYDDKNWDRAQR-CYQ-OH) and its retro-inverso isomer **2** (H-QYYCRQPARDWNKDDYLADD-OH) as the temperature and ψ values were systematically varied using acetonitrile-water (A and B) and methanol-water (C and D) isocratic systems. (Data from Ref. 20.)

temperatures with an RPC silica-based sorbent containing immobilized *n*-octadecyl groups at different ψ values with water-acetonitrile and water-methanol mixtures containing 0.09% TFA. Similar trends in the $\log k'_i$ versus $1/T$ data, involving nonlinear (parabolic) van't Hoff plots, have been previously documen-

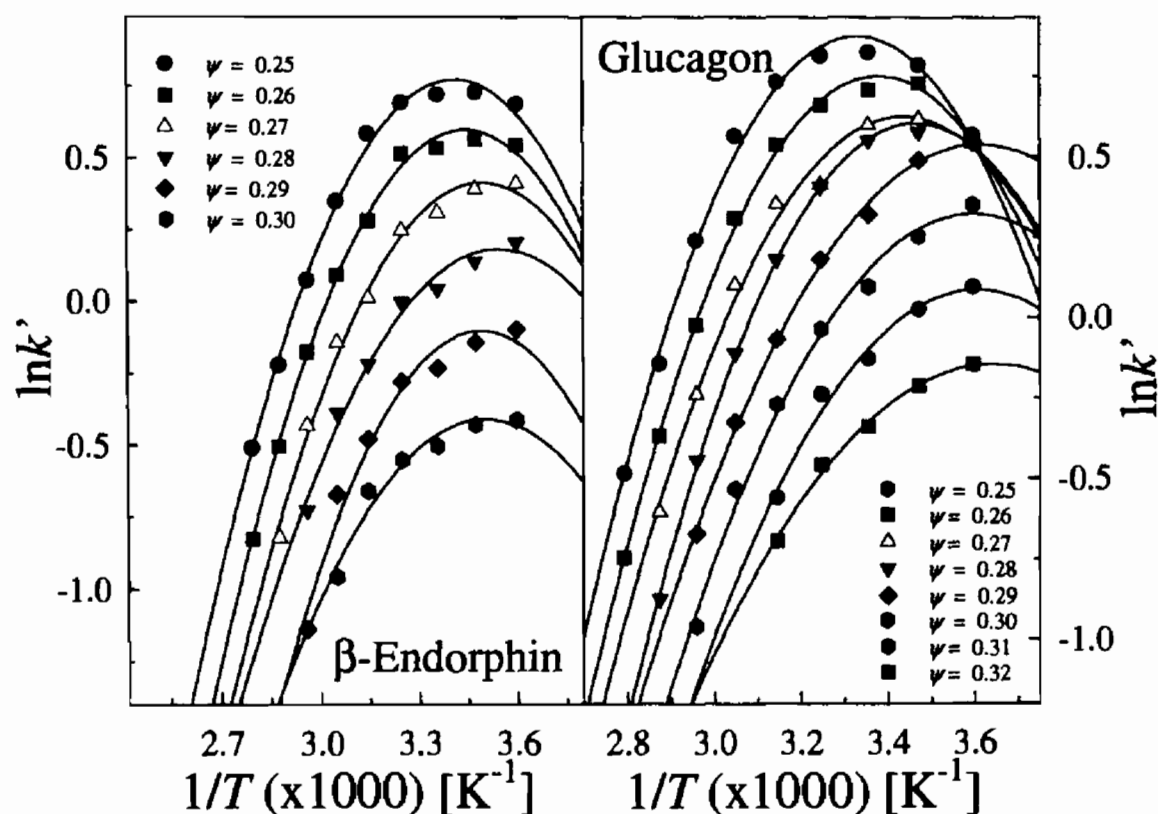


FIGURE 27 Van't Hoff plots of $\log k'_i$ versus $1/T$ for the hormonal polypeptides β -endorphin, over the organic solvent modifier range from $\psi = 0.25$ to $\psi = 0.30$ using acetonitrile-water mixtures containing 0.1% TFA, and glucagon, over the range from $\psi = 0.25$ to $\psi = 0.32$ using acetonitrile-water mixtures containing 0.1% TFA, with an *n*-butylsilica at temperatures $T = 278$ – 358 K. The plots show the experimental data and the lines of best fit according to the relationship given as Eqs. (51)–(57). (Data from Ref. 133.)

ted [346–348] with larger polypeptides and small globular proteins, such as insulin, cytochrome *c* variants, or the transcription factors *fos* and *jun*. The characteristic shapes for the plots of $\log k'_i$ versus $1/T$ for such polypeptides and proteins can thus be interpreted in terms of the extended mean value theory that forms the basis of Kirchoff's law based on Eqs. (51)–(57). Concomitantly, it is also possible to calculate the corresponding $\Delta H_{\text{assoc},i}^\circ$, $\Delta S_{\text{assoc},i}^\circ$, and $\Delta C_{p,i}^\circ$ values from these nonlinear dependences of $\log k'_i$ versus $1/T$ for different solvent compositions. According to Eqs. (48)–(57), in cases where the interaction of a peptide or protein with nonligates is associated with $\Delta C_{p,i}^\circ$ values that are not constant with temperature, this behavior is consistent with participation of heterothermic processes.

These heterothermic processes have been documented in a variety of investigations. For example, in Fig. 28 (for an acetonitrile-water system) and Fig. 29 for a methanol-water system) experimental findings illustrating this

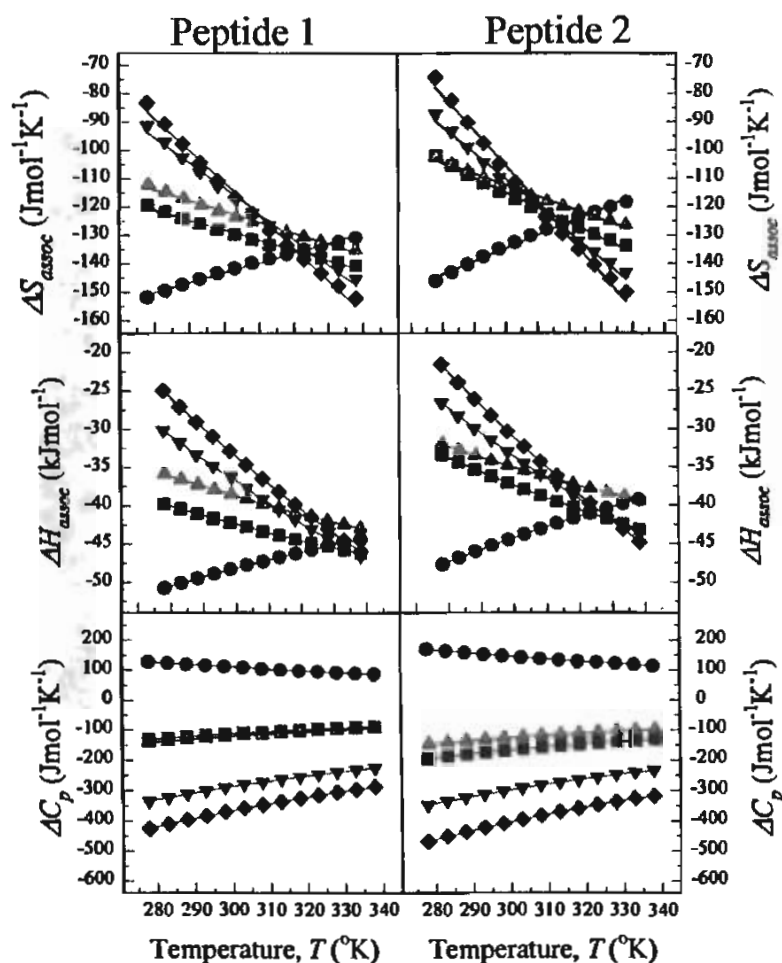


FIGURE 28 Plots of $\Delta H_{\text{assoc},i}^{\circ}$, $\Delta S_{\text{assoc},i}^{\circ}$, and $\Delta C_{p,i}^{\circ}$ versus T for the L- α -polypeptide **1** (H-DDALYDDKNWDRAPQRCYYQ-OH) and its retro-inverso isomer **2** (H-QYYCRQ-PARDWNKDDYLADD-OH) with acetonitrile-water mixtures at different ψ values. The code for the ψ values used in the generation of these data is shown in Fig. 25, and the temperature range encompassed $T = 278$ to 338 K in 5 K increments. (Data from Ref. 20.)

behavior are shown, again using polypeptides **1** and **2** as the exemplars. Essentially linear dependences were observed between $\Delta H_{\text{assoc},i}^{\circ}$, $\Delta S_{\text{assoc},i}^{\circ}$, or $\Delta C_{p,i}^{\circ}$ and T for the interaction of these 20-mer polypeptides with *n*-octylsilica RPC sorbents. Although the $\Delta H_{\text{assoc},i}^{\circ}$ and $\Delta S_{\text{assoc},i}^{\circ}$ values were negative, the slopes of these $\Delta H_{\text{assoc},i}^{\circ}$ versus T and $\Delta S_{\text{assoc},i}^{\circ}$ versus T plots were dependent on the volume fraction, ψ , of the solvent composition, resulting in these plots intersecting at convergent values near $T = 320$ – 330 K. Similarly, the slopes of the $\Delta C_{p,i}^{\circ}$ versus T plots for both the acetonitrile and the methanol systems were also essentially linear. As noted earlier, acetonitrile is known to be a poor hydrogen bond acceptor as a solvent, resulting in the generation of solvent clusters with water-acetonitrile mixtures, whereas methanol is a good hydrogen bond acceptor [165–169]. These solvent differences give rise to significant

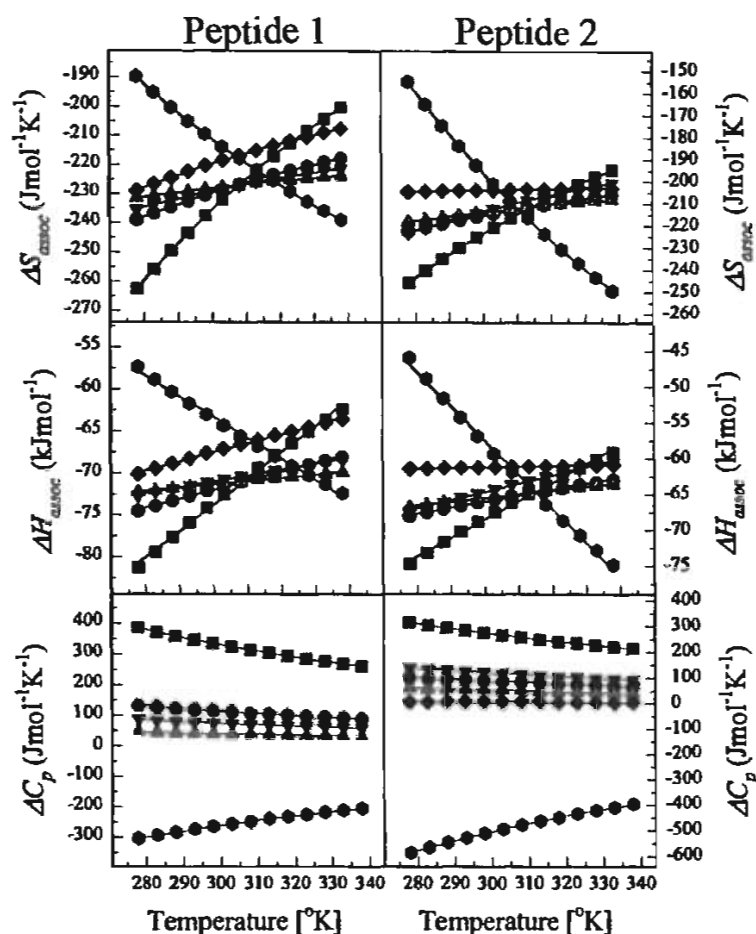


FIGURE 29 Plots of $\Delta H_{\text{assoc},i}^{\circ}$, $\Delta S_{\text{assoc},i}^{\circ}$, and $\Delta C_{p,i}^{\circ}$ versus T for the L- α -polypeptide **1** (H-DDALYDDKNWDRAPQRCYYQ-OH) and its retro-inverso isomer **2** (H-QYYCRQ-PARDWNKDDYLADD-OH) with the methanol-water mixtures at different ψ values. The code for the ψ values used in the generation of these data is shown in Fig. 25, and the temperature range encompassed $T = 278$ to 338 K in 5 K increments. (Data from Ref. 20.)

changes in the hydrogen-bonding characteristics and solvation state of peptides (or proteins) and their ligates, as demonstrated in the preceding case. As also evident from the data shown in Figs. 28 and 29, these differences in solvation properties were reflected as different slopes in the plots of $\Delta H_{\text{assoc},i}^{\circ}$, $\Delta S_{\text{assoc},i}^{\circ}$, or $\Delta C_{p,i}^{\circ}$ versus T . Because of the molecular organization of binary water-acetonitrile mixtures, a peptide or protein will be less constrained at higher temperatures in water-acetonitrile mixtures than for the hydrogen-bonding methanol-based mixtures; i.e., the peptide or protein will appear to undergo a greater extent of unfolding with a acetonitrile-water system at higher temperatures, consistent with the helix-inducing properties of this solvent with peptides and proteins at low temperatures. In reality, in methanol-based eluents, the peptides or proteins may

be less structured at lower temperatures, and this will result in smaller changes in conformation as the temperature is increased.

The consequences of these types of thermodynamic behavior manifested by the peptides and proteins in their interactions with the different classes of RPC and HIC ligates are clearly important, because similar behavior underpins the so-called enthalpy-entropy compensation effect observed for protein folding in bulk solution [349–354]. When molecular associations between a peptide or protein and a nonpolar ligate system involve enthalpy-entropy compensation, then large enthalpy changes will be associated with stronger binding and a more restricted conformation for the peptide or protein in the bound state; i.e., a higher order will be induced with a concomitantly greater change in entropy. These observations have an important bearing on the interpretation of the molecular processes associated with surface-induced conformational changes of peptides and proteins in the presence of RPC and HIC environments. In particular, the retention behavior of peptides and proteins in different ligate systems can be rigorously explored from theoretical as well as experimental perspectives to gain insight into the thermodynamic and extrathermodynamic characteristics associated with the interaction, including the influence of the molecular properties and conformational effects manifested by peptides and proteins when they are placed into the hydrophobic milieu that characterizes an RPC or HIC system.

Resolution Effects in Interconverting and Static Systems

Analytical RPC and HIC techniques also have the ability to yield quantitative data on rate constants for protein folding and unfolding transitions as well as to detect conformational intermediates with relaxation half-times of similar magnitude to the mass transport time (i.e., $\tau_{\text{conf}} \approx \tau_{\text{mass transfer}}$, where $\tau_{\text{mass transfer}} > 10$ sec). These calculations also have important ramifications in the selection of conditions in a purification procedure. For example, if a protein were to undergo a two-stage interconversion in the mobile phase and at the surface of an RPC sorbent, then a retention cycle corresponding to a distribution process involving the two chromatographic phases and the protein in its native form, P_N and two unfolded states P_{UM} and P_{US} will occur via the corresponding transition states P_{UM}^* and P_{US}^* and can be represented as shown in Fig. 4. Various investigations have confirmed that conformational interconversions of globular proteins, e.g., lysozyme, α -lactalbumin, and myoglobin, can be readily followed in RPC and HIC systems where intermediates approximating the molten globule states of these proteins have been observed [18,103,104,106,146,314]. Different retention pathways can thus be envisaged, based on the composition and complexity of such distribution cycles, including the stochastic probability models and chemical reactor models. When the conformational changes are associated with relatively slow secondary conformational interconversion but rapid loss of the tertiary

structure, the near-equilibrium assumption may not apply and more complex peak shape profiles are often generated.

Such an example is illustrated for hen egg white lysozyme (HEWL) in Fig. 30. In this case, the emergence of distorted and multiple peak zones occurs for a notionally homogeneous preparation of this protein in terms of its composition of matter and mass spectral properties as a consequence of the chromatographic residency and temperature conditions. This complex interactive behavior evident with HEWL with RPC sorbents is typical of a similar behavior seen in bulk solution, which results in the generation of molten globule states. When the process $P_N \rightarrow P_U^*$ for a peptide or protein in the mobile phase and stationary phase can be represented as the reversible binding of two forms with first-order or pseudo-first-order interconversion kinetics, the resolution of the interconverting species can be evaluated [355] by treating the column as a “chemical reactor” with properties specified by the corresponding Damkohler number, D_a . Thus,

$$R_s = \frac{t_0 \Phi (k_{12}/k_{21} - k_{43}/k_{34}) (1 - e^{-D_a})}{4D_a(\sigma_1 + \sigma_2)} \quad (59)$$

and

$$D_a = \frac{L(\Phi k_{12}k_{23} + k_{43}k_{34})(k_{14} + k_{41})}{k_{14}k_{21}u_0} \quad (60)$$

where Φ is the phase ratio (V_s/V_m), $k_{i,j}$ are the respective rate constants for adsorption, desorption, unfolding, or refolding, σ_i is the peak width of component i , L is the column length, and u_0 is the linear velocity. The plate height increment due to slow kinetics of interconversion, H_{se} , can be determined from

$$H_{se,i} = \frac{2A}{D_a} \left[1 + \frac{(e^{-D_a} - 1)}{D_a} \right] \quad (61)$$

where

$$A = \frac{[L(k'_{i,1} - k'_{i,2})^2/K_m]}{[1 + k'_{i,2} + (1 + k'_{i,1}/K_m)]^2} \quad (62)$$

and $k'_{i,1}$ and $k'_{i,2}$ are the respective capacity factors for the two interconverting species. The Damkohler number, D_a , represents the ratio of the time taken by the peptide or protein in passage along the RPC or HIC column to the overall relaxation time for all conformation interconversions in the chromatographic system. When the D_a ratio is small, and as the limit approaches zero, the chromatogram for a four-component cycle will reflect the average macroscopic behavior of a fully unfolded form or, alternatively, two peaks separated by a time interval $t_m(k_{12}/k_{21} - k_{43}/k_{34})$. Conversely, when D_a is very large, kinetic effects associated with conformational interconversion essentially vanish. However, at

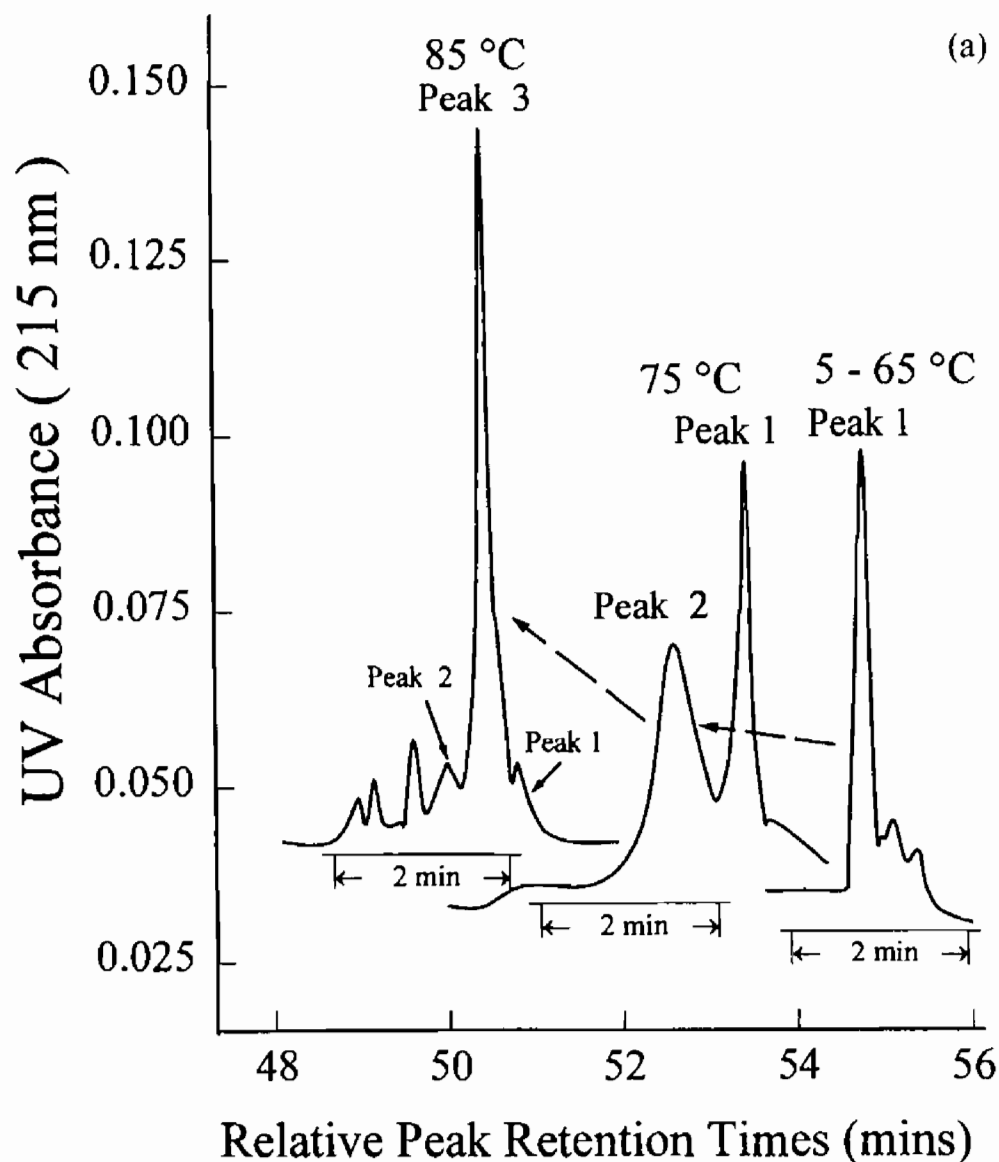


FIGURE 30 Elution profiles for hen egg white lysozyme (HEWL) (a and c) and reduced and carboxymethylated hen egg white lysozyme (cm-HEWL) (b and d) acquired using a linear gradient of 0.1% trifluoroacetic acid in water (buffer A) to 0.09% TFA in 65% aqueous acetonitrile (buffer B) and a gradient time of 90 min at a flow rate of 1 mL/min with C_{18} and C_4 reversed-phase sorbents. In (a) and (b), a wide-pore Bakerbond C_{18} sorbent, nominally of 5 μ m particle size and 30 nm average pore diameter, was packed into columns of dimensions of 250 $\times$ 4.6 mm ID and operated at 5–65°C, 75°C, and 85°C. The hatched arrows refer to the progressive conversion of peak 1 to peak 2 and peak 2 to peak 3. In (c) and (d), a wide-pore Bakerbond C_4 sorbent, nominally of 5 μ m particle size and 30 nm average pore diameter, was packed into columns of dimensions of 250 $\times$ 4.6 mm ID and operated at 5–65°C, 75°C, and 85°C. For clarity of presentation in (a), (b), and (c) the elution profiles for the 75°C and 85°C experiments have been offset by $\sim$ 2 min with regard to the 5–65°C experiments, and in (d) the 45°C, 65°C, 75°C, and 85°C experiments have been offset by $\sim$ 4 min from the 5–37°C experiment. (Data from Ref. 106.)

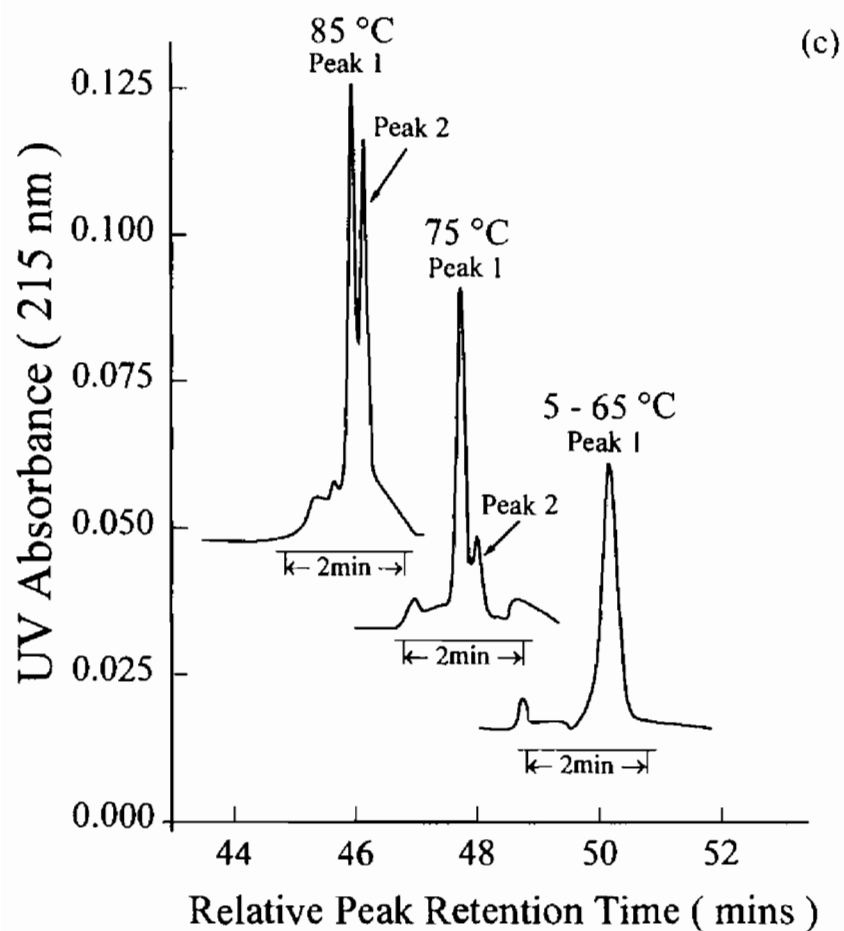
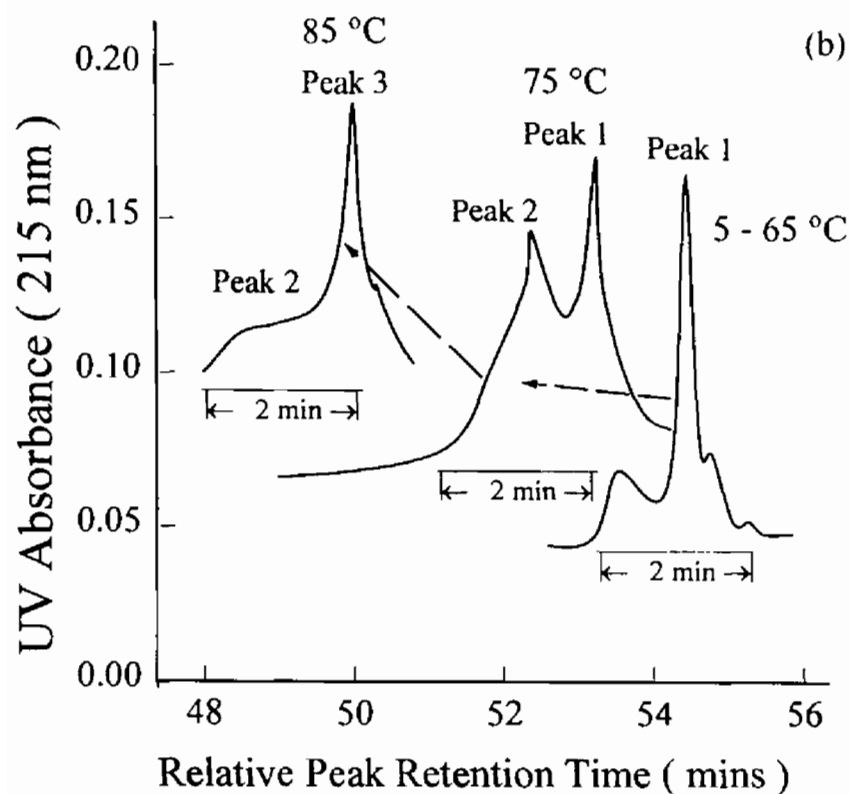


FIGURE 30 (continued)

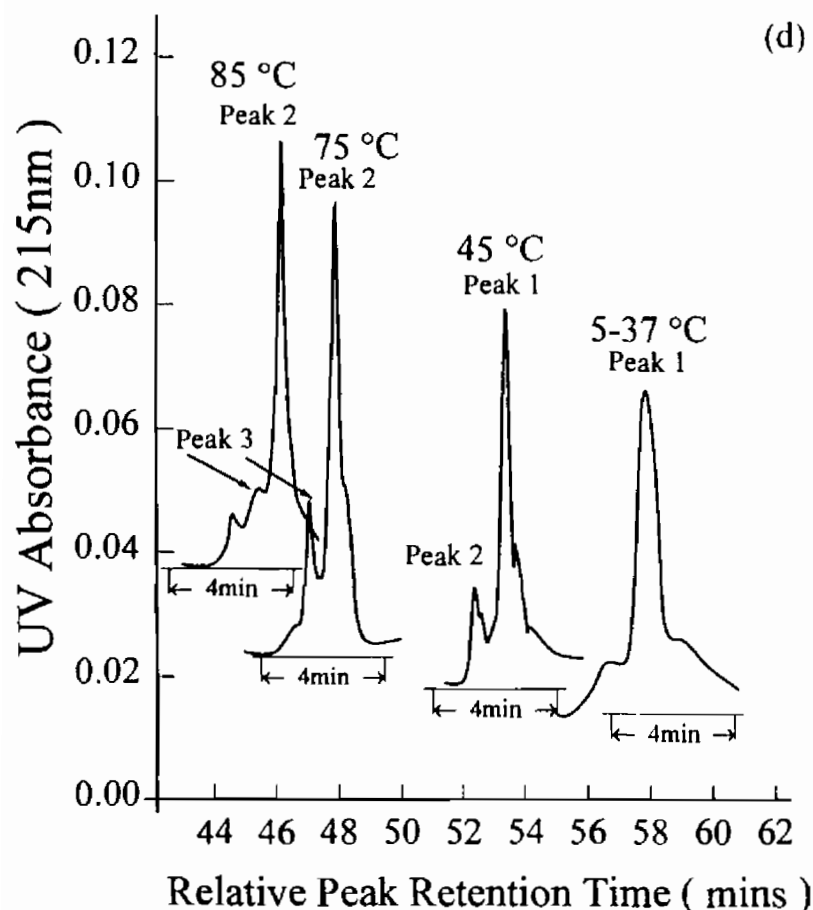


FIGURE 30 (continued)

intermediate reaction rates where $1 < D_a < 10$, complex elution profiles will be obtained, which are dependent on the intrinsic column efficiency, the equilibrium distribution of the two species P_N and P_U , the rate of interconversion of $P_N \rightarrow P_U$, and the type of chromatographic conditions used. The *cis-trans* interconversion of proline-rich peptides in RPC systems represents a classic example of this type of interconversion behavior.

When conformational reordering of a peptide or protein structure occurs in either the mobile phase or stationary phase, multizoning of the component into active and/or inactive zones can be anticipated, with the relative mole fraction abundance of each species time, concentration, and temperature-dependent. If the protein contains a cofactor or prosthetic group that is easily displaced under the chromatographic conditions, then the *apo* form of the protein will result. Such behavior has been observed with various heme-containing proteins and multimeric enzymes. A possible unfolding trajectory for horse skeletal muscle myoglobin involving the concomitant loss of the prosthetic group under the influence of inappropriate binding conditions is illustrated in Fig. 31.

A further type of multizoning phenomenon can arise with RPC and HIC sorbents from nonlinear isotherm behavior of the biosolutes, due to matrix

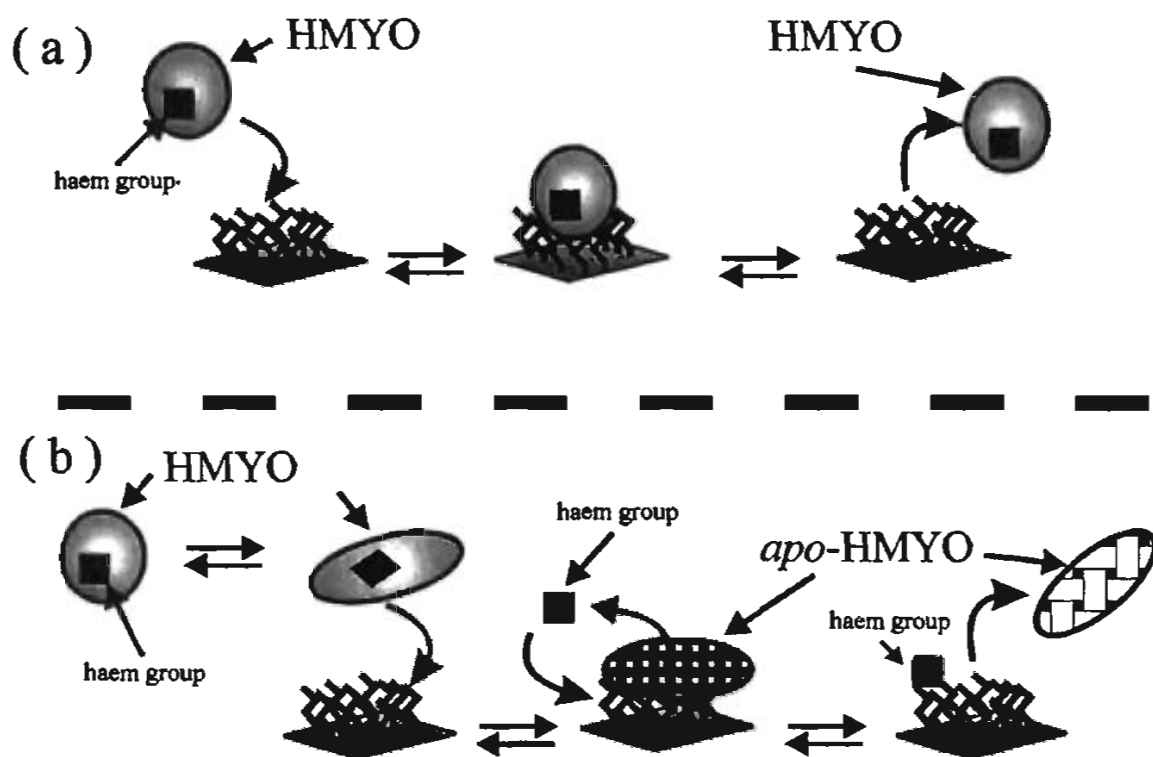


FIGURE 31 Schematic illustration of the possible unfolding trajectory associated with the binding of horse muscle myoglobin (HMYO) and concomitant loss of the heme group under the low-pH conditions of the RPC separation with an *n*-alkyl silica surface. (a) The binding of native or partially unfolded HMMYO species to the *n*-alkyl ligands, followed by elution without loss of the heme group (stylized as the square box, ■). (b) The binding of native or partially unfolded HMYO species to the *n*-alkyl ligands, followed by thermally induced changes in the folded state, loss of the heme group (■) while the protein remains adsorbed to the surface, and then desorption of the unfolded *apo*-HMYO species due to an increase in the organic solvent content in the mobile phase.

heterogeneity and nonuniformity of the nonpolar ligate distribution over the stationary phase surface. This effect is most noticeable between “virgin” and “conditioned” columns operated at analytical loadings, leading at the micro-preparative scale to catastrophic losses and nonreproducible recoveries. With improved manufacturing technologies now available, batch-to-batch variability does not represent the same level of problem as even 10 years ago, although with process scale RPC or HIC sorbents, a concern of some end users still remains the issue of batch equivalence.

When no secondary effects, i.e., when no temperature-mediated effects, conformational changes, slow chemical equilibrium, or pH-dependent ionisation processes, occur as part of the RPC or HIC chromatographic distribution event,

then the resolution, $R_{s,i}$, that can be achieved between components separated under these equilibrium or near-equilibrium conditions can be expressed as

$$R_{s,i} = \frac{1}{4} \left(\frac{L}{h_{e,i} d_p} \right)^{1/2} (\alpha - 1) \left(\frac{1}{1 + k'_i} \right) \quad (63)$$

or

$$R_{s,i} = \frac{1}{4} (N)^{1/2} (\alpha - 1) \left(\frac{1}{1 + k'_i} \right) \quad (64)$$

where α is the selectivity, N the number of theoretical plates, d_p the particle diameter, L the column length, and $h_{e,i}$, the reduced plate height. Equations (63) and (64) provide an important linkage between the three essential parameters that dictate the overall quality of the chromatographic resolution, namely the relative retention, expressed in terms of the capacity factor, k'_i ; the relative selectivity, α ; and the extent of peak dispersion, N_i , or $h_{e,i}$. Higher system performances arise in analytical separations when larger values of $R_{s,i}$ per unit time (or greater peak capacities, P_c) are achieved. When such criteria are met, this attribute translates in the analytical mode to lower cost per analysis. At a process level, high peak capacity performance in RPC and HIC separations can also be evaluated in terms of the throughput per system cost, i.e., as the cost of separating a kilogram of product at a predefined purity level per unit time. As evident from Eqs. (63) and (64), the resolution of an RPC or HIC separation of a complex mixture of peptides or proteins can be improved by adjusting the differences of k'_i values, by decreasing the mean particle diameter, by increasing the α values; or through increases in the N_i (and thus decreases in the $h_{e,i}$) values. For a particular analytical separation, each peptide or protein will have an optimal k'_i value for maximum resolution with a designated column, flow rate, and mobile phase composition. Similar considerations apply in preparative (overload) chromatography with multicomponent mixtures when resolution is enhanced following optimization of chromatographic selectivity and zone bandwidth.

OPERATIONAL CONSIDERATIONS

Basic Strategy Considerations

Important progress has been made in the application of gradient elution retention data for a particular peptide or protein mixture to the selection of isocratic elution conditions and vice versa based on a variety of predictive algorithms [302,356–360]. Furthermore, in large-scale RPC or HIC separations it is feasible in circumstances of regular retention behavior and high mass recovery to apply data derived from small-scale or analytical experiments as normalized integrals of

the elution volume, column performance, etc. to achieve scale-up of the chromatographic bed configuration or choice of the physical characteristics of the sorbent [46,240,241,361,362]. Such approaches underpin the current popularity of RPC procedures for the large-scale purification of synthetic or recombinant polypeptides [293–297,363,364]. However, in some cases when similar linear scale-up methods are applied to proteins, their biological activity may be lost due to unfavorable column residency effects and sorbent surface area dependences. It is thus mandatory that in the design of preparative RPC separation systems attention be specifically given to the issues of recovery of bioactivity. Often some key parameters can be easily controlled, i.e., by operating the preparative separation at lower temperatures such as at 4°C, by better pH selection, or by minimizing column residency times.

Inherent in all RPC and HIC investigations with peptides or proteins is the question of the end use to which the separated products will be applied. If the task involves purification solely for subsequent primary structure determination (i.e., essentially an analytical task at a semi-preparative scale), then control over preservation of bioactivity may not be necessarily relevant. Obviously, with a new or partially characterized protein, recovery of the component of interest with high mass and bioactivity balance is essential. For preparative methods where subsequent biological uses are contemplated, it is similarly mandatory that the design of the RPC or HIC separation system specifically address recovery issues. High recovery of bioactivity can usually be satisfied without sacrificing the obvious demands of selectivity through proper attention to the physicochemical consequences of the dynamic behavior of the polypeptide or protein of interest in bulk solution and at liquid-solid interfaces.

The application of multidimensional and multinodal techniques, particularly at the microscale level [253,365,366], in RPC and HIC separation of peptides and proteins has been in vogue for a number of years. Integral to these approaches with RPC systems has been the application of computer-assisted protocols for the optimization of the multidimensional chromatographic conditions, including the selection of mobile phase compositions that contain different types of ion-pairing systems, buffers, pH conditions, or organic solvent combinations [4,8,16,26,57,58,72,171,192,211,212,305,342,367,368]. Similarly, multidimensional procedures are equally pertinent to HIC separations whereby the same or different HIC sorbents of defined hydrophobicity gradation (ethyl, butyl, phenyl, etc.) can be used sequentially displacing salts of different lyotropicity, solvated radii, and electronegativities [19,146,155,179,215,369]. Moreover, in protein fractionation if an ion-exchange step is employed prior to an HIC procedure, advantage can be taken of the concomitant use of ions of different electronegativity and solvation state selected from the Hofmeister series. However, in the choice of these protocols care must be taken to ensure that the chromatographic conditions are fully compatible with the task in hand. If it is

solely an analytical or structural study, then advantage may be gained by driving the peptide or protein into one, soluble conformational state, but if the study has a biological endpoint, then the multidimensional conditions must be compatible with the recovery of bioactivity.

For HIC separations of proteins in the presence of kosmotropic salts, a commonly held belief is that only surface-exposed residues of the native protein(s) will be in contact with the chromatographic surface. Difference in selectivity between HIC and RPC separations of globular proteins can thus be attributed to the presence of different (conformational) species. In both HIC and RPC, an increase in retention occurs with the unfolding of a globular protein. In contrast, in IEC, when proteins are denatured a decrease in retention is frequently observed [370]. These differences between RPC/HIC and IEC can be rationalized on the basis that in the hydrophobic modes an increase in the nonpolar contact region(s) of the protein occurs, whereas in the IEC mode a decrease arises due to the loss of secondary/tertiary structure. Spectroscopic studies [371,372] exploiting *in situ* FT-NIR and Raman IR techniques have demonstrated that with both RPC and HIC, the interaction of the peptide or protein with the nonpolar surface invariably causes changes in conformation compared with the bulk solution scenario, although the magnitude of the changes differs. Moreover, the use of difference or derivative diode-array spectral analysis of the eluted peaks of proteins or peptides separated with RPC or HIC sorbents at different temperatures permits conformational states to be monitored. Application of these spectroscopic techniques has confirmed that as the RPC or HIC stationary phase becomes more hydrophobic, the possibility for conformational changes to occur with a globular protein at a given temperature will be greater. Moreover, the presence of mobile phase additives, such as metal ion cofactors in addition to kosmotropic salts, appears to stabilize protein conformation in both RPC or HIC [373] against thermal denaturation effects.

As apparent from the preceding discussion, enhancement of the hydrophobic interactions in the RPC and HIC of peptides and proteins is a characteristic feature with increasing temperature. To a very large measure, these interactions involve entropy-driven processes. With mixed-mode RPC or HIC phases, and particularly in the case of RPC with solvents that have high hydrogen-bonding potential, the enthalpy change upon adsorption may also be significant. Although such systems may often exhibit enthalpy-entropy compensation, when binary effects operate, the influence of temperature may become much less predictable. Adsorption studies with water-soluble organic polymers have typically revealed that for each polymer molecule adsorbed to the nonpolar surface, several solvent molecules are released, resulting in an overall increase in translational entropy of the system but a decrease in translational entropy of the adsorbed polymer. The loss of translational entropy for the bound polymer, because it remains in an essentially random coil state, represents only a minor

contribution to the overall free energy change on adsorption. Compared with simple isotactic synthetic polymers, proteins (and some larger peptides), on the other hand, will usually be in a folded state when exposed to an RPC or HIC sorbent, with substantial contributions to the overall free energy of the system from the conformational enthalpy and entropy terms, $\Delta H_{\text{conf},i}^\circ$ and $\Delta S_{\text{conf},i}^\circ$. At higher temperatures, an increase in conformational entropy of the peptide or protein is anticipated [374], with the favorable (small and negative) conformational free energy of a folded peptide or protein, $\Delta G_{\text{fold},i}^\circ$, dissipated as the temperature is increased, initially as a consequence of short-range perturbations of the secondary and tertiary structure that will be progressively lost due to increased solvation of the unfolding species. In the presence of RPC or HIC ligands, these processes may/may not be reversible under the experimental conditions, although an increase in translational entropy [375] may still arise on binding of such biopolymer to nonpolar sorbents. Concomitantly, several solvent molecules will be released as each peptide or protein molecule becomes adsorbed.

This suggests that in RPC and HIC the observed temperature dependences are due to both short- and long-lived conformational changes in peptides and proteins, either in the bulk solvent or following binding to the nonpolar sorbent, with the changes in state predominantly due to an increase in conformational entropy [374]. Currently, only limited data are available on the effect of changes in the conformational enthalpy of peptides or proteins in association with RPC or HIC sorbents. Several studies based on differential scanning microcalorimetry [376,377] have demonstrated that the enthalpy changes for small globular proteins on interaction with nonpolar ligates are of similar magnitude to those determined by chromatographic measurements. Depending on the structure of the peptide or protein and the chromatographic environment, these conformational effects may involve microenvironmental short-range forces or alternatively become progressively more energetic and involve substantial reorganizational changes in hydrogen bonding, solvent interactions, etc. When the former circumstances apply, the conformational changes may be reversible in the solvent, at the sorbent surface, or both. If the latter circumstances prevail, then the outcome can be denaturation either in solution or on the surface of the sorbent prior to desorption. When denaturation occurs *in situ* with RPC or HIC sorbents, failure to elute may be a common codicil.

Based on these considerations, in order to minimize conformational effects, RPC and HIC separations can be employed at low temperatures, particularly with less robust proteins, i.e., multimeric or multidomain proteins, and polypeptides without significant levels of secondary structure or disulfide connectivities. However, the downside of low-temperature operation may be zone broadening or multiple zones due to the presence of slowly interconverting species such as those associated with *cis/trans* proline isomerization. Such effects are evident in

Fig. 30, where the influence of temperature on the peak shape and relative retention of a globular protein, hen egg white lysozyme, in an RPC system is illustrated. Similar examples of bizarre peak shapes and retention aberrations have been documented in the scientific literature including the case described earlier with Ca^{2+} -depleted α -lactalbumin. This protein readily elutes as a relatively sharp peak from a weak HIC sorbent [methyl polyether (Cl) phase [192] at 5°C but fails to elute under the same gradient elution conditions at 25°C from a TSK Phenyl PW column [279]. A number of other studies have documented that, in general, RPC and HIC retention increases with temperature with globular proteins and for polypeptides with well-developed secondary structure [42,43,47,171,192–194,270,378–383].

Comparative Retention and Band-broadening Behavior in RPC and HIC

One of the general, but currently not firmly rationalized, observations about the RPC and HIC behavior of peptides and proteins is that their elution order is often different, yet the prevailing mechanism of dominant hydrophobic interactions for the adsorption and desorption steps is common. It can be argued that the mobile phase conditions in RPC may be much harsher, typically being low pH, or that the hydrophobicities of the current generation of RPC sorbents are higher. Larger surface tension changes could thus be needed to affect desorption so that, as a consequence, proteins migrate in RPC in their unfolded states. Yet, numerous isolation studies have demonstrated that with RPC techniques, proteins can be recovered in bioactive form, so whatever conformational perturbations occur in these cases, they are rapidly reversible. On the other hand, with HIC, the protein is typically exposed to kosmotropic conditions [384], while the sorbent surface is less hydrophobic. The conformational integrity of the protein thus is a matter of time—how long does each conformational state exist and what is its mole fraction abundance? Thus, in RPC and HIC it is conceivable that the same conformers (involving the same surface-exposed amino acid residues) may be present, but on balance different clusters of amino acid residues form the preferred binding sites for intercalation with the nonpolar ligates under RPC compared with HIC conditions.

This temporal dimension of RPC and HIC separations has only recently been appreciated in a more stochastic context and still remains a powerful opportunity underexploited in process-scale purifications of globular proteins or for that matter in microanalytical separations, including CEC applications. With careful attention to the magnitude of the temperature intervals, the thermal transition profiles of proteins and large polypeptides can be monitored in RPC and HIC from the change in k' as well as from the significant changes in the peak shape, as $4\sigma_v$. Illustrative of these band-broadening effects is the behavior of

horse heart myoglobin over the temperature range 278 to 358 K shown as Fig. 32. The conclusion can be drawn from such observations that “bizarre” peak shapes can arise in RPC (as well as HIC) separations of globular proteins under “inappropriate” conditions. Although this is true if the expectation of the investigator is to obtain only a high-efficiency separation with maximal peak capacity, in fact the appearance of multiple peak due to band-broadening changes and retention time shifts also has provided the important attribute for the analytical chemist and biophysicist to explore the conformational integrity and purity of proteins. Thus, complex band-broadening behavior has been reported for α -lactoglobulin, lysozymes, and other globular proteins in both RPC and HIC systems [18,103,104,146,171,192,194,346–348].

Monitoring Interactions and Conformational Effects

As peptide and protein conformational changes can occur either in the mobile phase or on the sorbent surface in both RPC and HIC, a panel of different experimental procedures are needed to further substantiate the molecular basis of these changes and to provide a detailed characterization of the underlying phenomena. Because of their inherent nature, most spectroscopic techniques readily provide information on the bulk solution component of these conformational processes. Thus, on-line photodiode array detection utilizing difference spectroscopy, absorbancy ratio spectroscopy, or second-derivative spectroscopy at different wavelengths can be employed to assess the extent of exposure of aromatic amino acid residues, and tyrosine exposure in particular, to the solvent for the eluted polypeptide or protein peaks. Wavelengths that are typically monitored include the regions of isobestic absorbance of the amide backbone (210–220 nm) or the phenylalanine (254 nm), tyrosine and tryptophan (280 nm), cystine (240 nm), or histidine (228 nm) side chains. Peak identification via peak-tracking algorithms [385,386], determination of peak purity [191,387], or assessment of the conformational integrity of a peptide or protein molecule in the effluent can be achieved with photodiode array detection. Thus, an increase in the 274 nm/292 nm absorbance ratio, with concomitant decrease in the 292 nm/254 nm ratio is indicative of enhanced Tyr exposure, and an increase in the 292 nm/254 nm ratio provides confirmation that the Trp residues are less buried due to their conformational motion from the interior to a more solvent-accessible location. Similarly, fluorescence detectors provide higher sensitivity, as well as greater selectivity, in the detection of peptides or proteins containing tyrosine and tryptophan residues through their intrinsic fluorescence. Moreover, the fluorescence spectrum of a protein can also yield insight into the extent of solvent exposure of tyrosine or tryptophan residues, thus permitting conclusions to be drawn about the extent of unfolding from a native structure.

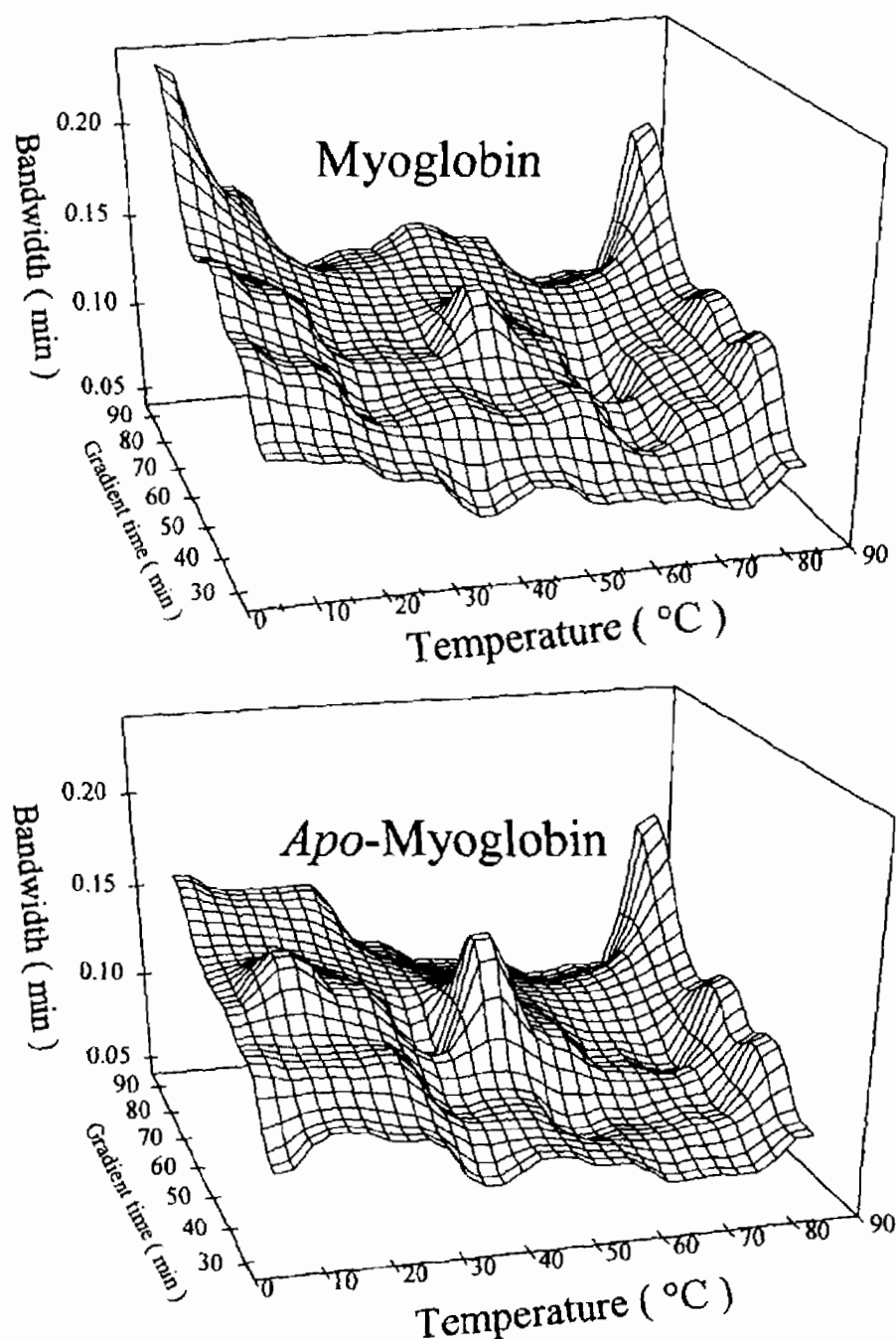


FIGURE 32 Dependence of the band-broadening behavior (as the experimental bandwidth, in min) of horse heart myoglobin and apo-myoglobin on the gradient time (proportional to the residency time) over the temperature range 278 to 358 K when chromatographed on (a) a C_{18} and (b) a C_4 reversed-phase sorbent. These band-broadening data were acquired using a linear gradient of 0.1% trifluoroacetic acid in water (buffer A) to 0.09% TFA in 65% aqueous acetonitrile (buffer B) with gradient times of 30–90 min at a flow rate of 1 mL/min with wide-pore Bakerbond C_{18} and C_4 sorbents, nominally of 5 μm particle size and 30 nm average pore diameter, packed into columns of dimensions of 250 $\times$ 4.6 mm ID. (Data from Ref. 106.)

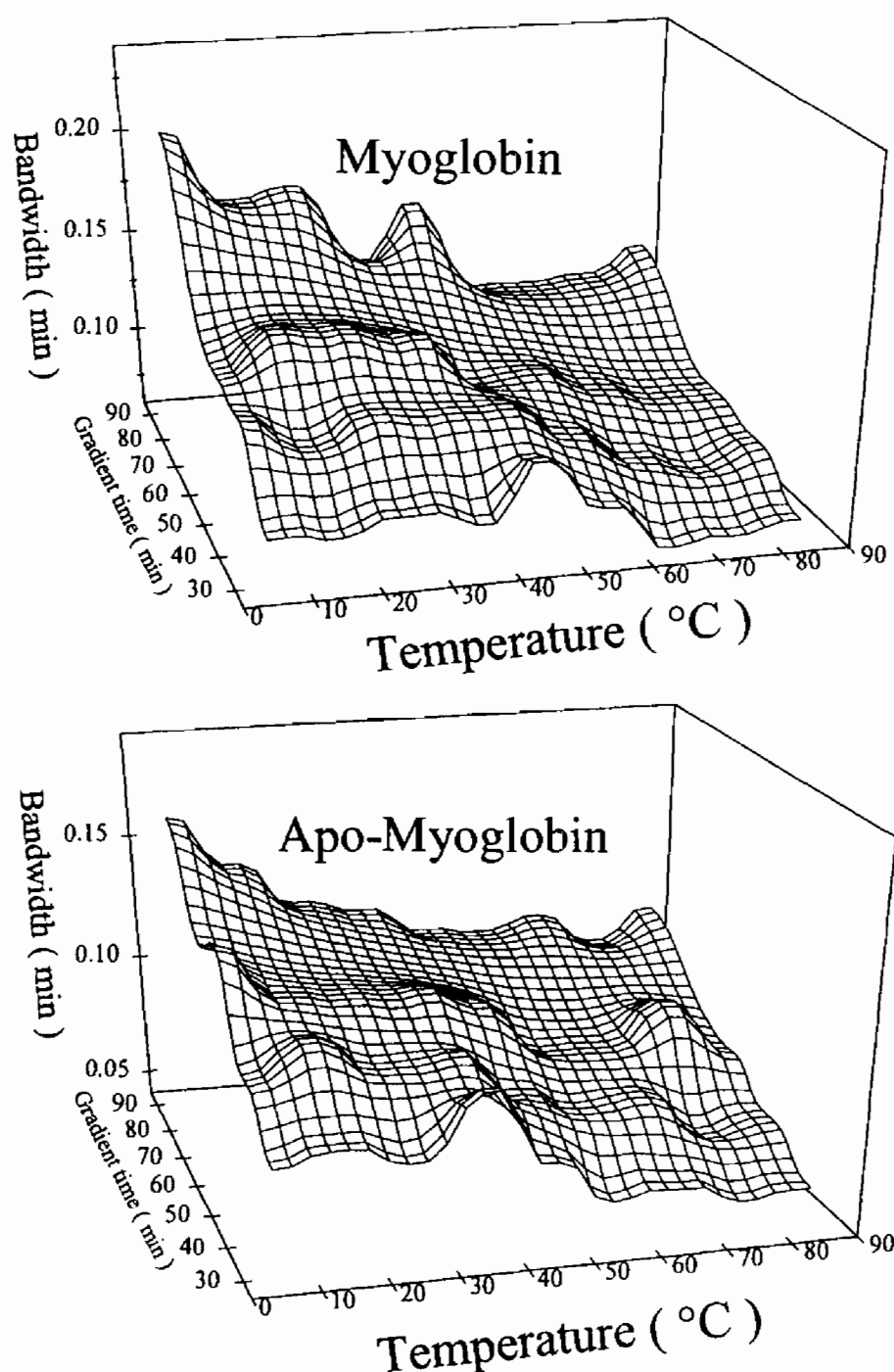


FIGURE 32 (continued)

Mass spectrometry (MS) in its different detection modes is now the ultimate method for the characterization of the mass, composition, and sequence of peptide or protein components in a mixture and is particularly well suited to the mobile phases employed in RPC. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopic methods, and to a lesser extent thermospray and fast atom bombardment

(FAB) mass spectrometry, can all be very successfully employed for these purposes. With microbore and capillary columns operated with or without postcolumn stream splitting, ESI-MS is typically operated at flow rates of $\leq 50 \mu\text{L}/\text{min}$ [388] or continuous-flow FAB-MS [389,390] operated at flow rates of $\sim 1\text{--}10 \mu\text{L}/\text{min}$, representing two very powerful approaches to characterize peptides derived from enzymatic digestions of proteins or following solid-phase synthesis. The routine application of tandem RP-HPLC-ESI-MS of large molecules, including proteins, via suitable stream-splitting devices and electrospray interfaces has greatly expanded over the last decade, permitting spectra to be acquired on line for proteins with molecular masses in excess of 200 kDa. Similarly, $^1\text{H} \rightarrow ^2\text{H}$ amide exchange studied with electrospray mass spectrometry (ESI-MS) has become [118,135,251] a powerful technique to address these issues.

Figure 33 shows the unfolding curves generated [116] when hen egg white lysozyme is thermally unfolded in a water-acetonitrile-0.1% TFA solution using

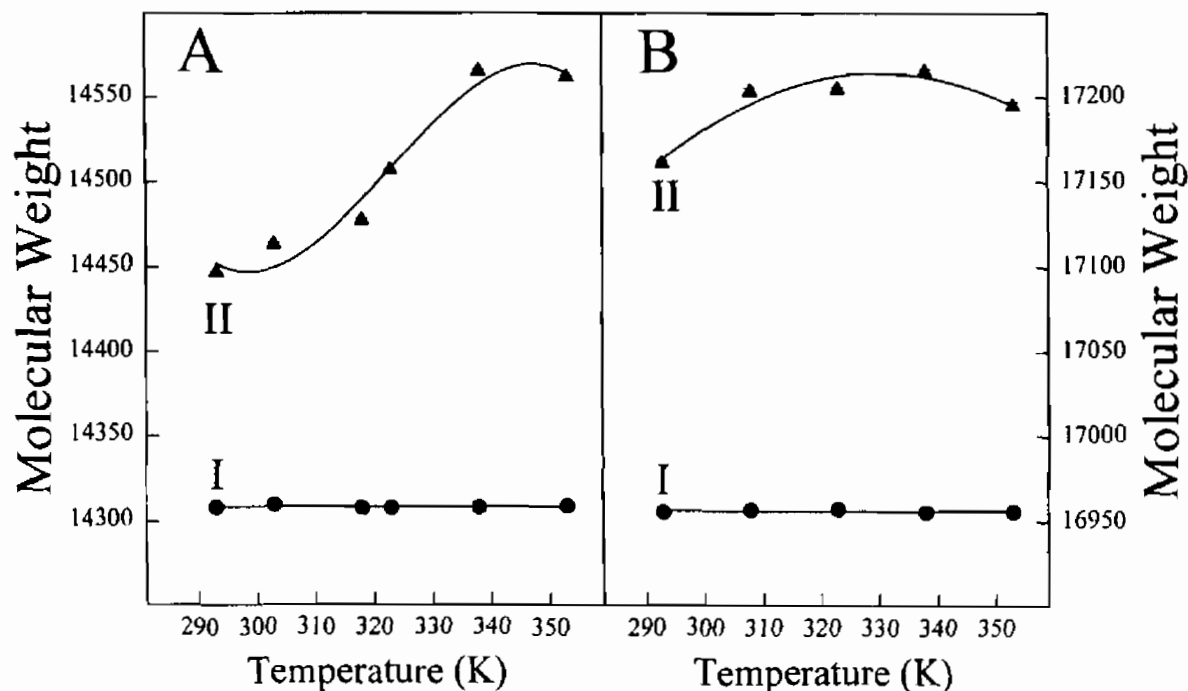


FIGURE 33 Plot of the molecular mass versus temperature for the unfolding of hen egg white lysozyme (HEWL) (A) and horse heart myoglobin (HMYO) (B) at different temperatures utilizing the hydrogen exchange $^1\text{H} \rightarrow ^2\text{H}$ approach. In this experiment, the constant mass of these proteins at different temperatures under the proton exchange condition is shown as I, and the progressive increase in molecular mass as the protected and less accessible hydrogen bonds are disrupted and exchanged by deuterons is shown in II. The difference between the molecular weight obtained under these conditions of $^1\text{H} \rightarrow ^2\text{H}$ exchange at any specified temperature equates with the number of exchangeable hydrogen bonds for HEWL or HMYO under the selected pH and solvent conditions. (Data adapted from Ref. 188.)

either ^1H - or ^2H -amide exchange conditions. In this example, experiments carried out in the ^1H environment do not show any mass gain for the protein on unfolding, while in the ^2H environment progressive amide and side chain protein exchange can be observed, monitoring the unfolding process. Not all peptides or proteins contain tryptophan residues, but when these are present, time-resolved fluorescence measurements also provide an important opportunity to assess conformational transitions. On-line circular dichroism can also be utilized to assess the α -helical, β -sheet, and random coil content of a polypeptide or protein as it elutes from an RPC or HIC column. With current technology on-line ^1H NMR techniques do not yet have the horsepower to address the conformational state of polypeptides or proteins as they elute from an RPC or HIC column. With the greater availability of very high field NMR spectrometers, such measurements may see the light of day in the near future and certainly within the next 20 years. Finally, when RPC and HIC methods are used in preparative studies with peptides and proteins, routine analysis of the recovered fractions of the column effluent is required. Commonly employed analytical methods include high-speed RP-HPLC, zonal and micellar electrokinetic high-performance capillary electrophoresis (HP-CZE and MECK-CZE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), enzymatic mapping, and assays of bio- or enzymatic activity and immunoassays, usually based on ELISA formats.

Besides conformational processes, other effects, such as isodesmic self-association, aggregation, or sample handling-induced denaturation (air-liquid interface frothing, excessive agitation/vortexing, or inappropriate buffer exchange), can all lead to multiple peak zones and more complex or bizarre elution profiles, in many cases concentration dependent. Aggregate formation can be monitored using LALLS, CD, and thermometric HPLC procedures. Mobile phase additives, such as allosteric cofactors, specific binding salts (i.e., citrate for NAD-dependent enzymes), or ion-pairing reagents that enhance the stability of the polypeptide or protein in its native conformation state, may remedy these problems and clearly have an important role in the RPC and HIC of these biomolecules. Sharper peak zones and better mass balances/biorecoveries with such optimized conditions can be anticipated even under conditions of elevated temperature. The combination of chromatographic information, drawn from the $\log k'$ versus ζ plots for RPC and HIC experiments, and data from these on-line spectroscopic modes thus readily generates an avenue to explore the participation of conformational events at least as they occur in the mobile phase. Direct measurements of differences between the spectroscopic properties for peptide or protein samples utilizing the elution profiles derived from the experiments with blank and packed columns offer information on the effect of the sorbent, provided the interconversion rates for the different conformational species are relatively slow in the mobile phase. As discussed earlier, when the interconversion kinetics for unfolding/refolding are fast, then a single peak zone would pass through the

column and the detector, with the spectral properties of the peak suggestive of a constant conformational state. Where the collective chromatographic and spectroscopic evidence is in accord, conclusions about the relative contributions of the mobile and stationary phases can be objectively drawn concerning the origin of a conformational change for a polypeptide or protein under the specific conditions.

PRACTICAL ASPECTS OF RPC AND HIC

Because of the high resolving power of these techniques, sample preparation is usually straightforward, provided the sample is appropriately filtered prior to loading to an RPC or HIC column. Selection of the operating variables in RPC and HIC separations of peptides and proteins follows a common set of criteria, with the choice of flow rate, mobile phase composition, and temperature the key parameters once a particular sorbent has been selected. With high-performance RPC and HIC sorbents, rapid separations of peptides and proteins can be carried out with crude feedstocks at low temperatures under conditions that favor the maintenance of biological activity. Both techniques can be employed to desalt samples. With HIC procedures in particular, high recovery of mass and activity has been repeatedly documented with globular proteins [8,15,110,271,391]. From a pragmatic perspective, HIC procedures currently offer greater flexibility at the preparative level than RPC methods for the fractionation of crude mixtures of proteins, and the reverse situation applies with crude peptides. Thus RPC and HIC can both be applied either early or late in a purification protocol depending on the task, readily permitting in both cases a purified product(s) to be obtained in the range from μg to g for each separation.

A particularly useful aspect of HIC procedures directly following ammonium sulfate precipitation of a biological extract containing the target protein or following an ion-exchange fractionation step is that the recovered fractions can be directly applied to the HIC sorbent with no dialysis required. Because RPC utilizes an organic solvent as the stronger eluent modifier, recovery of fractions from RPC separations is often straightforward, requiring only a simple lyophilization step. However, with HIC, water is used as the stronger solvent, and the recovered fractions will contain a considerable concentration of salt, which must be removed by membrane dialysis, gel filtration desalting steps, ultrafiltration, ion exchange, or other procedures [392]. In order to obtain eluted fractions with the separated peptides or proteins in higher concentrations, various approaches have been explored based on the use of the displacement mode of chromatography [393,394], a technique that with low-molecular-weight organic compounds can lead to an increase by orders of magnitude in the amount of material that can be purified in a single run. Moreover, contaminants present at low concentrations in a sample can be enriched in this manner and isolated in sufficient quantity to permit further characterization. However, despite extensive explorative work, the

application of displacement chromatography in the RPC or HIC separations of peptides or proteins has not yet gained much popularity due to the limited availability of suitable displacement compounds, although some initial success for some low-molecular-weight displacers with small peptides and standard proteins has been reported [395,396].

In the RPC and HIC separation of peptides and proteins, following the selection of mobile and stationary phase conditions, the other operating parameters must be chosen with care to achieve optimal resolution and column efficiency without sacrificing adequate control over the binding strength and conformation state. The efficiency of the chromatographic separation is determined in part by the mobile phase flow rate. Because peptides and particularly proteins have considerably smaller molecular diffusivities than small molecules, column efficiencies in the RPC and HIC separations of peptides and proteins are much more flow rate sensitive. Moreover, due to the dwell effect, the flow rate can influence the conformational integrity of a protein in RPC or HIC by affecting the time the molecule is adsorbed on the chromatographic surface. With very low flow rates, the longer contact between the protein and the nonpolar ligates permits relatively slow conformational changes of a protein to be detected as it unfolds. With micropellicular or perfusive sorbents, higher linear velocities can be employed due to the reduced diffusional resistances, resulting in higher efficiencies as well as reduced distortion of peak shapes compared with the more conventional porous sorbents.

As noted before, the effect of temperature on the retention of peptides and proteins is more complex than found for small molecules in RPC and HIC because both the molecular diffusivities and the conformational status change with temperature. For example, for small molecules in RPC, column efficiencies typically increase while retention decreases as the temperature is increased [397]. With peptides and proteins, on the other hand, retention often increases with temperature in the RPC or HIC modes under conditions that promote unfolding, although decreased retention times may arise at some specific temperature values for some proteins. Similarly, peak shape may also become impaired due to these conformational effects, despite the reduction in eluent viscosity and concomitant increase in the molecular diffusivity of the peptide or protein with temperature.

The retention and peak-broadening behavior of peptides or proteins is also much more heavily influenced by the choice of mobile phase conditions in isocratic or gradient elution than found for small molecules. Due to the relatively large contact area between a peptide or protein and the nonpolar chromatographic surface in RPC or HIC, the binding affinity is very sensitive to the concentration of the "stronger" solvent in the eluent, i.e., an organic modifier in RPC or water in HIC. Conformational changes, including aggregation or subunit disassociation, may also arise in the gradient elution mode due to changes in the composition of the eluent during the chromatographic run, with the mobile phase conditions at

the loading stage differing substantially from those required for elution. With inappropriate isocratic or gradient mobile phase compositions, the residence time effects may result in kinetically-controlled unfolding at the sorbent surface.

APPLICATIONS OF RPC AND HIC

As already noted, RPC and HIC separations of peptides and proteins provide opportunities complementary to the selectivities offered by ion-exchange or size exclusion chromatography. To date, the majority of the applications related to the high-resolution separations of peptides and proteins by RPC techniques at the laboratory scale as well as at the industrial level have been dependent on the use of silica-based sorbents. In contrast, the majority of the industrial/process applications of HIC have employed soft organic gels, whereas at the laboratory level, analytical or small-scale preparative HIC separations have tended to be based on high-performance sorbents derived from silica support materials. This division of applications reflects the fact that in RPC, proteins can unfold to a greater extent than in HIC, exposing a larger number of hydrophobic residues to the chromatographic surface. This advantage of RPC can be exploited in analytical applications and has proved fundamental to the understanding of the role of the hydrophobic effect in peptide or protein interactions with nonpolar surfaces.

Table 5 lists a selection of examples of polypeptides and proteins successfully separated under RPC conditions. Similarly, Table 6 shows different examples of proteins resolved by HIC procedures. These examples, and the ample scientific literature now available on the use of RPC and HIC procedures for the analysis and purification of peptides and proteins, lead to several substantive conclusions. First, the development of suitable RPC and HIC protocols has now permitted peptides and proteins to be separated faster, with greater efficiency and to a higher level of resolution than was previously possible with classical types of support systems, such as partition chromatography with Sephadex LH. Because the resolution and peak capacities are usually greater than found with ion-exchange or hydroxyapatite separations, the isolation of protein isoforms, enzyme zymogens, or protein muteins becomes less challenging or time-consuming. Moreover, RPC and HIC procedures represent very attractive separation options for the purification and analysis of recombinant proteins due to their potential advantages in GMP scale-up and rigorous analytical characterization. Thus, numerous examples of recombinant proteins of importance to the biotechnology industries have been examined by RPC methods, including recombinant human insulin [215], epidermal growth factor (rEGF) [398]; tissue plasminogen activator, (rt-PA) [399]; desulfatohiridin [400]; α -, β -, and γ -interferon (rIFN- α -, β -, and γ -) [401]; methionyl-human growth hormone (rMet¹-hGH) and human growth hormone (rhGH) [329,402]; interleukins

TABLE 5 Selected Examples of the Use of Laboratory Scale RP-HPLC Procedures for the Isolation of Biologically Active Polypeptides and Proteins

1. Acidocin-A related bacteriocin peptides from *Lactobacillus acidophilus* [429]
2. Alpha-amylase inhibitors from wheat [430]
3. Amyloid peptides from human insulinomas [431]
4. Angiogenin-2, and angiogenic protein from bovine serum and milk [432]
5. Antihypertensive peptides from *Lactobacillus helveticus* CPN4 extracts [433]
6. Atrial natriuretic peptide [434]
7. Bitistatin, a disintegrin isolated from the venom of the viper *Bitis arietans* [435]
8. Bone resorptive polypeptides [436]
9. Brain-derived neurotropic factor [437]
10. Bovine, porcine, and sheep inhibin from follicular fluid extracts [438–440]
11. Brochocin-C, an antibotulinal, two-peptide bacteriocin [441]
12. Bursicon, a cuticle sclerotizing neuropeptide from *Tenebrio molitor* [442]
13. *Buthus sindicus* scorpion venom peptides [443]
14. Callinectin, an antibacterial peptide from blue crab (*Callinectes sapidus*) hemocytes [444]
15. Carcinoembryonic antigen (CEA) domains [445]
16. *Centropomus striata* muscle prokallikrein [446]
17. Cholecystokinin-related polypeptides [447]
18. Colony stimulating factor-1 [448]
19. Comparison and characterization of the venoms of the *Parabuthus transvaalicus*, *P. granulatus*, and *P. villosus* scorpion species occurring in southern Africa [449]
20. CRISP-related proteins from *Picia pastdoris* extracts [450]
21. C-type natriuretic peptide from platypus *Ornithorhynchus anatinus* venom [451]
22. Cytochrome *ba*(3) subunit polypeptides from *Thermus thermophilus* hb8 [452]
23. Diuretic myokinin neuropeptides from the housefly, *Musca domestica* [453]
24. Enterocin I related bacteriocins from *Enterococcus Faecium* 6T1a [454]
25. Epidermal growth factor receptor [555]
26. Fibronectin type II domains (Cys²⁴–Cys⁶¹, Cys⁶⁹–Cys¹⁰⁹) of the major heparin-binding protein (PDC-109) of bull seminal plasma [456]
27. FSH releasing protein from bovine follicular fluid [457,458]
28. β -D-Galactoside binding lectin from cuttlefish, *Todarodes pacificus* [459]
29. Gliostatin and platelet endothelial cell growth factors [460]
30. Glucagon-like peptide-1 (GLP-1^{7–36}) amide, a potent insulin-releasing hormone [461]
31. Glutathione transferase *Triticum aestivum* GST 1 (Tagst 1) variants [462]
32. Granulin-like polypeptide from *Hirudo nipponia* [463]
33. Gonadotropin-releasing hormone [464]
34. Haemoglobin [α ¹¹⁴(gh2)Pro→Ser] [465]
35. Hemopoietic growth factors [466]

(continued)

TABLE 5 (continued)

-
36. Heavy and light chain polypeptides of the prophenoloxidase-activating isoenzymes (PPAE-1 and PPAE-2) of the silkworm *Bombyx mori* [467]
 37. Heparin binding growth factors from porcine, bovine brain and pituitary extracts [468–470]
 38. HIV-1 protease from *Escherichia coli* inclusion bodies [471]
 39. Honeybee (*Apis mellifera*) venom polypeptides [472]
 40. Honeybee (*Apis mellifera*) hypertrehalosemic peptides [473]
 41. Horned frog (*Ceratophrys ornata*) insulin and proglucagon-derived peptides [474]
 42. Human fibrinogen chains [475]
 43. Human growth hormone antagonist [476]
 44. Human hemoglobins variants [477]
 45. Human platelet-derived growth factor [478,479]
 46. Human proinsulin [480]
 47. Huwentoxin-II, a neurotoxic peptide from the venom of the Chinese bird spider *Selecnocosmia huwena* [481]
 48. Insulin receptor [483,483]
 49. Interleukin 2 [484]
 50. Interleukin-3 [485]
 51. Ion channel peptides [486]
 52. Kunitz-type trypsin inhibitor from *Enterolobium contortisiliquum* seeds [487]
 53. Leech neuropeptides [488]
 54. Leucocin H, a two-peptide bacteriocin [489]
 55. Low-molecular-weight glutenin subunits [490]
 56. Lumbricin I, proline-rich antimicrobial peptides [491]
 57. Mabinlin II related polypeptides [492]
 58. Maurotoxin, a four-disulfide-bridge scorpion toxin from the venom of the Tunisian chactoid scorpion *Scorpio maurus* [493]
 59. Mictocystin peptides [494]
 60. Murine epidermal growth factor mutants [495]
 61. Neuroparsin A, a polytropic neurohormone of the locust *Locusta migratoria* [496]
 62. Neurophysins from rat pituitaries [497]
 63. Nerve growth factor [498]
 64. Orphanin FQ/nociceptin neuropeptides [499]
 65. *Parabuthus schlechteri* scorpion venom polypeptides [500]
 66. Plant antimicrobial peptides [501]
 67. Plantaricin-related bacteriocins from *Lactobacillus plantarum* [502]
 68. Peptide YY^{1–36} and peptide YY^{3–36} from human blood [503]
 69. Proglucagon and prosomatostatin from the fruit-eating fish *Piaractus mesopotamicus* [504]
 70. Pituitary adenylate cyclase-activating polypeptide (PaCap) [505]
 71. α - and β -Polypeptides from the peripheral light-harvesting pigment-protein complex II of *Rhodobacter sulfidophilus* [506]

TABLE 5 (continued)

72.	Polypeptides fractionated from crude plant biomass [507]
73.	POMC peptides derived from rat pituitary extracts [508]
74.	Prolamin from Kodo millet (<i>Paspalum scrobiculatum</i>) [509]
75.	Prolamin, the major storage protein from selected varieties of foxtail millet (<i>Setaria italica</i> (L) beauv) [510]
76.	Proline-rich antimicrobial peptides from sheep and goat leukocytes [511]
77.	Proteinase inhibitor from the hemolymph of a solitary ascidian, <i>Halocynthia roretzi</i> [291]
78.	Pseudocholesterol inhibitors of cyclic nucleotide-gated ion channels [512]
79.	Rat pancreatic polypeptide hormones [513]
80.	Recombinant activin $\beta_A\beta_A$ from CHO cell supernatants [514]
81.	Recombinant rat fibroblast growth factor [515]
82.	Recombinant human factor VIII [428]
83.	Recombinant human proinsulin [480]
84.	Recombinant vasoactive intestinal peptide (VIP) analogues [516]
85.	Relaxin-like molecule from the male atlantic stingray, <i>Dasyatis sabina</i> [517]
86.	Retinal pigment epithelial protein [518]
87.	Scrapie prion proteins (PrP ^{Sc}) [519]
88.	Sea anemone toxin [520]
89.	Subunits of giant extracellular hemoglobins from earthworms <i>Pontodrilus matsushimensis</i> and <i>Pheretima communissima</i> [521]
90.	Sweet protein mabinlin II [522]
91.	Thymopoietin II (BTP-II) [523]
92.	α -Transforming growth factor [524]
93.	Transforming growth factor- α [525]
94.	Transforming growth factor- β [526–527]
95.	Type I threonine-rich fish antifreeze protein [528]
96.	Uterine milk protein from ovine allantoic fluid [529]
97.	Vasoactive intestinal peptide analogues [530]
98.	Venom protease with β -fibrinogenase activity from the Taiwan habu (<i>Trimeresurus mucrosquamatus</i>) [531]
99.	White muscle 65-kDa protein from warm temperature-acclimated goldfish (<i>Carassius auratus</i>) [532]
100.	<i>Xenopus laevis</i> oocyte immunoactive insulin [533]

[403,404]; and macrophage colony-stimulating factor (rM-CSF) [405]. Typically, in all of these applications, wide-pore RPC sorbents of moderate hydrophobicity have been employed in conjunction with either 0.1% TFA/acetonitrile-water or low pH, 1-propanol-water (or 2-propanol-water) mobile phase systems. Similarly, the resolution of the spinach, barley, and wheat isoforms of ferridoxins by HP-HIC using a TSK gel Phenyl 5-PW sorbent [406] in less than 30 min based on a gradient from 2 to 1 M ammonium sulfate is illustrative of this advantage.

TABLE 6 Selected Examples of the Use of Laboratory and Process Scale HIC-HPLC Procedures for the Isolation of Biologically Active Polypeptides and Proteins

Protein	Column	Mobile phase	Reference
<i>Enzymes</i>			
Rabbit reticulocyte hexokinase type I	Toyopearl Phenyl 650 S	A: 30% (w/v) ammonium sulfate, 3 mM 2-mercaptoethanol (2-MSH), 1 mM dithiothreitol (DTT), 5 mM glucose, 5 mM sodium potassium phosphate, pH 8.1	2
α -Amylase	Propyl SynChropak	B: buffer A without ammonium sulfate A: 1 M ammonium sulfate, 0.02 M potassium phosphate, pH 7.0 B: 0.1 M ammonium sulfate, 0.02 M potassium phosphate, pH 7.0	278
Microsomal cytochrome P450	Phenyl-Superose HR 5/5	A: 200 mM phosphate, 20% glycerol, pH 7.2 B: 100 mM phosphate, 0.4% cholate C: Emulgen 911	289
Calcium-independent phospholipase	Phenyl-Sepharose CL-4B	<i>Step elution</i> A: 0.15 M ammonium sulfate, 10 mM imidazole, 1 mM potassium phosphate, 1 mM EDTA-Tris, 0.1 mM phenylsulfonyl fluoride, 10% glycerol, 0.5 mM Triton X-100 1.5 g/mL leupeptin, 1 g/mL pepstatin, pH 6.8 B: Buffer A without ammonium sulfate	290

(continued)

TABLE 6 (continued)

Protein	Column	Mobile phase	Reference
Proteinase inhibitor	Ether-Toyopearl 650 M	A: 50% saturated ammonium sulfate, 0.5 M NaCl, 0.01% Brij-35, 50 mM tris-HCl, pH 8.0 B: Buffer A without ammonium sulfate	291
17 β -Hydroxysteroid dehydrogenase	Phenyl Sepharose	A: 0.6 M ammonium sulfate, 1 mM EDTA, 0.2 mM DTT, 20% glycerol, 40 mM Tris-HCl, pH 7.5 B: 1 mM EDTA, 0.2 mM DTT, 20% glycerol, 10 mM Tris-HCl, pH 7.5	326
Rate liver carbamoylphosphate synthetase I and ornithine transcarbamoylase	Ether-bonded stationary phase	A: 3 M ammonium sulfate, 0.5 M ammonium acetate, pH 6.0 B: Buffer A without ammonium sulfate	391
UDP-galactopyranose mutase	Toyopearl HICPAK-Butyl	A: 1 M ammonium sulfate, 1 mM DTT, 1 mM PMSF, 50 mM HEPES, pH 7.6 B: Buffer A without ammonium sulfate	534
NADP ⁺ oxidoreductase G6PDH from <i>Leuconostoc mesenteroides</i>	Superose HR 5/5	A: 1.7 M sodium sulfate, 1 mM EDTAQ, 50 mM sodium phosphate, pH 7.4 B: 50 mM sodium phosphate	535
Bovine pancreatic deoxyribonuclease F	Phenyl-Sepharose CL-4B	A: 4 M sodium chloride, 10 mM calcium chloride, 20 mM Tris-HCl, pH 7.5 B: 10 mM calcium chloride, 20 mM Tris-HCl, pH 7.5	536

<i>Chromobacterium viscosum</i> lipase on polyethylene glycol immobilized	PEG 10000-Sepharose CL-6B	<i>Isocratic:</i> A: 20% (w/v) ammonium sulfate B: 10 mM phosphate buffer, pH 7.0	537
Fungal aspartic proteinase rhizopuspepsin	Ultrapase C18 modified with Brij 76 (decaethyleneglycol <i>n</i> -octadecyl ether)	A: 3 M ammonium sulfate, 0.5 M ammonium acetate, pH 6.0 B: Buffer A without ammonium sulfate	538
<i>Antibodies</i>			
Monoclonal antibodies with light-chain heterogeneity produced by mouse hydridomas	TSK gel Phenyl-5PW	A: 1 M ammonium sulfate, phosphate- buffered saline (PBS), pH 7.4 B: Buffer A without ammonium sulfate	288
Natural human polyreactive monoclonal IgM antibody	Phenyl Superose HR 10/10	A: 1 M ammonium sulfate, 10 mM phosphate buffer, pH 7.4 B: 10 mM phosphate buffer, pH 7.4	539
Recombinant antibody to human IgE	<i>Analytical:</i> TSK-butyl-NPR <i>Preparative:</i> TSK Phenyl 5PW	<i>Analytical:</i> A: 2 M ammonium sulfate, 20% glycerol, 20 mM Tris, pH 8.0 B: A without ammonium sulfate <i>Preparative:</i> A: 2 M ammonium sulfate, 20% glycerol, 25 mM sodium phosphate, pH 7.0 B: A without ammonium sulfate	540
Reduction of bovine IgG (BGG) from MAb preparations	HIC SOURCE 15PHE	A: 0.75 M ammonium sulfphate, 0.1 M sodium phosphate, pH 7.0 B: 0.1 M sodium phosphate, pH 7.0	541
Anti-Klenow antibodies from goat serum	Econo-Pac HIC	A: 2.4 M ammonium sulfate, 20 mM phosphate, pH 6.8	542

(continued)

TABLE 6 (continued)

Protein	Column	Mobile phase	Reference
Murine monoclonal antigranulocyte IgG-1), IMMU-MN3	Bio-Gel TSK-Phenyl-5PW	B: Buffer A without ammonium sulfate A: 1 M ammonium sulfate, 20 mM phosphate, pH 7.5	543
F(ab')-2 fragments of mouse monoclonal antibodies (immunoglobulins G1)	TSK gel Phenyl-5PW	B: Buffer A without ammonium sulfate A: 1 M ammonium sulfate, phosphate-buffered saline (PBS: sodium phosphate buffer containing sodium chloride), pH 7.4	
7E3 X P4B6, a chemically cross-linked biospecific F(ab')-2 antibody that inhibits platelet aggregation	Biogel TSK Phenyl 5PW	B: Buffer A without ammonium sulfate A: 1 M ammonium sulfate, 100 mM sodium phosphate, pH 6.3	545
CD19 biospecific antibodies	Phenyl-Superose	B: Buffer A without ammonium sulfate A: 2 M ammonium sulfate, 100 mM sodium phosphate, pH 7.2	546
F(ab')-2-mu fragments from rat IgM monoclonal antibodies	TSK gel Phenyl-5PW	B: 1 mM ammonium sulfate, 100 mM sodium phosphate, pH 7.2 A: 1 M ammonium sulfate, phosphate-buffered saline (PBS), pH 7.4	547
<i>Recombinant proteins</i>			
Human macrophage colony-stimulating factor (M-CSF)	Phenyl-Sepharose CL-6B	A: 0.8 M ammonium sulfate, 0.01% PEG-6000 (v/v), 0.02 M phosphate buffer, pH 6.8 B: 0.01% PEG-6000 (v/v), 0.02 M phosphate buffer, pH 6.8	294

Recombinant human tumor necrosis factor alpha from an extract of <i>Escherichia coli</i>	Phenyl-Superose	A: 1.7 M ammonium sulfate, 0.1 M potassium phosphate, pH 7.8 B: Buffer A without ammonium sulfate	295
Recombinant human growth hormone preparation (rhGH)	TSK-Phenyl-5PW	A: 0.7 M sodium phosphate, 0.03 M Tris-HCl, pH 8.0 B: Buffer A without ammonium sulfate	381
Recombinant human growth hormone preparation (rhGH)	TSK-Phenyl-5PW	A: 0.7 M sodium phosphate, 0.03 M Tris-HCl, pH 8.0 B: 5% acetonitrile, 0.075% Brij, 35, 0.03 M Tris-HCl, pH 8.0	381
Staphylococcal nuclease	Phenyl-Superose HR5/5	A: 1.7 M ammonium sulfate, potassium phosphate, pH 7.0 B: Buffer A without ammonium sulfate	548
Native and recombinant <i>Leishmania mexicana</i> glycosomal glyceraldehyde-3-phosphate dehydrogenase	Phenyl-Sepharose	A: 1 M ammonium sulfate, 1 mM NAD, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylsulfonyl fluoride, 1 μ M leupeptin, 1 μ M pepstatin, 0.3 M Tris-HCl, pH 7.8 B: Buffer A without ammonium sulfate	549
Recombinant human serum amyloid A (SAA or rSAA)	Octyl-Sepharose CL-4B	A: 4 M guanidine-HCl in phosphate-buffered saline B: 4 M guanidine-HCl, 30% ethylene glycol, 0.01% NaOH	550
Active recombinant human gastric lipase	Phenyl-Sepharose CL-4B	A: 0.5 M NaCl, 50 mM sodium acetate, pH 5.0 B: 2 M NaSCN, 50% ethylene glycol, 50 mM sodium acetate, pH 5.0	551

(continued)

TABLE 6 (continued)

Protein	Column	Mobile phase	Reference
<i>Growth factors</i>			
Insulin-like growth factor-I	Phenyl-Superose HR 5/5	<i>Isocratic:</i> A: 1 M sodium chloride, 10 mM Tris, pH 7.0	552
Transforming-growth-factor- β 2-related polypeptide from bovine milk	Phenyl-Sepharose CL-4B	A: 0.6 M ammonium acetate, pH 5.0 B: Isocratic with 28% ethanol (v/v), 0.2 M ammonium acetate, pH 5.0 C: Isocratic with 40% ethanol (v/v), 0.2 M ammonium acetate, pH 5.0	553
Transforming growth factor-beta (TGF-beta)-like polypeptides	TSK Phenyl 5PW	A: 1.5 M ammonium acetate, pH 5.3 B: 0.02 M ammonium acetate, pH 5.3	554
Epidermal growth factor	TSK Phenyl 5PW	A: 0.5 ammonium sulfate, 20 mM sodium phosphate, pH 7.0 B: Buffer A without ammonium sulfate	555
Analogue of acidic fibroblast growth factor (aFGF)	Phenyl-Sepharose	A: 2 M ammonium sulfate, 20 mM sodium citrate, pH 6.5 B: A without ammonium sulfate	556
Recombinant human growth hormone (rhGH)	Two TSK Phenyl 5PW columns connected in series	A: 300 mM sodium citrate, 50 mM Tris, pH 7.8 B: Water	557
Early-pregnancy factor (EPF)	TSK Phenyl 5PW column	A: 2 M ammonium sulfate, 5 mM CaCl_2 , 0.01% NaN_3 , 0.1 M Tris-HCl, pH 7.0 B: Buffer A, 10% CAN (vol), but without $(\text{NH}_4)_2\text{SO}_4$	558

<i>Receptors</i>			
Neuropeptide Y ₂ receptor	Phenyl-Sepharose CL-4B	A: 1% octyl glucoside in 1 M K ₂ HPO ₄ , pH 7.4 B: 1% octyl glucoside in 10 mM K ₂ HPO ₄ , pH 7.4	335
Active human estrogen receptor	Synchropak-Propyl 300 column with silica guard column	A: 2 M ammonium sulfate, 10 mM potassium phosphate, 1.5 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, pH 7.4 B: A without ammonium sulfate	336
Human estrogen receptor	Polyether bonded, nonionic silica-based Spherogel CAA-HIC column	A: 2 M ammonium sulfate, 1.5 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 10 mM potassium phosphate, pH 7.4 B: A without ammonium sulfate	411
Alpha-1 thyroid hormone receptor (rTR-alpha-1)	Toyopearl Phenyl 650S	A: 0.7 M ammonium sulfate, 20% glycerol, 20 mM HEPES, 0.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, pH 8.0 B: Buffer A without ammonium sulfate	559
Short consensus repeat (SCR) oligomer of human complement receptor type 1 (C3b/C4b receptor, CD35)	Toyopearl Butyl 650M	A: 1 M ammonium sulfate, 100 mM sodium hydrogen phosphate, pH 7.0 B: Buffer A without ammonium sulfate	560
Glucocorticoid receptor	Phenyl-Sepharose	A: 2.5 M NaCl, 10 mM dithiothreitol, 20 mM Bis-Tris B: A, without NaCl C: B, with 50 mM CHAPS and 100 mM NaC D: As A, but 100 mM NaCl	561

(continued)

TABLE 6 (continued)

Protein	Column	Mobile phase	Reference
Calcitonin gene-related peptide (Cgrp) receptor	Phenyl-Sepharose CL-4B	Glucocorticoid receptor was eluted with CHAPS gradient CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid A: 1 M potassium phosphate, 6 mM sodium cholate, pH 7.5 B: 10 mM potassium phosphate, 6 mM sodium cholate, pH 7.5	562
Glucocorticoid type II receptor complexes	Shaltiel series of 1.5-mL columns [23]. Along with unmodified agarose the series included propyl, butyl, pentyl, hexyl, octyl, decyl, and dodecyl alkyl groups covalently attached to agarose	A: 0, 150, 300, 600, 900, 1200 mM KCl, 50 mM sodium molybdate B: 20 mM HEPES, 2 mM DTT, pH 7.6	563
Nuclear androgen receptor	Shaltiel hydrophobic columns with 1 mL of $\text{NH}_2\text{-(CH}_2\text{)}_n\text{-agarose}$ (n -alkyl chain length from $n = 1-10$)	<i>Isocratic:</i> 0.6 M NaCl, 0.01 M TES, 0.5 mM mercaptoethanol TES: 2-[[2-hydroxyl-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulfonic acid	564
<i>Therapeutic peptides/proteins</i>			292
Anti-CD4 monoclonal antibody MAX.16H5 for human therapy	BioPilot Phenyl-Sepharose HP 35/100	A: 0.5 M ammonium sulfate, 70 mM potassium phosphate, pH 7.2 B: Buffer A without ammonium sulfate	

Receptor-binding B subunit (EtxB) of toxins useful as a component of vaccines against enterotoxigenic <i>E. coli</i>	Phenyl-Superose HR5/5	A: 1 M ammonium sulfate, 20 mM Tris-HCl, pH 7.5 B: A without ammonium sulfate	293
Immunotoxin B3LysPE38, composed of the modified <i>Pseudomonas aeruginosa</i> exotoxin A (LysPE38) chemically linked to a murine monoclonal antibody (B3)	Phenyl-Sepharose Fast Flow (High Substitution)	A: 0.5 M ammonium sulfate, 1 mM EDTA, pH 7.4 B: A without ammonium sulfate	565
<i>Toxins</i>			
Alpha-toxin	Alkyl-Superose HR5/5	A: 45% ammonium sulfate, 20 mM sodium phosphate, pH 7.0 B: Buffer A without ammonium sulfate	566
Toxic shock syndrome toxin-1 (TSST-1)	Octyl Agarose	A: 1.5 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0 B: Buffer A without ammonium sulfate	567
Vacuolating toxins from <i>Helicobacter pylori</i>	Phenyl-Sepharose HR5/5	A: 0.5 M ammonium sulfate, 60 mM Tris, pH 7.7 B: Buffer A without ammonium sulfate	568

Similar purification outcomes have been achieved using HP-HIC methods with various labile enzymes such as ornithine transcarbamoylase and carbamoylphosphate synthetase I [391]; β -galactosidase from *Aspergillus oryzae* [407]; phospholipase C from *Trypanosoma brucei* [278]; glucose oxidase and catalase from *Penicillium chrysogenum* [408]; glycosidases from *Streptococcus pneumoniae* [409]; bovine cortical bone matrix acid phosphatase [410]; as well as other proteins including human pituitary prolactin [275] and isoforms of the human estrogen receptor [296,336,411]. Moreover, HIC has been successfully applied to the analysis of various rDNA derived proteins including t-PA [412], tumor necrosis factor [413], IGF-I [414], and hGH [415].

It is often difficult to establish that a recombinant protein manufactured in a novel cell system by genetically engineered approaches has the same tertiary structure as the authentic wild-type protein. Because detection by spectroscopic techniques such as circular dichroism (CD) of subtle conformational differences that may arise due to minor perturbations in the hydrophobic regions of a protein is often difficult, the observation of identical retention times for the rDNA and the wild-type protein by RPC and HIC procedures is a useful indicator of common three-dimensional structures. Moreover, when small structural variations arise due to amino acid residue additions, replacement, or chemical/enzymatic modifications, the use of RPC and HIC procedures under less denaturing elution conditions often favors the resolution of these species from the parent protein. The separation of recombinant Met¹-hGH from recombinant hGH is an example [385,404,415] where such approaches have been successfully applied.

A further area of wide application, particularly of RPC techniques, relates to the structural characterization of proteins. Since their introduction [58,72,416] in the late 1970s, the separation of enzymatic maps of proteins by RPC procedures has become an essential part of all structural studies. In this approach, a protein is digested with a protease (usually trypsin) and the resulting mixture of peptides is then readily separated by RPC to obtain a peptide map of the protein. Because the majority of enzymatically derived peptides from most proteins contain less than 20 amino acid residues, resolution difficulties encountered in the chromatography of the intact protein due to the presence of significant tertiary structure are largely avoided. Differences in the peptide maps for different samples of the protein digested with the same enzyme reveal compositional differences that may not be evident in the chromatographic profile of the intact protein, and differences in the peptide maps that arise when different enzymes are used for the digestion can reveal microheterogeneities that could have coalesced in a single peptide map system. Because the effects of amino acid substitution in small peptide fragments will be exaggerated in the peptide map as significant retention time changes, the detection of structural/sequential variations is thus relatively straightforward chromatographically, and can be subsequently confirmed by N-terminal sequencing, mass spectrometry, or amino acid analysis.

However, it should also be recalled that some peptides within complex maps may colute under a specified chromatographic regime, so alternative fragmentation procedures should always be examined in addition to selecting different mobile phase conditions.

Besides trypsin, other proteolytic enzymes such as thermolysin, Lys-C, V-8 protease, chymotrypsin, and pepsin have been successfully utilized to generate enzymic maps of proteins that were subsequently resolved by RPC techniques. Similarly, chemical cleavage methods such as cyanogen bromide, hydroxylamine, or reduction of disulfides can be employed. If the enzymatic or chemical fragmentation of a protein is carried out under conditions whereby the cystines remain intact, it is often possible to assign their positions by comparing the RPC results with those obtained for the same mixture reduced by β -mercaptoethanol or dithiothreitol. Because the sequence sites for fragmentation are well known and can be predicted with reasonable precision, empirical rules can be applied to facilitate the identification of the fragments following RPC separation from algorithmic analysis of the retention times [211,212,386,417–419]. Thus, deamidation of a peptide/protein usually results in a slight increase in retention time in RPC under low-pH elution conditions, whereas the generation of oxidation products such as methionine sulfoxide or cysteic acid results in significantly decreased retention. Such behavior is generally consistent with the intrinsic hydrophobicity properties of the amino acid side chains. Any variability in chromatographic performance will result in unacceptable deviations in retention times and peak shapes and thus lower levels of correlation. Special care should thus be taken with RPC enzymatic procedures to ensure reproducibility.

When mistranslation or posttranslational modifications occur, RPC procedures can usually be employed to separate the mutant peptide(s) from the peptide containing the wild-type sequence. Such approaches have been employed to resolve Met from norLeu variants of recombinant proteins, as well as to characterize differences in the secreted to stored forms of other recombinant proteins. Such procedures are particularly powerful to discriminate differences between the isoforms of glycoproteins. In this case, the combination of boronate or lectin affinity chromatography and RPC offers a powerful method for characterizing glycopeptides even with very complex enzymatic digest mixtures, such as the tryptic digest derived from recombinant tissue plasminogen activator (rt-PA), which is expected to contain 51 peptides [420]. In this application, the glycopeptides isolated by a ConA-lectin affinity column were rechromatographed under conditions used for the tryptic map of rt-PA. This approach was used to determine the position of elution of the glycopeptide components in the proteolytic map of this recombinant protein.

The very high resolving power of RPC enzymatic mapping procedures in terms of their simplicity and high information content has been amply demonstrated over the past decade, including studies with recombinant t-PA [399], basic

fibroblast growth factor [421], insulin-like growth factor-1 [414], interleukin-2 [422]; endomorphins [423], hGH [402,415,424], brain-derived neurotropic factor [425], and the seminal plasma cystine-rich secretory protein [426]. As a consequence, enzymatic mapping by RPC procedures has become an essential requirement for the characterization of rDNA-derived proteins and in quality control of therapeutic proteins as part of their Drug Masterfile registration. Moreover, the application of automated proteolytic mapping procedures will greatly streamline future investigations [427]. Detection of variants or contaminating species present at low levels (typically less than 2%) still represents a challenge with such analytical RPC methods. For example, RPC enzymatic mapping procedures do not reveal the presence of conformational variants although the consequences of proteolytic degradation products derived by proteases present in the cell culture or recovery process can be readily assessed [428]. Algorithmic scoring techniques, which include spectral as well as retention time and peak area analysis by principal component analysis, have been introduced and allow computer-controlled comparisons of enzymatic maps of different lots of a given recombinant protein [402,429].

CONCLUSIONS

In this chapter, an overview of the status of RPC and HIC separations of peptides and proteins has been presented. Several common themes inherent in these two powerful separation procedures have been highlighted. First, the retention processes that are manifested in RPC and HIC have a common theoretical basis in the hydrophobic effect, although RPC and HIC are not synonymous in terms of their selectivity outcomes. Second, the differences that are theoretically and experimentally evident between RPC and HIC provide a rich opportunity to explore the conformational properties, microheterogeneity, and surface anisotropy of globular proteins as well as peptides. Third, use of thermal effects and thermometric strategies offers a powerful avenue to explore the underlying thermodynamics of the interaction, utilizing RPC and HIC methods to probe different conformational states of the same molecule. Fourth, the principles governing retention and resolution apply equally to high-performance and classical soft gel sorbents in both the RPC and HIC modes.

From the perspective of the separation sciences, further refinement of RPC and HIC techniques can be expected to continue unabated due to their importance at the research as well as industrial levels. The development of synthetic RPC and HIC sorbents with tailored selectivities and greater stability over wider pH ranges can be readily envisioned. Alternatively, existing technologies for the surface modification of support materials will be adapted, such as the recent study with controlled pore glasses chemically coated with *N*-isopropylacrylamide copolymers for the HIC separation of proteins [430]. Because both RPC and HIC

techniques provide new opportunities to assess from biophysical and biochemical perspectives the relative conformational stability and associated information on the secondary and tertiary structure of globular proteins, in solution and at nonpolar interfaces, these procedures are expected to grow in importance, further complementing more conventional spectroscopic techniques. Because of their ability to assess the intrinsic hydrophobicity of peptides and proteins, RPC and HIC methods may become even more useful as analytical procedures par excellence in the characterization of the structure-function relationships of peptides and proteins and thus provide a basis for considerable expansion of knowledge on proteins and protein design in the postgenomic era of biotechnology.

This postproteomic revolution, manifested as the age of zeomies/atomic biology, will be the engine that drives biotechnology, with the versatility, high separating efficiency, and unique selectivities of RPC and HIC methods providing essential contributions and pivotal solutions, not only for the development of peptide- and protein-based pharmaceuticals or other commercially valuable products but also continuing to expand the horizons with new insights into fundamental problems in biorecognition and molecular folding. RPC and HIC are already recognized as major separation tools in peptide and protein chemistry, from the ultramicroanalytical to the industrial process-scale levels. From a regulatory perspective, the development of new quality control procedures will be increasingly dependent on faster, more sensitive separation procedures. New RPC and HIC methods will significantly contribute to these objectives. As already noted, many new types of microparticulate tailored RPC and HIC sorbents, with greater pH operation ranges and improved mass transfer kinetics, can be visualized. To meet these challenges, additional progress will be essential in miniaturized instrumentation, including novel micromachined systems for very high speed, nanoanalytical separations in the RPC or HIC modes. The opportunity to employ sequentially or concurrently hyphenated separation principles, such as advanced forms of CEC and affinity capture CEC modes [431,432] in microcolumn or alternatively multichannel micromachined systems, will also be realized. Greater utilization of capillary column systems in novel multichannel formats with gradient elution times of 30 sec to 1 min, will become routine for such ultraanalytical separations. Such developments will become the engine for the development of new detection systems, including micromachined devices.

Integration of several chromatographic and detection systems into a single instrument is now feasible at the research laboratory level. The commercial availability of such systems is only a matter of several years away. Already, various computer-assisted procedures can be employed to simultaneously run multiple equipment systems, change columns or adjust the composition of mobile phase eluents, while concurrently optimizing the resolution, peak spectral scanning, or delivering the peak effluent to a second, third, ... detector as a

multidimensional hyphenated separation. Full implementation of such control and monitoring systems will change how analysis and quality control are carried out online at the research laboratory as well as in larger scale production procedures with peptides and proteins. Moreover, the same principles and procedures will underpin the design and evaluation of new types of sorbents and chromatographic conditions. Possibly, however, the greatest excitement will come from experimental work on the structure-retention-function correlations that these new instrumental systems and associated experimental procedures will offer. Realization of this potential, in their various manifestations, will ensure that RPC and HIC procedures expand their fundamental importance as innovative tools in the *molecular chromatography* revolution over the next several decades. Exciting times are ahead for those lucky and committed investigators who pursue this path of innovation. Enjoy the fun and rewards!

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Metal Interaction Chromatography

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INTRODUCTION

Metal interaction chromatography is an HPLC technique that can separate many biopolymers because of their differential ability to form complexes with metal ions [1]. It employs a stationary phase with an appropriate metal immobilized via chelating functions bound to the surface. Retention and separation of the sample components occur largely by their interaction with the chelated metal. Although immobilized metal affinity chromatography (IMAC) is a common name for the technique, it is called metal interaction chromatography (MIC) in this book to conform to the nomenclature used for the other interactive chromatographic methods for biopolymer separation by HPLC.

Stationary phases with immobilized metals have been used for chromatographic separation of small molecules since Helfferich [2] introduced ligand

exchange chromatography in 1961. Initially, regular ion exchangers [3] had been employed as the supports for the metals, but they were replaced by resins with fixed chelating functions [4,5]. The most popular among such chelating resins is Chelex-100, which contains iminodiacetic acid (IDA) groups attached to a cross-linked polystyrene matrix. Fundamentals of ligand exchange chromatography and its application to the separation of small molecules have been reviewed in detail by Davankov and Semechkin [6]. Microparticulate siliceous bonded stationary phases with chelated metals have also been developed for HPLC of small molecules [7–12].

In 1975, Porath and his collaborators [13] successfully explored the possibility of separating proteins and nucleic acids on agarose-bound metal chelates [14,15] and termed the technique “metal chelate affinity chromatography.” Later he changed the name to “immobilized metal affinity chromatography [IMAC] to include all kinds of chromatography dealing with interactions between solutes and immobilized heavy metals, regardless of whether the latter are in the form of ions or not” [16]. Most often, IDA has been the chelating ligate and Cu^{2+} the bound ion in this technique, which has found increasing application in the separation of a wide variety of biopolymers. The importance of MIC as a tool for protein purification in biotechnology is evidenced by a number of reviews on the subject [1,17–27].

As with other branches of biopolymer HPLC, the concepts underlying MIC were soon applied to the HPLC of proteins based on the adaptation of chromatographic principles, established and tested successfully in conventional column chromatography with polysaccharide-based stationary phases, to HPLC columns packed with bonded stationary phases made of rigid microparticulate supports [12,28–33]. Sorbents with appropriate metals immobilized by suitable chelating functions at the surface of siliceous, resinous, or highly cross-linked agarose supports have been used for the separation of proteins by HPLC.

Although MIC has been increasingly used for protein purification since its introduction in the mid-1970s, the physicochemical phenomena underlying the retention process were not well understood immediately. This may have been an impediment to exploiting the full potential of MIC despite the strong need engendered by the emerging biotechnology sector for efficient protein separation methods. This chapter attempts to review MIC techniques and to provide analyses of the various factors involved in the retention process. The treatment includes the application of MIC for protein purification and isolation by both traditional column chromatography and HPLC because the results accumulated with agarose-based metal chelate stationary phases, although mainly qualitative, have paved the way to the application of the technique to the HPLC of proteins. The increasing use of HPLC should generate a sufficiently large body of quantitative data to augment the understanding of the chromatographic process and in turn facilitate the design of rapid and efficient separations.

INTERACTION SITES ON THE PROTEIN MOLECULE

Amino Acids

MIC is a general technique for protein analysis or purification because binding is due to surface amino acids or phosphate groups rather than the metal binding sites of metalloproteins [1]. Under nondenaturing conditions, only amino acid residues situated at the protein surface are implicated in the retention process because only they can interact with the chelated metal on the stationary phase. In the 1970s, Porath et al. [13] postulated that histidine, cysteine, and, to a lesser extent, tryptophan residues would interact with the chelated metal via electron donor atoms. The subject has been investigated by many research groups to explain further the mechanism of MIC. Sulkowski [18] examined the relationships between the retention behavior of proteins on metal-IDA columns and their histidine and tryptophan contents and found that ribonuclease (RNase)S-peptide (one histidine residue), RNase A (two accessible histidine residues), and RNase S-protein (three exposed histidine residues) were retained in the order of increasing number of histidine residues by a Cu^{2+} -IDA column using gradient elution with decreasing pH or increasing imidazole concentration. In addition, tuna cytochrome *c*, with no exposed histidine residues, was not retained, whereas horse cytochrome *c* with one histidine was strongly retained on Cu^{2+} -IDA [18]. Egg white lysozyme (one exposed histidine residue) is another protein that was strongly retarded on Cu^{2+} -IDA, and Peking duck lysozyme, which has no histidine, was not retained [18]. In a different study using a Cu^{2+} -IDA HPLC column, the retentive behavior of myoglobins from different sources was shown to be similarly based on exposed histidines when a decreasing pH gradient from 7.0 to 5.5 was used in that dog myoglobin (one exposed histidine residue) eluted first, followed by horse myoglobin (two exposed histidine residues), and sperm whale myoglobin (three exposed histidine residues) [12], as shown in Fig. 1.

These studies and others have shown that to a first approximation, proteins are retained according to the number of accessible histidine residues when MIC is used with borderline metal ions such as Cu^{2+} . MIC has thus been effective as a probe for histidine topography in proteins [34]. Histidine sequences added by protein engineering have been very effective as purification tags with MIC. A His-Trp sequence was used to purify luteinizing hormone-releasing hormone (LHRH) and proinsulin on Ni^{2+} -IDA [35]. In another study, the Zn^{2+} binding site from the active center of human carbonic anhydrase, which consists of three histidine side chains, was grafted onto recombinant serum retinal-binding protein to allow purification using Cu^{2+} [36]. When affinity tags from a peptide library were selected by MIC using Cu^{2+} , 13 tags containing two histidines, one with four histidines, and another with one histidine surrounded by hydrophobic residues were selected in the process [37].

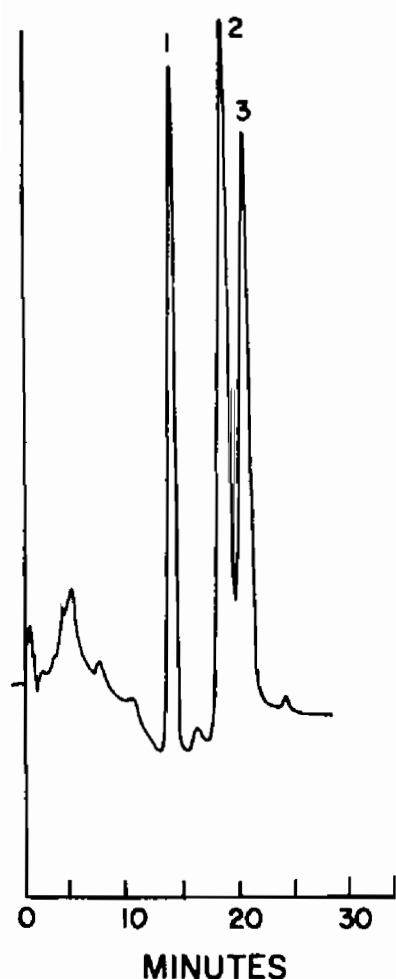


FIGURE 1 MIC of myoglobin mixture on silica-bound Cu(II)-IDA. Column, 100×4.6 mm, $5\text{-}\mu\text{m}$ IDA-Vydac in Cu(II) form; 20 min linear gradient 0–100% B in A; eluent A, 0.05 M acetic acid/MES/Hepes buffer, pH 8.0, containing 0.08 M Na_2SO_4 and 2×10^{-6} M Cu(II); eluent B, 0.05 M acetic acid/MES/Hepes buffer, pH 5.5, containing 0.05 M ammonium acetate and 2×10^{-6} M Cu(II); flow rate, 1 mL/min; temperature, 25°C . 1, Dog myoglobin; 2, horse myoglobin; 3, sperm whale myoglobin. (From Ref. 12.)

Because histidine is a rare amino acid ($\sim 2\%$ abundance), incorporating it into genetically engineered proteins is an attractive option for selective purification [25,38–40]. The spacing of histidine residues in the tag can strengthen the binding. For example, two histidines separated by three amino acids in an α -helix has high affinity for metals [18,23]. The attachment of amino acid sequences such as His-Tyr- NH_2 , His-Gly-His, and His-Trp to the N-terminus of genetically engineered proteins has been used to bring about the isolation of biosynthetic proteins by MIC due to strong binding to Co^{2+} -, Ni^{2+} -, and Cu^{2+} -IDA columns [41]. The pK_a of an individual histidine residue is related to its position and environment, which explains why pH gradients can effect separations so well [40].

The nature of histidine binding is specific to the metal ion [42]. On Cu^{2+} , all histidine-containing peptides were retained above pH 5. They were also retained on Ni^{2+} ; however, the absence of a free α -amino group resulted in a 5- to 10-fold reduction in the retention on Ni^{2+} but had little effect on Cu^{2+} [42]. Peptides containing only one histidine exhibit Langmuir-type isotherms, indicating monolayer binding, whereas those with multiple accessible histidines appear to bind to multiple sites, which may increase the affinity up to 1000-fold [43]. Under certain conditions, such as high ionic strength, proteins are more likely to associate with previously bound molecules and further increase total protein binding on Cu^{2+} [44].

The role of other amino acids in retention by MIC is exemplified by Peking duck lysozyme (no histidine residues), which is slightly retained on Cu^{2+} -IDA at neutral pH [18], a fact that was attributed to metal interaction with its tryptophan residues. Similarly, avidin, which has at least two exposed tryptophan residues, was found to bind very strongly to Cu^{2+} -IDA [18]. It has been suggested that aromatic amino acids may also enhance interactions with histidine [25]. When protoporphyrin IX was used as a chelating ligate, tryptophan had a very high affinity for Cu^{2+} , Fe^{3+} , and Ni^{2+} , probably due to a combination of metal ion and π - π interactions [45]. The binding affinity of tryptophan was much greater than that of either histidine or phenylalanine. Using conventional chelating ligates (e.g., IDA), tryptophan was found to contribute to binding only when the α -amino group had already anchored the peptide to the support [46]. This anchoring was shown to occur in a cytochrome *c* containing no histidine residues that bound to a Cu^{2+} -TSK chelate support at pH 7.5 [40]. Once a peptide was anchored to a Cu^{2+} support, negative binding contributions from glutamic acid or aspartic acid residues were exhibited [47] and retention could be predicted according to amino acid content.

In a study of MIC of proteins with surface-exposed thiol groups, it was suggested that cysteine-17 in human β -interferon was responsible for its strong binding to IDA stationary phases in the Co^{2+} , Ni^{2+} , Cu^{2+} , or Zn^{2+} form [48]. However, it is also possible that surface-exposed cysteines may oxidize in the presence of Cu^{2+} to form disulfide-linked dimers [25]. In a study of the binding of amino acids to a Cu^{2+} -IDA silica-based support, the binding strength of amino acids at pH 6 was $\text{His} \gg \text{Cys} > \text{Trp} > \text{Phe}$ [31]. Lysine and arginine also exhibited some binding that was not caused by the silica or the IDA ligate alone. In a study of MIC on Cu^{2+} and Ni^{2+} , cysteine-containing peptides that lacked histidine and tryptophan residues exhibited no binding in the absence of the free α -amino group [49]. Furthermore, single amino acids have been found to bind more strongly than tripeptides, which bind more strongly than dipeptides. Binding enhancement for the tripeptide seems to occur when the metal-binding amino acids are spaced by at least one other amino acid residue [24].

It should be pointed out that differences in molecular size, amino acid composition, and net charge between various proteins may also affect their retention in MIC so that the number of strongly binding amino acid residues in the molecule alone does not suffice to predict the elution order of different proteins on a given metal chelate column. For example, bovine pancreas RNase (two exposed histidine residues, $pI = 9.4$) was less retained than egg white lysozyme (one exposed histidine residue, $pI = 11.0$) on a Cu^{2+} -IDA column at pH 6.0 [50]. In another example, cytochrome *c* eluted before lysozyme on a Cu^{2+} -IDA column to allow ready separation even though both egg white lysozyme and horse heart cytochrome *c* have one exposed histidine residue [51]. Belew and Porath [52] showed that dipeptides with C-terminal histidines were not bound on a Cu^{2+} -IDA column, whereas those having N-terminal histidines were strongly retained.

Carbohydrates

Carbohydrate groups on the protein surface have been found to influence retention in MIC. The lower retention of ribonuclease B (RNase B) than ribonuclease A on Cu^{2+} -IDA has been attributed to the glycan moiety in RNase B [23]. Glycan is a high-mannose oligosaccharide that shields the two histidine residues of the enzyme so that they cannot interact with the Cu^{2+} -IDA ligates. Because RNase A is not glycosylated, there is no such impediment to metal interactions. The lack of retention of human α_1 -acid glycoprotein by Fe^{3+} -IDA has also been explained by steric hindrances of the potential interacting sites on the polypeptide by the carbohydrate envelope [53]. Electrostatic repulsion by negatively charged sialic acids in the glycan moiety of glycoproteins may also attenuate retention. Studies with sialic acid-rich glycoporphins from human red blood cells showed no retention of these membrane proteins at low ionic strength on Cu^{2+} -IDA despite their histidine residues [54]. However, at high salt concentration in the mobile phase (0.7 M NaCl in 25 mM phosphate buffer, pH 6.0), glycoporphins were strongly retained and separated with a decreasing salt gradient. Surprisingly, they were retained in the order of increasing number of histidine residues in the protein molecule as normally observed with other proteins when gradient elution with increasing salt concentration was used [54]. Evidently, at sufficiently high salt concentration in the mobile phase, not only is electrostatic repulsion between the protein and the chelated metal ligates attenuated but also salt-mediated hydrophobic interactions contribute additional retention to that caused by specific metal interaction. Such a mixed-mode retention mechanism is typical for membrane proteins that have relatively large nonpolar polypeptide domains. The use of 0.05% (w/v) sodium dodecyl sulfate (SDS) in the mobile phase was necessary for the separation of glycoporphins, but the detergent did not interfere with the metal interaction [54].

Phosphates

It is well known that phosphate forms very stable complexes with Fe^{3+} ; therefore, it is not unexpected that Fe^{3+} columns retain phosphorylated proteins. Andersson and Porath [55] found that at pH 3.1–5.0, differently phosphorylated ovalbumins were retained by Fe^{3+} -IDA and could be eluted and separated according to the number of phosphate residues by using an increasing pH gradient. The higher the degree of phosphorylation, the longer the retention on the Fe^{3+} -IDA column. When Muszynska et al. [56] investigated the chromatographic behavior of egg yolk phosvitin and certain histones, before and after dephosphorylation, they found that the dephosphorylated proteins were less retained by Fe^{3+} -IDA than the corresponding phosphorylated forms. Posewitz and Tempst [57] observed that selectivity and elution of phosphopeptides occurred most efficiently on a Ga^{3+} -IDA compared with other metals such as Fe^{3+} and Al^{3+} .

Metalloproteins

Further insight into the effect of protein structure on retention in MIC has been gained from comparative studies on the retention of metalloproteins and their corresponding apoproteins on metal chelate stationary phases. Apocarbonylpeptidase A (CPD) was not retained on either naked IDA or Zn^{2+} - or Co^{2+} -IDA [58]. On the other hand, both the apoenzyme (CPD) and holocarbonylpeptidase [CPD(Zn)], which contains zinc, were retained on Cu^{2+} -IDA. CPD was also able to bind to an Ni^{2+} -IDA column, suggesting a selective interaction of CPD with Ni^{2+} chelates through the active site for zinc binding [58]. Moreover, CPD could be reactivated upon passing through tandem metal-IDA columns: Zn^{2+} -IDA, Co^{2+} -IDA, Ni^{2+} -IDA. In another study, if human serum transferrin was not saturated with iron, it was retained by both Co^{2+} - and Zn^{2+} -IDA [59]. Similarly, the affinity of transferrins for Ni^{2+} - and Cu^{2+} -IDA also decreased with increasing iron occupancy in the protein molecule [59]. It was postulated that two proximal histidine residues in transferrin in coordination with ferric ions may be involved in the MIC binding.

STATIONARY PHASE

The stationary phase of MIC supports consists of metal ions coordinated by chelating ligates affixed to a neutral bonded phase; for HPLC, the matrix must also be rigid. Figure 2 illustrates the hypothetical surface of an HPLC stationary phase for MIC of proteins. The stationary phases for high-performance MIC of biopolymers must meet the following criteria:

1. The support should be mechanically and chemically stable.
2. The pores of the support must be large enough for the chelated metal to

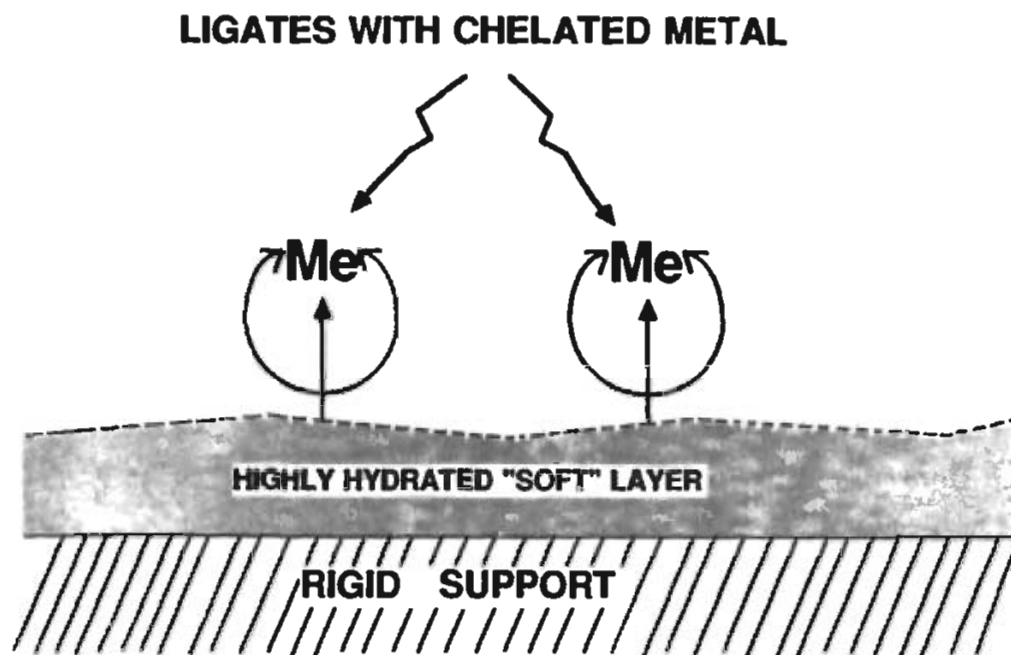


FIGURE 2 Surface of the stationary phases used for MIC of proteins by HPLC.

- be accessible to biopolymeric solutes or, alternatively, nonporous or micropellicular [60,61].
3. The support should have a highly hydrated "soft" surface that will not interact with the biopolymers to be separated without the chelating ligates.
 4. The chelating functions fixed at the surface should hold the metal of interest sufficiently strongly to prevent leaching under normal MIC operating conditions.
 5. Chelation of the metal should not be so strong as to shield it completely from interacting with the biopolymeric solutes.
 6. Removal of the chelated metal from the stationary phase should be easily achieved with a solution of a suitable chelating agent to allow the reuse of the stationary phase either in naked form (without metal) or with another chelated metal.

Ligates

The chelating ligate in MIC has two critical aspects. It must bind the metal ions tightly enough to prevent leaching, yet also allow some of the coordination sites of the metal to remain free to form adequate bonds to the solutes. Iminodiacetic acid (IDA) has been the most popular ligate for MIC, at least partially because of its commercial availability on chromatographic supports. High-performance commercially available supports for MIC include TSKgel Chelate 5PW, Chelat-

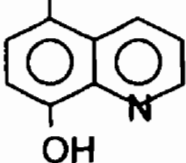
ing Sepharose Fast Flow, HiTrap Chelating, and Fractogel EMD Chelate 650(s). In conventional column chromatography, IDA moieties are often covalently attached to a polysaccharide support such as agarose or cross-linked dextran using a dioxirane coupling agent [13]. Alternatively, an organic moiety containing primary amino groups can be bound to agarose and subsequently reacted with bromoacetic acid [62]. The direct coupling method was found to yield a superior stationary phase in terms of the Cu^{2+} -binding capacity [62].

The popularity of stationary phases with IDA ligates in protein HPLC is readily explained by the fact that they nearly meet the criteria described before for an ideal MIC stationary phase. The intermediate strength of the metal-IDA complexes renders them sufficiently stable for use as stationary phase ligates and at the same time allows easy removal of the chelated metal from the column by washing with EDTA or another suitable chelating agent [31,32]. This permits the convenient regeneration or reloading of the IDA stationary phase with other metal ions. Perhaps most important, more than one coordination site on the chelated metal remains available for interaction with proteins or other solutes due to the intermediate strength of metal complexes with the tridentate IDA.

Porath and coworkers [14] have synthesized several other chelating stationary phases on agarose support, such as tris(carboxymethyl)ethylenediamine (TED) for immobilization of various metal ions (Fe^{3+} , Al^{3+} , Ga^{3+} , In^{3+} , and Tl^{3+}). Novarose TED was used to chelate Eu^{3+} to purify calmodulin, a calcium-binding protein that required calcium in the mobile phase to effect binding [63]. Sorbents with nitrilotriacetic acid (NTA) as the chelating ligates have also been used in various metal chelate forms for the separation of peptides and proteins [64]. 8-Hydroxyquinoline (8-HQ), which has been employed extensively for the chromatography of metal ions, was effective for immobilizing hard metal ions [65]. Ethylene-*N,N'*-diacetic acid (EDDA) exhibited stable chelation, yielding about $2 \mu\text{mol}/\text{m}^2$ of surface coverage [33]. Protoporphyrin IX formed very stable complexes with metals; binding constants were 10^{29} M^{-1} compared with $10^{7.3} \text{ M}^{-1}$ for IDA and $10^{10.4} \text{ M}^{-1}$ for NTA [45]. 3-Bromopentanedione also formed stable complexes that did not leach when used with Cu^{2+} [66]. 1,4,7-Triazacyclononane (tacn) had higher stability constants than IDA, 8-HQ, or NTA and yielded different retentive behavior [67]. The efficacy of IDA, 8-HQ, and O-phosphoserine (OPS) as chelating ligates for hard metal ions was tested for a series of model proteins [68]. Generally, at pH 5.5, the binding strength was $8\text{-HQ} > \text{IDA} > \text{OPS}$, whereas at pH 8, $8\text{-HQ} > \text{OPS} \geq \text{IDA}$. Both IDA and OPS bound more strongly at pH 8 than at pH 5.5 due to formation of metal ion-hydroxide ion complexes [68]. Selected chelating ligates employed in stationary phases for the separation of proteins by MIC are shown in Table 1.

The nature of the chelating ligate dictates both the stability of the metal complex and the strength of the metal-solute affinity. IDA is tridentate, which leaves multiple coordination sites on the metal to bind to the protein, whereas

TABLE 1 Chelating Ligates of the Stationary Phase in MIC

Name	Abbr.	Structure	Ref.
Iminodiacetic acid	IDA	$-\text{N}(\text{CH}_2\text{COOH})_2$	13
Tris(carboxymethyl)-ethylenediamine	TED	$-\text{N}-\text{CH}_2\text{CH}_2-\text{N}(\text{CH}_2\text{COOH})_2$ $\quad \quad \quad $ $\quad \quad \quad \text{CH}_2\text{COOH}$	14
Glycine hydroxamic acid	GH	$-\text{NHCH}_2\text{CONHOH}$	69
Nitrilotriacetic acid	NTA	$-\text{N}(\text{CH}_2\text{COOH})_3$	64
Ethylenediamine- <i>N,N'</i> -diacetic acid	EDDA	$-\text{N}-\text{CH}_2\text{CH}_2-\text{NH}-\text{CH}_2\text{COOH}$ $\quad \quad \quad $ $\quad \quad \quad \text{CH}_2\text{COOH}$	33
8-Hydroxyquinoline	8-HQ		65
O-Phosphoserine	OPS	$-\text{NH}-\text{CHCOOH}$ $\quad \quad $ $\quad \quad \text{CH}_2\text{OPO}_3^{2-}$	70
Protoporphyrin	ProP	Protoporphyrin IX	45

TED is pentadentate, forming a stronger bond with the metal ion but leaving fewer sites on the metal for binding to the solutes. Consequently, TED-metal ion complexes are more stable than those with IDA, but they also have weaker affinity for proteins. NTA and EDDA, on the other hand, are tetradentate. The logarithmic stability constants of EDDA with metal ions were almost twofold higher than those of IDA [33]. 8-HQ is bidentate yet it yields high stability constants with hard metal ions [65]. One consequence of weak metal ion binding is that metal ion transfer (MIT) can take place either from a metal-containing protein to a chelating ligate without a bound metal or from an MIC support to the apo form of metalloenzyme [27]. The likelihood of this occurrence is related to the binding constants and the extent of metal complexation.

The nature of chelating ligates plays an important role in determining the retentive properties of the stationary phase [13,15,16,45,68,69]. Generally, protein retention is greater on metal-IDA than on metal-TED [16] stationary phases under comparable conditions. This is expected because the hexacoordinate metal-TED complex possesses only one coordination site available for interaction with proteins, whereas three coordination sites are available in the weaker complex with tridentate IDA as shown in Fig. 3. If a metal ion is maximally

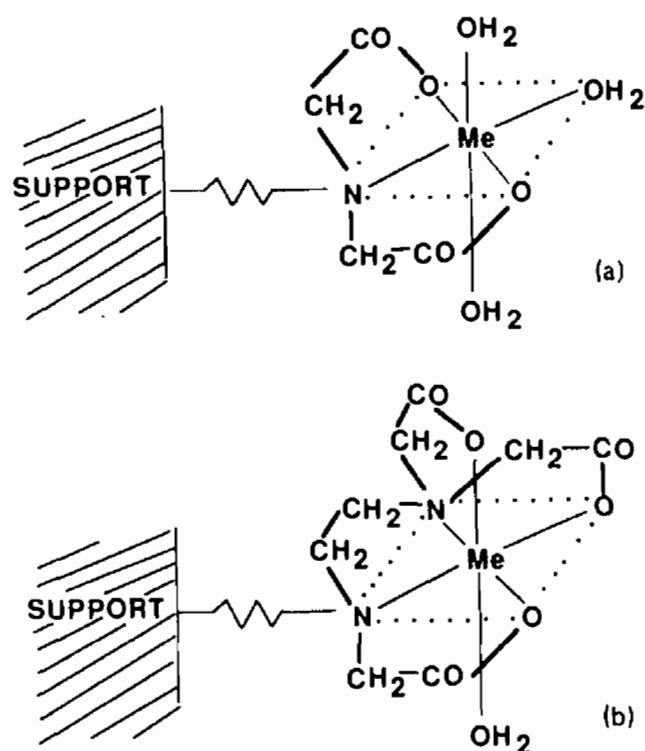


FIGURE 3 Schematic illustration of the surface-bound metal chelates. (a) Tri-dentate IDA and (b) pentadentate TED, both with a coordination number of 6.

coordinated in the complex with the chelating ligate, it will not interact with proteins, yielding low or nonexistent adsorption capacity.

The experimental evidence leads to the conclusion that the metal complexes at the stationary phase surface should be of intermediate strength. The complex should not be so strong that the metal does not possess sufficient residual binding capacity (affinity) for proteins. On the other hand, most weakly chelating ligates (mono- or bidentate) should be avoided because they yield unstable chelates, giving rise to metal bleeding and formation of metal-protein complexes in the mobile phase.

Spacer Arm

The metal ions of MIC supports are connected to the matrix via a spacer arm, which may be composed of only the chelating ligate or an additional moiety to help distance it from the surface. Most linkages are 3–12 atoms [1]. The tentacle structure of Fractogel Chelate, which utilizes a hydrophilic polymer as a spacer, has higher loading than some conventional supports [71]. A C-1 polyether has also been used as a leash from silica to IDA [12]. The nature of the ligate and spacer may give the support reversed-phase or normal-phase attributes.

Support Matrix

Although the requirements for the ligate and the metal complex are the same for all MIC sorbents, adsorbents prepared from soft polysaccharide gels cannot be used in HPLC due to their low mechanical stability. Most microparticulate stationary phases used in MIC of proteins by HPLC have been synthesized with IDA or other chelating functionalities covalently bound to a hydrophilic silane attached to the surface of macroporous silica [12,28–31,33,45,61]. IDA has also been bonded onto 160 Å and 1000 Å zirconia [72]. Commercially available products are generally 20–60 µm cross-linked agarose with the exception of 10 µm TSK gel Chelate 5-PW [32] and Fractogel EMD Chelate [71], which are resin-based; the iminodiacetic functions are attached to the soft surface of a macroreticular resin or agarose support. A magnetic agarose that is convenient for batch methods is also available [73].

The pore diameter is also an integral parameter in an MIC support for biopolymers. The access of proteins and resultant retention and binding capacity are dependent on the pore diameter. When nonporous and 1000 Å silica were compared, the porous support had higher capacity and retention for lysozyme but concanavalin A was somewhat restricted [61]. In another study comparing TSK gel Chelate with Sepharose Fast Flow, the frontal analysis was much sharper for the smaller particle diameter TSK gel despite the higher loading on the larger pore of the agarose support [74]. On both supports, flow rate did not affect elution volume. The TSK column lifetime has been shown to be greater than 900 hr or 1000 injections [75].

Metal Ions

Nature of the Metal

There is ample evidence that the retention of proteins in MIC is greatly affected by the nature of the chelated metal on the stationary phase. The concept of hard-soft ions of Pearson [76] based on the polarizability of the interacting metal ions was employed by Porath and coworkers [13,14,35] to provide a framework for the chromatographic behavior observed on different metal chelate stationary phases. “Hard” metal ions, such as Mg^{2+} , Ca^{2+} , Fe^{2+} , Al^{3+} , Ga^{3+} , and In^{3+} , associate strongly with oxygen, whereas “soft” metal ions, such as Cd^{2+} , Hg^{2+} , and Tl^{3+} , are most easily polarized in an electric field and have a preference for sulfur. Ions of the “intermediate” type, such as Cu^{2+} , Zn^{2+} , Ni^{2+} , and Co^{2+} , preferentially coordinate with amino and imino nitrogen but also interact with oxygen and sulfur; these are also called “borderline” metal ions. Coordinate bonds formed with soft metals are essentially covalent, whereas those formed with hard metals are ionic [24]. Chelates of intermediate metal ions such as Cu^{2+} - or Zn^{2+} -IDA bind proteins rich in histidine such as sperm whale myoglobin [18] and lymphotoxin [23]. Thiol-containing human β -interferon was reported to bind

specifically to intermediate or soft metal chelates [48]. The interpretation of protein retention on an MIC column is not always straightforward in terms of the nature of the metal because different kinds of mechanisms are involved in protein-metal chelate interactions, including Coulombic interaction and coordination bonding with the chelated metal. Furthermore, at relatively high salt concentrations in the eluent, a common condition in MIC, hydrophobic interactions with both the chelating ligates and the chelated metal may affect retention.

The adsorption capacity of stationary phases containing different divalent metal ions chelated to IDA-agarose has been found to decrease for human serum proteins in the order $\text{Cu}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Mn}^{2+}$ [13]. Certain interferons, depending on their molecular structure, were bound to a Cu^{2+} -IDA column but not to Zn^{2+} -IDA [77], whereas other interferons had higher affinity for Zn^{2+} than for Cu^{2+} [78]. When a series of peptides was run on IDA- Cu^{2+} , $-\text{Ni}^{2+}$, and $-\text{Zn}^{2+}$, the retention on Cu^{2+} and Ni^{2+} was similar but that on Zn^{2+} was significantly lower [47]. On the Ni^{2+} column, some of the calcitonins were less retained than on Cu^{2+} whereas PTH peptides were more highly retained. The retention was shown to be caused by the metal ions and not the base support because the metal-free adsorbent showed little retention. Human serum transferrin, human lactoferrin, and hen ovotransferrin were all retained on IDA-agarose with Ni^{2+} and Cu^{2+} chelates but not with Co^{2+} and Zn^{2+} chelates. On the other hand, only human serum transferrin was retained on the corresponding Co^{2+} -IDA columns [59]. The effect of metal chelated to IDA-silica on the retention of various proteins was extensively studied by HPLC [31], as shown in Fig. 4. The proteins investigated showed the strongest and weakest interaction with the Cu^{2+} -IDA and the Zn^{2+} -IDA stationary phases, respectively, at pH 5.0 and 6.0 with an increasing salt gradient.

The relatively low formation constants of hard metal ions with IDA have restricted their general use in MIC. TED ligates have greater affinity for metal ions, especially for iron: $\text{Fe}^{3+} > \text{Cu}^{2+} > \text{Ni}^{2+} > \text{Zn}^{2+} \approx \text{Co}^{2+} > \text{Fe}^{2+} > \text{Ca}^{2+}$ [1]. The use of alternative chelating ligates such as TED [63], EDDA [33], 8-HQ [65], and *O*-phosphoserine [70] has broadened the scope of MIC to include more hard metal ion applications due to their stable chelation with these alternative ligates. In a comparative study of the use of IDA, 8-HQ, and OPS chelates of hard metal ions for MIC, it was shown that the net charge on the complex varied with metal and pH, resulting in differential protein retention [68]. The pH differences were thought to be due to the formation of metal ion-hydroxide ion complexes at pH 8 that are more negatively charged than those complexed with water at pH 5.5. The differences in charge among ligates and metal ions are related to the number of dentation sites. The effective negative charge at pH 8, which can range up to -4 for Yb^{3+} -OPS complexes or -3 for Fe^{3+} -OPS or Al^{3+} -OPS complexes, may result in binding to lysine or arginine residues. Substitution of hydroxide for water may also result in changes in coordination number [68]. The generally weaker interactions of proteins with certain MIC supports may be compensated

METAL	pH	RETENTION VOLUME [ml]									
		0	5	10	15	20	25	30	35	40	
Zn ²⁺	6.0	+	x			□	◇	○			
	5.0	+			□ x ◇	○					
Ni ²⁺	6.0	+	x			□	◇	○			
	5.0	+				x □	◇	○			
Fe ²⁺	6.0	+					x	□		◇ ○	
	5.0	+						□		x ○ ◇	
Fe ³⁺	7.0	+	x			□			◇ ○		
	6.0	+					□ x		○		
	5.0						+	□	○	x, ○	
Cu ²⁺	6.0					□					
	5.0				□						
—	6.0	+	x			□	◇	○			
	5.0	+				□ x	◇ ○				

FIGURE 4 Retention volume of proteins on a silica-bound IDA column without and with various chelated metals. Column, 100 × 4.6 mm, 5 μm IDA-Vydac; linear gradient 0.05 M sodium chloride in 25 mM phosphate buffer, at pH 5.0, 6.0, and 7.0; flow rate, 1 mL/min; temperature, 25°C. The proteins are (+) ovalbumin, (□) α-chymotrypsinogen A, (x) β-lactoglobulin A, (◇) cytochrome c, and (○) lysozyme. (From Ref. 31.)

for by the selectivity offered by the metal ions. For example, chelates of hard metal ions, e.g., Fe³⁺-IDA, have been found to bind phosphoproteins such as albumin [55], phosvitin, and phosphorylated histones very strongly [56]. It is probable that aspartic acid and glutamic acid are involved in binding of proteins by hard metal ions as evidenced by the binding of tuna heart cytochrome *c* by Fe³⁺-8-HQ [65]. On stationary phases prepared from TED-agarose, serum proteins were strongly adsorbed at pH 7.6 with Tl³⁺ as the chelated metal but not with Al³⁺, Ga³⁺, and In³⁺ [14].

Cu²⁺ is one of the most popular metal ions for proteins. The equilibrium dissociation constants of Cu²⁺-IDA for most proteins are about 10⁻⁵–10⁻⁶ M and for imidazole are about 10⁻⁴ M [24]. Retention of proteins on borderline metal ions can be correlated with that of imidazole. The affinity of Cu²⁺ is 15 times that for Ni²⁺, which is more than 3 times that for Zn²⁺ or Co²⁺ [25]. It should be noted, however, that the highest affinity does not necessarily result in the best separations because selectivity varies, with the individual proteins.

Amount of Chelated Metal

In a metal interaction stationary phase, it is believed that the metal-IDA functions are 1 : 1 complexes. The experimental results listed in Table 2 on the uptake of three

TABLE 2 Metal Uptake by IDA-Silica (5- μm Vydac) from 15 mM Aqueous Salt Solutions^a

Metal salt	Metal uptake by 1 g of adsorbent		log K ^b (free solution)
	mg	μmol	
Cupric nitrate	14.4	227	10.57
Cobalt sulfate	14.4	244	6.94
Ferric chloride	12.0	215	10.72

^aThe metal solution was pumped through the column (100 $\times$ 4.6 mm) until the metal concentration was the same in the influent and the effluent. The amount of bound metal was determined by analysing the fresh and spent-load solutions by atomic absorption.

^bThe logarithmic stability constants are from Ref. 79.

different metals by an IDA-silica stationary phase indeed showed that the number of micromoles of metal ions bound per gram of adsorbent was about the same. Thus, the bound metal concentration at saturation can be used to measure the density of chelating ligates (number of μmol of chelating ligates per gram of adsorbent) on a given metal interaction stationary phase. Some of the metal ions adsorbed to the ligates may be only weakly bound and these should be removed before chromatography by washing with 0.001–0.01 M imidazole, 0.1–1 M glycine, or a similarly effective solvent [24]. In one case of Cu^{2+} -IDA gels that adsorbed 30–50 μmol of Cu^{2+} , about 30–50% was removed during the wash step [24].

In a study by Belew et al. [74], the total concentrations of immobilized Cu^{2+} on a TSK gel Chelate 5-PW column and a Chelating Sepharose Fast Flow column, each containing IDA functions, were measured as 20 $\mu\text{mol}/\text{mL}$ and 30–40 $\mu\text{mol}/\text{mL}$ of adsorbent, respectively. These values compare to the capacity of 50–70 $\mu\text{mol}/\text{mL}$ exhibited by silica-based stationary phases of 300 Å mean pore diameter and 60–80 m^2/g specific surface area [31]. Because Sepharose and the TSK gel had higher mean pore diameters and lower surface areas, accessibility of the chelated metal may have been greater for them than for the silica-based stationary phases. In that case, the differences in operational capacities could be significantly smaller than indicated by the preceding values.

The surface concentration of the chelated metal in the stationary phase plays an important role in MIC of proteins [1]. As seen in Table 3, the magnitudes of both the retention and the selectivity increase with the amount of bound metal. Selectivity reversal can even occur upon increasing the surface concentration of chelated copper in an IDA stationary phase as shown in Fig. 5. This comparison

TABLE 3 Retention Factors k' and Relative Retention α of Two Proteins on Fe(III)-IDA-Silica at Different Levels of Fe(III) Loading^a

Fe(III) conc. [mM] (loading solution)	Retention factor, k'		Selectivity α
	Cytochrome c	Lysozyme	
0.0	5.10	4.91	1.04
0.5	13.70	10.80	1.27
13.0	40.10	21.80	1.84

^aMobile phase, 25 mM phosphate buffer, pH 6.0, containing 0.1 M $(\text{NH}_4)_2\text{SO}_4$.

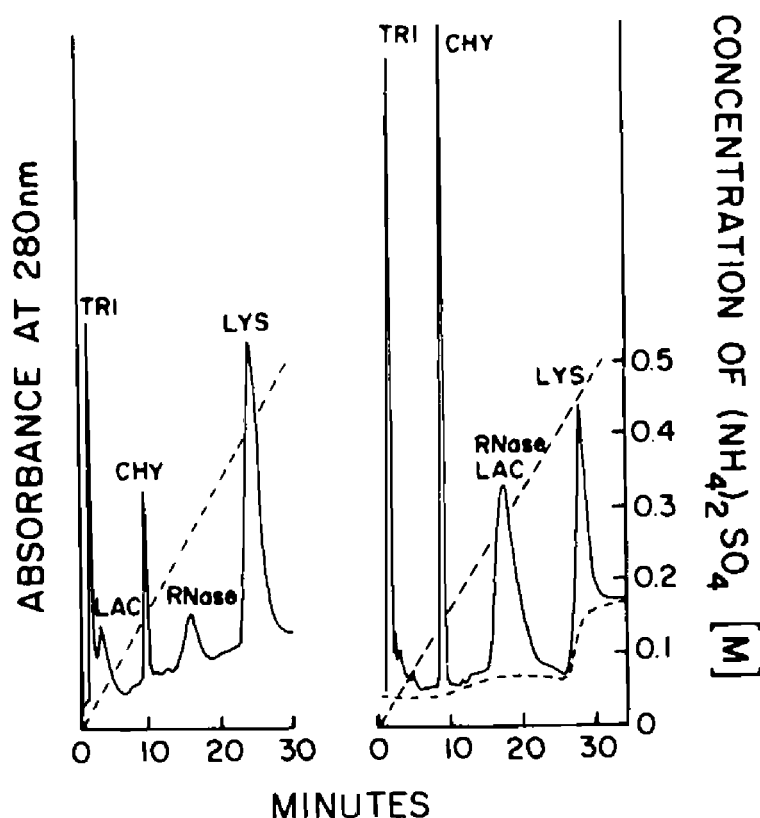


FIGURE 5 Chromatograms of proteins on a silica-bound Cu(II)-IDA column with different surface densities of chelated metal. Column, 5- μm IDA-Vydac in Cu(II) form, 100 $\times$ 4.6 mm; (a) light-metal load and (b) heavy-metal load; 30 min linear gradient 0–0.5 M $(\text{NH}_4)_2\text{SO}_4$ and 1–20 mM glycine in 25 mM phosphate buffer, pH 6.0; flow rate, 1.0 mL/min; temperature, 25°C. Proteins: TRI, trypsin inhibitor; LAC, β -lactoglobulin A; CHY, α -chymotrypsinogen A; RNase, ribonuclease A; LYS, lysozyme.

of the chromatograms obtained with columns that differ only in the surface concentration of Cu^{2+} -IDA shows that the retention of basic proteins such as RNase A and lysozyme, as well as that of β -lactoglobulin, a slightly acidic protein, increased with the surface density of chelated copper. In contradistinction, the retention of α -chymotrypsinogen A, a basic protein, was lower in the column with the higher content of chelated copper, whereas trypsin inhibitor, a strongly acidic protein, was not retained on either column. These observations suggest that the free IDA functions at the surface of a metal interaction stationary phase serve as cation-exchange sites that also contribute to retention when the ionic strength of the eluent is sufficiently low. Thus, this type of metal interaction stationary phase can behave as a strong cation exchanger for acidic and basic proteins lacking exposed amino acids for specific metal interaction.

Proteins with only one accessible histidine had maximum binding capacities at 5–10% of the maximum copper loading [43]. Proteins with multiple accessible histidines, which appear to interact with more than one metal ion, required a higher surface concentration of copper ($\geq 50\%$ of the maximum) to achieve significant binding [43]. The ligate densities of IDA on 1000 Å and nonporous silica, using Cu^{2+} as the metal, were related to the binding capacities of lysozyme and concanavalin A [61]. For the nonporous support, an increase in binding was seen with that of ligate density, whereas maximum binding at 20–50% ligate density was observed for the porous matrix. The maximum was attributed to accessibility within the pores [61].

MOBILE PHASE

Mobile phase is a critical parameter in MIC due to the potential of mixed mechanisms including ion exchange, hydrophobic interaction, and metal interaction. The salt composition and concentration, pH, and additives all determine whether any given solute will bind. Modulating any or all of the parameters subsequently causes elution and/or separation.

Salt

Composition

Although varying the concentration of salt in the eluent usually modulates the retention of proteins in MIC, the nature of the salt also plays an important role. It was found that stationary phases with chelates of soft and intermediate metals bind protein more strongly in the presence than in the absence of antichaotropic salts [21]. In a study using EDDA-silica supports, the elution strength of sodium salts on an Fe^{3+} column was chloride < acetate < formate < phosphate for several proteins, whereas the strength on a Co^{2+} column was acetate < phosphate <

TABLE 4 y -Intercepts of Logarithmic Plots of Retention Factor vs. Molarity

Protein	y -intercept			
	Na chloride	Na formate	Na acetate	Na phosphate
Iron-free transferrin	-2.07	-2.55	-3.36	-4
Cytochrome <i>c</i>	-2.04	-2.44	-2.84	-2.78
Lysozyme	-1.94	-1.68	-1.41	-2.15
Lactoferrin	-1.06	-0.88	-0.89	-1.51
Selectivity (α)				
Cytochrome <i>c</i> /transferrin		8.85	6.75	
Lysozyme/cytochrome <i>c</i>		5.29	2.66	

Column: Fe^{3+} -EDDA. y -intercept study in 10 mM Na acetate, pH 5.5 with varying salt concentration. Selectivity in 0.12 M sodium acetate or formate, pH 5.5.

Source: Ref. 33.

chloride [33]. The effects on individual proteins, as shown in Table 4, were not equal, resulting in large changes in selectivity between salts. In another paper, acetate and phosphate were suggested to yield qualitatively stronger binding than EDTA and citrate as buffers [1]. In a comparative study of IDA bound to TSK G5000 polymer or Sepharose 6B, it was found that metal ions leaked from the former when Tris or glycine was used as the buffer; substitution of phosphate eliminated the problem [80]. When the hard metal ion Eu^{3+} was immobilized on TED, the best buffer for calmodulin was composed of phosphate and citrate [63].

Concentration

Salt concentration is a major factor in retention using MIC, but the relationship is complex because of the variety of mechanisms that may be involved. There may be an ion-exchange or ion-exclusion dimension caused by the negative functionalities of the chelating ligates. There may also be hydrophobic interaction caused by the ligates and/or spacer arms, especially when antichaotropic salts are used. Increasing salt concentration diminishes ionic interactions but may increase those caused by hydrophobicity. The combination of these mechanisms results in the relationship [31]:

$$\log k = A + B \log m + Cm$$

where m is the salt molality, B is the limiting slope at sufficiently low salt concentrations, and C is the limiting slope at sufficiently high salt concentrations. The B term relates to the ionic interactions and the C to hydrophobic interactions. When only ionic interactions are involved, linear curves such as those seen in Fig. 6 for proteins using formate and acetate on an Fe^{3+} column are observed [33].

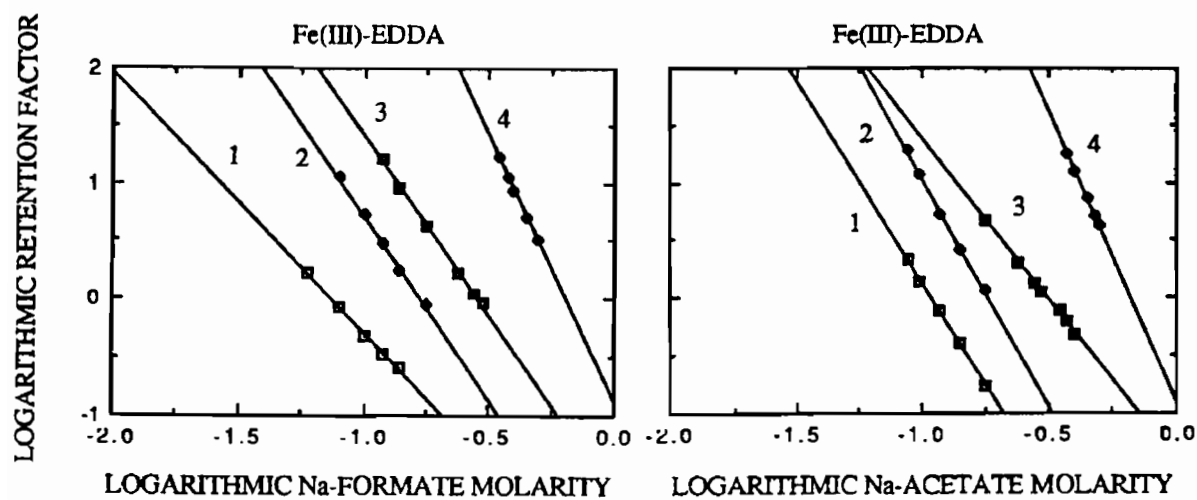


FIGURE 6 Plots of logarithmic retention factor of proteins versus logarithmic salt molarity. Column, 100×4.6 mm ID; flow rate, 1.0 mL/min; temperature, 25°C ; isocratic elution with 110 mM buffer, pH 5.5, at different salt concentrations. Proteins: 1, iron-free transferrin; 2, cytochrome c; 3, lysozyme, 4, lactoferrin.

When an antichaotropic salt such as ammonium sulfate is used, retention reaches a minimum before increasing when hydrophobic interaction takes place.

As a rule, salt concentration greater than or equal to 0.1 M reduces ionic interaction and that greater than about 0.5 M permits adsorption to the metal [24]. The high salt concentrations also suppress protein-protein interactions, which makes MIC a useful procedure for early-stage protein purification [1]. It has been shown for lysozyme on both Sepharose [44] and silica [81] MIC supports that at low ionic strengths (≤ 0.2 M), monolayer binding of a Langmuirean model occurs, whereas at high ionic strength (≥ 0.5 M) positive cooperativity between proteins and groups is present, resulting in multiple site binding indicative of a Freundlich model. Peptides were also seen to exhibit some changes in retention when sodium chloride concentration was increased from 0 to 2 M, but the effect varied with the individual peptide [47]. Neurotensin showed little variation whereas gastrin exhibited a major increase in retention.

The effect of antichaotropic salt concentration on protein retention by an Fe^{3+} -IDA column was investigated by isocratic elution at different concentrations of ammonium sulfate in 25 mM phosphate buffer at pH 6. With increasing salt concentration, retention first decreased and then increased; the retention minimum of proteins such as cytochrome *c*, lysozyme, and β -lactoglobulin was in the range 0.5–1.0 M ammonium sulfate in the eluent [31]. The magnitude of salt-mediated hydrophobic interactions, i.e., the magnitude of retention at high salt concentrations, was smaller on Fe^{3+} -IDA than on naked IDA [31]. This observation suggests that strong solvation with water molecules of the chelated metal renders the surface of the stationary phase highly hydrated, i.e., less hydrophobic in contact with aqueous eluents. As a result of this mixed retention behavior, the proteins could be eluted and separated either by increasing or by decreasing salt gradients. Not all proteins exhibited such behavior; the retention of ovalbumin, an acidic protein, always increased with the salt concentration in the domain investigated [31].

The effect of the concentration of sodium chloride on the retention of calmodulin on Cu^{2+} -IDA was examined with a decreasing pH gradient [23]. The increased concentration enhanced the retention of calmodulin, as shown in Fig. 7. Calmodulin, an acidic protein ($\text{pI} = 3.8\text{--}4.1$), was not retained by Cu^{2+} -IDA stationary phases when the ionic strength of the eluent was low but was retained upon the addition of sodium chloride to the eluent. These observations can be explained by ion exclusion or repulsion between the negatively charged protein and the stationary phase surface at low ionic strength. At high salt concentration, coulombic repulsion is screened and the retention of the protein occurs via metal interactions. This effect was maximized at about 1 M and increasing the salt concentration from 1 to 2 M decreased retention somewhat. The effect of increased salt differs for individual proteins, as shown in a study of glycoporphins

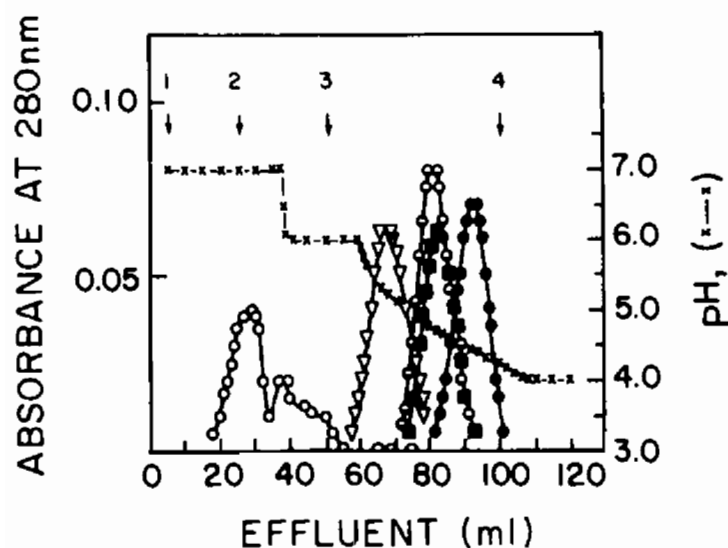


FIGURE 7 Montage of chromatograms of calmodulin on agarose-bound Cu(II)-IDA column under different elution conditions. Column chelating Sepharose 6B in Cu(II) form; decreasing pH gradient 0–4.0 in 20 mM phosphate buffer with no (○), 0.1 M (▽), 0.5 M (●), 1.0 M (●), and 2.0 M (□) NaCl; flow rate, 0.2 mL/min. (From Ref. 23.)

from human erythrocytes, for which the extent of the increase in retention on a Cu^{2+} -IDA column with salt concentration differed radically [54].

A general conclusion of these studies is that on both hard and soft metal ions, the retention of acidic proteins tends to increase with the salt concentration, whereas the retention of basic proteins first decreases and then increases with the salt concentration. Such effects are specific, however, for individual biopolymers, salts, and metal ion–chelating ligate functionalities.

pH

Protein binding to the stationary phase in MIC is strongly affected by the pH, offering a powerful means to modulate retention and to bring about separation. Because binding on soft metal ions is primarily via histidine residues, decreasing the pH below its pI of 6 decreases retention. As a rule, intermediate and soft metals such as Cu^{2+} , Zn^{2+} , Ni^{2+} , and Tl^{3+} exhibit a greater affinity for proteins when the pH of the eluent is higher, whereas hard metals such as Fe^{3+} , Ga^{3+} , Al^{3+} , and In^{3+} bind proteins more strongly at low pH than at high pH. Cytochrome *c*, lysozyme, and α -chymotrypsinogen A were found to be more retained at pH 6.0 than at pH 5.0 on a Zn^{2+} -IDA column, whereas on Fe^{3+} -IDA the reverse behavior was observed with the three proteins plus ovalbumin, as illustrated in Fig. 4 [31]. When Kato et al. [32] investigated the effect of pH on the separation of transferrin and carbonic anhydrase by Zn^{2+} -IDA, they found that the proteins were more strongly retained at pH 7 than at pH 9.

A decreasing pH gradient using phosphate from pH 7 to 3.8 on a Cu^{2+} -IDA TSK column resulted in excellent resolution of peptides [47,75], as illustrated in Fig. 8. In another study, a decreasing pH gradient resulted in excessive leakage of Zn^{2+} and Ni^{2+} on a similar matrix [80]. A pH gradient using a mixed buffer of acetic acid, MES, and HEPES proved to be highly reproducible and yielded excellent peak shapes for proteins [12], as was seen in Fig. 1.

For hard metal ions, pH appears to change the charge of the complex [68]. When this results in a negative charge, binding may be due to lysine or arginine residues rather than anionic amino acids. Human serum proteins were strongly adsorbed on Al^{3+} , Ga^{3+} , Tl^{3+} , or In^{3+} chelates with TED-agarose at pH 5.5 when the ionic strength of the eluent was low, but they were readily eluted upon increasing the salt concentration in the eluent [14]. In contrast, at pH 7.6, the serum proteins were retained only by Tl^{3+} -TED. At pH 5.5, Fe^{3+} -TED was found to bind serum proteins more strongly than Ni^{2+} -TED, whereas at pH 8.1 the opposite behavior was observed [14].

The effect of pH on binding in MIC is related to the solute, the metal ion, and the chelating ligate. In a study of EDDA-metal columns, pH had a major

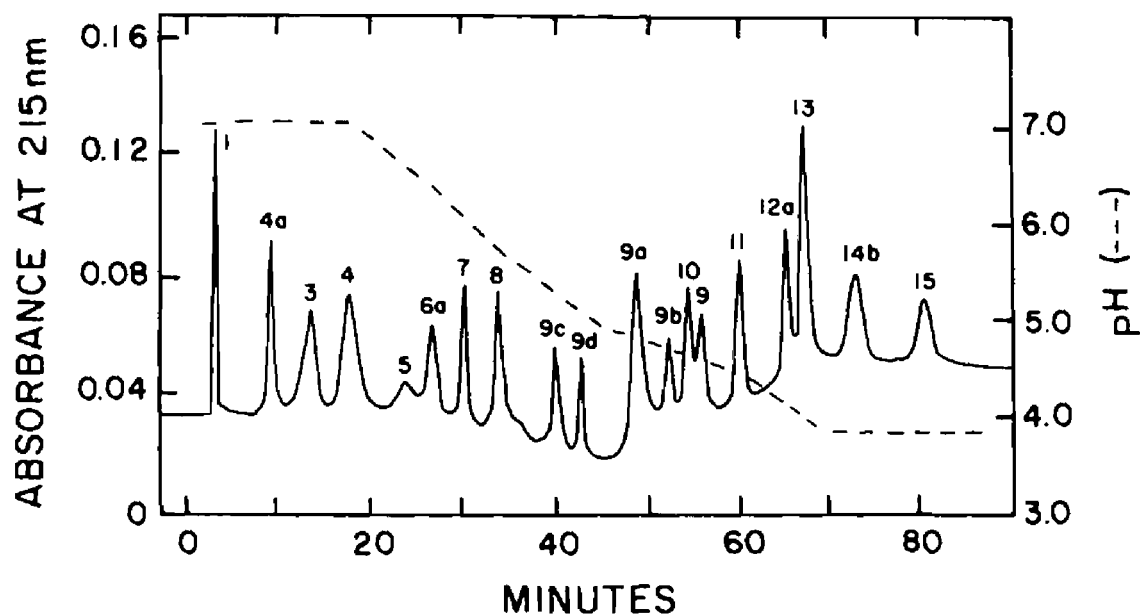


FIGURE 8 Peptide separation by MIC on Cu(II)-IDA . Column, TSK gel chelate 5-PW, 75×7.5 mm; gradient elution from 0.02 M phosphate (0.5 M NaCl), pH 7.0 to 0.1 M phosphate (0.5 M NaCl), pH 3.8; flow rate, 1 mL/min. Peptides: 1, neurotensin; 3, oxytocin; 4, $[\text{Leu}^5]$ enkephalin; 4a, sulphated $[\text{Leu}^5]$ enkephalin; 5, mastoparan; 6a, Tyr-bradykinin; 7, substance P; 8, somatostatin; 9, human calcitonin; 9a, $[\text{Asn}^{1,2}]$ human calcitonin; 9b, human calcitonin (17–32); 9c, $[\text{Asn}^{1,7}]$ eel calcitonin; 9d, eel calcitonin (11–32); 10, bombesin; 11, angiotensin II; 12a $[\text{Trp}(\text{for})^{25,36}]$ human GIP (21–42); 13, LH-RH; 14b, human PTH (13–34); 15, angiotensin I. (From Ref. 75.)

effect on retention for holo transferrin and iron-free transferrin [33]. Retention drastically decreased at pH levels over 5.5 on Co^{2+} and Zn^{2+} columns and over pH 6 on Fe^{3+} . On the Zn^{2+} column, cytochrome *c* experienced a decrease in retention as pH was increased over pH 5, but retention increased again when the pH was over 6.

Additives

Competitive Displacers

Another means to modify the retention of proteins in MIC is the inclusion of a competing complexing agent such as imidazole [24,82], histidine [82], histamine [83], glycine [31,32,83], ammonia [12], glutamate [65], or phosphate [65] in the mobile phase. Protein retention decreases with increasing concentration of the additive because of competition for the metal chelate binding sites. Imidazole is more effective at displacing proteins from Co^{2+} , Ni^{2+} , Cu^{2+} , or Zn^{2+} MIC columns than glycine, yet less prone to scavenge metal ions from the supports [24]. It can be used in step or gradient elution protocols. Imidazole gradients were effective for peptide analysis on Cu^{2+} , Ni^{2+} , and Zn^{2+} TSK Gel Chelate 5PW columns [47]; however, most peptides had lower retention than when pH gradients were used. On hard metal ions, imidazole had no effect on the elution of calmodulin [63], whereas citrate, malonate, and phosphate worked well as displacers. Histidine is also a good displacing agent, as would be expected by the histidine binding mechanism. When different histidine concentrations up to 15 mM in the mobile phase were investigated concerning their effects on the retention of certain proteins on an Fe^{3+} -IDA column isocratically eluted with 25 mM phosphate buffer (pH 6.0) containing 0.15 M ammonium sulfate, the logarithmic retention factors were found to depend linearly on the histidine concentration [31]. With eluents lean in histidine (<3 mM), lysozyme and α -chymotrypsinogen A were retained less strongly than cytochrome *c* and β -lactoglobulin A, respectively, whereas the retention order was reversed with eluents rich in histidine (>5 mM) [31].

In another chromatographic system, phosphoserine was used in the mobile phase to elute phosphorylated histones from an Fe^{3+} -IDA column [56]. Glutamate and phosphate were more effective than malonate in removing tuna heart cytochrome *c* from hard metal ions chelated by 8-HQ [65]. This protein, which does not have surface histidines, binds via glutamic acid and aspartic acid residues. Citrate, malonate, and phosphate were all effective at removing calmodulin from hard metal ions immobilized on TED [63]. The addition of a competing agent to the mobile phase can greatly alter the selectivity in MIC and give rise to selectivity reversal, i.e., change in elution order, particularly when gradient elution is employed. In many cases, relatively high concentrations of a

competing agent may be required to elute a protein from a given column. The retention of proinsulin variants was individually effected when ammonia, glycine, propylenediamine, and ethylenediamine were used as displacing agents on Ni^{2+} -IDA [35]. MIC columns may be adversely affected by processes using competitive agents in the mobile phase that may leach out the metal from the column, as reported for the use of amino acid additives in the eluent with Ni^{2+} -IDA [77] and Zn^{2+} -IDA [32] columns. In such cases, it is necessary to reload the column with the metal after each gradient run.

Metal Ions

Metal ions can also be used to modulate the retention of proteins in MIC. When included in the mobile phase, they reduce retention by competing with the chelated metal on the stationary phase for the metal binding sites on the protein molecule. In one example, calcium acetate in the eluent at low concentrations was employed as a competing agent to elute strongly binding proteins from Cu^{2+} -IDA [51]. Eluents containing magnesium chloride have also been used to debind phosphorylated histones from an Fe^{3+} -IDA column [56]. Further, traces of copper ions were added to the mobile phase to attenuate protein retention on a Cu^{2+} -IDA column [12]. Inclusion of Ca^{2+} in the mobile phase resulted in the retention of calmodulin, a calcium-binding protein, on a series of hard metal-TED columns [63].

Organic Solvents

The inclusion of organic solvents in the mobile phase can alter the retention behavior of proteins in MIC. Figure 9 illustrates how the retention of several proteins on an Fe^{3+} -IDA column changed upon addition of methanol to the mobile phase [31]. Because the effects differed substantially among the proteins, selectivity could be drastically changed. On a Cu^{2+} -IDA column, the retention of glycoporphins was found to increase with the methanol concentration of the eluent [54]. Acetonitrile was effective at reducing the retention of amino acids on MIC supports with protoporphyrin IX as the chelating ligate [45]. The use of a suitable organic solvent in the eluent at low concentration, where the integrity of the protein molecule is not affected, can therefore be a powerful means to modulate retention in MIC.

Detergents

Certain other additives to the mobile phase have been tested in MIC. It was found that the inclusion of urea or detergents such as sodium dodecyl sulfate and Tween 80 in the mobile phase considerably decreased the adsorption of proteins on Ni^{2+} -TED agarose [16]. In the separation of glycoporphins, 0.05% SDS was required in the mobile phase to achieve elution [54]. Likewise, 7 M urea was required for the analysis of proinsulin variants on a Ni^{2+} -IDA MIC column [35].

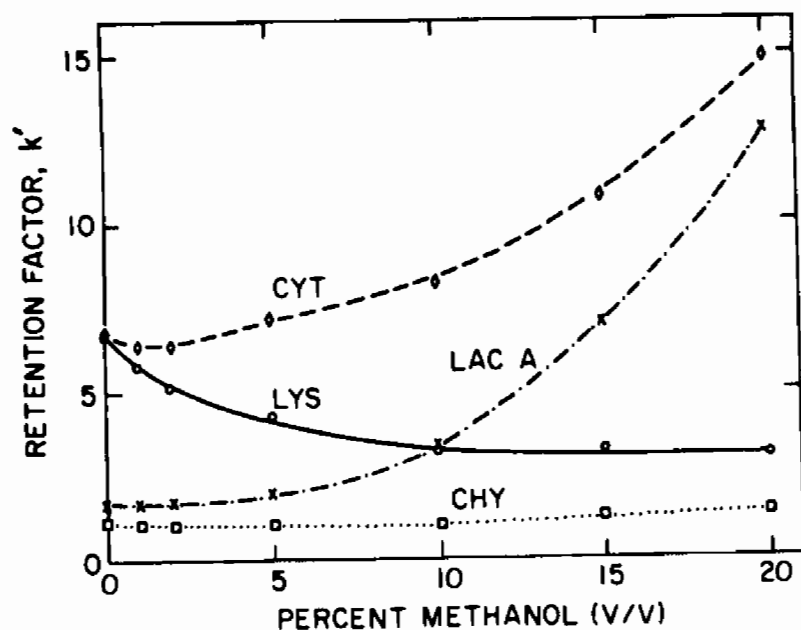


FIGURE 9 Effect of methanol concentration of the eluent on the retention of proteins on silica-bound Fe(III)-IDA. Eluent, 25 mM phosphate buffer; pH 6.0, containing 0.15M ammonium sulfate and methanol at different concentrations. Symbols are as in Fig. 4. (From Ref. 31.)

OPERATIONAL ASPECTS

The principal steps in MIC procedures consist of:

1. Loading the metal ion
2. Washing off loosely bound ions
3. Applying the sample
4. Eluting with a step and/or linear gradient
5. If necessary, stripping with EDTA and regenerating

The chelating stationary phase can be loaded with the metal by either frontal chromatography or perfusion with a solution of the metal in a recirculating system until equilibrium is reached. A third method entails the injection of a slug of a relatively concentrated solution of the metal salt into the column using an appropriate sample loop [32]. The surface concentration of the chelated metal can be limited by selection of an appropriately low metal concentration or inclusion of a competing agent such as an amino acid in the loading solution [50]. It is essential that any excess unchelated metal be removed from MIC columns prior to use to avoid contamination of proteins during the ensuing chromatographic separation. Generally, metal ions that are not tightly bound can be removed with a wash of the starting buffer containing imidazole or glycine at a concentration dependent on the chelating ligate and metal ion or extensively washing the column with elution buffer [24].

The metal chelated on the stationary phase can be exchanged for another metal by flushing the column with an EDTA solution or another complexing agent to strip off the first metal. The resultant naked chelating stationary phase can then be loaded with the second metal by one of the methods described previously.

Proteins are fractionated on MIC columns by changes in pH, salt concentration, or competing agents. These can be accomplished separately or in combination using step or linear gradients. For borderline metals such as Cu^{2+} or Zn^{2+} on IDA ligates, the sample is usually loaded onto the column at sufficiently high pH and the individual components eluted by solutions of appropriate salt concentration and pH [12–14,47,84–91]. A decreasing pH gradient for protein fractionation is employed preferentially with stationary phases having soft metal–IDA chelates [77,78,84,86,92]. In these cases, elution occurs when protonation of side-chain amino groups of the protein, particularly those of histidine, weakens their interaction with the chelated metal on the stationary phase. Hard metal ions or alternative ligates may have different pH requirements [63,68]. Increasing salt [93] or pH [55] gradients are used for protein fractionation on columns containing hard metal chelates, e.g., Fe^{3+} –hydroxamic acid. Gradient elution with increasing concentration of a suitable competing agent such as imidazole [24,47,82], histidine [94,95], histamine [83], glycine [16,35,83], citrate [63], or ammonia [16,35] may replace the pH or salt gradient or be used subsequently for the elution of strongly binding proteins by MIC.

When necessary, the last step may be the removal of the most strongly binding proteins and the metal from the column by EDTA solution [13,87,88]. Certain proteins, such as the lectin from *Dolichos biflorus* on Ca^{2+} -IDA, bind so strongly to the chelated metal that they require elution with EDTA solution [96]. If contamination of the protein with metal ions removed by the competing agent from the stationary phase (metal ion transfer) is a problem, it can be avoided by loading only the first two thirds of the column with the metal by frontal chromatography and leaving the remainder free to bind the released metal ions [83]. Alternatively, trace amounts (2×10^{-6} M) of the same metal chelated on the stationary phase can be added to the eluent to prevent metal bleeding caused by a strong eluent such as ammonium acetate [12], although this is not the preferable technique [80].

APPLICATIONS

Porath et al. [13,24] have developed sophisticated schemes for the fractionation of proteins with a series of columns, each loaded with a different metal. In one example, a sample of human serum proteins was passed through tandem Zn^{2+} -IDA and Cu^{2+} -IDA columns in a mobile phase of sufficiently high pH [13]. Thereafter, the bound proteins were eluted from each column separately by stepwise elution with appropriate buffers. Albumin, haptoglobins, β -lipoproteins,

and prealbumin were retained by and subsequently separated on the Zn^{2+} -IDA column, whereas α_1 -antitrypsin, acid glycoproteins, ceruloplasmin, α_2 -macroglobulin, and hemoglobin were separated on the Cu^{2+} -IDA column. In another study, four TED columns [14], each loaded with a different metal, were used in series for the separation of human serum proteins. Most of the proteins were adsorbed on the first Al^{3+} -TED column and only a few reached the following columns, which were loaded with Ga^{3+} , In^{3+} , and Tl^{3+} .

MIC has been widely used for protein purification using both conventional and HPLC columns [26,27,97]. Copper and zinc are the most commonly used metals for protein separation and IDA is the most frequently employed ligate. MIC has been used extensively for the isolation, purification, and characterization of interferons using Zn^{2+} -, Ca^{2+} -, Co^{2+} -, or Ni^{2+} -IDA stationary phases [48,77,78,85,98–100]. It was found that human fibroblast interferon binds strongly to Zn^{2+} -IDA, unlike human leukocyte interferon, which was not retained on such a column [78]. Syrian hamster interferon did not bind to Zn^{2+} -IDA but was retained on Cu^{2+} -IDA [77]. The latter column also strongly bound human fibroblast and human leukocyte interferons [85]. Figure 10 illustrates the separation of mouse L-cell interferons on a Cu^{2+} -IDA agarose column [48].

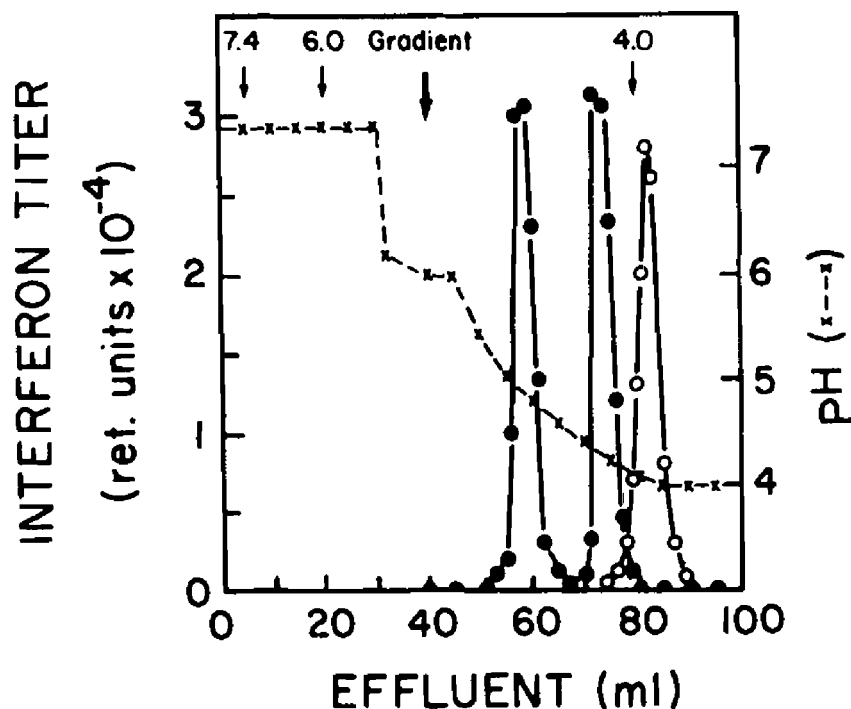


FIGURE 10 Chromatography of mouse interferons on agarose-bound Cu(II) -IDA column. Sample introduction was followed by stepwise elution with 0.02 M sodium phosphate (1.0 M NaCl), pH 7.4, and thereafter with 0.1 M sodium acetate (1.0 M NaCl), pH 6.0. The chromatogram was obtained by gradient elution with the same acetate solution at decreasing pH. (●) Glycosylated; (○) nonglycosylated. (From Ref. 48.)

Nakagawa et al. [75] employed MIC for the separation of peptides on HPLC columns with Cu^{2+} , Ni^{2+} , or Zn^{2+} chelated by an IDA stationary phase using either gradient elution with increasing imidazole concentration or decreasing pH. Best results were obtained with the Cu^{2+} -IDA column and either elution scheme, as previously shown for the separation of a peptide mixture with decreasing pH gradient in Fig. 8 [75]. In a further study by this group, 67 synthetic biologically active peptides were examined for their adsorptive properties on Cu^{2+} -, Ni^{2+} -, and Zn^{2+} -IDA on an HPLC support [47]. They were also assessed on the metal-free matrix to distinguish interactions caused by mechanisms other than MIC. The efficacy of pH versus imidazole gradients was assessed and prediction of retention on the basis of amino acid composition was described [47].

Another application of MIC is the purification of monoclonal antibodies from mouse ascites fluid by HPLC on an IDA column charged with Zn^{2+} , as illustrated in Fig. 11 [80]. Calmodulin, a calcium-binding protein, could be effectively purified on a Eu^{3+} -TED column only with a mobile phase containing

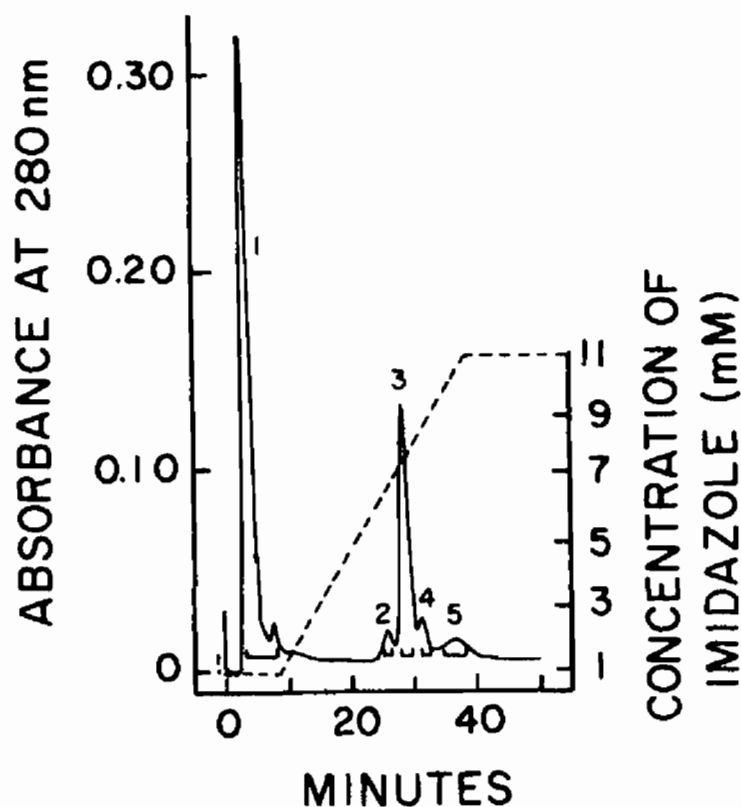


FIGURE 11 Purification of a monoclonal antibody on Zn(II) -IDA. Column, TSK gel chelate 5-PW in the Zn(II) form 75×7.5 mm; starting eluent, 20 mM phosphate buffer (0.5 M NaCl, 1 mM imidazole), pH 7.0; gradient former, the same, but with 20 mM imidazole; flow rate, 1 mL/min; 20°C ; sample, mouse ascites fluid raised against canine parvovirus, peak, 3 is IgG. (From Ref. 80.)

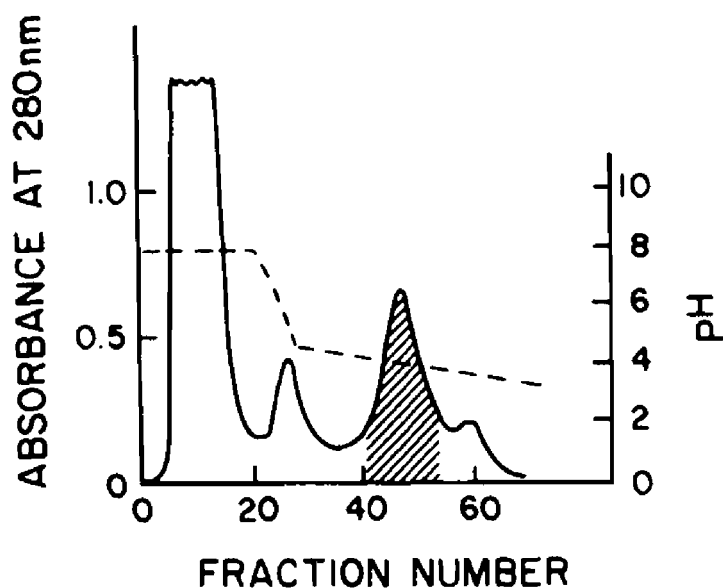


FIGURE 12 Chromatography of human milk proteins. Column, Cu(II)-IDA agarose, 5.5×3.2 cm; decreasing pH gradient from 8.2 to 2.8 in Tris/acetate containing 0.5 M NaCl. The shaded peak contains the lactoferrin. (From Ref. 84.)

each of the components sodium chloride, calcium, and citrate [63]. Human milk lactoferrin, an iron-binding protein that inhibits bacterial growth, has been purified on Cu^{2+} -IDA agarose [84], as shown in Fig. 12.

MIC has found great importance in the purification of genetically engineered proteins obtained by recombinant DNA technology [26,35–41]. The attachment in the biosynthetic process of specific metal-chelating peptides to the N-terminus of proteins allows their subsequent isolation by MIC. The strong specific binding of the peptide moiety to an appropriate MIC column facilitates the isolation and purification of the chimeric protein. Amino acid sequences such as His-Tyr- NH_2 , His-Gly-His, and His-Trp have been identified as having strong binding to Co^{2+} -, Ni^{2+} -, and Cu^{2+} -IDA stationary phases [25,35,40].

When proteins are adsorbed via metal interaction to a suitable stationary phase, their molecular integrity is not affected drastically, allowing their recovery without loss of their biological activity. Superoxide dismutase, a metalloenzyme containing copper and zinc, fully retained its biological activity when purified on Cu^{2+} -IDA agarose without loss of its metal and biological activity [17]. The purification of carboxypeptidase G was carried out on Zn^{2+} -IDA-silica with good yield and retention of biological activity [28]. Recovery of proteins using a Zn^{2+} -IDA stationary phase on macroreticular resin was higher than 85% and the enzymatic activity of lipoxidase was 90% [32]. General protein recoveries from TSK Chelate have been 86–100% [32].

In addition to chromatographic applications, metal chelates covalently attached to a suitable support have been employed for immobilization of

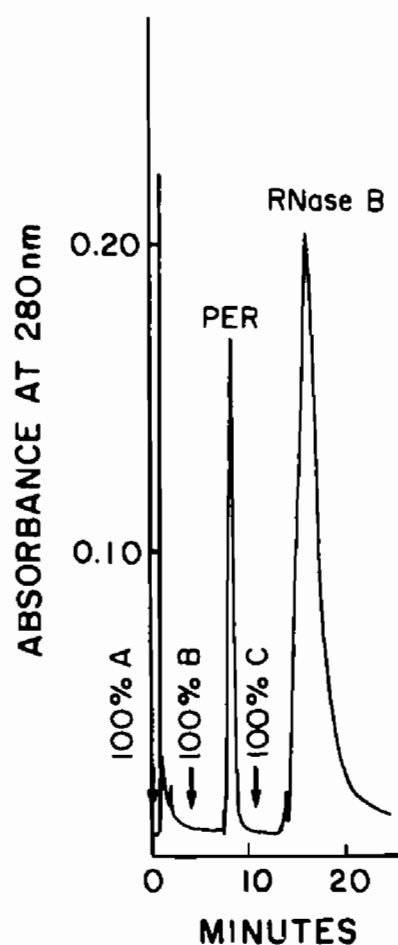


FIGURE 13 Separation of peroxidase (PER) and ribonuclease B (RNase B) on an HPLC column containing concanavalin A immobilized by metal interactions. Column, Con A/Cu(II)-IDA containing 84 mg of Con A; 100 × 4.6 mm; stepwise elution with A = 0.2 M NaCl in 25 mM phosphate buffer, pH 6.0; B = 0.1 M methyl- α -D-glucopyranoside in A; C = 0.3 M sodium borate in A; flow rate, 1 mL/min; temperature, 25°C. (From Ref. 50.)

enzymes. Porath and coworkers [101] adsorbed alkaline phosphatase, maltose dehydrogenase, and lactate dehydrogenase on cobalt, zinc, or copper chelates of IDA-agarose. Concanavalin A was successfully immobilized on Cu^{2+} -IDA on 300 Å silica and used to purify peroxidase and ribonuclease B, as shown in Fig. 13 [50]. Enzymes immobilized by this technique retained their catalytic activity without leaching out under the conditions used for the enzymatic reaction; subsequently they could be easily desorbed using EDTA without loss of activity.

MULTIMODAL CHROMATOGRAPHY

Certain ion-exchange columns can be used for protein separations by either ion-exchange chromatography (IEC) at low salt concentrations in the eluent or

hydrophobic interaction chromatography (HIC) at high salt concentrations [102]. When the use of a given column can be extended to other modes of retention, the term "multimodal" is used. Multimodal employment of an IDA-silica column illustrates the versatility and flexibility of this approach. Without the metal, the IDA column can be used for the separation of proteins as a rather "strong" cation-exchange support at low ionic strength or as a hydrophobic interaction support at high antichaotropic salt concentrations in the eluent [12,31]. With chelated metal, the same IDA column separates proteins by MIC or at sufficiently high salt concentrations in the eluent with an HIC mechanism [12]. Because the chelated metal on a given MIC column can be readily exchanged, the selectivity of the chromatographic system can be conveniently altered further [31,51]. When a Cu^{2+} -IDA stationary phase was used to immobilize concanavalin A, it was suitable for affinity chromatography of glycoproteins and glycoconjugates [50], as shown in Fig. 13. The flexibility of IDA or other MIC stationary phases means that they can be used in different ways to solve a variety of separation problems. The advantages of using the same column in multiple modes rest primarily with the possibility of altering its selectivity to suit the need in solving a given separation problem. In this fashion, the number of columns needed to carry out protein separation can be reduced.

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Preparative Chromatography

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PHYSICOCHEMICAL PROPERTIES OF BIOMOLECULES

Proteins and Peptides

Proteins have been defined by Hoppe Seyler as egg white bodies composed of amino acids, and in the first half of this century, all proteinogenic amino acids were identified. Later, the nature of the chemical bond between the amino and acid groups was discovered. Currently, the research activities of protein chemists are in the areas of side-chain modification and structure-function relationship. The amino acid backbone of a protein determines its unique properties, size, shape, solubility, and function. The properties may be influenced by the side-chain modification, which may result in improvement of solubility by prevention of aggregation or enhancement of the thermal stability of the protein. Side-chain modification also plays a pivotal role for the biological activity; e.g., the highly glycosylated peptide hormone erythropoietin exhibits no activity in the nonglycosylated form [1]. It is essential to overexpress and purify proteins in their authentic structure in order to prevent loss of biological activity. Only for sequencing purposes, for peptide mapping, and for determination of the immunointegrity of proteins can proteins be purified under denaturing conditions [2].

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In all other cases, when the biological activity has to be preserved, the environmental conditions for purification are very limited, usually requiring room temperature or below, salt concentrations in the range of an osmotic pressure around 400 Mosmol, and a pH close to 7.0. In cases where these conditions cannot be maintained, the protein should be kept in its unfriendly environment as briefly as possible. Usually small peptides are much more stable than proteins, and they can be dissolved in organic solvents and withstand harsh conditions.

Protein solutions are amphoteric colloidal solutions; they are different from real solutions. Colloidal solutions show the phenomenon of opalescence and they are viscous, but the osmotic pressure is very low. The actual solubility of a protein is a unique property of the substance and varies with the side-chain modifications. Computer algorithms are available to calculate the solubility, but the sequence is not the sole feature in determining the solubility, and the programs are limited to the prediction of hydrophobic and hydrophilic patches at the surface of the protein molecule [3]. Very often hydrophobic residues are packed into the core of a protein. Therefore, for reversed-phase chromatography (RPC), it is necessary to open the protein coil by denaturing agents [4,5]. These properties allow the separation of unfolded, partially folded, and completely folded proteins by RPC [6] and other methods using the hydrophobic patches on a protein as a recognition site for the stationary phase. This separation method has become a very important tool in the biotechnology industry because many proteins are overexpressed and deposited as inclusion bodies [7] and they can be solubilized only under denaturing conditions [8]. Denatured proteins must later be refolded to attain their native conformation. This process is usually not efficient and the misfolds have to be removed (Fig. 1).

The isoelectric point, another very characteristic parameter of a protein, is also very crucial for chromatography, especially ion-exchange chromatography, and for preservation of the biological activity. Proteins have the lowest solubility at a pH close to the isoelectric point. The conformation of a protein is often stabilized by disulfide bridges, metal ions, and prosthetic groups. Separation conditions have to be found where the stability is not affected. Chelating agents, chaotropic salts, and reducing conditions may destroy the conformation. All these boundary conditions, which have to be observed to develop an optimal purification system, make protein purification an art. At this time, theoretical engineering considerations have not successfully solved this problem.

Polynucleotides

Large-scale purification of polynucleotides was not required previously. Molecular biology experiments are usually done on the microgram and submicrogram scale. Purification of plasmids in the range of 100 mg may satisfy the worldwide

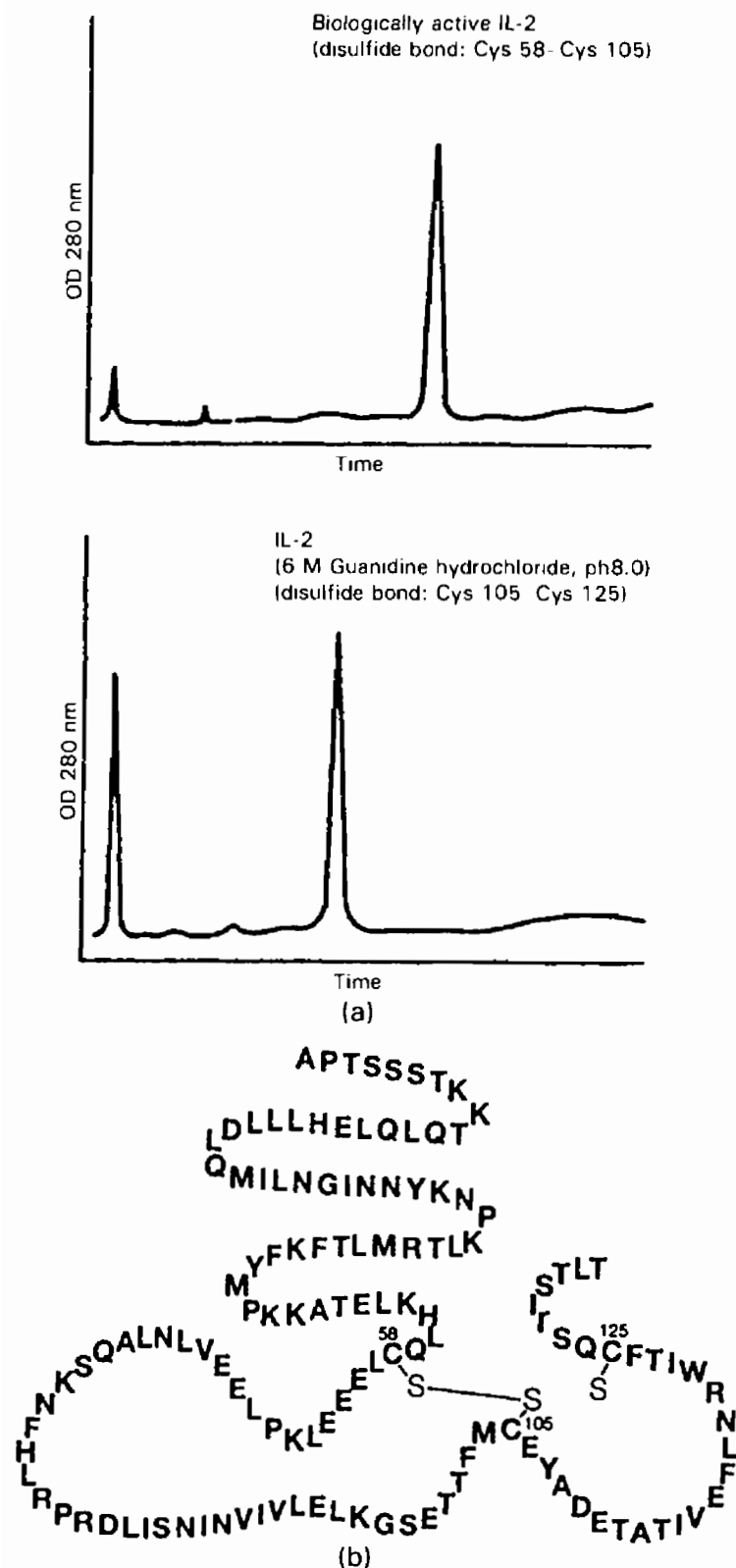


FIGURE 1 (a) Chromatographic behavior of the correctly folded (Cys 58-Cys 105) and misfolded (Cys 105-Cys 125) cytokine interleukin-2 on a reversed-phase column (Brownlee Aquapore 20 μ m, 4.6 $\times$ 30 mm, 0.1% aqueous TFA and acetonitrile). (b) Amino acid sequence of interleukin-2. (Data courtesy of R. Plot and E. Wasserbauer, Novartis Research Institute, Vienna, Austria.)

demand for a certain plasmid for gene therapy or genetic vaccination. The rapid development of gene therapy [9,10] and genetic vaccination [11] has required moderate amounts of purified DNA and viruses.

Polynucleotides, either deoxyribonucleic acids or ribonucleic acids, are determined by the sequence of four different nucleotides, AGTC for DNA and AGUC for RNA. The conformational change of a circular piece of DNA is less critical for maintenance of biological activity compared with proteins. This category of biopolymers can be purified under nondenaturing conditions such as organic solvents, high salt, high temperature, and harsh pH. Conditions leading to nicks in the strands must be avoided because nicks are required for separation into the two different strands. For certain molecular cloning experiments, separation of the two DNA strands is necessary. Nicks are rapidly produced by endonucleases. Chemical conditions leading to nicks in strands are usually not used for purification of DNA or RNA. In one aspect similar to proteins, polynucleotides are colloidal solutions. They are negatively charged due to their content of phosphodiester groups, which is one major difference from proteins, which can be highly negatively or highly positively charged. Nuclear DNA is usually accompanied by histones, proteins that are positively charged. These protein-DNA complexes form the nucleosome complex, which has been shown to demonstrate the complexity of the structure of nuclear DNA (Fig. 2).

In gene therapy, the injected DNA must replicate in the target cells to develop its biological function; therefore, it is mandatory to remove DNA with

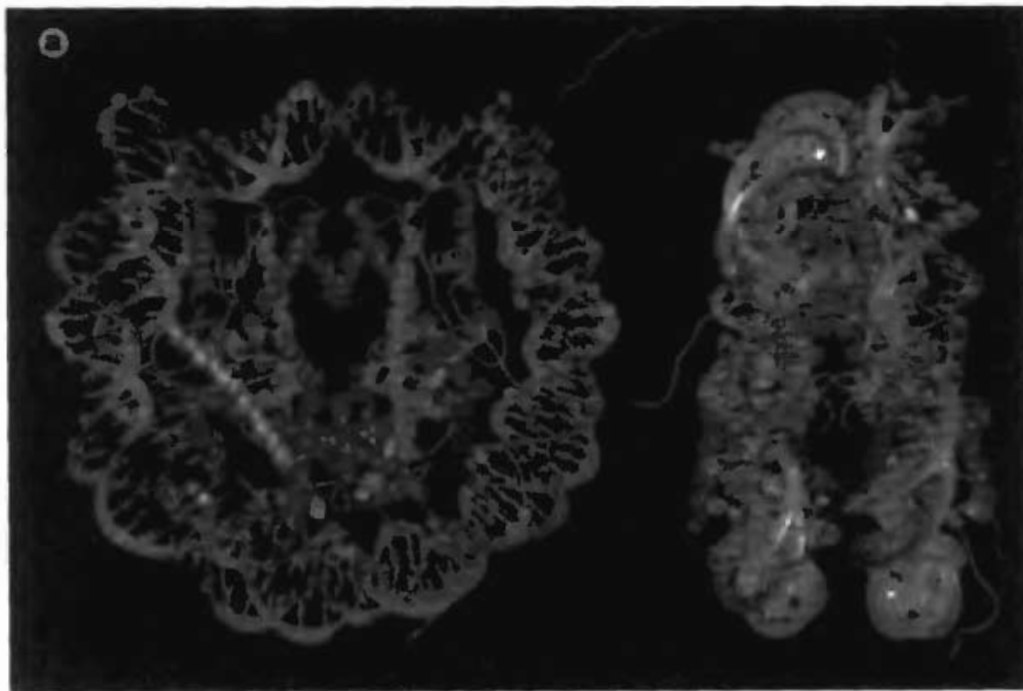


FIGURE 2 Structure of the nucleosome core as proposed by Luger et al. [131].

incorrect sequences. In contrast to application of a protein therapeutic, such a contaminant may be amplified in the patient. This problem drives the high demands on the purification strategy for nucleic acids.

Polysaccharides

Polysaccharides are completely different from proteins and polynucleotides, although they are also polymers composed of repetitive subunits, the monosaccharides. Chromatographic procedures are usually not used for the purification of polysaccharides, whose major use is in the area of nutrition. Chromatography is considered an operation that is too expensive for this purpose. This is even more true in the paper and pulp industry. Polysaccharides serve as valuable raw materials for the preparation of chromatography media (agarose, cellulose, and chitosan). Polysaccharides such as agarose and cellulose are water soluble, usually not charged, and noninteractive with proteins. These properties made them successful candidates for chromatography material.

SOURCES OF BIOMOLECULES

Biomolecules can be used for therapeutic purposes, fine chemicals, food additives, or catalysts in organic synthesis. The biomolecule can be either the end product or an aid in the production process, such as proteinaceous ligands for affinity chromatography. Future applications for biopolymers may be in the area of electronics, substituting for conventional semiconductors [12], or in the area of novel material design [13]. In the past, the major sources of biomolecules were body fluids, tissues of animal and plant origins, and microorganisms. Presently, proteins for therapeutic purposes and use as fine chemicals are mainly produced by recombinant DNA technology.

Maintenance of Biological Activity

Irrespective of whether a biomolecule is extracted from a natural source or from a recombinant organism, the main focus during the production process is the maintenance of biological activity. This goal determines the whole purification strategy. There are some general rules, but it should be noted that the protective environment for a biomolecule can vary in a wide range. To maintain the biological activity of an enzyme, receptor, hormone, metabolite, or second messenger, five rules should be obeyed: (1) reduction of temperature, (2) addition of stabilizers, (3) inhibition of enzymatic degradation processes, (4) maintenance of natural redox potential and pH value, and (5) prevention of adsorption to surfaces.

Temperature generally slows down the biological and chemical degradation processes. In the laboratory, the initial recovery steps are often carried out on ice

or dry ice. Sometimes the starting material is even kept under liquid nitrogen before it is subjected to purification. Almost all protein purification processes are carried out either at room temperature or at 4°C. Cohn fractionation, which is widely used for blood plasma, is carried out below the freezing point [14]. Although preservation of biological activity may require low temperatures, chromatography performance is improved with increased temperature. An optimum balance between speed and degradation must be sought [15]. Temperature has a significant influence on the performance of expanded bed adsorption. Bed height and related separation power are highly correlated with temperature [16].

Stabilizers are often used for preservation of biological activity. Glycerol and other polyols are very common. The addition of substrate and ligands to stabilize enzymes and receptors is practical only on a small scale; therefore, on a larger scale these additives have to be replaced. Detergents are also very popular. Some detergents, such as Triton X-100, have a high absorption at 280 nm. Their use may be banned in the future because degradation products in wastewater plants have shown hormonal activity [17]. They must also be avoided because the ultraviolet (UV) adsorption of the buffer will strongly interfere with the absorption of the proteins at 280 nm.

Inhibition of enzymatic and chemical degradation processes is the most efficient way to protect the biomolecule during separation. Depending on the nature of the biomolecule, proteolytic enzymes, glycolytic enzymes, DNases, and RNases may have to be inhibited. Table 1 lists an example of a cocktail that is

TABLE 1 Frequently Used Protease Inhibitors in Protein Purification at Small Scale

Protease inhibitor	Specificity of inhibitor	Working concentration
PMSF (Phenylmethyl sulfonyl fluoride)	Serine protease	0.1–1 mM
EDTA, EGTA	Metalloprotease	0.1–1 mM
Benzamidin	Serine protease	1 mM
Pepstatin A	Aspartic protease	1 µg/mL
Leupeptin	Thiolprotease	1 µg/mL
Aprotinin	Serine protease	5 µg/mL
Antipain	Thiolprotease	1 µg/mL
TPCK (<i>N</i> -tosyl-L-phenylamine-chloromethyl ketone)	Chymotrypsin, serine and cysteine proteases	70–100 µg/mL
TLCK (<i>N</i> α- <i>p</i> -tosyl-L-lysine chloromethyl ketone)	Trypsin, serine, and cysteine proteases	37–50 µg/mL

used on a small scale for inhibition of proteases. Many of these additives are extremely expensive and must be partially replaced at large-scale operation. The inhibitors are also very toxic and must be cautiously applied when biopolymers for therapeutic purposes or food additives are produced.

Sometimes the intermediate products are less stable than the starting material. For example, a natural inhibitor for a certain degradation process can be removed during a process. For large-scale purifications, a careful examination of the hold step is necessary.

Maintenance of the redox potential and pH of the environment can be very tricky. The chromatography conditions to get the appropriate separation sometimes require buffers with extreme pH values, e.g., glycine-HCl buffer at pH 2.7 for elution of antibodies from protein G affinity chromatography [18,19]. Also, when products are extracted from cells, the redox potential changes dramatically. It is well known that protein solutions can be denatured by excessive shaking or stirring due to dissolved oxygen. Redox buffer systems are often required. The most popular reducing agents are β -mercaptoethanol, oxidized glutathione γ (-Glu-Cys-Gly), reduced glutathione, dithioerythritol (DTE, dihydroxybutane 1,4-dithiol), DL-dithiothreitol (DTT), and thioglycerol α (-monothioglycerol, 3-mercapto-1,2-propanediol). Heavy metal ions may catalyze oxidation processes, necessitating the addition of chelating agents and use of glass vessels or enamelled steel vessels to prevent the release of heavy metal ions into the product solution. Prevention of adsorption to surfaces is very important when diluted material is processed. Some biomolecules show high affinity for glass and polished stainless steel [20]. To avoid adsorption, either high-quality plastics have to be used or the glass surfaces have to be coated with silicon. "Siliconizing" of glass surfaces is a very simple process and should be employed in all critical cases.

Preparation of a Tissue Homogenate

For each separation purpose—diagnostic, research, large-scale production, forensic chemistry, etc.—the disintegration of a tissue has to be carried out in a different way. When only limited material is available, especially for diagnostic purposes, a small sample of tissue is homogenized and preparative chromatography is carried out. In all cases, it is essential that the material have a postmortem status and the ATP pool be significantly reduced. The latter could lead to significant contributions to the biochemical decomposition of the tissue.

Homogenization of a tissue sample includes the following steps:

1. Perfusion of the organ with a salt buffer.
2. Removal of fat and connective tissue.
3. Mechanical mincing using very sharp tools to avoid squeezing and release of enzymes. Frozen tissue should be broken and ground, if

possible. Enzymatic disintegration by addition of enzymes such as elastase and trypsin accelerates the whole process and preserves the biological activity.

4. Disintegration is carried out with a homogenizer (Potter or Dounce), a mixer (Waring blender), or pulverization with quartz sand in a mortar. Explosion decompression has also been applied.

The disintegrated tissue has to be used immediately. Preserving agents can be added either before disintegration or afterward. Fat is removed by addition of silicon dioxide and finally the homogenate is clarified by centrifugation or filtration.

Disintegration of Single Cells

Bacteria and yeast cells can be disintegrated by three different methods, which are frequently combined. These methods are chemical, mechanical, and physical disintegration. Addition of organic solvents such as toluol, acids, caustic agents, or detergents destroys the cell wall. Grinding with glass beads in swing mills or ball mills is very efficient for yeast. Bacteria are often homogenized by high-pressure homogenization, extrusion, ultrasound, and explosion decompression [21,22]. Mechanical methods produce a local velocity gradient, which leads to cavitation. During cavitation, a jet stream knocks a hole into the cell. Subsequently, the cell juice is released into the supernatant. Combination of the mechanical methods with physical methods, such as osmotic shock, freeze-thaw cycles, or addition of detergents, accelerates the disintegration process [23]. Biological methods with enzymes and enzyme mixtures, such as lysozyme for *Escherichia coli* and lyticase for *Saccharomyces cerevisiae*, are very efficient, but because of the price of these enzymes, these methods are restricted to the laboratory scale [24]. In addition, when therapeutic proteins are produced, these enzymes have to fulfill regulatory requirements for production of therapeutic proteins. Application of lytic bacteriophages has also been studied [25] but never used in practice.

The homogenate of single cells can also be clarified with centrifuges and microfilters. Sometimes a flocculation agent is used to improve the clarification. The fines in a clarified homogenate can sometimes lead to unstable chromatography processes because they may clog the chromatography bed.

Tracing the Biological Activity

It is essential to know both the origin of biological activity and whether contaminants are released from other cell organelles. Lysosomes must not be destroyed during homogenization because they contain many enzymes responsible for degradation of biopolymers. As long as the lysosomes are intact, these enzymes cannot attack the biopolymer of interest. Sometimes proteins or nucleic

acids have to be extracted from organelles and not from cytoplasm. *E. coli*, however, expresses the proteins of interest into the cytoplasm or into the periplasmic space. In order to trace bioactivity, the latent activity has been defined as a measure of the intactness of the cell organelles. For each cell organelle where a marker enzyme is available, the latent activity is defined as

$$\frac{\text{Enzyme activity with detergent} - \text{enzyme activity without detergent}}{\text{enzyme activity with detergent}} \times 100 \quad (1)$$

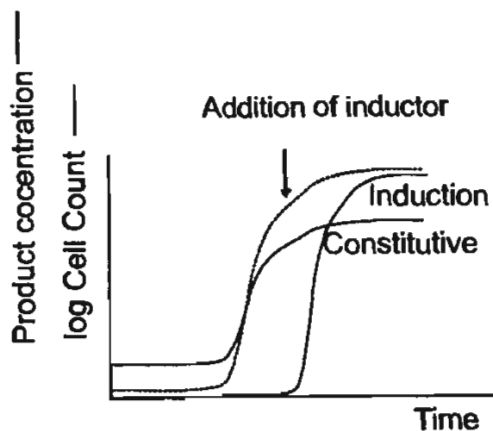
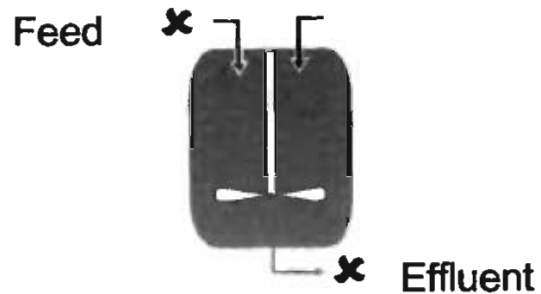
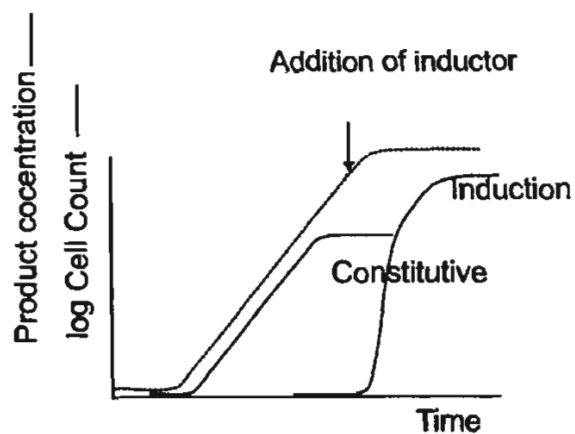
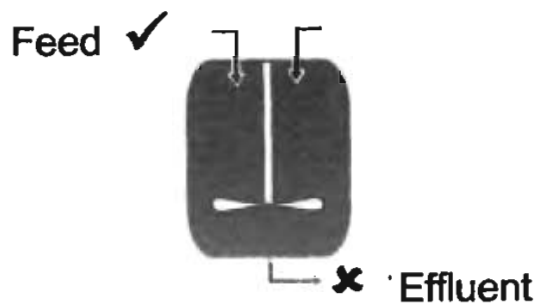
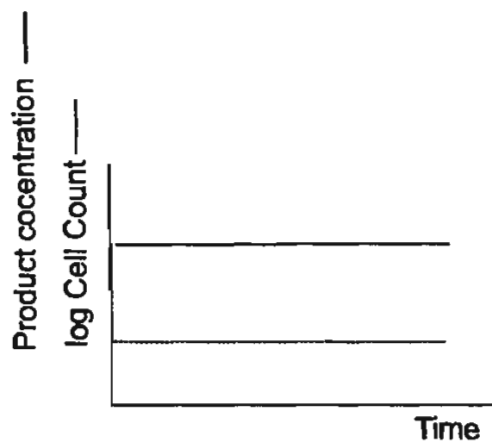
Fermentation Strategies for Genetically Modified Microorganisms

The fermentation process and downstream processing are usually unlinked. The fermentation broth is harvested and stored, and the recovery is carried out later. In rare cases, when the product is not stable, purification is carried out immediately after fermentation. The construct of the expression plasmid also influences the purification strategy. It is important to take this fact into account when an efficient and sustainable process must be designed. The expression plasmid determines whether a protein is secreted into the culture medium, into the periplasm, or stays in the cytoplasm. It also plays a pivotal role in the half-life of a protein. Although it may sound odd, the N-terminal amino acid is responsible for the half-life of a protein in the cell [26,27]. The most important features determining the functionality of an expression system are listed in Table 2. The expression system, together with the mode of operation, produces a characteristic product concentration profile with time (Fig. 3).

Recombinant bacteria and yeast cells are efficiently cultivated in a fed-batch mode [28–30]. Old-fashioned processes use the simple batch mode. The fed-batch mode has higher productivity because the physiological requirements of the cells can be better adjusted in fed-batch culture. Animal cells are often cultivated in continuous mode [28]. The cells are immobilized or retained by special devices in the fermenter and the culture medium is continuously fed to the bioreactor. In this case, the product can be harvested continuously. So far, continuous purification processes have not been used on an industrial scale to

TABLE 2 Important Properties of an Expression System for Recombinant Protein Production

Category	Property
Type of expression vector	Plasmids, mitochondria, genome
Destination of expressed protein	Intracellular, periplasm, extracellular
Regulation of protein biosynthesis	Constitutive expression, induction

Batch*Fed-Batch**Continuous*

a large extent. The fundamental bottlenecks in the application of continuous bioprocesses are comprehensively reviewed by Heijnen et al. [31].

Biomolecules from Transgenic Animals and Plants

Transgenic animals have been considered highly productive systems for the production of proteins. The expression of cDNA is targeted either to the blood (human hemoglobin in pigs [32]) or to the mammary glands. The expression of a therapeutic protein into milk has been used for several blood clotting proteins such as factor VIII (FVIII), FIX, protein S, and antithrombin III [33,34]. Also, antibodies have been produced by transgenic animals. The initial recovery from transgenic milk is very simple if the protein can withstand conditions around pH 4.7. The milk is defatted by centrifugation using conventional technologies in the dairy industry; then casein is precipitated by titration of the skim milk to pH 4.7. The whey is adjusted to the appropriate pH and further processed. Separation of heterologous protein from the homologous protein is an extremely difficult task and can be accomplished only by chromatographic means. Sometimes heterodimers between the host protein and the overexpressed heterologous protein have been observed [35]. This is the case when a human protein with a sequence almost identical to the cognate host sequence is expressed. Displacement chromatography is a method of choice for separation of heterodimers.

Transgenic plants have been developed to improve the quality of food, to improve resistance to pests, and to prolong or shorten the time required for ripening. They are considered to be a valuable production system for edible vaccines [36,37]. In this case, downstream processing is not required. Therapeutic

FIGURE 3 Fermentation modes for recombinant bacteria, yeast, and animal cells. On the left-hand side, the feed streams and harvesting streams are schematically shown for the batch, fed-batch, and continuous cultivation of microorganisms. On the right-hand side, the product concentration and the cell density are shown. For batch and fed-batch a discontinuous product concentration profile is obtained. With constitutive expression of a product, the product concentration is dependent on the cell density. Product is present in the culture supernatant during the whole production cycle and thus more susceptible to degradation. When the product formation is induction controlled, the production concentration raises sharply after addition of the inductor. The residence time of the product in the bioreactor is reduced. For continuous culture a constant product concentration profile is maintained over the entire production cycle. The residence time of the product in the bioreactor depends on the harvesting time and is shortest for all fermentation modes when harvesting is continuously performed. Changing the mode of production can highly influence the composition of feed for preparative chromatography.

proteins such as monoclonal antibodies have been expressed in transgenic plants [38]. In this case, downstream processing is a challenge because disintegration of plant organs such as leaves, roots, and stems is more complicated than the processing of milk. Some tannins may inactivate proteins by nonspecific precipitation; therefore, after disintegration, the tannins have to be removed as quickly as possible. The addition of polyvinylpyrrolidone is very efficient in removal of these denaturing compounds. Production of other biopolymers, such as tailor-made polysaccharides, may be a major future area for transgenic plants [39–41].

Requirements for Therapeutic Proteins

The most detailed requirements for the quality of biomolecules, and instructions on how to proceed, have been released for therapeutic proteins. There are three important parameters for a therapeutic protein—purity, consistency, and potency. All quality issues are based on these three cornerstones. In the United States, biological macromolecules are termed biologics.

In the United States, biologics are defined in Code of Federal Regulations (CFR 21) subchapter F as products with the following characteristics: "... 'Biological product' means any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man. ..." However, the regulatory affairs for drugs (CFR 21 subchapters C and D) are very similar to those for biologics.

The guidelines for medicinal products derived from biotechnology of the Commission of the European Community are clearer than the definition in CFR 21, but they also do not consider future developments.

In some European countries, biologics are defined as products derived from:

1. Human blood, other human body fluids, or human tissue
2. Animal blood
3. Microorganisms or components of microorganisms
4. Animals or microorganisms for active or passive immunization
5. Monoclonal antibodies
6. Recombinant DNA products

For production, purification and quality control, preclinical testing, etc., a series of recommendations and requirements must be obeyed. The Office of Biologics released "Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology" and "Points to Consider in the Manufacture and Testing of Microclonal Antibody Products for Human Use." Also, the ad hoc Working Party on Biotechnology/Pharmacy, as a consulting committee of the Commission of the European Community, released a

series of recommendations concerning biologics. Purity, removal of adventitious agents, consistency, potency, etc. are well defined and in general follow the same principles. A very superficial summary of the requirements for therapeutic proteins is given in Table 3.

Removal of Adventitious Agents

Removal of DNA, viruses, and bacterial endotoxins is the most challenging task for downstream processing of therapeutic proteins. When animal cell-derived products are purified, endotoxins are not the major concern. Demonstration of efficient removal of viruses and virus-like particles is very important because they may carry infectious viruses, although the risk is extremely low. Usually the starting material has a very low virus load. By spiking the cell culture supernatant, the removal of viruses is simulated. Clearance factors are calculated from these small-scale experiments. A certain safety factor is always included in a purification sequence; therefore, a one-step procedure will never have a chance to be licensed for production of therapeutic proteins. In contrast to the viral load, the DNA and endotoxin load of the starting material can be very high. Nevertheless, small-scale experiments with spiked starting materials are carried out to demon-

TABLE 3 Requirements for Therapeutic Proteins^a

Main parameters	Value/property
Purity	
Specific protein content	>99.9%
Dimer and oligomer content	<1.0
Ligand leakage	Usually less than 1 ppm
Virus content	Absence of virus
DNA content	<10 pg/dose
Pyrogen content	1 EU/dose
Prion content	Absence of prions
Consistency	
Microheterogeneity	Allowed, but consistent
Impurities	Allowed, but consistent
Potency	
Folding	Correctly folded
Mutations	Correctly expressed, no mutations
Processing	Correctly processed

^aThe detailed regulations may vary from country to country and some regulations are product related. The figures provided are only a rough guideline.

strate the efficient clearance of these adventitious agents. A correct scale-down is very important to obtain figures that reflect the industrial situation.

Besides scale-down, the nature or the spiking material is very critical. DNA and endotoxin are not chemically well-defined products. The shape, size, and composition of the original starting material can differ from those of the material used for the spiking experiments. We have circumvented this problem by *in vivo* radiolabeling of hybridoma DNA. The cell culture has been fed with radiolabeled nucleotides. The extracted DNA was used for the spiking experiments. Removal of endotoxins has been solved. The charge and hydrophobicity of endotoxins allow efficient removal by ion-exchange chromatography, hydrophobic interaction, and other adsorption techniques. Validation of the removal of adventitious agents is a core business of scale-up and the engineering of therapeutic protein purification schemes.

ANALYTICAL VERSUS PREPARATIVE CHROMATOGRAPHY

Information versus Production

The most important difference between analytical and preparative chromatography is the desired outcome of the separation process. In analytical chromatography, we want to generate information. In preparative chromatography, we want to generate a product. Sometimes people are confused because preparative chromatography can also be performed on an analytical system. The scale is not the characteristic of preparative chromatography. For biochemists, microgram quantities are often sufficient; they are solely interested in the product and not in the retention behavior (information). The differences between analytical and preparative chromatography are summarized in Table 4. In preparative chromatography, we are interested in the purity and the recovery of the product. In analytical chromatography, we are interested in resolution. We want to separate our compounds and identify them unequivocally by their retention behavior. If this is not possible, we have to apply hyphenated techniques. In preparative chromatography, we need capacity as well as selectivity, which is why affinity chromatography has become so popular. The high selectivity allows operation in an adsorption-desorption mode. Large particles can be used to build up only low back pressure. The low back pressure allows installation of simpler equipment and safety precautions are not as important. The large particles, together with the large particle size distribution, have beneficial effects on the economics of the process.

Giddings [42] has defined two categories in chromatography, ideal and nonideal chromatography. Ideal chromatography assumes equilibrium conditions with no extra column band-spreading effects. In preparative chromatography, we usually have highly concentrated samples and the extra column effects cannot be

TABLE 4 Preparative Chromatography Compared with Analytical Chromatography.^a

	Analytical	Preparative
Purpose	Analysis	Production
Output	Information	Products
Concentration	Low	Varies
Feed rate	Low	High (usually)
Isotherm	Linear	Nonlinear (usually)
Competition ^a	Insignificant	Significant
Efficiency	High	Low
Optimization	Resolution speed requires efficiency and selectivity	Purity, concentration throughput costs Requires selectivity

^aEspecially in overloaded situations, the solutes compete for the free binding sites and sample displacement is observed.

neglected. The two categories will be combined with one type of adsorption isotherm—linear or nonlinear. In preparative chromatography, we work under nonlinear conditions. From the theoretical point of view, we deal with the worst case: nonideal, nonlinear conditions. Nevertheless, for theoretical considerations, preparative chromatography has often been simplified to ideal chromatography; however, simplification to linear adsorption behavior cannot be justified.

Adsorption Isotherms

In preparative chromatography, we deal with favorable adsorption isotherms, which means that the concentration in the stationary phase does not increase at the same rate as does the concentration in the mobile phase. The Langmuir adsorption isotherm originally described for adsorption of gases on plane surfaces [43] has frequently been used to describe phase equilibrium in chromatography of biomolecules [44].

$$C_s = \frac{aC_m}{1 + bC_m} \quad (2)$$

Although the physical meaning of the Langmuir adsorption isotherm is limited, the experimental data can often be very accurately approximated. C_s is the stationary phase concentration, C_m is the mobile phase concentration, and a and b are empirical parameters reflecting the maximal adsorption capacity and the equilibrium binding constant. It has to be noted that the Langmuir adsorption

isotherm assumes that there is a homogeneous distribution of the binding sites, all binding sites are equivalent, and equilibrium can be attained. For high concentrations or situations with high surface coverage, these assumptions are no longer valid [45].

In some stationary phases, especially for ion-exchange chromatography, a rectangular adsorption isotherm is often observed. Even at very dilute concentration, the whole sample is in the adsorbed state. The Langmuir-Freundlich adsorption isotherm [44] has been used quite successfully for approximation of such adsorption conditions

$$C_s = \frac{aC_m^n}{1 + bC_m^n} \quad (3)$$

In affinity chromatography, a certain type of linear adsorption isotherm has often been observed. The linear adsorption isotherm is different from the linear part of the thermodynamic behavior of a favorable isotherm in that the linearity originates from the interaction between the ligand and protein.

As a consequence of the deviation of the linearity of the adsorption isotherm, a symmetrical peak is modified to a tailed peak. Excessive peak tailing may lead to a dilute product or to product losses because the peak has to be cut after a certain threshold has been reached.

In preparative chromatography, the influence of salt, or, more generally speaking, the mobile phase modifier, on adsorption has to be taken into account [46]. The aforementioned equations do not reflect this situation. We have developed an empirical approach where we have combined the conventional isotherms and the dependence on salt using a logistic dose-response function [44]. Velayudhan and Horvath [47] have described a similar approach—the mobile phase modified adsorption isotherm. They assume that the maximum binding capacity is constant; however, this assumption does not reflect real conditions in preparative chromatography.

Hysteresis between adsorption and desorption has been observed frequently, but attempts at rational explanations are very rare [48]. Once the biopolymer has been adsorbed onto the surface, desorption cannot be achieved under similar conditions. Molecular rearrangement is probably responsible for this behavior.

Modes and Steps in Preparative Chromatography

In principle, the modes and steps in preparative chromatography do not differ from those in analytical chromatography. Four modes are used (Fig. 4): isocratic elution, linear gradient elution, stepwise elution, and displacement development. Frontal analysis is not used for purification of biomolecules, but it is used for

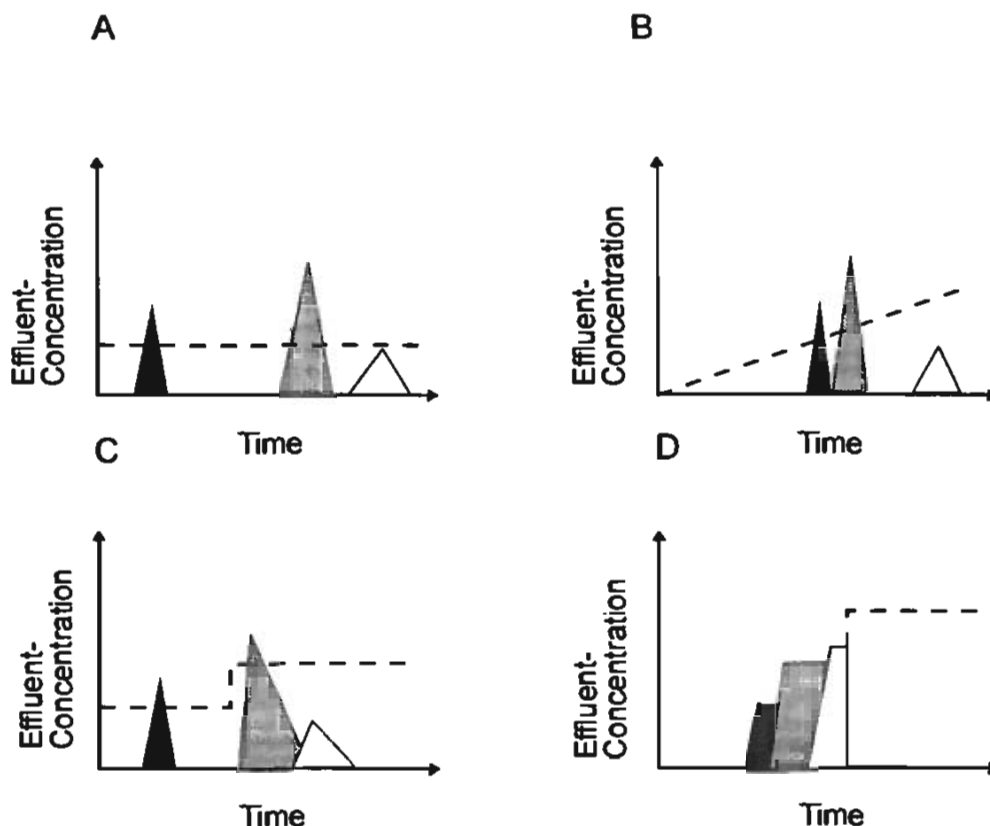


FIGURE 4 Different modes in liquid chromatography of biomolecules: (A) isocratic elution, (B) linear gradient elution, (C) stepwise elution, and (D) displacement development.

determination of dynamic capacity and as an alternative method for determination of the static equilibrium capacity. For size exclusion, the isocratic mode must be used because of the nature of the separation process. Displacement development does not yet have industrial application because nontoxic and inexpensive displacers with the appropriate selectivity are usually not available. The following steps in preparative chromatography are very common regardless of the mode. The first step is the equilibration of the stationary phase, after which feed is applied and the unbound material is washed out. In some cases, a second or third washing step is applied prior to elution. After elution, the column is regenerated and a cleaning step may be added. For the next cycle, the column has to be equilibrated again.

The buffers used in preparative chromatography of biomolecules do not inhibit the growth of microorganisms; therefore, a preservation buffer has to be applied when the column is stored. The preservation of a column is also a very delicate problem because many of the preservatives are very toxic for humans. Only a limited number of compounds are available for use in the production of therapeutic proteins. Sterilization of chromatography columns has been discussed

in the past but it has never been considered as an essential step. Sanitation is essential [49,50].

Equipment

The equipment for preparative chromatography is slightly different from that for analytical chromatography with respect to the extra column volume. To keep the cost under a reasonable limit, the pressure must be kept as low as possible. Consequently, a large tubing diameter is chosen to reduce back pressure that is caused by friction on the walls. According to Taylor, tubes introduce a symmetrical Gaussian broadening (σ_t^2) of the band profile. The band spreads under the combined action of the molecular diffusion and the parabolic axial flow velocity profile. For a fully developed laminar flow profile, this broadening can be described by

$$\sigma_t^2 = \frac{t_R r^2}{24 D_M} \quad (4)$$

where t_R is the retention time, r is the radius of the tube, and D_M is the molecular diffusion coefficient.

Safety devices such as air sensors and pressure transducers are built into preparative HPLC units, together with a series of valves. These devices create dead volume and contribute to the extra column volume. A large-scale chromatography unit is composed of valves for selection of buffers and feed solutions, at least two pumps, the separation column, and, in most cases, at least one detector. Instead of a fraction collector, a combination of valves is often used. These sources of dead volume create typical washout kinetics, which contribute exponentially to the band-broadening processes. For the industrial scale, the equipment is mainly customer designed. For medium scale, modular units are available [51]. Attention should be paid to extra column volume when systems are compared. Extra column effects are an important parameter of the quality of a system and should be considered when a system is purchased.

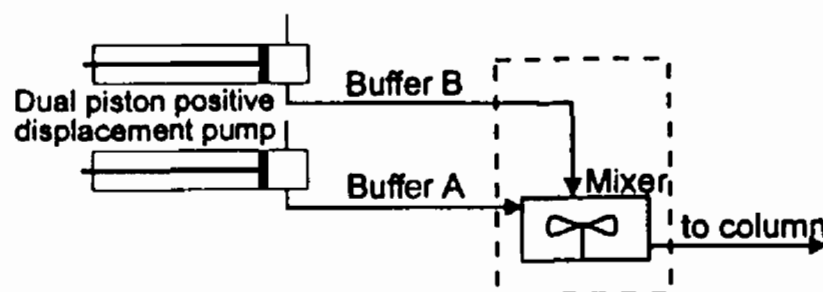
Wisniewski [52] has extensively reviewed the equipment for large-scale HPLC. The old question of low-pressure or high-pressure mixing is now outdated. Due to advances in electronic and process control instruments, the reliable blending of solutions should not be a problem anymore. The mixer has to be characterized when scale-up is considered or when a purification method is transferred to another separation unit. We have described a simple function to characterize a mixer [53]. The system is characterized by a simple blending of two buffers—a salt buffer and the normal running buffer. When salt is used, a conductivity monitor is necessary. The column is disconnected from the system

and buffer blending is carried out. The response curve is fitted by the following equation:

$$I = \frac{2 \exp\left(\frac{t-b}{a}\right)}{\exp\left(\frac{t-b}{a}\right) + 1} - 1 \quad (5)$$

I_0 , the initial salt concentration or conductivity of buffer A, and $I_{\max}$, the salt concentration of buffer B, are normalized to $I_{\max}$. The a is a lumped parameter that is composed of the symmetrical broadening during mixing and the exponential time constant of the mixer, assuming an exponential washout characteristic; t is the time and b is the delay time of the system. The same equation can be used to describe the quality of a step gradient. In this case, the equation does not

A



B

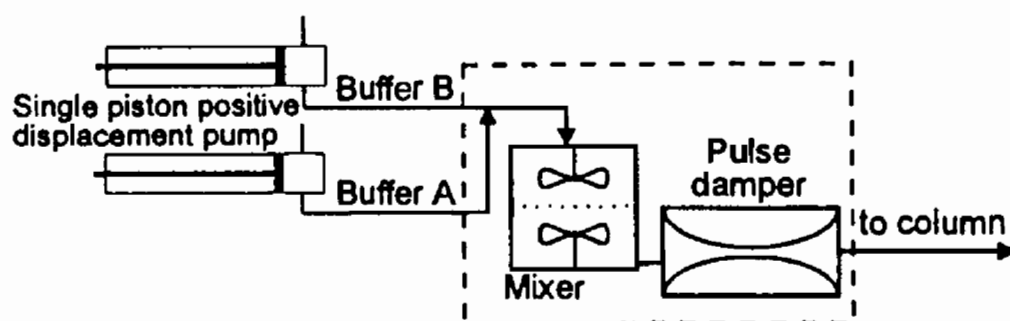


FIGURE 5 Schematic drawing of the high-pressure mixing system. (A) Inline mixer as normally used in HPLC systems. To provide the best mixing effect, the line with the buffer of higher density enters the mixer from the top while the less dense buffer is pumped in radially. (B) Inline mixer as normally used in a ProSys Workstation. Two mixers are connected together in series, separated by a seal with several bores. Both solutions are pumped radially into the first mixer and leave the system from the second (From Ref. 53.)

contain a scale-dependent variable and, therefore, the parameter a should be identical at different scales.

When a system is working with a pulse damper (as shown schematically in Fig. 5), the delay time of the system changes with the flow rate. With increasing flow rate, the pressure changes and the damper expands, resulting in a higher internal volume. Currently, systems are on the market that cover an extremely broad range of flow rates. This may be convenient for the chromatographer, but several compromises result. First, as a rule, the mixer volume should be of the same order of magnitude as the volume (millilitres) per minute pumped through the mixer. If the tubing and mixer are not changed, the system will operate at optimal conditions at a low flow rate but suboptimal ones at high flow rates. Conversely, if the system is operated at optimal conditions at high flow rates, then the high extra column volume will produce additional peak broadening at low flow rates. At this time, optimal equipment is not available for working at superhigh velocities in the preparative mode (separation is completed in less than 1 min). At these high flow rates, the detector time constant has to be carefully adjusted but commercially available equipment for these purposes does not yet exist.

MEDIA FOR PREPARATIVE CHROMATOGRAPHY

Differences Between Analytical and Preparative Chromatography

The most important differences between preparative media and analytical media are the particle size and the particle size distribution. For incompressible fluids, the Hagen-Poiseuille law can be applied for packed beds under laminar flow conditions. The permeability (K_0) of a chromatography bed is calculated as

$$K_0 = \frac{u\eta L}{\Delta p} \quad (6)$$

where u is the linear velocity, η the dynamic viscosity, L the column length, and Δp the pressure drop. Permeability is also dependent on the particle diameter and can be described according to the Konzeny-Carman equation:

$$K_0 = \left(\frac{d_p^2}{180\delta^2} \right) \left(\frac{\varepsilon_f^3}{(1 - \varepsilon_f)^2} \right) \varphi \quad (7)$$

where d_p is the particle diameter, δ is a shape factor usually between $1 \leq \delta \leq 1.3$, and φ is the porosity of the packed bed.

The terms $(1/180\delta^2)(\epsilon_f^3/(1 - \epsilon_f)^2)\phi$ are combined into a column resistance factor (Φ). This parameter is known as the Knox quality of a column. Rearranging Eqs. (6) and (7) shows the relationship between pressure drop and particle size:

$$\Delta p = \frac{u\eta L}{\Phi d_p^2} \quad (8)$$

Several companies are now manufacturing monodisperse particles that have a lower pressure drop than particles with nonuniform diameters. These materials are expensive and at the moment their applications are limited to small scale.

Categories Related to the Principle of Mass Transport and Retardation Forces

A plethora of media have been produced for preparative chromatography [54,55]. Initially, natural polymers such as agarose, cellulose, and dextran were the base materials [56]. Polymethacrylate-based material was developed at the same time, but these sorbents are not suitable for high-pressure applications. Consequently, silica-based materials were introduced for the separation of proteins and other biopolymers. Because silica tends to adsorb proteins irreversibly, several coating procedures have been developed. After more than two decades of extensive development work in academia and industry, media for chromatographic separations can be divided into eight different categories according to their principle of mass transport (Table 5). Media with nonuniform shape and hydrophilic gels in beaded form exhibit the lowest nonspecific adsorption, but unfortunately they cannot be used for high-pressure applications. Improved polysaccharide gels and synthetic macroporous hydrophilic supports are the most common materials for HPLC applications of biopolymers. Matrices with connective pores [57,58], monoliths [59–61], and gel-filled mineral porous material [62] are the latest developments. These materials show good mass transfer properties (can be operated at high flow rates) together with high physical stability. Spiral-wound cartridges made from textiles have also been described for preparative chromatography of biomolecules. These cartridges may be considered to have similar mass transfer properties to monoliths [63].

Another way to describe media is according to the retardation forces (Table 6). Other than covalent chromatography, all the modes listed in Table 6 are used for preparative chromatography of biomolecules. Reversed-phase chromatography is not used for the separation of large proteins because of its denaturing separation conditions and the use of organic solvents as eluents, requiring explosion-proof environments. Another more practical description of chromatography media is according to the conditions for elution, binding, and regeneration, as shown in Table 7.

TABLE 5 Media Categories with the Main Features for Preparative Applications

Media category	Composition	Main properties	Example
Nonuniform shape	Fibrous cellulose	Usable in batch mode, low-cost material, low flow rate, low non-specific adsorption	DE-cellulose, Whatman
Hydrophilic gels in bead form	Dextran	Limitations in flow rate due to compressibility, low non-specific adsorption	Sephadex, AP Biotech
Improved polysaccharide gels	Cross-linked agarose	Large pores, moderate compression	Sepharose FF, Source, AP Biotech
Synthetic macroporous hydrophilic supports	Polymethacrylates, trisacryl, polyvinyl	Very large pores, moderate compressibility, stable in acids, nonbiodegradable	Mar coprep, Biorad; Toyopearl, Toso Haas
Matrix with convective pores	Polystyrene coated with hydrophilic polymers	Very large pores to allow convection of macromolecules	Poros, Perspective
Tentacular sorbents	Porous synthetic particles with "active" polymeric tentacles	Rapid sorption-desorption kinetics, ligand availability, high capacity by surface diffusion	Fractogel, Merck XL; Sepharose, Pharmacia
Monoliths	Block polymerisate of various methacrylates and agglomerated silica	Extremely high speed	Uno, BioRad; CIM, BIA
Gel-filled mineral porous material	Silica filled with dextran gels. Composite porous particles filled with viscoelastic synthetic gels	High sorption capacity at high flow rates, high resolution, noncompressibility	HyperD, BioSeptra

TABLE 6 Types of Chromatography Classified by the Nature of Retardation Forces and Principle of Separation

Name	Action principle	Separation by
Adsorption chromatography	Surface binding	Molecular structure
Ion-exchange chromatography	Ionic binding	Surface charge
Molecular sieve chromatography (gel filtration, size exclusion)	Size exclusion	Molecular size and shape
Affinity chromatography	Biospecific adsorption/desorption	Molecular structure
Hydrophobic (interaction) chromatography	Hydrophobic complex formation	Hydrophobicity and hydrophobic patches
Covalent chromatography (Metal) chelate chromatography	Covalent binding Coordination complex	Functional groups Complex formation with transition metals
Reversed-phase chromatography	Hydrophobic complex formation	Hydrophobicity

In a separation process for a therapeutic protein, efficient cleaning of the system has the same priority as the elution or binding conditions. The medium should be resistant to harsh regeneration conditions, but, on the other hand, it must also be inert and exhibit low nonspecific adsorption.

Media for Ion-Exchange Chromatography

The introduction of this type of chromatography was based on the observation that natural aluminosilicates (zeolites) retarded salt ions dissolved in water. In 1935, Adams and Holmes had already produced artificial ion exchangers by condensation of phenolic acids and formaldehyde. These ion exchangers were not suited for separation of biopolymers because the nonspecific adsorption was too high. In the 1960s and 1970s, ion exchangers based on cellulose, Sepharose, and methacrylate were developed [64–66]. These ion-exchangers are compatible with proteins and therefore suitable for the separation of proteins and other biopolymers. Their swelling properties with salt in the running buffer and their high back pressure when operated at higher flow rates led to the development of media with a higher degree of cross-linking. Cross-linking reduces both swelling and the theoretical number of sites for the introduction of functional groups. We observed the highest number of functional groups in “old-fashioned” ion exchangers such

TABLE 7 Physicochemical Conditions Used for Protein Chromatography Media

Type of chromatography	Common adsorption conditions	Common elution conditions	Regeneration conditions
Cation exchange	Low ionic strength, pH below protein pI	Increase of ionic strength, increase pH	Acid/base washing, high concentration salts
Anion exchange	Low ionic strength, pH above protein pI	Increase of ionic strength, decrease pH	Acid/base washing, high concentration salts
Hydrophobic	High ionic strength	Ionic strength decrease (ammonium sulfate)	Alkaline washing, organic acids, chaotropics, aqueous-miscible solvents
Hydroxyapatite	Dilute phosphate buffers, pH 6.8	Increase phosphate buffer concentration	Sodium hydroxide
Size exclusion	Any ionic strength, any pH, low feed	Isocratic separation	Salt washing, dilute alkaline washing
Affinity	Close to physiological conditions except special cases	pH changes, ionic strength changes, competitive elution	Salt washing, specific displacement treatments
Reversed phase	Hydro-organic mixtures	Organic modifiers: acetonitrile, methanol, etc.	Solvents, glycols, urea, acidic solutions

as DEAE-Sephadex or DE-Cellulose. Up to 400 mmol of groups are present in 1 cm³ of swollen ion-exchange gel. Cross-linking improved the media, but because of the particle size and distribution, the resolution has been rather low.

Silica matrices, already popular for other HPLC applications, have been coated and derivatized and successfully applied for protein chromatography [67]. Another development has been the production of monodisperse particles, the so-called Monobeads. These beads became very popular in the area of biochemistry for rapid protein and DNA purification [68]. Mono S and Mono Q, both from Amersham-Pharmacia, reduced separation times to several hours or, in many applications, to less than 1 hr. At the same time, Toyopearl was introduced by Toyo Soda (Tosoh) [69]. These media had a very regular shape and were targeted to low-pressure chromatography. These media have been derivatized with various ligands using various particle diameters [70–73]. They have also been modified by grafting longer chains onto the surface [74], producing higher capacity. This modification is manufactured by Merck under the name Tentacle Gels. A compilation of chromatography media suitable for the separation of biopolymers is given in Table 8. CIP stability is the resistance to conditions for cleaning in place.

Ion-exchange chromatography has become so popular for protein chromatography because of its high capacity that a wide range of applications have been described. In cases in which the protein of interest has a different isoelectric point than the main impurities, very high enrichment factors can be achieved. Ion-exchange chromatography usually works with very simple buffers that are compatible with proteins. Elution is effected with salt. Stepwise elution can generate a massive product reconcentration in the eluate. These beneficial technological properties, together with the low price of ion exchangers, explain why ion-exchange chromatography is the primary mode in the biotechnology industry.

Ion-exchange membranes have also been successfully used in the biotechnology industry [75,76]. Sartorius has grafted their microfiltration membranes so that the high ligand density in the pore space is responsible for the high capacity. So far, these ion-exchange membranes have been successfully applied for the removal of endotoxins and DNA [77]. DNA and endotoxin removal can be accomplished by ion-exchange columns as well as membranes. The good binding capacity for DNA and endotoxins is a strong argument for placing an ion-exchange step in the downstream processing scheme.

Media for Hydrophobic Interaction Chromatography

The support matrices used for ion-exchange chromatography have been modified by different means and successfully used for hydrophobic interaction chromatography (HIC), which also has a broad field of applications for the purification of

TABLE 8 Commercially Available Media for Ion-Exchange Chromatography

Trade name	Matrix	Particle size (μm)	Ligand	Capacity (mg/mL gel)	pH stability	CIP stability	Remarks	Supplier
Streamline SP	6% cross-linked agarose containing crystalline quartz	200	Sulfopropyl	70 (lysozyme)	3–13	3–14	For capture direct from crude feedstock.	AP Biotech
Streamline DEAE	6% cross-linked agarose containing crystalline quartz	200	Diethylaminoethyl	55 (BSA)	2–13	2–14	For expanded bed For capture direct from crude feedstock.	AP Biotech
DEAE Sephadex A-25	Cross-linked dextran	45–190	Diethylaminoethyl	n.a.	2–10	2–14	For expanded bed For batch processing, economical	AP Biotech
DEAE Sephadex A-50	Cross-linked dextran	100–370	Diethylaminoethyl	n.a.	2–11	2–15	For batch processing, economical	AP Biotech
CM Sephadex C-25	Cross-linked dextran	65–235	Carboxymethyl	n.a.	4–10	3–13	For batch processing, economical	AP Biotech
CM Sephadex C-50	Cross-linked dextran	110–400	Carboxymethyl	n.a.	4–10	3–13	For batch processing, economical	AP Biotech
SP Sephadex C-25	Cross-linked dextran	40–125	Sulfopropyl	n.a.	4–10	3–13	For batch processing, economical	AP Biotech
QAE Sephadex A-25	Cross-linked dextran	40–125	Quaternary aminoethyl	n.a.	2–10	2–13	For batch processing, economical	AP Biotech
Q Sepharose Big Beads	Highly cross-linked 6% agarose	100–300	Quaternary aminomethyl	60 (BSA)	2–12	2–14	For capture in very large volumes	AP Biotech

SP Sepharose Big Beads	Highly cross-linked 6% agarose	100–300	Sulfopropyl	n.a.	4–13	3–14	For capture in very large volumes	AP Biotech
DEAE Sepharose Fast Flow	Highly cross-linked 6% agarose	45–165	Diethylaminoethyl	110 (HSA)	2–13	1–14	Large scale	AP Biotech
CM Separose Fast Flow	Highly cross-linked 6% agarose	45–165	Carboxymethyl	50 (ribonuclease)	4–13	3–12	Large scale	AP Biotech
Q Separose Fast Flow	Highly cross-linked 6% agarose	45–165	Quaternary aminomethyl	120 (HSA)	2–12	1–14	Large scale	AP Biotech
SP Separose Fast Flow	Highly cross-linked 6% agarose	45–165	Sulfopropyl	121 (HSA)	4–13	3–14	Large scale	AP Biotech
Q Sepharose HP	Cross-linked agarose	34	Quaternary aminomethyl	122 (HSA)	2–12	1–14	High resolution, intermediate scale and polishing	AP Biotech
SP Sepharose HP	Highly cross-linked 6% agarose	34	Sulfopropyl	55 (ribonuclease)	4–13	3–14	High resolution, intermediate scale and polishing	AP Biotech
CM Sepharose HP	Highly cross-linked 6% agarose	34	Carboxymethyl	0.06–0.08 mmol/mL	2–13	2–14	High resolution, intermediate scale and polishing	AP Biotech
SOURCE 15Q	Polystyrene/divinylbenzene	15	Quaternary aminoethyl	45 (BSA)	2–12	1–14	High resolution, preparative purification	AP Biotech
SOURCE 30Q	Polystyrene/divinylbenzene	30	Quater + D41nary aminoethyl	40 (BSA)	2–12	1–14	High resolution, preparative purification	AP Biotech
SOURCE 15S	Polystyrene/divinylbenzene	15	Sulfopropyl	75 lysozyme	2–12	1–14	High resolution, preparative purification	AP Biotech
SOURCE 30S	Polystyrene/divinylbenzene	30	Sulfopropyl	80 (lysozyme)	2–12	1–14	High resolution, preparative purification	AP Biotech

(continued)

TABLE 8 (continued)

Trade name	Matrix	Particle size (μm)	Ligand	Capacity (mg/mL gel)	pH stability	CIP stability	Remarks	Supplier
Fractogel EMD TMAE (M)	Polymer	40–90	Trimethylamino-ethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD TMAE (S)	Polymer	20–40	Trimethylamino-ethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD DEAE (M)	Polymer	40–90	Diethylaminoethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD DEAE (S)	Polymer	20–40	Diethylaminoethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD DMAE (M)	Polymer	40–90	Dimethylamino-ethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD DMAE (S)	Polymer	20–40	Dimethylamino-ethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD SO ₃ ⁻ (M)	Polymer	40–90	Sulfonate	130 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD SO ₃ ⁻ (S)	Polymer	20–40	Sulfonate	130 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD COO ⁻ (M)	Polymer	40–90	Carboxymethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck
Fractogel EMD COO ⁻ (S)	Polymer	20–40	Carboxymethyl	100 (BSA)	n.a.	n.a.	High capacity, allow high flow rate	Merck

POROS 50 HQ	Polystyrene-divinylbenzene	50	Quaternized polyethyl- amineimine	55 (BSA)	n.a.	n.a.	PerSeptive
POROS 50 PI	Polystyrene-divinylbenzene	50	Polyethyleneimine	45 (BSA)	n.a.	n.a.	PerSeptive
POROS 50 HS	Poly + B58styrene-divinylbenzene + B46	50	Sulfoethyl	75 (lysozyme)	n.a.	n.a.	PerSeptive
Macro-Prep DEAE	Polyacrylamide	50	Diethylaminoethyl	35 (BSA)	1–14	n.a.	Bio-Rad
Macro-Prep High Q	Polyacrylamide	50	Quaternary aminomethyl	40 (BSA)	1–14	n.a.	Bio-Rad
Macro-Prep Q	Polyacrylamide	50	Quaternary aminomethyl	15 (BSA)	1–14	n.a.	Bio-Rad
Macro-Prep High S	Polyacrylamide	50	Sulfonate	70 (IgG)	1–14	n.a.	Bio-Rad
Macro-Prep S	Polyacrylamide	50	Sulfonate	50 (IgG)	1–14	n.a.	Bio-Rad
Macro-Prep CM	Polyacrylamide	50	Carboxymethyl	35 (hemoglobin)	4–14	n.a.	Bio-Rad
TSK-GEL-DEAE-5PW	Hydrophilic resin	20–40	Diethylaminoethyl	20–40 (lysozyme)	n.a.	n.a.	Bio-Rad
TSK-GEL-SP-5PW	Hydrophilic resin	20–40	Sulfoethyl	20–40 (BSA)	n.a.	n.a.	Tosoh
TSK-GEL-DEAE-3SW	Silica	10	Diethylaminoethyl	n.a.	n.a.	n.a.	Tosoh
TSK-GEL-CM-3SW	Silica	10	Carboxymethyl	n.a.	n.a.	n.a.	Tosoh
Toyopearl DEAE-650S	Polymer	20–50	Diethylaminoethyl	25–35 (BSA)	1–14	1–14	Tosoh
Toyopearl DEAE-650M	Polymer	40–90	Diethylaminoethyl	25–35 (BSA)	1–14	1–14	Tosoh
Toyopearl DEAE-650C	Polymer	50–150	Diethylaminoethyl	25–35 (BSA)	1–14	1–14	Tosoh
Toyopearl Super Q-650S	Polymer	20–50	Quaternary aminopolymer	105–155 (BSA)	1–14	1–14	Tosoh
Toyopearl Super Q-650M	Polymer	40–90	Quaternary aminopolymer	105–155 (BSA)	1–14	1–14	Tosoh

(continued)

TABLE 8 (continued)

Trade name	Matrix	Particle size (μm)	Ligand	Capacity (mg/mL gel)	pH stability	CIP stability	Remarks	Supplier
Toyopearl Super Q-650C	Polymer	50–150	Quaternary aminopolymer	105–155 (BSA)	1–14	1–14		Tosoh
Toyopearl QAE-550C	Polymer	50–150	Quaternary aminoethyl	60–80 (BSA)	1–14	1–14		Tosoh
Toyopearl CM-650S	Polymer	20–50	Carboxymethyl	40–60 (hemoglobin)	1–14	1–14		Tosoh
Toyopearl CM-650M	Polymer	40–90	Carboxymethyl	35–55 (hemoglobin)	1–14	1–14		Tosoh
Toyopearl CM-650C	Polymer	50–150	Carboxymethyl	40–60 (hemoglobin)	1–14	1–14		Tosoh
Toyopearl SP-650S	Polymer	20–50	Sulfopropyl	40–60 (lysozyme)	1–14	1–14		Tosoh
Toyopearl SP-650M	Polymer	40–90	Sulfopropyl	40–60 (lysozyme)	1–14	1–14		Tosoh
Toyopearl SP-650C	Polymer	50–150	Sulfopropyl	35–55 (lysozyme)	1–14	1–14		Tosoh
Toyopearl SP-550C	Polymer	50–150	Sulfopropyl	80–120 (lysozyme)	1–14	1–14		Tosoh
BAKERBOND polyethylenimine (WAX)	Silica	40	Polyethylenimine	n.a.	n.a.	n.a.	High-performance semipreparative	J.T.Baker
BAKERBOND DEAM (WAX)	Silica	40	Diethylamino-methyl	n.a.	n.a.	n.a.	High-performance semipreparative	J.T.Baker
BAKERBOND QUAT	Silica	40	Diethyl-methyl-aminomethyl	n.a.	n.a.	n.a.	High-performance semipreparative	J.T.Baker
BAKERBOND (Carboxyl)	Silica	40	Carboxyethyl	n.a.	n.a.	n.a.	High-performance semipreparative	J.T.Baker
BAKERBOND Sulfonate	Silica	40	Sulfonate	n.a.	n.a.	n.a.	High-performance semipreparative	J.T.Baker
DE-51	Cellulose	n.a.	Diethylaminoethyl	30 (BSA)	2–9	n.a.		Whatman
DE-52	Cellulose	n.a.	Diethylaminoethyl	130 (BSA)	2–9.5	n.a.		Whatman

DE-53	Cellulose	n.a.	Diethylaminoethyl	150 (BSA)	2–12	n.a.		Whatman
CM-52	Cellulose	n.a.	Carboxymethyl	210 (lysozyme)	3–10	n.a.		Whatman
SE-52	Cellulose	n.a.	Sulfoxyethyl	195 (lysozyme)	2–12	n.a.		Whatman
SE-53	Cellulose	n.a.	Sulfoxyethyl	210 (lysozyme)	2–12	n.a.		Whatman
Q ThruPut Plus		60–160	Quaternary aminoethyl	n.a.	n.a.	–14	Clarified feed, downstream	Sterogene
SP ThruPut Plus		60–160	Sulfopropyl	n.a.	n.a.	–14	Clarified feed, downstream	Sterogene
DEAE ThruPut Plus		60–160	Diethylaminoethyl	n.a.	n.a.	–14	Clarified feed, downstream	Sterogene
CM ThruPut Plus		60–160	Carboxymethyl	n.a.	n.a.	–14	Clarified feed, downstream	Sterogene
Q ThruPut		100–200	Quaternary aminoethyl	n.a.	n.a.	–14	Partially clarified, lipid containing	Sterogene
SP ThruPut		100–200	Sulfopropyl	n.a.	n.a.	–14	Partially clarified, lipid containing	Sterogene
DEAE ThruPut		100–200	Diethylaminoethyl	n.a.	n.a.	–14	Partially clarified, lipid containing	Sterogene
CM ThruPut		100–200	Carboxymethyl	n.a.	n.a.	–14	Partially clarified, lipid containing	Sterogene
Upstream Q		200–300	Quaternary aminoethyl	n.a.	n.a.	–14	Unc clarified, viscous lipid containing	Sterogene
Upstream SP		200–300	Sulfopropyl	n.a.	n.a.	–14	Unc clarified, viscous lipid containing	Sterogene
Upstream DEAE		200–300	Diethylaminoethyl	n.a.	n.a.	–14	Unc clarified, viscous lipid containing	Sterogene
Upstream CM		200–300	Carboxymethyl	n.a.	n.a.	–14	Unc clarified, viscous lipid containing	Sterogene
Q Cellthru BigBead Plus		300–500	Quaternary aminomethyl	n.a.	n.a.	–14	Mammalian cell culture, fermentations up to 20% wet solids	Sterogene

(continued)

TABLE 8 (continued)

Trade name	Matrix	Particle size (μm)	Ligand	Capacity (mg/mL gel)	pH stability	CIP stability	Remarks	Supplier
SP Cellthru Bigbead Plus		300–500	Sulfopropyl	n.a.	n.a.	–14	Mammalian cell culture, fermentations up to 20% wet solids	Sterogene
DEAE Cellthru Bigbead Plus		300–500	Diethylaminoethyl	n.a.	n.a.	–14	Mammalian cell culture, fermentations up to 20% wet solids	Sterogene
CM Cellthru BigBead Plus		300–500	Carboxymethyl	n.a.	n.a.	–14	Mammalian cell culture, fermentations up to 20% wet solids	Sterogene
Q 9 Cellthru BigBead Plus		800–1100	Quaternary aminomethyl	n.a.	n.a.	–14	Bacterial and yeast fermentations up to 20% wet solids	Sterogene
SP 9 Cellthru Bigbead Plus		800–1100	Sulfopropyl	n.a.	n.a.	–14	Bacterial and yeast fermentations up to 50% wet solids	Sterogene
DEAE 9 Cellthru Bigbead Plus		800–1100	Diethylaminoethyl	n.a.	n.a.	–14	Bacterial and yeast fermentations up to 50% wet solids	Sterogene

CM 9 Cellthru BigBead Plus		800–1100	Carboxymethyl	n.a.	n a	–14	Bacterial and yeast fermentations up to 50% wet solids	Sterogene
Q HyperD F	Gel-in-a-shell	35	Quaternary aminomethyl	80 (BSA)	n.a.	n.a.	Rigid open porous bead structure	BioSeptra
Q HyperD M	Gel-in-a-shell	60	Quaternary aminomethyl	80 (BSA)	n.a.	n.a.	Rigid open porous bead structure	BioSeptra
S HyperD F	Gel-in-a-shell	35	Sulfonate	80 (BSA)	n a	n.a.	Rigid open porous bead structure	BioSeptra
S HyperD M	Gel-in-a-shell	60	Sulfonate	80 (BSA)	n a	n.a.	Rigid open porous bead structure	BioSeptra
DEAE HyperD F	Gel-in-a-shell	35	Diethyl aminoethyl	80 (BSA)	n.a.	n.a.	Rigid open porous bead structure	BioSeptra
CM HyperD F	Gel-in-a-shell	35	Carboxymethyl	80 (BSA)	n.a.	n.a.	Rigid open porous bead structure	BioSeptra
Q HyperDTM 10	Gel-in-a-shell	10	Quaternary aminomethyl	80 (BSA)	n a	n.a.	Direct scalability	BioSeptra
Q HyperDTM 20	Gel-in-a-shell	20	Quaternary aminomethyl	80 (BSA)	n.a.	n.a.	Direct scalability	BioSeptra
S HyperDTM 10	Gel-in-a-shell	10	Sulfonate	80 (BSA)	n a	n.a.	Direct scalability	BioSeptra
S HyperDTM 20	Gel-in-a-shell	20	Sulfonate	80 (BSA)	n a	n.a.	Direct scalability	BioSeptra
DEAE Trisacryl M	Poly <i>N</i> -acryloyl-2-amino- 2-hydroxy 1,3-propanediol	40–80	Diethyl aminoethyl	n.a.	n.a.	n.a.	Extremely hydrophilic surface	BioSeptra
CM Trisacryl M	Poly <i>N</i> -acryloyl-2- amino-2-hydroxy 1,3-propanediol	40–80	Carboxymethyl	n.a.	n a.	n.a.	Extremely hydrophilic surface	BioSeptra
SP Trisacryl M	Poly <i>N</i> -acryloyl- 2-amino-2-hydroxy 1,3-propanediol	40–80	Sulfopropyl	n a	n.a.	n.a.	Extremely hydrophilic surface	BioSeptra

(continued)

TABLE 8 (continued)

Trade name	Matrix	Particle size (μm)	Ligand	Capacity (mg/mL gel)	pH stability	CIP stability	Remarks	Supplier
DEAE Trisacryl LS	Poly <i>N</i> -acryloyl-2-amino-2-hydroxy 1,3-propanediol	80–160	Diethyl aminoethyl	n.a.	n.a.	n.a.	Extremely hydrophilic surface	BioSeptra
CM Trisacryl LS	Poly <i>N</i> -acryloyl-2-amino-2-hydroxy 1,3-propanediol	80–160	Carboxymethyl	n.a.	n.a.	n.a.	Extremely hydrophilic surface	BioSeptra
SP Trisacryl LS	Poly <i>N</i> -acryloyl-2-amino-2-hydroxy 1,3-propanediol	80–160	Sulfopropyl	n.a.	n.a.	n.a.	Extremely hydrophilic surface	BioSeptra
DEAE Trisacryl Plus M	Poly <i>N</i> -acryloyl-2-amino-2-hydroxy 1,3-propanediol	40–80	Diethyl aminoethyl	n.a.	1–14	1–14	Excellent resistance to alkalinity	BioSeptra
SP Trisacryl Plus M	Poly <i>N</i> -acryloyl-2-amino-2-hydroxy 1,3-propanediol	40–80	Sulfopropyl	n.a.	1–14	1–14	Excellent resistance to alkalinity	BioSeptra
DEAE Trisacryl Plus LS	Poly <i>N</i> -acryloyl-2-amino-2-hydroxy 1,3-propanediol	80–160	Diethyl aminoethyl	n.a.	1–14	1–14	Excellent resistance to alkalinity	BioSeptra
SP Trisacryl Plus LS	Poly <i>N</i> -acryloyl-2-amino-2-hydroxy 1,3-propanediol	80–160	Sulfopropyl	n.a.	1–14	1–14	Excellent resistance to alkalinity	BioSeptra
DEA Spherosil M	Polymer-grafted silica	40–100	Diethyl aminoethyl	n.a.	n.a.	n.a.	Pore size (1000 Å)	BioSeptra
QMA Spherosil M	Polymer-grafted silica	40–100	Quaternary?	n.a.	n.a.	n.a.	Pore size (1000 Å)	BioSeptra

DEA Spherosil LS	Polymer-grafted silica	100–300	Diethyl aminoethyl	n.a.	n.a.	n.a.	Pore size (1000 Å)	BioSeptra
QMA Spherosil LS	Polymer-grafted silica	100–301	Quaternary?	n.a.	n.a.	n.a.	Pore size (1000 Å)	BioSeptra
DEAE Spherox M	Silica encapsulated by dextran	40–100	Diethyl aminoethyl	n.a.	n.a.	n.a.		BioSeptra
CM Spherox M	Silica encapsulated by dextran	40–100	Carboxymethyl	n.a.	n.a.	n.a.		BioSeptra
SP Spherox M	Silica encapsulated by dextran	40–100	Sulfopropyl	n.a.	n.a.	n.a.		BioSeptra
DEAE Spherox LS	Silica encapsulated by dextran	100–300	Diethyl aminoethyl	n.a.	n.a.	n.a.		BioSeptra
CM Spherox LS	Silica encapsulated by dextran	100–300	Carboxymethyl	n.a.	n.a.	n.a.		BioSeptra
SP Spherox LS	Silica encapsulated by dextran	100–300	Sulfopropyl	n.a.	n.a.	n.a.		BioSeptra
NucleoGEL SAX 1000-8/77	Polymer	8	Quaternary poly-ethylenimine	>40 mg (BSA)	1–13	n.a.		Macherey-Nagel
NucleoGEL SAX 4000-8/77	Polymer	8	Quaternary poly-ethylenimine	>20 mg (BSA)	1–13	n.a.		Macherey-Nagel
NucleoGEL SAX 1000-10	Polymer	10	Quaternary poly-ethylenimine	>40 mg (BSA)	1–13	n.a.		Macherey-Nagel
NucleoGEL SAX 4000-10	Polymer	10	Quaternary poly-ethylenimine	>20 mg (BSA)	1–13	n.a.		Macherey-Nagel
NucleoGEL SCX 1000-8/77	Polymer	8	Sulfonate	>40 mg (BSA)	1–13	n.a.		Macherey-Nagel
NucleoGEL SCX 4000-8/77	Polymer	8	Sulfonate	>20 mg (BSA)	1–13	n.a.		Macherey-Nagel
NucleoGEL SCX 1000-10	Polymer	10	Sulfonate	>40 mg (BSA)	1–13	n.a.		Macherey-Nagel
NucleoGEL SCX 4000-10	Polymer	10	Sulfonate	>20 mg (BSA)	1–13	n.a.		Macherey-Nagel

(continued)

TABLE 8 (continued)

Trade name	Matrix	Particle size (μm)	Ligand	Capacity (mg/mL gel)	pH stability	CIP stability	Remarks	Supplier
SepraSorb TM QM	Monolithic cellulose		Quaternary aminomethyl	62 mg (BSA)	n.a.	n.a.	High flow rate, omit column pretreatment step	Sepragen
SepraSorb TM DE	Monolithic cellulose		Diethylaminoethyl	59 mg (BSA)	n.a.	n.a.	High flow rate, omit column pretreatment step	Sepragen
SepraSorb TM SE	Monolithic cellulose		Sulfoxyethyl	34 mg (BSA)		n.a.	High flow rate, omit column, pretreatment step	Sepragen
SepraSorb TM CM	Monolithic cellulose		Carboxymethyl	51 mg (BSA)	n.a.	n.a.	High flow rate, omit column pretreatment step	Sepragen
YMC-Pack AX	Silica with polymer shell	6.5	Weak anion exchanger	6.6 mg (BSA)	2–8	n.a.		YMC

YMC-Pack CM	Silica with polymer shell	6,5	Carboxymethyl	2.6 mg (BSA)	2–8	n.a.		YMC
Amicon PAE-300	Silica	10, 20, 50	Weak anion exchanger	100 mg (BSA)	n.a.	n.a.		Millipore
Amicon PAE-1000	Silica	10, 20, 50	Weak anion exchanger	70 mg (BSA)	n.a.	n.a.		Millipore
Cellufine A-200	Cellulose	45–105	Diethyl aminoethyl	105 mg (BSA)	1–14	n.a.		Millipore
Cellufine A-500	Cellulose	45–105	Diethyl aminoethyl	78 mg (BSA)	1–14	n.a.		Millipore
Cellufine A-800	Cellulose	45–105	Diethyl aminoethyl	60 mg (BSA)	1–14	n.a.		Millipore
Cellufine C-200	Cellulose	45–105	Carboxymethyl	148 mg (Lys)	1–14	n.a.		Millipore
Cellufine C-500	Cellulose	45–105	Carboxymethyl	83 mg (BSA)	1–14	n.a.		Millipore
Hypersil WP300 SAX	Silica	5	Quaternary ammonium	n.a.	n.a.	n.a.		Shandon HPLC
CIM-DEAE	Polymethacrylate	Monolith	Diethyl aminoethyl	25 mg (BSA)	1–14	1–14	Extremely high flow rate	BIA
CIM-QE	Polymethacrylate	Monolith	Quaternary aminomethyl	25 mg (BSA)	1–14	1–14	Extremely high flow rate	BIA
CIM-SP	Polymethacrylate	Monolith	Sulfopropyl	30 mg (Lys)	1–14	1–14	Extremely high flow rate	BIA

biopolymers. As most proteins require a high salt concentration in the loading buffer for HIC, the technique is limited by the solubility of proteins in these buffers. Because proteins tend to precipitate in the required equilibration buffer, repetitive injection of loading buffer, and sample dissolved in normal buffer, has been developed to circumvent this problem. This method of for loading has never really been accepted by industry.

HIC is very well suited in combination with ion-exchange chromatography. Because biopolymers are eluted from ion-exchange chromatography with salt, simply addition of salt to the eluate of the ion exchanger generates the conditions for loading onto the HIC column. Elution is normally accomplished by using a buffer with a low salt content. The use of ammonium sulfate in loading buffers is a strong limitation for very large scale preparations. Ammonium sulfate causes severe disposal problems in wastewater plants because generation of H_2S is induced. The disposal costs often prevent the installation of an HIC step even though the separation efficiency is excellent. A compilation of HIC media used for separation of biopolymers is given in Table 9.

Media for Affinity Chromatography

Affinity chromatography, which was first described by Cuatrecasas et al. [78], has become the most efficient separation tool in biotechnology. Two types of media must be distinguished: ready-to-use affinity media and individual media. Ready-to-use media with generic ligands are commercially available. The most common generic ligand is protein A, a cell wall protein of *Staphylococcus aureus*. This protein binds various classes of IgG and other immunoglobulins [79] and has been successfully applied for industrial production of monoclonal antibodies. A list of commercially available ready-to-use affinity chromatography media is given in Table 10.

In the area of affinity chromatography, the users often prepare the media by immobilizing a ligand on a so-called preactivated matrix. The preactivated matrix carries reactive groups that enable the covalent coupling of the ligand. Because the direct immobilization of the ligand often leads to steric hindrance effects, an additional molecule called a "spacer" is introduced between the matrix and ligand. The choice of ligands is the most interesting and important part of affinity chromatography. Once a ligand has been identified, its handling becomes very simple.

Basis of Molecular Recognition and Specificity

The binding reaction between a biopolymer (ligate) and a molecular counterpart (ligand) results from the sum of various types of interactions. Noncovalent interactions such as van der Waals and electrostatic interactions, hydrogen bonding, and the size and shape of the molecules contribute to molecular

TABLE 9 Commercially Available Media for Hydrophobic Interaction Chromatography

Trade name	Matrix	Particle size (μm)	pH stability	CIP stability	Ligand	Capacity (mg/mL gel)	Remarks	Supplier
Butyl Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	45–165	3–13	2–14	Butyl	7 IgG or 27 HSA	For capture and intermediate purification	AP Biotech
Octyl Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	45–165	3–13	2–14	Octyl	n a.	For capture and intermediate purification	AP Biotech
Phenyl Sepharose 6 Fast Flow (high sub)	Highly cross-linked 6% agarose	45–165	3–13	2–14	Phenyl	30 IgG or 36 HSA	Very efficient for capture	AP Biotech
Phenyl Sepharose 6 Fast Flow (low sub)	Highly cross-linked 6% agarose	45–165	3–13	2–14	Phenyl	10 IgG or 24 HSA	Very efficient for capture	AP Biotech
Phenyl Sepharose High performance	Highly cross-linked 6% agarose	34	3–13	12–14	Phenyl	20 IgG or 24 HSA	Intermediate purification of polishing, high resolution	AP Biotech
Phenyl Sepharose CL-4B	Highly cross-linked 4% agarose	45–165	3–12	2–14	Phenyl	n a	Traditional for all applications	AP Biotech
Octyl Sepharose CL-4B	Highly cross-linked 4% agarose		3–13	2–14	Octyl	n.a.	Traditional for all applications	AP Biotech
SOURCE 15ETH	Polystyrene/divinylbenzene	15 Monosized	2–12	2–14	Ether	n a	For polishing, high resolution at high loading and high flow rates	AP Biotech
SOURCE 15ISO	Polystyrene/divinylbenzene	15 monosized	2–12	2–14	Isopropyl	n.a	For polishing, high resolution at high loading and high flow rates	AP Biotech

(continued)

TABLE 9 (continued)

Trade name	Matrix	Particle size (μm)	pH stability	CIP stability	Ligand	Capacity (mg/mL gel)	Remarks	Supplier
SOURCE 15PHE	Polystyrene/divinylbenzene	16 monosized	2–12	2–14	Phenyl	n.a.	For polishing, high resolution at high loading and high flow rates	Pharmacia
Fractogel EMD Propyl (S)	Polymer	20–40	1–14	n.a.	Propyl	35 ovalbumin		Merck
Fractogel EMD Phenyl (S)	Polymer	20–40	1–14	n.a.	Phenyl	20 ovalbumin		Merck
POROS 50 P	Polystyrene/divinylbenzene	50	1–14	n.a.	Phenyl ether	8 BSA		PerSeptive
POROS 20 PE	Polystyrene/divinylbenzene	20	1–14	n.a.	Phenyl ether	8 BSA		PerSeptive
POROS 20 HP2	Polystyrene/divinylbenzene	20	1–14	n.a.	High-density phenyl	12 BSA	For weakly hydrophobic molecules	PerSeptive
POROS 20 ET	Polystyrene/divinylbenzene	20	1–14	n.a.	Ethyl ether	4 BSA	For high-molecular-weight molecules	PerSeptive
Macro-Prep methyl HIC	Methacrylate based	50	1–14	n.a.	Methyl	>25 HSA		Bio-Rad
Macro-Prep t-Butyl HIC	Methacrylate based	50	1–14	n.a.	Tertiary butyl	>15 HSA		Bio-Rad
TSK-GEL Phenyl-5PW	Polymer	20–40	n.a.	n.a.	Phenyl	10–30 lysozyme		Tosoh
TSK-GEL Ether-5PW	Polymer	20–40	n.a.	n.a.	Ether	10–30 lysozyme		Tosoh
Toyopearl-Ether-650S	Polymer	20–50	2–12	n.a.	Ether	10–30 lysozyme		Tosoh
Toyopearl-Ether-650M	Polymer	40–90	2–12	n.a.	Ether	10–30 lysozyme		Tosoh
Toyopearl-Butyl-650S	Polymer	20–50	2–12	n.a.	Butyl	30–50 lysozyme		Tosoh

Toyopearl-Butyl-650C	Polymer	40–90	2–12	n.a.	Butyl	30–50 lysozyme	Tosoh
Toyopearl-Butyl-650M	Polymer	50–150	2–12	n.a.	Butyl	30–50 lysozyme	Tosoh
Toyopearl-Phenyl-650S	Polymer	20–50	2–12	n.a.	Phenyl	30–50 lysozyme	Tosoh
Toyopearl-Phenyl-650M	Polymer	40–90	2–12	n.a.	Phenyl	30–50 lysozyme	Tosoh
Toyopearl-Phenyl-650C	Polymer	50–150	2–12	n.a.	Phenyl	30–50 lysozyme	Tosoh
BAKERBOND HI-Propyl	Silica	15	2–7	n.a.	Isopropyl	n.a.	J.T.Baker
Phenyl Agarose 4XL/6XL	4% or 6% cross-linked agarose	45–165	3–13	2–14	Phenyl	34–42/47–63 BSA	Affinity Chromatography Ltd
Butyl Agarose 4XL/6XL	4% or 6% cross-linked agarose	45–165	3–13	2–14	Butyl	5–15/15–25 BSA	Affinity Chromatography Ltd
Hexyl Agarose 4XL/6XL	4% or 6% cross-linked agarose	45–165	3–13	2–14	Hexyl	13–23/20–30 BSA	Affinity Chromatography Ltd
Octyl Agarose 4XL/6XL	4% or 6% cross-linked agarose	45–165	3–13	2–14	Octyl	18–28/30–40 BSA	Affinity Chromatography Ltd
Decyl Agarose 4XL/6XL	4% or 6% cross-linked agarose	45–165	3–13	2–14	Decyl	25–35/40–50 BSA	Affinity Chromatography Ltd
YMC-Pack HIC PAE-1000L	Silica	6, 5	2–8	n.a.	Multiple propyl	5.0 mg ovalbumin	YMC
Cellufine Butyl	Silica	10, 20, 50	n.a.	n.a.	Similar to DEAE	n.a.	Millipore
Cellufine Phenyl	Cellulose	53–125	3–14	n.a.	Butyl	n.a.	Millipore
Cellufine Octyl	Cellulose	53–125	3–14	n.a.	Phenyl	n.a.	Millipore
Hyperprep PEP 300 C4, C8, C18	Cellulose	53–125	3–14	n.a.	Octyl	n.a.	Millipore
	Modified silica	8, 15	2.2–8.8	n.a.	C4, C8 C18	n.a.	Shandon HPLC

TABLE 10 Commercially Available Media for Affinity Chromatography

Trade name	Matrix	Ligand	Ligand density	pH stability	Particle size (μm)	Target molecules or groups	Supplier
Glutathione Sepharose 4B	4% agarose	Glutathione	n.a.	n.a.	45–165	S-transferase and glutathione-dependent proteins	AP Biotech
Calmodulin Sepharose 4B	4% agarose	Calmodulin	n.a.	n.a.	45–165	Calmodulin-regulated proteins	AP Biotech
Con A Sepharose 4B	4% agarose	Concanavalin A	10–16 mg/mL	4–9	45–165	Immunoglobulins, hormone receptors, polysaccharides, glycoproteins, membrane proteins, lipoproteins	AP Biotech
Lentil lectin Sepharose 4B	4% agarose	Lentil lectin	2 mg/mL	3–10	45–165	Membrane proteins	AP Biotech
Agarose Wheat Germ Lectin	Agarose	Wheat germ lectin	1–2 mg/mL	n.a.	n.a.	Glycoproteins, polysaccharides, membrane proteins	AP Biotech
Wheat Germ Lectin Sepharose 6MB	6% agarose	Wheat germ lectin	5 mg/mL	n.a.	200–300	Glycoproteins, polysaccharides, membrane proteins	AP Biotech
Arginine Sepharose 4B	4% agarose	L-Arginine	14–20 $\mu\text{mol/mL}$	2–13	45–165	Coagulation proteins	AP Biotech
ECH-Lysine Sepharosa 4 Fast Flow	Highly cross-linked 4% agarose	L-Lysine	12 $\mu\text{mol/mL}$	4–13	45–165	Nucleic	AP Biotech

Lysine Sepharose 4B	4% agarose	L-Lysine	4–7 $\mu\text{mol/mL}$	2–11	45–165	Nucleic acids	AP Biotech
Gelatin Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	Gelatin	5 mg/mL	3–10	45–165	Fibronectin	AP Biotech
Gelatin Sepharose 4B	4% agarose	Gelatin	4.5–8 mg/mL	3–10	45–165	Fibronectin	AP Biotech
Heparin Sepharose CL-6B	Cross-linked 6% agarose	Heparin	~ 3 mg/mL	5–10	45–165	Growth factors, protein synthesis factors, hormone receptors, coagulation proteins, lipoproteins, enzymes	AP Biotech
Heparin Sepharose 6 Fast Flow	Highly cross-linked 6% agarose	Heparin	~ 5 mg/mL	4–12	45–165	Growth factors, protein synthesis factors, hormone receptors, coagulation proteins, lipoproteins, enzymes	AP Biotech
Benzamidine Sepharose 6B	6% agarose	<i>p</i> -Aminobenzamidine	n a.	2–13	45–165	Serine protease	AP Biotech
IgG Sepharose 6 Fast Flow	Highly cross-linked 6% agarose	IgG	n a.	3–10	45–165	Protein A	AP Biotech
Blue Sepharose CL-6B	Cross-linked 6% agarose	Cibacron Blue 3G	~ 2 $\mu\text{mol/mL}$	4–12	45–165	Growth factors, coagulation proteins, NAD ⁺ -dependent, ATP-dependent	AP Biotech

(continued)

TABLE 10 (continued)

Trade name	Matrix	Ligand	Ligand density	pH stability	Particle size (μm)	Target molecules or groups	Supplier
Blue Sepharose 6 Fast Flow	Highly cross-linked 6% agarose	Cibacron Blue 3G	~ 7 μmol/mL	4–12	45–165	Growth factors, coagulation proteins, NAD ⁺ -dependent, NADP ⁺ -dependent, ATP-dependent	AP Biotech
Chelating Sepharose 6B	6% agarose	Iminodiacetic acid groups	n.a.	3–13	45–165	Chelating Interaction	AP Biotech
Chelating Sepharose 4 Fast Flow	Highly cross-linked 6% agarose	Iminodiacetic acid groups	n a.	3–13	45–165	Chelating Interaction	AP Biotech
rProtein A Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	Recombinant protein A	~ 6 mg/mL	3–9	60–165	Immunoglobulins	AP Biotech
Protein A Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	Protein A	~ 6 mg/mL	3–9	45–165	Immunoglobulins	AP Biotech
Protein A Sepharose CL-4B	Cross-linked 4% agarose	Protein A	~ 3 mg/mL	3–9	45–165	Immunoglobulins	AP Biotech
Protein G Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	Recombinant protein G	~ 2 mg/mL	3–9	45–165	Immunoglobulins	AP Biotech
GammaBind G Sepharose	4% agarose	Recombinant gamma Bind G type 3	~ 3 mg/mL	3–9	45–165	Immunoglobulins	AP Biotech
GammaBind Plus Sepharose	Highly cross-linked 6% agarose	Recombinant gamma Bind G type 3	~ 3 mg/mL	3–9	45–165	Immunoglobulins	AP Biotech

Red Sepharose CL-6B	Highly cross-linked 6% agarose	Procion Red HE-3B	~ 2 μ mol/mL	4–12	45–165	Immunoglobulins	AP Biotech
DNA-Agarose (denatured) single stranded	4% agarose copolymerised with ligand	Denatured calf thymus	1–3 mg/mL	n.a.	45–165	DNA polymerase, RNA polymerase, T4 polynucleotide kinase, exonuclease, other deoxyribonucleases	AP Biotech
DNA-Cellulose (nativeDNA) double stranded	Cellulose	Native calf thymus DANN	0.25–1.5 mg/mL	n.a.	45–165	Glucocorticoid receptor, DNA polymerase, DNA binding proteins, exonuclease	AP Biotech
DNA-Cellulose (denatured)	Cellulose	Denatured calf thymus DANN	0.25–1.5 mg/mL	n.a.	45–165	DNA polymerase, RNA polymerase, DNA binding proteins, DNases, exonuclease	AP Biotech
2'5' ADP Sepharose 4B	4% agarose	2'5' ADP	2 μ mol/mL	n.a.	45–165	NADP-dependent dehydrogenases	AP Biotech
5' AMP Sepharose 4B	4% agarose	5' AMP	2 μ mol/mL	n.a.	45–165	NAD-dependent dehydrogenases, ATP-dependent kinases	AP Biotech
7-MethylGTP Sepharose 4B	4% agarose	7-Methyl-GTP	0.2 μ mol/mL	n.a.	45–165	Eukaryotic mRNA cap-binding protein	AP Biotech

(continued)

TABLE 10 (continued)

Trade name	Matrix	Ligand	Ligand density	pH stability	Particle size (μm)	Target molecules or groups	Supplier
Poly(A) Sepharose 4B	4% agarose	~ 100 nucleotides long polyadenylic acid	~ 0.25 mg/mL	n.a.	45–165	mRNA-binding proteins, poly(A)-binding RNA, DNA-dependent RNA polymerase, antibodies to nucleic acids and tubulin assembly protein	AP Biotech
Poly(U) Sepharose 4B	4% agarose	~ 100 nucleotides long polyuridylic acid	~ 0.5 mg/mL	n.a.	45–165	mRNA, reverse transcriptase, interferon, and nucleic acids from plants	AP Biotech
AGPOLY(I)-POLY(C) Type 6	4% agarose	Poly(I) poly(C)	1–4 mg/mL	n.a.	45–165	Interferon induction	AP Biotech
AGPOLY(U) Type 6	4% agarose	Poly (U)	2–4 mg/mL	n.a.	45–165	Detection of nucleotide sequences, isolation of ribosomes, mRNA and polynucleotide 5'-triphosphatase	AP Biotech
Affi-Prep Protein A	Pressure stable polymer	Protein A	2 mg/mL	2–10	n.a.	Immunoglobulins	Bio-Rad

Affi-Gel Blue gel	Cross-linked agarose	Cibacron Blue F3GA	1.9 mg/mL	2–10	n.a.	Nucleotide-requiring enzymes, albumin, other proteins	Bio-Rad
CM Affi-Gel Blue gel	Cross-linked agarose	Cibacron blue F3GA, and CM groups	n.a.	2–11	n.a.	Albumin and serum proteins	Bio-Rad
DEAE Affi-Gel Blue gel	Cross-linked agarose	Cibacron Blue F3GA, and DEAE groups	n.a.	2–10	n.a.	Albumin and serum proteins	Bio-Rad
Affi-Gel Heparin gel	Cross-linked agarose	Heparin	~ 0.6 mg/mL	5–10	n.a.	Growth factors, coagulation factors, enzymes, lipase, proteases	Bio-Rad
Affi-Prep polymyxin	Pressure stable polymer	Polymyxin	2–4 mg/mL	2–10	n.a.	Endotoxin removal	Bio-Rad
Affi-Gel 501 gel	Cross-linked agarose	Organomercurial	3–10 μ mol/mL	4–8	n.a.	Sulfhydryl proteins	Bio-Rad
Affi-Gel 601 gel	Polyacrylamide gel	Boronate	1.05 mEq/g	2–10	n.a.	cis-Hydroxyl containing molecules	Bio-Rad
Toyopearl AF-Chelate-650M	Methacryl	Iminodiacetic acid	35 μ mol/g	2–14	65	Chelating interaction	AP Biotech Tosoh
Toyopearl AF-Heparin-650M	Methacryl	Heparin	n.a.	2–14	65	Growth factors, coagulation factors, enzymes, lipase, proteases	Tosoh

(continued)

TABLE 10 (continued)

Trade name	Matrix	Ligand	Ligand density	pH stability	Particle size (μm)	Target molecules or groups	Supplier
Toyopearl AF-Blue-650ML	Methacryl	Cibachron Blue F3GA	10 μmol/g	2–14	65	Nucleotide-requiring enzymes, albumin, other proteins	Tosoh
Toyopearl AF-Blue-650MH	Methacryl	Cibachron Blue F3GA	18 μmol/g	2–14	65	Nucleotide-requiring enzymes, albumin, other proteins	Tosoh
Toyopearl AF-Red-650ML	Methacryl	Procion Red HE-3B	7 μmol/g	2–14	65	Enzymes and other proteins	Tosoh
Toyopearl AF-Protein A-650M	Methacryl	rProtein A	4 μg/mL	2–14	65	IgG	Tosoh
Fractogel EMD Chelat-650 (S)	Polymer	Iminodiacetic acid groups	n.a.	n.a.	20–40	Chelating Interaction	Merck
Fractogel EMD TA 650-(S)	Polymer	Sulfur containing	n.a.	n.a.	20–40	Proteins with thiophilic regions and peptides with aromatic amino residues	Merck
Fractogel EMD Heparin 650-(S)	Polymer	Heparin	n.a.	n.a.	20–40	Growth factors, coagulation factors, enzymes, lipase, proteases	Merck

Mimetic Affinity Media	6% cross-linked agarose	Synthetic dye	2.5 $\mu\text{mol/mL}$	2–13	45–165	Growth factors, coagulation proteins, NAD^+ -dependent, NADP^+ -dependent, ATP-dependent	Affinity Chromatography Ltd
Mimetic Red 3 A6XL	6% cross-linked agarose	Synthetic dye	2.5 $\mu\text{mol/mL}$	2–13	45–165	Growth factors, coagulation proteins, NAD^+ -dependent, NADP^+ -dependent, ATP-dependent	Affinity Chromatography Ltd
Mimetic Red 3 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd
Mimetic Orange 2 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd
Mimetic Orange 3 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd
Mimetic Yellow 1 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd
Mimetic Yellow 2 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd
Mimetic Green 1 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd
Mimetic Blue 2 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd

(continued)

TABLE 10 (continued)

Trade name	Matrix	Ligand	Ligand density	pH stability	Particle size (μm)	Target molecules or groups	Supplier
Mimetic Blue 1 A6XL	6% cross-linked agarose	Synthetic dye	n.a.	n.a.	n.a.		Affinity Chromatography Ltd
Aminobenzamidine Agarose 6XL	6% cross-linked agarose	Aminobenzamidine	7.4 $\mu\text{mol/mL}$	<9	n.a.	Serine proteases and esterases	Affinity Chromatography Ltd
Arginine Agarose 6XL	6% cross-linked agarose	L-Arginine	5 $\mu\text{mol/mL}$	n.a.	n.a.	Proteases	Affinity Chromatography Ltd
Lysine Agarose 6XL	6% cross-linked agarose	L-Lysine	10 $\mu\text{mol/mL}$	n.a.	n.a.	Proteases and nucleic acids	Affinity Chromatography Ltd
Heparin Agarose 6XL	6% cross-linked agarose	Heparin	n.a.	n.a.	45–165	Growth factors, coagulation factors, enzymes, lipase, proteases	Affinity Chromatography Ltd
IgG Agarose 4XL	4% cross-linked agarose	Human IgG	n.a.	n.a.	45–165	Protein A, protein G, recombinant gene fusion proteins	Affinity Chromatography Ltd
Protein A Agarose 4 XL	4% cross-linked agarose	Protein G	2.5 $\mu\text{mol/mL}$	2–12	45–165	IgG	Affinity Chromatography Ltd
Protein G Agarose 4XL	6% cross-linked agarose	Protein A	2.0 $\mu\text{mol/mL}$	2–11	45–165	IgG	Affinity Chromatography Ltd
Aminophenyl Boronate Agarose 6XL	4% cross-linked agarose	Aminophenyl boronate	n.a.	3–11	45–165	Carbohydrates, nucleic acids, proteinases	Affinity Chromatography Ltd

PROSEP-A High Capacity	High silica glass	Protein A	n.a.	<8	n.a.	IgG	Bioprocessing
PROSEP-RemTox	High silica glass	Synthetic small ligand	n.a.	n.a.	n.a.	Endotoxins	Bioprocessing
PROSEP-Thiosorb	High silica glass	Thiol	n.a.	<8	n.a.	–SH-containing proteins	Bioprocessing
PROSEP-Heparin	High silica glass	Heparin	n.a.	n.a.	n.a.	Growth factors, coagulation factors, enzymes, lipase, proteases	Bioprocessing
PROSEP-Chelating I	High silica glass	IDA	n.a.	n.a.	n.a.	Chelating interaction	Bioprocessing
Protein A HyperD™	Gel-in-a-shell	Protein A	n.a.	n.a.	n.a.	IgG	BioSeptra
Heparin HyperD™	Gel-in-a-shell	Porcine heparin		n.a.	70	ATII, FIX, growth hormones	BioSeptra
Blue HyperD™	Gel-in-a-shell	Basilene Blue Dye	n.a.	n.a.	n.a.	Growth factors, coagulation proteins, interferons, HSA	BioSeptra
Lysine HyperD™	Gel-in-a-shell	L-Lysine	n.a.	n.a.	70	Plasminogen, Ref. glycoproteins, rRNA	BioSeptra
Blue Trisacyl M	Poly <i>N</i> -acryloyl-2-amino-2-hydroxyl-1,3-propanediol	Basilene Blue Dye	n.a.	n.a.	40–80	Growth factors, coagulation proteins, interferons, HSA	BioSeptra
Blue Trisacyl Plus LS	Poly <i>N</i> -acryloyl-2-amino-2-hydroxyl-1,3-propanediol	Basilene Blue Dye	n.a.	n.a.	80–160	Growth factors, coagulation proteins, interferons, HSA	BioSeptra
Gelatin Ultrogel	5% agarose	Gelatin	15–20 mg/mL	n.a.	60–180	Fibronectin, enzymes	BioSeptra

(continued)

TABLE 10 (continued)

Trade name	Matrix	Ligand	Ligand density	pH stability	Particle size (μm)	Target molecules or groups	Supplier
Phospho Ultrogel A6R	6% cross-linked agarose	Phosphate	n.a.	n.a.	60–140	Kinases, ATPases, DNA polymerases, dehydrogenases	BioSeptra
NucleoGEL AFC Protein A77	Hydrophile	Protein A	n.a.	1–13	10	IgG	Macherey-Nagel GmbH
Amicon Matrex Blue A	Agarose	Ciba Blue F3GA	n.a.	2–12	50–150	Enzymes and other proteins	Millipore
Amicon Matrex Blue B	Agarose	Blue Dye (proprietary)	n.a.	2–12	50–150	Enzymes and other proteins	Millipore
Amicon Matrex Red A	Agarose	Procion Red HE-3B	n.a.	2–12	50–150	Enzymes and other proteins	Millipore
Amicon Matrex Orange A	Agarose	Orange A (proprietary)	n.a.	2–12	50–150	Enzymes and other proteins	Millipore
Amicon Matrex Green A	Agarose	Green A (proprietary)	n.a.	2–12	50–150	Enzymes and other proteins	Millipore
Amicon PBA-10	Agarose	Phenyl boronate	10–15 $\mu\text{mol/mL}$	2–12	50–150	1,2-diol contained biomolecules	Millipore
Amicon PBA-30	Agarose	Phenyl boronate	30–15 $\mu\text{mol/mL}$	2–12	50–150	1,2-diol contained biomolecules	Millipore
Amicon PBA-60	Agarose	Phenyl boronate	60–100 $\mu\text{mol/mL}$	2–12	50–150	1,2-diol contained biomolecules	Millipore
Cellufine Sulfate	Cellulose	Sulfate	6–8 mg Lysine/mL	0.1 N NaOH	45–105	Virus, heparin-binding proteins	Millipore
Cellufine Chelate	Cellulose	Imidodiacetate	22–30 $\mu\text{mol Zn}^{2+}$	0.1 N NaOH	125–210	Chelating interaction	Millipore
HiPAC Protein A	Silica	Protein A	n.a.	n.a.	30	IgG	Shandon HPLC
HiPAC Protein A 4000	Silica	Protein A	n.a.	n.a.	30	IgG pore size 4000 Å	Shandon HPLC
HiPAC Protein G	Silica	Protein G	n.a.	n.a.	30	IgG	Shandon HPLC

HiPAC Hydrazine	Silica	Hydrazine	n.a.	n a.	30	Glycoproteins immobilization	Shandon HPLC
POROS 50 A	Polystyrene-divinylbenzene	Recombinant protein A	30 mg/mL	1–14	50	Immunoglobulins	PerSeptive
POROS 20 A	Polystyrene-divinylbenzene	Recombinant protein A	30 mg/mL	1–14	20	Immunoglobulins	PerSeptive
POROS 20 G	Polystyrene-divinylbenzene	Recombinant protein G	15 mg/mL	1–14	20	Immunoglobulins	PerSeptive
POROS 20 HE	Polystyrene-divinylbenzene	Heparin	15 mg/mL lysozyme	1–14	20	Growth factors, coagulation factors, enzymes, lipase, proteases	PerSeptive
POROS 20- MC	Polystyrene-divinylbenzene	Imidodiacetate	15 mg/mL myoglobin Cu ²⁺ -form	1–14	20	Growth factors, coagulation factors, enzymes, lipase, proteases	PerSeptive

recognition. Although the individual contributions to the binding strength are very small, the overall binding strength can be quite high and reach dimensions equivalent to those of covalent bonds. In some cases, the separation of the ligate-ligand complex is possible only by applying slightly denaturing conditions. As mentioned before, the principle of affinity chromatography was described for the first time by Cuatrecasas et al. [78]. Currently, the technical term affinity chromatography is divided into several subcategories such as immunoaffinity chromatography, pseudoaffinity chromatography, metal chelate affinity chromatography, and others.

Because terms such as affinity, avidity and specificity are often confusing, we will attempt to define these terms. Specificity is the nature of the molecular recognition and is based on the unique features of the interaction between two molecules. Only certain species of molecules will interact. Specificity is a stochastic term. Affinity, a thermodynamic term, is a measure of the binding strength. The reverse conclusion, that high affinity also implies high specificity, is not valid. The binding of proteins to a hydrophobic surface is a good example. The binding strength is very high but not specific. This strong binding does not underlie the principle of molecular recognition. Avidity, often used by immunologists, is an expression for affinity under operational conditions.

Screening of Ligands

Affinity chromatography is very simple if a specific ligand is available. The search for novel ligands has been improved in the past decade. Previously, ligands were selected from natural sources such as enzyme-substrate, enzyme-inhibitor, enzyme-cofactor, antibody-antigen, DNA binding, protein-oligonucleotide, lectin-sugar moiety, hormone-receptor, complexes, etc. Development of monoclonal antibodies and recombinant DNA technologies have broadened the spectrum of ligands. Today we are not restricted to the ligands found in nature. Novel methods of combinatorial chemistry and development of biological peptide libraries provide an unlimited source of ligands. A library is defined as a mixture of peptides with different amino acid sequences. Either the mixture is encoded by the DNA sequence or the peptides are bound to various formats of a solid carrier.

In cell-linked libraries, the peptide is presented at the surface of a virus or a cell [80]. Each cell presents only one individual peptide sequence at the surface. A panning cycle enriches these cells, carrying peptides with high affinity. The cells are passed over a column or incubated in a vial coated with the target protein. The cells displaying a peptide with a high affinity bind and the others are washed off. The bound cells or viruses are eluted and propagated. After several panning rounds, a population with high-affinity peptides survives. Individual colonies of the organisms can be selected, and special construction of the expression plasmid of the peptide allows rapid identification of the sequence of the peptides.

Combinatorial chemistry has become a fast-growing discipline and it would exceed the scope of this chapter to explain all its methodological approaches. Li et al. [81] have developed synthetic ligands for antibody purification by combinatorial chemistry. These ligands mimic the binding of protein A to antibodies. Combinatorial peptide chemistry has also been successfully applied for de novo generation of ligands. Houghten et al. [81a] have described multiple-peptide synthesis such as the tea-bag method. Meanwhile, the methods have been refined and improved. Modern multiple-peptide synthesis allows synthesis of peptides on cellulose sheets, on pins, or on chips so that up to 40,000 peptides can be synthesized in parallel.

Immobilization of Ligands

The methods for immobilization can be divided into methods for inorganic and organic matrices. The immobilization procedure consists of several steps:

1. Introduction of a group that can be activated (necessary only for inorganic matrices)
2. Activation of the matrix
3. Coupling of a spacer (optional)
4. Coupling of the ligand
5. Blocking of the residual active groups
6. Washing out of unreacted residuals and reaction by-products (very critical when the affinity medium is used for purification of a therapeutic protein)

Inorganic chromatography matrices such as glass, silica, aluminum oxide, titanium oxide, zirconium oxide, bentonite, and zeolites do not possess groups that can be activated. Therefore, amino groups are usually introduced by reaction with aminoalkylethoxysilanes or aminoalkylchlorosilanes. These amino groups can be further activated and used for reaction with a spacer or directly with the ligand.

Many organic polymers already possess an amino, carboxyl, or hydroxy group. BrCN activation was very popular, but this activation did not give a very stable linkage between the ligand and matrix. The *N*-hydroxyl succinimide (NHS) activation has displaced the BrCN activation because it is very well suited for immobilization of peptides, proteins, and oligonucleotides. A peptide bond is formed between a carboxy group on the matrix and the amino group on the ligand. The activation of the carboxy groups is started by addition of *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide (EDC) and NHS, forming an active ester. When a compound with an NH_2 group is added, the peptide bond is formed. The optimal pH for this reaction is about pH 8.5. The immobilization does not solely react with the N-terminal amino group, and ϵ -amino groups and other reactive groups are also affected. If free sulphydryl groups are available, the

reaction prefers this pathway; however, the bonds formed with SH groups are not very stable and excessive bleeding of ligand during operation has been observed.

Immobilization of a protein via the carboxyl group can be easily accomplished by simultaneous reaction of EDC with the protein. The formed product is then incubated with the matrices in the presence of NHS. Using this reaction, the intermolecular cross-linking is substantially reduced.

Ligand leakage is a severe problem in affinity chromatography and must be carefully validated when the matrix is used for production of therapeutic proteins. Leakage is more critical when a toxic ligand (dye in dye affinity chromatography) is used. A list of preactivated matrices and matrices with generic ligands is given in Table 11.

Fusion Proteins

Fusion proteins are artificial proteins composed of a peptide or protein fragment and a protein. The fusion is made on the DNA level by combining both coding sequences. Often a sequence encoding a special peptide fragment used as a proteolytic cleavage site is inserted between fusion partners. Fusion proteins have been designed for various purposes. One fusion partner may serve as a recognition site for a generic affinity ligand to exploit highly selective affinity techniques when a ligand for the target protein is not available. Alternatively, a fusion partner can be used for rapid detection; for this purpose, the enzymes β -galactosidase and luciferase have been widely used. Although promising, fusion protein technology has not been generally successful in industrial applications because the cleavage of the fusion partner is a critical step. Fusion proteins are very popular in the biochemistry laboratory because they represent a rapid method for overexpression and purification of proteins. Examples of protein fusions are reviewed by Kaul and Mattiasson [82] and listed in Table 12.

Media for Size Exclusion Chromatography

Size exclusion chromatography (SEC) has a broad application for rapid desalting and buffer exchange in the production of proteins and other biopolymers. Other applications for SEC are separation of dimers from monomers or degradation products. Usually SEC is avoided for production purposes because it works at a lower rate than other modes and the feed is diluted during the separation process. Applications where the product is eluted in the void volume are preferred because the sample becomes less diluted. Another drawback is the viscous fingering that is often observed with highly concentrated solutions where the introduced sample plug forms finger-like structures. These structures have been visualized by nuclear magnetic resonance (NMR) techniques [83]. It is obvious that under these conditions, additional band broadening will occur and the sample will be further diluted. To avoid viscous fingering, the concentration of the sample

TABLE 11 Commercially Available Preactivated Media for Immobilization of Ligands

Trade name	Matrix	Target molecules to be coupled	Functional group	Ligand density ($\mu\text{mol/ml gel}$)	Particle size (μm)	Supplier
CNBr-Activated Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	Proteins, peptides	$-\text{NH}_2$	n.a.	45–165	AP Biotech
NHS-Activated Sepharose 4 Fast Flow	Highly cross-linked 4% agarose	Proteins, peptides	$-\text{NH}_2$	n.a.	45–165	AP Biotech
CNBr-Activated Sepharose 4B	4% agarose	Proteins, peptides	$-\text{NH}_2$	n.a.	45–165	AP Biotech
CH Sepharose 4B	4% agarose	Proteins, peptides	$-\text{NH}_2$	10–14 μmol	45–165	AP Biotech
AH Sepharose 4B	4% agarose	Amino acids, carboxylic acids	$-\text{COOH}$	6–10 μmol	45–165	AP Biotech
Epoxy-activated Sepharose 6B	4% agarose	Proteins, carbohydrates, thiol compounds, amines	$-\text{NH}_2$, $-\text{OH}$, $-\text{SH}$	19–40 μmol	45–165	AP Biotech
Activated CH Sepharose 4B	4% agarose	Amino acids, peptides	$-\text{NH}_2$	n.a.	45–165	AP Biotech
EAH Sepharose 4B	4% agarose	Amino acids, carboxylic acids	$-\text{COOH}$	7–11 μmol	45–165	AP Biotech
ECH Sepharose 4B	4% agarose	Amino acids, peptides	$-\text{NH}_2$	n.a.	45–165	AP Biotech
Agarose Adipic Acid Hydrazide	Agarose	Aldehydes	$-\text{CHO}_2$	1–6 μmol	45–165	AP Biotech
Thiopropyl Sepharose 6B	6% agarose	Thiol compounds	$-\text{C}=\text{O}$, $-\text{C}=\text{C}$, $-\text{N}=\text{N}$, $-\text{SH}$	$\sim 20 \mu\text{mol}$	45–165	AP Biotech
Toyopearl AF-Tresyl-650M	Methacryl	Amines, thiols (incl proteins)	$-\text{NH}_2$, $-\text{SH}$	80 μmol	65	Tosoh
	Methacryl	Proteins, carbohydrates, thiol compounds, amines	$-\text{NH}_2$, $-\text{OH}$, $-\text{SH}$	800 μmol	65	Tosoh

(continued)

TABLE 11 (continued)

Trade name	Matrix	Target molecules to be coupled	Functional group	Ligand density ($\mu\text{mol/ml gel}$)	Particle size (μm)	Supplier
Toyopearl AF-Formyl-650M	Methacryl	Proteins	$-\text{NH}_2$	60 μmol	65	Tosoh
Toyopearl AF-Amino-650M	Methacryl	Carboxylic acids	$-\text{COOH}$	100 μmol	65	Tosoh
Affi-Gel 102 Gel	Cross-linked agarose	Amino acids, ketoacids, carboxylic acids	$-\text{COOH}$	n.a.	n.a.	BioRad
Aminoethyl Bio-Gel	Aminoethylpolyacrylamide	Amino acids, ketoacids, carboxylic acids	$-\text{COOH}$	n.a.	n.a.	BioRad
CM-Bio Gel A	Agarose	Amines, amino acids, peptides	$-\text{NH}_2$	n.a.	n.a.	BioRad
Affi-Prep 10	Acrylic	Proteins	$-\text{NH}_2$	n.a.	n.a.	BioRad
Affi-Prep Hz	Polymer	Proteins	$-\text{NH}_2$	n.a.	n.a.	Bio-Rad
Cellufine Formyl	Cellulose	Proteins	$-\text{NH}_2$	n.a.	125–210	Millipore
Cellufine Amino	Cellulose	Carboxylic acids	$-\text{COOH}$	n.a.	125–210	Millipore
Cellufine Carboxyl	Cellulose	Proteins	$-\text{NH}_2$	n.a.	125–210	Millipore
HiPAC Aldehyde	Silica	Proteins	$-\text{NH}_2$	n.a.	30	Shandon HPLC
HiPAC Hydrazine	Silica	Glycoproteins	$-\text{CHO}$	n.a.	30	Shandon HPLC
Fratogel EMD Epoxy	Polymer	Proteins, carbohydrates, thiol compounds, amines	$-\text{NH}_2$, $-\text{SH}$, $-\text{OH}$	n.a.	20–40	Merck

Fratogel EMD Aziacton	Polymer	Proteins, peptides	–NH ₂	n.a.	20–40	Merck
Reacti-Gel (GX)	6% cross-linked agarose	Proteins, peptides	–NH ₂	n.a.	n.a.	Pierce
Reacti-Gel (HW-65F)	Polymer	Proteins, peptides	–NH ₂	n.a.	n.a.	Pierce
Reacti-Gel (25DF)	Cross-linked dextran	Proteins, peptides	–NH ₂	n.a.	n.a.	Pierce
Reacti-Gel (GF-2000)	Ultrogel	Proteins, peptides	–NH ₂	n.a.	n.a.	Pierce
POROS OH	Polystyrene- divinylbenzene	For initial activation with CNBr, tresyl chloride etc.	–OH	n.a.	20, 50	PerSeptive
POROS AL (Aldehyde)	Polystyrene- divinylbenzene	Proteins, peptides	–CHO	n.a.	20	PerSeptive
POROS EP (Epoxide)	Polystyrene- divinylbenzene	Proteins, carbohydrates, thiol compounds, amines	–NH ₂ , –SH –OH	n.a.	20	PerSeptive
POROS NH (Amino)	Polystyrene- Divinylbenzene	Glycoproteins, carbohydrates	–OH	n.a.	20, 50	PerSeptive
POROS HY (Hydrazide)	Polystyrene- divinylbenzene	For site-specific coupling of IgG or other glycosylated molecules	–OH	n.a.	20	PerSeptive

TABLE 12 Fusion Proteins for Purification and Detection of Proteins

Affinity tail	Affinity tag or type of chromatography	Limitations	References
Peptides			
Arg-Gly-Arg-Gly-Gly-Arg	Anhydro-trypsin	Coelution of contaminants	131
Poly-Cys	Covalent chromatography	High Cys concentration down-regulates the expression rate	
Poly-Phe	Hydrophobic interaction chromatography		132
Poly-His	Ni-chelate chromatography	Decrease of pH may destroy protein	133
Poly-Arg	Cation-exchange		134
FLAG-peptide	Anti-FLAG antibody	Two different antibodies, M1 and M2 are available. M1 works only when the N-terminal amino acid is accessible	135
Calmodulin-binding peptides	Calmodulin		136
Proteins			
Glutathione-S-transferase	Glutathione		137, 138
Protein A binding domain	IgG	Leakage of IgG into product	139
Maltose-binding protein	Amylose		140
Cellulose-binding protein	Cellulose	Irreversible binding, elution by enzymatic cleavage	141
Thioredoxin	Monoclonal antibody against denatured	Prevents inclusion body formation	142
Streptavidin	Biotin		143
Protein A/luciferase	IgG	Immunoassays	144
Protein A/glutathione-S-transferase	Glutathione, IgG	Immunoprecipitation	145

or/and the flow velocity must be reduced—both of which tend to low productivity.

Resolution in SEC is very dependent on the column packing quality. Kato et al. [69] have demonstrated with a model system that resolution depends on the packing method (slurry packing or gravitational packing) and on the packing velocity. As a general trend, they observed higher resolution at higher velocities. Media for SEC are similar to the supports used for ion-exchange and affinity chromatography. These matrices are composed of either agarose or cross-linked dextran, methacrylates, or polyacrylamides.

A compilation of media used for separation of biopolymers is given in Table 13.

Media for Reversed-Phase Chromatography

Media for reversed-phase chromatography differ from the media for the other types of chromatography because they have to be resistant to organic solvents. Commercially available media are composed of either a silica matrix or a polystyrene backbone. After derivatization, the silica matrices still have silanol groups left on the surface. The quality of the column is determined by the extent of shielding these groups. The term for this shielding is end capping. Fully end-capped and partially end-capped media are available. Linear alkanes from C1, C4, C8, C18, and CN groups are most widely used as functional groups (Table 14). Reversed-phase chromatography is very useful for the separation of peptides, either from synthesis or after fragmentation of proteins.

Media for Adsorption Chromatography

Adsorption chromatography using bare silica is not applicable for proteins. Hydroxyapatite has gained some value for protein purification but its mechanism is not fully understood. Proteins are retarded by electrostatic interactions and ion bridging. Addition of Ca^{2+} improves resolution in many applications. Two hydroxyapatite media are on the market. A composite material between trisacryl and small hydroxyapatite particles has excellent flow rate properties and can be regenerated with NaOH, but generally the capacity for proteins is rather low [19]. Pentax (now manufactured by BioRad) is a ceramic hydroxyapatite with good flow properties and high mechanical stability. This material has been widely used for antibody purification from various sources.

Other inorganic matrices such as zirconia [84] are under development but have not yet been launched to the market. An overview of commercially available matrices for adsorption chromatography is shown in Table 15.

TABLE 13 Commercially Available Media for Size-Exclusion Chromatography

Trade name	Matrix	Particle size (μm)	pH stability	Pore size ($\text{\AA}$)	Fractionation range, Dalton (globular protein)	Remarks	Supplier
Superdex Peptide	Cross-linked agarose and dextran	13	3–12	n.a.	100–7000	High performance for peptides and small molecules	AP Biotech
Superdex 30 prep grade	Cross-linked agarose and dextran	34	3–12	n.a.	Up to 10,000	For polishing of peptides and small proteins	AP Biotech
Superdex 75 prep grade	Cross-linked agarose and dextran	34	3–12	n.a.	3000–70,000	For separation of recombinant DNA	AP Biotech
Superdex 200 prep grade	Cross-linked agarose and dextran	34	3–12	n.a.	10,000–600,000	Particularly for separation of Mab from dimmers and lower molecular weight contaminants	AP Biotech
Superose 12 pep grad	Highly cross-linked agarose	10	3–12	n.a.	1000–300,000	High flow rate	AP Biotech
Superose 6 prep grad	Highly cross-linked agarose	13	3–12	n.a.	5000–5,000,000	High flow rate	AP Biotech
Sephacryl S-100 high Resolution	Allyl dextran and <i>N,N'</i> -methylene bisacryamide	50	3–11	n.a.	1000–100,000	High resolution, industrial scale applications	AP Biotech
Sephacryl S-200 high Resolution	Allyl dextran and <i>N,N'</i> -methylene bisacryamide	50	3–11	n.a.	5000–250,000	High resolution, industrial scale applications	AP Biotech
Sephacryl S-300 high Resolution	Allyl dextran and <i>N,N'</i> -methylene bisacryamide	50	3–11	n.a.	10,000–1,500,000	High resolution, industrial scale applications	AP Biotech

Sephacryl S-400 high Resolution	Allyl dextran and <i>N,N'</i> -methylene bisacryamide	50	3–11	n.a.	20,000–8,000,000	High resolution, industrial scale applications	AP Biotech
Sepharose 2B	2% agarose	45–165	4–9	n.a.	70,000–40,000,000	Industrial scale separations of very large molecules and virus particles	AP Biotech
Sepharose 4B	4% agarose	45–165	4–9	n.a.	60,000–20,000,000	Industrial scale separations of very large molecules and virus particles	AP Biotech
Sepharose 6B	6% agarose	45–165	4–9	n.a.	10,000–4,000,000	Industrial scale separations of very large molecules and virus particles	AP Biotech
Sepharose CL-2B	Cross-linked 2% agarose	45–165	3–13	n.a.	70,000–40,000,000	Industrial scale separations of very large molecules and virus particles	AP Biotech
Sepharose CL-4B	Cross-linked 4% agarose	45–165	3–13	n.a.	6,000–20,000,000	Industrial scale separations of very large molecules and virus particles	AP Biotech
Sepharose CL-6B	Cross-linked 6% agarose	45–165	3–13	n.a.	10,000–4,000,000	Industrial scale separations of very large molecules and virus particles	AP Biotech
Sephadex G-25 Coarse	Cross-linked dextran	100–300 dry	2–13	n.a.	1000–5000	For desalting and buffer exchange	AP Biotech

(continued)

TABLE 13 (continued)

Trade name	Matrix	Particle size (μm)	pH stability	Pore size ($\text{\AA}$)	Fractionation range, Dalton (globular protein)	Remarks	Supplier
Sephadex G-25 Medium	Cross-linked dextran	50–150 dry	2–13	n.a.	1000–5000	For desalting and buffer exchange	AP Biotech
Sephadex G-25 Fine	Cross-linked dextran	20–80 dry	2–13	n.a.	1000–5000	For desalting and buffer exchange	AP Biotech
Sephadex G-25 Superfine	Cross-linked dextran	20–50 dry	2–13	n.a.	1000–5000	For desalting and buffer exchange	AP Biotech
Sephadex G-1	Cross-linked dextran	40–120 dry	2–13	n.a.	Up to 700	For desalting and buffer exchange	AP Biotech
Sephadex G-15	Cross-linked dextran	40–120 dry	2–13	n.a.	Up to 1500	For desalting and buffer exchange	AP Biotech
Sephadex G-50 Coarse	Cross-linked dextran	100–300 dry	2–13	n.a.	1500–30,000	For desalting and buffer exchange	AP Biotech
Sephadex G-50 Medium	Cross-linked dextran	50–150 dry	2–13	n.a.	1500–30,000	For desalting and buffer exchange	AP Biotech
Sephadex G-50 Fine	Cross-linked dextran	20–80 dry	2–13	n.a.	1500–30,000	For desalting and buffer exchange	AP Biotech
Sephadex G-50 Superfine	Cross-linked dextran	20–50 dry	2–13	n.a.	1500–30,000	For desalting and buffer exchange	AP Biotech
Sephadex G-75	Cross-linked dextran	40–120 dry	2–13	n.a.	3000–80,000	For desalting and buffer exchange	AP Biotech
Sephadex G-Superfine	Cross-linked dextran	20–50 dry	2–13	n.a.	3000–70,000	For desalting and buffer exchange	AP Biotech
Fractogel EMD BioSEC 650 (S)	Polymer	20–40	2–13	n.a.	n.a.		Merck
Bio-Gel P-2 gel	Polyacrylamide	45–90	2–10	n.a.	100–1800	Rapid carbohydrate, peptide, and protein purification	Bio-Rad

Bio-Gel P-4 gel	Polyacrylamide	90–180	2–10	n.a	800–4000	Carbohydrate, peptide separation and protein desalting	Bio-Rad
Bio-Gel P-6 gel	Polyacrylamide	90–180	2–10	n.a	800–6000	Protein and polypeptide purification	Bio-Rad
Bio-Gel P-6DG gel	Polyacrylamide	90–180	2–10	n.a.	1000–6000	Desalting and buffer exchange	Bio-Rad
Bio-Gel P-10 gel	Polyacrylamide	90–180	2–10	n.a.	1500–20,000	Purification of proteins, polypeptides and DNA	Bio-Rad
Bio-Gel P-30 gel	Polyacrylamide	90–180	2–10	n.a	2500–40,000	Purification of proteins, polypeptides and DNA	Bio-Rad
Bio-Gel P-60 gel	Polyacrylamide	90–180	2–10	n.a.	3000–60,000	Purification of proteins, polypeptides and DNA	Bio-Rad
Bio-Gel P-100 gel	Polyacrylamide	90–180	2–10	n.a.	5000–100,000	Purification of proteins, polypeptides and DNA	Bio-Rad
Bio-Gel A 0.5m gel	Cross-linked agarose	150–300	4–13	n.a.	<10,000–500,000	Purification of proteins, polypeptides and DNA	Bio-Rad
Bio-Gel A 1.5m gel	Cross-linked agarose	150–300	4–13	n.a.	<10,000–1,500,000	Purification of proteins, polypeptides and DNA	Bio-Rad

(continued)

TABLE 13 (continued)

Trade name	Matrix	Particle size (μm)	pH stability	Pore size ($\text{\AA}$)	Fractionation range, Dalton (globular protein)	Remarks	Supplier
Biuo-Gel A 5m gel	Cross-linked agarose	150–300	4–13	n.a.	10,000–5,000,000	Purification of proteins, polypeptides and DNA	Bio-Rad
Biuo-Gel A 15m gel	Cross-linked agarose	150–300	4–13	n.a.	40,000–15,000,000	Purification of proteins, polypeptides and DNA	Bio-Rad
Biuo-Gel A 50m gel	Cross-linked agarose	150–300	4–13	n.a.	100,000–50,000,000	Purification of proteins, polypeptides and DNA	Bio-Rad
TSK-G2000SWxy	Silica	20 μm	2–7.5	125	5000–100,000		Tosoh
TSK-G3000SWxy	Silica	20 μm	2–7.5	250	10,000–500,000		Tosoh
TSK-2000PW	Polymer	20 μm	2–12	125	<5000		Tosoh
TSK-2500PW	Polymer	20 μm	2–12	<200	<8000		Tosoh
TSK-3000PW	Polymer	20 μm	2–12	200	500–800,000		Tosoh
TSK-4000PW	Polymer	22 μm	2–12	500	10,000–1,500,000		Tosoh
TSK-5000PW	Polymer	22 μm	2–12	1000	<10,000,000		Tosoh
TSK-6000PW	Polymer	25 μm	2–12	>1000	<200,000,000		Tosoh
TOYPEARL-HW-40C	Polymer	50–100	2–12	50	100–10,000		Tosoh
TOYPEARL-HW-40F	Polymer	30–60	2–12	50	100–10,000		Tosoh
TOYPEARL-HW-40S	Polymer	20–40	2–12	50	100–10,000		Tosoh
TOYPEARL-HW-50F	Polymer	30–60	2–12	125	500–80,000		Tosoh
TOYPEARL-HW-50S	Polymer	20–40	2–12	125	500–80,000		Tosoh

TOYPEARL-HW-55F	Polymer	30–60	2–12	300	1000–700,000		Tosoh
TOYPEARL-HW-55S	Polymer	20–40	2–12	300	1000–700,000		Tosoh
TOYPEARL-HW-65F	Polymer	30–60	2–12	1000	40,000–5,000,000		Tosoh
TOYPEARL-HW-65S	Polymer	20–40	2–12	1000	40,000–5,000,000		Tosoh
TOYPEARL-HW-75F	Polymer	30–60	2–12	>1000	500,000–50,000,000		Tosoh
Ultrigel AcA 202	Mixed polyacrylamide and agarose	60–140	n a	n.a	1000–15,000		BioSeptra
Ultrigel AcA 54	Mixed polyacrylamide and agarose	60–140	n.a.	n.a.	5000–70,000		BioSeptra
Ultrigel AcA 44	Mixed polyacrylamide and agarose	60–140	n.a.	n a.	10,000–130,000		BioSeptra
Ultrigel AcA 34	Mixed polyacrylamide and agarose	60–140	n.a.	n.a.	20,000–350,000		BioSeptra
Ultrigel AcA 22	Mixed polyacrylamide and agarose	60–140	n.a.	n.a.	100,000–1,200,000		BioSeptra
Ultrigel A6	Agarose	60–140	n.a	n.a.	25,000–2,400,000		BioSeptra
Ultrigel A4	Agarose	60–140	n.a.	n a.	55,000–9,000,000		BioSeptra
Ultrigel A2	Agarose	60–140	n.a.	n a.	120,000–25,000,000		BioSeptra
Trisacryl GF 05 M	Poly- <i>N</i> -acryloyl-2-amino-2-hydroxyl-1, 3-propanediol	40–80	n.a.	n.a.	300–2500	For desalting	BioSeptra
Trisacryl GF 05 LS	Poly- <i>N</i> -acryloyl-2-amino-2-hydroxyl-1, 3-propanediol	80–160	n.a.	n.a.	300–2500	For desalting	BioSeptra

(continued)

TABLE 13 (continued)

Trade name	Matrix	Particle size (μm)	pH stability	Pore size ($\text{\AA}$)	Fractionation range, Dalton (globular protein)		Remarks	Supplier
Trisacryl GF 2000 LS	Poly- <i>N</i> -acryloyl-2-amino-2-hydroxy-1, 3-propanediol	40–80	n a.	n.a.	10,000–15,000,000			BioSeptra
NucleoGEL aqua-OH 40-10	Hydrophile	10	2–12	300	100 (exclusion limit)		Polymer, polysaccharide, cellulose derivative	Machery-Nagel GmbH
NucleoGEL aqua-OH 50-10	Hydrophile	10	2–12	1000	1000 (exclusion limit)		Polymer, polysaccharide, cellulose derivative	Machery-Nagel GmbH
NucleoGEL aqua-OH 60-10	Hydrophile	10	2–12	4000	2000 (exclusion limit)		Polymer, polysaccharide, cellulose derivative	Machery-Nagel GmbH
NucleoGEL GFC 300-10	Hydrophile	10	1–13	300	100 (exclusion limit)		Polymer and protein	Machery-Nagel GmbH
NucleoGEL 1000-10	Hydrophile	10	1–13	1000	1000 (exclusion limit)		Polymer and protein	Machery-Nagel GmbH
NucleoGEL GFC 4000-10	Hydrophile	10	1–13	4000	20,000 (exclusion limit)		Polymer and protein	Machery-Nagel GmbH
Cellufine GCL-25	Cellulose	45–105, 25–45	2–12	n.a.	3000		Peptides, nucleic acids	Millipore
Cellufine GCL-90	Cellulose	45–105, 25–45	2–12	n.a.	35,000		Proteins	Millipore
Cellufine GCL-300	Cellulose	45–105, 25–45	2–12	n a	90,000		High-MW polysaccharides	Millipore
Cellufine GCL-1000	Cellulose	45–105, 25–45	2–12	n a.	600,000		Enzymes	Millipore
Cellufine GCL-2000	Cellulose	45–105, 25–45	2–12	n a.	3,000,000		IgG, high-MW proteins	Millipore
Cellufine GC-700	Cellulose	45–105, 25–45	2–12	n a.	300??, ??000		Enzymes, IgG, proteins	Millipore
Cellufine GH-25	Cellulose	45–105, 25–45	2–12	n a	n.a.		Desalting	Millipore

TABLE 14 Commercially Available Media for RPC

Trade name	Matrix	Particle size (μm)	pH stability	CIP stability	Capacity (mg/mL gel)	Remarks	Supplier
SOURCE 15RPC	Polystyrene/ divinylbenzene	15	1–12	1–14	10 BSA	Controlled pore size, scales prep. separations	AP Biotech
Sephasil C8 prep grade	Silica	12	2–7/5	n.a.	n.a.		AP Biotech
Sephasil C18 prep grade	Silica	12	2–7 5	n.a.	n.a.		AP Biotech
POROS 50 R1	Polystyrene/ divinylbenzene	50	1–14	1–14	6 lysozyme	Retention similar to conventional C4	PerSeptive
POROS 50 R2	Polystyrene/ divinylbenzene	50	1–14	1–14	10 lysozyme	Retention similar to conventional C8/18	PerSeptive
POROS 20 R1	Polystyrene/ divinylbenzene	20	1–14	1–14	6 lysozyme	Retention similar to conventional C4	PerSeptive
POROS 20 R2	Polystyrene/ divinylbenzene	20	1–14	1–14	10 lysozyme	Retention similar to conventional C8/18	PerSeptive
Oligo R3	Polystyrene/ divinylbenzene	30	1–14	1–14	30 lysozyme	For phosphodiester and phosphorothioate oligonucleotides	PerSeptive
LiChrospher WP 300 RP-18	Polymer	12, 15	2–7 5	n.a.	n.a.	Pore size 300 Å	Merck
BAKERBND (Octadecyl)	Silica	15	2–7	n.a.	n.a.	High performance	J.T.Baker
BAKERBOND (Octyl)	Silica	15	2–8	n.a.	n.a.	High performance	J.T.Baker
BAKERBOND (Butyl)	Silica	15	2–9	n.a.	n.a.	High performance	J.T.Baker

(continued)

TABLE 14 (continued)

Trade name	Matrix	Particle size (μm)	pH stability	CIP stability	Capacity (mg/mL gel)	Remarks	Supplier
BAKERBOND (Dipheyl)	Silica	15	2–10	n.a.	n.a.	High performance	J.T.Baker
PLRP-S	Polystyrene/ divinylbenzene	15–20	1–14	n.a.	n.a.	With pore size 300, 1000, 4000 Å	Polymer Laboratories
Kromasil-10-C8	Silica	10	1.5–9.5	n.a.	n.a.		Eka Chemicals AB
Kromasil-5-C8	Silica	5	1.5–9.5	n.a.	n.a.		Eka Chemicals AB
Kromasil-10-C18	Silica	10	1.5–9.5	n.a.	n.a.		Eka Chemicals AB
Kromasil-5-C18	Silica	5	1.5–9.5	n.a.	n.a.		Eka Chemicals AB
Kromasil-10-C4	Silica	10	1.5–9.5	n.a.	n.a.		Eka Chemicals AB
Kromasil-5-C4	Silica	5	1.5–9.5	n.a.	n.a.		Eka Chemicals AB
NucleoGEL RP 300-8/77	PV/DVB	8	1–13	n.a.	n.a.	Peptide and protein	Macherey-Nagel GmbH
NucleoGEL RP 1000-8/77	PV/DVB	8	1–13	n.a.	n.a.	Peptide and protein	Macherey-Nagel GmbH
NucleoGEL RP 4000-8/77	PV/DVB	8	1–13	n.a.	n.a.	Peptide and protein	Macherey-Nagel GmbH
NucleoGEL RP 300-10	PV/DVB	10	1–13	n.a.	n.a.	Peptide and protein	Macherey-Nagel GmbH
NucleoGEL RP 1000-10	PV/DVB	10	1–13	n.a.	n.a.	Peptide and protein	Macherey-Nagel GmbH
NucleoGEL RP 4000-10	PV/DVB	10	1–13	n.a.	n.a.	Peptide and protein	Macherey-Nagel GmbH
YMC-Pack Protein RP	Silica	n.a.	n.a.	n.a.	n.a.	Peptide and protein	YMC
Amicon RP C8	Silica	5	n.a.	n.a.	n.a.		Amicon
Hyperprep PEP 300 C4, C8, C18	Silica	8, 15	2.2–8.8	n.a.	n.a.		Shandon HPLC
Hypersil WP300 C4, C8	Silica	5, 10	n.a.	n.a.	n.a.		Shandon HPLC
Kromasil, C4, C8, C18	Silica	5, 10	n.a.	n.a.	n.a.		Eka Chemicals AB

TABLE 15 Commercially Available Media for Hydroxyapatite Chromatography

Trade name	Matrix	Particle size (μm)	CIP pH stability	Capacity (mg/g gel)	Remarks	Supplier
Macro-Prep Ceramic Hydroxyapatite Type I	Calcium phosphate	20; 40; 80	5.5–14 autoclavable	>25 lysozyme	Removing nucleic acid from pharmaceutical products and for isoenzyme separation	Bio-Rad
Macro-Prep Ceramic Hydroxyapatite Type II	Calcium phosphate	20; 40; 80	5.5–14 autoclavable	>12.5 lysozyme	Removing nucleic acid from pharmaceutical products and for isoenzyme separation	Bio-Rad
Bio-Gel Hydroxyapatite HAT	Tiselius material	n.a.	n.a.	10 BSA; >500 calf thymus DNA	Removing nucleic acid from pharmaceutical products and for isoenzyme separation	Bio-Rad
Bio-Gel Hydroxyapatite HTP	Tiselius material	n.a.	n.a.	10 BSA; >800 calf thymus DNA	Removing nucleic acid from pharmaceutical products and for isoenzyme separation	Bio-Rad
Bio-Gel DNA Grade Hydroxyapatite HTP	Tiselius material	n.a.	n.a.	10 BSA; >500 calf thymus DNA	Removing nucleic acid from pharmaceutical products and for isoenzyme separation	Bio-Rad
Hydroxyapatite	40.5% Ca, 18.8% P (density 2.96 mg/mL)	4–10; 5–40	>5.5 autoclavable	18 lysozyme	Reasonable binding capacity for proteins	Merck
HA Ultrogel	Agarose trapped hydroxyapatite	60–180	n.a.	n.a.	Chemically and mechanically stable	BioSeptra

OPTIMIZATION OF PREPARATIVE CHROMATOGRAPHY

The parameters of preparative chromatography that can be adjusted by the chromatographer for optimization are listed in Table 16. Other parameters cannot be modified by the user but are rather related to the nature of the chromatography medium. When an optimization routine does not yield the expected results, it is best to switch to another medium, based on either a different mass transfer principle or another matrix material. The adsorption process that occurs at a chromatography surface is very complex and is poorly understood. This is especially true for non-specific adsorption. This is why it is necessary to carry out the optimization with the real solutions. Experiments with artificial samples often do not result in conditions that can be transferred to the real situation. The most important targets for optimization are purity and productivity. After the required purity has been achieved, the productivity can be optimized. Productivity includes costs, column size, and operation time. It also includes the lifetime of the column material.

Particle Size

in preparative chromatography, we want to increase the particle diameter to reduce the back pressure, which is the determining factor for the equipment costs. As mentioned before, purity is the most important issue. The back pressure decreases with the square of the particle diameter, which also reduces the separation efficiency. The empirical relationship between the number of plates (N), the column length (L), the velocity (u) and the particle diameter (d_p) is given by

$$L_1 = \left(\frac{N_1}{N_2} \right) \left(\frac{u_1}{u_2} \right) \left(\frac{d_{p,1}}{d_{p,2}} \right) L_2 \quad (9)$$

TABLE 16 Main Parameters That Modify Resolution in Preparative Chromatography

Adjustable and modifiable by the user	Column support properties
pH of mobile phase	Type of sorption groups
Ionic strength of mobile phase	Site accessibility and density
Elution gradient slope	Particle size and distribution
Column geometry	Sorption capacity
Flow rate	Polymer properties
Temperature	Size of the pores
Loading volume and concentration	Nonspecific binding

where the subscripts 1 and 2 represent columns that are packed with particle size 1 and 2. Equation (9) provides an estimate for column length when the particle size, plate number, and eluent velocity are changed in scaling up a given separation. If a separation problem cannot be solved with larger particles, switching to a support with better mass transfer (Table 6) may help.

Productivity, Resolution, and Loading Volume

A very important issue in optimizing preparative chromatography is the improvement of loading at a defined resolution. Mao and Heam [85] have developed an algorithm called the sectional model that is based on a tank in series model to calculate the optimal productivity. Although increasing the loading volume can destroy the resolution, reducing the velocity may compensate for the losses in resolution by improving efficiency. It is a game with several variables. They have solved the problem by drawing contour plots where the operating regions can be visualized. Column loading is plotted as a function of flow rate; the production rate and the yield are superimposed. For a certain yield and production rate, the operating variables can be determined.

Yamamoto and Borgberg [85a] have presented an interesting optimization algorithm for linear gradient elution systems. They have calculated or graphically determined an isocost curve that indicates different chromatographic conditions having the same costs. The goals for this optimization are scale-up in linear gradient elution and constant resolution. The separation is optimized to save buffer consumption and time. These assumptions may not be valid for every process. For example, when an expensive buffer is used, the buffer consumption should be minimized, or when a process exceeds a certain period of time (e.g., a shift), then a reduction of time may become very important in saving money. Because identical resolution is projected for columns with different lengths, a different gradient slope is calculated for each column and, consequently, a different elution volume. The procedure for the calculation of resolution in gradient elution chromatography is described in detail by Yamamoto et al. [86,87]. The constraints and requirements are a fixed gel volume, the same resolution (a function of gradient slope and flow rate), pressure limit as a function of geometry, and the isocost curve. After the separation time is plotted versus the elution volume, the curve for pressure limit is superimposed. One can operate only in the area above the pressure limit. The slope of the isocost curve is determined as

$$\text{Slope} = \frac{\text{price per liter buffer} \times \text{elution volume}}{\text{price per hour}} \quad (10)$$

Applying separation conditions where a separation must be completed within one shift, the intercept at the time axis is set to 8 hr and the slope is calculated

Separation time vs. elution volume at fixed bed volume

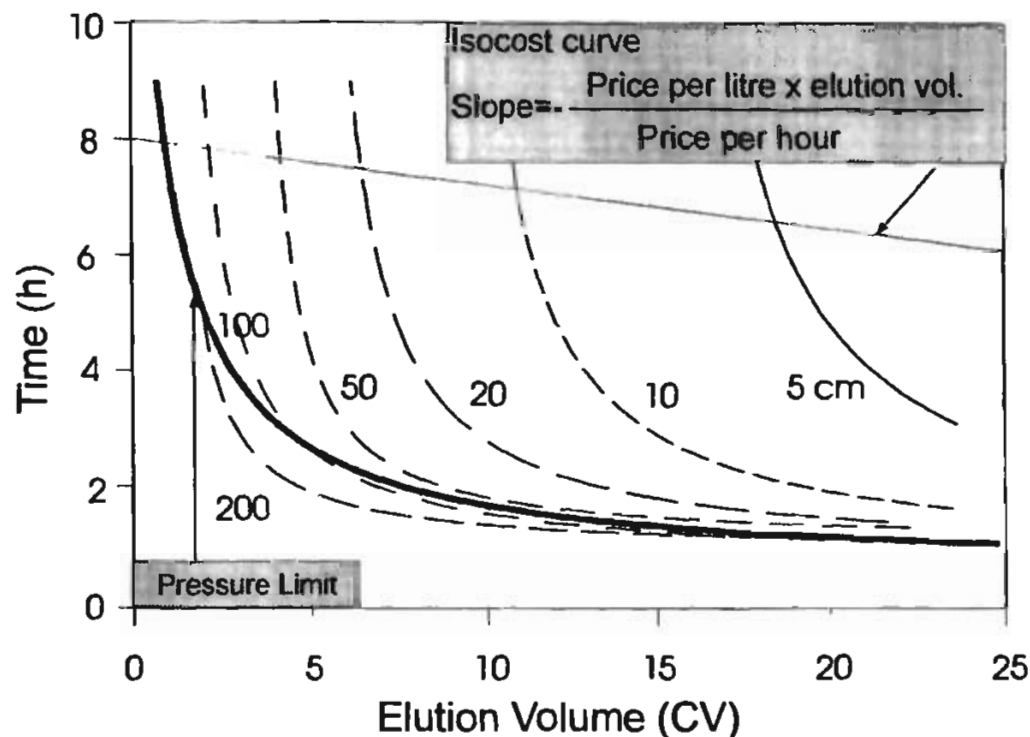


FIGURE 6 Optimization of linear gradient elution assuming the same resolution, at different column lengths using an isocost line. (Courtesy of S. Yamamoto and U. Borgberg.)

according to Eq. (10). All separation conditions crossing with the isocost line have the same operating costs, as illustrated in Fig. 6.

Rational Design Strategies

Although the rational design of preparative chromatography has been advanced, the plethora of parameters make the strategy complicated. First it is necessary to define the goal, which may seem to be trivial at first glance. Conventional optimization of chromatography is an iterative process; some assumptions must be made by the user that are never explicitly stated. To cope with such a situation, either a self-learning system, such as a neural network, is necessary or the user must clearly express the variable parameters and the boundary conditions in mathematical equations. A decision tree for computer-assisted optimization of chromatography is shown in Fig. 7.

Leser et al. [88,89] have described basic rules for the rational design of downstream processing. The rules include two important mathematical parameters—the separation coefficient and the economic separation coefficient.

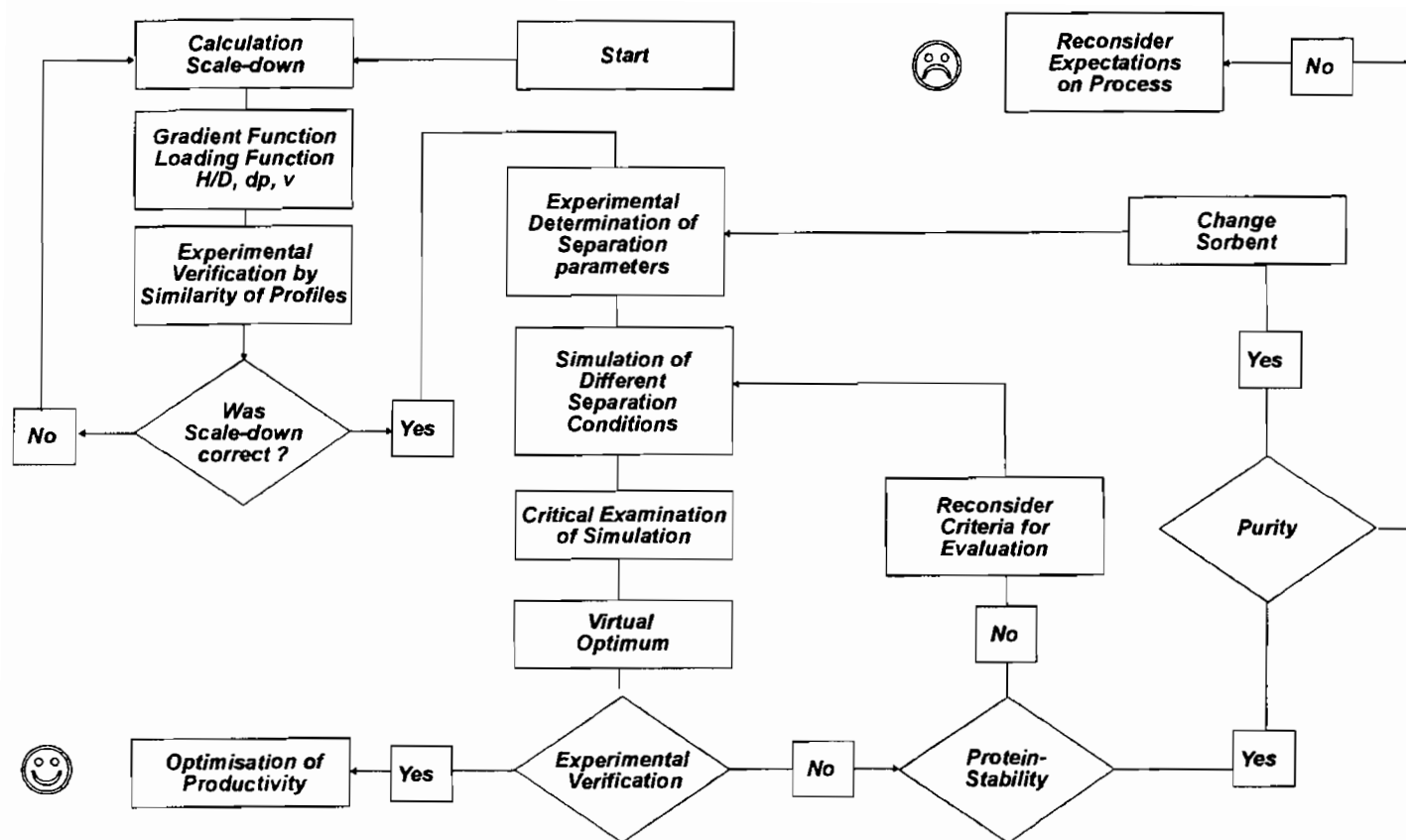


FIGURE 7 Route for computer-assisted optimization of chromatography for a process starting from a large-scale process running under suboptimal conditions.

The separation coefficient is a function of the efficiency of the process and the deviation factor accounting for the differences among the physicochemical properties. The deviation factor is a function that represents the behavior of proteins in chromatographic separations. The economic separation factor is a function of the separation coefficient and the cost factor, which is a measure of the cost of the purification operation. A knowledge-based computer algorithm is being developed to allow the design of a purification scheme that fulfills the main aims of protein purification—highest possible yield, prespecified level of purity, and use of minimal resources and costs.

Evolutionary systems have been developed in chemical process engineering to overcome some of the uncertainties of heuristic methods and to use, whenever available, simulation tools to improve the design. The evolutionary synthesis of separation sequences includes the following tasks: generate an initial separation sequence, identify the rules that will govern the evolutionary steps, and determine the evolutionary strategy by comparing different candidate sequences. Neural networks have been used for developing purification strategies for antibiotics. For protein separation, these methodologies are still under development. A further description of rational design approaches would exceed the scope of the chapter.

SCALE-UP CONSIDERATIONS

Advances in the development of modern chromatography materials and modern equipment have turned scale-up into a less critical issue than it was in the past. Usually, nonchromatographic parameters such as consistency of starting material or limited stability create problems. The scale-up of purification is usually aligned with the scale-up of fermentation. Different reactor types are often used for small and large scale, which may lead to a different composition of the impurities, product concentration, and even molecular structure. Maiorella et al. [90] demonstrated that bioreactor types and culture conditions influence the isoelectric pattern of a monoclonal antibody. This has a big impact on purification because different isoelectric patterns may lead to different solubilities and binding behavior to ion exchangers or other sorbents affected by ionic interactions.

It is also very important to pay attention to the residence time and the process time during scale-up. When the residence time is changed during scale-up, the products from the different scales may have different properties. Usually the residence time increases with scale; therefore, a protein has a longer contact time for adsorption and molecular reorientation at the surface of the chromatography sorbent. It may also diffuse much deeper into the particle. As a consequence, the protein may bind tighter and may not be released under the same conditions worked out on the laboratory scale. As a rule, keep the residence time constant if possible and do not change the temperature; temperature influences the binding strength [91,92].

Packing of Large-Scale Columns

Packing of large-scale columns is a tricky process for silica-based material. It is much easier for chromatography media based on synthetic or natural polymers. The chromatography beads can be added into the column as a dry powder, or a slurry can be poured or pumped into the empty column. The procedures are called dry packing and slurry packing. The most frequently used dry packing method, so-called dynamic axial compression, is described as follows. The column is filled with the dry powder and then the piston (adapter) is slowly brought upward. The column should be vibrated, which can be simply done by tapping with a hammer. The whole procedure has to be thoroughly standardized [93]. During the dry packing procedure, a lot of air is generated in the packed bed and must be thoroughly removed later by the liquid phase. Slurry packing is much easier than dry packing. After the bottom of the chromatography column is covered with liquid phase, the slurry is pumped or poured (when smaller columns are used) into the column. The column is then closed and the liquid phase is pumped through the column with a higher flow rate than used for separation. The slurry rapidly compacts to the final packed bed height and then the adapter of the column is lowered so that it touches the surface of the packed bed. Finally, the pump is turned off.

After packing, the quality of the packed bed must be checked. This can be done by injection of a small pulse of salt or a small organic molecule. The number of plates (N) should be determined, as well as the asymmetry factor. The asymmetry factor and the theoretical plates are very sensitive measures of the quality of packing, but once the column is packed, it will not last forever. The packed bed undergoes a change in its “structure” and at some point, the column will collapse. It is not very easy to predict the time point of collapsing because this is a chaotic process [94].

Several authors [95,96] have reported that the quality of packing measured as plate height decreases with increased column diameter. They have suggested that wall effects are responsible for the higher separation efficiencies of columns with smaller bores. Because the wall supports the chromatographic beads, larger chromatography particles show this effect to be less pronounced. For very large scale columns, only particles with large diameters and/or polymer-based material are used. In both cases, packing is less critical.

Constant Diameter

Keeping the diameter constant will keep the process time and residence time constant during scale-up. Under such conditions, the separation efficiency, yield, peak width, and buffer consumption, normalized with respect to the total column volume, should be maintained over all the scales. The best way to proceed is to

carry out the traditional optimization routine at a small scale. Afterward, the column must be increased in length until a certain value is determined by pragmatic issues such as availability of columns and the required throughput. When the column length is changed, the dynamic capacity and the separation efficiency must be determined again, as these values may change. Once these values are known, the further scale-up is very simple. The feed volume of the large column ($V_{F,2}$) is calculated as

$$V_{F,2} = V_{F,1} \left(\frac{A_2}{A_1} \right) \quad (11)$$

where $V_{F,1}$ is the optimized feed volume of the smaller column and A_2 and A_1 are the column cross-sectional areas of the large and small columns. With this relationship, all other volumes such as the peak volume, elution volumes, and the overall buffer consumption will change. With increasing diameter, the separation efficiency may slightly decrease, but this can be compensated for by increasing the column length, recognizing that the residence time will also change.

Constant H/d_p Ratio

Sometimes chromatography is scaled up by keeping the ratio between plate height (H) and particle diameter (d_p) constant. According to Eq. (7), the column length must be proportional to the square of the particle diameter if the same separation efficiency is to be maintained. Therefore the efficiency will change if the particle diameter is varied. It is not wise to use the H/d_p rates for scale-up. Similarly a constant L/r ratio is unsatisfactory. In both cases, the residence time is changed and the separation conditions cannot be predicted [97].

Changing the Mode of Mass Transport

Because some technical problems are attributed to the scale of chromatographic separation, engineers have concentrated on improving the mass transport and mode of mass transport so that the whole process could be run faster. Other separation modes are membrane chromatography [98] and combinations of microfiltration and adsorption (CARE = continuous affinity recycle extraction; see next section). Sepracor has developed an adsorption process based on hollow fibers [99]. The ligand is immobilized at the surface of the membrane and the feed is pumped through the hollow fibers with subsequent steps as described under "Modes and Steps in Preparative Chromatography." According to their theoretical considerations, the process should be extremely fast; however, the system was withdrawn from the market because it was not economically successful. The nonspecific adsorption in the hollow-fiber membranes was extremely high and rapid fouling occurred.

Merck KGa and Azko have also developed a hollow fiber-based module for rapid separation using a membrane chromatography principle. This system is operated in the dead-end mode. It is too early to judge whether this system will be successful in large-scale operations. Sartorius has also developed membrane modules that are handled on a smaller scale, similar to chromatography columns. Microfilters are derivatized with functional groups (sulfopropyl, DEAE, etc.) in a proprietary way. In order to obtain high capacity, the microfilter pores are filled with some sort of functionalized gel. The membranes are packed into a filter holder and the separation is carried out extremely fast. Selective removal of contaminants, such as bacterial endotoxins, has been successfully proved on an industrial scale. For very large scale, they have developed spiral-wound membranes that have a lower separation efficiency than those run in the flat-membrane mode. It must be concluded that the packed bed is the system with the highest efficiency. That means that the buffer consumption is the lowest and the elution volume is the lowest with the highest product concentration compared with all other modes! Other goals or other engineering aspects may indicate the use of a non-packed bed adsorption strategy.

Radial Chromatography

Instead of running a column in the axial direction, radial chromatography runs it radially to enhance the speed of separation. A specially designed column, manufactured by the filter company Cuno, is composed of a layer of chromatography medium placed on a grid and wound on a central tube. The running buffers are perfused through this cartridge in an axial direction and the liquid collected through the central tube. Although the performance of these cartridges was excellent, they did not survive on the market. When the same housings used for filtration were used for chromatography, the high dead volume was responsible for significant extra-column band spreading and in some applications, the resolution obtained in the cartridge was completely destroyed [100]. Another radial chromatography system has been designed by Sepragen. The specially designed column allows the loading and operation of the column in a radial direction (Fig. 8). The bed is stabilized by this special design and, therefore, optimally suited for soft gels.

CONTINUOUS PREPARATIVE CHROMATOGRAPHY

The current status of downstream processing of fermentation broths (microorganisms, animal or human cells, extracts from plants) is a multistage process. First, cells and cell fragments are separated from the fermentation broth by centrifugation or microfiltration. The supernatant is then processed by a sequence of different process steps: precipitation, ultrafiltration, and different chromato-

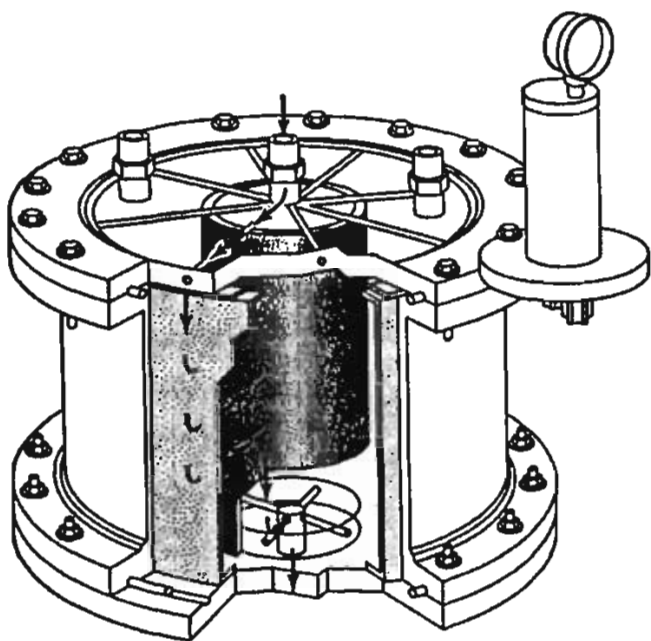


FIGURE 8 Explosion drawing of a radial flow column manufactured by Sepragen. The sample and buffers flow from the outside into the center, along the column radius. Because of the large surface area and relatively small bed depth, these columns allow high flow rates. (Courtesy of Sepragen Inc.)

graphic methods such as ion-exchange, affinity, hydroxyapatite, size exclusion, and hydrophobic interaction chromatography. Most of these steps are carried out batchwise; however, when the process solutions exceed certain volumes, continuous is superior to batchwise processing because storage of intermediate products is not necessary.

In contrast to conventional packed bed adsorption, the processing of turbid solutions and suspensions is possible with this design of process equipment. Particles such as cells and cell fragments are removed from the matrix by continuous washing, partly by countercurrent washing, preventing fouling of the matrix. Affinity chromatography can be used as the first and highly selective purification step. Using conventional gels, this is not always possible because affinity ligands are sometimes very expensive and the gels have short shelf lives even if the fermentation broth has been preclarified.

The regeneration and decontamination of the matrix is usually a very time-consuming process step and overloading leads to reduction of column lifetime [101]. In a continuous process, all steps are performed simultaneously. The contact time between the biomolecule and the ligand is very short, which prevents denaturation of the product caused by longer contact. Regeneration can be done under less harsh conditions than usual and ligand utilization is much better.

Continuous packed bed adsorption, such as true moving bed and simulated moving bed [102–105], involves reduced process times compared with batchwise

chromatographic methods, but these methods are susceptible to fouling, as with conventional packed beds. In addition, reduction of process steps cannot be achieved. Expanded bed [106–112] and magnetically stabilized bed [113,114] can be used with turbid solutions and suspensions, but they are batchwise methods and show the same disadvantages regarding the process time as the packed bed adsorption. For fluidization of the bed, one has to apply high flow rates. In some cases, the biomolecule cannot be captured within one cycle and the process solution must be recycled.

SMB (Simulated Moving Bed) and TMB (True Moving Bed)

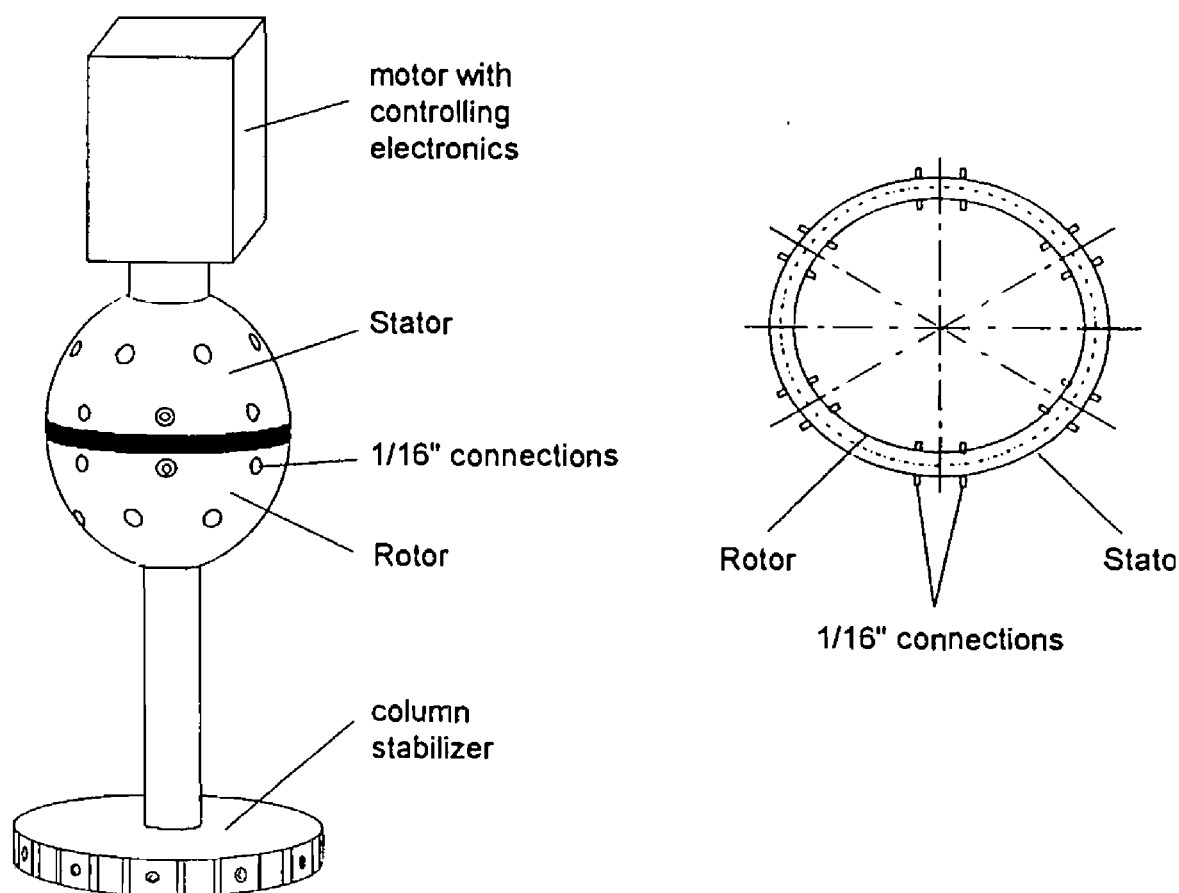
In a true moving bed process, the solid phase (adsorbent) and the fluid phase (eluent) are moved in countercurrent directions. Free adsorbent is fed at the top of the column and the mobile phase is pumped in an upward direction. The adsorbent slides downward and selectively adsorbs compounds from the feed, which is introduced into the system at the middle part of the column. At the lower part of the column, the loaded adsorbent is racked off. Compounds without affinity for the adsorbent will travel upward with the mobile phase and can be harvested at the top of the column. TMB can separate a mixture into two different components; therefore, the system is capable of separating only binary and pseudobinary mixtures.

Simulated moving bed chromatography shows similarity to the TMB process. The feed stream is divided into two different streams and, therefore, a mixture can be separated into two phases. Analogously to the TMB process, feed-stream, eluent-feed, raffinat, and extract exits are hooked up in such a system. Raffinat is the leftover after extraction. In SMB, the entrances and exits of liquid phase are located at four different positions. In a defined time interval, the switch time, the entering and the exiting positions will be switched by one position in the direction of the eluent stream. Consequently, a countercurrent is simulated between adsorbent and eluent. The real countercurrent is only a discrete process at the time point of switching. Between the switch rhythm, in the silence period, the SMB is a series of normal packed beds connected together. The switching of the valves produces a pseudostationary (cyclic) state. Analogously to the TMB approach, only binary and pseudobinary mixtures can be separated. The features of TMB, SMB, and annular chromatography (see the following) are compared in Table 17.

SMB has been successfully exploited for the separation of chiral drugs. Systems are generally operated on an industrial scale, but recently scale-down was successful. Knauer has developed an intelligent switching valve for laboratory scale experiments (Fig. 9) that allows installation of SMB columns on a laboratory scale as a very compact unit. In the past, exact switching, together with reproducible packing, was not possible and generated unstable processes. There-

TABLE 17 Properties of Continuous and Quasi-continuous Chromatographic Processes

Properties	Annular chromatography	True moving bed	Simulated moving bed
Parameter for optimization	3	5	5
Number of components to be separated	More than 3	2	2
Mode of separation	Continuous	Continuous	Quasi-continuous
Possibility of gradient elution	Yes	No	Yes

**FIGURE 9** Laboratory switching valve for SMB designed and manufactured by Knauer, (Courtesy of Knauer.)

fore, biotechnological applications for SMB were very rare compared with applications for chiral compounds, steroids, and other molecules from chemical synthesis.

Annular Chromatography

Annular chromatography is a truly continuous chromatography process [115–117]. Annular chromatography as it currently exists was developed at Oak Ridge National Laboratory in the group of Charles Byers [118–121]. Improved versions fulfilling the demand of hygienic design have been introduced into the market by the company PRIOR AG under the trade name P-CAC (Prior Continuous Annular Chromatograph). The apparatus (see Fig. 10) consists of two rotating cylinders. The annulus is filled with the chromatography medium. The top of the bed is covered by a layer of glass beads. The liquid phase perfuses the rotating

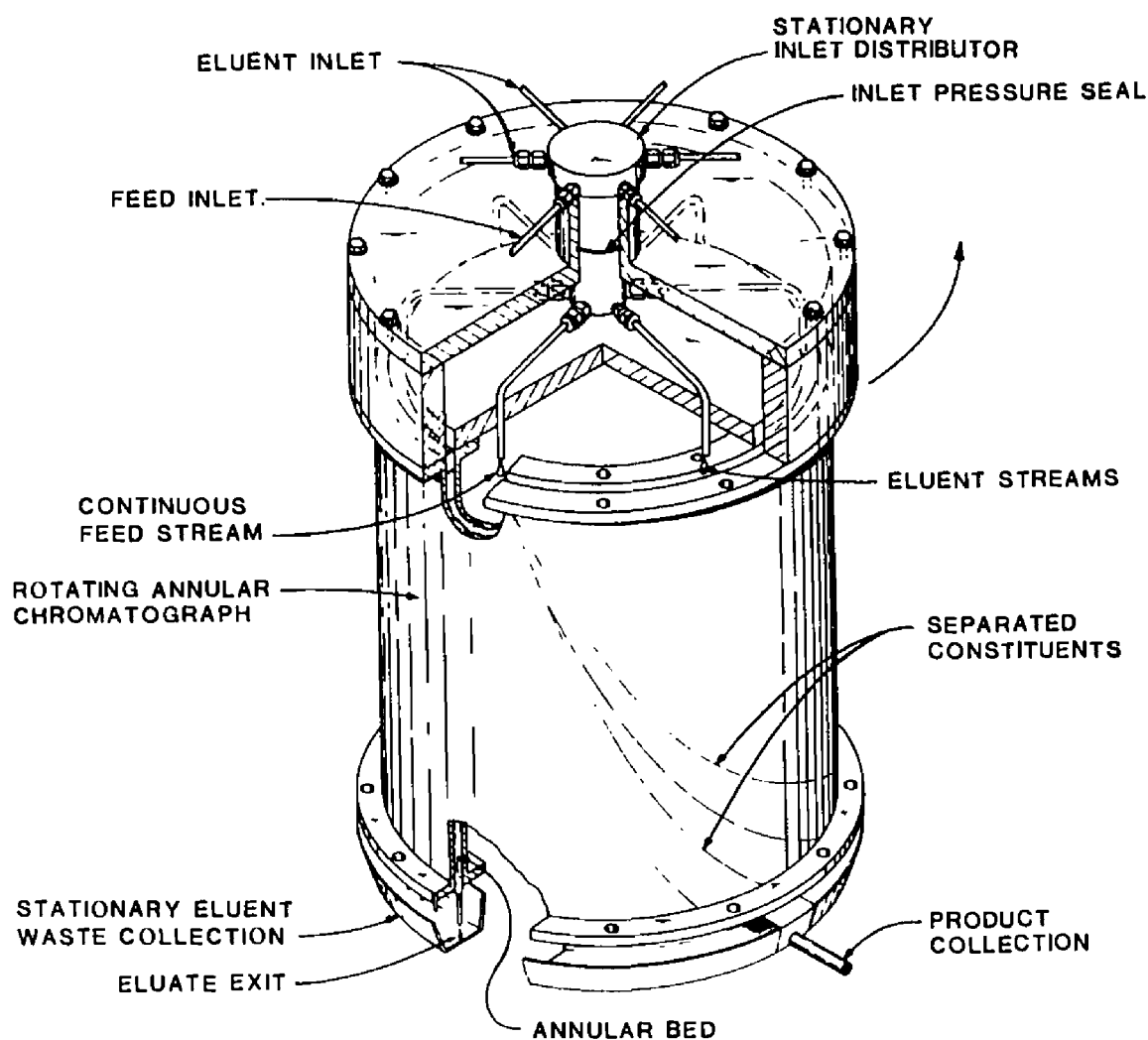


FIGURE 10 Schematic drawing of an annular chromatograph according to the Prior P-CAC design. (Courtesy of Prior AG.)

annular bed. The sample is continuously introduced by a nozzle, which dives into the layer of glass beads. Elution and regeneration buffers can be introduced by additional nozzles that are positioned at another angle of the annulus, allowing operation in all modes—isocratic, gradient, and displacement. Continuous regeneration is possible, which is a major advantage over other continuous and pseudocontinuous chromatography systems.

At the bottom of the packed annular bed, a series of outlets are positioned that are sealed with membranes for retention of the chromatography medium. The different liquid streams can be collected at these outlet ports. When a sample is introduced into the annular bed, the various components are retained according to their distribution coefficient. While they are moving downward, the bed is rotating and the different components form helical trails until they leave the column at the bottom. If the perfusion rate, rotation speed, and sample feed are kept constant, a stationary state is achieved. The separation is influenced by the rotation speed in that the faster the rotation, the flatter the winding of the helical bands. The resolution is thus improved. The maximum rotation speed is obtained when the band with the highest distribution coefficient catches up with the fastest band. The perfusion rate and the sample feed rate also influence the resolution.

The modeling of annular chromatography is very similar to that of conventional chromatography. The one-dimensional continuity equation is transformed into a two-dimensional one by making the following change of variables:

$$\theta = \omega t \quad (12)$$

with ω the rotation rate (deg/hr) and t the time; θ is the displacement from the feed point. Analogously to conventional packed bed chromatography, retention is expressed in degrees instead of by retention time or volume. When a standard chromatographic method is transferred to annular chromatography, the retention data are converted into θ -values by assuming a certain rotation rate. Carta and Stringfield [122] have developed an exact analytical solution of the continuity equation for a fixed bed and with the use of Eq. (9), their equation can be transformed. Assuming no interactions between species and linear adsorption isotherms, the transformed equation can be applied to each component in the feed mixture independently to compute concentration profiles for the individual components. A simple computer program is available to handle the series computations leading to concentration profiles.

Annular chromatography has been successfully applied for desalting of proteins [123] and many ion-exchange and affinity separations are currently under investigation. Assuming the same number of plates per column length unit, the productivity of fixed bed chromatography and annular chromatography should be identical; however, recent studies showed that annular chromatography had a higher plate number than fixed bed (J. Wolfgang, A. Prior, and A. Herting, personal communication). The support of the concentric walls may account for

the better performance of annular chromatography because with the same amount of gel, a higher dynamic capacity is reached.

Continuous Membrane Chromatography

Diving Belt

Niven and Surlock [124] developed a continuous separation process working with a nylon belt. The belt was carried through different baths that contained the starting solution and the buffers for equilibration, elation, and regeneration (Fig. 11). In principle, turbid solutions can be processed continuously, although the authors did not mention that in their publication. The main disadvantage of the Niven-Scurlock process is the carryover of starting solution and buffers from one

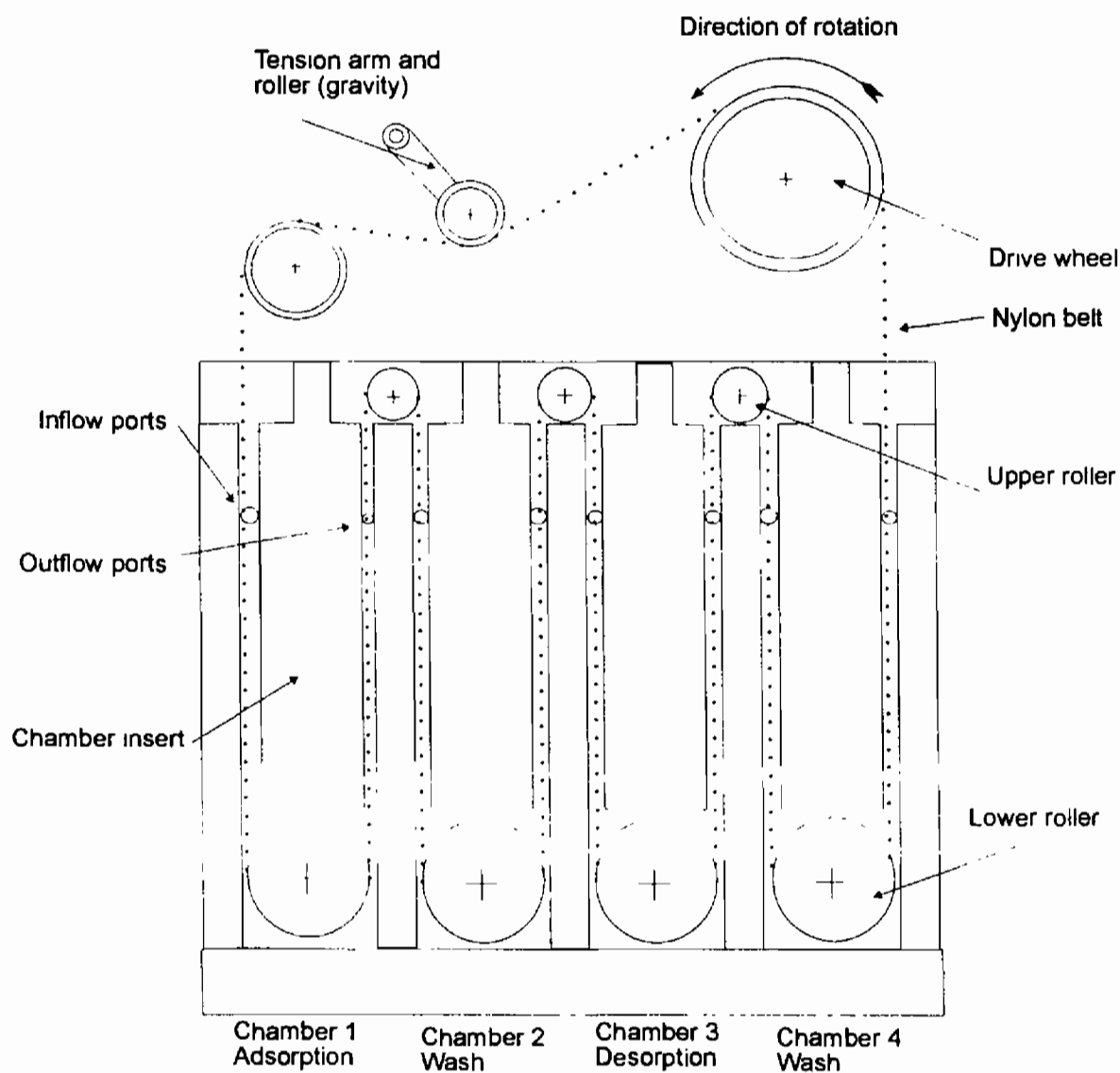


FIGURE 11 Schematic drawing of the process for continuous affinity adsorption developed by Niven and Surlock [124].

bath to the next one. In addition, the binding capacity of the affinity belt is only partly utilized because the solutions enter the belt only by diffusion due to the lack of connective flow through the nylon belt. At the loading step, this disadvantage can be compensated for by enlargement of the bath, but at the elution step, this would lead to dilution of the product. Complete extraction of the product from the suspension can be obtained only for ligands exhibiting very high affinity constants.

Continuous Affinity Recycle Extraction (CARE)

The CARE process combines batch adsorption of proteins to conventional affinity resins with microfiltration [125]. In the first reactor, the resin is in contact with a homogenized bacteria-suspension. The reactor is connected with a microfiltration unit. The product binds to the affinity resin and contaminants (proteins, cell fragments, etc.) are removed with the filtrate stream. The loaded gel is subsequently transferred to a second reactor, where the product is desorbed from the resin and removed with the filtrate stream of the microfiltration unit. The resin remains in the retentate vessel and is transferred back to the first reactor (Fig. 12).

Product can always be detected in the filtrate stream of the first reactor. In the published work, the ratio of the product concentration in the filtrate stream of the first and second microfiltration units was 1 : 20; therefore, 5% of the product was lost during the loading step. Fouling of the resin cannot be prevented because the homogenate is in contact with the resins and blocks the pores. The purification is not very effective, and contaminants cannot be completely removed because the adsorption and washing steps are carried out in one reactor. High purification factors can be achieved if the volume of the washing buffer exceeds the volume of the loaded solution (>10 : 1). In any case, the main disadvantage of the CARE process is the loss of product during loading.

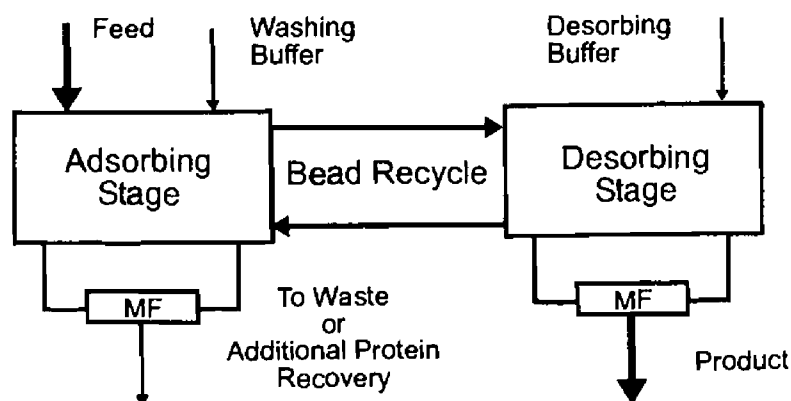


FIGURE 12 Schematic drawing of the CARE process [125].

Luong et al. [126] published research describing affinity cross-flow filtration similar to the CARE process. The system consisted of three reaction vessels and two cross-flow filtration units. In the first vessel, the product was loaded onto an affinity resin in a batchwise manner. Contaminants were removed by cross-flow microfiltration and the washed affinity resin was transferred to the second vessel, where the product was dissociated from the resin. The product was separated from the resin in the second cross-flow microfiltration unit. In the third vessel, the affinity medium was regenerated and equilibrated for the next loading step. A continuous process mode is feasible but would be very complicated. The process is not very well suited for the processing of suspensions and turbid solutions because cells and the affinity resin are retained by the membrane. Instead of an affinity resin, an affinity emulsion can be used, but the properties of the process will not be changed.

Continuous Affinity Belt

A chemically inert belt can be modified in such a way that biomolecules can be selectively captured from a fermentation broth using affinity ligands or electrostatically charged groups immobilized on the belt. The endless affinity belt is driven by two driving shafts. The process solutions (loading, washing, elution, and regeneration) are applied to the belt simultaneously, which results in a continuous process. The fermentation broth flows vertically on the belt and penetrates the matrix, which generates mass transport by convective flow. When the flow rate, the reaction time of the biomolecule with the ligand, and the chart speed of the belt are accorded correctly, a short residence time and complete recovery of the biomolecule can be achieved [127]. The microbial cells are retained at the surface of the belt. At the adjacent site, where the belt turns around, the cells are removed by a fine dosed liquid jet. The next step is the removal of the unbound contaminants by washing the belt in the reversed direction from that applied during loading. Nonspecifically bound molecules and cell fragments are removed by this back-flushing process (Fig. 13).

This washing step prevents fouling of the affinity belt, which may be a main advantage over existing processes such as the CARE process, the Niven-Scurlock process [124], affinity cross-flow filtration [126], and others. The next step is the elution of the biomolecule from the affinity belt. The elution is done in the reversed flow direction, which can decrease the elution volume. Finally, the belt is regenerated and equilibrated to be ready for the next cycle. In contrast to the Niven-Scurlock process, cross-contamination of the process solutions is minimized due to the vertical flow direction. The flow rate, the optimal residence time of the process solutions, and the chart speed of the belt can be adjusted by varying the size of the loading device and introduction of negative pressure under the belt. The whole system is very flexible in terms of different process conditions, which

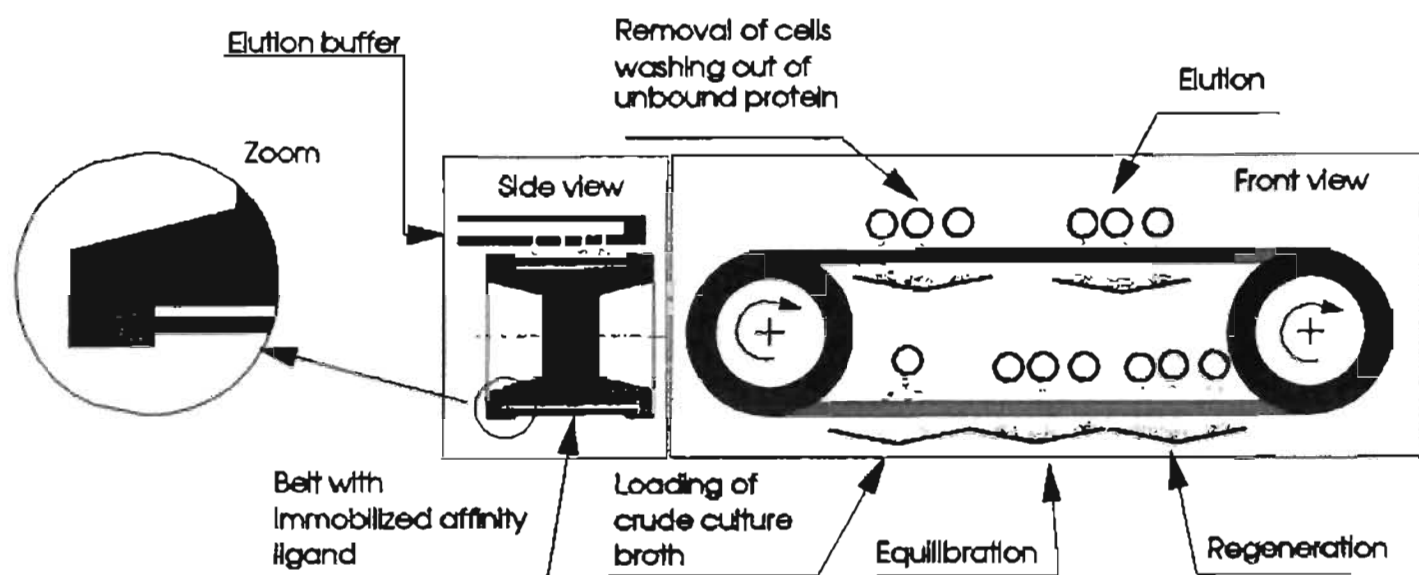


FIGURE 13 Schematic drawing of a diving-belt chromatograph according to Jungbauer and Hahn [127].

depend on the ligand-ligate interaction, the belt material, and the properties of the fermentation broth.

Countercurrent Chromatography (CCC)

Countercurrent chromatography is based on the distribution of substances in two liquid phases [128,129]. The liquid is fed into a coiled tube that is moved along an orbital trajectory. Due to centrifugal power, the liquids move in a counter-current. For proteins and many other biomolecules, this method is not practical because of denaturation in a nonaqueous phase. In aqueous two-phase systems, at least one phase exhibits high viscosity and, therefore, mass transfer between the two phases is limited. Similar problems occur with reversed micelle extraction as were observed with the aqueous two-phase extraction [130]. CCC has not been used for large-scale purification of proteins and other biopolymers.

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Sample Preparation

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INTRODUCTION

It is rarely the case that an analyst is presented with a sample in a suitable form for injection onto an HPLC column. Most often the sample must be manipulated in some fashion to concentrate the compound of interest, remove interferences, or modify the sample matrix to achieve compatibility with the separation conditions. Inadequate sample preparation will usually compromise HPLC column performance and, in worse cases, lead to equipment failure and reduced column lifetime. For “clean” samples, such as relatively pure polypeptides, preparation may require only simple procedures such as filtration, concentration, or dilution. When HPLC methods are used early in purification schemes, however, the complexity of the samples requires more sophisticated manipulations. In these cases, sample preparation can be the most time-consuming and labor-intensive element of the purification process; hence, sample handling techniques that lend themselves to automation are highly desirable, especially in industrial laboratories. An additional consideration in purification schemes for peptides, proteins, and nucleic acids is prevention of inactivation, degradation, or modification of the biomolecules during the process [1]. Oxidation can be prevented by addition of

compounds such as β -mercaptoethanol or dithiothreitol to the samples. Proteolysis can be controlled with reduced temperatures, addition of proteolytic inhibitors, or operation at pH values where proteases are inactive. Recovery of proteins sensitive to metals may require use of chelating agents, whereas the use of such additives in the purification of metalloproteins may be inadvisable. In designing multistep purification procedures for biomolecules, delivering a sample from one step in a form compatible with the successive step can minimize recovery losses due to intervening manipulations such as desalting and concentration. If sample preparation includes steps to modify (e.g., derivatization) or degrade (e.g., protease digestion, cleavage of oligosaccharides) the analyte or to stabilize it (e.g., reductive alkylation of cystines), conversion of the sample to the required pH or ionic strength for these procedures may be part of the sample workup.

Although most HPLC applications utilize one or more sample preparation procedures, it is best to keep sample preparation as simple as possible [2]. Some simple procedures that have found success for protein and peptide samples are precipitation and chromatographic methods. This chapter reviews the commonly used methods and devices for sample preparation in chromatography of biomolecules, particularly proteins. The proper choice and execution of the methods can greatly affect the success of the analysis, especially in terms of recoveries [1,3,4]. Planning the sequence of procedures can minimize time and maximize efficiency; a flowchart for sample preparation strategies was presented in *LC-GC* [5]. Table 1 summarizes some of the typical requirements for the preparation of a sample for injection onto an HPLC column.

Some traditional methodologies for these operations are laborious, frequently providing poor recoveries. Fortunately, many improved procedures that are rapid and simple, with increased yields, are available. In some cases,

TABLE 1 Objectives in Sample Preparation

Prefractionation
Extraction
Removal of contaminants
Solubilization
Detergent removal
Concentration
Desalting
Dilution
Removal of particulates
Preservation of structure
Preservation of biological activity

commercial products allow parallel processing of multiple samples, sometimes on line as part of an automated chromatographic method.

PREFRACTIONATION

The first step in sample preparation for HPLC is to ensure that the sample is in a suitable physical form, that is, in a liquid state. After that is achieved, the objectives of sample prefractionation are twofold. The first goal is to simplify the mixture so that the separation is within the peak capacities of available HPLC columns. This can be attained by separating the target class of biomolecules from extraneous molecules or fractionating a complex protein mixture into subclasses for separate processing. The other goal of sample prefractionation is to eliminate sample components that would bind irreversibly to the stationary phase, changing column selectivity or otherwise degrading chromatographic performance. The sample prefractionation techniques that accomplish these aims are listed in Table 2.

Precipitation

Precipitation is often employed as a simple first step in a purification procedure, particularly in the case of large-volume samples containing many components [1]. Precipitation can concentrate the analyte protein and simplify the sample matrix. This step may also denature or remove sample components that might compromise recovery, e.g., proteases. Conditions can be chosen to precipitate selectively the analyte protein. For example, the sample pH can be adjusted to the pI of the analyte to effect isoelectric precipitation. Isoelectric precipitation may be facilitated by the addition of organic solvents [6]. Fractional precipitation can be accomplished by addition of high concentrations of a salt such as ammonium sulfate to salt out the protein of interest selectively. Ammonium sulfate is typically the salt of choice because of its high solubility, low heat of solution, low density (important when recovering precipitated protein by centrifugation), and recovery of proteins without denaturation [7]. Protein precipitation in

TABLE 2 Prefractionation Techniques

Precipitation
Liquid Extraction
Liquid-Solid
Liquid-Liquid
Solid Phase Extraction (SPE)
Batch
Cartridge

ammonium sulfate is typically favored at pH values near the isoelectric point of the target protein and at higher temperatures [6]. Fractional precipitation can also be achieved using organic solvents such as ethanol or acetone [1,6,7]. Organic solvent precipitation is carried out in the cold (typically at 0°C) to reduce protein denaturation.

Organic polymers can also be used to precipitate proteins, although most polymer solutions are too viscous to be useful. Polyethylene glycol (PEG) is the most widely used polymer for protein purification because of its low viscosity at relatively high concentrations. An advantage of precipitation with PEG rather than ethanol is its reduced tendency to denature protein; following precipitation, PEG can be removed from the protein by ion-exchange or size exclusion chromatography or by ultrafiltration [8]. Nonspecific precipitation of all proteins in a sample can be accomplished by addition of perchloric or trichloroacetic acid or by addition of a nonpolar organic solvent such as acetonitrile. The latter techniques are frequently used to deproteinate a sample prior to analysis of a small molecule such as a drug or drug metabolite.

Precipitation has some disadvantages as a purification technique for proteins. For dilute protein solutions, yields may be poor [1,9]. Also, the sample may be recovered in a state incompatible with a following purification step. For example, a protein fraction recovered after ammonium sulfate precipitation will require desalting prior to ion-exchange chromatography.

Liquid Extraction

Liquid-Solid

The first step in preparing a solid sample for HPLC is usually homogenization in preparation for extraction [5]. Extraction of the relevant components of the solid can be accomplished by a variety of techniques including Soxhlet extraction, accelerated extraction methods, and supercritical flow [10]. Soxhlet extraction, which leaches a specific component from a sample by refluxing in an appropriate solvent, is the benchmark classical technique for extraction of small molecules from a matrix [5,10].

Supercritical fluid extraction (SFE) is gaining popularity as a prefractionation technique because it can reduce extraction time with concomitant good recoveries and ease of automation [11]. SFE frequently employs carbon dioxide, which is converted into a supercritical state by pressure and temperature. Adjustment of these parameters and addition of modifiers and cosolvents yield selective extractions of specific classes of molecules [11–13]. Like Soxhlet extraction, SFE is typically used for extraction of small molecules.

Liquid-Liquid

When solutions contain classes of compounds with radically different solubilities, they can often be isolated with classical extraction techniques. In liquid-liquid

extraction methods, two solvents that are not miscible are vigorously agitated, usually by shaking or stirring. These procedures can be manual or automated to include simple separatory funnel extractions, countercurrent chromatography, and on-line procedures [14]. Liquid extraction is especially useful for very hydrophobic proteins, protein aggregates, or lipids that are primarily soluble in organic solvents or aqueous-organic mixtures. In some cases, a single solvent extraction can be used to remove the desired protein or protein class; alternatively, a series of extractions with different organic solvents or aqueous-organic mixtures can be employed to effect class separations. This latter approach has been used for fractionation of mixtures of hydrophobic membrane proteins and structural proteins. For example the four major classes of cereal proteins (albumins, globulins, prolamins, and glutelins) can be sequentially extracted with water, dilute salt, and ethanol-water [4]. Similarly, the four small subunits of yeast cytochrome *c* oxidase (a seven-subunit oligomeric membrane protein) have been selectively extracted from the holoenzyme with water-acetonitrile (60:40) followed by water-acetonitrile-isopropanol (50:25:25) [15].

Liquid-liquid extraction using water-soluble polymer solutions provides a mild, nondenaturing extraction system for proteins [1,7]. The most familiar example is partition between a relatively hydrophilic dextran phase and a relatively hydrophobic PEG phase. The selectivity of the two phases can be modulated by addition of salts; very high selectivity can be achieved by binding an affinity ligand to a fraction of the PEG polymers. In the case of affinity liquid-liquid extraction, recovery of protein requires modification of the phase to dissociate the bound protein from the polymer, e.g., by a pH change.

Solid-Phase Extraction (SPE)

Batch

Batch extraction with solid-phase sorbents has been widely used for prefractionation of biological mixtures containing small proteins and peptides [4]. The technique is inexpensive, can be applied to large volumes, and is amenable to handling viscous samples that would plug solid-phase cartridges or analytical HPLC columns. The steps in batch extraction are listed in Table 3.

As with most solid-phase extraction techniques, the selected sorbent must first be conditioned with a suitable solvent to wet the phase. Methanol is commonly used for bonded-phase sorbents and hexane for unbonded silica. The conditioned sorbent is then mixed with the sample so that specific molecules can bind to it, typically requiring gentle stirring in the cold for several hours. Characteristically, low-molecular-weight species (<40,000 Da) bind more tightly than large species. Binding can be followed by one or more extraction steps with suitable solvents, such as aqueous salt solutions or aqueous-organic solvent mixtures, to remove the undesirable components. Final elution of the biomole-

TABLE 3 Steps in Solid-Phase Extraction

Sorbent selection
Sorbent conditioning
Sample binding
Extraneous substance extraction
Sample elution

cules of interest is achieved batchwise in a small volume of a strong eluent following collection of the sorbent by filtration or centrifugation. Alternatively, the sorbent can be packed into a column and the desired molecules eluted in a very small volume with step or gradient elution. Solid-phase batch extraction has been applied to purification of human immune interferon [16], gibbon interleukin-2 [17], and murine B-cell stimulatory factor [18].

The requirements for the adsorbent (e.g., particle size and uniformity, porosity, mechanical stability) are much less stringent than for chromatography packings, so materials for batch extraction can be much less expensive and more affordable for large-scale applications. It should be noted that batch extraction can be used to adsorb a contaminant selectively while the unbound target protein remains in solution.

Cartridge

Solid-phase extraction employing fully porous microparticulate silica or polymers, with or without covalently bonded stationary phases, has been used in the analysis of low-molecular-weight compounds in clinical, environmental, food, and agricultural samples for over a decade. Typically, up to a few hundred milligrams of 20- to 50- μ m porous support is packed into a minicolumn, a syringe barrel, or a plastic cartridge that can be fitted to a tuberculin or Luer-Lok type of syringe. Sample application, extraction, and elution are achieved by passage of liquids through the cartridge bed with positive pressure or vacuum. The advantages of solid-phase extraction are its ease of use and the wide variety of commercially available sorbents and cartridge types, permitting selection of the appropriate phase for any particular sample preparation problem. Also, vacuum and pressure manifolds are available for simultaneous processing of multiple samples [4]. This rapid fractionation is advantageous when bioactive peptides or intermediates in peptide metabolism might be lost or degraded by more extensive isolation procedures.

The steps in solid-phase cartridge extraction are the same as those listed in Table 3 for batch extraction. The choice of the sorbent is dependent on the nature

of the fractionation desired. Compounds may be discriminated on the basis of charge using cation- or anion-exchange sorbents or on relative surface hydrophobicity using one of a variety of alkyl, aromatic, or nitrile phases. The most commonly used phases are silica, octadecyl, octyl, and ion-exchange supports, although many others have also been implemented, including mixed phases [2,19].

Approaches to developing a solid-phase sample cleanup method for low-molecular-weight compounds are generally applicable to biopolymers [4]. It is best to have the analytical chromatographic method including column, mobile phase, and gradient parameters established so that the effectiveness of the extraction method can be assessed. Using all available information about the sample and matrix, the general cleanup strategy, such as selective retention of sample or interfering compounds, should be determined. At this point, any constraints on the extraction method dictated by the sample chemistry or HPLC method should be identified. These might include the ultimate concentration or volume of the sample or the solvent composition, including pH, ionic strength, organic modifier concentration, and volatility. The final sample volume should be small (a minimum of two bed volumes is generally required to elute sample from the sorbent) and the sample solvent should be weak in strength relative to the HPLC mobile phase.

The selection of the appropriate sorbent depends on the amino acids on the interactive surface of the desired peptide or peptide class relative to the functional groups of interfering substances. Chromatographic data on the sample are useful in predicting its behavior in solid-phase extraction. For example, polypeptides with basic side chains can be selectively retained on a sulfopropyl cation-exchange phase and selectively excluded from an anion exchanger. For peptides with significantly hydrophobic contact regions, octyl or phenyl phases would be appropriate. Very hydrophobic polypeptides that are soluble in organic solvents can sometimes be retained on polar sorbents in the presence of nonpolar organic solvents.

Composition of the sample matrix may also affect the choice of sorbent, particularly if the extraneous substances are in high enough concentration to interfere with sample binding. For example, the presence of concentrated salts would reduce retention on ion-exchange sorbents and enhance retention on nonpolar sorbents; desalting or sample dilution of such solutions would be required prior to SPE by ion exchange. Certain detergents can reduce polypeptide binding by competing for active sites on ion-exchange sorbents, and others may reduce surface tension on nonpolar sorbents. The HPLC method may suggest that a sorbent different from that of the analytical column is preferable for reasons of solvent strength. For example, because the aqueous salt solutions used to elute samples from ion-exchange sorbents are weak solvents in reversed-phase systems, sample fractions can be introduced directly onto a reversed-phase HPLC column with no intermediate manipulations.

Once the sorbent type has been selected, extraction of the sample should be confirmed. The cartridge should be conditioned with 1–2 bed volumes of a solvent such as methanol, then prepared for sample introduction by passage of 10–20 bed volumes of the sample solvent. If an ion-exchange phase is used, it should first be converted to the proper counterion, then equilibrated at the appropriate pH. Because of the slow diffusion rates of macromolecules, samples should be applied at relatively low flow rates, preferably less than 10 mL/min; a cartridge syringe device can generate flow rates of 200 mL/min [3]. After sample application, the bed should be washed twice with 10–20 bed volumes of sample solvent followed by analysis of both washes by HPLC. Absence of sample components in the chromatogram indicates adequate extraction by the sorbent, whereas appearance of samples in the wash implies that a more retentive sorbent is needed.

The last steps in SPE are to wash out extraneous bound substances with suitable solvents and, finally, to elute the sample in a small volume with a strong solvent. Typical wash solvents and sample elution solvents are given in Table 4.

Elution conditions for SPE columns follow the same principles as analytical columns of the same mode. Because proteins interact at multiple sites with chromatographic supports, the transition from complete sorption to full desorption occurs over an extraordinarily narrow range of solvent strengths; therefore, in many cases it is possible to find elution conditions where the desired protein or protein class is recovered while unwanted material is separated by significantly stronger or weaker retention on the sorbent. Similarly, sequential elution steps can be used to fractionate a complex protein sample into subfractions. In both ion-exchange and nonpolar interactions, retention is strongly dependent on the ionic state of the sample; therefore, manipulation of solvent pH provides a powerful

TABLE 4 Solvents for Sorbent Extraction

Sorbent class	Weak solvents (wash)	Strong solvents (sample elution)
Nonpolar	Water Low ionic strength buffers Low % organic in water or buffer	High % polar organic (methanol, propanol, or acetonitrile) in water or buffer
Polar	Nonpolar organic (hexane or methylene chloride)	Polar organic solvents Aqueous buffers or acids
Ion exchange	Water Low ionic strength buffers Cation exchange, pH < pK Anion exchange, pH > pK	Cation exchange, 2 pH units > pK Anion exchange, 2 pH units < pK High-ionic-strength buffers

tool for modulating retention of both interfering substances and the molecules of interest. Release of strongly retained samples from ion-exchange sorbents may be achieved by increasing ionic strength, changing pH, or using a counterion with stronger selectivity. For example, a protein strongly retained in chloride buffer may be eluted with phosphate. Commercially available sorbents that use silica as the support material may excessively retain peptides or proteins containing basic side chains because of silanol interactions. In such cases, addition of a competing base such as triethylamine or tetramethylammonium salt to the eluent may improve recovery. Sorbents for SPE with pore diameters in the range 60–100 Å will possess low loading capacities for proteins and peptides, which are excluded from most of the sorbent surface.

A number of automated sample processing systems for SPE are available. In some cases, sample wash and elution steps are performed automatically off line and eluted samples are transferred to HPLC autosampler vials. In other systems, the processor functions as an on-line autosampler by employing cartridges compatible with high-pressure operation. This permits samples to be eluted from the sorbent directly onto the HPLC column via an automated injection valve. Conditioning and wash steps may be manually performed off line or automatically by the processor or an autosampler interfaced to it. In on-line methods, the choice of sorbent and extraction solvents will depend strongly on the HPLC method because the initial mobile phase composition of the HPLC method must quantitatively transfer the sample from the sorbent to the head of the analytical column. It must be both a strong eluent for the SPE sorbent and a weak eluent for the HPLC column; thus, coordination of the SPE sorbent to the HPLC column chemistry is required. Ion-exchange prefractionation should be ideal for automated processing prior to a reversed-phase separation because the typical initial mobile phase composition for reversed-phase chromatography (RPC) (dilute aqueous acid with low percentages of organic modifier) will effectively strip sample from an ion-exchange chromatography (IEC) sorbent. If a reversed-phase SPE support is desired for use with an octadecyl analytical column, a less hydrophobic sorbent, such as, C_8 or C_3 , would be required and quantitative transfer would need to be confirmed. The requirement for correct matching of SPE sorbents and analytical columns suggests that all manual off-line methods may not be easily adapted to on-line processing.

Applications of SPE for peptide and protein samples abound in the literature. In one interesting example, a series of bonded-phase sorbents was used to fractionate pituitary peptides from biological tissues [20]. Initially, an acid extract was passed through reversed-phase (C_{18}) cartridges to remove hydrophobic proteins. The pH of the eluent was then adjusted and the sample was run through tandem cation- and anion-exchange cartridges that bound basic peptides and acidic peptides, respectively; neutral peptides passed unretained through the cartridge set. Peptides retained on each cartridge could be eluted with appropriate

solvents. Four fractions of a complex tissue extract, which could each be conveniently analyzed by RPC without interference or damage to the column by unwanted substances, were thus obtained. Optimum fractionation in this kind of system is highly dependent on both pH and phase selection. In this case, at pH 7, weakly basic peptides failed to bind to the cation exchanger and eluted unretained with the neutral fraction.

On-line multicolumn prefractionation is an efficient but complex method of isolating fractions for HPLC analysis [1]. Switching devices can implement the transfer of fractions to other extraction columns or to an analytical column. Each solvent used for extraction must be compatible with the subsequent step. Such on-line procedures minimize sample loss from manual manipulations. On-line multicolumn techniques are most effective for high-volume repetitive procedures where the time involved in method development is inconsequential due to throughput. If cross-contamination must be avoided, columns or cartridges must be discarded after each use; In other cases, regeneration is possible.

SPE has also provided a direct approach for removal of contaminants from proteins excised from two-dimensional electrophoresis gels [21]. An empty cartridge containing the gel material was connected to a short reversed-phase cartridge column. With passage of 0.1% aqueous trifluoroacetic acid (TFA) through the coupled columns, the protein was transferred from the gel and bound to the reversed-phase column whereas gel contaminants, buffer, and stain eluted in the void volume. Proteins were analyzed by gradient elution with TFA-acetonitrile. This procedure is well suited for recovering small peptides that are often lost in electroblotting.

SOLUBILIZATION

Any molecules that are separated by HPLC must first be soluble in the mobile phase. For some biomolecules, particularly membrane proteins, additives must be used to achieve this [22]. Integral membrane proteins are very hydrophobic species that lie embedded in the lipid bilayers of cell membranes. They are composed of relatively hydrophilic regions that are located on the membrane surfaces and one or more hydrophobic sequences that are associated with the interior hydrophobic portions of the membrane lipid. Disruption of this organization during purification usually leads to aggregation and precipitation unless detergents are added. Detergents compete with the lipids for the hydrophobic regions of the protein, thus maintaining its soluble state in aqueous environments, usually in the form of protein-detergent or mixed protein-lipid-detergent micelles. In a typical purification scheme, loosely associated hydrophilic peripheral proteins are first removed from the membrane with salt or chaotropic agents, and then integral membrane proteins are liberated by the addition of the detergent.

The choice of detergent used for solubilization of membrane proteins depends on both the protein and the subsequent method of purification. Some detergents with applications in membrane protein purification are listed in Table 5.

A systematic approach to the design of a solubilization procedure includes selecting the detergent, buffer, and temperature and testing for solubilization [23]. Major considerations in detergent selection are the constraints imposed by the subsequent chromatographic method and any requirements for recovery of biological activity. The critical micelle concentration (CMC) is the concentration of a detergent monomer at which micelles, i.e., spherical aggregates of detergent molecules, begin to form. Detergents with high CMC are easier to remove by dialysis than those with low CMC. Some detergents, such as Triton and Nonidet, are ultraviolet (UV) active and thus interfere with absorbance detection at wavelengths less than 280 nm. A reduced (hydrogenated) form of Triton X-100 is commercially available from Sigma. It has properties similar to those of the nonreduced form and no absorption in the middle to low UV [24]. If ion-exchange chromatography is to be used, ionic detergents should be avoided. Most

TABLE 5 Detergents Used for Solubilization of Membrane Proteins

Detergent	CMC (mM)
Ionic	
Sodium dodecyl sulfate	8.13 (water) 2.3 (0.05 M NaCl) 0.51 (0.5 M NaCl)
Sodium cholate	13–15
Sodium deoxycholate	4–6
Sodium taurodeoxycholate	2–6
Nonionic	
Triton X-100	0.24–0.3
Nonidet P40	0.29
Triton X-114	0.2
Tween 80	0.012
Emulphogen BC-720	0.087
Octylglucoside	25
Brij 35	0.091
Dodecyl dimethylamineoxide	2.2
Amphoteric	
CHAPS	4–6
Zwittergent 3–12	3.6

Source: Ref. 23.

detergents will bind to reversed-phase and size exclusion columns via hydrophobic interactions and, if not removed prior to chromatography, are likely to alter permanently the bonded phase and, thus, the selectivity of the column. If an HPLC column is used for the analysis of detergent-containing materials, it is best if it is dedicated to this application.

Sodium dodecyl sulfate (SDS) binds strongly to proteins and is one of the most effective solubilizing agents; however, it tends to denature proteins irreversibly with loss of biological activity. Bile salts have been found to be more effective than nonionic detergents in dissociating protein complexes [4]. Bile salts such as sodium cholate and deoxycholate, and their derivatives such as CHAPS, are known for their gentleness and are often used when solubilization without denaturation is desired; however, they form aggregates which precipitate at acidic pH [22]. If divalent cations are required for maintenance of biological activity, sodium cholate should be avoided [4].

In general, solubilization is best achieved at high ionic strength (0.1–0.5 M salt) in a 25 mM buffer with a pH close to the pK of the buffer. The salt and buffer must be compatible with the detergent; for example, SDS will precipitate in potassium phosphate but is soluble in sodium phosphate. If ion-exchange chromatography is to be used subsequently, the buffer and salt ions should not have greater strength than the mobile phase counterions. Usually, solubilization is carried out at reduced temperatures. After the detergent, salt, and buffer conditions have been selected, the effectiveness of solubilization can be determined by centrifuging after incubation to separate solubilized from insoluble material for subsequent assay. A range of detergent concentrations (0.1–3.0% in the protein-salt-buffer solution) should be tested with gentle stirring in the cold for 1 hr. Successful solubilization may depend on finding the optimal detergent/protein ratio by this procedure. Protein solubilization occurs at or near the CMC for most detergents. If biological activity is lost during detergent solubilization, addition of a stabilizer such as glycerol, dithiothreitol, or EDTA may be necessary.

Effective solubilization of a membrane protein may require a detergent that is incompatible with subsequent steps in the purification process. For example, competition with membrane lipids to form protein-detergent micelles may require a high concentration of a detergent with low CMC and high aggregation number, such as Triton. Removal of such detergents by dilution or dialysis is often difficult; however, it may be necessary because of its UV absorption or other properties. It is sometimes possible to exchange the detergent for one more compatible, such as a bile salt [25]. In such a strategy, protein-detergent micelles are first separated from detergent micelles, detergent-lipid micelles, and detergent monomers by SEC. Detergent exchange is then achieved by running the detergent-protein fraction through an SEC column with a mobile phase containing a detergent of high CMC and low aggregation number. The mobile phase detergent will disperse the solubilization detergent into monomers, as well as

replace it in the protein-detergent micelle. Detergent exchange can be accomplished using other chromatographic media by injecting the protein-detergent complex on the column, washing the column with about 20 column volumes of the new detergent, followed by appropriate elution conditions for the new protein-detergent complex [26].

Addition of chaotropic agents to the solubilization mix may be required if protein-protein interactions are strong [27]. Urea and guanidine hydrochloride can be used. However, urea complexes with nonionic detergents and some detergents (nonionic detergents, linear alkyl sulfobetaines) are insoluble at moderate guanidine hydrochloride concentrations. Combinations of CHAPS and guanidine hydrochloride have been recommended for difficult applications.

DETERGENT REMOVAL

General

Removal of detergents from extracted or solubilized hydrophobic proteins may be necessary prior to subsequent purification steps [26]. Detergent micelle formation may complicate concentration procedures or detergents may degrade column performance and interfere with UV detection in HPLC separations. Techniques for removal of detergents from proteins are listed in Table 6. Dialysis can be used to remove detergents with high CMC values from proteins after dilution of the detergent below the CMC, but this is generally a time-consuming technique.

Extraction

Chloroform extraction has been shown to be effective in removing SDS from proteins electroeluted from SDS polyacrylamide gels [28]. After elution, the aqueous fractions were mixed with methanol and extracted with chloroform. The SDS was removed by the chloroform and the extracted protein was washed extensively with methanol and then dried to remove any residual chloroform.

TABLE 6 Methods for Detergent Removal

Dialysis
Solvent extraction
Ion-pair extraction
Adsorption on polystyrene resin
Adsorption on ion-retardation resin
Binding to affinity matrix

Ion-pair extraction has also been used to remove SDS from detergent-protein complexes [29]. Dry samples were extracted with a solution of ion-pairing agent in an organic solvent, such as acetone/triethylamine/acetic acid/water or heptane/tributylamine/acetic acid/butanol. Sufficient water to promote formation of the alkylammonium-SDS ion pair was required in the extractant or protein sample; therefore, in the second solvent system, water had to be added to about 1%. A single extraction removed up to 95% of the total SDS; protein was recovered as a precipitate that could be separated from the extractant by low-speed centrifugation. Washing with acetone or heptane removed any residual extractant or SDS. Salts in the sample had to be removed prior to extraction due to interference with SDS removal. This procedure can be adapted to extraction of small volumes of aqueous protein-SDS solutions by 1:20 dilution using extractant prepared without water. A two-step extraction quantitatively removes the detergent with recovery of protein that generally exceeds 80%. This extraction procedure also removes the Coomassie Blue stain from proteins recovered from SDS polyacrylamide gels.

Adsorption

Adsorption on neutral polystyrene resins by batch or by column is a popular technique for removal of surfactants from aqueous solutions of proteins. Because this technique removes only detergent micelles, the detergent concentration will be reduced only to the CMC. Amberlite XAD-2 resin and Biobeads SM have been used for removal of Triton X-100 by both batch and column methods [3].

Ion-retardation resins, which consist of acrylic acid polymerized inside a strong anion-exchange resin on a polystyrene divinylbenzene matrix [30], are also effective for removal of SDS from proteins. Passage of a protein-SDS complex through the resin results in complete retention of SDS and elution of protein with 80–90% recovery [31]. The capacity of the resin for SDS is more than 2.2 mg/g, which effectively reduces the SDS level to less than one molecule of SDS per protein molecule. Because SDS binds tenaciously to the resin, it cannot be removed and the resin must be discarded after use. In the presence of buffers, adsorption of SDS by an ion-retardation column is reduced, resulting in incomplete removal of detergent from the protein. This can be circumvented by prior removal of buffer by SEC or, more conveniently, by the addition of a few grams of size exclusion gel to the head of the ion-retardation resin bed to retard the buffer [4].

Affinity Binding

Removal of detergents using Extractigel, an affinity matrix developed for detergent removal, is based on interaction with proprietary affinity ligands after

selective permeation of the detergent into the gel [32]. Proteins larger than 10,000 Da are excluded and pass through in the void volume; therefore, operation in the column mode rather than batch format is required. The technique cannot be used for polypeptides below the 10,000 Da exclusion limit because they permeate the matrix and bind to the ligand. Dilute protein solutions ($\leq 50 \mu\text{g/mL}$) may yield poor recoveries due to nonspecific binding. In such cases, it is recommended that a protein such as bovine serum albumin be added to the sample to saturate nonspecific binding sites. This affinity product has been used for removal of both ionic and nonionic detergents [4].

CONCENTRATION

General

Biological samples are frequently very dilute by the time HPLC procedures are employed; therefore, concentration is necessary for detection as well as general volume reduction. Commonly used techniques for sample concentration are listed in Table 7.

Conventional techniques, such as lyophilization and evaporative concentration, are lengthy and may cause chemical modification or loss of sample by nonspecific adsorption if the sample is taken to near dryness. Ultrafiltration and chromatographic concentration are more rapid and gentle, minimizing the problems encountered in conventional approaches.

Precipitation

Fractional precipitation is a useful tool for biomolecule purification because it can be very selective for specific classes of molecules. Precipitation of proteins may be initiated by pH manipulation; high salt concentration, most notably with ammonium sulfate; high concentration of organic; or addition of certain chemicals such as perchloric acid [1,7]. Similarly, RNA can be specifically precipitated with lithium chloride [1]. The success of such techniques depends on the goals. If

TABLE 7 Sample Concentration Techniques

Lyophilization
Vacuum evaporation
Precipitation
Dialysis against concentrator resins
Ultrafiltration
Chromatography

the objective is to remove the proteins or nucleic acids and analyze the components of the supernatant, these can be very effective procedures. If the aim is to analyze the precipitated molecules, success will depend on their quantitative reconstitution and preservation of biological activity. Sample loss can occur during precipitation by formation of insoluble aggregates.

Ultrafiltration

Ultrafiltration is based on the selective passage of low-molecular-weight sample components through a porous membrane filter of appropriate porosity while high-molecular-weight species, such as proteins that cannot penetrate the pores, become concentrated. Centrifugation is the driving force. Filters are available with molecular weight cutoffs in the range of 10,000–30,000 Da [33,34]. Ultrafiltration devices that are designed for use in fixed-angle rotors permit unrestricted solvent flow through the membrane and prevent the sample from reaching complete dryness. These devices permit milliliter samples to be rapidly concentrated up to 80-fold with low nonspecific adsorption and generally good recoveries.

Chromatography

In chromatographic concentration, the strategies for choosing columns and packings are similar to those employed for solid-phase extraction. The sample, diluted in a large volume of a weak solvent, is concentrated onto the column and then eluted in a small volume of strong solvent. For example, low-pressure ion-exchange gels packed into minicolumns were used to concentrate samples up to 30-fold prior to SEC [35]. The necessity of using a solvent of low ionic strength to allow quantitative binding of protein to an ion exchanger may require appropriate dilution before application to the column.

Short (3–10 cm) HPLC columns can also be used for rapid sample concentration. Microbore reversed-phase columns have been used for preconcentration of proteins prior to microsequence analysis, after collection and dilution of milliliter fractions from conventional HPLC columns [36]. Gradient elution at low flow rates (0.1–0.2 mL/min for 2-mm-ID or 0.02–0.04 mL/min for 1-mm-ID columns) permits recovery of proteins in volumes as small as 25 μ L, with concentration factors up to 80-fold. The high capacities of porous microparticulate packings enable 50–100 μ g to be loaded onto microbore columns as long as the sample is introduced in a weak solvent.

DESALTING

Removal of salt from a protein solution may be necessary prior to chromatography. For example, injection of high concentrations of salt would result in irreproducibility in retention of early eluting peaks on an ion-exchange column. Concentrated salts may also be incompatible with some organic mobile phases in reversed-phase chromatography. Common desalting techniques are listed in Table 8.

Dialysis is the traditional but time-consuming method for removing salts, chaotropic agents, and detergents from protein fractions. The speed and efficiency can be improved by using hollow fiber techniques [1]. Size exclusion chromatography is a fast and effective alternative to dialysis for desalting using small-pore, cross-linked, dextran or polyacrylamide gels so that proteins are excluded while salts are retarded by permeation. Desalting gels are available as low-pressure prepacked columns [33,34] or packed centrifuge tubes with centrifugation as the driving force [37]. Size exclusion HPLC columns can also be used for rapid desalting; however, operation with low-ionic-strength mobile phases may result in adsorptive interactions for some proteins.

Octyl and octadecyl reversed-phase sorbents are frequently used for desalting aqueous protein fractions because salts and chaotropic agents are unretained under conditions where proteins exhibit strong interactions. After sample application, salts and other small polar compounds can be washed from the sorbent with the weak solvent, followed by elution of protein with an aqueous-organic mixture. Desalting can be performed off line using minicolumns or cartridges or on line in an HPLC gradient method after elution with a volume of weak solvent. Reversed-phase desalting is advantageous if it must be followed by a concentration step because the elution solvent is generally volatile.

Ion-retardation resins, as discussed previously for detergent removal, are also used for desalting [30]. These resin copolymers consist of adjacent anionic and cationic sites that, in the absence of counterions, exist in a self-adsorbed state. On application of a protein-salt solution to the resin, ionic species in the sample adsorb to the resin in an ion-exchange process, disrupting the resin self-association. Salts of certain monovalent ions such as sodium chloride, potassium chloride, and Tris-HCl are retained, whereas proteins can be readily eluted with

TABLE 8 Desalting Techniques

Dialysis
Size exclusion chromatography
Reversed-phase chromatography
Ion retardation
Ultrafiltration

water. Sodium acetate, sodium citrate, sodium phosphate, and ammonium sulfate do not compete as effectively with the self-adsorption and thus they may not be completely removed from the proteins. After desalting, the resin can be regenerated by treatment with 1 M hydrochloric acid followed by 1 M ammonium hydroxide containing 0.5 M ammonium chloride and, finally, by extensive washing with water.

The ultrafiltration devices used for sample concentration can also be effective for desalting [38]. A sample that has been concentrated by ultrafiltration should be diluted 10-fold in buffer or water and subjected again to ultrafiltration; each ultrafiltration cycle removes about 90% of the salt, resulting in reduction by 99% in two or three cycles.

FILTRATION

Filtration is an essential process in sample preparation. For complex samples, it may be implemented at more than one step in the procedure, but it is always one of the final steps before injection onto an HPLC column. Particulate matter will not only damage HPLC columns, it may also interfere with other purification steps. Usually the liquid from the filtration is collected, but sometimes the precipitate is the desired fraction to undergo further processing.

The primary considerations in filtration are the pore size and composition of the filters [39]. The choices are based on the specific analyte and sample matrix. As mentioned previously, filters of 0.45 μm should be used prior to injection. The composition of the membrane must be compatible with both the sample and the solvent. Mismatch with a solvent can result in disintegration of the filter or leaching of some of its components. Mismatching with a sample can result in adsorption; for example, proteins may bind to either very hydrophobic or very polar surfaces.

SAMPLE PREPARATION CONSIDERATIONS FOR CAPILLARY ELECTROPHORESIS

The foregoing discussions have focused on preparation of samples for chromatography. Capillary electrophoresis (CE) is a relatively new analytical technique that combines the separation processes of electrophoresis with the instrumental characteristics of HPLC. Most of the considerations in preparing a sample for introduction into a CE system are identical to those for HPLC, and many of the same strategies and manipulations can be employed for capillary electrophoresis. However, there are some unique properties of CE that must be kept in mind when preparing samples for analysis.

The first consideration is the sensitivity of the CE injection process to sample ionic strength. When injecting samples electrokinetically, the presence of

salt in the sample will reduce injection efficiency in direct proportion to the sample ionic strength. Therefore salt will reduce overall detection sensitivity, and the presence of variable salt levels in samples that are to be compared for analyte concentration will make accurate quantitation impossible. Displacement injection techniques (pressure, vacuum, gravity) are less sensitive to sample ionic strength, but salt levels can still affect quantitative accuracy. If samples are known to contain high or variable salt concentrations, they must be desalted or brought to similar ionic strengths prior to analysis. It should be noted that simple dilution to reduce ionic strength is not satisfactory because dilution will not change the ratio of salt to analyte ions. If the salt concentration is unknown, dialysis or buffer exchange is strongly advised. In the case of isoelectric focusing, the presence salt in the sample will cause compression of the ampholyte pH gradient, resulting in loss of resolution and increased risk of protein precipitation. Excessive sample salt levels will be evidenced by high currents during the focusing process.

The second consideration in CE sample preparation is the importance of sample pH. In capillary zone electrophoresis (CZE), analyte mobility is based on mass and charge, so migration velocity is highly dependent on the pH of the medium. In electrokinetic injection, variations in sample pH can change analyte mobility and therefore affect injection efficiency. In displacement injection, a differential in sample and run buffer pH is sometimes used to effect zone sharpening or stacking in order to increase detection sensitivity [40]; variations in sample pH alter stacking efficiency.

On-tube detection is almost universally used in CE, and the short detection path lengths provided by fused silica capillaries result in a 100- to 400-fold reduction in detection sensitivity compared with HPLC. Although various techniques are employed in CE to regain most of this sensitivity, detectivity remains a serious concern. Therefore concentration steps are often necessary in sample preparation for CE. It should be emphasized that some concentration techniques such as evaporation and lyophilization will not change the sample ion/salt ion ratio for nonvolatile salts, so no effective concentration is achieved when electromigration injection is used. The use of transient isotachophoresis has been proposed for on-line preconcentration of proteins prior to CZE separation [41,42].

One of the advantages of CE is the ability to analyze extraordinarily small sample volumes (injected volumes are typically in the nanoliter range, and total sample volumes of a few microliters are easily accommodated by automated CE systems). Workup of microliter sample volumes is difficult because many HPLC sample preparation methods and devices are scaled for milliliter volumes. Fortunately, many sample preparation devices for handling small volumes have become commercially available in the last few years. These include miniature desalting columns and ultrafiltration cartridges, microdialyzers, and small-scale sorbent extraction devices. Miniaturized techniques for desalting and concentrat-

ing CE samples using polyacrylamide gel devices have been described by Hjertén et al. [43]. Laio and Zhang [44] in the same group described techniques, for on-line desalting of proteins by ion-ampholyte exchange prior to capillary isoelectric focusing.

CONCLUSIONS

The ideal sample preparation scheme will minimize the final HPLC separation problem, reduce the risk of HPLC column contamination, and provide good analyte recovery. At the same time, the procedure should be as simple and rapid as possible. The number of steps can be minimized by coupling steps with complementary rather than similar selectivity. The product of each step should be directly compatible with the successive step to minimize interstep manipulations such as desalting or concentration. Many devices are commercially available to streamline, automate, and miniaturize many steps in sample preparation.

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Gradient Elution Separation of Large Biomolecules

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INTRODUCTION

Gradient elution is used more often than not when biological samples are to be separated by HPLC in a retentive mode, i.e., except for size exclusion chromatography. The reason is that samples of biological origin are often quite complex, and different sample components may vary widely in their relative retention. This in turn precludes the use of isocratic separation, where it is necessary that all compounds elute within a fairly narrow retention range, e.g., $1 < k' < 20$. In gradient elution, on the other hand, conditions can usually be arranged so that each band passes through the column with an average k' value between 1 and 20. This in turn means improved resolution, better band shape, and/or easier detection of individual compounds in the sample mixture.

Most chromatographers have a good understanding of the basis and application of isocratic HPLC. Workers in the life sciences have adapted these principles to the special requirements of their individual samples, as described elsewhere in this book. Separations that use gradient elution, however, are inherently more complicated. All the variables present in isocratic elution

(column, mobile phase composition and flow rate, temperature) apply to gradient separations as well; in addition, the composition of the mobile phase varies during gradient elution. Fortunately, the situation is not as difficult as might be imagined. The reason is that the separation of any two adjacent bands in gradient elution is governed by exactly the same factors that influence separation in "corresponding" isocratic systems. Corresponding isocratic and gradient systems are characterized by the same sample, column, and mobile phase components; other variables (e.g., flow rate, temperature) also have the same values. For example, a 50% propanol-water mobile phase (isocratic elution) might be replaced by a 40–60% propanol-water gradient, with other conditions the same. As we will see in Eq. (4) and related discussion the choice of an appropriate gradient time t_G then allows the duplication of the isocratic separation of any two adjacent bands by gradient elution. This means that we can use essentially the same approach to obtain a satisfactory separation by either isocratic or gradient elution.

In this chapter we will show how gradient elution is related to isocratic separation. As a result, it will be possible to apply all of our knowledge of isocratic separations to the understanding and control of corresponding gradient systems. This way of looking at gradient elution will also provide us with a clear picture of how different separation variables affect gradient elution. Some useful gradient elution techniques for improving the final separation of peptides, proteins, and other large biomolecules will also be explored.

BASIC PRINCIPLES OF GRADIENT ELUTION

The following discussion is a practical summary of theory and experimental observation. For details, see Refs. 1–3a.

Reversed-Phase HPLC

We will start with reversed-phase systems, as these are somewhat easier to understand. However, the general principles are the same for ion-exchange, hydrophobic interaction, affinity, and other retentive HPLC methods. Linear gradients with increasing % organic solvent in the mobile phase will be assumed, i.e., the volume fraction ϕ of some organic solvent (e.g., acetonitrile or propanol) will change with time (t) during the gradient as

$$\phi = \phi_0 + \left(\frac{t}{t_G}\right)(\phi_f - \phi_0) \quad (1)$$

Here, ϕ_0 is the initial value of ϕ , ϕ_f is the final value, and t_G is the gradient time (time during which ϕ is changing). The relationships are illustrated in Fig. 1.

Usually the sample will be injected at time $t = 0$, at which time the gradient is also begun. In most cases the retention of the various sample components at the beginning of the gradient ($t = 0$) will be quite strong, i.e., the k values (k_0) of

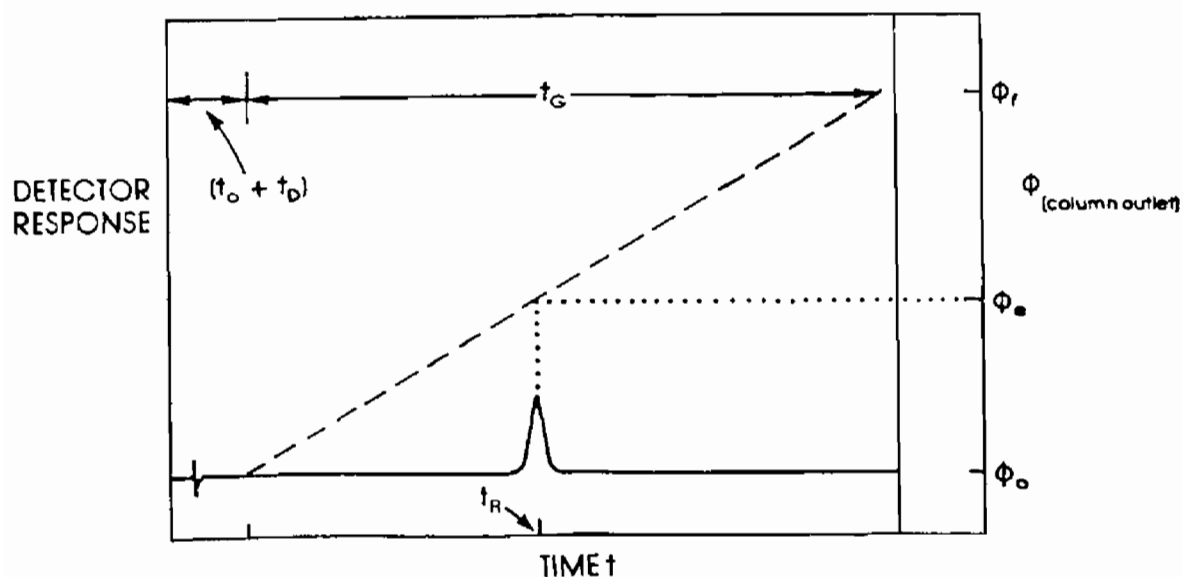


FIGURE 1 Separation of a compound by gradient elution, showing various related quantities.

different sample components will be large. As a result, a sample compound will initially remain near the column inlet after the gradient is begun. As the value of ϕ increases, however, the k value of the compound decreases according to the relationship:

$$\log k = \log k_w - \phi S \quad (2)$$

Here k_w refers to the value of k for water as mobile phase ($\phi = 0$) and S is a constant for a given compound and organic solvent.

When the value of k [Eq. (2)] becomes sufficiently small (usually $k < 20$, the band begins to move through the column at an appreciable rate. Eventually it leaves the column at a retention time t_R (see Fig. 1). This situation is illustrated in Fig. 2a, where the fractional migration r of compound X through the column (y axis of Fig. 2a) is shown as a function of time (t) after the start of the gradient (solid curve). The dashed curve of Fig. 2a shows the corresponding change in k during the separation. Figure 2b shows the actual migration of two proteins (cytochrome *c* and hemoglobin) during reversed-phase separation, observed directly by using glass-walled columns (the two proteins are colored).

The separation of adjacent bands in gradient elution can be described in the same terms used for isocratic separation. Resolution R_S can be expressed as [5]:

$$R_S = \frac{1}{4}(\alpha - 1)N^{1/2} \left(\frac{k}{1 + k} \right) \quad (3)$$

Here α is the separation factor for the two bands, N is the column plate number, and k is the average retention factor for the two bands. In gradient elution, the

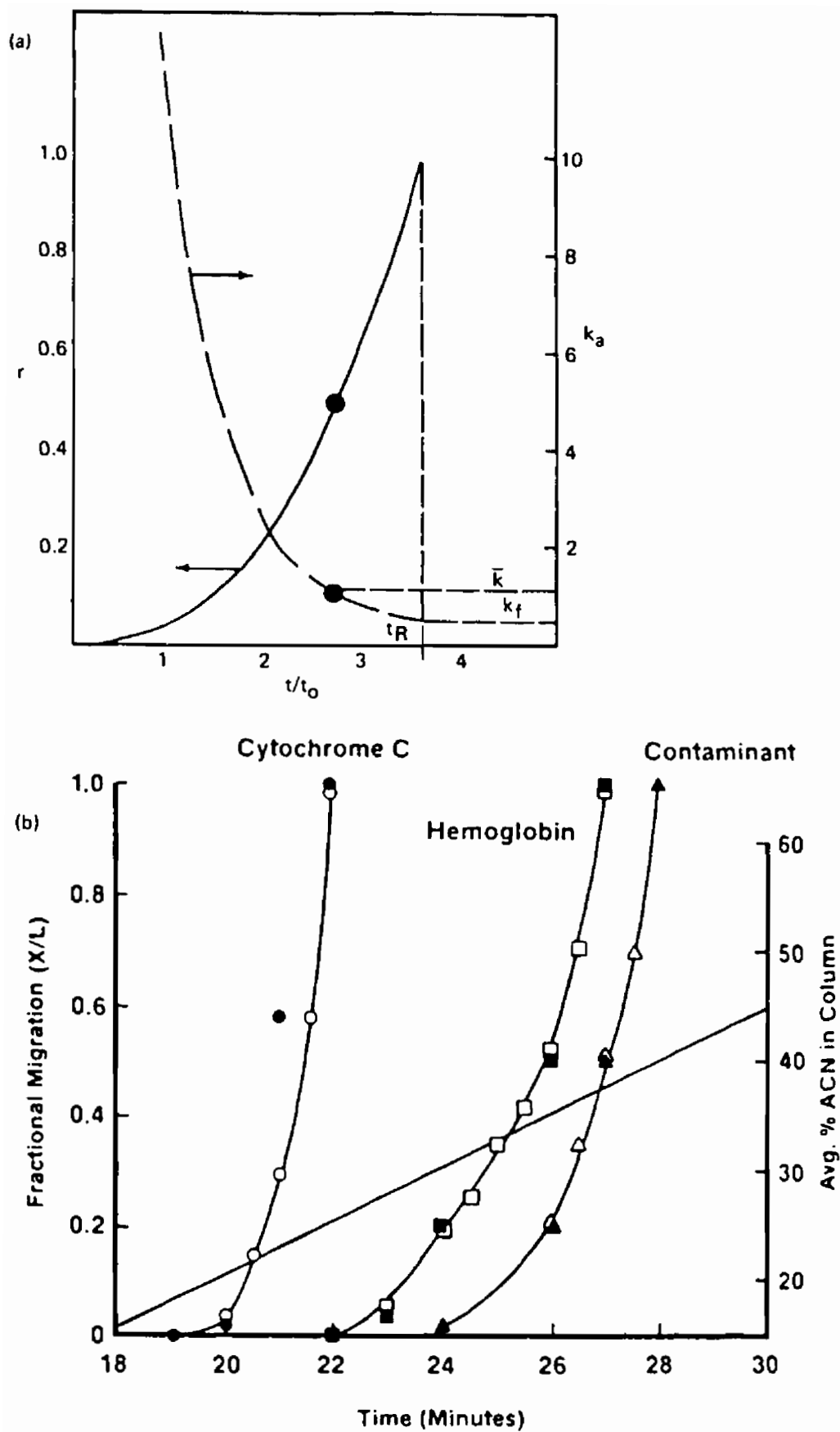


FIGURE 2 Migration of sample bands through the column in gradient elution. (a) Hypothetical example (from Ref. 1) and (b) proteins separated by reversed-phase HPLC (from Ref. 4).

same relationship [Eq. (3)] holds exactly, except that k is replaced by the average value of k during migration of a band through the column. This average k value (k^*) is defined as shown in Fig. 2a; it is the value of k when the band has moved halfway through the column. The value of k^* is given for any system that obeys Eq. (2) by

$$k^* = \frac{0.87 t_G F}{V_m \Delta\phi S} \quad (4)$$

Here F is the mobile phase flow rate, V_m is the column dead volume (equal to column dead time $t_0 \times F$), and $\Delta\phi$ is the change in ϕ during the gradient (equal to $\phi_f - \phi_0$). Thus the average sample retention during gradient elution (k^*) depends on the choice of experimental conditions (t_G , F , V_m , $\Delta\phi$) and the sample (value of S). Steeper gradients (faster change in % organic per unit time or volume) give smaller values of k^* . When gradient conditions are adjusted to give some average value of k^* for two adjacent bands, then a similar separation should be found with isocratic elution for a value of ϕ that gives $k = k^*$ for the same two bands (other conditions equal, so that values of N are similar).

The dependence of sample retention (k^*) on gradient conditions (t_G , $\Delta\phi$), flow rate, and column volume is a source of frequent confusion. For example, if we decrease flow rate in the isocratic separation of a protein sample, the sample resolution almost always improves—because the column plate number is increased [Eq. (3)], and values of k and α are not affected. In gradient elution, on the other hand, a decrease in flow rate often leads to a decrease in resolution for some bands and an increase in resolution for others. This is illustrated in Fig. 3 for the separation of a lysozyme digest, where all conditions are the same in these two runs except for flow rate. In Fig. 3a, for a flow rate of 0.5 mL/min, the two bands (arrows) that elute at about 28 min are well resolved. However, with a flow rate of 1 mL/min (Fig. 3b, arrow, 17 min), these two bands merge into a single band. The opposite situation is observed for two bands (arrows) that elute at about 42 min in Fig. 3a and 31 min in Fig. 3b.

Although an increase in column length increases N and resolution in isocratic separation (other conditions the same), this is not generally true in gradient elution. Here an increase in column length usually does not lead to a consistent increase in sample resolution if other variables are held constant. This is illustrated in Fig. 4a–c for the separation of a protein mixture on columns of different length: 4.5 cm (a), 0.63 cm (b), and 0.16 cm (c). The observed resolution is about the same for all three columns. The reason is that an increase in column length simultaneously results in an increase in N and a decrease in k^* [Eq. (4)], just as an increase in F causes a decrease in N and an increase in k^* . Because resolution increases with both N and k^* [Eq. (3)], these two factors effectively cancel each other. However, some band pairs may be better resolved and others more poorly resolved, just as for the case of flow rate variations (Fig. 3).

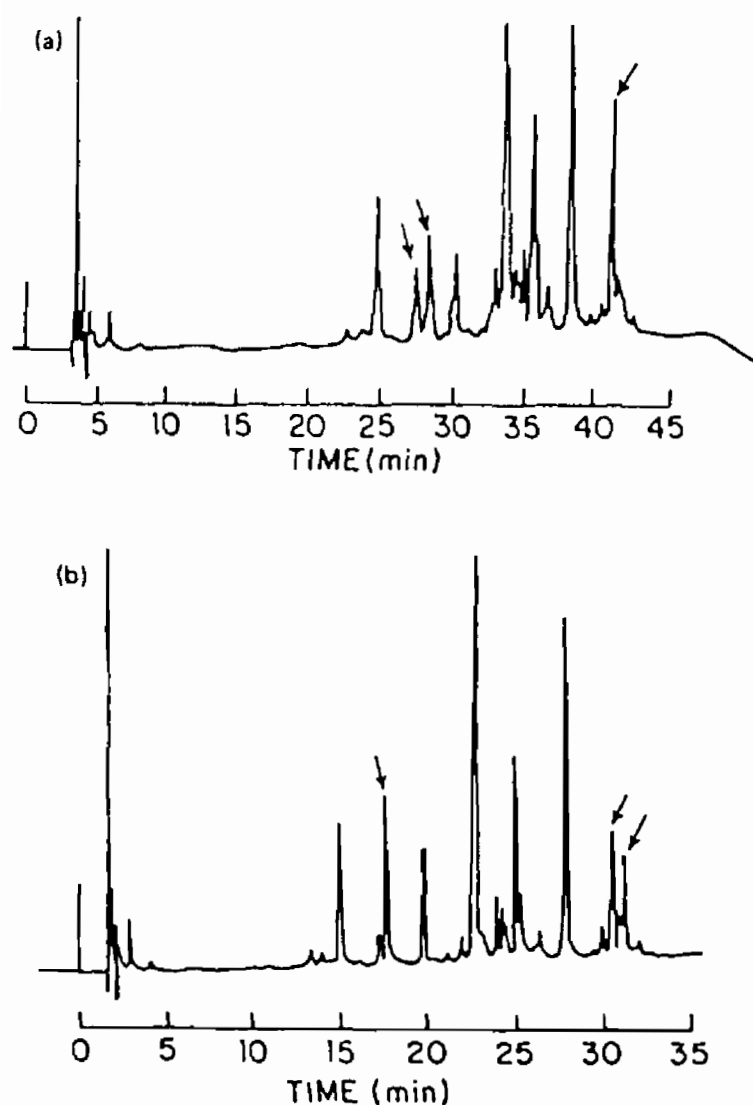


FIGURE 3 Flow rate effects in gradient elution. Separation of peptide mixture (lysozyme digest) by reversed-phase gradient elution. Gradient time 30 min; 8–40% acetonitrile-water (0.1% TFA) gradient; C_8 column. (a) 0.5 mL/min and (b) 1.0 mL/min. (From Ref. 6.)

In gradient elution, a change in flow rate or column length will often not improve the overall separation if other conditions are held the same. However, an increase in gradient time usually leads to a consistent increase in average resolution. This is illustrated in Fig. 5 for the separation of a protein mixture where only t_G is varied. A systematic increase in sample resolution is observed in going from Fig. 5d (48 min) to Fig. 5a (396 min). The reason is that when only t_G is changed [Eq. (4)], k^* for all bands increases but N remains constant. This usually means better overall resolution [Eq. (3)]. We also see that peak heights decrease as t_G increases in Fig. 5. The reason is that larger values of k^* [Eq. (4)] mean wider, more dilute bands—just as in isocratic elution.

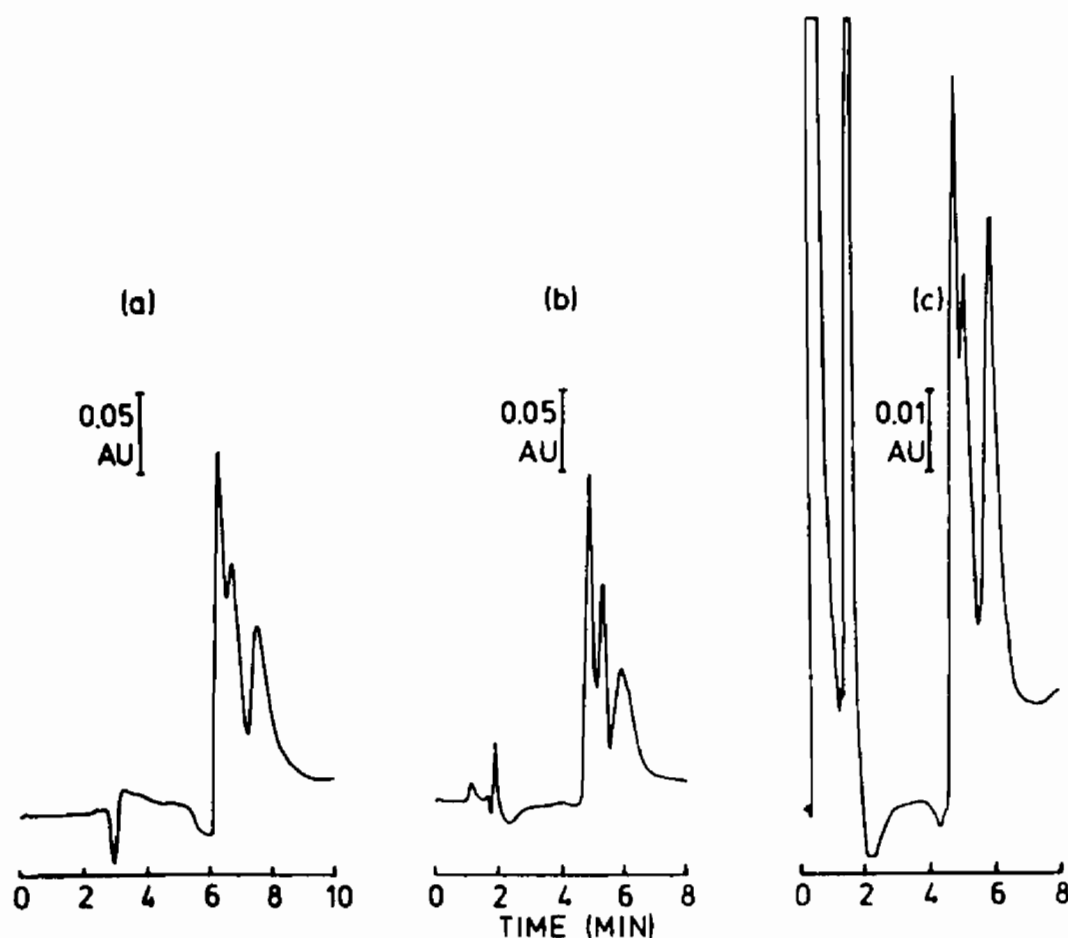


FIGURE 4 Column length effects in gradient elution. Separation of ribonuclease A, cytochrome c, ovalbumin. C_8 columns of varying length, 0–100% propanol-water gradient; 0.5 mL/min. (a) 4.5 cm column; (b) 0.63 cm; (c) 0.16 cm. (From Ref. 7.)

When the quantity $(t_G F / V_m \Delta \phi)$ is held constant while individual conditions are varied, values of k^* for each sample band remain the same. This is analogous to isocratic separations (constant k values), when the mobile phase, column, and temperature remain unchanged; i.e., relative retention does not vary in either case (gradient or isocratic elution). Under conditions where k^* values remain constant in gradient elution, if we increase column length and/or decrease flow rate, the plate number usually increases; the resolution of every pair of adjacent bands therefore also improves.

This is illustrated in Fig. 6 for the gradient separation of a lysozyme digest (sample of Fig. 3). Flow rate and column length (L) are varied from Fig. 6a to Fig. 6d in such a way as to increase N continuously (by decrease in F or increase in L). Sample resolution systematically increases from 6a to 6d, but band spacing is not changed. If the sample weight per gram of column packing is kept constant, all sample bands will increase in height as N increases (k^* assumed constant). Again, this is analogous to isocratic separations, when the mobile phase and

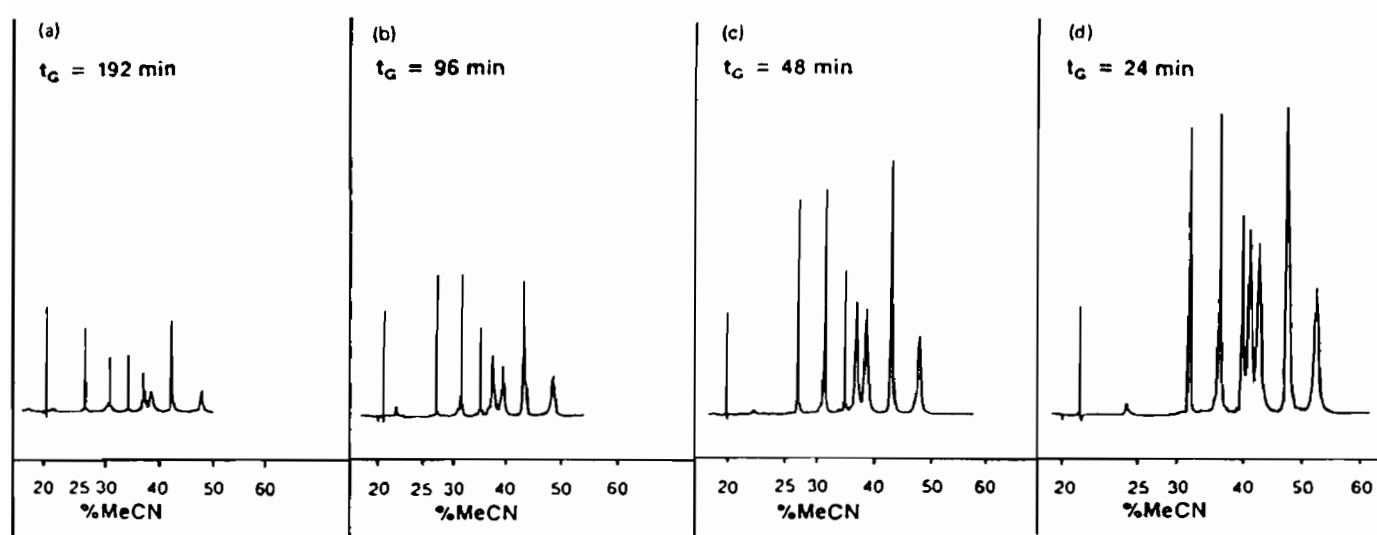


FIGURE 5 Effect of gradient time on separation. Sample, seven proteins with molecular weights of 12,000–80,000; C_3 column; 20–68% acetonitrile-water gradients; 0.25 mL/min. (a) 384 min gradient, (b) 192 min, (c) 96 min, and (d) 48 min. (From Ref. 8.)

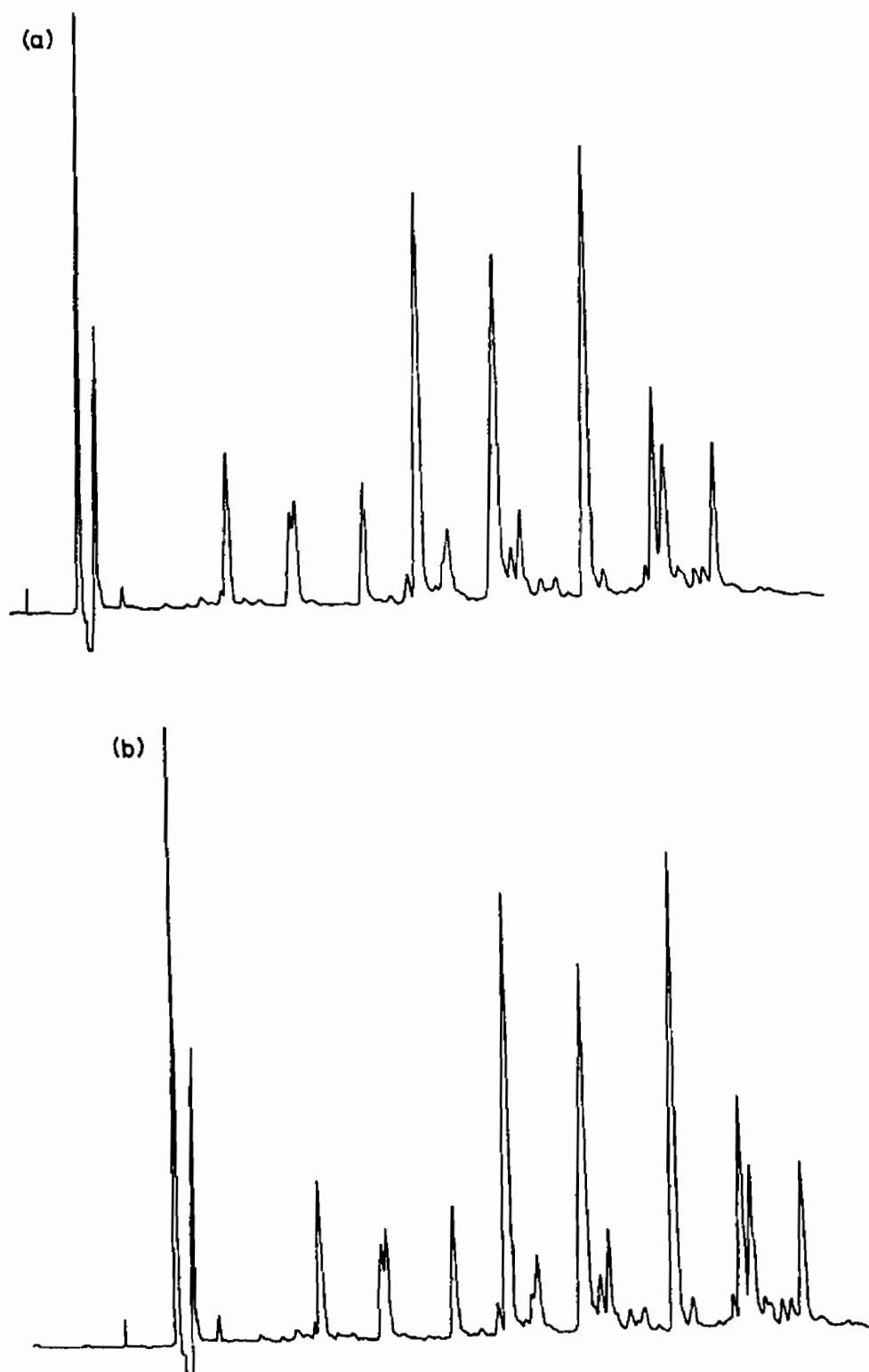


FIGURE 6 Effect of flow rate and column length on separation in gradient elution, with $(t_G F/V_m)$ held constant. C_8 column; 8–40% acetonitrile-water gradient. (a) $t_G = 15$ min, 8-cm column, 2 mL/min, (b) 30 min, 8-cm, 1 mL/min. (From Ref. 9.)

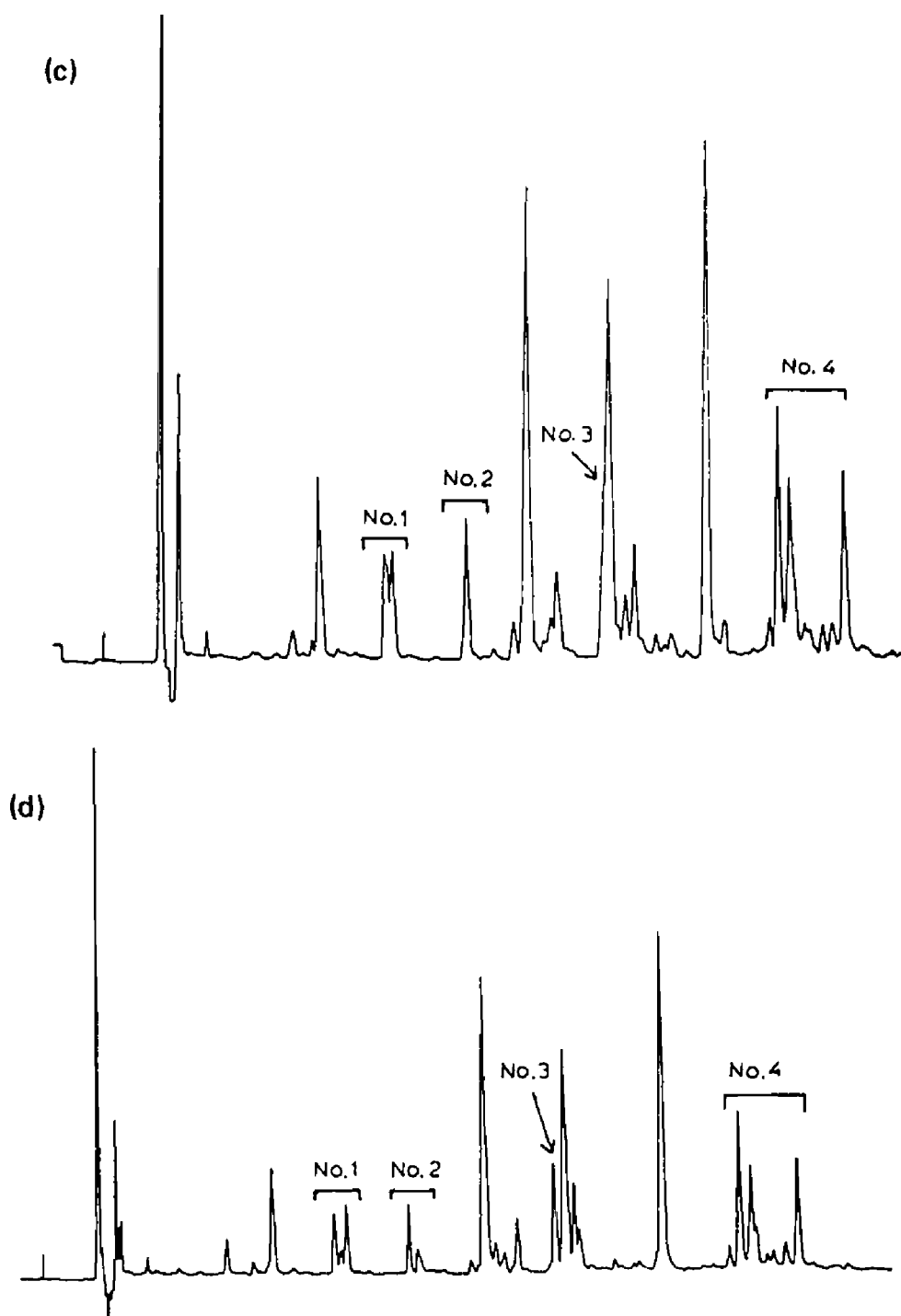


FIGURE 6 Effect of flow rate and column length on separation in gradient elution, with $(t_G F/V_m)$ held constant. C_8 column; 8–40% acetonitrile-water gradient. (c) 60 min, 8-cm, 0.5 mL/min, and (d) 60 min, 16-cm, 1 mL/min. (From Ref. 9.)

temperature are held constant, but flow rate is decreased or column length is increased.

Generally the various bands in a gradient separation have about the same width. The reason is that each band migrates through the column in similar fashion (e.g., Fig. 2b) whenever values of S (and therefore k^*) are similar; see Eq. (4). For typical peptide or protein samples, values of S for the individual compounds are often not widely different (see following section).

Changing the initial or final values of ϕ (mobile phase % organic) usually has no effect on separation, providing that values of t_G are adjusted in proportion to changes in $\Delta\phi$ [Eq. (4)]. This is illustrated in Fig. 7 for the separation of the

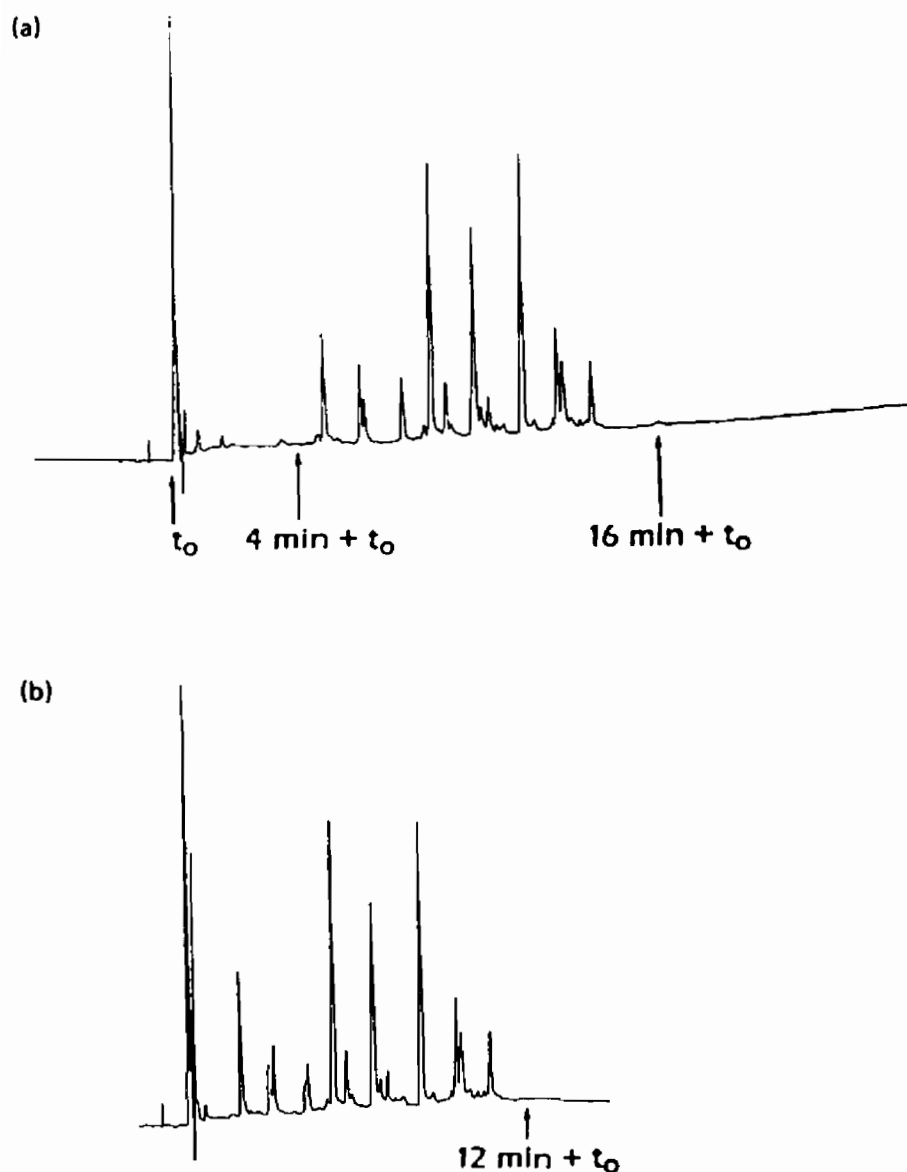


FIGURE 7 Effect of gradient range on reversed-phase separation of lysozyme digest, with $t_G/\Delta\phi$ and k^* held constant. C_8 column, 1.9 mL/min. (a) 5–60% acetonitrile-water (0.1% TFA), 30-min gradient, (b) 12–34%, 12 min. (From Ref. 9.)

lysozyme digest from Fig. 3. In Fig. 7a, a 5–60% acetonitrile-water gradient is used, with a gradient time of 30 min. In Fig. 7b the gradient is 12–34% acetonitrile, and the time is 12 min ($t_G/\Delta\phi$ is the same). Essentially the same separation is seen in both cases. For sufficiently large values of ϕ at the beginning of the gradient, however, initial sample bands may elute with $k^* \approx 0$. This can then result in a loss of resolution at the beginning of the chromatogram.

Ion-Exchange HPLC

Gradients where the concentration of salt in the mobile phase increases linearly with time will be assumed. The basis of gradient separations using either reversed-phase or ion-exchange conditions is in general similar. Changes in gradient time t_G , flow rate F , column volume V_m , and gradient range ($\Delta\phi$ in reversed phase) have similar effects on separation. The value of k^* in ion exchange systems is given by

$$k^* = 0.87 t_G \frac{F}{V_m} \left(\log \frac{C_2}{C_1} \right) Z \quad (5)$$

Here C_1 is the total salt concentration (salt plus buffer) at the beginning of the gradient, C_2 is the total salt concentration at the end of the gradient, and Z is the effective charge on the sample molecule. Values of Z will be different for different sample molecules, just as values of S for different compounds vary in reversed-phase systems.

From Eq. (5) it can be seen that $\log(C_2/C_1)$ in ion-exchange corresponds to $\Delta\phi$ in reversed-phase HPLC. Increases in either of these quantities lead to a proportionate decrease in k^* , with predictable effects on separation; see the preceding discussion for reversed-phase HPLC. Likewise, an increase in t_G or F , or a decrease in V_m , also leads to larger k^* values in ion exchange.

Hydrophobic Interaction Chromatography

Aqueous mobile phases with a linearly decreasing salt gradient are normally used for hydrophobic interaction chromatography (HIC). The basis of HIC and reversed-phase separations is essentially similar, and a change in gradient conditions has the same result for both methods. Values of k^* are given by Eq. (4). Here the quantity $\Delta\phi$ is replaced by ΔC , the difference in initial versus final salt concentrations. Some additional examples of the effects of gradient conditions on separation will be given.

Figure 8 shows the HIC separation of a protein mixture for two different gradient times: 120 min in 8a and 60 min in 8b. Here the flow rate is varied inversely to gradient time, so that k^* remains constant. It is seen that band spacing does not change [predicted by Eq. (4)], and resolution is slightly better in the separation of Fig. 8a. This is a result of an increase in N as a result of the lower flow rate [Eq. (3)].

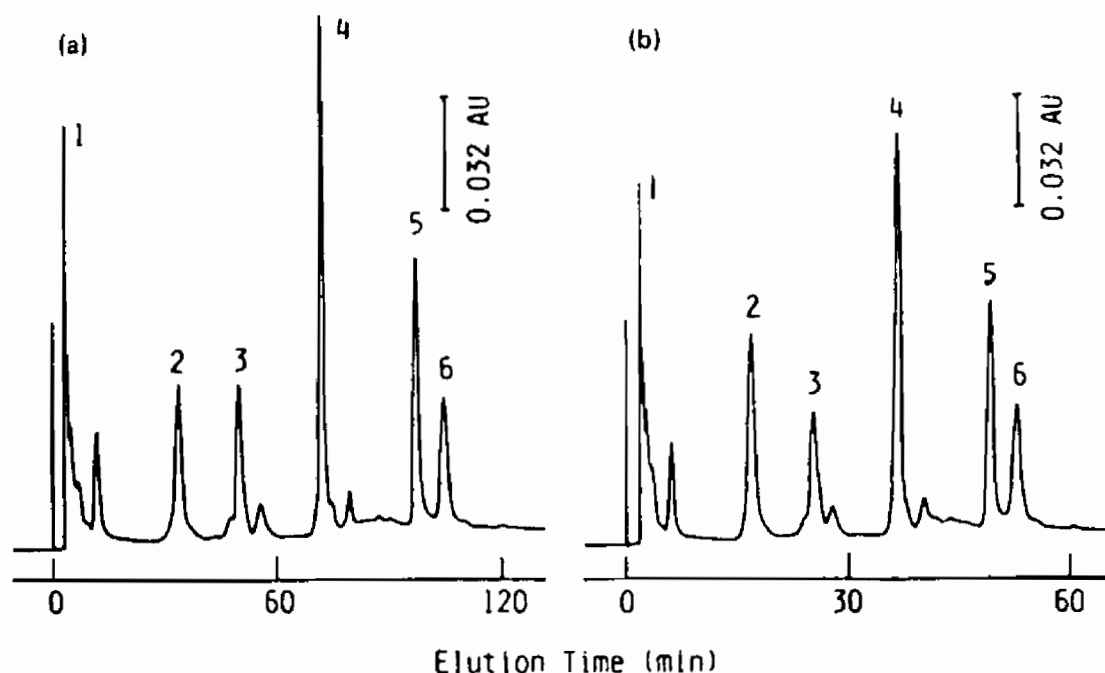


FIGURE 8 Combined effect of gradient time and flow rate ($t_G F$ constant) on separation of protein mixture by HIC. TSK gel Phenyl 5-PW column, ammonium sulfate gradient 1.8–0.0 M. (a) 120-min gradient, 0.5 mL/min, (b) 60 min, 1.0 mL/min. (From Ref. 10.)

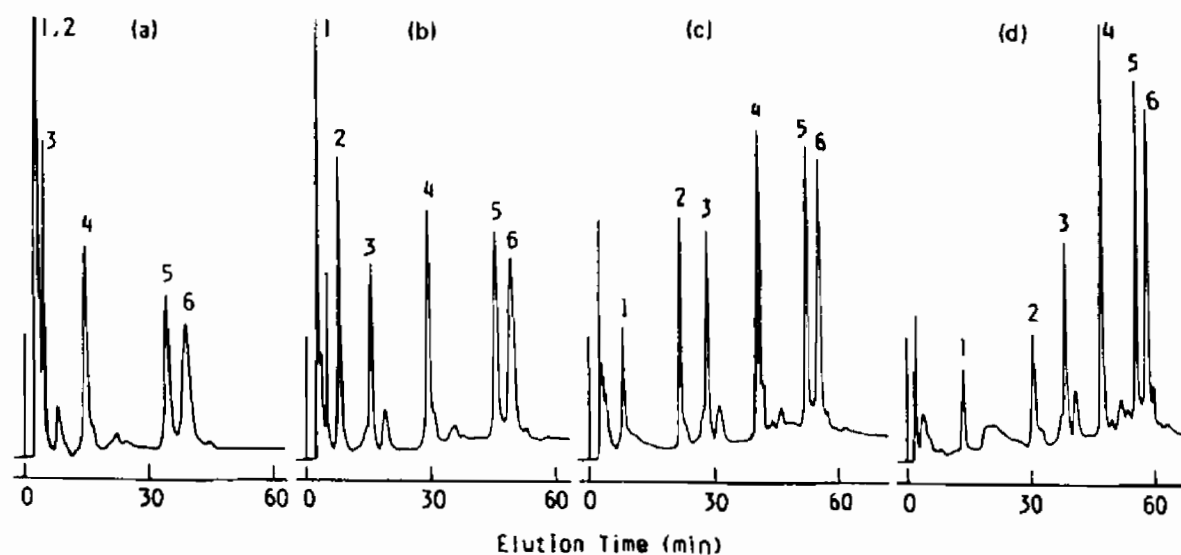


FIGURE 9 Effect of change in gradient range (other conditions constant) on the separation of protein mixture by HIC. TSK gel Phenyl 5-PW column, ammonium sulfate gradient in 60 min, 1 mL/min; (a) 1.0–0 M gradient, (b) 1.5–0.0 M, (c) 2.0–0 M, and (d) 2.5–0 M. (From Ref. 10.)

If the gradient range is varied while other conditions are kept the same, predictable effects on separation result. This is illustrated in Fig. 9 for the same HIC system as in Fig. 7. In this case, the initial salt concentration is increased in going from 9a to 9d. A lower initial salt concentration as in 9a corresponds to a stronger mobile phase or lower values of k at the start of the gradient. This results in the early elution of some bands in 9a and b, with poor resolution of these proteins. The initial salt concentrations in 9c and d are large enough to avoid this problem, so that bands 1 and 2 are now well resolved. At the same time, because other conditions are held constant while $\Delta\phi$ is increasing, the average value of k^* for later bands decreases in going from 9a to 9b. This results in a decreasing resolution of bands 4–6 as the initial salt concentration increases.

As we will see, it is usually better to avoid multiple changes in the gradient, as in Fig. 9—unless these are combined so as to hold k^* constant. One reason is that the resulting changes in resolution are harder to keep track of and to understand.

SPECIAL CHARACTERISTICS OF PEPTIDES, PROTEINS, AND OTHER LARGE MOLECULES

There is no fundamental difference between the separation of large and small molecules or of biological and nonbiological compounds. This is true for both isocratic and gradient elution. Several workers have misinterpreted the gradient elution effects described above and suggested that there is something inherently different about the gradient separation of large molecules such as proteins and synthetic polymers. A full discussion of this issue is presented elsewhere [3,11,12] and will not be repeated here. It is now possible to carry out quantitative predictions of retention and bandwidth for the separation of proteins by reversed-phase, ion-exchange, or hydrophobic interaction HPLC. Predicted results are in generally good agreement with experimental data [13,14].

However, certain aspects of the separation of large molecules—specifically proteins—should be pointed out. First, the molecular weights of these compounds are generally about two orders of magnitude larger than those of typical small molecules that are more often separated by HPLC. Second, protein molecules can have an organized tertiary structure. Each of these properties of protein molecules must be taken into account when designing HPLC separations.

Molecular Weight Effects

Large molecules diffuse more slowly, and this in turn requires lower flow rates and/or smaller column-packing particles if large values of N are to be attained. However, with sufficiently small particles and low flow rates, quite respectable values of N are possible, e.g., 20,000 for various proteins [13].

Another consequence of increased protein molecular weight is a corresponding increase in values of S (reversed phase and HIC) and Z (ion exchange). This is most notable in the case of S values for reversed-phase systems (e.g., Ref. 15). Thus molecules with molecular weights of 100–1000 have S values in the range of 3–10, but proteins can have S values of 50 or more. The origin of these large S values has been attributed to a displacement model of retention in reversed-phase HPLC [16,17] That is, during the retention process a larger sample molecule displaces a greater number of solvent molecules from the surface of the column packing. Large S values also mean that isocratic retention is quite sensitive to small changes in mobile phase composition (value of ϕ). Equation (6) shows that an 80,000-Da protein will have an S value of about 70, which means that a 2% change in the mobile phase water content will change k by a factor of 25.

The practical consequence of larger values of S (or Z) can be seen in Eq. (4). If other gradient conditions are maintained the same, large S values mean lower values of k^* with possibly adverse consequences on separation. Therefore it is usually desirable to use shallower gradients with proteins than with small molecules. There is a rough dependence of S on protein molecular weight [18]:

$$S \approx 0.48M^{0.44} \quad (6)$$

Usually it is desirable to start out with k^* values of 5–10. If we know the approximate molecular weight of our sample, it is possible to use Eqs. (4) and (6) to estimate suitable gradient conditions (for $5 \geq k \geq 10$). This means that our first gradient separation should provide significant separation of the sample, particularly if conditions that favor large N values are also used (low flow rates, longer columns, small particles).

Values of Z for proteins are somewhat larger (e.g., $Z \approx 4$) than for small molecules. However, the molecular weight dependence of Z values is usually far smaller than in the case of reversed-phase S values. A good discussion of protein Z values is given in Ref. 19. Values of S in HIC are usually smaller than in reversed phase, e.g., $S \approx 2$ [20], when salt concentration C is expressed as molarity.

Conformational Effects

A variety of unusual and unfavorable phenomena have been observed in the separation of peptides and proteins by gradient elution:

1. After an initial gradient run with a particular sample, a repeat of the gradient run without injecting a sample (blank gradient) may result in elution of some of the same bands that appeared in the initial run. This has been referred to as “ghosting” (see, for example, Refs. 21–23).

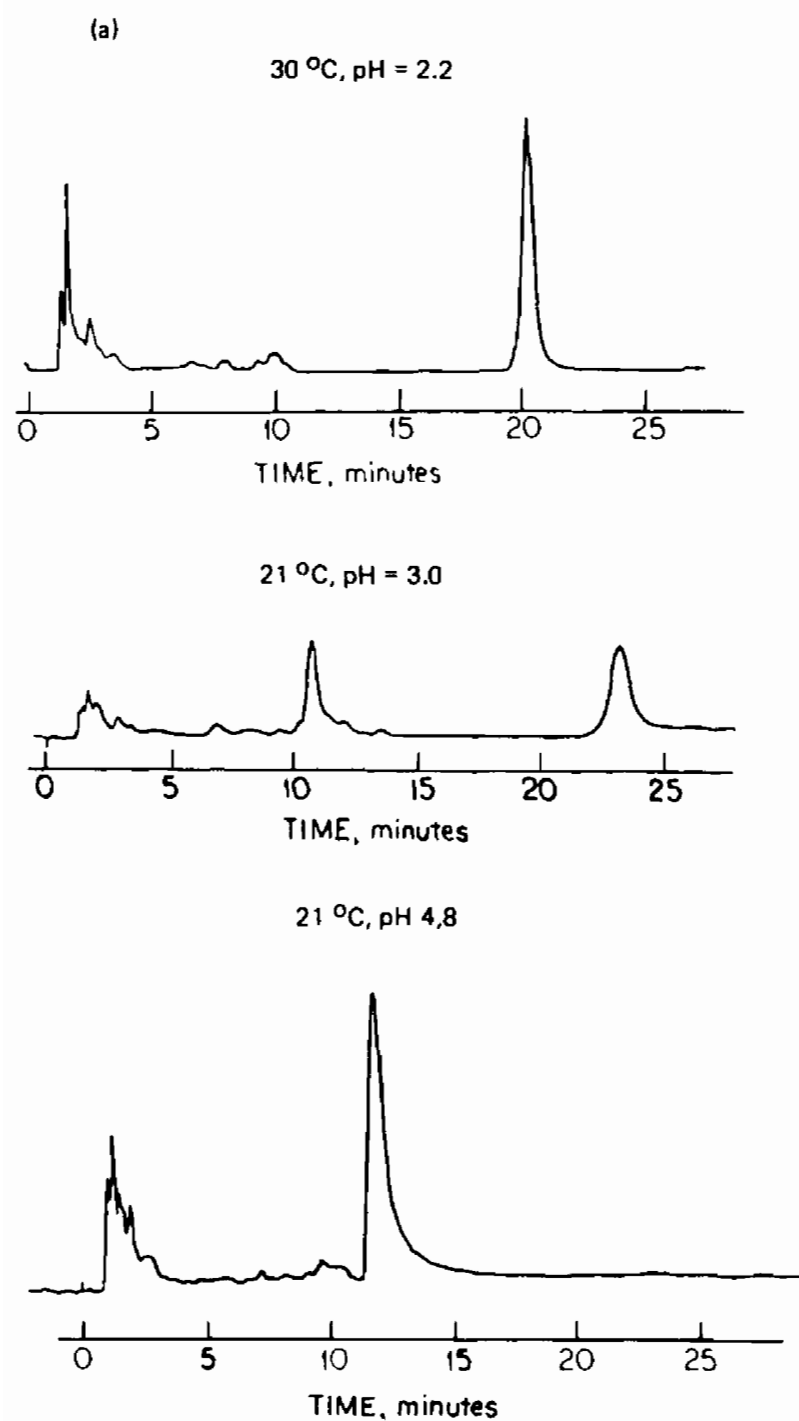


FIGURE 10 Protein conformation and HPLC separation. (a) Two bands for the reversed-phase gradient elution separation of papain; native conformer at ~11 min, denatured conformer at ~22 min. (From Refs. 24 and 28.)

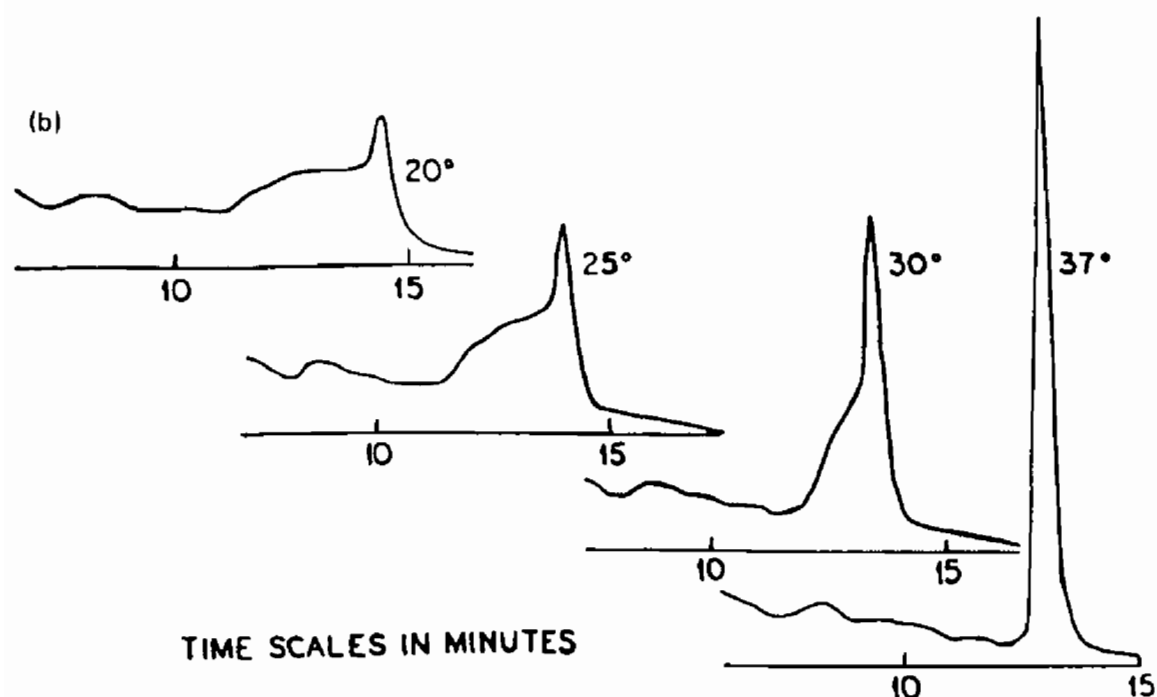


FIGURE 10 Protein conformation and HPLC separation. (b) reversed-phase gradient elution separation of ribonuclease as a function of temperature. (From Refs. 24 and 28.)

2. The gradient separation of a single protein has been observed to result in two or more separate bands (see Refs. 24–26).
3. A protein band may be excessively wide, tail badly, or be otherwise distorted (see Refs. 27–30).
4. The recovery of different compounds is not infrequently low (see Refs. 31 and 32).

Changes in conformation of the protein molecule have been implicated in each of these separation problems (see review of Ref. 33). It is well known that proteins commonly exist in either native (folded) or denatured (random coil) conformations. This offers a simple explanation for the appearance of two separate bands following the injection of a single (pure) protein. Figure 10 illustrates an example of this kind. Here the enzyme papain was separated by reversed-phase gradient elution using different conditions of mobile phase pH and temperature (as noted in Fig. 10a). Higher temperatures and/or lower pH favor the denatured protein, which is observed to elute at about 22 min. The native protein elutes at about 11 min.

Figure 10b shows a similar reversed-phase separation of the protein ribonuclease as a function of temperature. The denatured protein is favored at higher temperatures, and a single, sharp band is observed at 37°C (where the protein is totally denatured). At lower temperatures the sample is a slowly

interconverting mixture of native and denatured proteins, giving rise to the broad, badly shaped band observed at 20, 25, and 30°C.

Most of the separation problems listed earlier are associated with reversed-phase HPLC. The main problem is that a single compound may coexist in several forms (conformational states), which in turn leads to effects such as those illustrated in Fig. 10. The use of conditions that favor complete denaturation of the sample often improves separation in these cases, e.g., higher temperatures, extremes of pH, treatment of the sample (or addition to the mobile phase) by various denaturants: urea, guanidine, surfactants, etc. [34]. Similar problems have been observed in HIC separation [29]; here the use of conditions (e.g., lower temperatures) that favor the native conformer seems preferable. With HIC it is usually possible to find conditions that disfavor any denaturation of the sample.

SYSTEMATIC APPROACH TO A FINAL SEPARATION

Developing a satisfactory separation for a peptide or protein mixture is seldom a simple procedure. However, recent theory (as reviewed in this chapter) can be combined with the experience of practical workers to make this ordeal less painful. Some samples can be adequately resolved within the first one or two tries. In these cases, it is necessary only to avoid a poor choice of conditions in the initial separation. Other samples may require several trial separations before a satisfactory result is obtained. In general we should proceed in such a way that samples are separated with as few trial runs as possible. Table 1 summarizes one such approach. (Specific conditions for separating various biological macromolecules are presented elsewhere in this book. The following discussion is intended to be more general in nature.)

TABLE 1 Recommended Steps in Developing a Gradient Elution Separation for Peptide or Protein Samples

1. Choose an appropriate HPLC method (ion exchange, HIC, etc.) and initial conditions (column, solvents, pH, etc.).
2. Carry out an initial separation under conditions that yield k^* values in the range of 5–10, with a reasonably high plate number.
3. Reduce $\Delta\phi$ if there is room at the beginning or end of the chromatogram.
4. If the chromatogram is crowded, increase the column plate number (slower flow and/or longer column) while holding $(V_m \Delta t_G F)$ constant.
5. If necessary, change band spacing in order to improve sample resolution by varying k^* .
6. If further changes in band spacing are required, consider changes in other experimental conditions or the use of a different HPLC method.

Selecting an HPLC Method

Biological samples frequently require the use of more than one separation procedure. Often size exclusion separations are used initially in order to narrow the molecular weight range of the sample before using some other HPLC method. Separations by reversed-phase HPLC usually provide higher resolution fractionation (larger peak capacities) than is the case for ion exchange or HIC. On the other hand, reversed-phase HPLC is inherently denaturing, so that bands leaving the column are usually biologically inactive. However, peptides with molecular weights of less than 25,000 can usually be restored to the native conformation following separation. For these reasons, reversed-phase chromatography is usually a first choice for the separation of samples with molecular weights <25,000.

Ion-exchange and hydrophobic interaction chromatography often provide good recoveries (e.g., >90%) of biologically active material, and for this reason these methods are preferred for larger molecular weight samples. Complex samples generally benefit by the use of two or three of these HPLC methods (reversed phase, ion exchange, HIC) in tandem.

The Initial Separation

The choice of experimental conditions can have a profound effect on the quality of the first separation. Specifically, attention needs to be given to (1) the column, (2) the mobile phase, and (3) gradient conditions. The choice of column for different samples is discussed elsewhere in this book. Samples with larger molecular weights (M) generally benefit from larger column-packing pore diameters, e.g., 15 nm for samples where $M > 5000$.

The pH of the mobile phase should be ~ 2 for peptides with $M > 10,000$. Typical gradients are 5–60% organic, which provides elution of most peptides and proteins. Ion-exchange separations generally utilize NaCl or sodium acetate gradients from 0 to 0.5 M, with a pH of about 7. HIC separations often use (decreasing) ammonium sulfate gradients, e.g., from 2 to 0 M. The pH is usually maintained near 7. In each case (ion exchange or HIC) the buffer is present throughout the gradient at a concentration of about 0.02–0.05 M.

Gradient conditions should be selected initially to provide values of k^* in a generally optimum range, e.g., $k^* \approx 5$. From Eqs. (4) and (6) we then have

$$\frac{t_G F}{\Delta\phi V_m} \approx 3M^{0.44} \quad (7)$$

For example using a 15×0.46 cm column ($V_m \approx 1.5$ mL), a gradient range of 5–60% acetonitrile ($\Delta\phi = 0.55$), and a flow rate of 1 mL/min (a reasonable starting value), we would estimate that the gradient time t_G should equal about 3 hr for a sample with $M = 20,000$ and about 1 hr for a sample with $M = 2000$.

In similar fashion, for ion exchange or HIC we can estimate preferred gradient conditions:

$$\text{(Ion exchange)} \quad t_G \frac{F}{V_m} \log \frac{C_2}{C_1} \approx 30 \quad (8)$$

$$\text{(HIC)} \quad t_G \frac{F}{V_m} \Delta C \approx 8 \quad (9)$$

Thus for ion-exchange HPLC, with a salt gradient from 0.1 to 0.6 M (including buffer), a flow rate of 0.5 mL/min, and the same column ($V_m = 1.5$ mL), the preferred gradient time would equal about 70 min. Likewise, for HIC with a salt gradient of 2 (C_1) to 0 (C_2) M, a flow rate of 0.5 mL/min, and the same column, the optimum gradient time is predicted to be about 50 min.

Adjusting the Gradient Range

Sometimes our first separation will be adequate. More often we will want to improve resolution, while holding separation time (run time) to a minimum. Assuming that resolution must be improved, the next step should be to optimize the gradient range. However, this can logically be combined with a following step (options 4–6 of Table 1) in improving the separation.

Figure 7 illustrates the advantage of narrowing the gradient range in terms of the reduction in run time. Here the gradient time is reduced by 2.5-fold without any loss in resolution. This saving in run time can be traded for an increase in overall sample resolution, as we will see next.

Increasing the Column Plate Number

A decrease in flow rate and/or increase in column length generally results in an increase in the column plate number (N). If values of k^* are held constant while increasing N , the result will be increased resolution for all adjacent bands—with no change in relative retention. This was illustrated in the series of runs shown in Fig. 6, where N increases in the sequence a–d. For higher flow rates, the advantage of an increase in column length (with k^* constant) can be small [3]. However, for lower flow rates, an increase in column length usually provides a dramatic increase in sample resolution. If flow rate is decreased and/or column length increased, the gradient time must be increased proportionately (for k^* to remain constant).

An increase in N should be pursued to the point where there is room (empty space) in the chromatogram to accommodate roughly two to three times the

number of bands that are resolved. An advantage of increasing N while holding k^* constant is that the resolution of all bands increases regularly with increase in N . If we simply increase gradient time, as in Fig. 5, there will be a general increase in resolution, but individual band pairs may actually show poorer resolution.

A strategy based on improving resolution by increasing N can be subject to two possible limitations. First, as column length is increased and/or flow rate decreased, the gradient time increases. Apart from the inconvenience of long separations, there is clear-cut evidence that long gradient times often promote sample losses. Thus the recovery of purified compounds may be lower when t_G values are large. Second, it is not infrequently noted that an increase in column length and/or decrease in flow rate does not improve resolution as rapidly as might be expected on theoretical grounds. This has been attributed in selected cases to “non-well-behaved” systems [3]. That is, various separation artifacts—some of them discussed earlier (conformational effects)—may intervene to limit overall resolution. This is discussed in more detail in Ref. 3.

Changing Band Spacing by Varying k^*

Controllable changes in band spacing represents the single most powerful technique for resolving complex mixtures. It has only recently been appreciated that a change in conditions such that k^* is varied [Eqs. (4) and (5)] leads to frequent and significant changes in band spacing. This seems especially pronounced for the reversed-phase HPLC separation of peptides and proteins, but similar effect can be seen for other samples and HPLC methods.

One example of changes in band spacing as a result of change in k^* has already been discussed for the lysozyme digest separations of Fig. 3. A more striking illustration is provided by Fig. 11 for the separation of a peptide mixture that results from the tryptic hydrolysis of myoglobin. Figure 11a shows the two chromatograms for flow rates of 0.5 and 1.5 mL/min, respectively. Numerous changes in band spacing can be seen in these two runs. Figure 11b shows the separation of six compounds that elute at 23–35 min in the 0.5 mL/min run and at 10–15 min in the 1.5 mL/min run. This increase in flow rate results in the merging of bands 5 and 5a, the splitting of bands 6 and 6a, a the reversal of bands 6b and 7. It can be seen that at some flow rate (<0.5 mL/min) all six bands could be separated in a single run.

Figure 12 is another example of how a change in gradient conditions can alter band spacing, in this case for a mixture of four proteins (oxidized and reduced forms of interleukin-2; $M = 14,000$). The gradient conditions in the two runs are as follows:

Variable	Fig. 12a	Fig. 12b
Gradient time t (min)	60	80
Gradient range (%)	41–60	45–51
Flow rate (ml/min)	0.5	2.0
k^* (avg)	3	50

In Fig. 12a the four compounds are all seen to be well resolved, and they elute in the sequence A-ox > B-ox > A-red > B-red. In Fig. 12b the sequence is changed to A-ox > A-red > B-ox > B-red, and two bands are unresolved (A-red and B-ox). Thus, the selection of optimum gradient conditions as in Fig. 12a

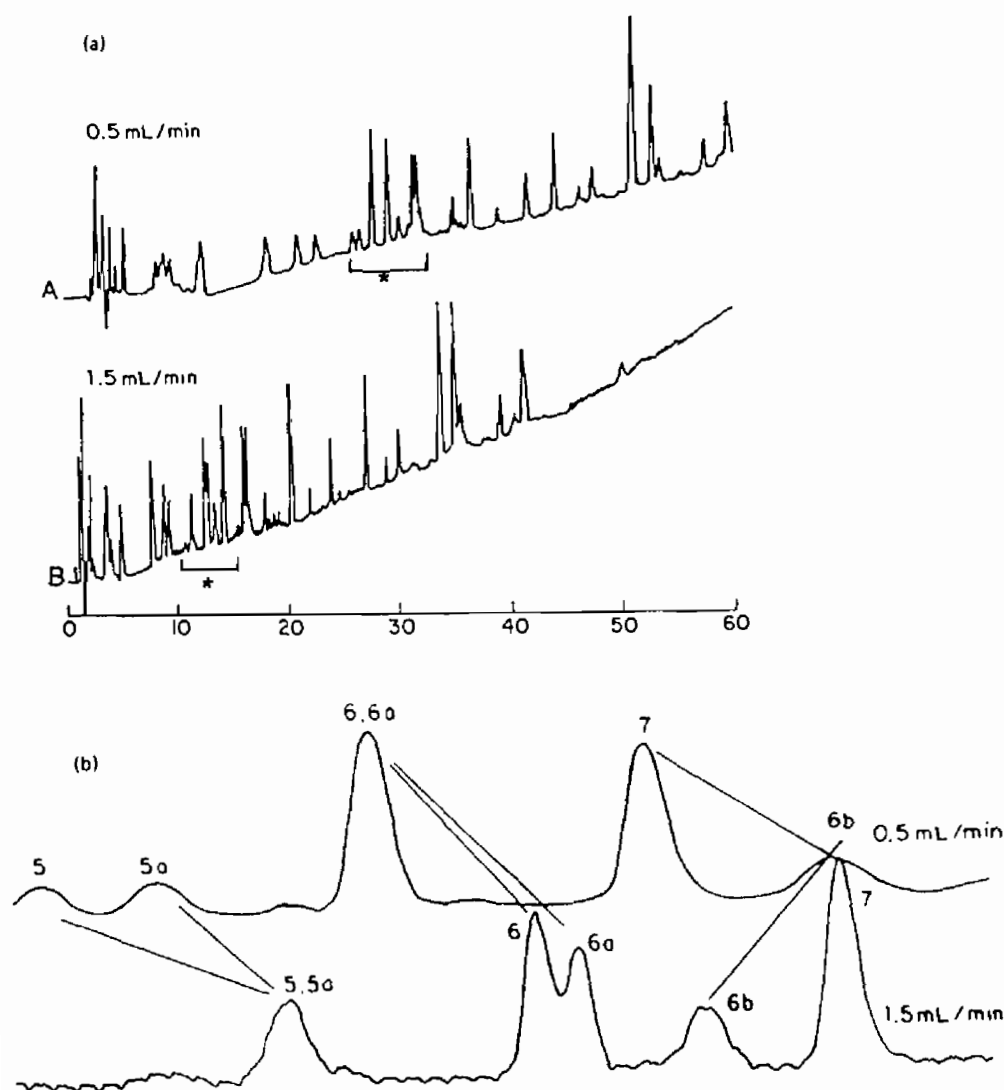


FIGURE 11 Separation of tryptic digest of myoglobin using reversed-phase HPLC with an acetonitrile-water gradient (0.1% trifluoroacetic acid added). (a) Whole chromatograms for 0.5 and 1.5 mL/min and (b) part of chromatograms from (a), indicated by *. (From Ref. 6.)

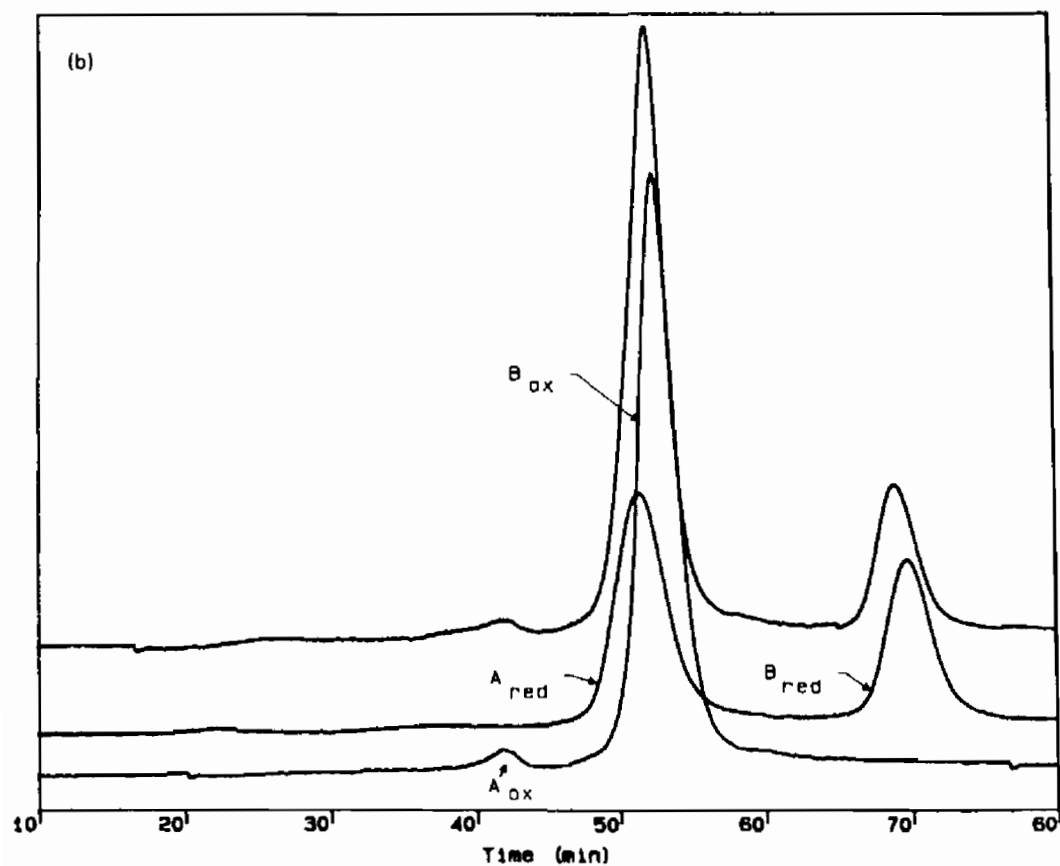
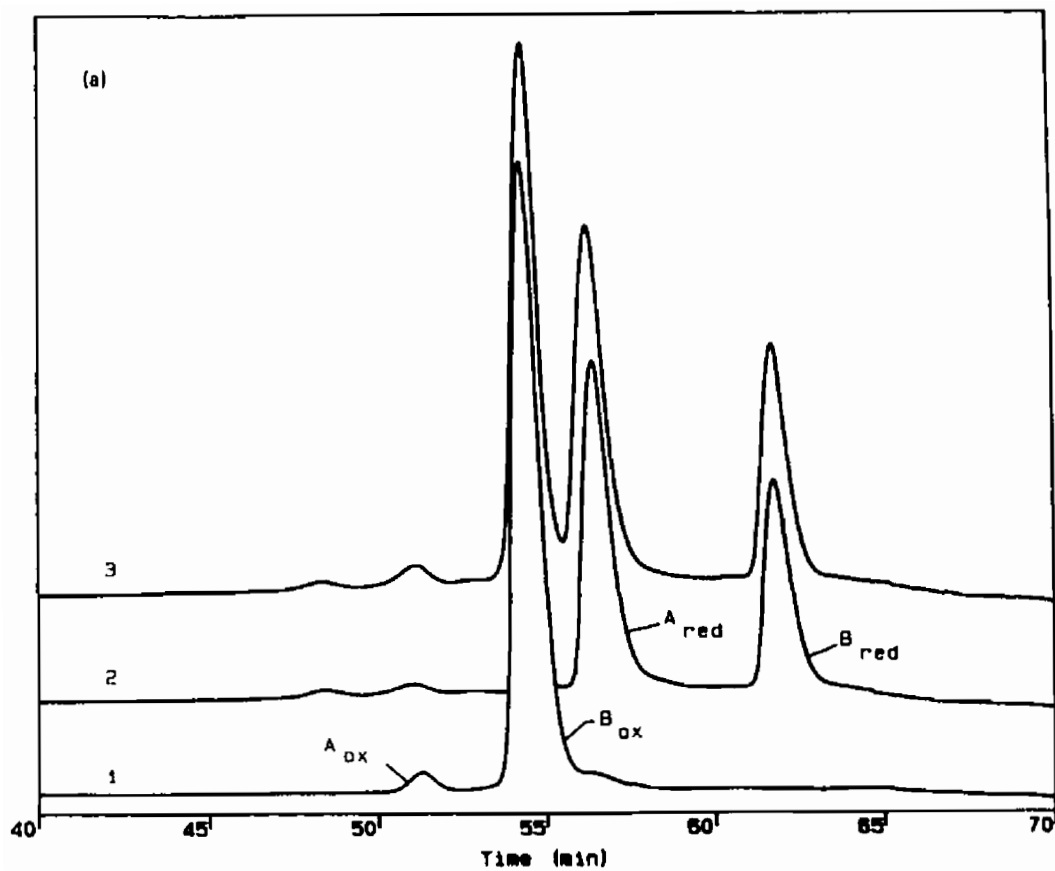


FIGURE 12 Separation of four oxidized/reduced forms of interleukin-2 by reversed-phase gradient elution. Gradient conditions given in text. (a) k^* equal 3 and (b) $k^* = 50$. (From Ref. 35.)

allows a better separation of this sample. Note also that the better separation in this case involves a *steeper* gradient (smaller k^*), which is the opposite of what most workers would expect.

The 35 proteins making up the 30S and 50S ribosomal-protein complexes ($6000 > M > 30,000$ D) also show striking changes in band position with changes in k^* . A suitable choice of conditions in combination with a nonlinear gradient allows almost all of these proteins to be resolved in a single reversed-phase run [36]. In this case, it was advantageous to vary the value of k^* at different parts of the gradient by changing gradient time (and k^*) during the run. This is illustrated in Fig. 13 for the separation of nineteen 30S ribosomal proteins. Figure 13a shows the separation of the entire sample in a gradient time of 240 min. Figure 13b shows the separation of the various protein groups (A–C) defined in (a) as a function of gradient tune. The best separation of groups A and C is obtained with a 240-min gradient, and the best separation of group B results for a 60-min gradient. This suggests the use of a segmented gradient that is first flat (for group A), steep (for group B), and finally flat again (for group C). Such a gradient was in fact able to resolve all of the 30S proteins in a single gradient run with $R_s \geq 0.7$ [36]. The best previous separation of this sample provided only 13 single-protein peaks. Similar results were obtained for a sample that contained thirty-five 50S ribosomal proteins. In the latter case, it was possible to separate 34 of the 35 peaks by optimizing gradient time. For ion exchange [37] and HIC [9],

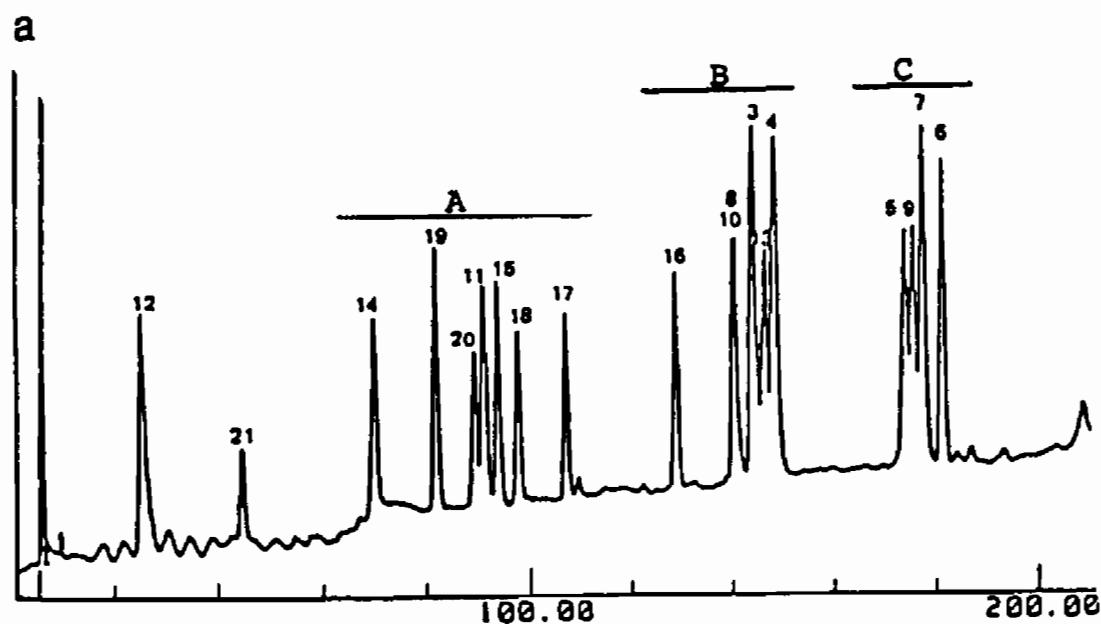


FIGURE 13 Separation of 30S ribosomal protein subgroups A–C as a function of gradient time. Conditions: 24–46% acetonitrile/0.1% TFA in water; 0.7 mL/min; 25×0.46 -cm C_3 column (Bio Series Protein Plus). (a) Whole chromatogram for 240 min gradient; (b) separation of protein groups A–C (see [a]) as a function of gradient time. (From Ref. 36.)

b

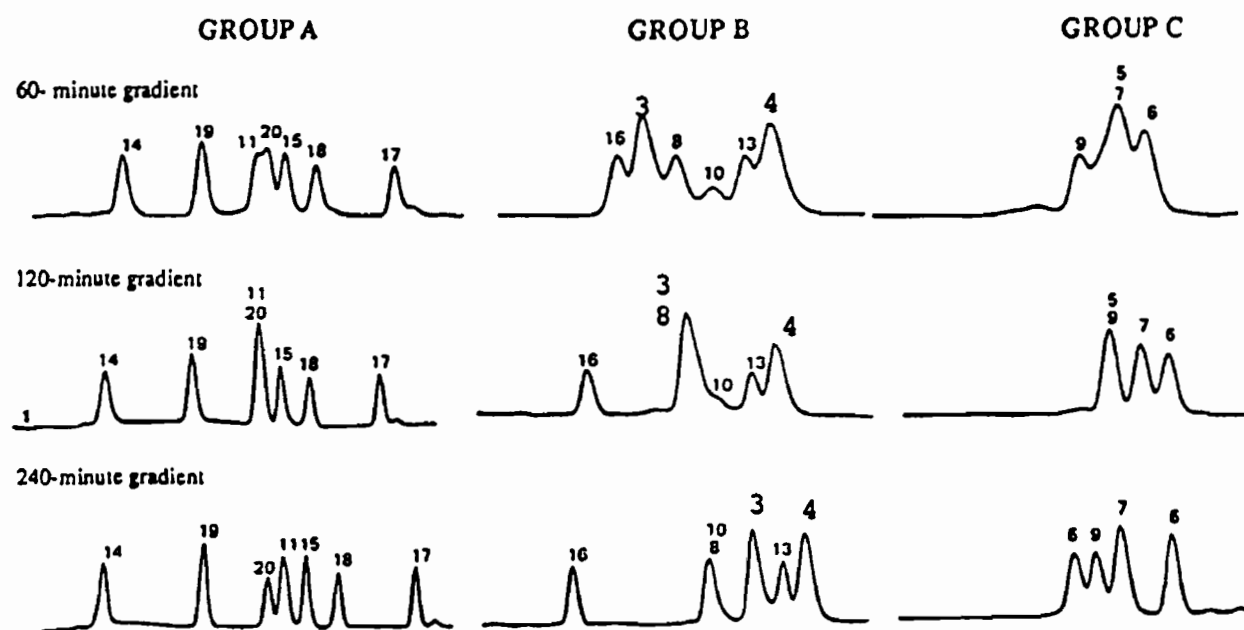


FIGURE 13 (continued)

it appears that changes in gradient time are less effective in controlling band spacing.

Other Ways of Changing Band Spacing

Achieving a successful separation in reversed-phase gradient elution is often successful, merely by changing gradient time or steepness—as illustrated in the preceding examples. Column temperature is another variable that can be used to further adjust band spacing and optimize sample resolution in the separation of peptide and protein samples [38,39]. This is illustrated in Fig. 14 for the separation of a tryptic digest of rec-human growth hormone (rhGH). Figure 14a shows the separation of the sample with a 0–60% acetonitrile (ACN) gradient in 120 min; bands 7–15 are the most difficult to separate and will be discussed

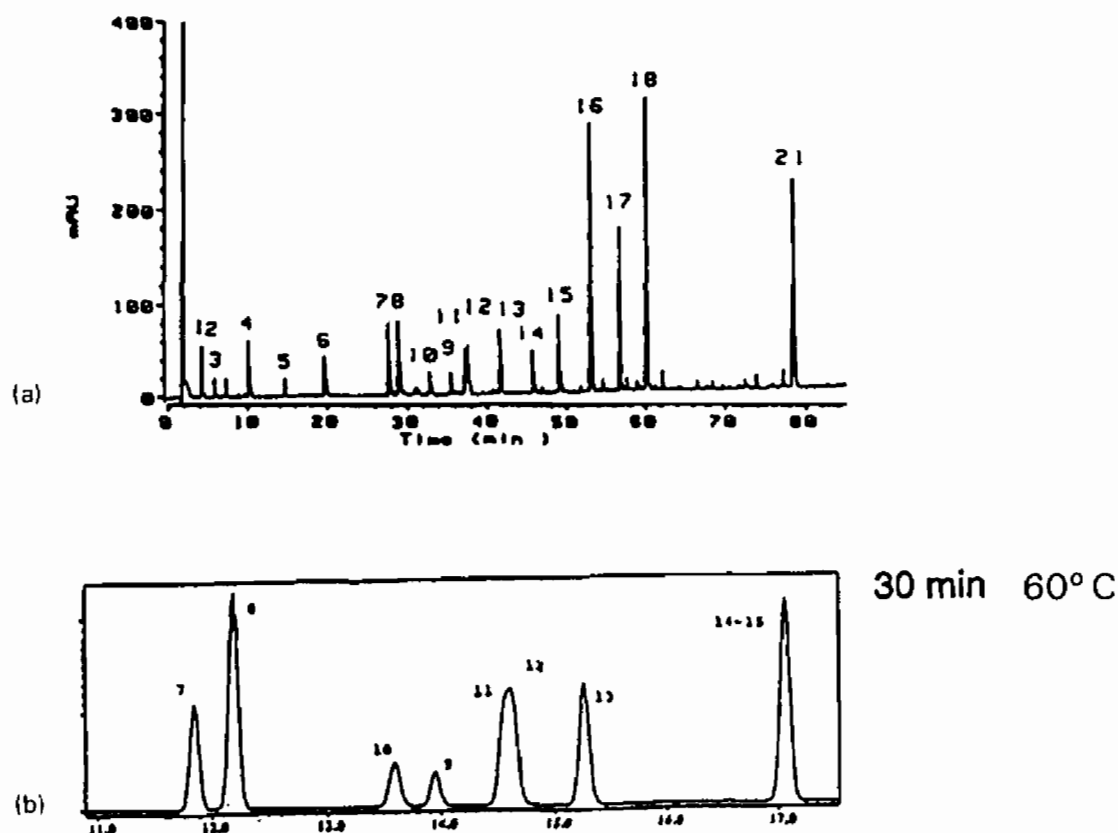


FIGURE 14 Separation of rhGH tryptic digest as a function of gradient time and temperature. Conditions: 0–60% acetonitrile/0.1% TFA in water; 1.0 mL/min; 15 × 0.46-cm, 300-Å-pore C₈ column (Zorbax SB-C8). (a) Separation of entire sample at 60°C and 120 min; (b–d) separation of bands 7–15 at 40°C and indicated gradient times; (e,f) separation of bands 7–15 in a time of 60 min and indicated temperatures. (From Ref. 38.)

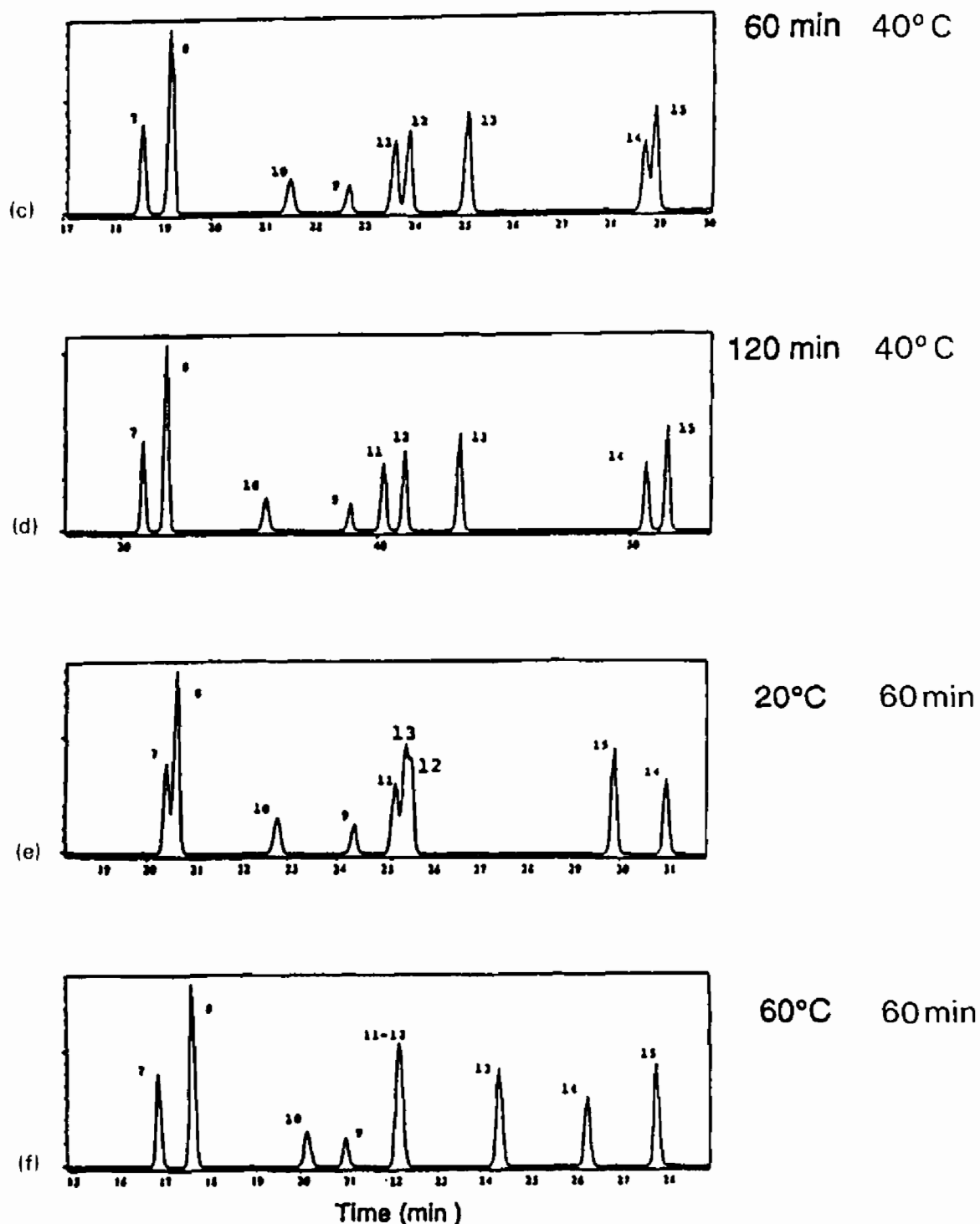


FIGURE 14 (continued)

further. The separation of bands 7–15 is shown in Figs. 14b–f as a function of gradient time and temperature. It is obvious that either variable (t_G or T) has a major effect on band spacing and resolution, with acceptable separation being observed at 40°C and a gradient time of 120 min (Fig. 14d). The same study

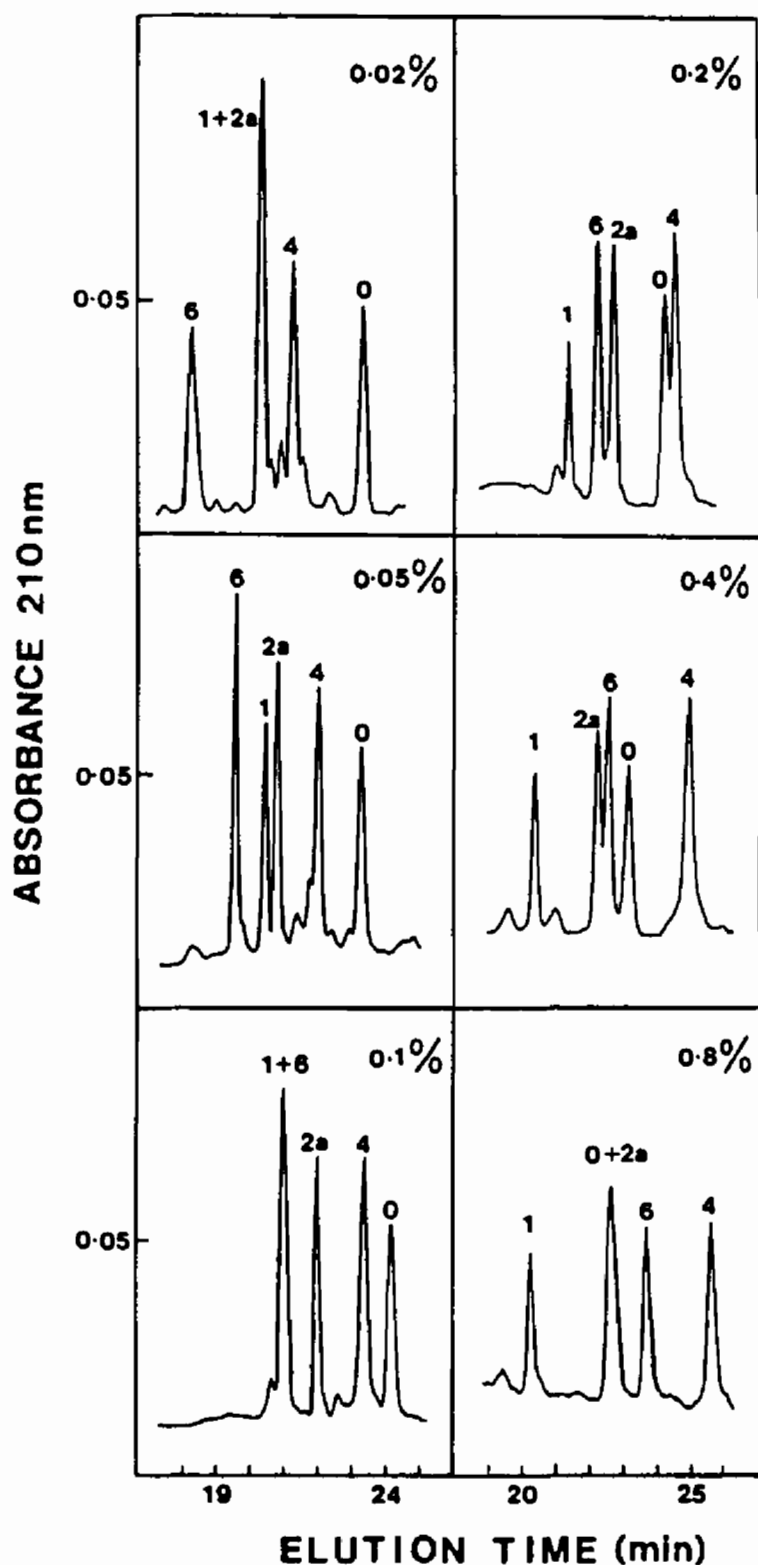


FIGURE 15 Separation of basic peptides as a function of TFA concentration. Conditions: 25 × 0.46-cm C₁₈ column (Syn Chropak C₁₈); gradient at 1%B/min; A solvent, water plus indicated TFA concentration; B solvent, acetonitrile plus indicated TFA concentration; 26°C; 1.0 mL/min. Sample peptides contain 4–10 amino acid residues; number for each band indicates number of basic amino acids in molecule (equal to positive charge on molecule). (From Ref. 40.)

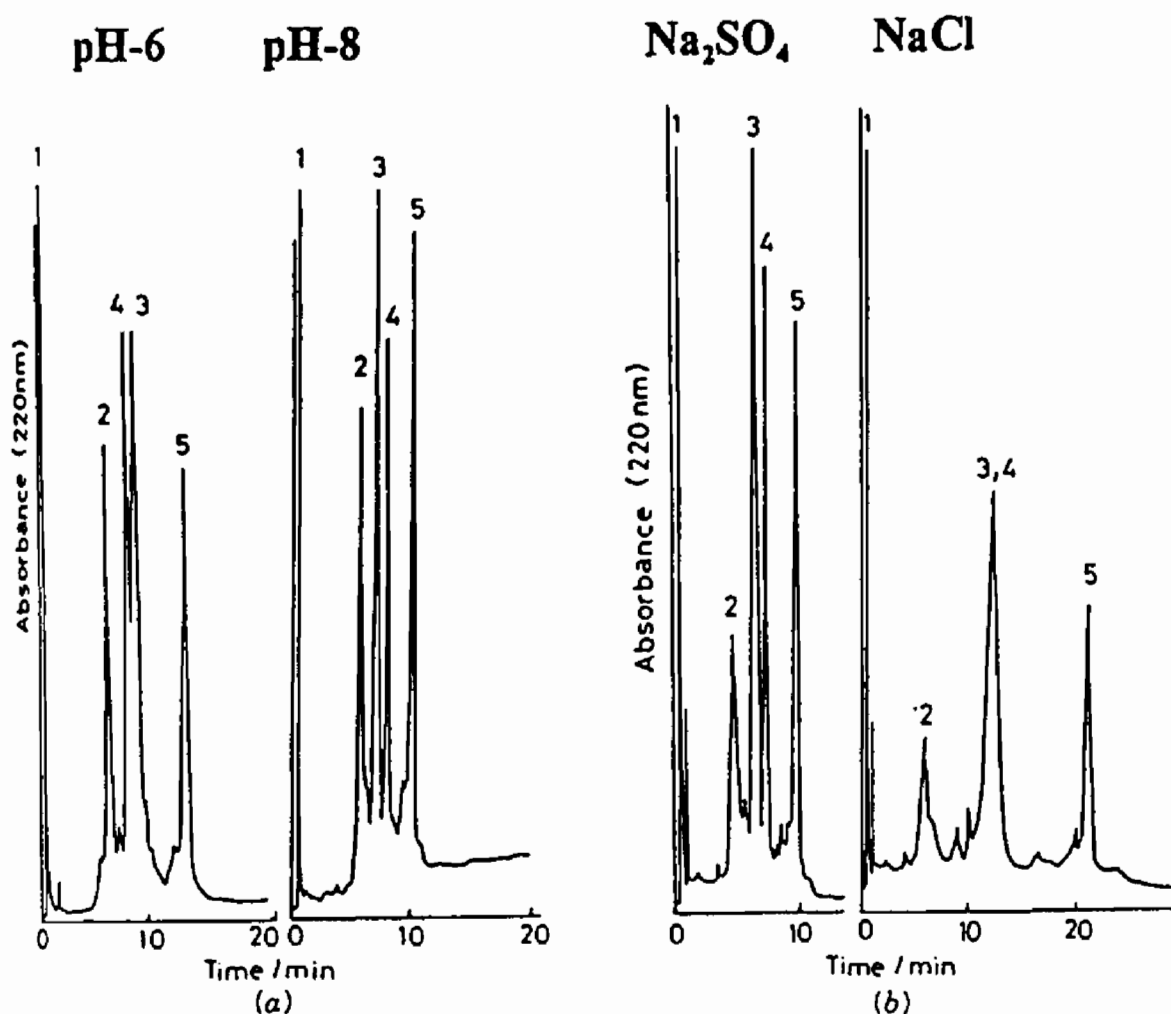


FIGURE 16 Effects of pH and salt type on band spacing in the anion-exchange separation of a protein sample: 1, myoglobin; 2, transferrin; 3, α_1 -acid glycoprotein; 4, α -lactalbumin; 5, soybean trypsin inhibitor. Conditions: 5×0.4 -cm WAX column (300 Å pore) (Shimadzee Shim-pack); 0–100% B in 20 min; 1.0 mL/min. (a) A solvent, 0.02 M phosphate buffer; B solvent, 0.5 M phosphate buffer; (b) A solvent, 0.02 M phosphate buffer; B solvent 0.5 M NaCl or Na₂SO₄. (From Ref. 41.)

[38,39] also demonstrated similar changes in selectivity for other peptide digests and a cereal protein sample as a function of temperature or gradient time.

Other variables that can be used to change band spacing and optimize separation include (1) mobile phase pH, (2) ionic strength, and (3) ion pairing. Trifluoroacetic acid (TFA) is often used as a low-pH buffer for the separation of peptides and proteins, and TFA also serves as a weak ion-pairing reagent. As such, an increase in TFA is expected to result in large changes in band spacing for samples whose components differ in molecular charge. An example is shown in Fig. 15 for the separation of a peptide mixture whose components contain varying numbers of basic amino acid residues. At low pH, the positive charge on the

molecule (0–6, indicated for each band in Fig. 15) is determined by these basic amino acids. As the mobile phase TFA concentration in Fig. 15 is varied from 0.02 to 0.8%, major changes in band spacing and separation result. The best separation in this example is for a TFA concentration of 0.05%.

A change in pH or salt type in ion-exchange gradient elution will often affect the band spacing for peptide or protein samples. Examples of this are shown in Fig. 16 for a mixture of five proteins. The relative retention of bands 3 and 4 can be reversed by either a change in pH or a change in the salt anion from sulfate to chloride.

COMPUTER SIMULATION FOR HPLC METHOD DEVELOPMENT

The theory summarized in this chapter and elsewhere [1–3a,18] has enabled the development of computer programs that allow so-called *computer simulation*. In this approach, two to four experimental gradient runs are first carried out for the reversed-phase or ion-exchange separation of a biological macromolecular sample. After the results of these initial experimental runs are entered into the computer, separation times of analytes can be predicted based on initial and final %B, gradient time and shape, column dimensions, flow rate, column-packing particle size, and temperature [42–47].

Computer Simulation with Two Starting Experiments

In its simplest application, computer simulation makes use of two initial experimental runs with only gradient time varying. Separation as a function of gradient conditions (initial and final %B, gradient time, gradient shape) can then be predicted by the computer without any further “real” experiments. This approach will be illustrated for the optimisation of the separation of the rhGH peptide sample of Fig. 14. Computer simulation allows the trial-and-error prediction of separation as a function of experimental conditions, but a more effective approach is first to examine a *resolution map*: a plot of the resolution R_s for the most overlapped (*critical*) band pair in the sample as a function of gradient time. A resolution map for the separation of the rhGH peptide sample is shown in Fig. 17b. (Note that a different column is used for the separations of Fig. 17 than those of Fig. 14; as a result, somewhat different separations are obtained in Figs. 14 and 17.)

The separation of the hard-to-separate bands 9–15 is illustrated in Fig. 17b as a function of gradient time. For a gradient time of about 50 min, bands 9 and 10 are completely overlapped ($R_s = 0$); for a gradient time of about 110 min, bands 9–15 are separated with a maximum resolution of $R_s = 1.2$; and for a gradient time of about 200 min, bands 11 and 12 are completely overlapped ($R_s = 0$). The resolution map of Fig. 17b immediately tells us which gradient

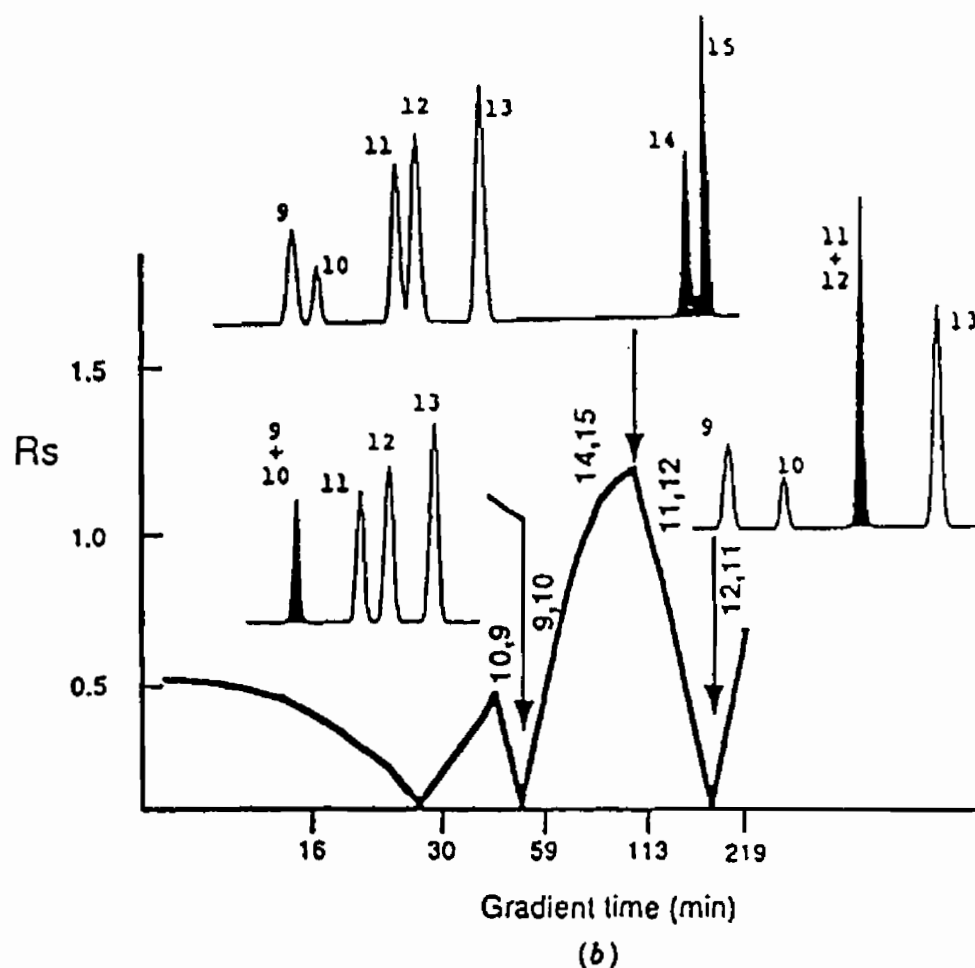
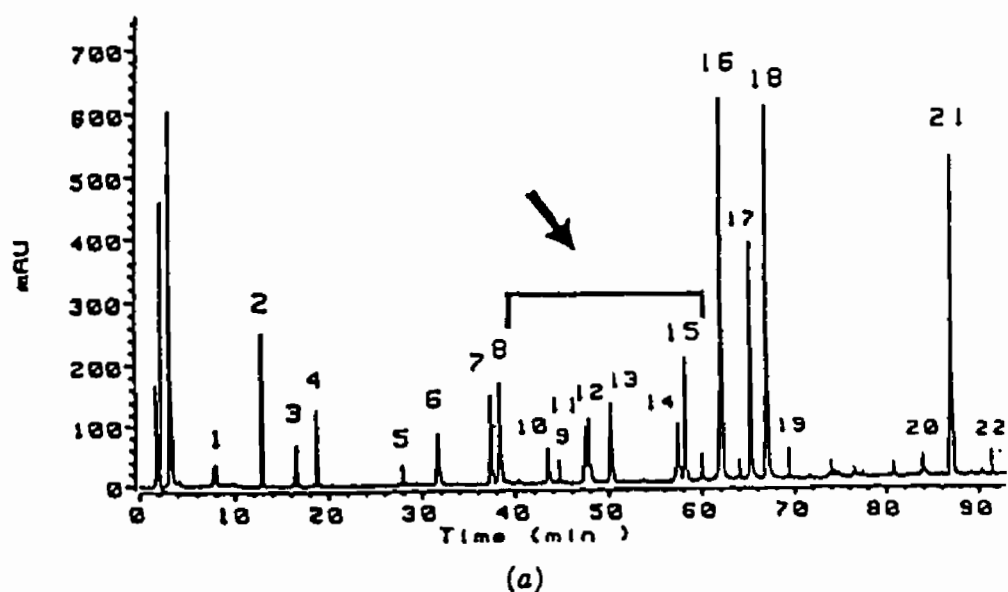


FIGURE 17 Resolution map for the separation of rhGH tryptic digest. Conditions: 15 × 0.46-cm Nucleosil C₁₈ column; gradient of 0–60% B; solvent A is 0.1% TFA in water; solvent B is 0.08% TFA in acetonitrile; 1.0 mL/min; 40 °C. (a) Separation for 120-min gradient; (b) resolution map as a function of gradient time (see text for details), DryLab software. (From Ref. 48.)

time will provide maximum resolution for this sample (assuming other conditions are not also changed). Once an optimum gradient time or steepness is known, the separation can be further improved by shortening the gradient (increase of initial %B or decrease of final %B), using segmented gradients, and/or changing column conditions (column length, flow rate, particle size). All of these changes in separation conditions can be predicted by the computer. When the desired separation has been achieved by computer simulation, an experimental run with the same conditions can be carried out to confirm the computer prediction.

Resolution maps for the rhGH peptide sample are shown in Fig. 18 for three different temperatures (columns, of Fig. 14), so these maps cannot be compared with the resolution map of Fig. 17b. For each temperature (20, 40, 60°C), two experimental runs were required to obtain a resolution map at that temperature. For a temperature of 20°C, maximum resolution ($R_s = 0.9$) is obtained for a gradient time of 90 min. At 40°C, maximum resolution ($R_s = 2.6$) is obtained with a 170-mm gradient. At 60°C, a maximum resolution of $R_s = 2.9$ is obtained for a gradient time of 260 min. If we require baseline separation of all peaks ($R_s = 1.5$), the shortest run time is provided for a temperature of 40°C (80-min gradient; see 40°C map of Fig. 18).

Computer simulation was first developed for reversed-phase separations. However, computer simulation can also be used for the reliable prediction of ion-exchange separations as a function of gradient conditions [49,50]. For a further discussion of computer simulation for the systematic development of optimized gradient separations for peptide and protein samples, see Ref. 51.

Computer Simulation with Four Starting Experiments

In its most recent development [45,46] computer simulation was carried out based on experimental runs with two different temperatures and two different gradient times. The computer software then allows a rapid selection of the optimum gradient time and temperature from a two-dimensional resolution map, similar to the use of several one-dimensional resolution maps for different temperatures (as in Fig. 18). Application of this software to the separation of the rhGH peptide sample [45] predicted an optimum temperature of 30°C in a run time of only 26 min (segmented gradient). Similar optimization of temperature and gradient time was also studied for 37 peptides from the tryptic digest of recombinant tissue plasminogen activator (rt-PA) [47]. For the latter (more complex) sample, the best resolution that could be obtained for the separation of all 37 bands was only $R_s = 0.5$ (serious overlap of two or more bands) at 57°C with a 90-min gradient. However, by optimizing conditions for different peptides in the sample, it was possible with *two* different assay runs to achieve an overall resolution of all bands with $R_s \geq 1.0$. These two separations are shown in Fig. 19.

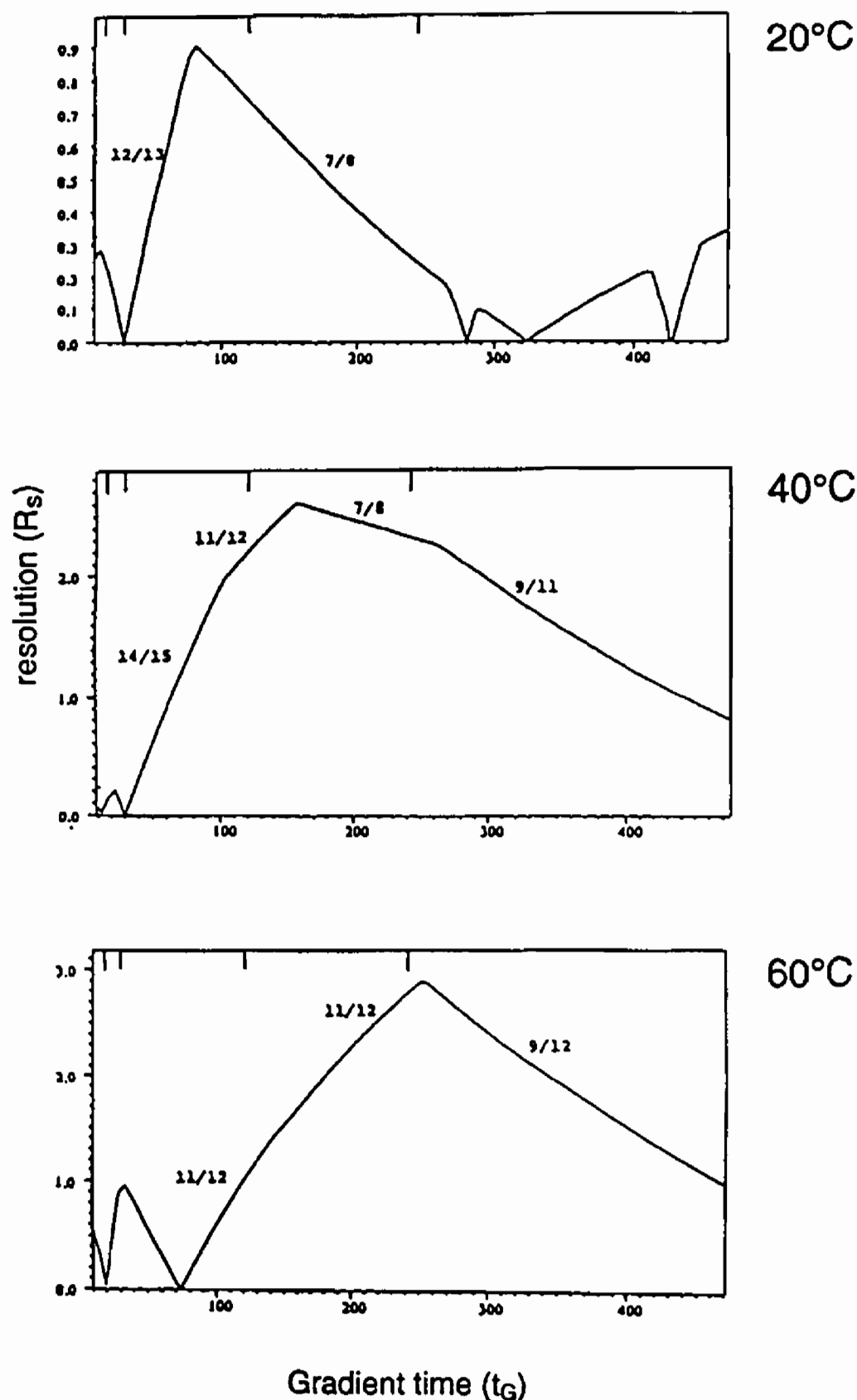


FIGURE 18 Resolution maps for the separation of rhGH tryptic digest as a function of temperature; DryLab software. Conditions: 15×0.46 -cm Zorbax-SB- C_8 column; gradient of 0–60% B; solvent A is 0.1% TFA in water; solvent B is 0.08% TFA in acetonitrile; 1.0 mL/min; 40°C. (From Ref. 38).

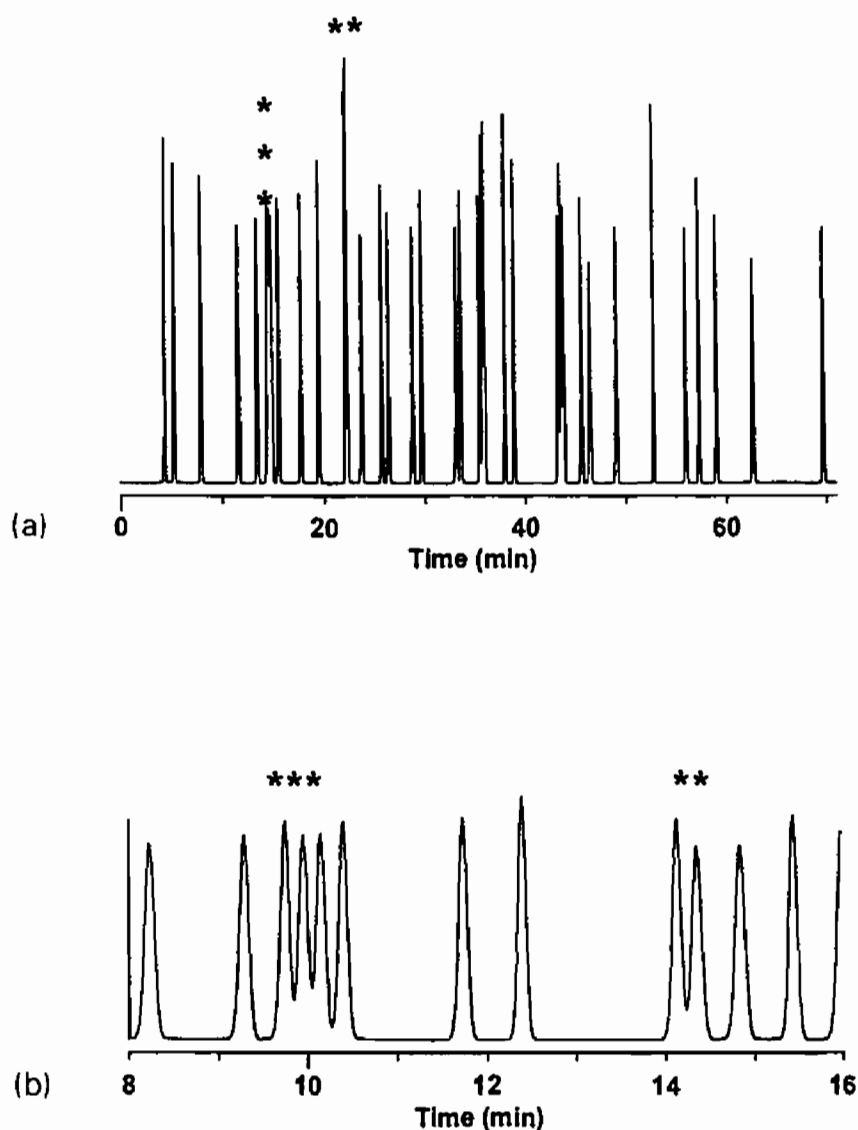


FIGURE 19 Separation of rt-PA digest sample using two different assay procedures. Conditions: 15 × 0.46-cm Zorbax-SB-C₈ column; gradient of 0–60% B; solvent A is 0.1% TFA in water; solvent B is 0.08% TFA in acetonitrile; 1.0 mL/min. (a) Separation of 32 peptides at 54°C with a 115-min gradient; five peaks marked * are not resolved with $R_s \geq 1.0$; (b) separation of remaining five peptides (marked with *) at 66°C with a 61-min gradient. DryLabR computer simulations. See text and Refs. 39 and 47 for details.

In the separation of Fig. 19a, the peaks that are not resolved with $R_s = 1.0$ or greater are marked with an asterisk (*); in the companion separation of Fig. 19b, the peaks that were not resolved in the separation of Fig. 19a are marked with an *. The development of two (or more) runs to achieve the separation and analysis of all sample components is easily carried out by means of computer simulation [47].

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Analytical HPLC of Peptides

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INTRODUCTION

Focus of This Chapter

High-performance liquid chromatography (HPLC) has proved remarkably versatile for the isolation/purification of peptides varying widely in their sources, quantity, and complexity. Indeed, it is reasonable to say that the development of high-performance packings has revolutionized the efficiency and speed for separations of molecules in general, and peptides in particular, considering the consistently poor resolution of the latter when employing traditional chromatographic techniques, e.g., size exclusion chromatography (SEC) and ion-exchange chromatography (IEC) in glass columns packed with stationary phases based on soft gels, paper electrophoresis, etc.

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Due primarily to this tremendous development of high-performance chromatographic techniques over the years in terms of scale, instrumentation, and column packings, with a concomitant array of mobile phases employed by various researchers for specific applications in all major modes of HPLC employed for peptide separations, it is all too easy to feel overwhelmed when one approaches peptide applications in HPLC for the first time. Whether one is employing high-performance approaches for routine peptide separations or for such applications lie in such state-of-the-art areas as capillary methods, proteomic and genomic research, biospecific interactions, or even current efforts to achieve microfluidic separations on a chip, the fundamentals of chromatographic protocols remain the same. Thus, this chapter is aimed at laboratory-based researchers, both beginners and more experienced chromatographers, who wish to learn about peptide analysis by HPLC. Standard analytical applications in HPLC of peptides will be stressed (as opposed to micro- or preparative scale chromatography); in addition, only nonspecialized columns, mobile phases, and instrumentation readily available and easily employed by the researcher are described in detail.

Characteristics of Peptides

The amino acids are the fundamental units, or building blocks, of both peptides and proteins. The distinction between a peptide, polypeptide, or protein is somewhat arbitrary with peptides usually defined as containing ≤ 50 amino acid residues. Molecules containing more than 50 amino acid residues usually have a stable three-dimensional structure in solution and are referred to as proteins. The side chains or R groups of the amino acids are generally classified according to their polarity (nonpolar or hydrophobic versus polar or hydrophilic). The polar side chains are divided into three main groups: uncharged polar, potentially positively charged or basic side chains, and potentially negatively charged or acidic R groups. Within any single group, there are considerable variations in the size, shape, and properties of the side chains. Peptides containing both ionizable acidic and basic side chains have a characteristic isoelectric pH and the overall net charge and polarity of a peptide in aqueous solution will vary with pH. Thus, hydrophobicity/hydrophilicity as well as the number of charged groups present become important factors in the separation of peptides.

Conformation can be an important factor in peptides as well as proteins and, thus, should always be a consideration when choosing the conditions for chromatography. Although secondary structure (e.g., α -helix) is generally absent even in benign aqueous conditions for small peptides (up to approximately 10 residues), the potential for a defined secondary or tertiary structure increases with increasing peptide length and, for peptides containing more than 20–30 residues, folding to internalize hydrophobic residues is likely to become a significant conformational feature. In addition, even small peptides may exhibit significant secondary structure, especially if such structures are somehow constrained,

making the HPLC retention behavior of such peptides different from what might be expected if such molecules were unconstrained. Similarly, the presence of a disulfide bridge(s) would be expected to affect conformation, and thus a peptide's retention time in HPLC could be altered from its retention time in the fully reduced state.

Peptide Detection

Ultraviolet (UV) detectors are, by far, the most commonly used detectors for peptide analysis. Peptide bonds absorb light strongly in the far ultraviolet (<220 nm; Fig. 1 [1]), providing a convenient means of detection (usually at 210–220 nm). In addition, the aromatic side chains of tyrosine, phenylalanine, and tryptophan absorb light in the 250- to 290-nm ultraviolet range; it should be noted, however, that these aromatic residues are not present in all peptides.

The HPLC (specifically, reversed-phase chromatography or RPC) elution profiles shown in Fig. 1 demonstrate the effect of varying detection wavelength (200 to 230 nm in 10-nm steps) on the sensitivity of detection of a mixture of six 10-residue peptides. The sensitivity of peak detection decreases dramatically as the detection wavelength increases from 200 nm (~850 mAU for the first eluted peak) to 230 nm (~15 mAU for the same peptide). The decrease in absorbance of the peptide bond is clear from the UV spectrum (200 to 250 nm) of the first eluted peak shown at the bottom of Fig. 1. Although from Fig. 1, wavelengths below 210 nm clearly offer enhanced sensitivity, several practical considerations preclude the use of wavelengths below this value. Indeed, the use of wavelengths below 210 nm tends to be the exception rather than the rule because such detection can suffer from interference due to impurities present in buffers, solvents, or even the sample, which may obscure the peak(s) of interest.

HPLC-grade solvents are the norm for most analytical purposes, especially where detectors are being used at high sensitivity; however, chemical groups or bonds in the pure solvents themselves may also contribute significantly to UV absorbance at wavelengths below 210 nm. The common use of 210 nm as the preferred detection wavelength for most HPLC applications is a good compromise between detection sensitivity and potential detection interference. It should be noted, however, that wavelengths in the range of 220 to 230 nm are frequently employed in preparative applications [2,3], where the use of more sensitive detection wavelengths would result in overloading of the detector response (usually above an absorbance of 2.0 to 2.5 AU). The development of multi-wavelength detectors, enabling the simultaneous detection of peptide bond absorbance at low UV wavelengths and aromatic side-chain absorbance of varying detection sensitivity, has proved to be of immense value for the rapid separation and identification of peptides and proteins.

Although fluorescence [4–6] and electrochemical [5–7] techniques have also been employed for specific peptide analyses, the average research laboratory

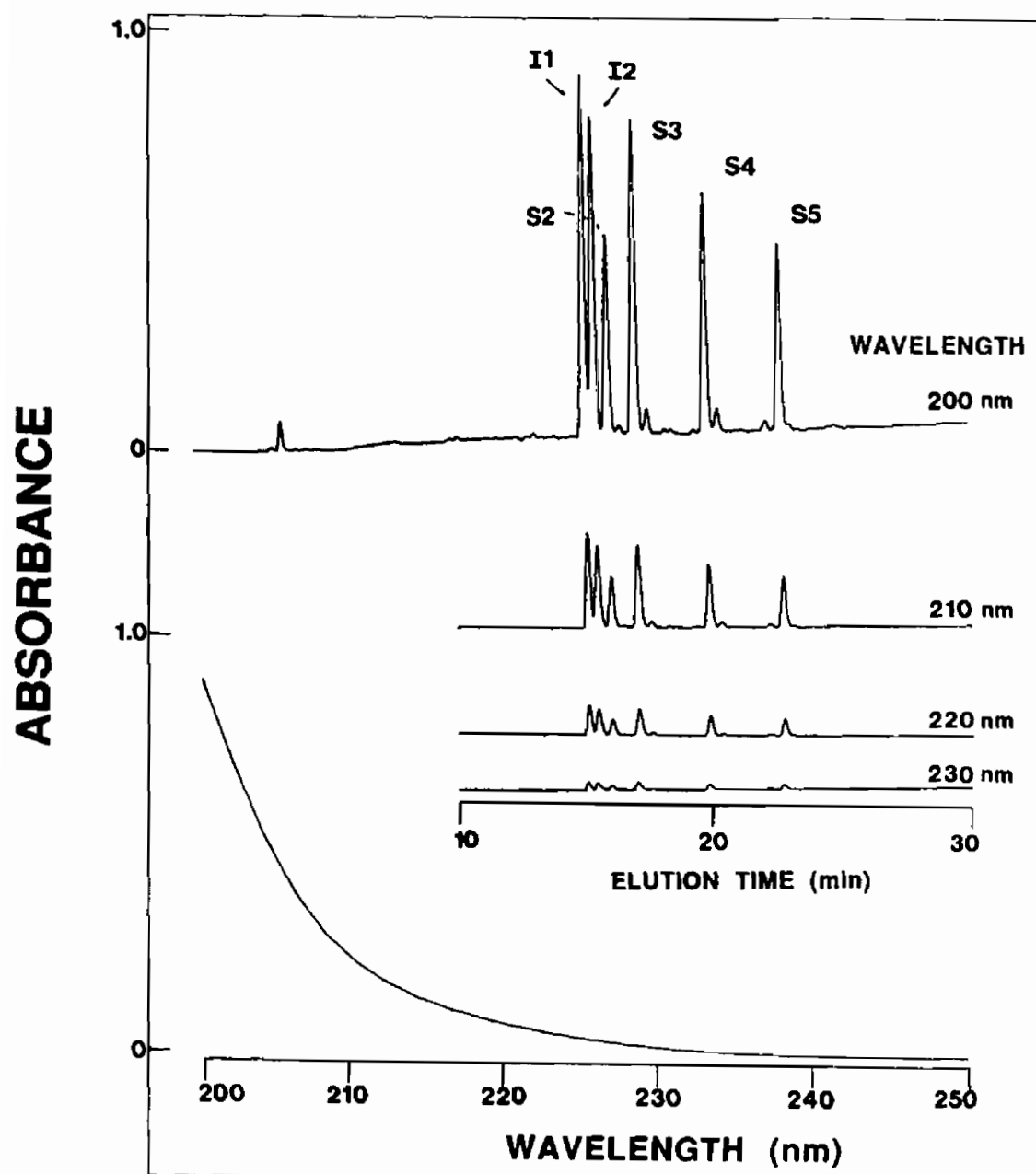


FIGURE 1 Effect of varying detection wavelength on sensitivity of UV detection of peptides during RPC. Column: SynChropak RP-P C₁₈ (250 × 4.6 mm ID, 6.5 μm particle size, 300 Å pore size; SynChrom, Lafayette, IN). Top and middle: elution profiles of synthetic 10-residue peptides (sequences shown in Table 1) at wavelengths of 200, 210, 220, and 230 nm. Bottom: spectrum (200–250 nm) of first eluted peptide, I1 (n.b., no aromatic residues are present in the peptides). (From Ref. 1.)

is much more likely to possess a UV detection system for peptide HPLC. Indeed, Lockhart et al. [6], comparing the preceding three types of detector, determined that, for routine peptide applications, the UV detector is superior to electrochemical and fluorescence detectors with respect to convenience of operation. Finally, it should be noted that all of the peptide applications illustrated in the present chapter utilized UV detection systems.

Separation Goals

High-performance liquid chromatography has proved very versatile in the isolation of biologically active peptides from a wide variety of sources. Investigation of the properties of peptides is vital in structure-function studies of proteins and it is often necessary to isolate a few peptides from a complex proteolytic and/or chemical cleavage mixture. The wide use of automated solid-phase peptide synthesis in recent years has also called for efficient isolation of peptides from various impurities. The complexity of peptide mixtures may vary considerably depending on the source. The number of peptide fragments obtained from a protein cleavage, for example, depends on the nature and efficiency of the enzymatic or chemical agent used. Peptides obtained from biological tissues are often found only in very small quantities and may require extensive purification. An efficient peptide synthesis should result in only a small number of synthetic impurities. However, these impurities are usually closely related to the peptide of interest (deletion, terminated, or chemically modified peptides), perhaps missing only one amino acid residue, and may be difficult to separate.

As a general rule, the approach to separation must be tailored to the separation goals; i.e., purification of a single peptide from a complex mixture will require a different approach from that necessary for separating all components of a mixture. Whereas the former approach may require only the application of a single HPLC mode, the latter will require a combination of separation modes (multistep or multidimensional HPLC) for efficient resolution of all desired peptides.

COLUMN SELECTION

Modes of HPLC Used in Peptide Separations

The three major modes of HPLC employed in peptide separations utilize differences in peptide size (size exclusion HPLC or SEC), net charge (ion-exchange HPLC or IEC), or hydrophobicity (reversed-phase HPLC or RPC). Within these modes, mobile phase conditions may be manipulated to maximize the separation potential of a particular HPLC column. RPC remains by far the

most widely used mode of HPLC. The ability of this technique to separate peptides of closely related structures has made it an extremely powerful high-speed analytical and preparative tool. Also, the flexibility of RPC (parameters such as pH, mobile phase, counterion, temperature, etc. can be varied for optimum resolution) makes it the obvious choice for the separation of one peptide from a complex mixture. The most favored RPC columns for separation of most peptides and smaller proteins remain silica-based supports containing octyl (C_8) or octadecyl (C_{18}) functionalities, although C_3 sorbents have been used for more hydrophobic peptides and proteins [8–10].

IEC is being increasingly used for peptide separations as commercially available HPLC packings capable of retaining both highly charged and weakly basic or acidic peptides have been introduced. In addition, the scope of IEC packings for peptide separations has been expanded with the demonstration of efficient separations of closely related peptides, including peptides with identical net positive charge, on a strong cation-exchange column (CEC) via a mixed-mode hydrophilic interaction/cation-exchange mechanism termed HILIC/CEC [11–15], described later in this chapter. Despite the introduction of a size exclusion column designed specifically for the separation of peptides in the molecular weight range 100–7000 [16], which perhaps promises to raise the profile of this HPLC mode for peptide analysis, the practical value of SEC columns is generally still rather limited for resolution of peptides (up to 50 residues). Much less used peptide separation techniques include affinity chromatography [17,18] and hydrophobic interaction chromatography (HIC) [19], which are not pursued further in this chapter.

A big advantage of peptide over protein separations lies in the wider range of mobile phases available for the former. Because peptides generally lack a unique conformation, the researcher may utilize chromatographic conditions for peptide separations that may denature proteins. The major limit of mobile phase conditions for peptide separations depends mainly on the nature and stability of the column support. It is a testament to the excellent characteristics of silica that silica-based supports are still the most widely used, despite promising advances in efforts to develop and improve other packing supports. Thus, the rigidity of microparticulate silica enables the use of high linear flow velocities of mobile phases. In addition, favorable mass transfer characteristics have allowed rapid analyses to be performed. However, most silica columns are limited to a pH range of 2.0–8.0. The silica matrix is rapidly dissolved in the presence of basic eluents and the hydrophobic bonded phase of RPC columns (generally octyl or octadecyl functionalities covalently linked via a siloxane bond to the silica support) is progressively cleaved at low pH. It should be noted, however, that considerable success has been achieved in developing more pH-stable silica-based RPC packings either through shielding the silica matrix from dissolution at pH values greater than 7.0 [20] or by protecting the siloxane bond from hydrolysis at acidic pH [21–24]. The majority of silica-based supports for RPC and IEC in

analytical and semipreparative applications comprise 5- to 10- μm spherical particles with pore sizes ranging from ~ 50 to several thousand angstroms ($\text{\AA}$). In general, 300- $\text{\AA}$ pore size matrices give better resolution and recovery for peptides and proteins [25,26]. Because pore diameter has little effect on peptide separations [8] and most researchers who separate peptides also separate proteins, the 300- $\text{\AA}$ support is the best buy for IEC and RPC columns.

Despite the preponderance of silica-based supports, packings based on organic polymers are becoming increasingly used in all modes of HPLC. These materials, the most common of which are formed from cross-linked polystyrene-divinylbenzene (PSDV) [27–29], are characterized by a broad pH tolerance (often pH 0–14). Other polymeric supports that show some promise include those based on polymethacrylate or vinyl alcohol copolymers (useful overviews of silica- and polymer-based supports may be found in Refs. 29–31). In addition, alumina-based [29,32,33] and zirconia-based [29,34,35] supports are currently receiving much attention. Although packings based on synthetic polymers have been frequently successfully used in SEC [16,36–39] and IEC [38,40–43] of peptides, the application of such supports to RPC has been somewhat limited, frequently because of poor mass transfer properties as well as a tendency for the packing to be adversely affected by the presence of organic solvent, e.g., shrinkage or swelling of PSDV in varying levels of acetonitrile. However, the application of such supports to RPC of peptides is growing [28,29,44,45] and, in our hands, specific PSDV-based RPC packings have clearly shown a distinct improvement for such applications.

There is, of course, a constant effort to develop novel stationary phases for improved bioseparations in terms of speed and/or selectivity [46]. Interesting developments in this area include monoliths as stationary phases (cast as a non-silica-based homogeneous phase into chromatography columns as a single, continuous piece) [47–49]; silica-based RPC packings with alkyl stationary phases containing polar-linked groups or embedded polar groups (particularly useful for separations of ionizable compounds) [50]; and spherical porous silicas in the micrometer to submicrometer size range [51], such supports having particular applications for miniaturized high-resolution techniques. In addition, packings based on nonporous silica particles, generally in the diameter range of 1–2 μm , continue to find a niche in all the major modes of HPLC used for polypeptide separations [52], often allowing efficient separations of peptides and proteins in under 5 min [53–55] or even separations requiring <1 min [56], although such high-speed applications frequently require specialized instrumentation (e.g., able to withstand column back pressures considerably higher than those encountered with conventional porous supports) and detection systems to take full advantage of such stationary phases.

Column sizes in terms of internal diameter (ID) employed for peptide separations generally fall in the range of 2–3 mm (narrow bore) and 4–4.6 mm (analytical), with optimum flow rates lying in the range of, respectively,

0.2–0.5 mL/min and 1–2 mL/min [57], depending on the HPLC mode (i.e., IEC or RPC) and the specific sample mixture of interest. Such columns have the advantage of being compatible with standard LC instrumentation and efficient separations are generally achievable in well under an hour; in addition, the narrower bore columns offer greater detection sensitivity, reduced mobile phase volume, and reduced sample loading [57], the latter characteristic being particularly important when sample amounts are limited. Smaller internal column diameters, such as those characteristic of microbore columns (typically 1 mm ID [57,58]) and capillary columns (0.15–0.5 mm ID [58,59]) offer even greater advantage in terms of detection sensitivity and speed (capillary chromatography separations are often measured in seconds). However, specialized instrumentation, which is not the focus of this chapter, is generally required for such micro-LC applications, particularly in terms of pumps able to maintain accurately optimum flow rates ranging from 0.1 to 0.2 mL/min for microbore separations [57] and, more particularly, 0.5–5 μ L/min for capillary columns [59]. In addition, the instrument must be able to generate accurate gradients over very short run times for gradient elution LC.

Column Selection for Multistep HPLC

It is a large requirement to expect a single technique to resolve very complex mixtures of peptides. A more efficient separation of all components of a mixture will be obtained by the combined use of separation modes that utilize different selectivities (size, charge, hydrophobicity). Because a portion of the chromatographic sample is usually unavoidably lost at each separation step, it is important to minimize the number of steps required for satisfactory peptide resolution. Hence, for this multistep approach, it is necessary to be careful in the selection of columns and the order in which they are used. This concept was well demonstrated by Mant and Hodges [60], who combined the different selectivities of SEC, IEC, and RPC to resolve completely the major peptide fragments from a cyanogen bromide cleavage of rabbit skeletal troponin I. The initial separation by SEC (Bio-Rad TSK 250 column) simplified the subsequent strong cation-exchange (SynChropak S300 column) and RPC (SynChropak RP-P C₁₈ column) steps. In addition, the use of a volatile eluent (0.1% aqueous trifluoroacetic acid) for the size exclusion step enabled the peptide fractions to be applied immediately to the cation-exchange column, thereby avoiding an intermediate desalting step.

Strong cation exchange and RPC are probably the most useful modes of HPLC for multistep peptide separations. The utility of strong cation-exchange sorbents, generally modified with sulfonate functionalities, lies in their ability to retain their negatively charged character in the acidic to neutral pH range. Most peptides are soluble at low pH where the side-chain carboxyl groups of acidic

residues are protonated, thus emphasizing any basic, positively charged character of the peptides. Apart from the powerful resolving capability of RPC, the use of volatile mobile phases at low pH enables it to act as a final desalting step. This is particularly important when preparing peptides for amino acid analysis and microsequencing or during large-scale purification of peptides. The use of acidic eluents for IEC and RPC also helps to extend the lifetime of silica-based packings, which, as noted before, are still the most widely used materials for peptide separations. In addition, extended use of silica-based columns with aqueous solvents at pH 6–7 accelerates silica dissolution.

Although size exclusion columns are of great potential value in the early stages of a peptide purification protocol, high-performance SEC columns with the correct fractionation range are not generally commercially available at present, despite some progress in this direction [16]. The high cost and rapid loss of peptide separations at the lower end of the fractionation range of such columns, designed mainly for protein separations, make their purchase prohibitive.

The reader is directed to Refs. 40, 42, 43, 60, and 61 for selected practical approaches to multistep HPLC of peptides, a brief review of which can be found in Ref. 62. Proteomics research has led to an increasing demand for high-throughput separation of proteins and their identification. Such multistep and fully automated HPLC systems are in development [55,63]. The coupling of HPLC to electrospray ionization (ESI) mass spectrometry for the identification of peptides and proteins is revolutionizing proteomic research. However, we are focusing on the basic understanding of HPLC in this chapter.

Requirement for Peptide Standards

It is important in HPLC to achieve good run-to-run reproducibility in peptide separations. Differences in peptide retention may arise from a variety of factors, including instrumentation variations, column aging, temperature variations, and subtle differences in the composition of the mobile phase solvent and buffers. In addition, the resolving ability of packings may vary from manufacturer to manufacturer or from batch to batch of packing from the same manufacturer. The need for standards to monitor column performance is well established. Organic molecules such as the alkylphenone series [64] have been used in monitoring reversed-phase columns. Proteins are often used to demonstrate the utility of ion-exchange and size exclusion columns, in the latter case often to calibrate the column for molecule weight determinations. However, peptide standards are best suited for monitoring peptide retention behavior in HPLC because it is preferable to use standards that are similar both structurally and in size to the sample of interest. Among other things, peptide standards allow the researcher to monitor column performance (efficiency, selectivity, and resolution), run-to-run reproducibility, column aging, instrumentation variations, and

the effect of varying run parameters (e.g., the flow rate in SEC, IEC, and RPC or the gradient rate in IEC and RPC), mobile phase additives, and temperature. In addition, which is very important, peptide standards allow the researcher to identify nonideal peptide retention behavior on a particular HPLC column as well as to develop approaches for manipulation or suppression of such nonideal behavior through changes in the mobile phase. Peptide standards designed specifically to monitor the peptide-resolving capability of SEC, IEC, and RPC are commercially available and are discussed in detail in later sections.

SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Mobile Phase Selection

Aqueous size-exclusion chromatography is generally employed for two purposes: peptide-protein separations and/or molecular weight determinations. Unique tertiary or quaternary structures can be demonstrated by molecular weight determinations in the presence and absence of denaturants in SEC [8,16,65–69]. In addition, SEC in the presence of varying levels of denaturant is proving useful in monitoring peptide/protein unfolding [70–72].

Separation of peptides by a mechanism based solely on peptide size, i.e., ideal SEC, occurs only when there is no interaction between the solutes and the column matrix. Although high-performance size exclusion columns are designed to minimize nonspecific interactions, most modern SEC columns are weakly anionic (negatively charged) and slightly hydrophobic, resulting in deviation from ideal size exclusion behavior [25,37]. Because electrostatic effects are minimized above an eluent ionic strength of about 0.05 M, aqueous phosphate buffers (pH 5.0–7.5), containing 0.1–0.4 M salts, are commonly employed as the mobile phase for SEC of peptides. Hydrophobic interactions are promoted under conditions of high salt concentrations; therefore, ionic strengths above 0.6 M should generally be avoided [25,37,73]. Agents such as 6 M guanidine hydrochloride, 8 M urea, and 0.1% sodium dodecyl sulfate (SDS) have also been employed in mobile phase solvents, both to suppress nonspecific interactions and to denature solutes for estimation of molecular weights [16,25,37,65]. Optimum flow rates for analytical size exclusion columns are generally in the range of 0.2–1.0 mL/min for the best compromise between separation time and efficiency of resolution. In addition, sample volume must be kept as small as possible.

The major disadvantage of nonvolatile eluents is the necessity for their removal prior to further peptide purification or analysis. In addition, the presence of denaturing compounds such as guanidine hydrochloride and SDS precludes the use of low-UV wavelengths (210 nm) for monitoring column effluent. A volatile eluting solvent enables lyophilization of peptide fractions prior to immediate analysis or their direct application to ion-exchange or reversed-

phase columns. Both silica-based and polymer-based SEC columns have occasionally demonstrated the utility of 0.1% aqueous (aq.) trifluoroacetic acid (TFA) with or without an organic solvent (acetonitrile, trifluoroethanol) for the separation of peptides and proteins. For instance, Lau et al. [8] reported a linear relationship between $\ln$ molecular weight and retention time for peptides in the range of 900–8000 daltons on a TSK G3000SW column with an eluting solvent of 0.1% aq. TFA, suggesting that nonspecific interactions with the packing were minimal with the peptides tested.

In the authors' own laboratory, a TSK G2000SW column demonstrated pure size exclusion resolution of five basic peptide standards (800–4000 daltons) using 0.1% aq. TFA as eluting solvent (Fig. 2) [37]; a linear relationship was observed between $\ln$ molecular weight and retention times of the peptides. Rivier [74] reported a similar linear relationship for peptides over a molecular mass range of 1000–44,000 daltons on a Waters PAC I-125 column, using an aqueous triethylammonium buffer (0.25 M, pH 2.25) containing 15–30% acetonitrile. Similar results have been obtained using volatile solvents on TSK G3000SW (900–8000 daltons) [2] and TSK G3000PW (300–134,000 daltons [36] and 300–7000 daltons [75,76]) columns. Apart from their low-UV transparency, the presence of such organic modifiers decreased nonspecific adsorption and increased overall solubility of peptides. It should be noted, however, that in the authors' experience with a range of both silica- and non-silica-based SEC columns, the luxury of volatile mobile phases is by far the exception rather than the rule, with nonspecific peptide/packing interactions almost always requiring nonvolatile salt additives to the mobile phase. Indeed, such additives are also frequently required to prevent solute aggregation [37].

Ideal versus Nonideal SEC

The majority of reports in the literature concerning SEC of peptides and proteins tend to describe chromatographic conditions designed to ensure a pure size exclusion process, although it is often overlooked that the nonideal properties of size exclusion columns can be advantageous in the separation of peptides [37,77]. Excellent examples of this were reported by Yasukawa et al. [78], who separated various hydrophilic and hydrophobic dipeptides on a non-silica-based column by isocratic elution with 50 mM sodium phosphate buffer (pH 7.0). The peptides, retained by adsorption, were eluted in the order of increasing hydrophobicity. Similarly, Mant et al. [37] demonstrated that the separation of five peptide standards of 10, 20, 30, 40, and 50 residues was markedly improved on a SynChropak GPC-60 column when advantage was taken of the nonspecific adsorptive properties of the column rather than when the column was utilized under conditions of ideal SEC. Thus, what is occasionally viewed as a column limitation may become a useful analytical tool.

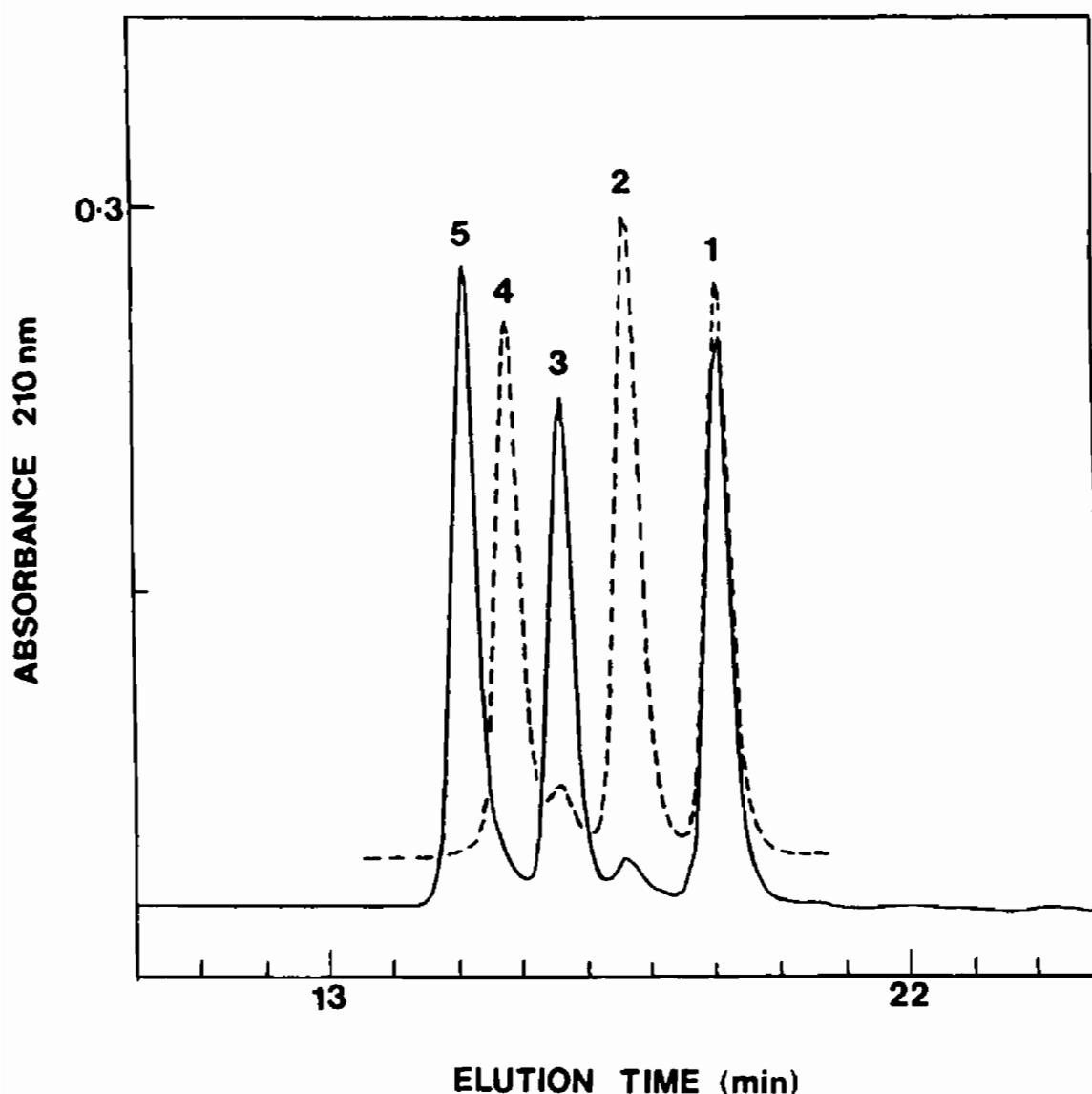


FIGURE 2 SEC of a mixture of synthetic peptide standards. Column: Altex Spherogel TSK 2000SW [300 × 7.5 mm ID; Beckman Instruments (Canada) Inc., Burnaby, BC]. Mobile phase: 0.1% aqueous TFA; flow rate, 0.5 mL/min; temperature, 26°C. The sequences of the five synthetic peptide standards (denoted 1, 2, 3, 4, and 5; 10, 20, 30, 40, and 50 residues, respectively) are shown in Table 1.

Despite the occasional advantage of nonideal column behavior for specific peptide applications, such nonspecific interactions must be suppressed if ideal peptide/protein elution behavior is required, i.e., predictable elution times based on solute size only. The ability to predict the position and/or elution order of peptides during SEC of a peptide mixture would greatly simplify preliminary separation of peptides of interest from a chemical or proteolytic protein digest. Under conditions of ideal SEC, large peptide fragments can be quickly identified and removed. Thus, it is important to achieve a linear relationship between $\ln$

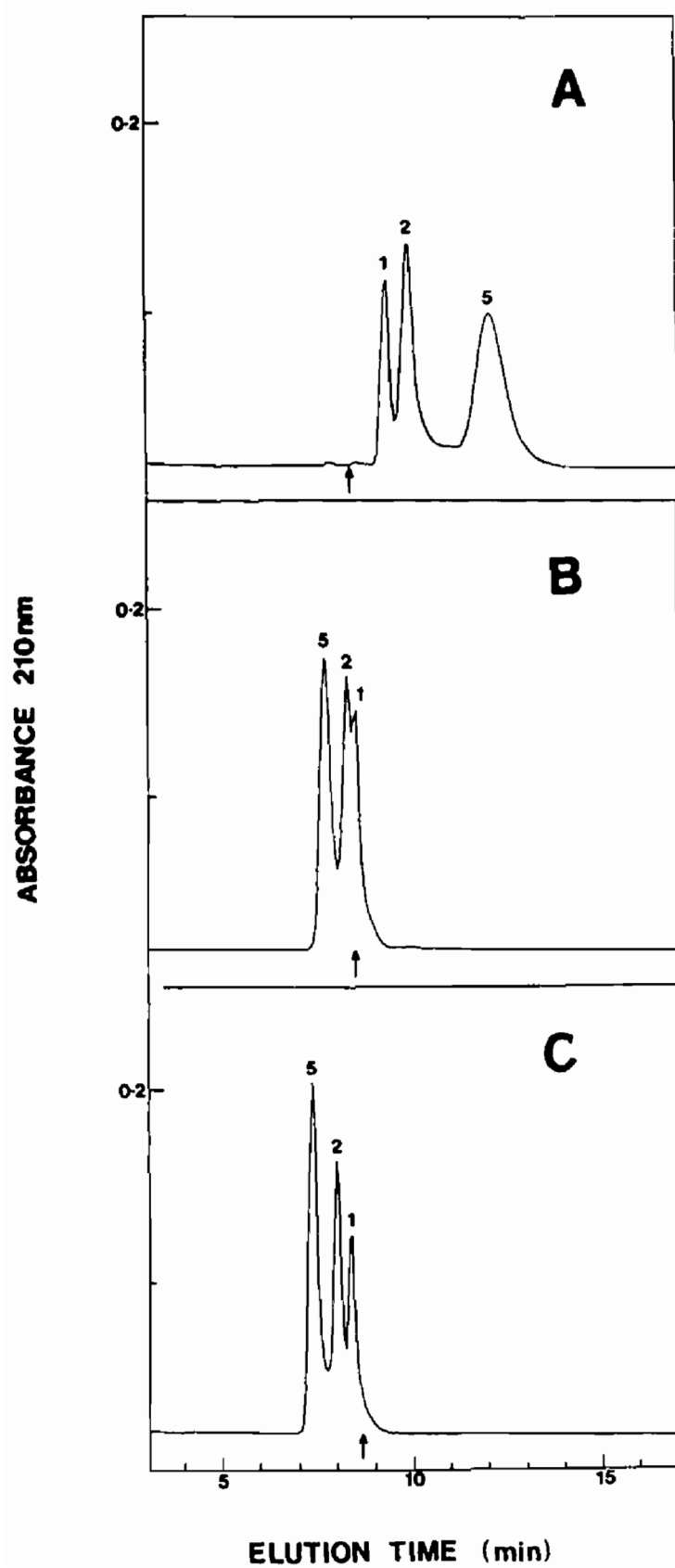
molecular weight and retention time over a wide molecular weight range. Under nondenaturing conditions, many proteins and large peptides may deviate from ideal size exclusion behavior due to conformational effects. Indeed, this laboratory could obtain a linear $\ln$ molecular weight–retention time relationship for myoglobin and its cyanogen bromide fragments (2500–17,000 daltons), on Superose 12 and TSK G2000SW columns, only under highly denaturing conditions (50 mM KH_2PO_4 , pH 6.5, 0.5 M KCl, 8 M urea). Thus, if the conformational character of a peptide-protein mixture in a particular mobile phase is uncertain, SEC should be carried out under highly denaturing conditions to ensure ideal size exclusion behavior.

Peptide Standards

Standards for Monitoring Nonideal SEC Behavior

A series of synthetic peptide standards ($\text{Ac-[G-L-G-A-K-G-A-G-V-G]}_n\text{-amide}$, where $n = 1\text{--}5$), designed to monitor both nonideal and ideal SEC behavior, have proved extremely beneficial in enabling rapid development of the optimal conditions for SEC of peptides [37]. The increasing size of the peptide standards (800–4000 daltons) enables an accurate molecular weight calibration of a column during ideal SEC; the increasingly basic character of the standards (+1 to +5) makes them sensitive to the anionic character of a size exclusion packing; the increasing hydrophobicity of the polymer series enables a determination of column hydrophobicity. In addition, the high glycine content of the standards minimizes or eliminates any tendency toward α -helix formation. Figure 2 shows the elution profile of the five standards on a TSK2000SW column, which exhibited ideal SEC behavior when eluted with 0.1% aq. TFA. However, as such standards can demonstrate, SEC packings vary in the magnitude of their nonspecific interaction characteristics. For example, a SynChropak GPC-60 silica-based column and a Superose 12 agarose-based column both exhibited nonideal behavior when the standards were applied under these conditions [37].

The utility of the peptide standards in monitoring nonideal SEC behavior on a SynChropak GPC-60 column is clearly illustrated in Fig. 3. In Fig. 3A, the separation of standards 1, 3, and 5 (+1, +3, +5 net charge, respectively) appears to be based on an ion exchange rather than a size exclusion mechanism. The elution order is reversed from that expected of a size exclusion mechanism, with the smallest peptide being eluted first ($n = 1$; 10 residues) and the largest peptide being eluted last ($n = 5$; 50 residues). In addition, all three peptides were retained longer than the total permeation volume of the column. As the ionic strength of the mobile phase increases, these electrostatic effects are gradually overcome, until an essentially ideal size exclusion mechanism is apparent at a salt concentration of 200 mM (Fig. 3C).

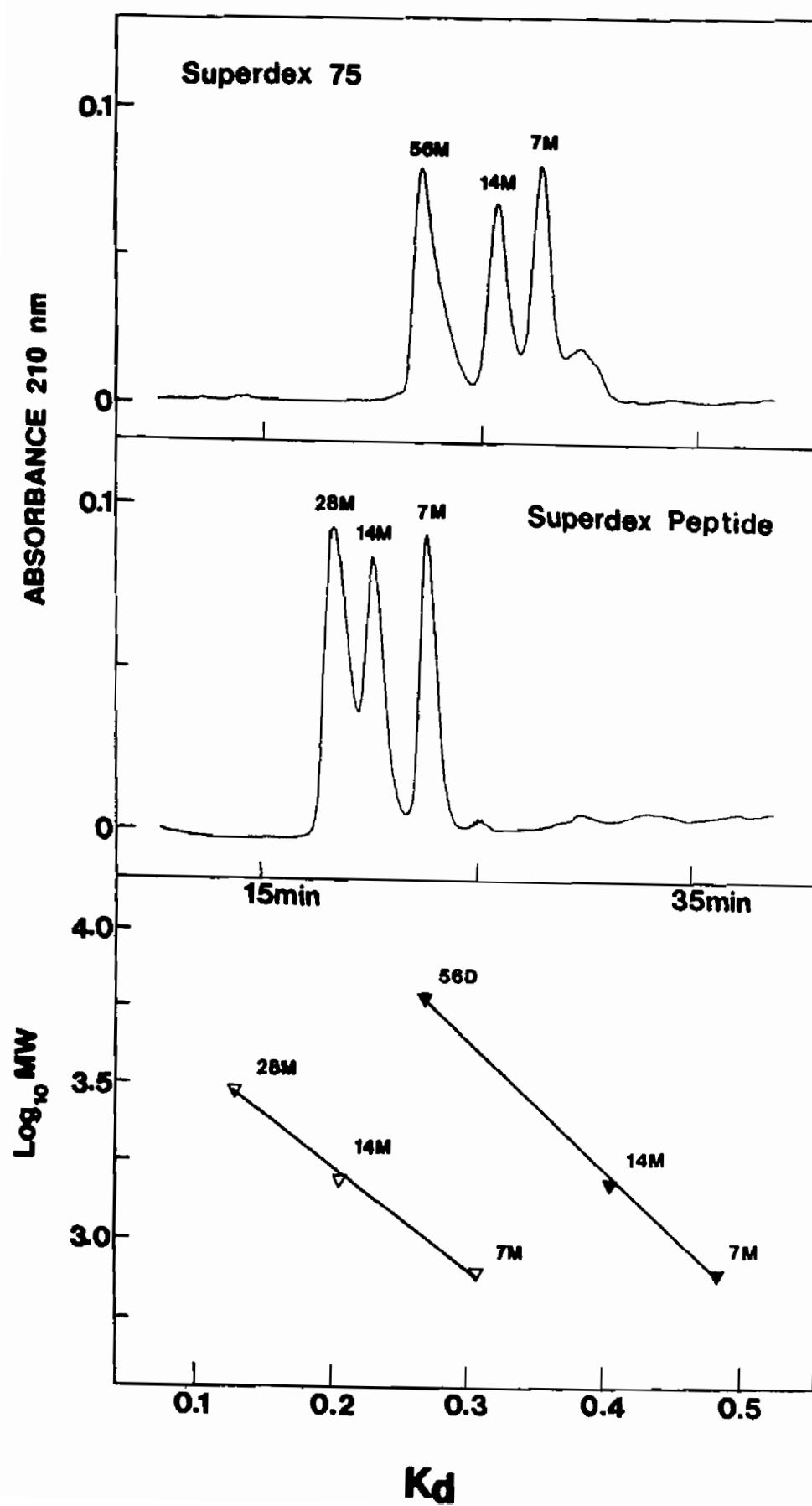


Peptide Conformation Calibration Standards

There is an increasing awareness of the value of SEC for monitoring the effect of environment on protein oligomerization state. Indeed, high-performance SEC has proved to be a particularly rapid and facile approach to monitoring the oligomerization state of α -helical coiled-coils, an important protein structural motif that includes two-stranded and multiple-stranded (or multiple-helix) coiled-coils [65–68,79,80]. Unlike the size exclusion standards described previously, which were designed to exhibit negligible secondary structure under any mobile phase conditions employed, a series of synthetic model amphipathic α -helical peptide standards was introduced, designed as peptide conformation calibration standards for SEC as well as monitors of the effects of mobile phase conditions on the oligomeric states of α -helical coiled-coil peptides [16]. The sequence of the standards is Ac-[K-L-E-A-L-E-A]_n-amide, where $n = 1$ (7 residues), $n = 2$ (14 residues), or $n = 4$ (28 residues); note that only the 28-residue peptide is long enough to form a two (or more) stranded α -helical coiled-coil; the smaller standards are always in their monomeric forms.

The value of these standards was reported by Mant et al. [16], who demonstrated that the 28-residue peptide standard was eluted from a Pharmacia Superdex 75 column as a trimer (three-stranded α -helical coiled-coil) under nondenaturing conditions, as a dimer (two-stranded α -helical coiled-coil) under mildly denaturing conditions, and in its monomeric form under highly denaturing conditions. Figure 4 compares the elution behavior of the peptide standards on a Pharmacia Superdex 75 column (top) and a Pharmacia Superdex Peptide column (middle) in the presence of 30% trifluoroethanol. The lower panel of Fig. 4 compares the $\log_{10}$ MW versus K_d plots for the two columns. Under these conditions, the 28-residue standard is eluted as a dimeric coiled-coil (denoted 56 D in Fig. 4) on the Superdex 75; in contrast, this peptide is eluted in its monomeric form (denoted 28 M in Fig. 4) on the Superdex Peptide column, suggesting that the column matrix influences the oligomeric state of the 28-residue standard. Thus, in addition to sensitivity to mobile phase variations, the value of these calibration standards extends to sensitivity of the 28-residue peptide to the particular SEC packing employed for its elution.

FIGURE 3 Nonspecific interactions in SEC. Column: SynChropak GPC 60 (300 × 7.8 mm ID; SynChrom, Lafayette, IN); Mobile phase: 0.1% aqueous TFA containing either 10 mM KCl (A), 50 mM KCl (B), or 200 mM KCl (C); flow rate, 1 mL/min; temperature, 26°C. Peptides 1, 2, and 5 are synthetic peptide standards containing 10, 20, and 50 residues, respectively (sequences shown in Table 1). The arrow denotes the elution time for the total permeation volume of the column.



Selected Peptide Separations in SEC

Figure 5 shows the excellent resolution of a mixture of six small peptides (ranging from ~450 to 2200 daltons) on a Pharmacia Superdex Peptide column, a column introduced in an effort to meet the needs of peptide separations over a separation range of 100–7000 daltons [16]. This result, achieved in a nondenaturing mobile phase, is a clear illustration of the potential of this column for small peptide separations. It is also worth noting that both the Superdex 75 (Fig. 4) and Superdex Peptide (Figs. 4 and 5) columns required salt (100 mM KCl in 50 mM phosphate buffer) in the mobile phase to suppress nonideal peptide elution behavior on these non-silica-based packings. The resolving capability of the Superdex Peptide column should be even more advantageous for purification of complex peptide mixtures by a multistep process (see earlier). Thus, an initial fractionation of such a mixture by the Superdex Peptide column should simplify considerably subsequent IEC and/or RPC steps.

Figure 6 is an excellent example of a protein digest requiring denaturing conditions for predictable elution behavior of subsequent peptide fragments. Thus, Mant et al. [37] could obtain a linear $\log_{10}$ MW versus peptide retention time relationship for myoglobin and its cyanogen bromide fragments (2500–17,000 daltons), together with the five peptide standards described previously (Fig. 2), only under highly denaturing conditions (50 mM KH_2PO_4 , pH 6.5, 0.5 M KCl, 8 M urea) on both a TSKG2000SW silica-based column (Fig. 6) and a non-silica-based Pharmacia Superose 12 column. It is important to note that, although the five peptide standards were previously shown to exhibit ideal SEC behavior on the G2000SW column under nondenaturing conditions (Fig. 2), the myoglobin fragments clearly exhibited nonideal behavior in the absence of 8 M urea.

FIGURE 4 SEC of a mixture of synthetic peptide conformation standards. Columns: Superdex 75 HR 10/30 (300 × 10 mm ID) and Superdex Peptide HR 10/30 (300 × 10 mm ID), both columns from Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). Mobile phase: 50 mM aqueous KH_2PO_4 , containing 100 mM KCl and 30% trifluoroethanol (TFE), pH 7.0; flow rate, 0.5 mL/min; temperature, 25°C. The synthetic peptide mixture consisted of three peptides with the sequence $\text{Ac}-(\text{K-L-E-A-L-E-A})_n\text{-amide}$, where $n = 1, 2, 4$, with 7 residues (denoted 7 M, i.e., 7-residue monomer), 14 residues (denoted 14 M, i.e., 14-residue monomer), or 28 residues (denoted 28 M or 56 D, i.e., 28-residue monomer or 56-residue dimer, respectively). The bottom panel compares the resulting $\log_{10}$ MW versus K_d relationship on the two columns. The K_d values were calculated from the expression $K_d = V_e - V_0 / V_t - V_0$, where V_e is the elution volume of the solute, V_0 is the void volume of the packing (obtained from the elution time of blue dextran), and V_t is the accessible volume of the column (obtained from the elution time of β -mercaptoethanol). (From Ref. 16.)

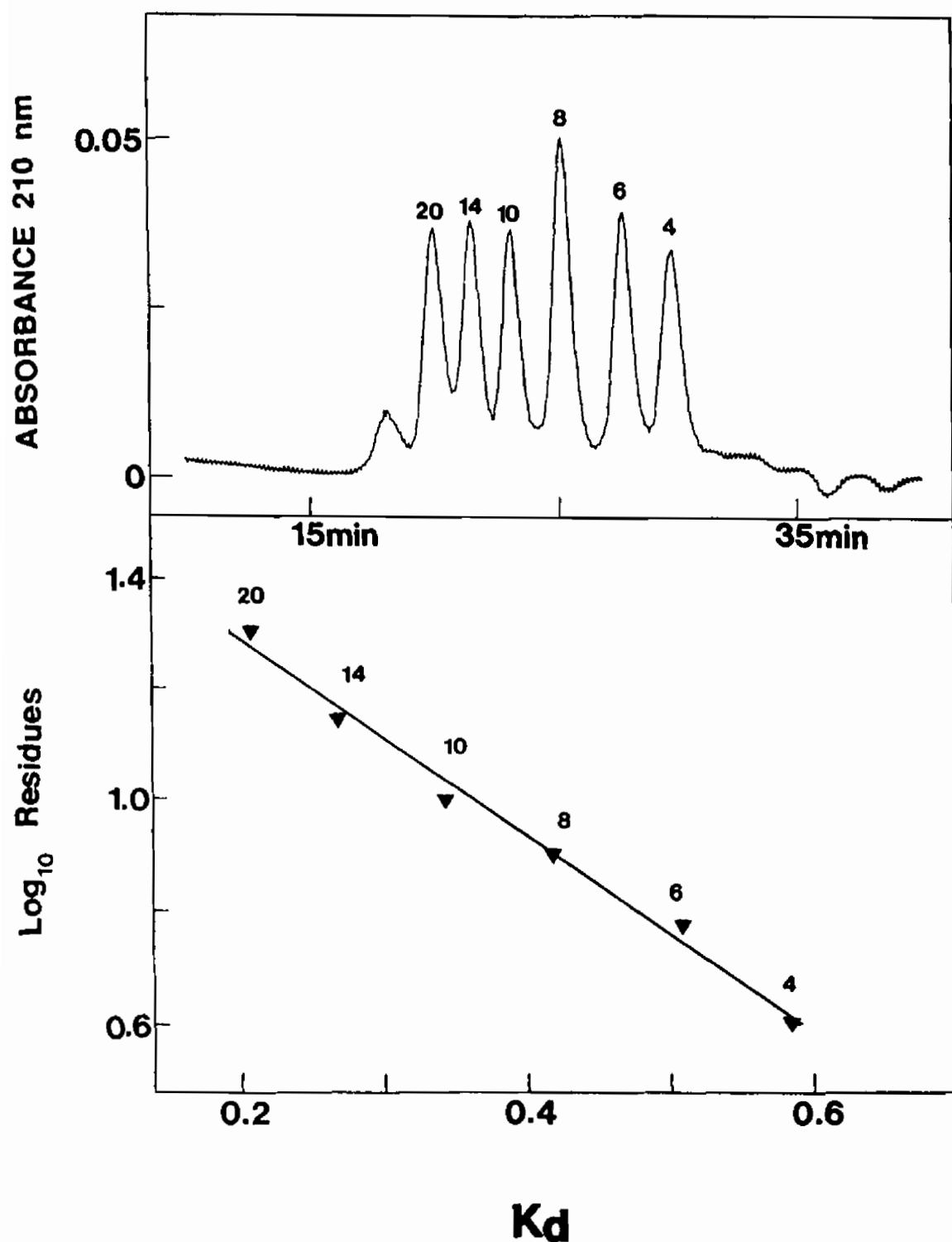


FIGURE 5 SEC of small peptides. Column: Superdex Peptide (see Fig. 4). Mobile phase: 50 mM aqueous KH_2PO_4 , containing 100 mM KCl, pH 7.0; temperature, 25°C. The bottom panels shows the resulting $\log_{10}$ number of residues versus K_d relationship (K_d values were calculated as shown in Fig. 4). The numbers above the peaks in the elution profile and on the bottom plot denote the number of residues in the peptides (sequences shown in Table 1) (From Ref. 16.)

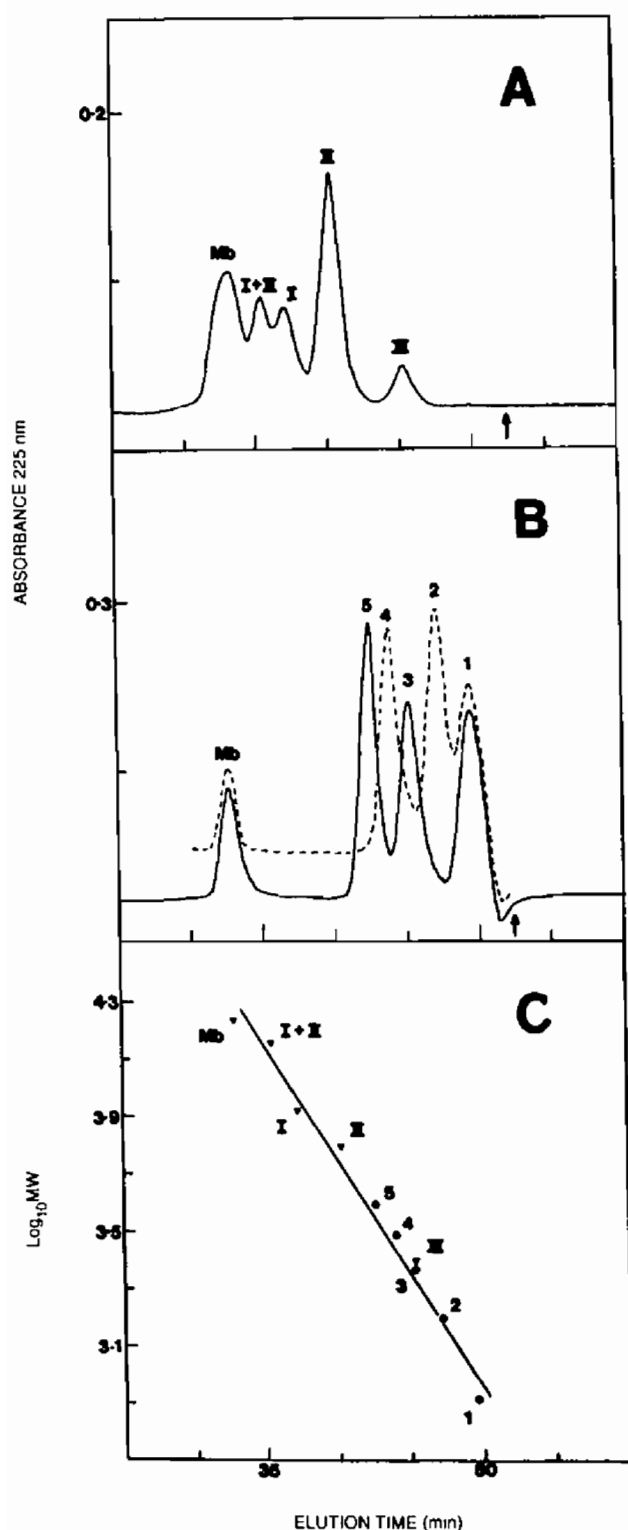


FIGURE 6 Ideal SEC of myoglobin fragments and a mixture of synthetic peptide standards. Column: same as Fig. 2. Mobile phase: 50 mM KH_2PO_4 , containing 500 mM KCl and 8 M urea, pH 6.5; flow rate, 0.2 mL/min; temperature, 26°C. (A) Elution profile of horse heart myoglobin (Mb) and its cyanogen bromide cleavage fragments (I, II, I + II, III); (B) elution profile of horse heart Mb and synthetic peptide standards 1–5 (sequences of standards are shown in Table 1); (C) plot of $\log_{10}$ MW versus elution time of Mb, cyanogen bromide fragments of Mb, and the five synthetic peptide standards. (From Ref. 37.)

ION-EXCHANGE CHROMATOGRAPHY (IEC)

Mobile Phase Selection

Although high-performance IEC has become increasingly popular for the analysis of proteins, less attention has perhaps been paid to the separation of peptides, due mainly to the availability of reversed-phase sorbents for such purposes and the necessity of sample desalting prior to further analysis. However, reversed-phase chromatography (RPC) and IEC are often complementary; i.e., their combined use can provide optimal separation of a peptide mixture or assess the purity of a peptide preparation. Both anion-exchange (AEC) [38,45,62,81–88] and cation-exchange (CEC) [38,41,45,60,61,89–92] columns have proved useful in peptide separations. Common anion-exchange packings consist of primary, secondary, and tertiary (weak AEC) or quaternary amine (strong AEC) functionalities adsorbed or covalently bound to a support; common cation-exchange packings consist of carboxyl (weak CEC) or sulfonate (strong CEC) functionalities bound to a support matrix.

The retention time of a peptide in either ion-exchange mode will depend on a number of factors including buffer pH and the nature and ionic strength of the anion or cation employed for displacement of acidic or basic peptides, respectively. Most ion-exchange separations are carried out using sodium or potassium ion as the cationic counterion and chloride ion as the anionic counterion. It has been suggested that continual use of high concentrations of halide ions may have a deleterious effect on stainless steel surfaces [93] and acetate is occasionally the anionic counterion of choice [84,93–95]. Other anions employed for specific peptide separations have included bicarbonate, borate, and formate [87,90,96]. Volatile pyridine–acetic acid buffers have also been used for resolution of peptide mixtures on cation-exchange and anion-exchange columns [82,97,98]. However, UV spectrophotometry below 250 nm is precluded by the use of pyridine.

Peptides may be removed from an ion-exchange sorbent by either gradient or isocratic elution. Linear gradient elution is generally the elution mode of choice when attempting to separate mixtures of peptides with a wide range of net charges. Gradient elution of peptides is usually performed with salt gradients of either sodium or potassium chloride in phosphate, tris, or citrate mobile phase buffers. Linear ionic strength gradients of buffer (0.05–0.5 M phosphate buffer) are also sometimes employed [81,83,93,99]. The authors' experience suggests that a linear sodium chloride gradient in 5–50 mM KH_2PO_4 buffer, pH 6.5 (AEC or CEC) or pH 3.0 (strong CEC), constitutes a suitable elution condition for most separations. In addition, the addition of 10–15% (v/v) of an organic modifier such as acetonitrile is recommended to suppress any undesirable hydrophobic interactions with the column (see later under "Peptide Standards"). Care should be taken in the choice of ionic strength of the starting buffer. If it is too high, weakly acidic or basic peptides that may otherwise be retained by anion-exchange

or cation-exchange sorbents, respectively, may be eluted with unretained compounds. Flow rates of 0.5–2.0 mL/min are favored for analytical ion-exchange separations.

The choice of gradient rate (increasing concentration of counterion per unit time) will be dictated by the complexity and charge distribution of the peptide mixture to be resolved, but an increase of 5–20 mM salt/min is suitable for most analytical purposes. The optimum separation of two peptides by gradient elution on anion- or cation-exchange sorbents will be obtained when there is a net charge difference of at least one unit between the two peptides. An advantage of peptide over protein separations in IEC lies in the fact that all charged groups in peptides are generally available to interact with the sorbent even under benign conditions. The tendency for proteins under benign conditions to assume tertiary structures causes shielding of charged residues so that only a portion of the protein surface interacts with the sorbent. Hence, the overall net charge on the protein will not be fully expressed unless highly denaturing conditions are employed. In contrast, peptides (<50 residues) are less prone to conformational masking of charged residues and their overall net charge is fully expressed under milder conditions. This was well demonstrated by Mant and Hodges [85], who showed that under benign conditions [linear KCl gradient (5 mM/min) in 5 mM KH_2PO_4 buffer] the elution times of several basic peptides (9–21 residues) on a strong cation-exchange column (SynChropak S300) were linear with respect to their net charge at pH 6.5. The same linearity was apparent when chromatographing the peptides at pH 3.0. Under these conditions, acidic residues are protonated and only the charge on the basic residues is expressed. Similar results have been obtained in our laboratory for a series of acidic peptides (–2 to –5 net charge) chromatographed on a strong anion-exchange column (SynChropak Q300) under conditions (pH 6.5) identical to those described for the S300 column. These results may be useful in correlating the overall charge of a peptide at a given pH with its amino acid composition.

A somewhat different form of gradient elution was described by Disdaroglu [84] in some interesting separations of acidic, neutral, and mildly basic peptides on a weak difunctional anion exchanger (MicroPak AX-10). Peptides are usually not retained well on this anion exchanger using aqueous buffers as mobile phases. This researcher employed mixtures of triethylammonium acetate (TEAA) and acetonitrile as the eluent. Acetonitrile promotes ionic interaction of peptides with the weak anion-exchange sorbent and peptides are eluted only with increasing concentration of TEAA in the mobile phase. The addition of acetonitrile to aqueous mobile phase buffers is also a feature of several more conventional ion-exchange separations [41,89,90].

Although less common, a pH gradient instead of the usual salt gradient may be used for the separation of peptides [83,90]. As the pH is linearly increased or decreased, the overall charge on a peptide will change as ionizable groups are

protonated or deprotonated, leading to differential elution of peptides depending on their overall acidic or basic residue composition. Although pH gradients are by necessity generally limited to a range of pH 2–8 on silica-based packings, this elution method may find more utility on the newer polymer-based cation-exchange and anion-exchange packings. The stability of these packings over a wide acidic and basic pH range presents the potential to employ pH gradients from conditions ensuring protonation of acidic side chains (<4.0) to those producing deprotonation of basic side chains (>9.0).

Optimal separation conditions for isocratic elution of peptides are generally more difficult to develop than those employed in gradient elution. However, isocratic elution has found use in separating peptides of similar net charge and, hence, poorly resolved by gradient elution. Cachia et al. [99] employed isocratic elution at pH 6.5 (50 mM KH_2PO_4 containing 0.3 or 0.4 M KCl) for almost complete resolution of two basic peptides of identical net charge (6+) on a weak cation-exchange column (SynChropak CM300). The ability to resolve the two peptides was most likely due to pK_a differences associated with different positively charged residues. The TEAA-acetonitrile systems of Disdaroglu [84] also produced several good isocratic separations of dipeptides and other larger, closely related peptides on the MicroPak AX-10 weak anion exchanger.

The high loading capacity of ion-exchange preparative columns can be extremely useful for preparative purification of peptides. However, the problem of subsequent sample desalting is a drawback of this technique if it is the sole method of purification. If RPC is used following IEC, the sample can be desalted in addition to providing a second mode of purification.

If a choice must be made concerning the type of ion-exchange column for general peptide applications, the researcher should purchase a strong cation-exchange column. The utility of such a column lies in its ability to retain its negatively charged character in the acidic to neutral pH range. Most peptides are soluble at low pH, where the side-chain carboxyl groups of acidic residues (glutamic acid and aspartic acid) and the free C-terminal α -carboxyl group are protonated (i.e., uncharged), thereby emphasizing any basic, positively charged character of the peptides. In addition, the use of acidic eluents helps to extend the lifetime of silica-based packings, still the most widely used material for peptide separations by IEC.

Peptide Standards

The value of peptide standards in IEC lies in allowing a quick comparison of the performance characteristics of different ion-exchange columns or assessment of the performance characteristics of a specific ion-exchange column. Thus, standards will confirm that the column can, indeed, retain charged species (the lower the net charge on the peptide that can be retained, the more versatile the column)

and standards can confirm that a particular mobile phase will elute charged species from an ion-exchange column. Standards can be used to assess the effect of pH variations on the resolving capability and load capacity of an ion-exchange column—a particularly important concern in strong cation-exchange chromatography, where the manipulation of mobile phases throughout the acidic to neutral pH range is frequently employed for peptide separations; finally, standards can be used to determine the presence of any nonspecific, i.e., hydrophobic, characteristics of a column packing.

A series of synthetic 11-residue cation-exchange peptide standards has proved to be extremely useful in satisfying the preceding criteria for both weak and strong cation-exchange packings [38,41,45,100]. The sequences of the standards are Ac-G-G-G-L-G-G-A-G-G-L-K-amide (denoted C1; +1 net charge), Ac-K-Y-G-L-G-G-A-G-G-L-K-amide (C2; +2 net charge), Ac-G-G-A-L-K-A-L-K-G-L-K-amide (C3; +3 net charge), and Ac-K-Y-A-L-K-A-L-K-G-L-K-amide (C4; +4 net charge). IEC, in conjunction with RPC, has proved useful during peptide mapping of protein digests; therefore, the 11-residue length of the standards was designed to approximate the average length of cleavage fragments from proteolytic (e.g., tryptic) digests of proteins. The uniform length of the peptides ensured that any potential chain length effect on peptide retention, if present, would be constant for all four peptide standards. Peptides C1–C4 contain, respectively, 1–4 basic residues (lysine) and no acidic residues; thus, over the pH range used for the majority of cation-exchange separations [pH 3.0 to 7.0 (strong CEC) or pH 4.5 to 7.0 (weak CEC)], the net charges of +1 to +4 for peptides C1–C4, respectively, do not change. In addition, the hydrophobicity of the standards increases from C1 to C4, with a concomitant increase in peptide sensitivity to potential hydrophobic-solute/packing interactions.

Monitoring the Effect of pH on Strong CEC Packings

Figure 7 shows the gradient elution chromatograms at pH 6.5 (top) and pH 3.0 (bottom) of peptide standards C1–C4 on a silica-based strong cation-exchange column. There was an initial 5-min isocratic elution with the starting buffer prior to the start of the gradient. At pH 6.5 (Fig. 7, top), standards C2, C3, and C4 (+2, +3, and +4 net charge, respectively) were removed by gradient elution, whereas peptide C1 (+1 net charge) was eluted during the initial isocratic elution. In contrast, at pH 3.0 (Fig. 7, bottom), only peptides C3 and C4 were eluted by the gradient, whereas both peptides C1 and C2 were eluted during the initial isocratic elution. In addition, the retention times of peptides C3 and C4 were reduced considerably. Ideally, there should have been no change in elution time of these peptides with a change in buffer pH. The observed effects apparently resulted from an unexpected reduction in the capacity of the column to retain charged species as the eluent became more acidic. This phenomenon has been detected to a greater or lesser extent on several of the silica-based columns in the authors'

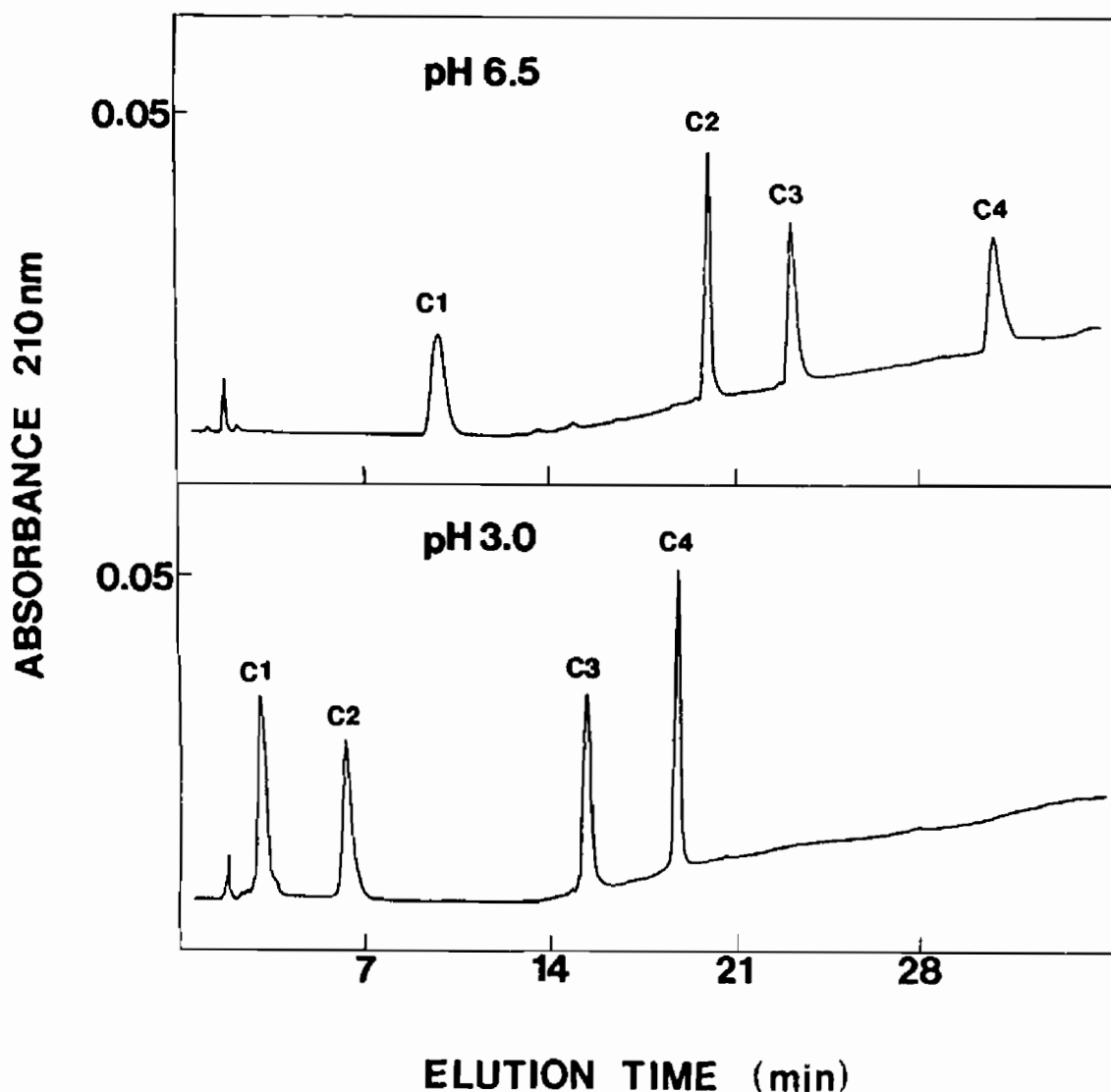


FIGURE 7 Monitoring effect of pH on a strong cation-exchange column with synthetic peptide standards. Column: SynChropak S300 (250 × 4.1 mm ID, 6.5 μm particle size, 300 Å pore size; SynChrom (Lafayette, IN). Mobile phase: linear AB gradient, where buffer A is 5 mM aqueous KH_2PO_4 [pH 6.5 (top) or pH 3.0 (bottom)] and buffer B is buffer A plus 1 M NaCl; gradient rate, 20 mM NaCl/min following 5-min isocratic elution with buffer A; flow rate, 1 mL/min; temperature, 26°C. Peptide standards C1, C2, C3, and C4 (see Table 1 for sequences) contain net charges of +1, +2, +3, and +4, respectively. (From Ref. 38.)

hands. However, a Mono S polymer-based strong cation-exchange column from Pharmacia has exhibited no such reduction in binding capacity at low pH. Protonation of the carboxymethyl functional groups of a weak cation-exchange column as the pH was lowered decreased the binding capacity of the sorbent, as illustrated by progressively decreasing retention times of the standards with

decreasing pH, as fewer carboxylate groups on the sorbent were available for binding [99].

Anion-exchange sorbents, too, may exhibit differential binding capacities at different pH values. Gooding and Schmuck [101] demonstrated that the quaternized support of a strong anion-exchange column (SynChropak Q300) yielded essentially unchanged retention times of proteins with decreasing pH. However, a weak anion exchanger (SynChropak AX300) demonstrated increasing protein elution times with decreasing pH. Although the cross-linked polyethyleneimine polymer adsorbed to the porous silica of this packing chromatographically behaved as if it were composed of tertiary amines, it also contained some primary and secondary amines. Increasing ionization of the sorbent with decreasing pH increased the basic character of the column. A series of acidic peptide standards, with no basic residues present, would accurately monitor such pH-influenced properties of anion-exchange columns. Such peptide standards, analogous to the preceding cation-exchange standards, have been designed, synthesized, and successfully tested in our laboratory.

Monitoring Effects of Hydrophobic Characteristics of CEC Packings

Figure 8 shows elution profiles of the peptide standards obtained on a Poly-Sulfoethyl Aspartamide column (column A) and a Mono S column (column B) at pH 6.5. The inclusion of 5% (top profiles) or 10% (bottom profiles) of acetonitrile (v/v) in the mobile phase buffers was found to be necessary to elute the four peptides from both columns within a reasonable time and with a reasonable peak shape. In the absence of acetonitrile, neither peptide C3 nor C4 (+3 and +4 net charge, respectively) were seen to be eluted from the two columns, but C2 (+2 net charge) was eluted as a broad, skewed peak. The improvement in peptide elution profiles of the four standards on the two columns on addition of acetonitrile to the mobile phase indicated that, in its absence, both column packings were exhibiting hydrophobic in addition to ionic characteristics. In the presence of 5% acetonitrile (Fig. 8, top profiles), all four peptide standards were eluted from the two columns in a similar time (~ 32 min). However, an increase in acetonitrile concentration to 10% in the mobile phase (bottom panels) substantially improved the peak shape of the peptides, particularly the more hydrophobic C3 and C4 (+3 and +4 net charge, respectively). In addition, peptide retention times, particularly those of C3 and C4, generally decreased with increasing acetonitrile concentration. The improvement in peak shape of the peptides on the Mono S continued with a further increase in acetonitrile concentration to 40% (data not shown).

From Fig. 8 (column A, bottom profile; PolySulfoethyl Aspartamide), it is apparent that, when hydrophobic interactions were effectively suppressed, the retention times of the peptide standards increased linearly with increasing net

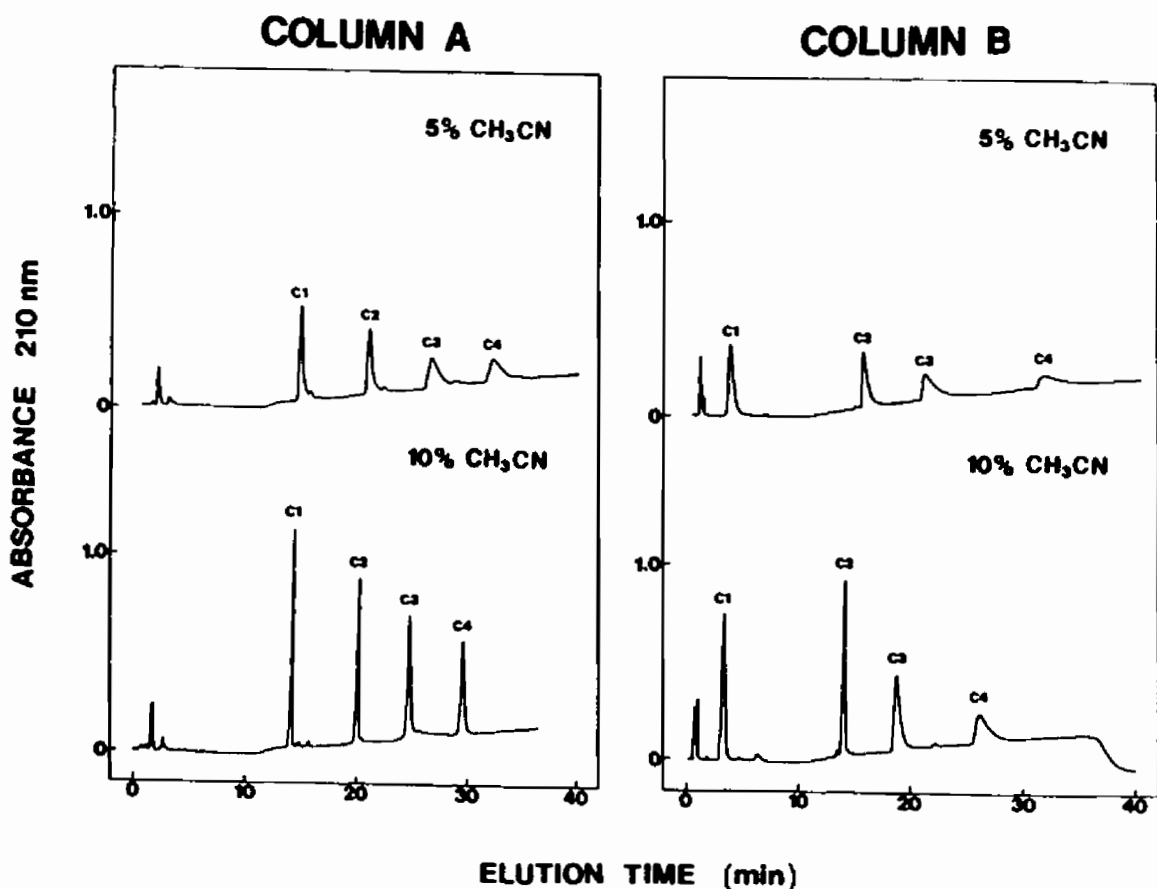


FIGURE 8 Monitoring effect of nonspecific, hydrophobic interactions on strong CEC columns: Columns: A, PolySulfoethyl Aspartamide (200 × 4.6 mm ID, 5 μ m particle size, 300 Å pore size; PolyLC, Columbia, MD); B, Mono S HR 5/5 (50 × 5 mm ID, 10 μ m; Pharmacia, Dorval, Canada). Mobile phase: linear AB gradient, where buffer A is 5 mM aqueous KH_2PO_4 , pH 6.5, and buffer B is buffer A plus 1 M NaCl, both buffers containing 5% (top) or 10% (bottom) acetonitrile (v/v); gradient rate, 20 mM NaCl/min, following 5-min isocratic elution with buffer A; flow rate, 1 mL/min; temperature, 26°C. The sequences of peptide standards C1, C2, C3, and C4 (+1, +2, +3, and +4 net charge, respectively) are shown in Table 1.

charge. Thus, the differences in retention times for adjacent peptide peaks (eluted by the gradient and not during the initial isocratic elution with buffer A) were essentially equal. The greater difference in retention time between C3 and C4 compared with between C2 and C3 on the S300 column at pH 6.5 (Fig. 7, top) indicated the presence of hydrophobic interactions even though all four peptides were removed from the column in the absence of acetonitrile in the mobile phase. This was confirmed when, following addition of acetonitrile to the mobile phase, standards C2, C3, and C4 were eluted in a linear fashion. Interestingly, the results of Fig. 7 suggest that, of the three columns (Figs. 7 and 8), the silica-based S300

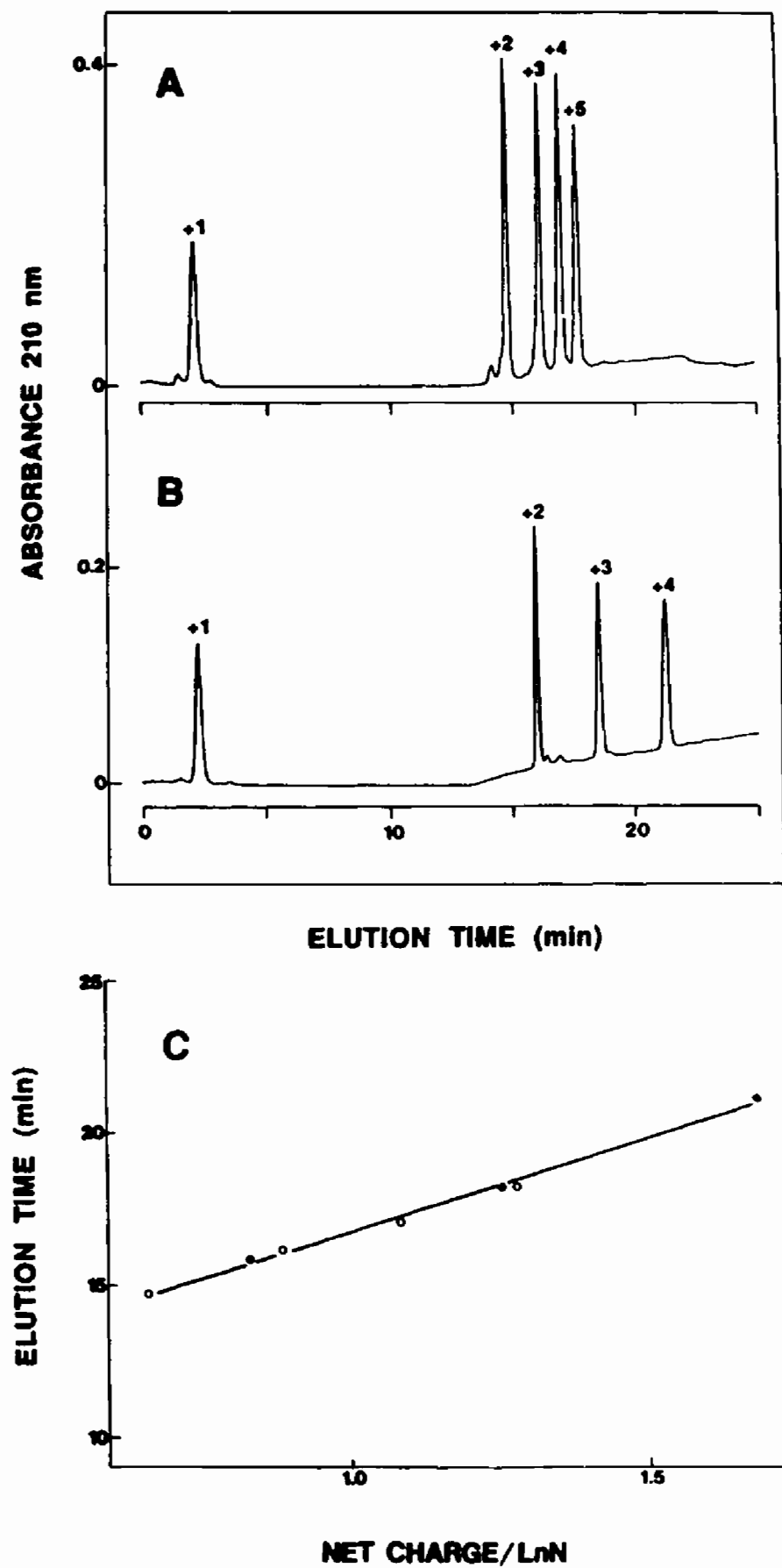
packing exhibited a less hydrophobic character than the silica-based PolySulfoethyl Aspartamide packing or the polymer-based Mono S because all four peptide standards were eluted from the S300 in the absence of acetonitrile.

The results shown in Figs. 7 and 8 not only demonstrate the utility of the cation-exchange standards in assessing column performance but also indicate the necessity of a routine addition of low levels of an organic modifier such as acetonitrile to the mobile phase buffers to ensure ideal IEC peptide elution behavior.

Monitoring Effect of Polypeptide Chain Length and Charge Density on Peptide Elution Behavior During Strong Cation-Exchange Chromatography

One aspect of IEC of peptide mixtures that is frequently overlooked is the potential effect of factors such as polypeptide chain length and charge density on peptide elution behavior. As demonstrated by Burke et al. [41], once any nonspecific interactions with an IEC packing have been suppressed, chain length and charge density must be taken into account when attempting to predict/rationalize peptide retention times. Figure 9A shows the strong cation-exchange elution profile of the five size exclusion peptide standards shown previously in Fig. 2, with chain lengths of 10, 20, 30, 40, and 50 residues (+1, +2, +3, +4, +5 net charge, respectively); Fig. 9B shows the elution profile of the four cation-exchange 11-residue peptide standards C1 to C4 (+1 to +4 net charge, respectively). Mobile phase conditions were designed to ensure the suppression of any nonideal behavior on this non-silica-based Mono S column, hence the addition of 40% acetonitrile (v/v) to the mobile phase buffers. From Fig. 9, it is clear that similarly charged species are not necessarily eluted at similar times. For instance, the 50-residue peptide (+5 net charge; panel A) was not retained as long as 11-residue peptides with net charges of +3 and +4 (panel B); similarly, the 40-residue peptide (+4 net charge; panel A) was eluted prior to an 11-residue peptide with a net charge of +3 (panel B). The two series of peptide standards differ significantly in their range of both peptide chain length (10–50 residues versus 11 residues; panels A and B, respectively) and charge density (+1 net charge per 10 residues for the peptides in panel A versus +1 to +4 net charge per 11 residues for the peptides in panel B).

Such results can be rationalized [41] as a linear relationship between peptide elution time and net charge/ $\ln N$ (Fig. 9C), where N is the number of residues the peptide contains. Such linearization of peptide retention behavior in IEC is useful in correlating the overall net charge on a peptide at a given pH with its amino acid composition. It should be noted that the simple relationship between peptide elution time and net positive charge during strong cation-exchange chromatography reported by Mant and Hodges [85] and Crimmins



et al. [102], i.e., no correction for peptide chain length, was probably due to the limited range of chain length of the peptide mixtures utilized: 12–21 residues and 7–13 residues, respectively. The study by Burke et al. [41] suggested that the polypeptide chain length effect becomes significant for predictive concerns only when the peptides are >20 residues in length.

HYDROPHILIC INTERACTION/CATION-EXCHANGE CHROMATOGRAPHY (HILIC/CEC)

General Principles

The term hydrophilic interaction chromatography (HILIC) was originally coined to describe separations based on solute hydrophilicity [103]. Thus, separation by HILIC, in a manner similar to normal-phase chromatography (to which it is related), depends on hydrophilic interactions between the solutes and a hydrophilic stationary phase; i.e., solutes are eluted in order of increasing overall hydrophilicity (decreasing hydrophobicity). Characteristic of HILIC separations is the presence of a high initial organic modifier concentration to promote hydrophilic interactions between the solute and the hydrophilic stationary phase.

The major process governing peptide retention behavior on ion-exchange columns involves ionic interactions between the column matrix and the peptide solutes; however, in our hands, all such columns have also exhibited some hydrophobic character, leading to long peptide retention times and peak broadening [41]. Although most researchers prefer to avoid separations based on such mixed-mode ionic-hydrophobic column behavior by adding an organic solvent, such as acetonitrile, to the mobile phase buffer, this laboratory has demonstrated that manipulation of the acetonitrile concentration enables considerable flexibility

FIGURE 9 Effect of peptide chain length and charge density during strong CEC of synthetic peptides. Column: Mono S (see Fig. 8). Mobile phase, linear AB gradient, where buffer A is 5 mM aqueous KH_2PO_4 , pH 6.5, and buffer B is buffer A plus 500 mM NaCl, both buffers containing 40% acetonitrile (v/v); gradient rate, 20 mM NaCl/min, following 10-min isocratic elution with buffer A; flow rate, 1 mL/min; temperature 20°C. (A) Mixture of five synthetic SEC standards (10, 20, 30, 40, and 50 residues; +1, +2, +3, +4, and +5 net charge, respectively; sequences shown in Table 1); (B) mixture of four synthetic 11-residue CEC standards (sequences shown in Table 1; denoted C1, C2, C3, and C4 for +1, +2, +3, and +4 net charge, respectively); (C) plot of observed peptide elution time versus peptide net charge, divided by the logarithm of the number of residues (net charge/ $\ln N$). (From Ref. 38.)

in the separation of basic (potentially positively charged) peptides on a strong cation-exchange column [11–15,23,45].

Concerning the aforementioned matrix hydrophobicity, it should be noted that different ion-exchange matrices exhibit differing degrees of hydrophobic characteristics [41]. In order to gain the full benefit of peptide separations by the HILIC mode, it is important to overcome unwanted hydrophobic properties of the matrix with as low a level of organic modifier (acetonitrile) as possible; i.e., the ion-exchange matrix should be as hydrophilic as possible. In this way, there is a greater organic modifier range open to the researcher to effect mixed-mode HILIC/CEC peptide separations.

Mobile Phase Selection

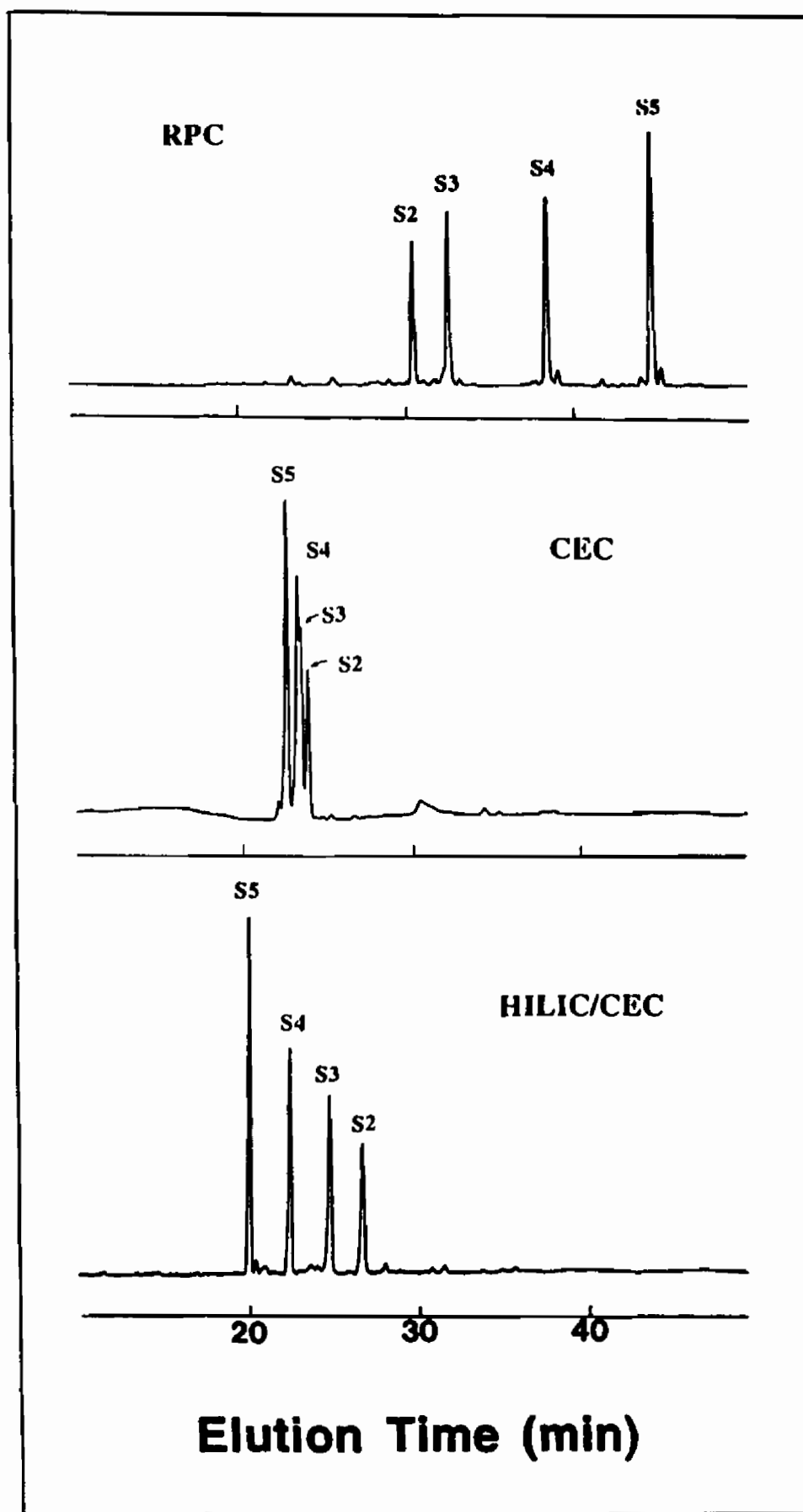
Typical conditions for mixed-mode HILIC/CEC are a linear increasing NaClO_4 gradient (2–20 mM $\text{NaClO}_4/\text{min}$) at pH 3, with both mobile phase buffers containing 15–80% acetonitrile. Thus, the cation-exchange column separates peptides based on net positive charge and this separation mode is overlaid by the presence of acetonitrile overcoming undesirable hydrophobic interactions with the column while promoting favorable hydrophilic interactions. NaClO_4 is suitable for this mixed-mode approach due to its excellent solubility characteristics in aqueous solution even in the presence of high concentrations of organic modifier [12,104]. The choice of a relatively low pH is governed by the desire to maximize the basic character of the peptide solutes to enhance ionic interactions with the negatively charged strong-cation-exchange matrix. Thus, at pH 3, any acidic (potentially negatively charged) residues (Asp, Glu) will be mainly protonated, i.e., uncharged. In addition, a full positive charge on the basic residue His ($\text{p}K_a = 6.5$) is assured at low pH. Less obvious, perhaps, is the need to be cautious with the pH of the mobile phase when considering basic residues such as Lys ($\text{p}K_a \approx 10$) and Arg ($\text{p}K_a \approx 12$). Through the use of synthetic peptide models, Sereda et al. [28] demonstrated that the hydrophobic environment characteristic of RPC (hydrophobic packings and organic modifiers) had a profound effect on the $\text{p}K_a$ values of ionizable groups, these values being decreased to 5.8, 7.4, and 7.3 for His, Lys, and Arg, respectively. Although an ion-exchange matrix is clearly far less hydrophobic than a reversed-phase packing, it was felt that the presence of high concentrations of acetonitrile (up to 90% characteristic in HILIC/CEC mobile phases) generally necessitates the use of relatively low pH conditions to ensure full protonation (i.e., a full positive charge) of basic side chains. As a side benefit, silica-based ion-exchange columns tend to be more stable over a period of time if utilized at pH 3 compared with pH values around neutrality.

The general principles of HILIC/CEC are demonstrated in Fig. 10 with a comparison of the separation of synthetic peptide RPC standards S2–S5 by RPC (top), CEC (middle), and HILIC/CEC (bottom). Because of their lack of secondary structure, these peptides were useful to demonstrate the basic principles distinguishing RPC from HILIC/CEC. This four-peptide mixture contains peptides with the same net positive charge (+2) and subtly increasing hydrophobicity ($S2 < S5$). Note that the only difference between the CEC and HILIC/CEC runs was the presence of 10% (v/v) acetonitrile in the former and 80% (v/v) in the latter.

From Fig. 10, peptides were, of course, eluted from the RPC column (top) in order of increasing hydrophobicity. Under characteristic cation-exchange conditions (middle) [the presence of 10% (v/v) acetonitrile helps to eliminate unwanted hydrophobic interactions between solutes and the column matrix (see earlier)], the four peptides were very poorly resolved, as expected given the identical net charge on the peptides. Interestingly, the low concentration of acetonitrile (10%) has already induced hydrophilic interactions with the matrix in that the elution order is already opposite to that of RPC (the most hydrophobic peptide was eluted first and the most hydrophilic last). In contrast, under HILIC/CEC conditions (bottom), the elution order remains the same but the peptides are now well resolved. Clearly, to effect a separation of these peptides on the cation-exchange column, an increased concentration (80%, v/v) of acetonitrile was required in the mobile phase in order to promote hydrophilic interactions with the column matrix to complement the ionic interactions.

Figure 11 demonstrates the effect of acetonitrile concentration on the elution behavior of a mixture of 12 peptides (10–11 residues) with negligible secondary structure. With 20% acetonitrile in the mobile phase (Fig. 11A), the overall elution order of all 12 peptides is essentially based on increasing net positive charge ($+1 < +2 < +3 < +4$) with ionic interactions dominating the separation process (the number above the peaks denotes the number of positive charges each peptide contains). At 50% acetonitrile (Fig. 11B), ionic interactions are still dominant because peptides are still generally eluted in order of increasing net positive charge. However, a hydrophilic interaction mechanism also becomes more substantial, as evidenced by the improvement in the separation of peptides of like charge. Also, note the elution of peptide l4 before the less charged (but more hydrophilic) b3 and a3, a clear example of how more highly charged peptides may still be eluted prior to less highly charged peptides if the latter are significantly more hydrophilic than the former. A further increase in acetonitrile concentration to 90% (Fig. 11C) results in the most dramatic change in column selectivity with the hydrophilic interactions now dominating the separation process, producing a much different elution profile than those seen at lower acetonitrile concentrations. For instance, peptide i1 (+1 net charge) is eluted after

Absorbance 210 nm

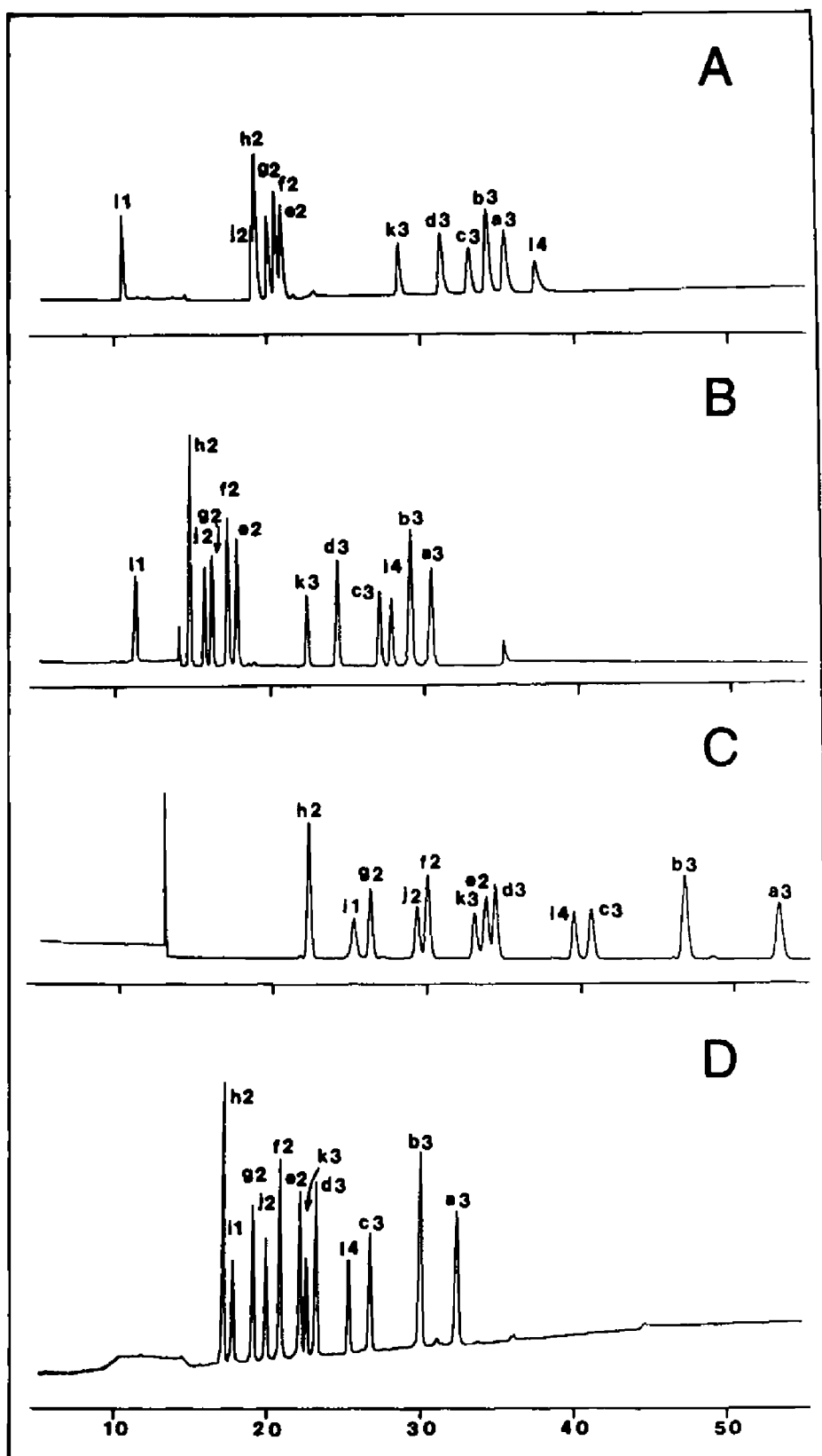


the less hydrophilic h2 (+2 charge); similarly, e2 (+2 net charge) is eluted after the much less hydrophilic k3 and, most dramatically, peptide l4 (+4 net charge) is eluted before the more hydrophilic c3, b3, and a3 (+3 net charge). The flexibility of HILIC/CEC is also demonstrated in Fig. 11D, where a double gradient (increasing salt gradient with concomitant decreasing acetonitrile gradient) is employed to effect the separation, thus maintaining dominant hydrophilic (over ionic) interactions while reducing analysis time (relative to the chromatogram shown in Fig. 11C) but retaining good column selectivity. From Fig. 11D, this is achieved by a lower level of acetonitrile in buffer B (50% as opposed to 90% in buffer A), which leads to a decrease in peptide retention relative to that effected by maintaining 90% acetonitrile in both mobile phase buffers (Fig. 11C). The peptide elution order obtained with this combined salt and acetonitrile gradient is almost identical (save for a reversal of e2 and k3) to that shown in Fig. 11C but is obtained in about two thirds of the time and with sharper peaks.

It should be noted that the double-gradient approach of Fig. 11D is a good starting point when first applying mixed-mode HILIC/CEC to a peptide mixture. Initial run conditions similar to those described for Fig. 11D (except for a lower pH, e.g., pH 3, to ensure the maximum degree of positively charged character of the peptides) are recommended. The observed elution profile may then be optimized by varying gradient rate and/or mobile phase acetonitrile concentration as required. In addition, if acidic (potentially negatively charged) residues are present in the peptides, the pH may be manipulated to modulate the positively charged character of the peptides and, thus, vary the strength of ionic interactions between peptides and the cation-exchange matrix.

FIGURE 10 General principles of RPC versus HILIC/CEC. Columns: Zorbax SB300-C₈ reversed-phase column (150 × 4.6 mm ID, 5-μm particle size, 300-Å pore size; Hewlett-Packard, Little Falls Site, DE); PolySulfoethyl Aspartamide strong cation-exchange column (200 × 4.6 mm ID, 5 μm, 300 Å; PolyLC, Columbia, MD). Mobile phase for RPC: linear AB gradient, where buffer A is 20 mM aqueous triethylammonium phosphate (TEAP), pH 3.0, and buffer B is eluent A containing 60% acetonitrile (v/v), both eluents containing 100 mM NaClO₄; gradient rate, 0.5% acetonitrile/min; flow rate, 1 mL/min; temperature, 30°C. Mobile phase for CEC: linear AB gradient, where buffer A is 20 mM aqueous TEAP, pH 3.0, containing 10% acetonitrile (v/v) and buffer A is buffer B containing 400 mM NaClO₄; gradient rate, 5 mM NaClO₄/min, following 5-min isocratic elution with buffer A; flow rate, 1 mL/min; temperature, 30°C. Mobile phase for HILIC/CEC: same conditions as for CEC, except for 80% acetonitrile (v/v) in both buffers. The sequences of the peptide standards are shown in Table 1. (From Ref. 13.)

Absorbance 210 nm



Elution Time (min)

Selected Peptide Separations in HILIC/CEC

Figure 12 compares RPC with HILIC/CEC for separation of peptide mixtures of highly amphipathic α -helical peptide analogs (left) and amphipathic, cyclic β -sheet peptide analogs (right). For both these peptide mixtures, HILIC/CEC is clearly superior to RPC for resolution of the peptides. For the α -helical peptides (left) during RPC, the identical hydrophobic preferred binding domains of the peptides (the single residue substitutions having been made in the hydrophilic faces of the helices, which are oriented away from the hydrophobic stationary phase) bind to the hydrophobic matrix, resulting in coelution of all four peptides. In contrast, all four peptides are well resolved by HILIC/CEC even though all peptides have the same net positive charge, with the substitution sites in the hydrophilic faces of the peptides able to interact intimately with the cation-exchange matrix and, hence, influence the retention behavior of the four analogs. The elution order is in order of decreasing hydrophobicity with the Leu analog being eluted first followed by the Val, Thr, and Ser analogs. Similarly, for the amphipathic, cyclic β -sheet peptide analogs (right), the hydrophilic faces of the peptides, in which residue substitutions have been made, are able to interact strongly with the cation-exchange packing, thus effecting a separation superior to that with the RPC mode. The arrows denote useful selectivity differences between the RPC and HILIC/CEC runs, highlighting the complementarity of the two approaches.

FIGURE 11 Effect of acetonitrile concentration column selectivity during HILIC/CEC of peptides. Column: PolySulfoethyl Aspartamide strong cation-exchange column (200 $\times$ 4.6 mm ID, 5- μ m particle size, 300-Å pore size; PolyLC, Columbia, MD). Mobile phase: (A–C) linear AB gradient, where buffer A is 5 mM aqueous TEAP, pH 7.0, and buffer B is buffer A plus 250 mM NaClO₄, both buffers containing 20% (A), 50% (B) or 90% (C) acetonitrile (v/v); gradient rate, 5 mM NaClO₄/min; flow rate, 1 mL/min; temperature, 20°C. Mobile phase: (D) linear AB gradient, where buffer A is 5 mM aqueous TEAP, pH 7.0, containing 90% acetonitrile (v/v) and buffer B is 5 mM aqueous TEAP, pH 7.0, containing 250 mM NaClO₄ and 50% acetonitrile (v/v); gradient rate, linear increasing salt gradient of 5 mM NaClO₄ and a linear decreasing acetonitrile gradient of 0.8% acetonitrile/min; flow rate, 1 mL/min; temperature, 26°C. The numbers over the synthetic peptide (10–11 residues each) peaks denote the number of positive charges they contain. Peptides i1, j2, k3, and l4 are the same as cation-exchange standards C1, C2, C3, and C4, respectively (sequences shown in Table 1); peptides e2, f2, g2, and h2 are the same as S2, S3, S4, and S5, respectively (sequences shown in Table 1 and Fig. 16); a3, b3, c3, and d3 are the nonacetylated analogs (i.e., free N-terminal α -amino group) of S2, S3, S4, and S5, respectively. (From Ref. 12.)

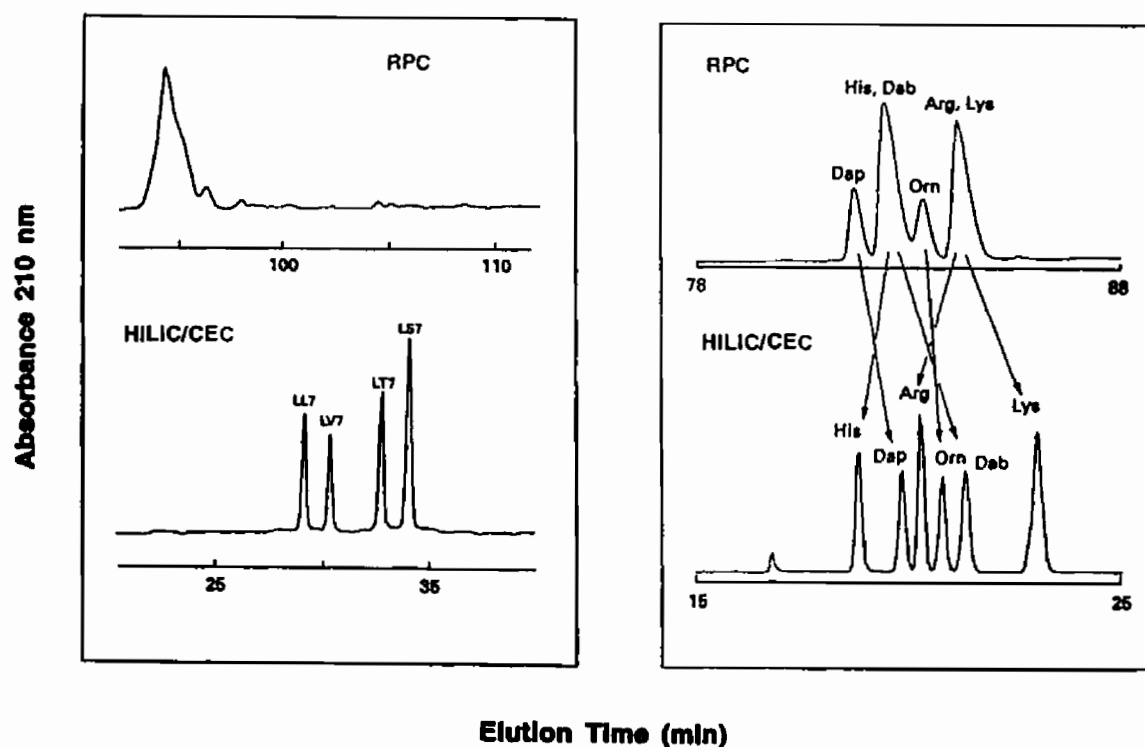
AMPHIPATHIC α -HELICAL PEPTIDESAMPHIPATHIC, CYCLIC β -SHEET PEPTIDES

FIGURE 12 RPC versus HILIC/CEC of amphipathic α -helical peptides (left) and amphipathic, cyclic β -sheet peptides (right). Columns: RPC column, same as Fig. 10; strong cation-exchange column, same as Fig. 11. Mobile phase for RPC: linear AB gradient, where eluent A is 0.05% aqueous trifluoroacetic acid (TFA), pH 2.0 and eluent B is 0.05% TFA in acetonitrile; gradient rate, 0.5% acetonitrile/min; flow rate, 1 mL/min; temperature, 30°C. Mobile phase for HILIC/CEC: same as Fig. 10. (Left) Mixture of four 18-residue amphipathic α -helical peptides with single residue substitutions (Leu, Val, Thr, Ser) made in the hydrophilic face of the helix (sequences shown in Table 1). (Right) Mixture of six 10-residue amphipathic, cyclic β -sheet peptides with variations only in the hydrophilic face (sequences shown in Table 1), the arrows denote major selectivity differences between the two modes.

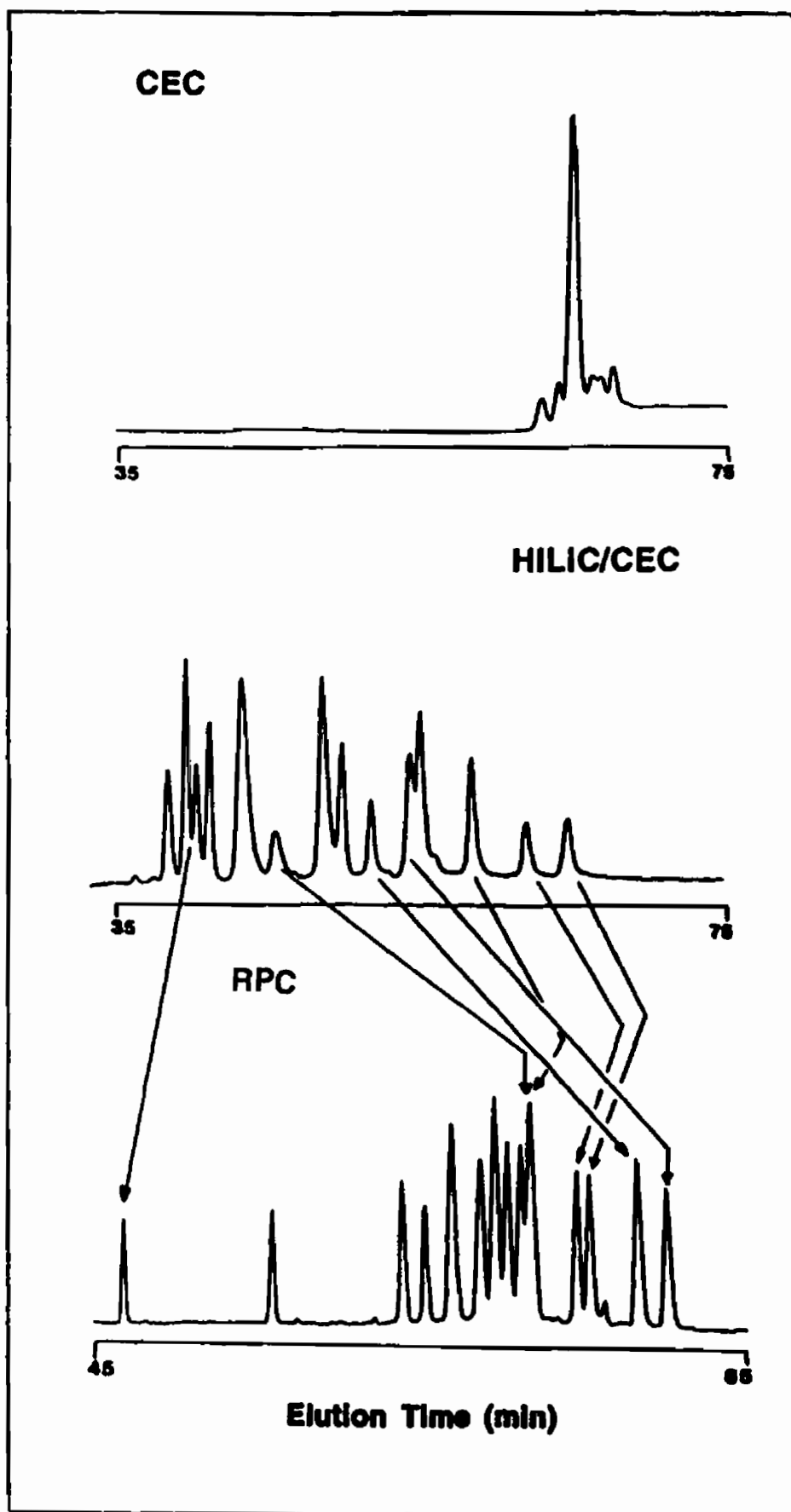
Figure 13 compares the separation by CEC (top), HILIC/CEC (middle), and RPC (bottom) of a mixture of 16 cyclic diastereomeric peptide analogs. Under straightforward CEC conditions (with 10% acetonitrile in the mobile phase to suppress any hydrophobic peptide-packing interactions), the peptides were essentially eluted as one major peak, a reasonable observation considering they all contain the same net positive charge (+4) under the conditions employed (pH 3). In contrast, under HILIC/CEC conditions, the separation is clearly much

improved with 14 out of 16 peaks now distinguishable; at the level of acetonitrile used for this result (90% and 80% acetonitrile in buffers A and B, respectively), hydrophobic interactions with the packing are highly unlikely, indicating that achievement of this separation resulted solely with differences in hydrophilicity of the analogs. A good separation of the 16 peptides was also effected by RPC, with the arrows between the HILIC/CEC and RPC runs denoting the more dramatic selectivity shifts between the two modes and demonstrating again the complementary nature of these two techniques.

Figure 14 outlines the purification and analysis of a 17-residue synthetic peptide containing an intrachain disulfide bridge. Following purification of the crude oxidized peptide mixture (top) by RPC, analysis of the purified product showed a single symmetrical peak (middle), indicating excellent peptide purity. However, mass spectrometry of this single peak showed, in addition to the expected mass, a second signal exhibiting a peptide mass 87 units less than the desired product, indicating deletion of one of the Ser residues. In RPC terms, Ser is classed as only a slightly hydrophilic group and hence contributes little to the retention behavior of a peptide during RPC; thus, it is not surprising that the deletion product is difficult to detect, let alone resolve, by RPC. However, Fig. 14 (bottom) again illustrates the efficacy of the HILIC/CEC approach in its ability to resolve two peptides inseparable by RPC. Indeed, this mixed-mode approach appears to enhance the hydrophilic contribution of a Ser residue, as the Ser-deletion impurity is now baseline resolved from the desired product. The Ser-deletion peptide is eluted prior to the desired product because loss of this residue has made the peptide less hydrophilic (i.e., more hydrophobic) than the native peptide (the more hydrophobic peptide is eluted first in HILIC mode).

The results of Fig. 14 suggested that the purification of the desired product would be best achieved by a multistep approach, i.e., an initial HILIC/CEC step followed by RPC of the desired peptide product. Another example of the resolving capability of the multistep HILIC/CEC and RPC protocol is illustrated in Fig. 15, which outlines the purification of a synthetic 21-residue amphipathic α -helical peptide from the peptide crude mixture. From the RPC elution profile (top) of the crude peptide, a major peak is obtained with a number of more hydrophilic and hydrophobic impurities, some of which are eluted very close to the main component. Subsequent attempts to improve the isolation of this main peak by varying the pH and/or the ion-pairing reagent used in the RPC mobile phase were unsuccessful, thus ruling out the possibility of an efficient one-step purification by RPC. Application of mixed-mode HILIC/CEC to the crude mixture resulted in the middle elution profile, which shows a major peak (P) as well as several other smaller, yet substantial, peptide components. Collection of the major peak followed by RPC produced the bottom elution profile, with the

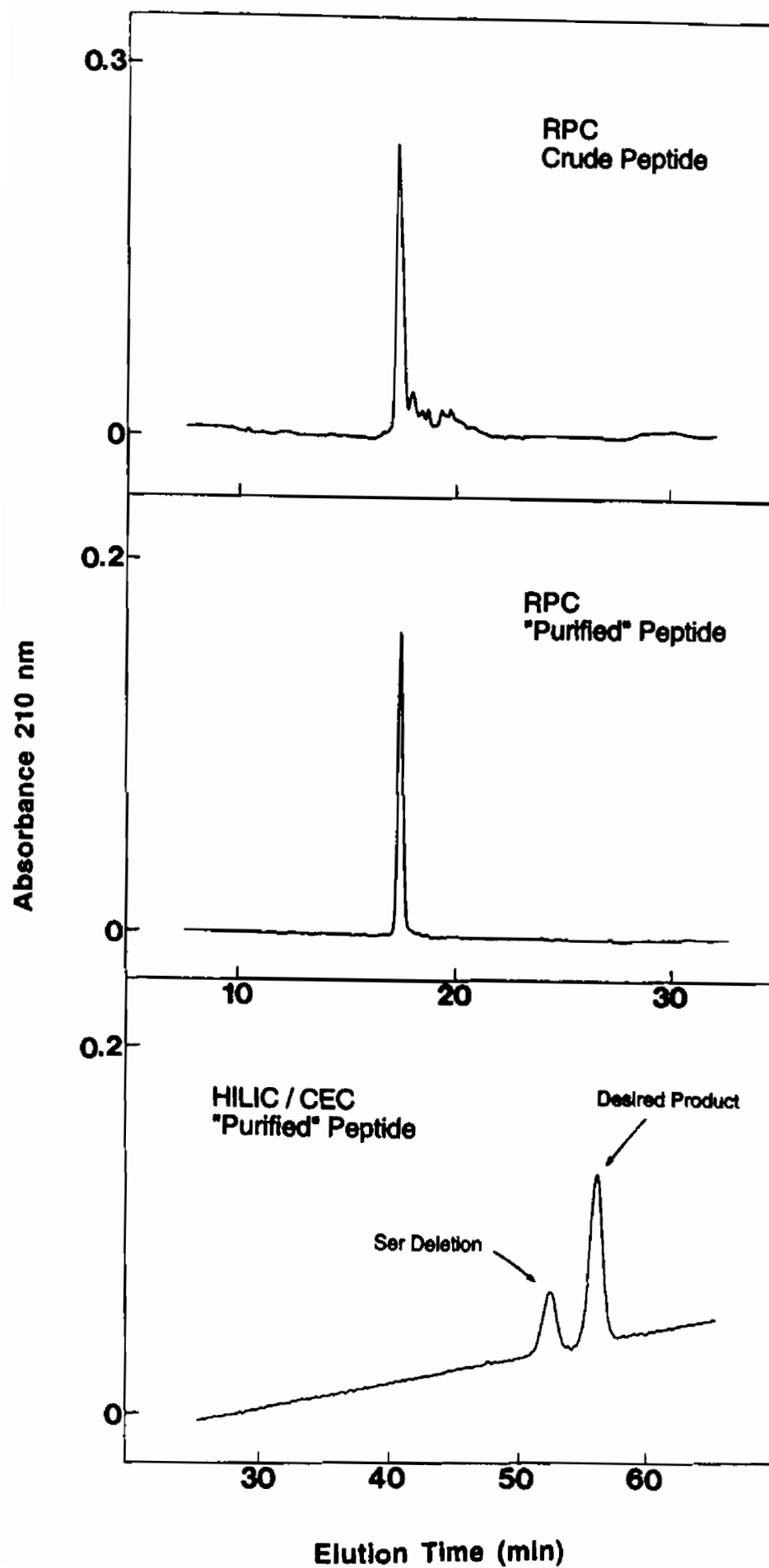
Absorbance 210 nm



major purified peak shown subsequently by mass spectrometry to be the desired product. Clearly, this two-step protocol was very successful in solving a difficult purification problem. Note that the middle separation, which efficiently removed from the desired product the impurities eluted close to this peptide during RPC (top), could not be achieved by IEC alone; i.e., the high acetonitrile level characteristic of HILIC-CEC was vital to the separation. Peak A was subsequently identified as a lysine-deletion impurity, which, not surprisingly, was well separated from the product of interest. What makes the HILIC/CEC resolution of the crude peptide mixture particularly noteworthy was the subsequent identification [15] of peak B as a peptide analog with acetylation of a single Ser at position 3 of the sequence; peak C represents a peptide analog with acetylation of a single Ser at position 10 or position 17 of the sequence or possibly a mixture of these two modified analogs (note that the Ser residues lie in the wide hydrophilic face of this amphipathic α -helical peptide). Thus, HILIC/CEC is able not only to resolve these Ser-modified analogues from the desired product but also to distinguish between the subtle differences in hydrophilicity and hydrophobicity arising from the sequence position of modification.

Clearly, HILIC/CEC offers a valuable, complementary alternative to RPC for peptide separations; indeed, such an approach often rivals RPC for resolution of specific peptide mixtures. Although this approach has only recently been introduced, it has already been successfully employed for protein separations where RPC alone was unable to effect the required resolution [105,106]. In addition, because the need to identify peptide mixture components as well as to separate and quantify them is great, e.g., HPLC in conjunction with electrospray mass spectrometry (HPLC-MS), the utility of HILIC/CEC

FIGURE 13 Separation of 16 cyclic diastereomeric 14-residue peptides by CEC (top), HILIC/CEC (middle), and RPC (bottom). Columns: RPC, same as Fig. 10; CEC and HILIC/CEC, same as Fig. 11. Mobile phase for CEC and HILIC/CEC, linear AB gradient, where buffer A is 20 mM aqueous triethylammonium phosphate (TEAP), pH 3.0, and buffer B is buffer A plus 400 mM NaClO₄; acetonitrile is present in both buffers at a concentration (v/v) of 10% (CEC), or present at a concentration of 90% (v/v) in buffer A and 80% in buffer B (HILIC/CEC); gradient rate, 2.5 mM NaClO₄/min, following 5-min isocratic elution with buffer A; flow rate, 1 mL/min; temperature, 30°C. Mobile phase for RPC, linear AB gradient, where eluent A is 0.05% aqueous trifluoroacetic acid (TFA), pH 2.0, and eluent B is 0.05% TFA in acetonitrile; gradient rate, 0.5% acetonitrile/min; flow rate, 1 mL/min; temperature, 70°C. The sequences of the peptides are shown in Table 1. The arrows denote major selectivity differences between the HILIC/CEC CEC and RPC modes. (From Ref. 14.)



separations will be enhanced even further with the development of volatile mobile phases.

REVERSED-PHASE CHROMATOGRAPHY (RPC)

Mobile Phase Selection

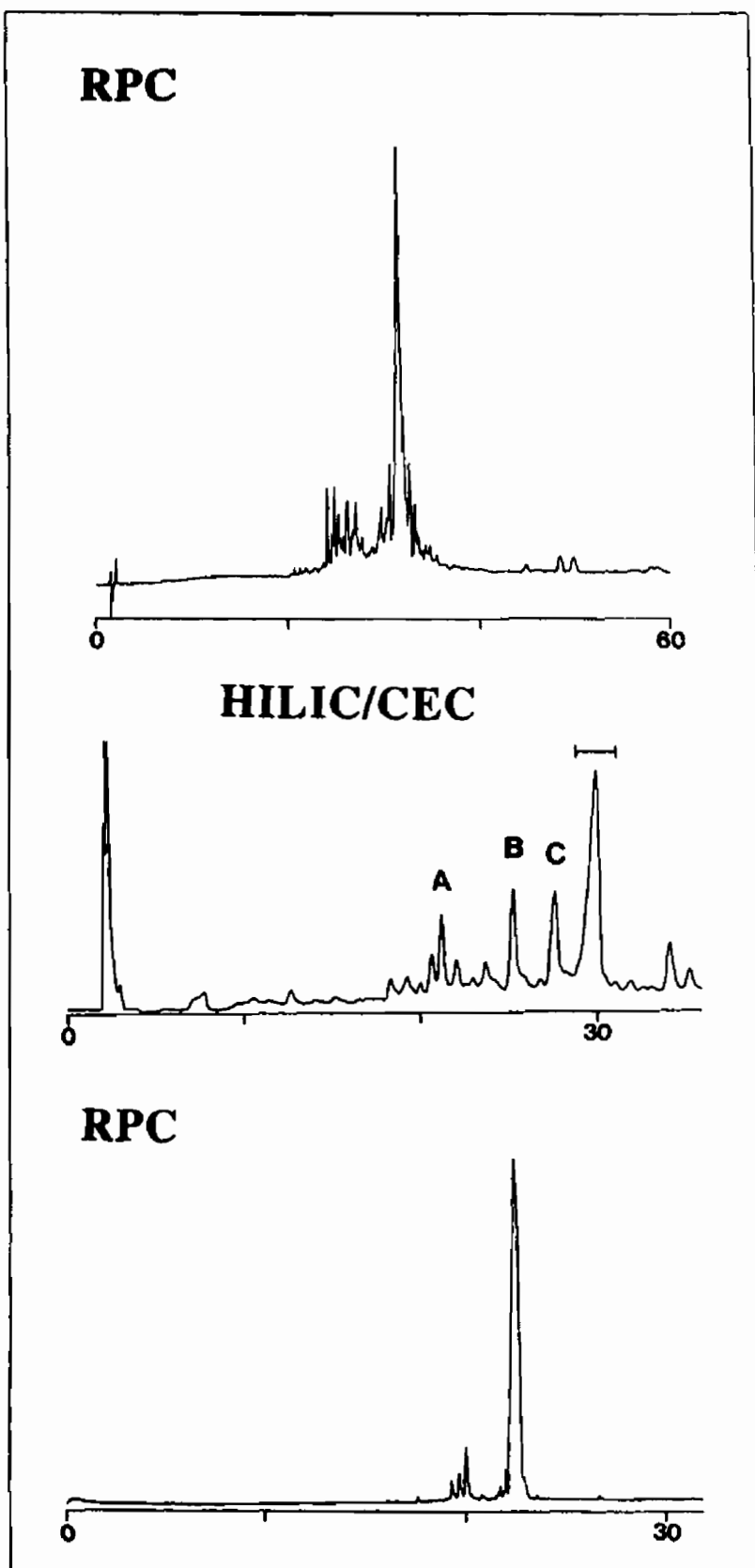
The excellent resolving power of RPC has made it the predominant HPLC technique for peptide separations, and its ability to separate peptides of closely related structures makes it extremely useful for high-speed, analytical and preparative applications. Not only is RPC usually superior to other modes of HPLC with respect to both speed and efficiency, but it also offers the widest scope for manipulation of mobile phase conditions to optimize peptide separations.

The choice of mobile phase must be dictated not only by its effect on the components of a peptide mixture but also by its effect on the reversed-phase sorbent. Reversed-phase silica-based columns may contain surface silanols that act as weak acids and are ionized above pH 3.5–4.0 [25,107]. These weak acids may interact with the basic residues of peptides chromatographed on reversed-phase columns and thus have an adverse effect on resolution, characteristically producing long retention times and peak broadening.

Although excellent resolution of peptide mixtures may be obtained at acidic or neutral pH, the majority of researchers have carried out RPC at pH <3.0. Apart from the suppression of silanol ionization under these conditions, silica-based columns are generally more stable at low pH. Although reports of peptide separations on pH-stable polymer-based columns are becoming more widespread [28,29,108–110], such packings still remain largely untested for routine peptide applications. In the authors' experience, the best approach to most analytical peptide separations is to employ TFA-H₂O to TFA-acetonitrile linear gradients (pH 2.0) at room temperature and a flow rate of 0.5–2.0 mL/min. Peptide

FIGURE 14 Analysis and purification of a 17-residue synthetic peptide by RPC and HILIC/CEC. Columns: RPC, same as Fig. 10; HILIC/CEC, same as Fig. 11. Mobile phase for RPC: linear AB gradient, where eluent A is 0.05% aqueous trifluoroacetic acid (TFA) pH 2.0 and eluent B is 0.05% TFA in acetonitrile; gradient rate, 1% acetonitrile/min; flow rate, 1 mL/min; temperature, 26 °C. Mobile phase for HILIC/CEC: linear AB gradient, where buffer A is 5 mM aqueous trimethylammonium phosphate (TEAP), pH 7.0, containing 65% acetonitrile (v/v), and buffer B is buffer A plus 400 mM NaClO₄, following 10-min isocratic elution with 30 mM NaClO₄; flow rate, 1 mL/min; temperature, 26 °C. The sequence of the peptide is shown in Table 1. (From Ref. 23.)

Absorbance 210 nm



Elution Time (min)

resolution can be optimized by varying the steepness of the acetonitrile gradient (generally 0.5–2.0% acetonitrile/min) and the volatility of TFA eliminates the need for subsequent sample desalting. Acetonitrile is the favored organic solvent for most peptide applications [23,38,45]; indeed, the best resolution of a peptide mixture is usually obtained between 15% and 40% of the organic solvent in the gradient [111], and this is generally achieved with acetonitrile. However, a more nonpolar solvent (e.g., isopropanol) [38,112–115] or a more polar solvent (e.g., methanol) [38,114,116,117] may be required for separations of more hydrophobic or more hydrophilic peptides, respectively. Ethanol has also proved occasionally useful as an organic modifier in RPC of peptides [118]. In addition, mixtures of these organic solvents (usually two) have been employed [119,120] because better peptide separations can occasionally be obtained with a mixture of organic modifiers rather than with a single organic solvent.

TFA is also effective in separating complex peptide mixtures because of its ion-pairing properties. Peptides are charged molecules at most pH values and the presence of different counterions will influence their chromatographic behavior. Differences in the polarities of peptides in a peptide mixture can be maximized through careful choice of ion-pairing reagent. Favored models for the mechanism of ion pair separations involve either formation of ion pairs with the sample solute in solution followed by retention of the solute molecules on a reversed-phase column [121,122] or a dynamic ion-exchange event in which the ion-pairing reagent is first retained by the reversed-phase column and then solute molecules exchange ions with the counterion associated with the sorbed ion-pair reagent [122–125]. Whatever the mechanism, the resolving power of ion-pairing reagents is affected by interaction with the ionized groups of a peptide. Anionic counterions will interact with the protonated basic residues of a peptide; a cationic ion-pairing reagent will show an affinity for ionized carboxyl groups. The actual effect on retention time of a peptide will depend strongly on the

FIGURE 15 Two-step HILIC/CEC and RPC purification protocol for 21-residue synthetic peptide. (Top) RPC of crude peptide. (Middle) HILIC/CEC of crude peptide. (Bottom) RPC of HILIC/CEC main peak. Columns: RPC, same as Fig. 10; HILIC/CEC, same as Fig. 11. Mobile phase for RPC: same as Fig. 14 except for a temperature of 30°C. Mobile phase for HILIC/CEC: linear AB gradient, where buffer A is 10 mM aqueous triethylammonium phosphate (TEAP), pH 6.5, containing 65% acetonitrile (v/v), and buffer B is buffer A plus 350 mM NaClO₄; gradient rate, 2.5 mM NaClO₄/min, following 10-min isocratic elution with buffer A; flow rate, 1 mL/min; temperature, 30°C. The fraction denoted by the bar in the HILIC/CEC elution profile (middle) was collected and subsequently purified by RPC (bottom). Impurities denoted A, B, and C are discussed in the text. The sequence of the peptide is shown in Table 1. (From Ref. 15.)

hydrophobicity of the ion-pairing reagent and the number of oppositely charged groups on the peptide. Hence, a hydrophobic counterion will, through interaction with charged groups in the peptide, increase further the affinity of the peptides for the reversed-phase sorbent; in contrast, a polar hydrophilic counterion, following ion-pair formation, would be unlikely to interact with the nonpolar sorbent [126].

The homologous series of alkylsulfonates offers a wide range of anionic ion-pairing reagents (e.g., butane- and hexanesulfonic acid) with gradually changing hydrophobicity [38,108,118,127–130]. These compounds are usually employed in aqueous buffers at a concentration of 2–20 mM. The most widely used anionic ion-pairing reagents at present still remain perfluorinated carboxylic acids, particularly TFA and heptafluorobutyric acid (HFBA) [38,126,131–133]. They are excellent solvents for most peptides, are completely volatile, and enable detection at wavelengths below 220 nm due to their low-UV transparency. They are usually employed at a concentration of 0.5–0.1% (v/v). It is important to be consistent in the level of TFA used in the mobile phase solvents. There is a concentration dependence effect on peptide retention [126,128,132,134,135] and peptide chromatographic profiles can vary quite markedly over a narrow range of TFA concentrations. This effect increases with increasing numbers of basic residues in a peptide. If the presence of TFA is not sufficient to resolve a particular peptide mixture efficiently, considerable flexibility in the degree of peptide retention and elution order may be achieved through the use of the equally volatile, more hydrophobic HFBA [38,126,134,136–138]. Hydrophilic anions such as phosphate are useful for hydrophobic peptide and proteins. The use of 0.1% aq. H_3PO_4 , for example, permits a significant decrease in the concentration of organic solvent in the mobile phase, thus reducing the possibility of denaturation or precipitation [139].

Hydrophobic cationic reagents include tertiary alkylamines [38,138,140–142], particularly triethylammonium phosphate. The use of these reagents (generally in the 2–10 mM concentration range) is, of course, limited to pH values above the $\text{p}K_a$ values of acidic side-chain groups ($\sim\text{pH}$ 4.0). The nature of interaction can range from exclusive ion-pair formation to dynamic ion exchange, depending on the type of additive [143]. Thus, cationic ion pairing can allow good control of the retentive behavior of a peptide [143]. Quaternary ammonium ions are also useful as cationic ion-pairing reagents [38,108,128,138,144]. Tetrabutylammonium phosphate has shown particular use as a strongly hydrophobic cationic counterion. It should also be noted that the concentration dependence effect described before for TFA (and, indeed, other anionic ion-pairing reagents) also applies to cationic reagents. Thus, varying the concentration of a particular ion-pairing reagent, instead of changing the reagent, can occasionally be of use in improving the resolution of peptide mixtures [126].

If it is desirable to carry out RPC at higher pH values, the addition of almost any salt to the mobile phase will block interactions of peptides with silanol groups present on the reversed-phase support, thereby improving the chromatographic profile [145]. Aqueous buffers, particularly phosphate buffers, have frequently been employed for reversed-phase peptide separations, enabling precise pH control [38,107,118,146–150]. A volatile ammonium acetate system has also proved popular for the separation of specific peptide mixtures [38,151–153]; a volatile ammonium formate system has also seen some use [154,155]. Amines are particularly effective in deactivating interactions between silanol groups and sample molecules, and the development of buffers based on triethylamine, particularly triethylamine phosphate (TEAP) [28,38,107,138,156–158] and triethylammonium formate (TEAF) [138,159–161], has enabled the effective resolution of a wide range of peptide mixtures. Triethylammonium formate has the advantage of volatility although it does not have the low-UV transparency of the triethylammonium phosphate buffer; however, the UV transparency of the TEAF system may be improved if dilute solutions (10–40 mM) are employed [138,159]. In addition, 0.1 M ammonium bicarbonate [109,162] and 0.02 M ammonium trifluoroacetate [163] have been employed in the pH range 7–11. It should be noted that above pH ~4.0 the presence of buffer salts may not be sufficient to eliminate silanol interactions entirely. Hence, it is often necessary to increase the ionic strength of the aqueous buffers by the further addition of 0.05–0.2 M salts, e.g., chlorides, sulfates, or a chaotropic reagent such as sodium perchlorate [38,107,146,147,149,164]. Interestingly, in our hands, a polymethacrylate-based C_{18} RPC column (throughout a pH range 2–11) and a PSDV-based RPC column (over pH ~9) both exhibited electrostatic interactions that required suppression to ensure ideal retention behavior of basic peptides (unpublished data). Such observations suggest that potential nonideal packing characteristics are not necessarily limited to silica-based packings and merit further investigation.

Simple unbuffered acid solutions have often been employed in peptide separations. One of the first solvents used in RPC of peptides was, in fact, dilute phosphoric acid [28,38,126,149,165]. Despite being nonvolatile, the low-UV transparency of H_3PO_4 enables sensitive detection of the peptide bond. In addition, the low pH of 0.1% aq. H_3PO_4 suppresses ionization of silanol groups and hence enhances peptide elution profiles. High concentrations (0.1–2.0 M) of acetic or formic acid, effective peptide solvents, have also been employed for RPC [111]. The addition of pyridine to these acids, although occasionally useful for some peptide separations, precludes UV detection below 250 nm [96]. In addition, the deleterious effect of high concentrations of acids on silica-based reversed-phase packings prohibits excessive employment of these systems [126]. Dilute hydrochloric acid has also been used for peptide separations at low pH [166,167]; however, the incompatibility of HCl with stainless

steel columns and fittings has precluded its widespread use. Other less-used acidic mobile phases include dilute perchloric acid [168], dilute acetic acid [169,170], and a TFA-acetic acid mixture [171].

The preference for gradient elution as the elution mode for RPC of peptides (and proteins) compared with isocratic elution lies in the predominant adsorption-desorption mechanism by which such solutes interact with the stationary phase [172,173]. The limited partitioning (or partitioning "window") of peptides with the reversed-phase packing, a consequence of multisite binding, makes the use of isocratic conditions impractical for separating mixtures of peptides exhibiting a wide range of hydrophobicities [173]. However, the reader is directed to some interesting RPC applications where isocratic elution has proved useful for monitoring synthetic impurities during solid-phase peptide synthesis [160,174]; for separation of peptides closely related in hydrophobicity, e.g., small peptides [175], angiotensin peptides [176], substance P and fragments [177], synthetic peptide analogs [178], and iodinated derivatives of peptide hormones [150,179,180]; and for separation of peptide diastereomers [181,182].

Peptide Standards

Efficiency data supplied by manufacturers with new reversed-phase columns are almost invariably derived from isocratic elution of small organic molecules, e.g., toluene, anthracene, methylbenzoate. Indeed, quite frequently, the purchase of a particular reversed-phase column will be based on such data, the reasoning being that high column plate counts should translate into efficient resolution of solute mixtures. Although this is a reasonable assumption for the most part, it has been our experience that there are pitfalls to this approach to column purchase when applying RPC to the separation of charged species such as peptides and proteins. Thus, occasionally a column has been purchased which, on paper, promised to be useful for difficult peptide separations but which subsequently produced poor separations and serious peak broadening due entirely to hitherto undetected (or unreported) electrostatic interactions with the hydrophobic reversed-phase matrix.

Although neutral compounds such as alkylphenones have been used as RPC standards [64] and, as already noted, other neutral molecules are employed for column efficiency measurements, it is preferable to use compounds that are structurally similar to the sample of interest and that presumably interact with the reversed-phase sorbent in a similar manner; in this way, a much more accurate assessment of a column can be made for a specific separation problem. For instance, as noted previously, peptides favor separation by an adsorption-desorption mechanism; in contrast, small organic molecules such as alkylphenones and toluene are separated mainly by a partitioning mechanism [164,173]. Indeed, even when RPC columns are assessed to be suitable for basic, polar (but

nonpeptide) applications [183], it is still recommended to employ peptide standards if peptides (or proteins) are the solutes of interest.

Peptide Standards for Monitoring RPC Column Performance

A series of five commercially available decapeptide standards (denoted S1–S5; sequences shown in Table 1) has shown great versatility in monitoring column (and, indeed, HPLC instrument) performance for peptide separations during RPC [23,38,45,126,184–189]. Each peptide contains two basic residues with no acidic residues present. The hydrophobicity of the standards increases only slightly between S2 and S5, enabling very precise determination of the resolving power of a reversed-phase column. The basic nature of the peptide standards (+2 for S2–S5, +3 for S1 over the acidic to neutral pH range) makes them ideal for monitoring the effectiveness of different anionic ion-pairing reagents. Figure 16 demonstrates the change in the chromatographic profile of the peptide standards on a C₈ column in different ion-pairing reagent systems. Increasing counterion hydrophobicity results in increased peptide retention times [126,131–134]. This is most pronounced in the case of the more highly charged S1, which shifts its relative position in the retention order of the standards when the hydrophilic ion-pairing reagent H₃PO₄ is replaced by the more hydrophobic TFA and the most hydrophobic HFBA.

The presence of the positively charged groups in the standards also makes them sensitive to any ionic, as opposed to hydrophobic, interaction with a reversed-phase sorbent. An excellent example of this is shown in Fig. 17, which illustrates the effect of nonspecific interactions on the elution profiles of the five standards on silica-based RPC columns when varying the gradient rate (1%, 1.5%, and 2% acetonitrile/min) of organic modifier. Varying the gradient rate of the organic modifier can be very effective in optimizing peptide separations, with this optimization generally achieved by decreasing the gradient rate, thereby improving solute resolution. However, as is clear from Fig. 17, predictable optimization depends upon the absence of nonspecific interactions.

From Fig. 17, the elution profiles on column II represent those that would be expected on a dependable column as the acetonitrile gradient rate is decreased from 2%/min to 1.5%/min and, finally, to 1%/min, i.e., a progressive improvement in the separation of the peptides as the gradient rate is decreased. In contrast, the separation of the peptides on column I actually worsens, with concomitant significant peak broadening (compared with column II), as gradient rate is decreased, where peptide S1 is coeluted with S3 at the lowest gradient rate of 1%/min. These results suggest that this column is exhibiting significant electrostatic interactions, with the anomalous behavior of S1 relative to the other four peptides probably due to the higher net positive charge on this peptide (+3) compared with S2 to S5 (+2), leading to a stronger electrostatic interaction with the stationary phase compared with the other four peptides. A key point

TABLE 1 Synthetic Peptides Used in This Chapter

Figure	Peptide sequence ^a
1,10,11,16,17,25	Ac-R-G-G-G-I-G-I-G-K-amide (I1); Ac-R-G-G-G-I-G-L-G-K-amide (I2); Ac-R-G-G-G-L-G-L-G-K-amide (S2); Ac-R-G-A-G-G-L-G-L-G-K-amide (S3, also denoted S1 when N-terminal residue is nonacetylated); Ac-R-G-V-G-G-L-G-L-G-K-amide (S4); Ac-R-G-V-V-G-L-G-L-G-K-amide (S5)
2,3,6,9,19	Ac-(G-L-G-A-K-G-A-G-V-G) _n -amide, where $n = 1, 2, 3, 4$, and 5 , i.e., 10, 20, 30, 40, and 50 residues, respectively
4	Ac-(K-L-E-A-L-E-A) _n -amide, where $n = 1$ (denoted 7 M), 2 (14 M) and 4 (28 M, 56 D)
5	F-I-P-K (4 residues); Ac-G-G-T-A-G-G-amide (6); P-Q-S-P-E-S-V-D-amide (8); L-K-A-E-I-E-A-L-K-A-amide (10); Ac-T-D-D-P-A-S-P-Q-S-P-E-S-V-D-amide (14); I-E-A-L-K-C-E-I-E-A-L-K-A-E-I-E-A-L-K-A-amide (20)
7,8,9,11,18	Ac-G-G-G-L-G-G-A-G-G-L-K-amide (C1); Ac-K-Y-G-L-G-G-A-G-G-L-K-amide (C2); Ac-G-G-A-L-K-A-L-K-G-L-K-amide (C3); Ac-K-Y-A-L-K-A-L-K-G-L-K-amide (C4)
12	Ac-E-L-E-K-L-L-X-E-L-E-K-L-L-K-E-L-E-K-amide, an amphipathic α -helical peptide, where X is substituted by leucine (LL7), valine (LV7), threonine (LT7), or serine (LS7), i.e., substitutions are made in the hydrophilic face of the helix; cyclo-V-X-L-Y-P-V-X-L-Y-P, where X is substituted by diaminopropionic acid (Dap), diaminobutyric acid (Dab), ornithine (Orn), lysine (Lys), arginine (Arg), or histidine (His)
13	Cyclo-(V-K-L-K-V-Y-P-L-K-V-K-L-Y-P), where 14 analogues were produced by single enantiomeric substitutions at each residue around the ring; an extra two analogues were produced by double or quadruple enantiomeric substitutions of the lysine (K) residues

- 14 Ac-K-C-K-S-T-Q-D-E-Q-F-I-P-K-G-C-S-K (contains an intrachain disulfide bridge)
- 15 Ac-K-I-S-A-L-K-E-K-I-S-A-L-K-E-K-I-S-A-L-K-E-amide
- 19 Ac-(G-K-G-L-G)_n-amide, where $n = 1, 2, 4, 6, 8,$ and 10 ; Ac-(L-G-L-K-E)_n-amide, where $n = 1, 2, 4, 6, 8,$ and 10 ; Ac-(L-G-L-K-L)_n-amide, where $n = 1, 2,$ and 4
- 20 Ac-T-D-L-L-G-amide (denoted O for O positively charged groups); Ac-V-S-K-T-E-T-S-Q-V-A-P-A-amide (1); peptides 2A and 2B (2) are S3 and S4, respectively (see above); peptide 3 (3) is the same as 2A but with a free α -amino group at the N-terminal; Ac-S-D-Q-E-K-R-K-Q-I-S-V-R-G-L-amide (4); Ac-G-K-F-K-R-P-P-L-R-R-V-G-amide (5); Ac-G-K-F-K-R-P-P-L-R-R-V-R-amide (6)
- 21 Ac-K-C-A-E-L-E-G-(K-L-E-A-L-E-G)_n-amide, where $n = 4$ (35B); Ac-K-K-G-(L)₁₆-K-K-A-amide (P22)
- 22 X-L-G-A-K-G-A-G-V-G-amide, where X is substituted by the residues denoted above the peaks
- 23 Ac-E-I-E-A-L-K-A-E-I-E-A-L-K-A-E-I-E-A-L-K-C-E-I-E-A-L-K-A-E-I-E-A-L-K-A-amide
- 24 Ac-X-L-G-A-K-G-A-G-V-G-amide, where X is substituted by the residues denoted above the peaks (N1, M1, etc.); Ac-E-A-E-K-A-A-K-E-X-E-K-A-A-K-E-A-E-K-amide, an amphipathic α -helical peptide, with substitutions at position 9 (residue AX9), denoting peptide analogue (AN9, AS9, AT9), this substitution being in the middle of the nonpolar face of the helix.

^a Peptide sequences are shown using the one-letter code for amino acid residues.

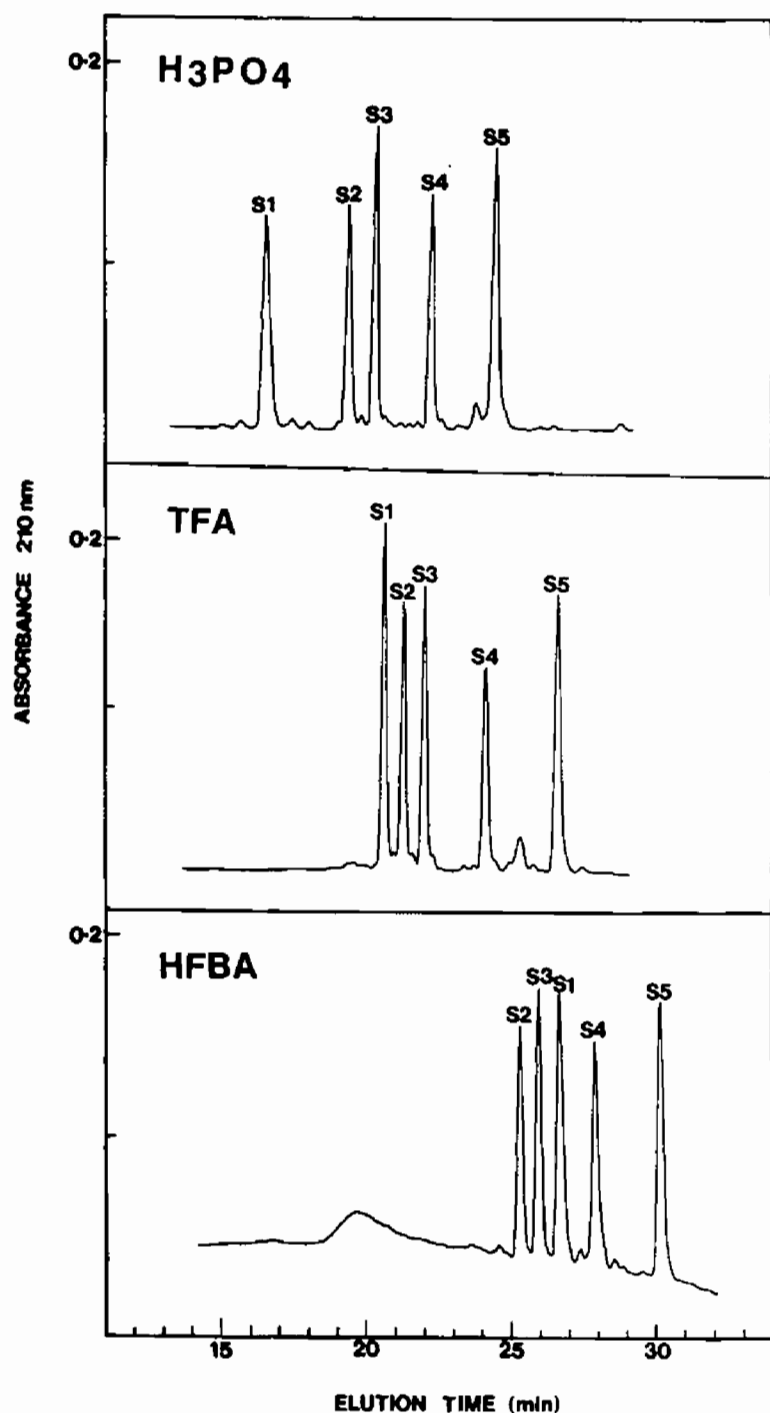


FIGURE 16 Effect of ion-pairing reagent on the RPC separation of a mixture of synthetic peptide standards at pH 2.0. Column: SynChropak RP-8 C₈ (250 × 4.1 mm ID, 6.5-μm particle size, 300-Å pore size; SynChrom, Lafayette, IN). Mobile phase: linear AB gradient, where eluent A is water and eluent B is acetonitrile, both eluents containing 0.1% H₃PO₄ (top), 0.1% TFA (middle), or 0.1% HFBA (bottom); gradient rate, 1% acetonitrile/min; flow rate, 1 mL/min; temperature, 26°C. The peptide standards have the sequence Ac-R-G-X-X-G-L-G-L-G-K-amide, where X-X is substituted by G-G (S2), A-G (S3), V-G (S4), or V-V (S5); the sequence for S1 is the same as S3 save for the presence of a free α-amino group.

ABSORBANCE 210 nm

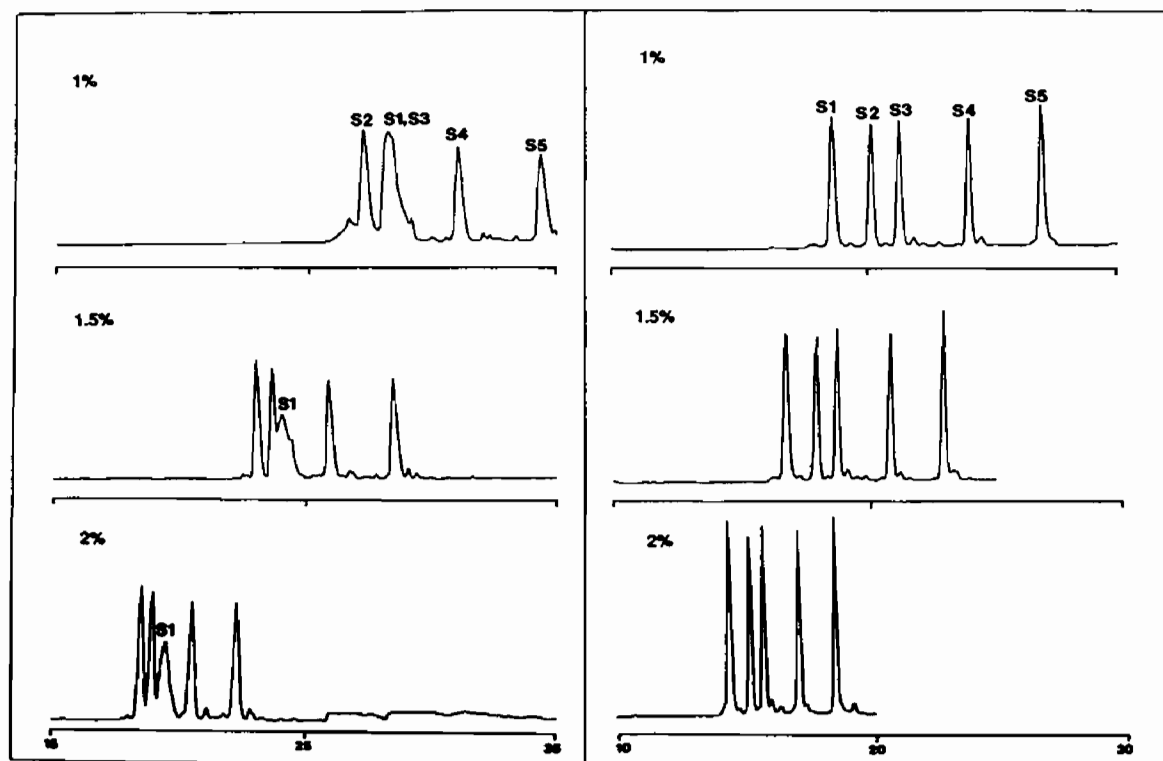


FIGURE 17 Effect of nonspecific interactions on gradient elution behavior of RPC peptide standards. Columns: column I, silica-based C₁₈ column (150 × 4.6 mm ID, 3-μm particle size, 80-Å pore size); column II, silica-based C₈ (220 × 4.6 mm ID, 7 μm, 300 Å). Mobile phase: linear AB gradient, where eluent A is 0.05% aqueous trifluoroacetic acid (TFA), pH 2.0, and eluent B is 0.05% TFA in acetonitrile; gradient rate, 1% (top), 1.5% (middle), and 2% (bottom) acetonitrile/min; flow rate, 1 mL/min; temperature, 26°C. The sequences of peptide standards S1–S5 are shown in Fig. 16 and Table 1.

about the results shown in Fig. 17 concerns the efficiency measurements reported by the manufacturers of the two columns (and subsequently confirmed in our laboratory) of 90,000 plates/meter for column I and 40,000 plates/meter for column II. Although column II proved to be a useful and efficient column for our purposes, the touted high efficiency of column I was negated by significant nonideal electrostatic behavior even at pH 2.0, probably due to the presence of isolated, highly acidic underivatized silanol groups that are not completely protonated even at this pH.

Peptide Standards for Monitoring Nonideal Column Behavior

The synthetic 11-residue peptide standards employed as cation-exchange standards (Figs. 7 and 8) were also developed to monitor the extent of silanol activity (i.e., the potential for nonspecific, ionic solute-packing interactions) on silica-based reversed-phase packings [38,45,107,190]; of course, such standards may also be applied to gauge the presence and extent of nonideal, electrostatic behavior on non-silica-based packings. The sequences for these peptide standards were shown previously, under "Ion-Exchange Chromatography," and in Table 1 [note that the peptides are designated as C1, C2, C3 and C4 (IEC) or 1, 2, 3 and 4 (RPC), depending on the mode of application]. Peptides 1–4 contain, respectively, 1–4 basic residues (Lys), with no acidic residues being present. Thus, over the pH range used for the majority of reversed-phase separations (pH 2.0 to 7.0), the net charges of +1 to +4 for peptides 1–4, respectively, do not change. The basic, positively charged character of the peptides ensures their sensitivity to any ionic, as opposed to hydrophobic, stationary-phase characteristics; this sensitivity increases with increasing basic character (peptide 1 to peptide 4) of the standards. The hydrophobicity of the standards also increases from Peptide 1 to Peptide 4.

Figure 18 demonstrates the application of these standards to monitoring silanol activity on two different silica-based columns at pH values of 2.0, 4.5 and 7.0. The contrast in performance between the two columns is striking. Column A exhibited significant ionic interactions with the basic peptide standards (apparent mainly from the retention behavior of peptides 3 and 4) over the entire pH range (pH 2.0–7.0) generally available to silica-based reversed-phase columns, even under conditions designed to suppress or block such interactions, i.e., low pH or the presence of amines or salts in the mobile phase. Peptides with charges of +3 and +4 are not uncommon and, thus, this column is clearly unsuitable for most peptide applications. Note that this column, which also appears to contain highly acidic (and thus unprotonated at pH 2.0) silanols, is a different column from that employed for Fig. 17 (column I). In contrast, the elution profiles obtained on column B were excellent at all three pH values. Some ionic interaction was apparent at pH 7.0 (greater peak broadening of peptide 4), even on this column, stressing once again the advantage of employing mobile phases at pH values

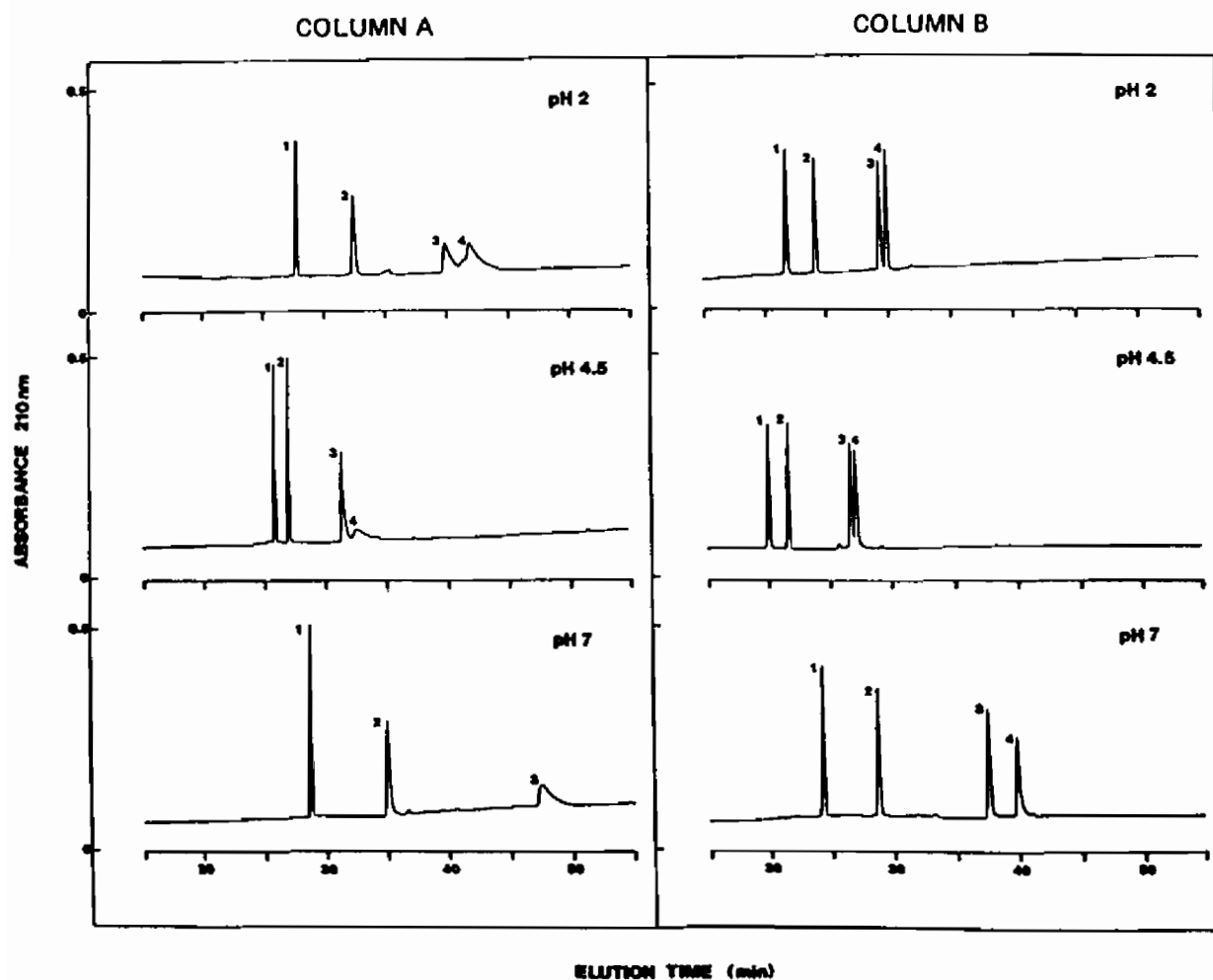


FIGURE 18 Monitoring of free silanols on commercial RPC columns with peptide standards. Columns: column A, silica-based C_{18} column (250×4.6 mm ID, $5\text{-}\mu\text{m}$ particle size, $300\text{-}\text{\AA}$ pore size); column B, silica-based C_8 column (220×4.6 mm ID, $7\text{-}\mu\text{m}$, $300\text{-}\text{\AA}$). Mobile phase: pH 2.0, linear AB gradient (1% acetonitrile/min), where eluent A is 0.05% aqueous trifluoroacetic acid (TFA), pH 2.0, and eluent B is 0.05% TFA in acetonitrile; pH 4.5, linear AB gradient (2% B/min, equivalent to 1% acetonitrile/min and 1 mM TEAP/min), where buffer A is 10 mM aqueous triethylammonium phosphate (TEAP), pH 4.5, and buffer B is 50% aqueous acetonitrile, containing 60 mM TEAP; pH 7.0, linear AB gradient (1.67% B/min, equivalent to 1% acetonitrile/min and 1.67% NaClO_4 /min), where buffer A is 10 mM aqueous $(\text{NH}_4)_2\text{HPO}_4$, pH 7.0, and buffer B is 60% aqueous acetonitrile containing 100 mM NaClO_4 . Flow rate, 1 mL/min; temperature, 26°C . The sequences of peptide standards 1, 2, 3, and 4 are shown in Table 1 (denoted C1, C2, C3, and C4, respectively). (From Ref. 107.)

<4.0, if at all possible. Most manufacturers attempt to cap underivatized silanol groups with reagents such as trimethylchlorosilane (TMS), and the effectiveness of their end-capping procedures may also be assessed through the use of the peptide standards at pH 7.0.

Prediction of Peptide Retention Times

The ability to predict the elution profiles of peptides of known composition greatly enhances the value of RPC. Although under certain circumstances sequence variations may significantly influence peptide retention, particularly for small, highly ionized peptides, there is a general assumption that the chromatographic behavior of a peptide is mainly or solely dependent on its amino acid composition; this assumption holds up well enough for small peptides (up to ~15 residues) [184,191]. Several research groups have determined sets of coefficients for predicting peptide retention on reversed-phase columns, based on computer-calculated regression analyses of the retention times of a wide range of peptides of varying composition [40,98,113,146–148,192–195].

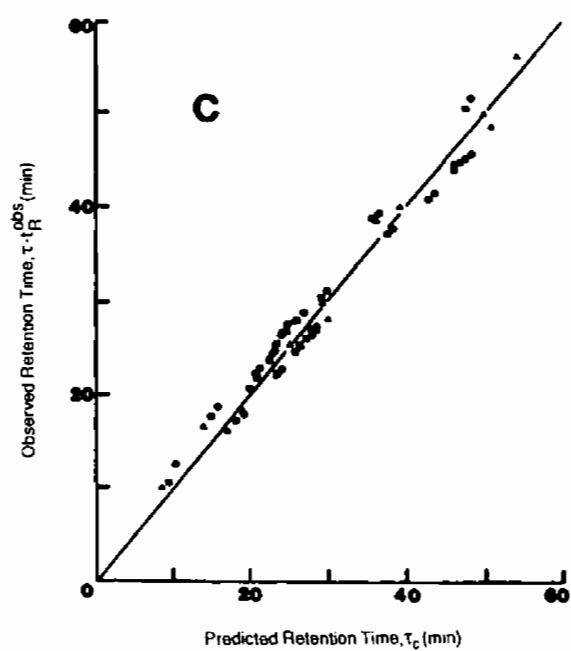
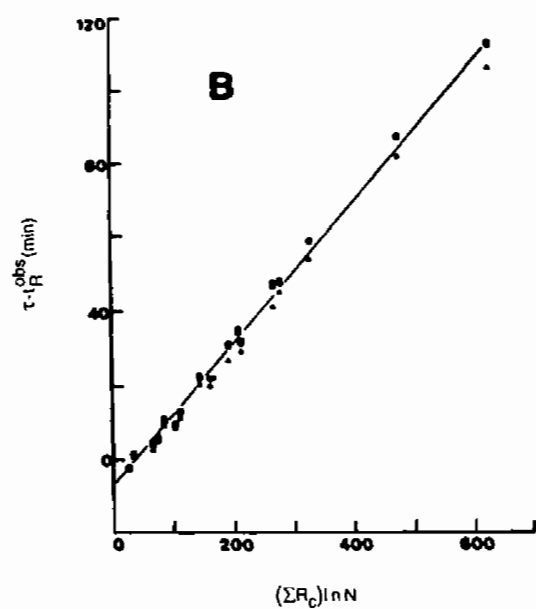
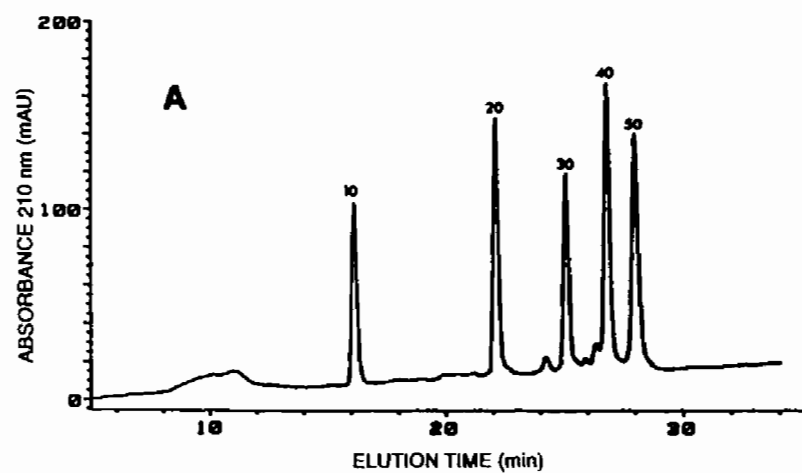
A more precise method for determining the contribution of individual amino residues was developed by Guo et al. [164,184]. Amino acid residue retention coefficients were determined for pH 2.0 and 7.0 by measuring their effect on retention of a model synthetic peptide, Ac-Gly-X-X-(Leu)₃-(Lys)₂-amide, where position X was substituted by the 20 amino acids found in proteins. The value of a predictive method in RPC is assessed by its accuracy in predicting the retention times of peptides not used to determine the retention coefficients. When the method was applied to retention time predictions for 58 peptides, the high correlation of predicted and observed retention times indicated good predictive accuracy for the range of peptides studied (2–16 residues) [184]. An internal peptide standard was always run with the peptides under investigation. The use of an internal standard allows the researcher to correct for any variations in HPLC apparatus, temperature, RP columns of any length or diameter, and RP packings of any *n*-alkyl chain length and ligand density. Peptide standards are essential for prediction of peptide retention times and cannot be replaced with nonpeptide compounds [164]. The coefficients at pH 2.0 were obtained by applying a linear TFA/H₂O to TFA-acetonitrile gradient at a flow rate of 1 mL/min and a gradient steepness of 1% acetonitrile/min. Of course, optimization of peptide separations may require variations of these parameters. Thus, Guo et al. [126] demonstrated a simple relationship between peptide retention in different ion-pairing systems and enabled the determination of rules for prediction of peptide retention times in one ion-pairing system from observed or predicted retention times in another system. In addition, Mant et al. [187] reported a simple, empirical approach for rapid assessment of the effect on

peptide separations of variations in flow rate and gradient rate as well as changes in column dimensions and packing type.

The retention time of a peptide is partially dependent on its molecular weight or, more strictly, on its polypeptide chain length [196]. This chain length effect is relatively unimportant in small peptides, but the accuracy of peptide retention time prediction based on a simple summation of amino acid residue retention coefficients decreases significantly beyond about 20 residues. This is clearly shown in the RPC elution profile (Fig. 19A) of the SEC peptide standards described previously (Fig. 2). The effect on peptide retention of increasing peptide length decreased progressively with each 10-residue addition. The intimate relationship between peptide hydrophobicity and chain length and their combined effect on peptide retention behavior in RPC was detailed by Mant et al. [196] and is summarized in Fig. 19B and 19C. Thus, these researchers demonstrated a linear relationship between predicted (τ) minus observed (t_R^{obs}) retention time versus the product of peptide hydrophobicity (expressed as $\sum R_c$, the sum of the coefficients of Guo et al. [164]) and the logarithm of the number of residues, $\ln N$ (Fig. 19B). Using the slope and intercept from such a plot, the retention behavior of peptides up to 50 residues in length can be accurately predicted. Figure 19C shows the good correlation between predicted and observed retention times for the peptides shown in Fig. 19A and three other series of synthetic peptide polymers (5–50 residues in length) varying widely in hydrophobicity (sequences shown in Fig. 19). Indeed, the results shown in Fig. 19B and combine the results of the four series of peptide polymers chromatographed on three different RPC columns, stressing the general validity of this predictive approach. This predictive process, together with those based on peptide standards for SEC (Figs. 3 and 6) and CEC (Fig. 9), subsequently formed the basis for an expert system [197,198] capable of stimulating HPLC peptide separations.

Peptide Conformation in RPC

Denaturation of a peptide or protein can be defined as a change in conformation from that which exists under benign conditions. If benign conditions are aqueous, separation by RPC can cause denaturation because of the nonpolar environment (progressively nonpolar mobile phase required for peptide elution and the nonpolar sorbent) as well as the low pH utilized for many separations. Most reversed-phase matrices have relatively high hydrocarbon loadings, causing strong binding of the peptide to the matrix, and the concentration of acetonitrile required to elute the peptide is usually greater than 10%. Both Lau et al. [65] and Mant et al. [199] successfully demonstrated the ability of sorbents to denature protein molecules. Lau et al. [65] used a synthetic 36-residue dimer that was extremely temperature stable ($t_{1/2} = 80^\circ\text{C}$). The coiled-coil quaternary structure



of this molecule was completely denatured on interacting with a reversed-phase sorbent, suggesting that this interaction with the sorbent was stronger than the hydrophobic interactions forming the coiled-coil. In a similar manner, Mant et al. [199] demonstrated that correlation of retention time behavior of larger peptides and proteins with their overall hydrophobicity required efficient polypeptide denaturation by the reversed-phase matrix.

Although many peptides can have a restricted conformation about certain peptide bonds, it is generally accepted that most peptides in an aqueous benign environment do not have a unique conformation. However, it has now been well established that a nonpolar environment such as that characteristic of RPC separations may induce helical structure in potentially helical molecules [200–205]. Hence, when applied to an RPC column, peptides in a peptide mixture may be expected to be bound to the hydrophobic stationary phase either as random coils or as α -helices, depending on both their length and α -helical potential. Such a situation would certainly be characteristic of peptide mixtures derived from, e.g., proteolytic digests of proteins.

Any time there is a conformation other than a random coil present on binding to the sorbent, it might be expected that deviations from predicted retention times would result. However, Zhou et al. [200] clearly established that, in the absence of a preferred binding domain such as the hydrophobic face of an amphipathic α -helix, the α -helical conformation of a peptide does not have any significant effect on peptide retention behavior in RPC. Thus, if the hydrophobic residues of a peptide are well distributed around the α -helical structure of the peptide, all residues in the α -helix are contributing to the overall hydrophobicity

FIGURE 19 RPC of a mixture of synthetic peptide polymers. Column: SynChropak RP-P C₁₈ (250 × 4.6 mm ID, 6.5- μ m particle size, 300-Å pore size). Mobile phase: linear gradient, where eluent A is 0.1% aqueous trifluoroacetic acid (TFA), pH 2.0, and eluent B is 0.1% TFA in acetonitrile; gradient rate, 1% acetonitrile/min; flow rate, 1 mL/min; temperature, 26°C. (A) Elution profile of five peptide polymers (10–50 residues; sequences shown in Fig. 2). (B) Plot of predicted minus peptide observed retention time ($\tau - t_R^{obs}$) versus the sum of the retention coefficients ($\sum R_c$) of Guo et al. [164] times the logarithm of the number of residues ($\ln N$). (C) Correlation of predicted and observed peptide retention times (derived from the plot shown in B, as described by Mant et al. [196]). The results shown in B and C are, in addition to the five peptide polymers shown in A, for three other series of peptide polymers (10–50 residues) with widely varying hydrophobicity (sequences shown in Table 1), run on the SynChropak RP-P C₁₈, a SynChropak RP-4 C₄ column (250 × 4.1 mm ID, 6.5- μ m particle size; 300-Å pore size), and an Aquapore RP-300 C₈ column (220 × 4.6 mm ID, 7 μ m, 300 Å; Brownlee Labs, Santa Clara, CA).

of the peptides that interact with the stationary phase. In fact, deviations from strictly predictable behavior of a particular peptide may alert the researcher to an interesting conformational feature of that peptide. Further, if it is possible to deduce the presence of interesting conformational characteristics of peptides from their retention behavior on the hydrophobic surface characteristic of RPC, it is possible that this characteristic may be expressed in the native protein where a similar hydrophobic environment prevails. Indeed, Zhou et al. [200] demonstrated the potential for predicting the behavior of amphipathic α -helices (an important protein structural motif) during RPC; clearly, the prediction of the retention behavior of peptides that form β -sheet-like structures when bound to a hydrophobic stationary phase would also be of value.

Selected Peptide Separations in RPC

Effect of Variation in Hydrophobicity of Anionic Ion-Pairing Reagents

Figure 20 demonstrates the peptide-resolving capability of RPC achievable by a simple variation of the hydrophobicity of the ion-pairing reagent. A mixture of basic peptides of similar size but containing varying numbers of positively charged groups (denoted by peptide numbers) was subjected to linear gradient elution on a C_{18} column in anionic ion-pairing systems of H_3PO_4 , TFA, and HFBA. From Fig. 20, the peptides are exhibiting increasing retention times with increasing counterion hydrophobicity: $H_2PO_4^-$ (Fig. 20A) < TFA $^-$ (Fig. 20B) < HFBA $^-$ (Fig. 20C). In addition, the elution order of the peptides changed from one counterion system to another because of the difference in magnitude of the effect of counterion hydrophobicity on peptides containing different numbers of positively charged groups. Thus, the greater the net positive charge of a peptide, the greater the effect on peptide retention of varying the anionic counterion; e.g., the elution order of peptides 1, 3, and 6 (containing 1, 3, and 6 positively charged residues, respectively) is reversed as the counterion is changed from $H_2PO_4^-$ (Fig. 20A) to HFBA $^-$ (Fig. 20C). Similar effects on peptide retention behavior through manipulation of anionic ion-pairing reagent hydrophobicity [TFA versus PFPA (pentafluoropropionic acid) versus HFBA] were also illustrated by Bennett [206]. The inset profiles shown in Fig. 20B show how varying the counterion (TFA $^-$) concentration can also produce useful selectivity changes; thus, the elution order of peptides 1, 3, and 6 (1, 3, and 6 positively charged residues, respectively) varied from 3, 6, and 1 at 0.01% TFA (Fig. 20B, left inset) to 1, 3, and 6 at 0.4% TFA (Fig. 20B, right inset). Such variations in counterion hydrophobicity and/or concentration may clearly be useful if contaminants of a desired peptide, e.g., from a synthetic crude mixture, varied in the number of positively charged residues they contain compared with the peptide of interest.

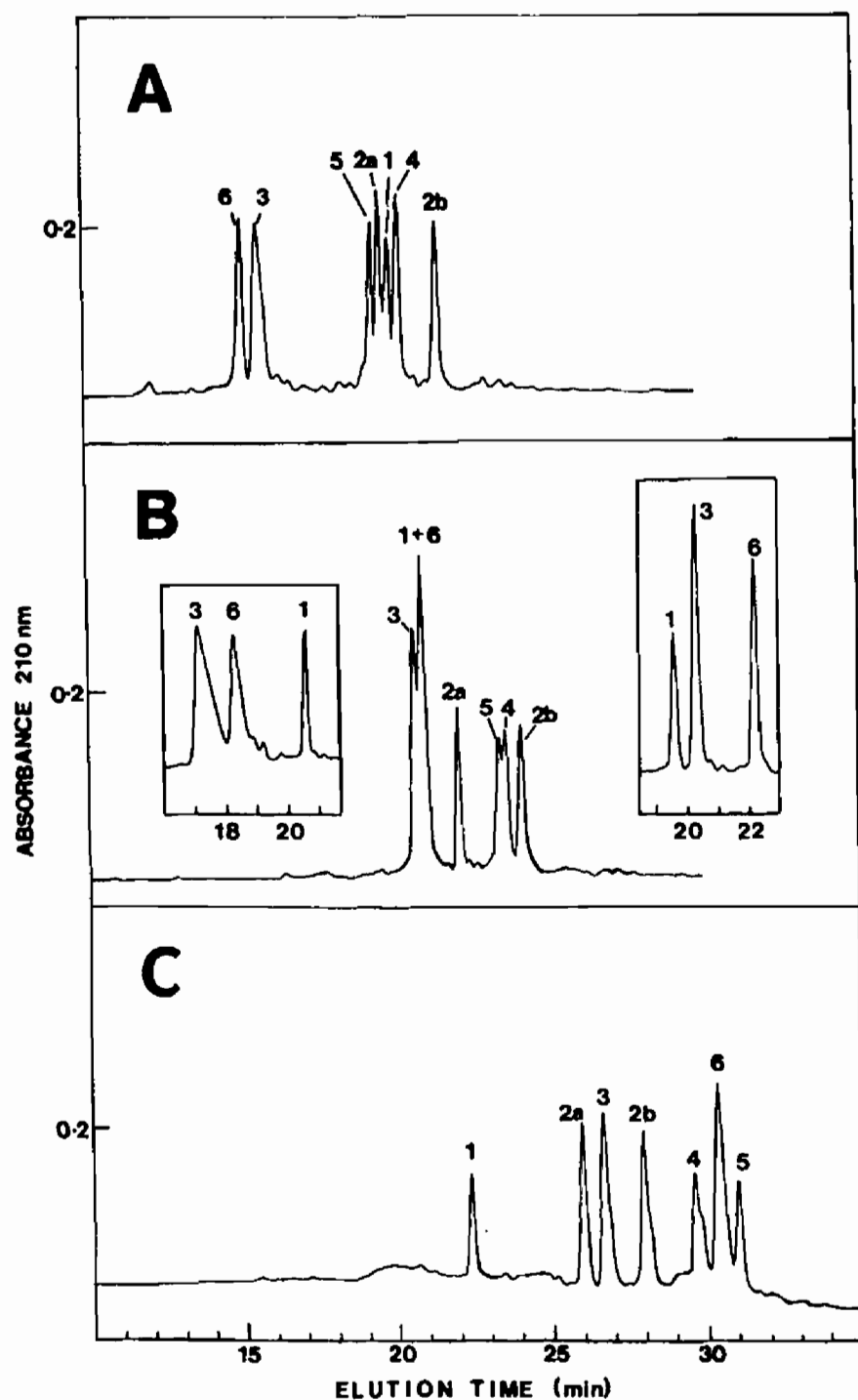


FIGURE 20 Effect of ion-pairing reagents on the RPC separation of a mixture of basic peptides in RPC. Column: SynChropak RP-P C₁₈ (250 × 4.1 mm ID, 6.5-μm particle size, 300-Å pore size; SynChrom, Lafayette, IN). Mobile phase: linear AB gradient, where eluent A is water and eluent B is acetonitrile, both eluents containing 0.1% H₃PO₄ (A), 0.1% trifluoroacetic acid (TFA) (B) or 0.1% heptafluorobutyric acid (HFBA) (C); gradient rate, 1% acetonitrile/min; flow rate, 1 mL/min; temperature, 26 °C. (B, insets) Left, 0.01% TFA in both eluents; right, 0.4% TFA in both eluents. The numbers above the peptide peaks (5–14 residues; sequences shown in Table 1) denote the number of positively charged groups they contain. (From Ref. 126.)

Effect of Variation in Stationary Phase Ligand

Figure 21 is an illustration of how reversed-phase packings containing ligands of different selectivity and/or hydrophobicity compared with the more common C_8 and C_{18} ligands may be useful for specific peptide applications. For example, Fig. 21 (left panels) indicates a useful role for a silica-based cyanopropyl (CN) packing in purifying organic contaminants, such as those encountered in solid-phase peptide synthesis (e.g., side-chain protecting groups, coupling reagents, cleavage reagents, and scavengers), from the peptide of interest. Thioanisole is a good scavenger and accelerator of the reaction cleaving the synthesized peptide from the resin support [207]; in many cases this scavenger is eluted with the peptide of interest from an alkyl-bonded reversed-phase column (Fig. 21, left, C_8 column). The dithiothreitol, DTT, is present to prevent disulfide bridge formation between the Cys-containing peptide molecules. In contrast, on the cyano column (Fig. 21, left, CN column), whereas the retention time of the peptide was similar to that exhibited on the C_8 columns, thioanisole was now barely retained, thus achieving an easy separation.

Figure 21 (right panels) shows the potential of cyano stationary phases for separation of extremely hydrophobic peptides. Such peptides, e.g., membrane-associated peptides, often pose special problems during RPC owing to their limited solubility and tendency to aggregate; in addition, they may be adsorbed irreversibly to some reversed-phase sorbents. Approaches to solving such problems include the use of mobile phases consisting of formic acid, water, and ethanol [208,209]; the addition of detergents to the mobile phase [210]; and the employment of isopropanol as organic modifier [9,211]. Despite reports of successful RPC purification of hydrophobic peptides, routine methods for such purifications are not yet well established. From Fig. 21 (right panels), the elution profile of a hydrophobic synthetic peptide P22 [Ac-(Lys)₂-Gly-(Leu)₁₆-(Lys)₂-Ala-amide] was improved on the cyano column compared with the C_8 column in several regards. First, about 12% less acetonitrile in the mobile phase was required to elute the peptide from the less hydrophobic CN column than from the C_8 column. In addition, a greater separation of P22 from impurities (I) was achieved on the CN column. Finally, the peak width of P22 on the CN column was less than that on the C_8 column (0.32 and 0.50 min, respectively). Similar results were obtained with synthetic peptide P26 [Ac-(Lys)₂-Gly-(Leu)₂₀-(Lys)₂-Ala-amide], which had peak widths of 0.52 and 0.38 min on the C_8 and CN columns, respectively. These results suggest that the employment of a CN column with an aqueous TFA-acetonitrile mobile phase is a simple and effective method for purification of many hydrophobic peptides.

Effect of Mobile Phase pH

Figure 22 demonstrates how pH variations may be used to effect RPC separations of peptide mixtures. The examples shown are for groups of nonacetylated peptide

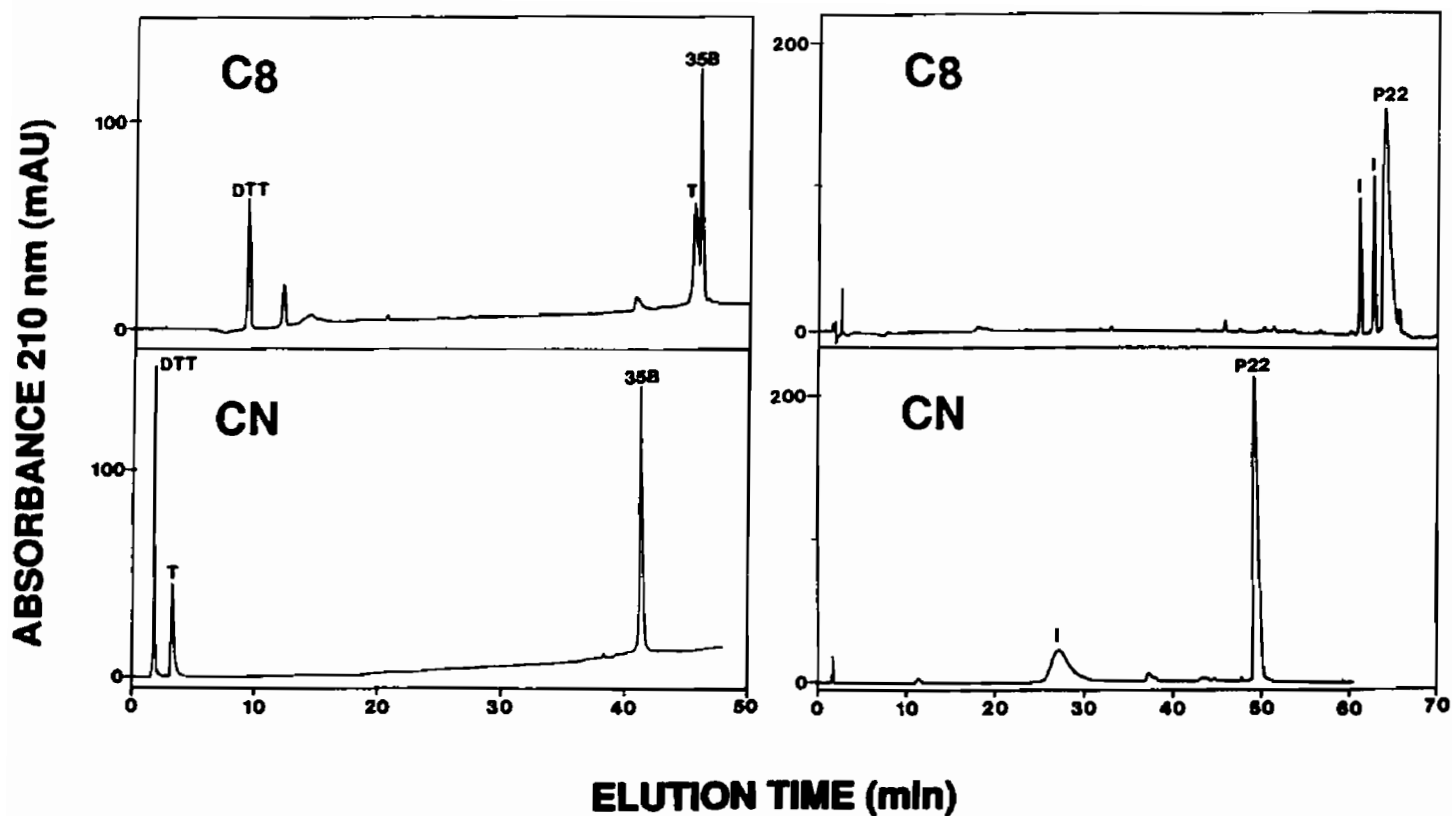


FIGURE 21 RPC of synthetic peptides on C8 and cyano (CN) columns. Column: Zorbax SB-C₈ (150 × 4.6 mm ID, 5-μm particle size, 94-Å pore size) and Zorbax SB-300 CN (150 × 4.6 mm ID, 6 μm, 300 Å) (Hewlett-Packard, Little Falls Site, DE). Mobile phase: linear AB gradient, where eluent A is 0.05% aqueous trifluoroacetic acid (TFA), pH 2.0, and eluent B is 0.05% TFA in acetonitrile; gradient rate, 1% acetonitrile/min; flow rate, 1 mL/min; temperature, 26°C. (Left) 35B denotes 35-residue peptide (sequence shown in Table 1); T denotes thioanisole; DTT denotes dithiothreitol. (Right) P22 denotes hydrophobic 22-residue peptide (sequence shown in Table 1). I denotes impurity; sample was dissolved in 70% eluent A/30% eluent B.

analogs. Thus, Fig. 22A and B show examples of profiles where specific peptide pairs are better separated at pH 2 than at pH 6.8. For example, the Leu and Met analogs are better separated at pH 2 than at pH 6.8. For example, the Leu and Met analogs are eluted as a doublet at pH 6.8, whereas at pH 2, these analogs are well separated. Similarly, the Trp and Phe analogs are coeluted at pH 6.8, whereas at pH 2, they are baseline resolved. In Fig. 22D, an example is shown in which a mixture of these nonacetylated analogs is better resolved at pH 6.8 than at pH 2. Thus, at pH 2, the Met and Ile analogs are not baseline resolved, whereas this is achieved at pH 6.8. Also from Fig. 22D, the Phe analog is much better separated

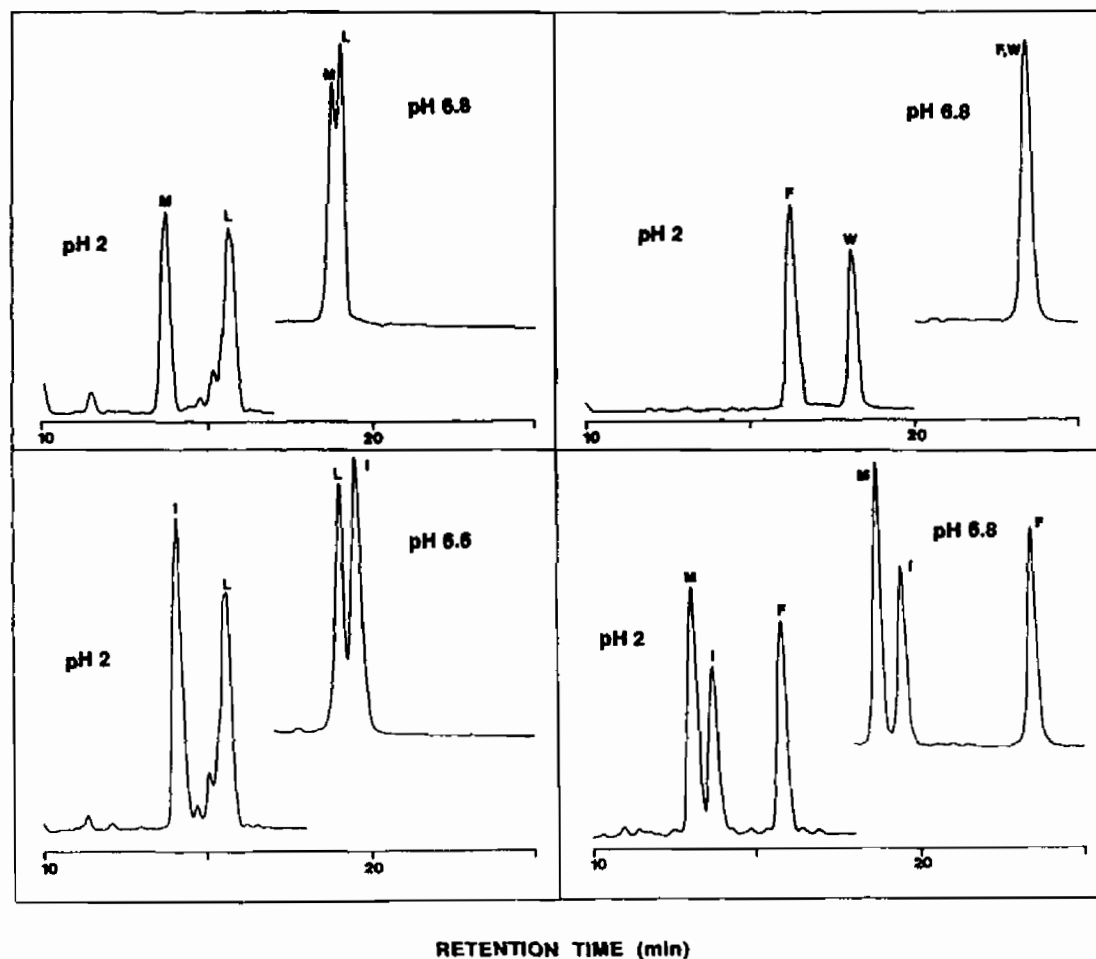


FIGURE 22 Effect of pH on RPC elution profiles of peptides. Column: Pep-S C₁₈/C₂ (250 × 4 mm ID, 5-μm particle size, 100-Å pore size, plus a 10 × 4 mm ID guard cartridge; Pharmacia, Baie d'Urfé, Canada). Mobile phase, pH 2.0, linear AB gradient (2% B/min, equivalent to 1% acetonitrile/min), where eluent A is 100 mM aqueous H₃PO₄ and eluent B is 100 mM H₃PO₄ in 50% aqueous acetonitrile; pH 6.8, linear AB gradient (2% B/min, equivalent to 1% acetonitrile/min), where buffer A is 100 mM aqueous triethylammonium phosphate (TEAP), pH 6.8, and buffer B is 100 mM TEAP in 50% aqueous acetonitrile. Flow rate, 1 mL/min; temperature, 26°C. The sequences of the synthetic peptide analogs are shown in Table 1 (note: all peptides have a free α-amino group). (From Ref. 28.)

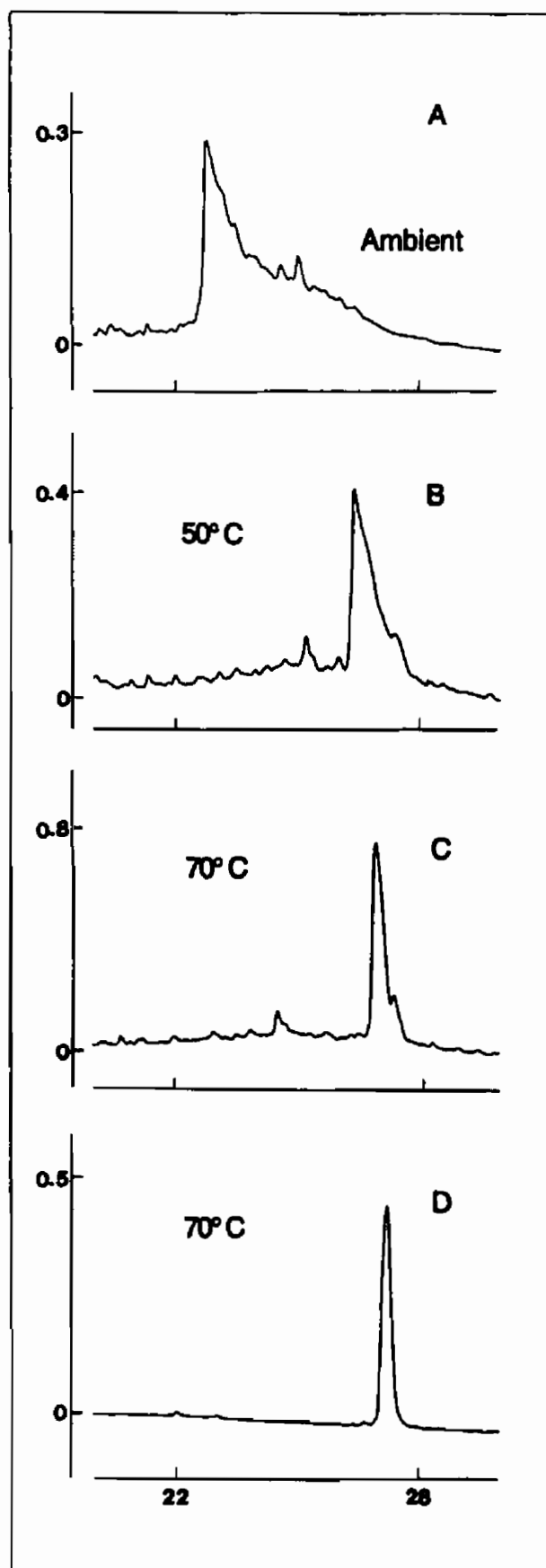
from the other two analogs at pH 6.8 than at pH 2. Figure 22C shows an inversion of elution order of a nonacetylated Ile-Leu pair at pH 6.8 and pH 2.

These interesting separations are probably the result of two factors: (1) the α -amino group affects the hydrophobicity of different N-terminal residue side chains to different extents at low pH and (2) the degree of deprotonation of the α -amino group ($\text{NH}_3^+ \rightarrow \text{NH}_2$) at higher pH varies, depending on the N-terminal side chain [28]. Taken together, such effects can be used to advantage for specific peptide mixtures. Other examples taking advantage of the charged character of potentially ionized side chains via pH manipulation of RPC mobile phases have also been reported [38]. It should be noted that the use of alkaline mobile phases (i.e., above pH 7.0) generally requires the use of a nonsilica packing and such separations over the pH 7.0 to 11.0 range have been reported [29,38,109,212,213]. However, in the authors' opinion, very high pH values have only limited use for peptide (and protein) purification by RPC.

Effect of Temperature Variation

Figure 23 shows an interesting example of the use of high temperature to effect a successful peptide purification, a result achieved due to the relatively recent commercial availability of RPC columns with excellent temperature stability [20–24]. The synthetic peptide being purified in Fig. 23 is an amphipathic α -helical peptide known to form a highly stable two-stranded α -helical coiled-coil [214]. At ambient temperatures (Fig. 23A), the poor elution profile may have suggested an unsuccessful synthesis, with the desired product at low yield and in the presence of many contaminants. However, LC mass spectrometry indicated essentially the same peptide mass throughout the major elution profile. As the temperature was raised to 50°C (Fig. 23B) and further to 70°C (Fig. 23C), there was a distinct improvement in the elution profile; indeed, the profile at 70°C indicated a yield of desired product of >90%. As noted previously [65,199], it is well known that the reversed-phase HPLC mode is highly denaturing to polypeptide tertiary and quaternary structure; thus, the hydrophobic stationary phases characteristic of RPC, as well as the nonpolar solvents required to elute solutes from such packings, serve to denature and to prevent peptide association or nonspecific aggregation (although, conversely, α -helicity is enhanced by such conditions [200–205]). In addition, temperature elevation serves to unfold polypeptides [199,215]. From Fig. 23A, it is possible that, at this ambient temperature, this peptide is present in different forms, ranging from a coiled-coil dimer through various unfolded (partially to fully denatured) forms. As the temperature is raised, the various unfolded forms of the peptide are all progressively denatured to produce the single monomeric species seen in Fig. 23C. Figure 23D shows the elution profile of the desired product following purification at 70°C. Such high-temperature separations have also proved useful in purifying peptides of high hydrophobicity/low solubility, where the elevation of temperature

Absorbance 210 nm



Elution Time (min)

helps to enhance the solubility of the peptide in the RPC mobile phase [23].

Effect of Salts in Mobile Phase

One approach to the manipulation of RPC profiles of peptides not often considered is the addition of salts to the mobile phase at low pH. Indeed, addition of salts (generally 50–100 mM) to mobile phases over a pH range of approximately 4–7 is traditionally designed to suppress negatively charged silanol interactions with positively charged solutes [104,107,164]. Figure 24 illustrates the potentially beneficial effect of salt on peptide separations at low pH by comparing separations of selected mixtures of peptides carried out in either an aqueous 0.1% TFA/acetonitrile, pH 2, mobile phase (Fig. 24A and C) or an aqueous 10 mM orthophosphoric acid (H_3PO_4)/acetonitrile, pH 2, mobile phase containing 100 mM sodium perchlorate (Fig. 24B and D). The negatively charged perchlorate ion acts as a hydrophilic anionic ion-pairing reagent and, thus, interacts with positively charged groups in peptides; the effect of perchlorate on the retention behavior of a particular peptide is then dependent on the number of positive charges the peptide contains. From Fig. 24A, peptides K1 and N1 and peptides I1 and AS9 are coeluted in the TFA system, whereas peptides AN9 and M1 are eluted as a doublet; in contrast, all six peptides are well resolved to baseline in the H_3PO_4 /perchlorate mobile phase (Fig. 24B). In a similar manner, from Fig. 24C, peptides R1 and G1 and peptides F1 and AT9 are coeluted, whereas peptides Y1 and AN9 are eluted as a doublet; from Fig. 24D, all six peptides are again baseline resolved by the H_3PO_4 /perchlorate mobile phase. Note that the retention times of all of the peptides increased in the H_3PO_4 /perchlorate system (Fig. 24B and D) compared with the TFA system (Fig. 24A and C), this increase being dependent on the magnitude of the positively charged character of a particular peptide.

FIGURE 23 Effect of temperature on RPC of a synthetic amphipathic α -helical peptide. Column: Zorbax Rx-C₈ (150 × 2.1 mm ID, 5- μm particle size, 300-Å pore size; Hewlett-Packard, Little Falls Site, DE). Mobile phase, linear AB gradient, where eluent A is 0.05% aqueous trifluoroacetic acid (TFA), pH 2.0 and eluent B is 0.05% TFA in acetonitrile; gradient rate, 2% acetonitrile/min; flow rate, 0.25 mL/min; temperature, ambient (A), 50 °C (B) or 70 °C (C and D). Profile D is that of the peptide following purification at 70 °C. The sequence of the peptide is shown in Table 1.

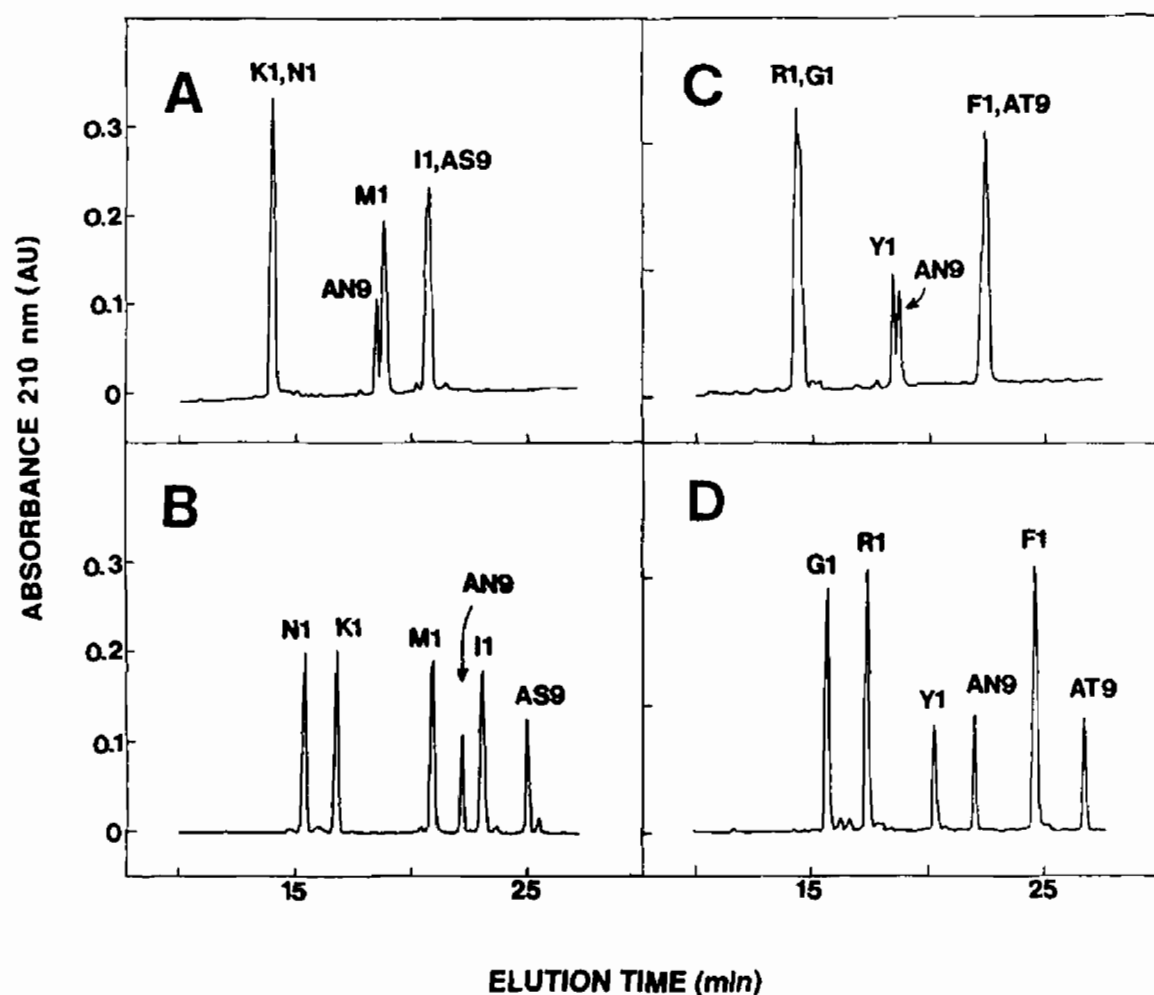


FIGURE 24 Effect of NaClO_4 on RPC of synthetic peptides at pH 2.0. Column: Zorbax 300SB- C_8 (150 $\times$ 4.6 mm ID, 5- μm particle size, 300- $\text{\AA}$ pore size; Hewlett-Packard, Little Falls Site, DE). Mobile phase: (A and C) linear AB gradient (1% acetonitrile/min) at a flow rate of 1 mL/min and a temperature of 26 $^\circ\text{C}$, where eluent A is 0.1% aqueous trifluoroacetic acid (TFA) and eluent B is 0.1% TFA in acetonitrile; (B and D) linear AB gradient (2% B/min, equivalent to 1% acetonitrile/min) at a flow rate of 1 mL/min and a temperature of 26 $^\circ\text{C}$, where eluent A is 20 mM aqueous H_3PO_4 and eluent B is 20 mM H_3PO_4 in 50% aqueous acetonitrile, both eluents containing 100 mM NaClO_4 . The sequences of the peptides are shown in Table 1. (From Ref. 104.)

RPC as a Probe of Stability, Folding, and Conformation of Peptides and Proteins

It is well known that the elution behavior of a peptide or protein during HPLC often offers important physical information about the sample of interest; e.g., SEC will estimate molecular weight and IEC will offer net charge information. However, the most potent probe of polypeptide structural characteristics is RPC. Although this area of research is not strictly a routine analytical application, the

major focus of this chapter, it is of great current interest and is expected to be even more so in the future. Indeed, such an application of RPC is an excellent example of where the characteristics of a particular HPLC mode have been utilized to great advantage in an originally unforeseen direction. Much of the efficacy of RPC in this particular area lies in the wealth of stationary phases and/or mobile phase conditions available to the researcher, not only to develop improved separation methodology but also to gauge the potential of relating peptide elution behavior with important physical features (e.g., the amphipathicity of α -helices or cyclic β -sheet peptides, destabilization of conformation) and/or biological activity (e.g., antimicrobial potency, receptor binding). Indeed, the recognition that the hydrophobic interactions between peptides or proteins and the nonpolar stationary phase reflect similar interactions between ligands and their receptors has enabled our laboratory [200,215–217], as well as other researchers [202,203,205,218,219], to demonstrate the general feasibility of equating polypeptide RPC elution behavior with in vitro and in vivo peptide and protein stability, conformation, and function.

Monitoring of Conformational Change by RPC

Although various techniques have been established for the study of surface-induced changes in peptide secondary structure, RPC has emerged as a sensitive analytical tool in monitoring conformational changes during the elution process [200–205,220,221]. In addition, this application of RPC can be enhanced further when performed in conjunction with circular dichroism (CD) [202,204] by providing quantitative estimates of peptide structure after interaction with a hydrophobic surface. CD studies of peptides bound to octadecyl (C_{18}) ligands covalently bound to glass plates have also provided a technique by which the conformation of peptides bound to a reversed-phase-like surface can be measured and then correlated with retention behavior [201,203]. Significantly, interactions between nonpolar residues, specifically between the nonpolar faces of amphipathic α -helical sequences in a protein (50% of α -helices found in proteins are amphipathic [222,223]), are the major driving force for protein folding and stability.

RPC of Peptides for De Novo Design of Antimicrobial Peptides

The ever-increasing development of bacterial resistance to traditional antibiotics has precipitated an urgent requirement for new antibiotics possessing novel modes of action as well as different cellular targets compared with existing antibiotics in order to decrease the likelihood of development of resistance.

For rational de novo design of α -helical and cyclic β -sheet antimicrobial peptides, it is known that optimization of the efficacy of such antibiotics depends on a balance of peptide features: (1) for amphipathic α -helical peptides, antimicrobial potency depends upon peptide amphipathicity [224,225], hydro-

phobicity [225,226] and helicity [227,228], such features coming into play only when the helical structure is induced in a hydrophobic environment such as a bacterial cell membrane [229] or, conversely, disrupted in an aqueous environment; (2) for cyclic amphipathic, β -sheet peptides, antimicrobial potency is related to the overall cationic and hydrophobic nature of the peptide, in addition to the amphipathicity of the β -sheet [230,231]; i.e., optimum antimicrobial, as opposed to hemolytic, activity has been found to require some disruption of the β -sheet. It turns out that RPC is an ideal system to measure hydrophobicity and amphipathicity of α -helical and β -sheet molecules, providing critical information in the design process.

TROUBLESHOOTING

The extended use of any column results in deterioration of resolution, often due to the accumulation of noneluted solutes such as hydrophobic peptides and proteins. The most general technique for removing retained solutes from all types of HPLC columns is gradient elution from 0.1% aqueous TFA to 0.1% TFA in isopropanol [25]. Repeated gradient elutions may be necessary for tightly bound solutes. If this general technique does not produce entirely satisfactory results, cleaning procedures specific to a particular type of HPLC column may be required.

SEC: Cleaning and Storage of Columns

For SEC columns, several column volumes of 20% dimethyl sulfoxide in water may prove effective in removing solutes. Flushing the column with denaturing solvents such as 6 M guanidine hydrochloride or 8 M urea or a detergent such as 0.1% SDS is another alternative. More vigorous conditions may be applied to polymer-based columns because flushing these packings with base (1–2 M NaOH) is possible. Both silica- and polymer-based columns are best stored in 10–20% aqueous alcohol (usually methanol) to prevent bacterial growth.

IEC: Cleaning and Storage of Columns

IEC columns are usually flushed with 1–2 M salt solutions to remove retained solutes. Chelating reagents such as 0.1 M EDTA have also proved useful. Polymer-based columns may also be flushed with 2 M NaOH followed by high salt elution. IEC columns are generally stored in 10–20% alcohol (usually methanol). These columns should not be stored in ionic solutions.

RPC

Cleaning and Storage of Columns

The general procedure of TFA/H₂O to TFA/isopropanol gradient elution described previously is usually the most effective cleaning procedure for RPC columns. Chelating reagents such as 0.1 M EDTA are also occasionally useful. Columns should be stored in a high concentration of organic solvent. The columns are then cleaned as well as stored after each day's use. The concentration of organic solvent should be significantly higher than the concentrations used in the mobile phase, and the authors recommend >50% aqueous organic solvent for most purposes. The storage solvent should not contain acidic mobile phase additives such as TFA. It is important to flush the column with a minimum of five column volumes of storage solvent to remove traces of acids efficiently.

When employing mobile phases containing salts for peptide applications, it is important to ensure that the column is salt free prior to storage in a solution containing organic solvents. This is particularly important when high salt and/or high organic solvent concentrations are employed in the mobile phase and storage solution, respectively. Thus, the column should be flushed with several column volumes of water prior to storage. This is true for SEC and IEC columns, in addition to RPC.

Regenerating Columns

Extensive use of silica-based reversed-phase columns, particularly under conditions of low pH, often leads to cleavage of covalently bonded hydrophobic ligands from the silica surface, subsequently resulting in an increased concentration of underivatized silanols. This leads to a decrease in ligand density, making the column less retentive, as well as an increased potential for ionic interactions with the positively charged residues of a peptide and, hence, a gradual deterioration in column performance. A number of researchers have demonstrated the viability of *in situ* silanization of deteriorated reversed-phase sorbents [186,188,232–234]. This procedure generally involves pumping a solution of the desired silane reagent through the column in an attempt to rederivatize exposed silanols and, hence, recover column performance. The potential of rederivatization of reversed-phase sorbents was clearly shown by Mant et al. [186] and Mant and Hodges [188] in their rederivatization of C₈ and C₁₈ reversed-phase columns. The procedure these authors developed was to rederivatize a deteriorated RPC packing with 20 mL (0.2 mL/min) of 10% (v/v) silane reagent in dichloromethane, also containing 7.5% (v/v) pyridine to neutralize the HCl released during the regeneration procedure. This procedure appears to have excellent potential for extending the useful lifetimes of columns (see Fig. 25, for example) and contributing appreciably to cost savings.

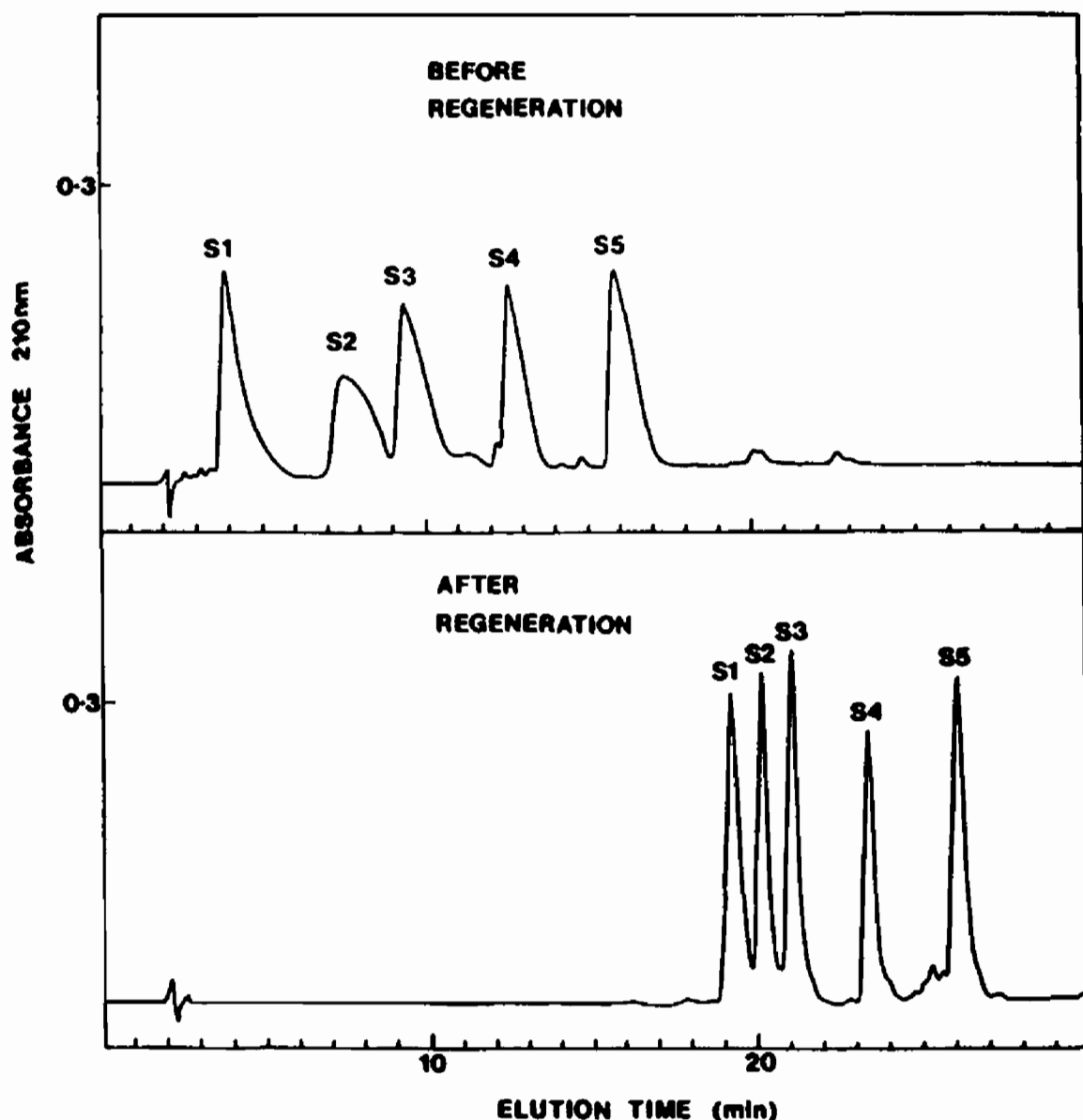


FIGURE 25 RPC of a mixture of synthetic peptide standards at pH 2.0 before and after column regeneration. Column: SynChropak RP-8 C₈ (250 × 4.1 mm ID, 6.5- μ m particle size, 300-Å pore size; SynChrom, Lafayette, IN). Mobile phase: linear AB gradient, where eluent A is 0.1% aqueous trifluoroacetic acid (TFA) and eluent B is 0.1% TFA in acetonitrile; gradient rate, 1% acetonitrile/min; flow rate, 1 mL/min; temperature, 26°C. The sequences of the peptide standards are shown in Fig. 16 and Table 1.

Handling of New Columns

Certain precautions should be observed when employing brand-new reversed-phase columns for the first time. The experience of this laboratory suggests that new silica-based reversed-phase packings may contain hydrophobic ligands that

are not covalently bonded to the silica support. Thus, gradient elution to high concentrations of organic solvent in the mobile phase may gradually elute these adsorbed ligands from the support, possibly resulting in their precipitation and blocking of lines or detector flow cells. The authors' experience with 10-mm-ID and 21.4-mm-ID C₁₈ columns also suggests that this problem is more serious with larger columns than with analytical sizes due to the significantly larger volume of packing material. This laboratory routinely washes new reversed-phase columns (not attached to the detector) with several column volumes of, sequentially, 100% 2-propanol, 50 : 50 (v/v) 2-propanol–water, and, finally, 100% water. The column is then ready for use.

FURTHER READING/INFORMATION

Several useful articles and reviews on HPLC of peptides can be found in Refs. 38, 235, and 236, with Ref. 237 representing a useful resource book in this area. For an extensive source of information on the early development of HPLC of peptides, the reader is directed to Ref. 238. A comprehensive practical overview on this topic is supplied by Ref. 239; the latter publication also supplied much useful information on preparative approaches to HPLC purification, as well as an overview of micro- and narrow-bore HPLC of peptides. An excellent and current publication dealing with high-performance analysis of biomolecules [240] also offers a sound introduction to capillary electrophoresis (CE), an important complementary technique to conventional HPLC.

Readily available journals that frequently contain up-to-date research articles on HPLC (and CE) of peptides include the *Journal of Chromatography*, the *Journal of Liquid Chromatography*, *Chromatographia*, *Bioseparation*, *Analytical Chemistry*, *Analytical Biochemistry*, and *Trends in Analytical Chemistry*. Peptide-based journals such as the *Journal of Peptide Research* also often contain useful peptide purification and analysis information. The symposium volumes from the International Symposium on the Separation and Analysis of Proteins, Peptides, and Polynucleotides (ISPPP) and International Symposium on Column Liquid Chromatography (HPLC '98, HPLC '99, etc.) meetings are a particularly good source of practical articles on peptide HPLC. For a continuing source of practical articles on peptide analysis by HPLC and CE (as well as a wealth of other aspects of these techniques), we recommend *LC.GC* (North America) [and its sister publication, *LC.GC* (Europe)], a monthly magazine on separation science. Indeed, this publication now carries a column dealing with Internet-related issues in the area of separation science, including descriptions of Web sites of interest to the chromatographer.

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HPLC of Membrane Proteins

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INTRODUCTION

Properties of Membranes and Membrane Proteins; Differences from Other Proteins

Biological membranes are organized structures that consist of phospholipids and proteins. They are responsible for the compartmentation of a wide variety of chemical activities in cells that are indispensable for life [1]. Membrane proteins with different chemical properties and functions are associated with a double layer of phospholipids (see Fig. 1). A phospholipid molecule consists of a hydrophilic head, e.g. phosphatidylcholine, and two hydrophobic tails, which are long hydrocarbon chains. It is energetically favorable for phospholipid molecules in water to form a bilayer and hence a spherical micelle structure. The hydrophobic tails occupy the inside of the bilayer while the hydrophilic heads are directed outward and make contact with the surrounding water. Proteins associated with the outer part of a lipid bilayer are called peripheral membrane

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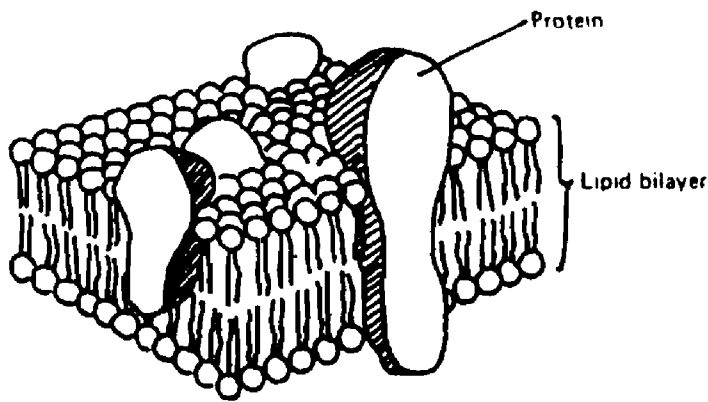


FIGURE 1 Integral membrane proteins embedded in a bilayer of phospholipid molecules.

proteins as opposed to those that interact with the hydrophobic inner portion of the membrane, which are called integral membrane proteins (see Fig. 2). A significant portion of an integral membrane protein, consisting mainly of hydrophobic amino acids, interacts with the hydrophobic tail of the lipids in the bilayer. Stretches of such amino acids existing in an alpha-helical conformation may span the membrane several times as shown by Deisenhofer et al. [2], who determined the first high-resolution structure of an integral membrane protein.

Other structural features that anchor a protein to a lipid bilayer may occur by posttranslational modification. Types of posttranslational modifications that have been recognized are: (1) attachment of lipid [3]; (2) fatty acid acylation of

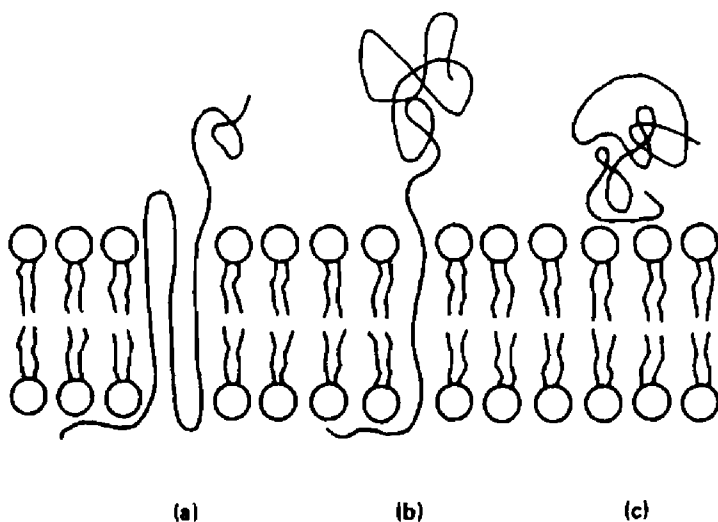


FIGURE 2 Various types of membrane proteins. Integral membrane proteins may cross the lipid bilayer several times (a) or once (b). Peripheral membrane proteins are associated with the hydrophilic outer part of the lipid bilayer (c).

the NH₂-terminal amino acid or formation of fatty acid thioesters with cysteine residues [3]; and (3) covalent attachment of the C-terminal part of the protein to phosphatidylinositol. The 1,2-diacylglycerol moiety serves as the hydrophobic anchor in the latter case [4]. This type of membrane anchoring has been observed in such widely different proteins as alkaline phosphatase, acetylcholinesterase, 5'-nucleotidase, Thy-1 glycoprotein that can be released from murine thymocytes, and the variant surface glycoprotein from *Trypanosoma brucei*. The latter protein contains *sn*-1,2-dimyristylglycerol [5]. Selective mobilization, i.e., conversion from the membrane form to the soluble form, is possible by the action of a specific phospholipase.

Integral membrane proteins are more hydrophobic than an average protein, especially when a significant part of the polypeptide chain interacts with the lipid bilayer. The hydrophobicity and molecular weight of a number of integral membrane proteins, poliovirus capsid proteins, and hydrophilic proteins are summarized in Table 1. The percentage of the hydrophobic amino acids, leucine,

TABLE 1 Hydrophobicity of Proteins and Peptides Based on the Percentage Leucine, Isoleucine, Valine, and Methionine (LIVM) and Their Molecular Weights

Protein/peptide	Molecular weight (kDa)	% LIVM	Ref.
Bovine ribonuclease	13.7	14.5	52
Ovalbumin	43	27.0	53
Average protein	—	20.2	6
Sendai virus			
F2	13–15	29.7	54
F1	50	29.6	54
M	38	30.3	55
HN	67	25.7	56
Bacteriorhodopsin peptide			
7	3.0	25.9	57
8	5.1	36.9	57
9	5.1	31.9	57
10	3.6	32.3	58
ω -subunit chloroplast energy complex	8	33.3	59
Cardiac membrane proteolipids	11	28.2	60
<i>sn</i> -1,2-Diacylglycerol kinase	13.3	36.9	28
Moloney MuLV p15 E	19.9	32.2	38
Polio virus			
VP1	33.5	20.9	61
VP2	30	21.4	61
VP3	26.4	26.1	61
VP4	7.3	29	61

isoleucine, valine, and methionine, was taken as a measure of the hydrophobicity of a protein. Of course, other amino acids also contribute to the hydrophobicity of a protein. The hydrocarbon portions of lysine, phenylalanine, tyrosine, and tryptophan also have some hydrophobic character. However, their contribution does not significantly affect the general picture of hydrophobicity derived from the percentage of Leu, Ile, Val, and Met (LIVM). The data show that an *average protein* (from 314 families; see Ref. 6) contains 20.2% LIVM. The fact that two common reference proteins, bovine pancreatic ribonuclease (RNase) and ovalbumin, have 14.5 and 27.2% LIVM, respectively, shows that there are considerable differences in the hydrophobic character of cytosolic proteins. For comparison, the hydrophobic character of the four tightly associated structural proteins of poliovirus, which are not integral membrane proteins, is seen to range from 20.9 to 29% LIVM. This chapter will focus on methods for the purification of integral membrane proteins by HPLC.

Properties of Detergents

An important step in the purification of integral membrane or transmembrane proteins is the solubilization by detergents. Membrane proteins behave as amphiphilic agents with their hydrophobic membrane-spanning region(s) inside and hydrophilic domains outside the membrane. The distribution of hydrophilic and hydrophobic regions determines the arrangement of the protein in the membrane of the cell or in the viral envelope. It is the amphiphilic character of these proteins that causes difficulties in their purification. Detergents are lipid-like substances with a hydrophilic head and a hydrophobic tail and therefore are also amphiphiles. Because they are able to compete with the phospholipids in a lipid bilayer, addition of an excess of an appropriate detergent to a protein-containing bilayer may result in extraction. There is a wide variety of detergents whose properties have been reviewed in detail [7–16].

Detergents can be classified according to their chemical properties:

1. *Ionic detergents* such as sodium dodecyl sulfate (SDS) and naturally occurring bile salts such as deoxycholate.
2. *Amphoteric detergents* such as zwitterionic substances, e.g., 3-((3-cholamidopropyl)-dimethylamino)-1-propane sulfonate (CHAPS).
3. *Nonionic detergents*, of which Triton X-100, X-114, Nonidet P40, decylpolyethylene glycol (DecylPEG), and octylglucoside are examples.

The principal difference between detergents within these classes is the balance between the hydrophilicity of the polar head and the hydrophobicity of the tail. The hydrophilic-lipophilic balance (HLB) value is used as an empirical parameter

to describe the amphiphilic properties of a detergent. To extract membrane proteins, detergents with HLB values >10 and <15 should be used.

A second parameter that may be important in the choice of a suitable detergent is its critical micelle concentration (CMC). The CMC represents the concentration of monomer at which micelles begin to form and an equilibrium between monomeric and micellar forms of the detergent is established. Triton X-100 has a low CMC and is difficult to remove. In contrast, the CMC of octylglucoside is 50 times higher. As a consequence, it is present as a monomer in a wider range of concentrations and can be removed more easily by dialysis. A convenient technique for determining CMC described by De Vendittis et al. [17] uses the absorption shift of either 8-anilino-1-naphthalenesulfonic acid or rhodamine 6G adsorbed to the monomeric form and micellar forms of a detergent. A number of commonly used detergents are listed in Table 2.

The type of detergent chosen for solubilization is determined by several factors. Extraction of membrane proteins with nonionic detergents has the advantage that the biological and immunological activity of the isolated protein is preserved. In addition, nonionic detergents have the advantage that they permit ion-exchange chromatography. This is counterbalanced by the disadvantage that many nonionic detergents have a low CMC and are difficult to remove. Another disadvantage of some nonionic detergents, e.g., polyoxyethylene detergents with alkylphenyl hydrophobic groups (Berol, Nonidet, Triton), is that they absorb ultraviolet light and interfere with the spectrophotometric determination of the elution pattern.

Extraction by ionic detergents may be the method of choice when maintaining the biological and immunological activity is of less importance. Ionic detergents, such as SDS, usually tend to denature proteins with irreversible loss of biological and probably some immunological activity. The hydrophobic regions of a transmembrane protein are likely to have strong affinity for the detergent, and therefore the number of SDS molecules bound will be higher than with hydrophilic proteins. Externally located hydrophilic domains of a protein may be denatured by SDS under conditions that would stabilize the domains that interact with the membrane. Extracts of membrane proteins obtained by SDS solubilization are suitable only for size exclusion chromatography (SEC). It is also important to note that the binding of SDS to proteins during extraction increases their molecular weight and should be taken into account when a particular size exclusion column is selected.

The use of bile salts has the limitation that at low pH they tend to form aggregates that precipitate. Although this problem may be circumvented by using extraction buffers and mobile phases at a pH greater than 7.8, the procedure is then unsuitable for chromatography on silica-based columns.

Amphoteric detergents are not as commonly used in the extraction of viral membrane proteins as they are with other membrane and receptor proteins [18].

TABLE 2 Detergents and Their Critical Micelle Concentration

Detergent	Description/synonym	CMC (mM)
Ionic		
Sodium dodecyl sulfate	H ₂ O	8.13
	0.05 M NaCl	12.30
	0.5 M NaCl	0.51
Sodium dodecyl- <i>N</i> -sarcosinate	Sarkosyl	13.7
Sodium deoxycholate	Bile salt	4–6
Nonionic		
Triton X-100	<i>tert</i> -C ₈ φE _{9.6} ^a	0.24–0.30
Nonidet P-40	NP40, <i>tert</i> -C ₈ φE ₉	0.29
Triton X-114	<i>tert</i> -C ₈ φE _{7–8}	0.2
Tergitol, Renex 690, Emulgens 911 and 913	Triton-like detergents	
Polyoxyethylene alkyl ether	C ₁₀ E ₅	0.69
Polyoxyethylene alkyl ether	C ₁₂ E ₅	0.049
Decyl-PEG	C ₁₀ E _{5–10}	
Berol 172 and 185	C ₁₀ /C ₁₂ E _n	
Emulphogen BC-720	C ₁₂ E ₈	0.087
Lubrol PX	C ₁₂ E _{9–10}	0.02–0.1
Thesit	C ₁₂ E ₉	0.09
Brij 35	C ₁₂ E ₂₃	0.091
Genapol X-100	C ₁₃ E ₁₀	0.15
Tween 20	Polyoxyethylene sorbitan monolaurate	0.059
Tween 80	Polyoxyethylene sorbitan monooleate	0.012
Octylglucoside	C ₈ glycoside	25.0
Dodecyl-β-D-maltoside	C ₁₂ maltoside	0.2
Hecameg	6- <i>O</i> -(<i>N</i> -heptylcarbamoyl)- methyl- <i>O</i> -D-glucopyranoside	19.5
Mega-10	<i>N</i> -(D-gluc-2,3,4,5,6-pentahy- droxyhexyl)- <i>N</i> -methyldeca- namide; decanoyl- <i>N</i> - methylglucamide	6.2
Amphoteric		
CHAPS	Bile acid derivative	4–6
Zwittergent 3–12 (sulfobetain SB-12)	Sulfopropylammonium compound	3.6
(<i>N</i> -Dodecyl- <i>N,N</i> -dimethyl- ammonio)undecanoate (DDMAU)	Alkyl carboxybetaine	0.13
Dodecyltrimethylamine oxide	LDAO (lauryldimethylamine oxide [C ₁₂ N ⁺ (CH ₃) ₂ O [−]]) pH > 7 (charge 0) pH < 3 (cationic)	2.1 8.1

^aC_xE_y, *x* refers to the number of C atoms in the alkyl chain and *y* to the average number of oxyethylene units; a phenyl ring is designated by φ; *tert*-C₈ refers to a tertiary octyl group.
Source: Data from Refs. 7–16.

The advantage of these detergents is that solubilized membrane proteins are still biologically active after extraction.

(Pre)Purification Procedures

Purification of hydrophilic proteins by chromatographic methods is often preceded by prepurification steps that involve salt fractionation and/or alcohol precipitation. Before starting the chromatographic purification procedure for an integral membrane protein, soluble proteins or peripheral membrane proteins attached to the membrane should be removed by treatment with (1) high salt concentrations (0.15–3.0 M NaCl or KCl), (2) basic or acid buffers, (3) chelating agents, (4) chaotropic ions, (5) 3,5-diiodosalicylate, or (6) high concentrations of guanidine-HCl or urea with a reducing agent. Further steps should take into account and take advantage of the special properties of integral membrane proteins, i.e., their tendency to interact extensively with a lipid bilayer or their solubility in organic solvents. These methods are generally directed toward extraction or release of the proteins from the bilayer by delipidation. After extraction with a detergent and subsequent centrifugation, the supernatant will contain the integral membrane proteins with detergent molecules attached to them.

Bordier [19] described an elegant way to extract proteins from a lipid bilayer by creating a two-phase system with Triton X-114. At 30°C, this detergent separates into an aqueous phase and a detergent phase. Integral membrane proteins can be found in the detergent phase and soluble proteins, including proteolytically degraded membrane proteins that lack their membrane-spanning region, show up in the aqueous phase. Parish et al. [20] developed a version of this methodology that is applicable to several other detergents. They used ammonium sulfate to lower the cloud point of these detergents to physiologically accepted levels. Six murine lymphocyte surface molecules partitioned into the Triton X-100 phase from 33–50% ammonium sulfate solutions. Another extraction method involves the use of organic solvents. In this case, two-phase systems are also created and membrane proteins that are soluble in the organic phase separated from proteins that are soluble in the aqueous phase as described for several cases [21].

Chromatographic Purification Strategy

The purification strategy to be followed after detergent extraction generally depends on the information that is available on the structure of the protein.

When the nucleotide sequence of the gene is available and the amino acid sequence and the molecular weight can be deduced, we suggest that the percentage of the hydrophobic amino acids, Leu, Ile, Val, and Met (% LIVM), should be determined. When the molecular mass is higher than 50 kD and the %

LIVM is around 30, severe difficulties can be expected when the protein is subjected to reversed-phase chromatography (RPC). Such proteins require at least 50% organic solvent for elution. These membrane proteins are eluted as broad peaks possibly as a result of repeated precipitation and dissolution [96]. In such cases, either SEC, ion-exchange chromatography (IEC), or a combination of both is preferable. Another concern in the purification strategy is maintenance of the biological or immunological activity. Generally, RPC is not advisable if the structural integrity of the protein has to be maintained [22]. Of course, this is of less importance when the purified protein is to be used in amino acid sequence studies. When the amino acid sequence is unknown and the molecular weight is the only information available about the protein, the same strategy can be applied, although with less certainty about the hydrophobicity of the protein. The size of the protein is an important aspect in the purification strategy. Rubinstein [23] pointed out that the larger the protein, the higher the concentration of organic solvent needed for its elution in RPC.

When specific polyclonal or monoclonal antibodies directed against the protein are available, immunoaffinity chromatography is an attractive and highly specific way to purify the protein. Recombinant DNA technology has provided the possibility of tagging a target protein with a specific ligand, e.g., six consecutive histidines. This facilitates subsequent purification by metal chelate chromatography [24,25]. Finally, in order to obtain a highly purified membrane protein, it is very often necessary to combine the preceding strategies.

COLUMN SELECTION

Modes of HPLC Available for Purification of Membrane Proteins

Several modes of HPLC are available for the purification of membrane proteins. Methods commonly used for the purification of soluble proteins serve as a starting point. Conditions for elution are then adjusted to the specific requirements to keep the membrane protein in solution. This means that a mixture of membrane proteins has to be solubilized by urea, guanidine-HCl, SDS, an organic solvent, or, if the conformation has to be maintained, by a nonionic detergent. In addition, it is often necessary to include a reducing agent such as dithiothreitol (DTT). The following modes of HPLC can be used for the purification of membrane proteins. They are based on:

1. Differences in size (SEC)
2. Electrostatic differences (IEC)
3. Biospecific interactions (immunoaffinity chromatography, IAC, and other types of affinity chromatography)
4. Hydrophobic interactions (RPC)

MOBILE PHASE SELECTION

Size Exclusion Chromatography

The peak capacity of SEC columns is relatively small; separation occurs in the volume between the void volume and the total permeation volume of the column. Preferably, SEC should be used in conjunction with one of the other modes of HPLC. Table 3 lists membrane proteins analyzed by SEC and the buffers used for elution. In all cases, a detergent or compounds that prevent aggregation are present during chromatography, with SDS being the most popular.

Nonionic detergents are probably the most suitable additives when further purification by IEC is required. Winkler et al. [26] used SDS in the elution buffer for SEC and were able to purify the eluted proteins further by RPC without prior removal of the SDS. We were less successful using a similar procedure for the purification of Sendai virus proteins. All Sendai virus membrane proteins containing SDS were eluted at the same position in RPC with low yields. In some cases [27,28], a volatile eluent has been used for chromatography, which facilitates further purification by one of the other modes of HPLC.

Analysis of the results from many systems shows that only in a limited number of cases can a pure protein be obtained directly from SEC. In general, it is necessary to apply another mode of HPLC for further purification.

Ion-Exchange Chromatography

Two general methods can be used for the elution of proteins from ion-exchange columns: (1) pH change, which results in weaker binding, and (2) increase of ionic strength, resulting in decrease of the electrostatic interaction between the protein and the column. Of these two, elution by changing the ionic strength is most easily controlled. In both methods, a nonionic detergent has to be present in the elution buffer to prevent aggregation. Table 4 summarizes gradient elution systems used for IEC of membrane proteins. Nonionic detergents are compatible with this type of chromatography; the gradient systems mentioned in the table also list the detergents. Triton X-100 used to be the most popular additive. Because the phenyl ring in Triton X-100 strongly absorbs at 280 nm, the ultraviolet (UV) detector had to be set at lower or higher wavelengths. Non-aromatic nonionic detergents, e.g., octylglucoside or polyoxyethylene alkyl ethers, are now widely available and are particularly suited as additives in IEC. However, the high price of octylglucoside and the concentration at which the best results are obtained (above the CMC of 0.7%) render it less attractive for inclusion in elution buffers.

Selective elution of integral membrane proteins was successful in an approach in which the concentration of the nonionic detergent was manipulated.

TABLE 3 Elution Buffers Used for SEHPLC of Membrane Proteins

Protein/proteins (from)	Elution buffer	Ref.
Ca ²⁺ -ATPase	20 mM Mops, pH 7.0, 50 mM NaCl, 0.1% SDS, Triton X-100, C ₁₂ E ₈ , Na deoxycholate, myristoylglycerophosphocholine	62
	20 mM Tes, pH 7.0, 100 mM Na/KCl, 10 mM MgCl ₂ , 1.5 mM CaCl ₂ , 10 mM EGTA, 5 mM DTT and various concentrations of C ₁₂ E ₈	63
	30 mM Hepes, pH 7.0, 10 mM imidazole, 0.1 mM CaCl ₂ , 200 mM Na acetate, 0.05% C ₁₂ E ₈	64
	100 mM Tes-Tris, pH 6.8, 25 mM NaCl or KCl, 0.01% C ₁₂ E ₈	65
Na ⁺ ,K ⁺ -ATPase	30 mM Hepes, pH 7.0, 10 mM RbCl, 1 mM ouabain, 0.04% C ₁₂ E ₁₀	66
	20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM MgCl ₂ , 1 mM DTT, 1 µg/mL leupeptin, 750 mM KCl, 1% octylglucoside	67
Arachidonoyl-diacylglycerol kinase		
Bacteriorhodopsin	30 mM Hepes, pH 7.0, 10 mM imidazole, 0.1 mM CaCl ₂ , 200 mM Na acetate, 0.05% C ₁₂ E ₈	64
Band 3 protein from erythrocyte membrane	5 mM Na phosphate, pH 7.0, 100 mM NaCl, 2–200× CMC C ₁₂ E ₈	68
Blood platelet membrane	100 mM triethanolamine HCl, pH 7.4, 0.5% Berol 185	69
Bovine rhodopsin	60 mM Na phosphate, pH 6.5, 0.15 M NaCl, 0.88% octylglucoside	70
Bovine diarrhea virus	100 mM K ₂ H phosphate, pH 7.0, 0.2 M NaCl, 0.5% Berol 185	71
Cell-CAM 105 (CAM = cell adhesion molecule)	PBS, pH 7.4, 0.2% octylglucoside or 0.25% CHAPS	72
Cytochrome <i>b₆f</i> complex (<i>Synechocystis</i> PCC 6803)	20 mM Mes, pH 6.5, 10 mM MgCl ₂ , 30 mM CaCl ₂ , 0.5 M mannitol, 0.03% dodecyl-β-D-maltoside	73
Reaction center photosystem I and II		

TABLE 3 (continued)

Protein/proteins (from)	Elution buffer	Ref.
Cytochromes (<i>E. coli</i>)	10 mM Tris-HCl, pH 7.5, 600 mM NaCl, 0.05% sarkosyl	74
sn-1,2-Diacylglycerol kinase	2-Propanol/heptane/water/TFA, 67/16.5/16.5/0.05	28
Dipeptidyl aminopeptidase	PBS, pH 7.4, 0.2% octylglucoside or 0.25% CHAPS	72
Equine infectious anemia virus (EIAV)	20 mM Na phosphate, pH 6.5, 6 M guanidine HCl 50 mM Na phosphate, pH 6.5, 0.1% SDS	75
Erythrocyte ghosts	100 mM Na phosphate, 0.21% SDS, 0.25% CHAPS, 0.08% reduced Triton X-100 or 0.003% Tween-20	76
Erythrocytes (human) (e.g., glycophorin C)	50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% C ₁₂ E ₉ or C ₁₂ E ₂₃	77
Glucose transporter	50 mM Tris-HCl, pH 7.0, 1 mM dithioerythritol, 1.5% octylglucoside	78
UDP-glucuronosyltransferase	25 mM K phosphate, pH 7.0, 30 mM KCl, 0.05% Emulgen 911	79
<i>Halobacterium halobium</i>	200 mM Tris-HCl, pH 8.0, 0.1% SDS or 100 mM Tris-HCl, pH 7.0, 100 mM NaCl, 0.1% SDS	80
	100 mM Tris acetate, 0.1–5% Triton X-100	81
Ia antigens	0.1% Triethylamine, pH 3.0, 0.2% Triton X-100	82
Influenza virus	100 mM Na phosphate, pH 7.0, 0.1% SDS, Brij 35, Lutensol ON 70D	83
$\alpha_1\beta_1$ -integrin	PBS, pH 7.4, 0.2% octylglucoside or 0.25% CHAPS	72
Intestinal peptide transporter	100 mM Na phosphate, pH 7.0, 0.03% Triton X-100 or 0.7% octylglucoside	84
Mannitol-specific enzyme II of the <i>E. coli</i> PEP-dependent phosphotransferase system	50 mM Tris-HCl, pH 7.0, 50 mM KCl, 1 mM DTT, 0.5% decylPEG, 1% octylPEG, 1.25% octylglucoside, 4% cholate	85

(continued)

TABLE 3 (continued)

Protein/proteins (from)	Elution buffer	Ref.
Plasma membranes (liver)/Morris hepatoma	8 mM Na phosphate, pH 7.1, 155 mM NaCl, 0.1% SDS 10 mM Tris-HCl, pH 7.1, 155 mM NaCl, 0.05% CHAPS	86 87
Membrane glycoprotein antigen (P815 mouse mastocytoma cell line)	10 mM Na phosphate, pH 7.4, 150 mM NaCl, 0.25% Na deoxycholate	88
<i>N</i> ⁵ -Methyltetrahydromethan- opterin:coenzyme M	50 mM K Mops, pH 7.0, 10 mM MgCl ₂ , 2 mM dithiothreitol, 0.1% dodecyl- β -D-maltoside	89
MHC class II-peptide antigen complexes	100 mM Na phosphate, pH 7.0, 0.025% CHAPS	90
Muscarinic acetylcholine receptor	200 mM Na phosphate, pH 7.0, 0.01 M carbamylcholine, 0.1% digitonin	91
<i>Mycoplasma gallisepticum</i>	20 mM Tris-HCl, pH 8.5, 1 mM DTT, 0.08% DDMAU	16
NADH oxidase	20 mM Tris-HCl, pH 8.2, 50 mM NaCl, 0.1% Triton X-100	92
Platelet-derived growth factor receptor fragments	100 mM Na phosphate, pH 7.0, 0.1% SDS; 10 mM Na phosphate, pH 7.0, 6 M guanidine HCl	93
Prostaglandin H ₂ synthase	10 mM Tes, 20 mM Na ₂ SO ₄ , pH 7.4, 0.5 M NaCl, 1% C ₁₀ E ₇	94
Rat lens	50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% SDS	95
Reaction center from <i>Rhodospseudomonas</i> <i>sphaeroides</i>	30 mM Hepes, pH 7.0, 10 mM imidazole, 0.1 mM CaCl ₂ , 200 mM Na acetate, 0.05% C ₁₂ E ₈	64
Sendai virus	50 mM Na phosphate, pH 6.5, 0.1% SDS 35 or 45% acetonitrile in 0.1% HCl, or 25 and 40% ethanol-1- butanol (4:1)	22, 96 27
	50 mM Na phosphate, 0.1% decylPEG, 0.1% decanoylmethylglucamide	97
Tick-borne encephalitis virus	50 mM Na phosphate, pH 6.5, 0.1% SDS	26
Vesicular monoamine transporter	200 mM Na phosphate, pH 6.9, 0.1% SDS, with and without TFA treatment	98

TABLE 4 Gradient Systems Used for IEHPLC of Membrane Proteins

Protein/proteins (from)	Gradient system	Ref.
ATPase complex	0–1 M NaCl in 5 mM MgCl ₂ , 20 mM Tricine, 0.03% octylglucoside, 1.5 mM DTE adjusted to pH 7.6 with NaOH	99
Blood platelet membrane	0–1 M NaCl in 0.5% Berol 185, 0.02 M triethanol	69
Bovine viral diarrhea virus	0–1 M NaCl in 50 mM Tris-HCl, pH 7.0, 0.18–0.20% Berol 172	100
	0–1 M NaCl in 20 mM piperazine acetic acid, pH 6.0, 0.18–0.20% Berol 172	100
	0–0.5 M NaCl in 20 mM ethanolamine, pH 9.2, 0.5% Berol 185	71
Chloroplast energy coupling complex	3–20 mM ammonium acetate in chloroform/methanol/water	59
Cytochrome <i>b₆f</i> complex (<i>Synechocystis</i> PCC 6803)	5–120 mM MgSO ₄ in 20 mM Mes, pH 6.5, 10 mM MgCl ₂ , 30 mM CaCl ₂ , 0.5 M mannitol, 0.03% dodecyl- β -D-maltoside	73, 73a
Reaction center photosystem I and II		
Cytochrome <i>c</i> oxidase	0–0.4 M NaCl or ammonium sulfate in 40 mM Tris-HCl, pH 7.5, 0.05% dodecyl- β -D-maltoside	101
Cytochrome P-450	0–800 mM Na acetate in 20 mM Tris-acetate, pH 7.2, 20% glycerol, 0.2% Emulgen	102
	NaCl gradient, Mono Q column, 10 mM Tris-HCl, pH 7.7, many detergents	103
	NaCl gradient, Mono S column, 10 mM K phosphate, pH 6.5	103
Epstein-Barr virus	NaCl gradient, 20 mM piperazine-HCl, pH 5.2, 0.5% Mega-9	104
Erythrocytes (human)	0–0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.054% C ₁₂ E ₈ , 0.049% C ₁₂ E ₇ , 0.045% C ₁₂ E ₆ , 0.041% C ₁₂ E ₅	105
	0–1 M NaCl, 50 mM Tris-HCl, pH 7.0, 0.9% octylglucoside and others	106

(continued)

TABLE 4 (continued)

Protein/proteins (from)	Gradient system	Ref.
Herpes simplex virus	0–0.5 M NaCl, 20 mM Tris-HCl, pH 7.8, 0.005–0.1% C ₁₀ E ₅	30
	0–0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% reduced Triton X-100	107
	0–0.05 M NaCl, 20 mM Tris-HCl, pH 7.8, 0.01–0.2% DDMAU	30a
Intestinal peptide transporter	NaCl gradient, Mono Q column, 10 mM Tris-HCl, pH 7.4, 0.03% Triton X-100, 0.7% octylglucoside	84
	NaCl gradient, Mono S column, 20 mM Na acetate, pH 4.5, 0.03% Triton X-100, 0.7% octylglucoside	84
<i>Leishmania</i>	Triton X-114 phase separation, 0–0.5 M NaCl in 10 mM Tris-HCl, pH 8.0, 0.03% Triton X-100	50
Measles virus	0–1 M NaCl in 20 mM piperazine, pH 9.5, 0.03% Triton X-100 or 0.88% octylglucoside	108
Membrane antigen (<i>Plasmodium falciparum</i>)	0–0.5 M NaCl, 20 mM Tris-HCl, pH 7.6, 0.01–0.1% C ₁₀ E ₅	31
Membranes (<i>E. coli</i>)	0–0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 0.054% C ₁₂ E ₈ , 0.049% C ₁₂ E ₇ , 0.045% C ₁₂ E ₆ , 0.041% C ₁₂ E ₅	105
N ⁵ -Methyltetrahydromethanopterin:coenzyme M	140–540 mM NaCl, 50 mM K Mops, pH 7.0, 10 mM MgCl ₂ , 2 mM dithiothreitol, 0.1% dodecyl-β-D-maltoside	89
<i>Mycoplasma gallisepticum</i>	0–1 M NaCl, 10 mM Tris-HCl, pH 8.5, 1 mM DTT, 0.08% DDMAU	16
Outer membrane proteins (OMPs from <i>E. coli</i>)	0–0.5 M NaCl in 10 mM Tris-HCl, pH 8.0, 0.05% C ₁₂ E ₈	105
Plasma membranes (liver)/Morris hepatoma	0–1 M NaCl in 10 mM Hepes, pH 6.1, 0.05% CHAPS	87
	0–1 M NaCl in 20 mM Hepes, pH 6.5, 0.1% Genapol X-100	109
Platelet-derived growth factor receptor	0–0.5 M NaCl in 20 mM Hepes, pH 7.4, 1 mM DTT, 1 mM EGTA, 10% glycerol, 0.2% Triton X-100	110
Protein tyrosine phosphatase	0–0.5 M NaCl in 10 mM Tris-HCl, pH 6.0, 0.1% CHAPS	111

TABLE 4 (continued)

Protein/proteins (from)	Gradient system	Ref.
Sendai virus	0–0.5 M NaCl in 20 mM Tris-HCl, pH 7.8, 0.1% Triton X-100	22
	0.15–1.5 M NaCl in 20 mM Na phosphate, pH 7.2, 0.1% Triton X-100	112
	0–0.5 M NaCl in 20 mM Tris-HCl, pH 7.8, 0.1% decylPEG or 0.1% octylglucoside	113
	0–0.5 M NaCl in 20 mM Tris-HCl, pH 7.8, 0.1% LDAO, SB12, C ₁₂ E ₅	114
	0–0.5 M NaCl in 20 mM Tris-HCl, pH 7.8, 0.013–0.1% C ₁₀ E ₅ , 0.001–0.1% C ₁₂ E ₅	29
	0–0.5 M NaCl in 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 1% octylglucoside	115
Submandibular and parotid gland (rat)		

At a low concentration of detergent, mainly hydrophilic proteins were eluted during the salt gradient [29]. A second step with a high concentration of detergent in the elution buffer resulted in elution of the membrane proteins. Integral membrane proteins of Sendai virus [29], herpes simplex virus [30,30a], and *Plasmodium falciparum* [31] were purified by this approach.

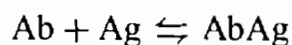
pH gradient elution, instead of elution by an increase in ionic strength, is used in the chromatofocusing technique developed by Sluyterman and Elgersma [32,33]. A pH gradient is generated within the column using buffers of low ionic strength [34], which results in the elution of proteins somewhat above their isoelectric points. This technique has also been applied for the purification of membrane proteins with soft gel columns [35,36]. Isozymes from mouse hepatic cytochrome P-450 in a buffer containing 0.5% Tergitol NP-10 were purified to some degree [35]. Although these results are promising, application of chromatofocusing in the HPLC mode will probably give better resolution. For most of the proteins mentioned in Table 4, gradient elution systems that were not harmful to the structural integrity of the protein were used, i.e., near-physiological conditions were used for the chromatography. Although finding the optimal conditions for IEC of a membrane protein may be time consuming, it is the method of choice for the purification of an intact membrane protein.

Affinity Chromatography

Affinity chromatography is a separation procedure in which the protein to be separated is selectively adsorbed by an immobilized secondary molecule. Undoubtedly the most specific method used to purify membrane proteins at the moment is immunoaffinity chromatography. In this method, the specificity of an antibody directed against a protein is used as a tool in the purification.

An important event that was responsible for the progress in immunoaffinity chromatography was the development of monoclonal antibodies in 1975 by Köhler and Milstein [37]. Monoclonal antibodies are directed to one specific region of a protein. The antibody binding region is called an epitope and usually comprises at least six to eight amino acid residues.

The essence of immunoaffinity chromatography is the antibody-antigen reaction. Multiple electrostatic, hydrophobic, van der Waals, and hydrogen-binding forces are responsible for the antigen-antibody reaction. These forces also affect the strength of the binding of antibody to antigen. The antigen-antibody reaction is represented by the equilibrium:



where Ab is the free antibody, Ag is the free antigen, and AbAg is the immunocomplex. Applying the law of mass action:

$$K_a = k_a/k_d = [\text{AbAg}]/[\text{Ab}][\text{Ag}]$$

where K_a is the affinity, binding, or association constant in M^{-1} , k_d is the dissociation rate constant (in sec^{-1}), and k_a is the association rate constant (in $\text{M}^{-1} \text{sec}^{-1}$).

For ideal immunoaffinity conditions, K_a should have a value between 10^4 and 10^8 M^{-1} . When $K_a < 10^4 \text{ M}^{-1}$, the affinity of antibody and antigen is too weak for purification. In contrast, when $K_a > 10^8 \text{ M}^{-1}$, the affinity is too strong and special elution conditions are needed. Even with conventional low-pressure systems, immunoaffinity chromatography is a relatively fast separation technique that usually results in the isolation of a pure product, in many cases without loss of biological and immunological activities.

Because applications in classical immunoaffinity chromatography using soft gel supports, i.e., coupling to CNBr-activated Sepharose, are numerous, we will address the use of such columns and the corresponding systems used for elution in the purification of membrane proteins. Most of the conventional immunoaffinity purifications described in the literature use monoclonal antibodies coupled either directly to Sepharose 4B or indirectly through reaction with protein A immobilized to Sepharose 4B. Occasionally, Sepharose Cl-4B or Sepharose Cl-6B is also used. Antibody density is generally from 5 to 10 mg of immunoglobulin G (IgG) per g of Sepharose 4B. Extensive washing is

recommended after coupling the immunoglobulin to the Sepharose. Washing buffers usually contain detergent, salt, and EDTA. Subsequently, the column is equilibrated with the buffer to be used for the solubilization and extraction of the membrane proteins.

Membrane proteins to be purified have to be solubilized from cells or from the envelope of virions. The most commonly used lysis or extraction buffer is 10 mM Tris-HCl, pH 7.6–7.8, supplemented with 0.10–0.15 M NaCl and 1–2% Triton X-100 or NP-40. Sometimes deoxycholate (up to 0.5%), 0.6 M KCl, and 0.5 mM $MgCl_2$ are added as well. To avoid proteolytic degradation during the extraction procedure, protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), EDTA, and aprotinin are added. After extraction, insoluble residues are removed by ultracentrifugation or by filtration, and the extract is then applied to the immunoabsorbent column. In some cases, it may be preferable to perform the adsorption batchwise and to pack the column afterward.

The conditions necessary to elute adsorbed membrane proteins may vary and are dependent on the affinity of the antibody for the antigen (see Table 5a). Disruption of antibody-antigen bonds can be achieved either by changing the ionic conditions of the mobile phase or by the introduction of additives such as chaotropic ions. In addition, polarity-reducing agents (10% glycerol, detergents)

TABLE 5a Elution Buffers Used in "Classical" Immunoaffinity Chromatography of Membrane Proteins

Buffers	Ref.
Acidic or basic buffers	
0.01 M citrate, pH 2.2, occasionally supplemented with 0.3 M NaCl	116, 117
0.1 M glycine-HCl, pH 2.5, plus 0.1% Triton X-100	118, 119
3% acetic acid in 0.1 M NaCl	120
0.15 M ethanolamine, pH 11.2, 0.1 M NaCl and 10% glycerol	121, 122
0.05–0.1 M diethylamine, pH 11.5, with 0.5% DOC, 0.5% NP-40, or 3.3% Brij 96	123, 124
Chaotropic buffers and denaturing agents	
3 M KSCN in various buffers, for example 10–50 mM Tris-HCl, pH 7.8, supplemented with 150 mM NaCl, 1 mM EDTA, 600 mM KCl, 0.5 mM $MgCl_2$, and 2% Triton X-100	119, 125–129
3 M NaSCN in 0.01 M Tris-HCl, pH 7.8, supplemented with 2% Triton X-100 or 0.05% sarkosyl	130, 131
4 M $MgCl_2$	132, 133
3 M NaCl	134
6 M guanidine-HCl in 0.1 M Tris-HCl, pH 7.6	117

are useful in the dissociation of hydrophobic reactions between antigen and antibody. Denaturing agents, i.e., high concentrations of urea and guanidine hydrochloride, are very effective in the elution but have the disadvantage of denaturing the protein. The application of stepwise or linear pH or salt gradients may be necessary to find the optimal elution conditions for a particular membrane protein.

Immunoaffinity chromatography using high-performance column supports has never become popular. The low price of soft gel supports, the convenient implementation of ready-for-use soft gels in an HPLC system, and the satisfactory speed of soft gel systems are most certainly reasons for this.

Other affinity chromatography methods for purification of membrane proteins are listed in Table 5b. Various tailor-made ligands can be used. Ni^{2+} -nitrilotriacetic acid is becoming more and more popular as a ligand in metal chelate chromatography. Using recombinant DNA technology, the target protein is provided with a His tail (six histidines). The high affinity of these histidines for metal chelate supports allows the purification of such a protein [24,25,25a].

Reversed-Phase Chromatography

The strategy for purifying a membrane protein largely depends on the nature of its association with the membrane. Peripheral membrane proteins are membrane bound through interactions with surface components of the lipid bilayer. After solubilization with compounds such as chaotropic agents, they resemble water-soluble proteins and may be purified accordingly. However, the large number of hydrophobic amino acids present in membrane-spanning regions generally makes integral membrane proteins more hydrophobic than an average water-soluble protein (see "Introduction" and Table 1). In aqueous media, intermolecular association of these hydrophobic patches on the protein surface may lead to aggregation.

Because the hydrophobic surface layer of RPC sorbents somewhat mimics the natural environment of membrane proteins, separation based on differences in hydrophobic interaction between hydrophobic regions of the protein and the hydrocarbonaceous ligands would seem the method of choice for the separation of these proteins. Moreover, in contrast to IEC and SEC, the presence of a detergent is not required because the organic solvent used for elution prevents aggregation and to some degree keeps the membrane protein in solution. RPC has been used for the purification of membrane proteins with a variety of elution systems that all include an organic solvent (Table 6). Hydrophilic proteins are generally eluted from the commercially available RPC packings with 10 to 40% organic solvent. In contrast, some of the much more hydrophobic membrane proteins often require solvent concentrations up to 60–70%.

TABLE 5b Elution Buffers Used in Affinity Chromatography (Not Immunoaffinity) of Membrane Proteins

Column ligand	Elution buffer	Ref.
Adenovirus	0.1 M Gly-HCl, pH 2.6, 0.1 M NaCl, 0.037% Triton X-100	135
Concanavalin A	2 mM Hepes, pH 7.4, 1 M NaCl, 10% glycerol, 1 M α -methylmannopyranoside	136
	10 mM Tris-HCl, 150 mM NaCl, 1 mM Ca^{2+} , 1 mM Mg^{2+} , 0.1% Genapol X-100, 0.1% octylglucoside, or 0.1% CHAPS,	137
	0.2 M α -methylmannopyranoside	
Cytochrome c	20–200 mM Tris-acetate, pH 7.0, 5% sucrose, 0.2 mM PMSF, 2 mM ascorbate, 0.04% Triton X-100	138
	20–28 mM NaCl, 20 mM Tris-acetate, pH 7.1, 1 mM EDTA, 5% sucrose, 0.5 mM ascorbate, 0.05% Triton X-100	139
Epidermal growth factor	5 mM ethanolamine, pH 9.7, 10% glycerol, 0.2% Triton X-100	140
Hexylglutathione	50 mM Hepes, pH 7.6, 1 mM EDTA, 5 mM β -mercaptoethanol, 10% glycerol, 0.1% Triton X-102, 0.1% Na deoxycholate, 15 mM probenecid	141
Ni^{2+} -nitrilotriacetic acid	150 mM imidazole in 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 50 mM NaCl, 50 μM PMSF, 10% glycerol and 0.1% octylglucoside	24
	100 mM imidazole in 50 mM Tris-HCl, pH 7.5, 10 mM β -mercaptoethanol, 0.5 M NaCl, 10% glycerol and 0.04% dodecyl- β -D-maltoside	25
	0–1 M imidazole in 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 2% Triton X-100	25a
Histone	20 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.1% Na cholate, 0.15% octylglucoside or 0.6% CHAPS	142
Leukotriene C ₂	50 mM Hepes, pH 7.6, 1 mM EDTA, 5 mM β -mercaptoethanol, 10% glycerol, 0.1% Triton X-102, 0.1% Na deoxycholate, 0.5 M NaCl, 5 mM reduced glutathione	141
Mannan	30 mM Na phosphate, pH 7.0, 0.025% Triton X-100, 0.5 M α -methylmannopyranoside	143

(continued)

TABLE 5b (continued)

Column ligand	Elution buffer	Ref.
Single-chain Fv + Strep tag	5 mM diaminobiotin or 5 mM lipoic acid in 50 mM Tris-HCl, pH 8.0, 0.025% dodecyl- β -D-maltoside, 1% octylglucoside or 0.1% LDAO	144
Heparin	0.15–2 M NaCl in PBS, pH 7.4, 0.1% C ₁₂ E ₈	144a

The limited solubility of proteins at high organic solvent concentrations is one of the main causes of the frequently observed broad peaks and poor recovery of membrane proteins with RPC. It is of interest to note that the application of a higher temperature, 50°C [38], and the use of mixtures of organic modifiers, such as acetonitrile-propanol [26,39] and ethanol-butanol [40], have been used to overcome this problem. Waterfield et al. [41] used a propanol gradient in 2 M guanidine-HCl containing 1 M acetic acid to purify the platelet-derived growth factor. This solvent system may also be useful for RPC of membrane proteins. 2-Propanol or acetonitrile in 60% formic acid, which was used for the isolation of poliovirus capsid proteins [42,43], may be of use when all other solvents fail. However, prolonged contact of a protein with high concentrations of formic acid may result in esterification of Ser and Thr residues or in cleavage at Asp-Pro bonds. Short exposure appears not to be harmful [39,43].

Mass Recovery

Recoveries in SEC, IEC, and IAC are generally higher than 80%. In contrast, recoveries in RPC vary from 0 to 100%. In the case of Sendai virus proteins [40], the yields for the reduced proteins F₁, F₂, M, and HN ranged from 5 to 100%. Yields were enhanced by using a column with a larger pore size, as discussed in the RPC application section.

TROUBLESHOOTING

Problems Associated with Membrane Proteins

One of the main problems currently associated with RPC of membrane proteins is the low recovery of protein. This is probably due to on-column precipitation caused by the decreased solubility of the proteins at the high organic solvent concentrations needed for their elution. RPC on a column with a large pore size (see Fig. 5) sometimes results in higher yields [40]. In order to increase the

TABLE 6 Elution Systems Used in RPHPLC of Membrane Proteins

Protein/proteins from	Gradient system (v/v)	Ref.
Alveolar epithelial cell apical membrane protein (type I)	A: formic acid–water, 3 : 2 B: formic acid–acetonitrile, 3 : 2	60a
Cardiac membrane proteolipids	A: chloroform/methanol/ water/TFA, 200 : 100 : 0.75 : 0.3; B: same components, 30 : 30 : 10 : 0.07	60
Chloroplast energy coupling complex	A: chloroform/methanol/ water/TFA, 200 : 100 : 0.75 : 0.3 B: same components, 30 : 30 : 10 : 0.07	59
Cytochrome P-450 fragments	A: 6 M formic acid, 0.13 M triethylamine, pH 1.5 B: 4 M formic acid, 0.09 M trimethylamine in propanol; other organic modifiers: acetonitrile and acetonitrile/propanol, 2 : 1	39
Cytochrome <i>c</i> oxidase	Acetonitrile gradient to 55% in 0.1% TFA	101
Herpes simplex virus	10–40% propan-2-ol in 50% formic acid	145
Ia antigens	A: 0.2% Triton X-100, 0.1% triethylamine, pH 3.0 B: acetonitrile	82
Influenza virus	Gradient of acetonitrile in 0.05% TFA	146
Moloney murine leukemia virus	Gradient of acetonitrile at 23°C followed by 1-propanol at 50°C, both in 0.1% TFA	38
<i>Mycoplasma hyopneumoniae</i>	A: formic acid–water, 65 : 35 B: formic acid–acetonitrile 65 : 35	48
Myelin glycoprotein P ₀	Acetic acid water (1 : 1), gradient acetic acid/2 propanol (1 : 1)	147
Rhodopsin (hydrophobic tryptic peptides)	A: 0.1% TFA, 0.08% morpholine, 0.1% octylglucoside (w/v) in water B: 0.1% TFA, 0.08% morpholine, 0.1% octylglucoside (w/v) in acetonitrile-1-propanol-water, 2 : 2 : 1, 55°C	148

(continued)

TABLE 6 (*continued*)

Protein/proteins from	Gradient system (v/v)	Ref.
Sendai virus	15–75% acetonitrile, 0.05% TFA Gradient of ethanol/butanol, 4 : 1 in 0.1% HCl	22, 96 40
Tick-borne encephalitis virus	Gradient of acetonitrile/ 2-propanol, 1 : 1 in 0.01 M TFA	26

recovery of both mass and biological activity, strategies that reduce the concentration of organic modifier needed for elution of the membrane protein should be employed.

Because the hydrophobic nature of a protein is determined by the total number of hydrophobic groups, it is to be expected that RPC of small membrane proteins will be more successful than similar chromatography of larger proteins. With equal percentages of hydrophobic residues, the organic solvent concentration necessary for protein desorption will increase with protein size. Thus, if conservation of the biological activity is not crucial, a reduction of the size of the protein will generally enhance protein recovery and separation efficiency. For instance, when Sendai virus membrane proteins were subjected to treatment with dithiothreitol to reduce inter- and intramolecular disulfide bonds, the resultant mixture contained membrane proteins with molecular masses between 13 and 67 kDa. Subsequent RPC of these proteins showed that they were eluted at lower organic modifier concentrations and in higher yields in the reduced than native forms.

A second approach used to elute membrane proteins with mobile phases of higher water content is to use an organic modifier of higher eluotropic strength, for example, 1- or 2-propanol instead of acetonitrile. For this purpose, mixed organic phases have also been used successfully. Generally, with mixtures of *n*-butanol in ethanol or in propanol, proteins are eluted at lower organic solvent concentration [22,40,44]. Improved recovery of both mass and biological activity was ascribed to the fact that the increase in eluent strength with mixtures of organic solvents is greater than the increase in the denaturing effect and possibly also than the protein-precipitating effect of the eluent.

A third possibility is to use solvents that contain phosphoric acid or hydrochloric acid instead of trifluoroacetic acid (TFA) as the ionic modifier. This generally results in elution at lower organic solvent concentration without affecting resolution [40,44].

Another problem associated with RPC of membrane proteins is the interference of detergents with UV detection. If UV monitoring is desired, UV-absorbing detergents such as Triton X-100 and NP-40 might be removed by

treatment with Amberlite XAD-2 or Biobeads SM-2 [40,45]. We favor the use of Amberlite XAD-2. The resin (500 mg/mL) was added to the extract and the mixture was rotated for 30 min at room temperature. Prior to chromatography, the resin was removed by centrifugation over a 2- μ m column frit mounted in a 1-mL Eppendorf tip. We found that subsequent RPC of Sendai virus proteins did not differ from chromatography with extracts obtained with UV-transparent detergents that had not been removed (unpublished results). The collected filtrate, a turbid solution probably consisting of aqueous dispersions of aggregated membrane protein, was then directly used for chromatography. In some situations, the membrane protein can be eluted before the strongly absorbing detergent peak(s) and removal of the latter is not essential. To increase resolution between the detergent and the protein, one may take advantage of the large difference between the solvent strength value (S) of the low-molecular-weight detergent and the higher S value of the protein [46]. Thus, a longer column will enhance resolution between a detergent and a protein [47].

In another approach, *Mycoplasma* membrane proteins were first precipitated from the detergent phase with 90% ethanol after Triton X-114 extraction. Subsequently, proteins were dissolved in 65% formic acid and separated with a formic acid–acetonitrile gradient [48].

Finally, on-column removal of detergent was achieved by having proteins retained on reversed-phase columns at high organic solvent concentrations, at which detergents are not retained and pass through the column. Proteins were then eluted with an inverse gradient, decreasing the organic solvent concentration after addition of TFA [49].

APPLICATIONS

HPSEC

Triton X-100 extracts of Sendai virus and several strains of the closely related Newcastle disease virus (NDV) were subjected to SEC. The elution patterns are shown in Fig. 3. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) showed that the tetramer and the dimer of the Sendai virus hemagglutinin-neuraminidase (HN) protein (272 and 136 kDa, respectively) and the monomeric fusion (F) protein (65 kDa) were present in peaks 1, 2, and 3, respectively. These peaks are followed by an extremely large peak that contains Triton X-100 micelles. The elution patterns of the other viruses show that there are notable differences when they are compared with that of Sendai virus. In some cases, for example, the strains La Sota and Texas, the most prominent protein eluting from the column is an HN monomer. In other cases, multimeric forms of the HN protein that differ from Sendai HN protein were eluted (strains Mukteswar, Florida, and Herts).

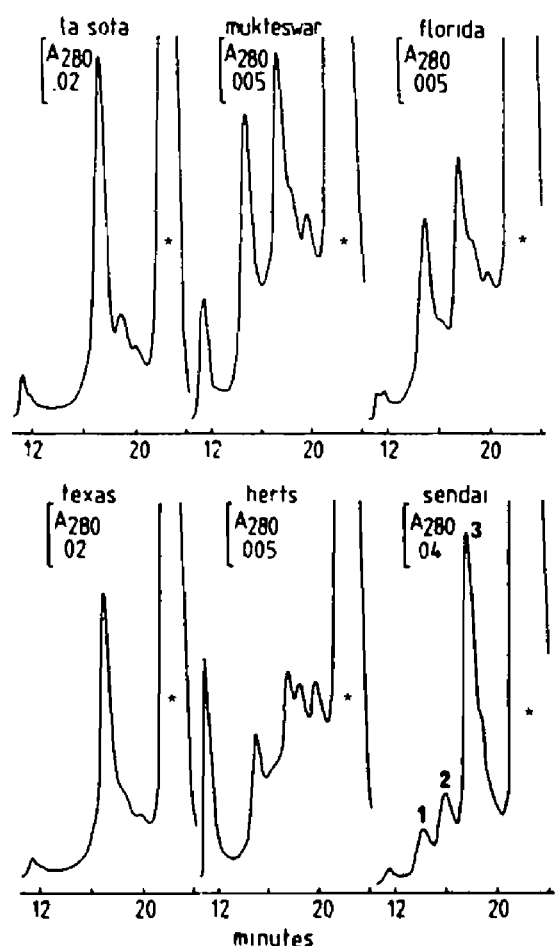


FIGURE 3 HPSEC of a Triton X-100 extract of purified Sendai and Newcastle disease virions. Virus was grown in 10-day-old embryonated chicken eggs and purified and extracted as described [5]. A TSK 4000SW (600 × 7.5 mm ID) column was eluted with 50 mM sodium phosphate, pH 6.5, containing 0.1% SDS. Samples were boiled for 2 min in 4% SDS prior to chromatography. The flow rate was 1 mL/min and the absorbance was monitored at 280 nm. 1, Tetramer of the hemagglutinin neuraminidase (HN) protein of Sendai virus; 2, dimer of HN; 3, Sendai virus fusion protein F; *, Triton X-100.

HPIEC

An interesting application utilizing IEC after detergent extraction with Triton X-114 [19] is described by Bouvier et al. [50]. The major integral membrane glycoprotein (p63) that is found on the surface of *Leishmania* promastigotes has been purified in three steps: (1) the specific extraction and phase separation of the integral membrane protein with Triton X-114, (2) DEAE-cellulose chromatography to exchange the 12% Triton X-114 present in the detergent phase for 0.03% Triton X-100, and (3) purification of the ^{125}I -labeled glycoprotein by IEC on a Mono-Q column. After equilibration with 10 mM Tris-HCl, pH 8.0 and 0.03% Triton X-100, elution was performed with a linear gradient of NaCl. Three

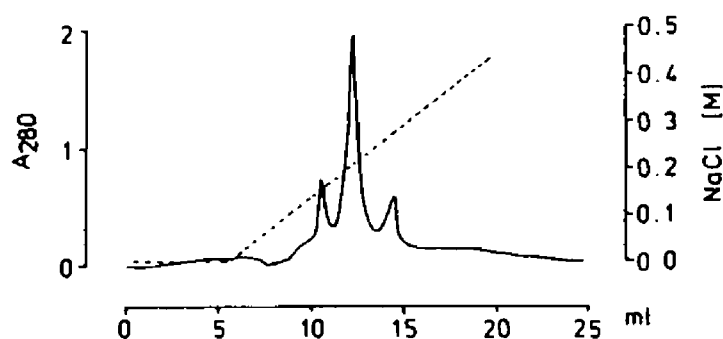


FIGURE 4 HPIEC of *Leishmania* membrane proteins. Pooled DEAE-cellulose fractions were diluted with 2 volumes of starting buffer, 10 mM Tris-HCl, pH 8.0, 0.03% Triton X-100, and applied to a Mono Q column (5 × 50 mm). Elution was performed with a gradient from 0 to 0.5 M NaCl (dotted line) at a flow rate of 1 mL/min. Absorbance was monitored at 280 nm. The first two peaks contained [^{125} I]*Leishmania* glycoprotein. (From Ref. 50.)

peaks were observed by monitoring the absorbance at 280 nm (Fig. 4). From the radioactivity measurements, it appeared that only the first two peaks contained the glycoprotein. The first peak contained a new hydrophilic form that migrated less far on SDS-PAGE than the micelle-associated form. A proteolytically derived form would have migrated farther. SDS-PAGE of the detergent phases of surface-iodinated promastigotes and promastigotes grown on ^3H -labeled myristate showed the same migration. This indicates that the membrane form of p63 contains one or more covalently bound myristyl residues. Like the variant surface glycoprotein of *Trypanosoma brucei* [5], the *Leishmania* protein interacts with the membrane with a hydrophobic tail containing a dimyristyl glycerol group. Detergent extraction of the IEC peaks with Triton X-114 and subsequent phase separation showed that the protein present in the first peak was found in the aqueous phase (the soluble form of the protein) and the second peak contained protein that showed up in the detergent phase (the protein including its hydrophobic dimyristyl glycerol tail).

RPC

The elution patterns obtained after RPC of a detergent extract of Sendai virus are shown in Fig. 5. A Sendai virus extract contains the tetramer and dimer of the HN protein, the F protein, and the matrix protein M (38 kDa). Disulfide bridges in these proteins were reduced with dithiothreitol, producing two components of the F protein, F1 and F2 (13–15 and 50 kDa, respectively), M protein, and the monomeric form of HN (67 kDa). This mixture was subjected to RPC on an (a) C_{18} column (100 Å), (b) C_1 column (250 Å), and (c) phenyl column (1000 Å). The elution patterns in Fig. 5 show that there is no problem in eluting the two

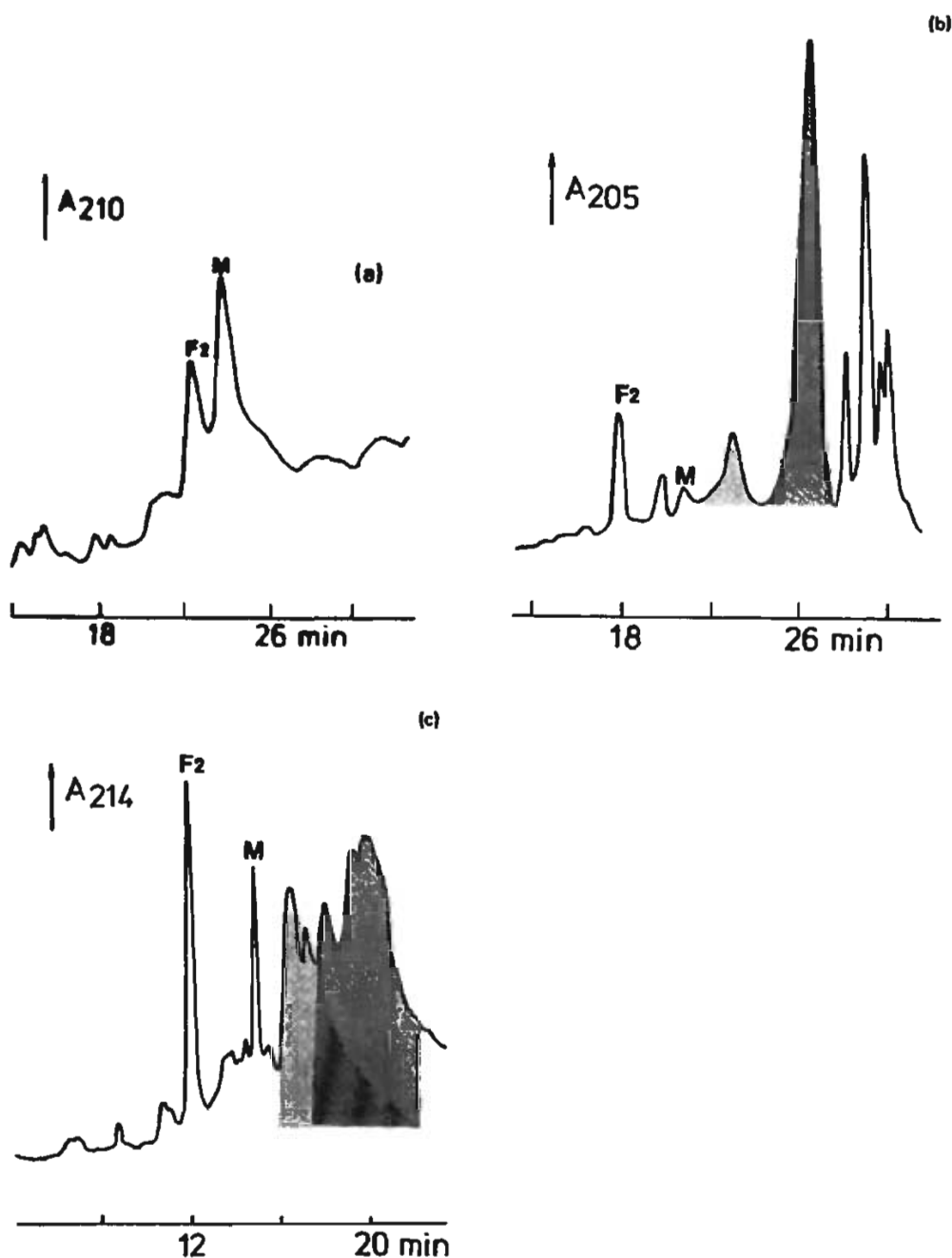


FIGURE 5 RPC of a Triton X-100 extract of purified Sendai virions that was reduced with dithiothreitol. (a) Nucleosil 10 C₁₈ column (300 × 4.6 mm ID), which has a pore size of 100 Å, was eluted with a 24-min gradient of 10–60% ethanol/1-butanol (4:1, v/v) in 0.1% HCl at a flow rate of 1 mL/min. The absorbance was monitored at 210 nm. (b) TMS-250 C₁ column (75 × 4.6 mm ID), pore size 250 Å, was eluted with a 25-min gradient from 25 to 75% acetonitrile in water containing 0.05% TFA; the flow rate was 1 mL/min and the absorbance was monitored at 205 nm. (c) Phe-5PW-RP column (50 × 4.6 mm ID) with pores of 1000 Å was eluted with a 24-min gradient consisting of 15–75% acetonitrile in water containing 0.05% TFA. The flow rate was 1 mL/min and the absorbance was monitored at 214 nm. Peak identification: F₂, the 13- to 15-kDa component of the F protein; M, the 38-kDa matrix protein; dotted area, HN protein; hatched area, F₁ protein.

smaller proteins in this mixture from any column. The two larger proteins are not eluted from the 100-Å column. They could be eluted in low yields in a second blank run [40]. Elution of the protein mixture is better with the 250-Å column, although the HN protein was obtained in relatively low yield [22]. The highest yields were obtained with the 1000-Å column. In addition to the problem of precipitation, these results indicate the importance of pore size when eluting relatively large membrane proteins.

Another application of RPC with membrane proteins is the purification of the inner core proteins and the envelope proteins of murine leukemia virus described by Henderson et al. [38]. Suspended purified virus was disrupted at pH 2.0 (TFA) by the addition of five volumes of saturated guanidine HCl. The resulting solution, which was slightly turbid, was injected. To separate this mixture of all known viral structural proteins on a μ -Bondapak phenyl column, an optimized solvent system was used (Fig. 6). A gradient of TFA in water (pH 2.0) to acetonitrile at 23°C was successively employed followed by a gradient to 1-propanol at 50°C. At 47% 1-propanol, the viral lipids were isocratically eluted. The most hydrophobic protein, p15(E), (see also Table 1), was found in the last peak eluting from the column. Recovery of the viral proteins was nearly quantitative.

CONCLUSIONS AND FUTURE PROSPECTS

HPSEC applied as a single technique for the isolation of membrane proteins will be useful only in special cases in which the desired protein has a large difference in molecular weight from the other components in a mixture, facilitating its separation from other proteins. HPSEC will be more useful in multidimensional chromatography when it is followed by one or more other modes of HPLC.

HPIEC is a versatile technique for the isolation of membrane proteins and its possibilities are still largely unexplored. A wide variety of detergents, salts, and pH values can be employed to obtain a pure protein under physiological conditions.

Affinity chromatography, in particular immunoaffinity chromatography, is the most specific method for purifying membrane proteins. However, a stable ligand, i.e., a monoclonal antibody and/or an engineered fragment thereof, has to be available. Metal chelate chromatography is also very useful when the target protein can be engineered to contain an additional His tail.

RPC is limited to relatively small membrane proteins, although the availability of large-pore-size column materials allows successful chromatography of proteins such as a 67-kDa Sendai virus membrane protein [51].

At present, a wide variety of systems for purification of membrane proteins is available and the choice of a particular solvent system and column will largely

depend on the protein to be purified and on whether an intact protein is required for further studies.

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HPLC of Cereal Endosperm Storage Proteins

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INTRODUCTION

Characteristics of Cereal Endosperm Storage Proteins

Of plant proteins, the endosperm proteins of cereal grains (family Gramineae) are perhaps most important nutritionally and functionally and have been investigated extensively [1–4]. Cereal storage proteins are, however, very difficult to study. Most are insoluble in water or saline solutions but soluble in aqueous alcohol solutions (prolamins) or in solutions containing acid, alkali, denaturants, detergents, or reducing agents (glutelins). These solubilities reflect the atypical amino acid compositions of these proteins (rich in glutamine, proline, and hydrophobic amino acids) and their aggregation through noncovalent and disulfide bonds into high molecular weight (MW) polymers. Each cereal protein class is also very heterogeneous due to gene duplication and mutation during evolution and, in wheat, to polyploidy. For example, wheat's prolamin (gliadin) and glutelin (glutenin) classes each contain at least 100 unique polypeptides. Such hetero-

*Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

geneity and homology place cereal proteins among the most difficult to isolate and characterize. Many traditional analytical methods were useless with these proteins, necessitating development of alternative technologies.

Goals of HPLC Separations

Knowledge of cereal protein compositions permits identification and selection of genotypes during breeding and marketing and prediction of end-use potential and quality. Many methods have thus been developed to purify and characterize these proteins. Early methods, however, were slow, labor intensive, difficult to reproduce or quantify, and lacked sensitivity and resolution. The availability of efficient high-performance liquid chromatography (HPLC) columns that can resolve proteins [5] provided new tools to the cereal chemist [6]. Reversed-phase (RP) HPLC of wheat and maize proteins was first reported by Bietz in 1983 [7], and size exclusion (SE) HPLC of wheat proteins in 1984 [8]. The next few years saw many rapid advances—HPLC methods for cereal proteins were rapidly developed and optimized and have matured. Today, HPLC is one of the most important tools to characterize cereal proteins; hundreds of publications describe its applications. A complete review of these studies is impossible here. To update my earlier review [9], this chapter will summarize the pioneering and most significant advances, showing examples of how HPLC can analyze prolamins and glutelins, the endosperm proteins of cereal grains. The reader is referred to a comprehensive treatise [10] and earlier articles [11–13] for detailed reviews.

METHODS FOR CEREAL PROTEIN HPLC

Extraction of Protein Samples

Extraction Conditions

The complexity of cereal storage proteins often necessitates atypical, multistep extraction procedures. In the classical 1907 procedure of Osborne [14], proteins were divided into four groups based on solubility. Albumins are soluble in water; globulins in dilute salt solutions but not water; prolamins are soluble in aqueous alcohols but not water or salt solutions; and glutelins are soluble in acid or alkali but not in alcohol, water, or salt solutions. Many variations of this procedure have since been developed, especially for prolamins and glutelins, which are most important nutritionally and functionally. Procedures for albumins and globulins are more standard than those for prolamins and glutelins. Also, albumins and globulins are less hydrophobic and smaller than prolamins and glutelins, so they have considerably different elution characteristics upon RP-HPLC and SE-HPLC.

Two extensive articles review methods for cereal protein extraction [15,16]. Here, I will discuss some important recent developments, especially as applied to

wheat, which presents a great challenge due to the complexity of its proteins and its hexaploid nature; wheat gluten may be nature's largest and most complex protein [17].

Prolamins. In the first detailed study of protein extraction conditions for HPLC, Bietz et al. [18] compared aqueous 70% ethanol, 55% 2-propanol, pH 3.1 aluminum lactate buffer, 2 M urea, and 2 M dimethylformamide as gliadin extractants. All were suitable, but urea and dimethylformamide also extracted some glutenin, making resolution appear poorer. Dimethyl sulfoxide (DMSO) is also an efficient extractant but may coextract starch and lead to subsequent problems [19]. Thus, 70% ethanol is often the preferred extractant for RP-HPLC, as for acid gel electrophoresis. Preextraction of albumins and globulins is usually unnecessary because these proteins are less abundant and most elute earlier than gliadins; in some cases, however, early-eluting albumins and globulins decrease significantly after extraction with 0.5 M NaCl [20]. Similarly, defatting is generally unnecessary. Using these procedures, even single kernels can be analyzed [7].

Extraction conditions must be tailored to both the separation method and the protein. For example, comparable gliadins are extracted with 50% and 70% ethanol, but use of 50% ethanol may prevent premature elution of weakly bound proteins during preparative RP-HPLC [21]. With complex mixtures such as those extracted from wheat with 70% ethanol, modifying initial extraction conditions and separating lower MW gliadins from coextracted glutenin oligomers can significantly affect subsequent RP-HPLC results [22]. For example, ω -gliadins are less hydrophobic than α , β , or γ -gliadins, so solvents varying in alcohol content can extract gliadin fractions with varying ratios of ω - to other gliadins [23]. Protein solubilities may also change because of previous treatments—for example, gliadins may be water soluble after exposure to 0.1% trifluoroacetic acid (TFA) [24].

In variations of these schemes, cereal proteins are extracted with alcohol solutions containing reducing agents. This is sometimes desirable because properties of monomeric prolamins and low-MW prolamins subunits of glutelins are often similar [25]. Thus, Marchylo and Kruger [26] recommended extraction of barley hordeins with 50% 1-propanol under reducing conditions for subsequent RP-HPLC, and Kruger et al. [27] solubilized wheat proteins with 50% 1-propanol containing dithiothreitol (DTT). Such procedures may give more complex chromatograms, however, because many alcohol-soluble glutelin subunits are uniquely different from monomeric prolamins.

Glutelins. Glutelins are polymers varying widely in molecular size. Some are soluble in aqueous alcohol, in dilute acids, or at elevated temperatures [25]. Native structures of these polymers, maintained by disulfide and noncovalent bonds, are important functionally but are difficult to characterize. For many

studies, dissociation of glutelins to their subunits is thus necessary. As already noted, sequential extraction is necessary to isolate glutelins free of albumins, globulins, and prolamins. Extraction procedures such as those used for electrophoresis are applicable [28]. Glutenin can also be simply isolated by first extracting nonglutenin proteins with 90% DMSO and then solubilizing glutenin from the remaining residue [29].

Burnouf and Bietz [19] first explored methods for solubilization, stabilization, and RP-HPLC analysis of wheat glutenin. For optimal separations, glutenin was extracted with solutions containing 8 M urea or 6 M guanidine hydrochloride and 5% 2-mercaptoethanol or 0.1% DTT; resulting thiol groups were stabilized by alkylation with 4-vinylpyridine; acidified reaction mixtures could then be analyzed directly by RP-HPLC.

Several studies have since shown, however, that selective precipitation or extraction of glutenin subunits, based on their different solubilities in aqueous alcohol solutions, is useful to fractionate reduced glutenin into alcohol-soluble (low-MW) and -insoluble (high-MW) subunits [30]. This can significantly enhance RP-HPLC resolution because analytical conditions for each protein type can be optimized [31–33].

Extraction of Unreduced Glutelins. Protein molecular sizes or size distributions often influence functional properties, necessitating extraction and characterization of native proteins. Various procedures have solubilized and fractionated unreduced wheat glutenin using dilute acetic acid [34], solutions differing in HCl concentration [35–37], or 50% and 70% 1-propanol [38].

The detergent sodium dodecyl sulfate (SDS) often efficiently extracts unreduced storage proteins. For SE-HPLC, proteins may be extracted with an SDS-containing buffer similar to the mobile phase. Usually the SDS concentration is increased to 1–2% and the sample is heated to ensure total complexing with protein [8,39]. To extract all proteins without reduction, SDS plus mechanical (ultrasonic) shear may also be used [40,41] but may decrease the size of native glutenin.

Protein Extraction from Other Cereals. Extraction procedures for proteins of other cereals are generally similar to those for wheat, but differences among grains necessitate some changes. Maize α -zeins are soluble in 55% isopropanol or 70% ethanol, but for RP-HPLC 70% ethanol containing 5% 2-mercaptoethanol and 0.5% sodium acetate is often preferred [42]; α - and early-eluting γ -zeins (actually glutelin subunits) can then be simultaneously analyzed, and reduction of disulfides facilitates quantitative extraction of α -zeins. Oat proteins have been sequentially extracted with 1.0 M NaCl, 52% ethanol, and borate buffer containing 1% SDS for RP-HPLC [43]. For rice, extraction of prolamins with 1-propanol is preferable to use of acetic acid or alcohol plus reducing agent because resulting

RP-HPLC patterns vary less with environment [44]. Detailed discussions of extraction procedures for cereal proteins have been published [15,16].

Other Considerations in Extracting Cereal Proteins. All components of these many protein solvents (water, organic phase, ion-pairing reagents, urea, reducing agents, salts, organic acids, and SDS) must be of high purity and free of contaminants that coelute with proteins. This can be easily ascertained by analyzing a solvent blank. Particulates must be removed from samples by membrane filtration (0.2–0.5 μm) or centrifugation (25,000–400,000 g for 10–25 min). Slower centrifugation may also suffice because guard columns and/or in-line filters remove any remaining particulates. Sample age may also be important. Proteins may aggregate and precipitate with time, as they pass through their isoelectric point upon equilibration with mobile phase, or if the mobile phase lacks essential solubilizing agents. Thus, all samples should be clarified shortly before analysis.

For many applications, extracted proteins can be analyzed directly by RP-HPLC with no additional processing because ionic components of solvents and samples elute in the excluded “solvent” peak. For large samples, however, care must be taken to avoid premature elution of loosely bound proteins due to excessive hydrophobicity of the extracting solvent itself.

Column Selection

Choosing the best column is the most important decision in HPLC. Reviews have discussed this in detail [45–48]. Marchylo [45] noted that column selection can be bewildering due to the many columns available but that many columns offer satisfactory performance, with some differences in resolution and selectivity. Verevacek and Huyghebaert [47] also reviewed commercial columns for IE-HPLC, SE-HPLC, and RP-HPLC of food proteins, along with column characteristics and manufacturers. Here I will briefly review the status and recent developments in columns for RP-, IE-, and SE-HPLC.

Reversed-Phase Chromatography

Of HPLC methods, RP-HPLC has been used most to analyze plant proteins. Its resolution equals or exceeds that of most methods; it is also fast, reproducible, sensitive, quantifiable, and gives good recovery. Most important, however, it complements other methods as it fractionates proteins based on different surface hydrophobicities. Selection of optimal RP-HPLC columns requires consideration of many factors, including support type, hydrophobic ligand, pore size, particle size, column dimensions, and silanization [46,48].

Initial RP-HPLC separations of cereal proteins, on 250×4.1 mm inside diameter (ID) columns packed with 300 Å C_{18} -coated silica 5- to 10- μm particles,

showed the advantage of wide-pore columns and their good stability and reproducibility [7]. Many additional wide-pore "protein" columns having C_{18} , C_8 , C_4 , C_3 , cyano, diphenyl, and cyanopropyl bonded phases were tested with wheat gliadins and glutenin subunits and barley hordeins [19,49–52]. Some differences in resolution and selectivity were apparent, but good separations were achieved on many commercial columns, with C_8 and C_{18} bonded phases generally preferred. Columns from different sources having comparable bonded phases gave similar separations. Column stability and age can affect reproducibility and resolution, however, and columns of the same type can vary among or even within manufacturers [45]; purchasing multiple columns of the same lot is desirable.

Shorter RP-HPLC columns combined with higher flow rates, steeper gradients, or elevated temperature are, however, also often acceptable [50,52,53]. Their resolution can equal that of longer columns because most proteins either bind tightly or not at all to hydrophobic ligands as gradient conditions change [45]. For example, Huebner and Bietz [54] used 1.2–3.2 mm $\times$ 15 cm C_4 columns to fractionate gliadins and glutenin subunits and to differentiate cultivars. Flow rate, sample size, analysis time, solvent consumption, and cost per analysis were reduced greatly, while excellent resolution was obtained. Some resolution may be sacrificed, but this may be acceptable, as for varietal identification. Further progress in developing rapid RP-HPLC methods for cereal proteins is undoubtedly possible—Chen and Horvath [55] showed that 1.5- to 2- μ m porous support particles provide very rapid analyses of proteins and that novel micropellicular and 4000-Å pore size supports, due to superior mass transfer, may be highly suitable for rapid and efficient HPLC of biological macromolecules.

Another important development in optimizing cereal protein RP-HPLC was reported by Marchylo et al. [56]. Sterically protected wide-pore monofunctional-silane bonded C_8 and CN columns (Zorbax RX-300) gave better resolution and reproducibility of gliadins and glutenin subunits than did conventional silica-based columns and were more stable. A typical separation is shown in Fig. 1.

Size Exclusion Chromatography

SE-HPLC was the first HPLC method applied to cereal proteins [8]. Unreduced wheat proteins were extracted with 0.1 M sodium phosphate buffer, pH 7.0, containing 2.0% SDS and separated on porous silica-based "TSK" columns. Separations were rapid, sensitive, reproducible, and quantitative. Molecular weights from SE-HPLC agreed well with those from other methods, and resolution was similar to that on earlier size exclusion supports.

SE-HPLC can predict wheat breadmaking quality from protein MW distributions [8]. Huebner and Bietz [57] found that quality could also be predicted from MW distributions of glutenin subunits. Dachkevitch and Autran

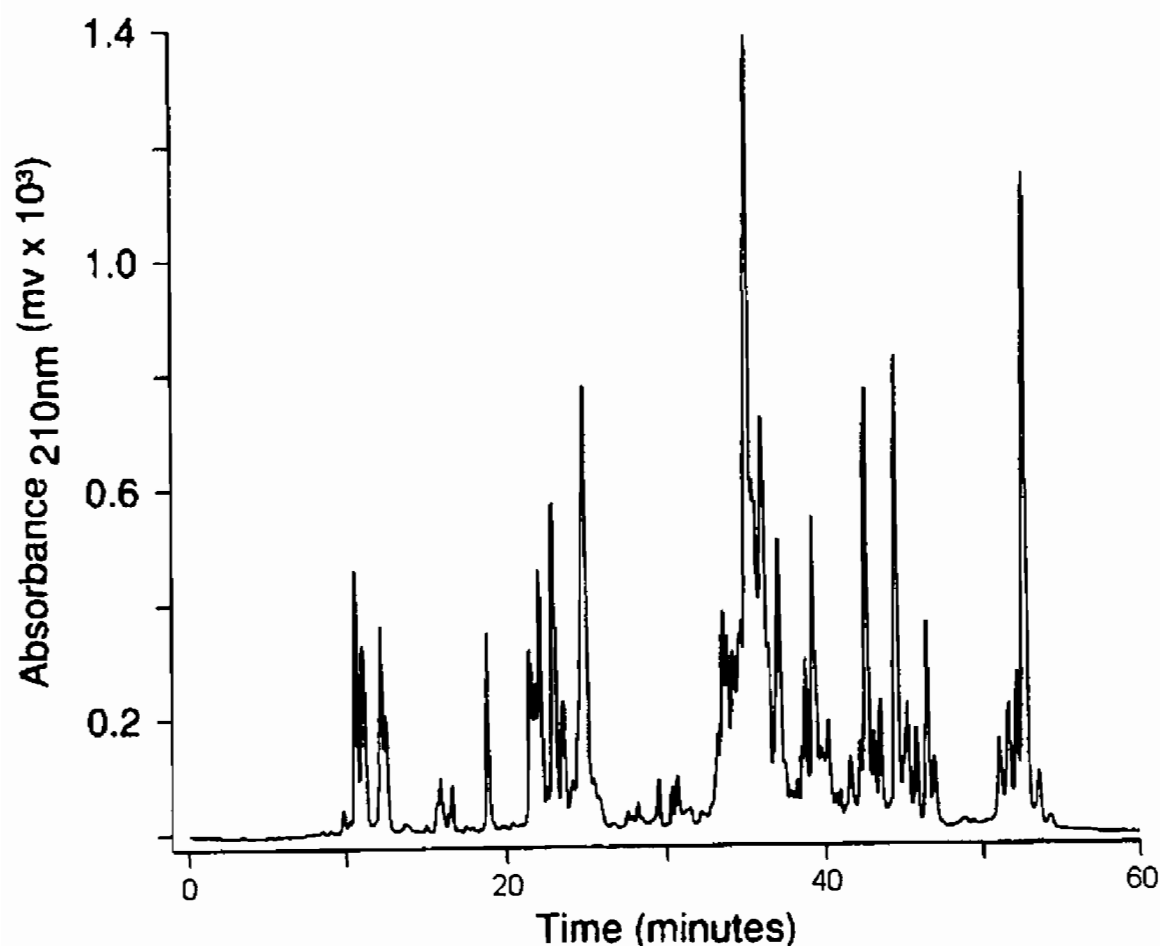


FIGURE 1 RP-HPLC on a Zorbax RX-300-C8 column of gliadins and glutenins extracted under reducing conditions from the wheat variety Neepawa. (From Ref. 56.)

[39] extended these studies and optimized conditions for quantitative extraction of stable samples for analysis, thus improving reproducibility.

SE-HPLC columns are larger and more expensive than RP-HPLC columns and have often had shorter lifetimes. This has led to additional method improvements and to use of alternative columns. Autran [58] has reviewed columns and methods for SE-HPLC of cereal proteins. For example, agarose-based columns are now widely used for SE-HPLC of wheat proteins [59]. In another major development, Batey et al. [60] showed that SE-HPLC resolution and column lifetime could be significantly enhanced by using less SDS (0.15%) in the extracting buffer and using 50% aqueous acetonitrile (ACN) containing 0.1% TFA as the elution solvent. Figure 2 shows typical results using these conditions.

Ion-Exchange Chromatography

Before HPLC, ion-exchange chromatography (IEC) on cellulose columns provided the best chromatographic separations of cereal proteins. Surprisingly,

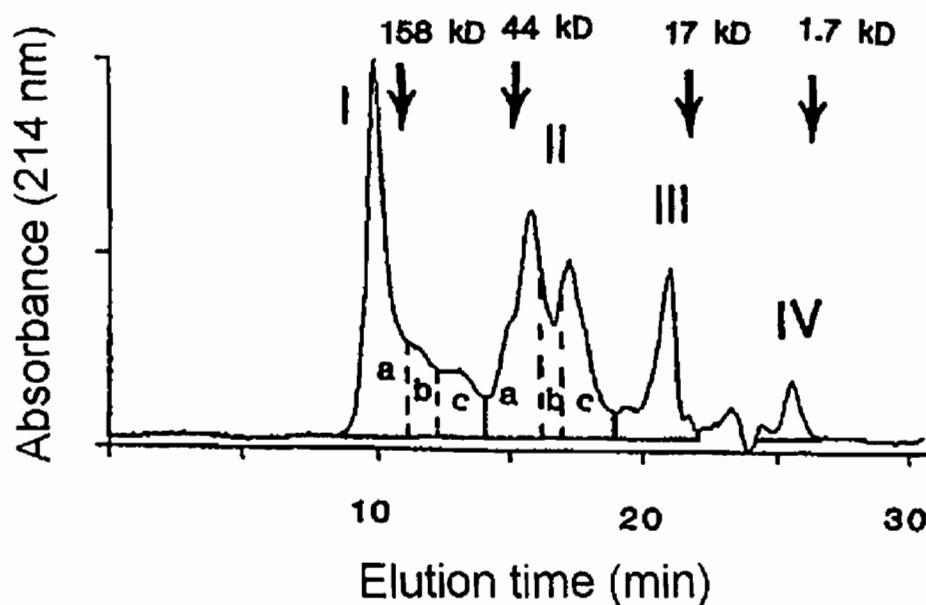


FIGURE 2 SE-HPLC profile of proteins extracted with 0.05 M phosphate buffer, pH 6.9, containing 2% SDS from the wheat cultivar Cook. Proteins were eluted with 50% acetonitrile and water containing 0.1% TFA from a Protein Pak 300 column at 0.5 mL/min. Elution times of MW markers are shown by arrows. (From Ref. 60.)

however, IE-HPLC of cereal proteins has lagged behind RP- and SE-HPLC, possibly because early IEC separations were partially mixed mode, depending upon factors besides ionic binding between solutes and support.

Batey [61] first used IE-HPLC, on a Pharmacia Mono-Q anion-exchange column, to fractionate wheat gliadins for varietal identification. More recently, Larre et al. [62] fractionated gliadins by rapid cation-exchange chromatography on a Mono-S FPLC column; separation speed and resolution were better than on a soft cation-exchange gel, and genotypes were distinguishable from chromatographic patterns. Chirido et al. [63] also separated wheat, rye, and barley prolamins by cation-exchange FPLC on Mono-S, achieving reproducible separations of good resolution that differentiated cultivars. Most recently, Huebner achieved still better separations of gliadins on a Vydac PROT-SCX column (Fig. 3) (F. R. Huebner, personal communication). Undoubtedly, these improvements in cereal protein IE-HPLC, combined with the complementary nature of this method, will lead to its increased use.

Practical Considerations for Cereal Protein HPLC

Mobile Phases

RP-HPLC. Early (~1980) RP-HPLC separations of noncereal proteins achieved excellent resolution using the ion-pairing agent trifluoroacetic acid (TFA) with aqueous gradients of acetonitrile (ACN), propanol, or other organic

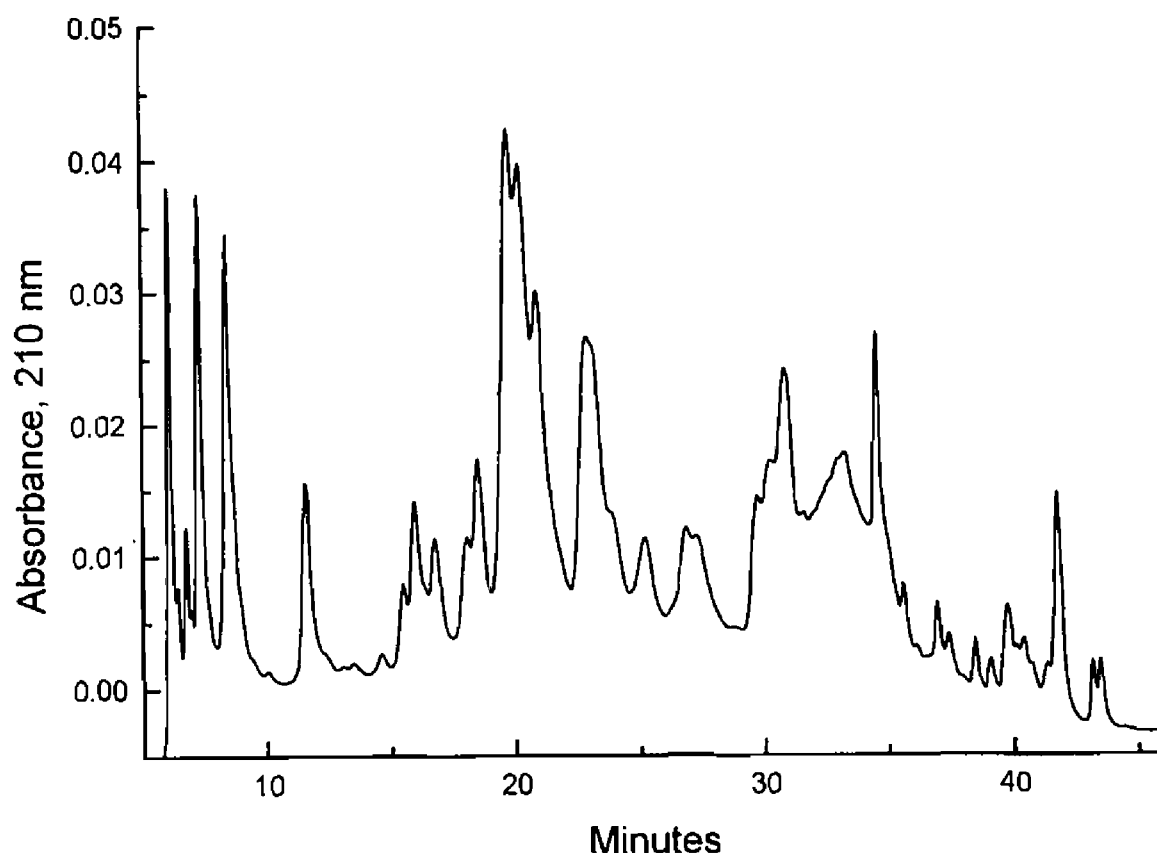


FIGURE 3 IE-HPLC of gliadins from the wheat variety Butte on a Vydac PROT-SCX column (0.75×5 cm). Proteins were extracted with 70% ethanol and eluted at 44°C with a multistep linear gradient of 0 to 0.25 M NaCl in a pH 2.5–2.65 solvent containing 30–35% ACN and 0.045–0.5% TFA (F. R. Huebner, personal communication).

solvents [64,65]. Comparable conditions were applied to cereal proteins by Bietz [7]. ACN gradients with $\sim 0.1\%$ TFA generally gave best results. ACN has low viscosity, is relatively transparent at ~ 210 nm, and generally provides better resolution than gradients of 1- or 2-propanol or methanol. TFA has relatively high absorbance at 210 nm but can be used at $\sim 0.1\%$. Higher TFA concentrations may modify selectivity [7], and inclusion of 0.5% TFA in RP-HPLC solvents enhances resolution of some cereal proteins (G. L. Lookhart, personal communication). Lower TFA concentrations (e.g., 0.05%) are, however, often used to prevent deamidation [66]. Sloping baselines can also be controlled by varying the TFA concentration in solvents [21]. Other ion-pairing agents (including heptafluorobutyric acid and SDS) [7] may modify selectivity but have been little used. Similarly, solvents near neutral pH, although little used, may provide excellent separations—Vensel et al. [67] reported superior separations of γ -gliadins with an acetonitrile/2-propanol (3:1) solvent system containing 0.02 or 0.05 M hexafluoroacetone and adjusted to pH 7.2 with ammonia.

Use of SDS in RP-HPLC solvents has been reported [7,68]. This is somewhat surprising because SDS binds strongly to proteins and may mask hydrophobic surfaces; nevertheless, SDS in RP-HPLC solvents can modify selectivity and enhance resolution, and SDS is a powerful solvating agent. Further studies are required to explain and optimize use of SDS in RP-HPLC. High concentrations of urea (5–6 M) and reducing agents, such as DTT, can also be incorporated into RP-HPLC solvents to ensure protein solubility, modify selectivity, and give excellent separations [69,70].

SE-HPLC. Early SE-HPLC studies of cereal proteins [8], primarily on silica-based columns, used SDS-containing buffers with or without reducing agents. MW estimates were as accurate as from other types of chromatography and electrophoresis; for wheat proteins, MW distributions could be related to breadmaking quality. Dachkevitch and Autran [39] confirmed the validity of SE-HPLC and showed its potential for predicting quality during breeding.

Singh et al. [40] also used an SDS-containing solvent to fractionate wheat proteins by SE-HPLC on silica-based columns, but column performance deteriorated quite rapidly [60]. The alternative solvent of 50% aqueous ACN containing 0.1% TFA (but no SDS) enhanced resolution and greatly increased column lifetime. Comparable solvents (with some variation in ACN and TFA concentrations) are also useful with agarose SE-HPLC columns [59]. Cereal protein SE-HPLC has been reviewed by Autran [58].

IE-HPLC. Many solvent systems are useful for IE-HPLC of cereal proteins. Factors to consider are buffer type and pH (to convert proteins to a charged state in which they bind to ionized support surface groups), ionic strength (for the elution gradient), and use of detergents, denaturants, or organic solvents (to maintain protein solubility). Batey [61] tested pH 8.0, 9.0, 9.5, and 10.4 solvents for anion-exchange HPLC of gliadins. The pH 10.4 solvent [0.01 M 3-(cyclohexylamino)propanesulfonic acid] was best, apparently because many cereal proteins are deficient in negatively charged amino acids and at pH 10.4 arginine loses its positive charge.

Because early ion-exchange separations of cereal proteins were generally best on cation-exchange columns, recent studies have tried cation-exchange HPLC separations. Larre et al. [62] separated gliadins with an NaCl gradient in a pH 3.3–3.8 acetate buffer containing 4 M urea. Chirido et al. [63] separated wheat, barley, and rye prolamins using an NaCl gradient in pH 3.0 citrate buffer containing 2 M urea. Huebner also eluted gliadins with an NaCl gradient, using pH 2.5–2.65 solvents containing 30–35% ACN and 0.045–0.5% TFA (Fig. 3). Batey [71] reviewed practical considerations, including buffer composition, involved in separating cereal proteins by IE-HPLC.

Solvent Composition, Preparation, and Use. All organic components of mobile phases (e.g., ACN and TFA) must be of high purity. Generally, "HPLC-grade" solvents are suitable, but spectral-grade solvents may not be suitable. Water quality is especially important. Bottled HPLC-grade water may be suitable but can deteriorate with time; the best water (especially for RP-HPLC) is produced by freshly processing distilled water in the laboratory with a device having mixed-bed ion-exchange, carbon (organic), and particulate (0.45 μm) filters. All solvents should be deaerated by vacuum, filtration (0.2–0.5 μm), and/or sonication and must be filtered immediately before use. A flat baseline is the ultimate test of solvent suitability; the presence of peaks or increasing baselines in blank runs indicates solvent problems.

Solvent uniformity is essential for HPLC reproducibility, especially with gradient elution. In early studies [7], equipment limitations often precluded use of pure water and ACN (+0.1% TFA) to generate gradients because of solvent outgassing. Instead, 15 and 80% aqueous ACN (+0.1% TFA) were used because all proteins eluted in this hydrophobicity range and outgassing was less severe. With such solvents, however, solute elution positions may change slightly with time due to evaporation of solvent components [53]. Mixed ACN-water-TFA solvents can also be difficult to prepare reproducibly. Outgassing during gradient formation is usually less severe with modern HPLC systems, however, so pure water and acetonitrile (+TFA) can generally be used, especially if maintained under helium and if the detector flow cell is slightly pressurized.

Temperature Effects

Column temperature is very important in optimizing HPLC separations, especially in RP-HPLC. Early RP-HPLC protein separations were often done at ambient temperature. Fluctuations in "room" temperature, however, significantly decreased run-to-run reproducibility of elution times [53]. Thus, maintaining constant column temperature (and, ideally, preheating solvents to this temperature) is essential for good reproducibility.

Similarly, early RP-HPLC studies of proteins suggested that resolution generally did not increase with temperature, so a maximum of $\sim 50^{\circ}\text{C}$ was generally used. Bietz and Cobb [53], however, found marked improvement in resolution of gliadins between 50 and 70°C (Fig. 4). Column selectivity was also modified, emphasizing that temperature variation may facilitate difficult separations. Variation of resolution and selectivity with temperature was shown in subsequent studies [43,52,72], with higher temperature separations generally being better. In an extreme example by Chen and Horvath [55], four standard proteins resolved in less than 10 sec at 120°C ! Sometimes, however, as for purothionins, lower temperatures enhance separations [73]. At any rate, the general importance of temperature in RP-HPLC and the possibility of improving

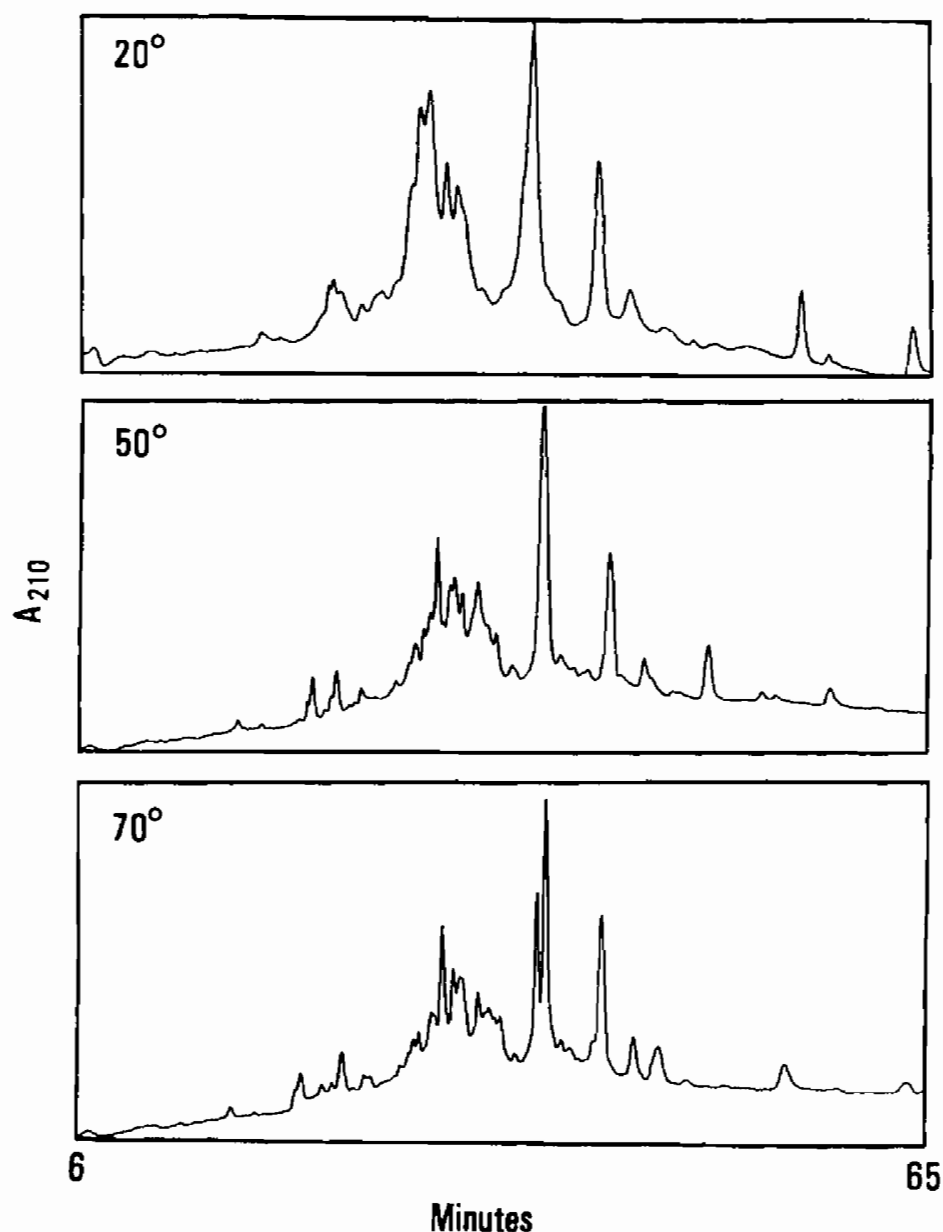


FIGURE 4 RP-HPLC of Anza gliadins at 20, 50, and 70°C. (From Ref. 53.)

or fine-tuning separations by varying temperature are now increasingly recognized [74].

Many factors, such as solvent viscosity, diffusion rates, and column type, contribute to temperature effects in protein RP-HPLC. Temperature may also directly affect protein conformation or aggregation. For example, whereas fresh extracts of maize zein resolved well upon RP-HPLC [42], even at low temperature, purified zeins, especially after lyophilization and storage, separated poorly [53] (Fig. 5). Resolution was restored, however, at 70°C. These results suggest that zeins may aggregate and polymerize via hydrogen bonds between glutamine

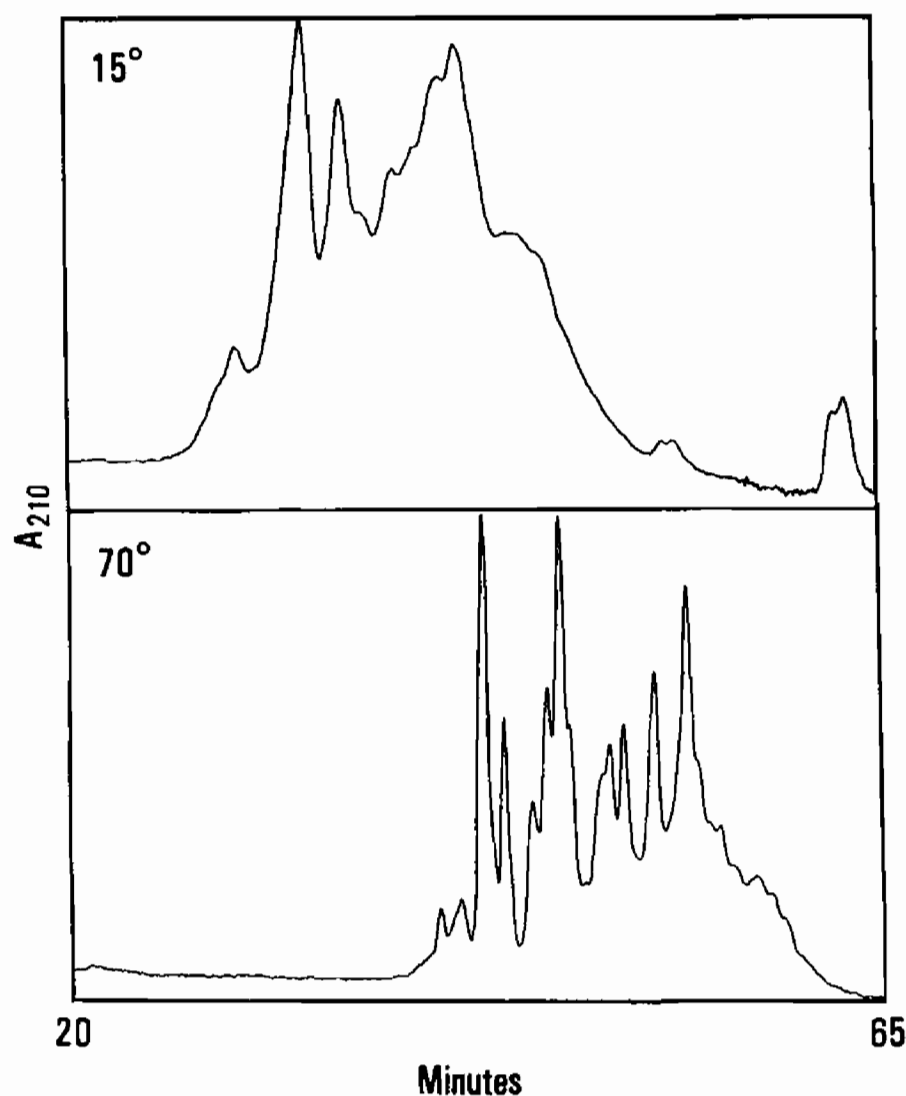


FIGURE 5 RP-HPLC of zein extracted with 70% ethanol, then dialyzed and lyophilized, and then redissolved in 70% ethanol for analysis at 15 and 70°C. (From Ref. 53.)

residues and that disruption of these bonds by higher temperature restores the proteins' monomeric nature.

Troubleshooting

Marchylo [45] presented an excellent discussion of how to recognize and deal with problems during cereal protein HPLC. A few of these important considerations and practical advice that may lead to and maintain good separations of cereal proteins by HPLC will be discussed.

Column Care. HPLC columns for plant protein analysis should be used according to manufacturers' recommendations. Extreme pH must be avoided for

silica, and columns should be stored in recommended solvents. In-line filters and/or guard columns should be used, and solvents and samples must be freshly filtered and/or centrifuged to rid them of particulates. The best test of column performance is periodic analysis of a complex, freshly prepared “standard” protein mixture similar to samples being analyzed, such as a 70% ethanol extract of a wheat flour. If resolution or selectivity changes, good performance may possibly be restored by washing the column (according to manufacturers’ recommendations) with solvents such as methanol, ACN, or 90% DMSO [19]. As a last resort, columns can be washed in the reverse direction, end frits can be cleaned or replaced, and packing near the column inlet can be replaced. Columns do not last forever, however—not even newer types [56]. Spare columns (preferably of the same lot) should be kept available.

Sample-Associated Problems. Because of the unusual nature of many cereal proteins, sample-associated problems may arise during HPLC. Proteins may precipitate during gradient elution or at their pI as they equilibrate with a different solvent. This may be detected by monitoring resolution and pressure and usually prevented by guard columns. When contamination does occur, washing columns according to manufacturers’ recommendations often restores performance. Protein solubility may also change with time due to aggregation. Samples are generally best analyzed soon after preparation; sample stability should be checked by repeat analyses during a period at least equal to that in which samples may reside in an autosampler before analysis. We have also occasionally observed “layering” phenomena as samples await analysis, making lower aliquots from a vial more concentrated. If this occurs, samples must be mixed shortly before analysis. If precipitates form during storage of samples, it is necessary that they again be filtered or centrifuged.

A more subtle problem is presented by proteins that associate or polymerize, noncovalently or covalently, but remain soluble. One example for zein was noted before (Fig. 5); a broad “envelope” underneath well-resolved peaks may suggest association or polymerization. Similarly, Huebner and Bietz [22] showed that medium-MW oligomeric wheat glutenin molecules eluted upon RP- HPLC as a broad peak but resolved as sharp peaks after disulfide bond cleavage.

Sample Preparation and Injection. Most cereal proteins are rich in glutamine and are subject to deamidation in acidic solvents. It is thus best to minimize exposure to acidic solvents, including ones containing TFA [21]. Similarly, alkaline extraction should be avoided because of potential peptide bond hydrolysis. As noted earlier, high temperature may also dissociate polypeptides. Most extracts are, however, quite stable [18–19,21].

Marchylo and Kruger [75] emphasized another important consideration for RP-HPLC: injection volume. If large samples in hydrophobic solvents are applied, some less hydrophobic proteins may not bind and will elute at the

column's void volume. This may be avoided by using solvents of minimum hydrophobicity and small (e.g., 5 μ L) injections (multiple if necessary).

Many cereal endosperm storage proteins—especially glutelins—are, in their native forms, disulfide-bonded oligomers or polymers. Even if soluble, such proteins may resolve poorly upon RP-HPLC and elute as broad peaks [22], in part due to their many possible polymeric forms. Reduction is necessary to release disulfide-bonded subunits from cereal glutelins or oligomeric prolamins for analysis [19,32,42,76]. Thus, protein resolution, extractability, and stability are often improved by extraction under reducing conditions. To prevent reoxidation and further enhance resolution, resulting cysteine residues may be stabilized by alkylation, usually with 4-vinylpyridine [19,21,29,70,76].

Achieving reproducible and quantitative extraction of unreduced high-MW glutelins for SE-HPLC is more difficult [8]. It is often hard to denature proteins completely before analysis and to ensure their stability during storage, but this can be achieved by extraction procedures involving SDS [39,58,77]. Sometimes, extractants containing both denaturants (e.g., urea) and detergents (e.g., SDS) are used to dissociate both hydrogen and hydrophobic bonds. Sonication is also effective to solubilize native polymeric glutelins [40].

Use of Standards. The need for reproducible HPLC separations within and between laboratories is complicated by different selectivities or resolution of columns varying in bonded phase, source, and age. To correct for such differences and monitor system performance, standardization is necessary. In SE-HPLC [8], this is readily achieved by analyzing standard proteins of known MW. The problem in RP-HPLC is, however, more difficult. Ideally, standardization between laboratories or between columns would use stable, commercially available compounds. Bietz and Cobb [53] tested alkylphenones as RP-HPLC standards, but use of such compounds has never been widely practiced, and few other suitable standards are available. More typically, within a laboratory, the best standard may be a fresh, easily prepared heterogeneous sample, such as a 70% ethanol extract of wheat flour, which gives a characteristic and complex chromatogram. Changes in resolution, retention times, or selectivity that indicate column or system problems are easily seen. Excellent studies have used such procedures to ensure long-term reproducibility of analyses, to normalize data based on characteristic peaks, to compare samples, and to identify genotypes automatically from their characteristic fingerprints [78–81].

Use of Standard Analytical Methods. To compare samples between laboratories and develop standard methods, agreement about optimal extraction and analysis methods would be desirable. The diverse aims and nonstandard nature of most separations, however, combined with inevitable changes in columns and optimization of procedures, make such a goal elusive. A collaborative study to determine optimal RP-HPLC analytical conditions for wheat

proteins [16] suggested that adoption of standard methods is unlikely but showed that optimal conditions for plant proteins are similar to those for other proteins. Procedures and results from different laboratories were also similar. For RP-HPLC, ACN-containing solvents (gradients from 15 to 70% ACN +0.05–0.1% TFA) are most common, with detection at ~ 210 nm to maximize sensitivity. Fixed-wavelength UV detection is normally used for proteins; photodiode-array detection at multiple wavelengths does not improve wheat varietal identification because most gliadins have similar spectral characteristics [82]. The C_4 , C_8 , and C_{18} phases bonded to ~ 5 - μ m spherical porous (~ 300 Å) silica particles are most effective, and sterically protected silica-based columns [56] offer improved stability. Columns of 4–5 mm ID, 15–25 cm long, are most common, but shorter, narrow-bore columns are increasingly used [54]. Separation times of 50 to 120 min generally give optimal resolution with 4–5 mm ID columns, but narrow-bore or microbore columns often achieve comparable separation efficiency in 10 to 20 min or less [54]. Most investigators use temperatures of 60–70°C for RP-HPLC. Using these conditions, RP-HPLC offers high resolution, sensitivity, and reproducibility; procedures are easy to learn and automate, and results can be readily quantified.

Data Storage and Analysis

In the early days of HPLC, displaying separations on a strip-chart recorder and manually calculating peak elution times and areas were the only options available. Few of the data were actually used. For complex separations, however, such data analysis is insufficient and interpretation of complex data is as important as the methods of separation. The availability of a mainframe computer, along with excellent programmers, thus led to development of programs that greatly improved storage, plotting, analysis, and comparison of data. Analog voltage readings (“raw” data) from the detector were periodically saved so that integration and plotting parameters could later be varied and optimized and data could be statistically analyzed.

An example of the capabilities of such programs is shown in Fig. 6 [83]. “Chromatographic comparison plots” enabled variable addition and subtraction of stored data sets. This permitted, for example, prediction of the composition of a hybrid from those of its parents. Similarly, a pattern of a known parent could be subtracted from that of a hybrid, identifying an unknown parent. Using similar strategies, components of mixtures could be identified and quantified, and chromosomal coding of polypeptides could be determined by comparing aneuploid lines. Another important program performed Gaussian deconvolution of unresolved chromatographic peaks, improving quantification of the data [83].

Today, however, most mainframe computers have been replaced by far more powerful minicomputers and personal computers that provide system control as well as data acquisition and analysis. Sophisticated software is also

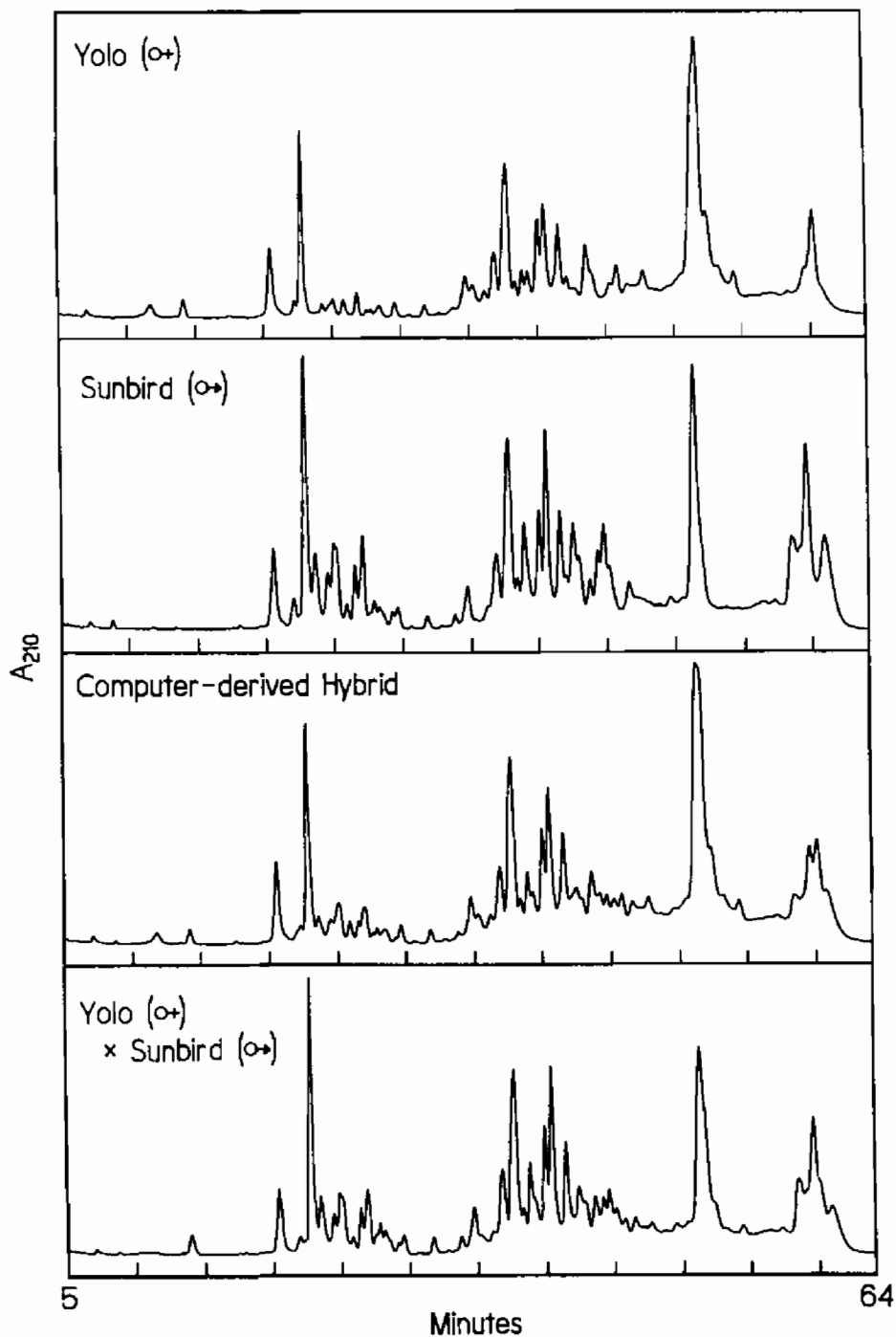


FIGURE 6 RP-HPLC patterns of gliadins from the wheat varieties Yolo and Sunbird, of their chromatograms added in a 2:1 ratio by computer, and of the actual hybrid of the two varieties. (From Ref. 85.)

available to interpret data. For example, the program Origin (Microcal Software, Inc.) provides excellent plotting, visualization, and analysis capabilities. GRAMS/386 (Galactic Industries Corp.) also offers sophisticated data analysis, including curve deconvolution, normalization of peak heights, and multivariate statistical analysis. It also permits multiple-peak x-axis adjustment to align

standard peaks between analyses (as recommended by Sapirstein et al. [78]). An example that used these programs to identify alleles that code wheat gliadins is shown in Fig. 7 [84].

Another major use of computers for HPLC data should be varietal identification (see later). Qualitatively, cereal storage proteins vary little within genotypes but significantly among different varieties, so they provide characteristic “fingerprints.” Varietal identification can be automated by computer comparisons with stored standard data. Scanlon et al. [80] showed that normalization of peak retention times provided sufficient precision for cultivar identification. Resulting data based on peak heights and times could be used in an automated library search to identify wheat varieties [81].

Similarly, cereal protein compositions are frequently related to grain or product functionality. Thus, it should be possible to correlate quality with the presence or amount of specific components. Finding such relationships in complex data sets is, however, not simple. Mosleth and Uhlen [85] first used the statistical procedure partial least squares (PLS) regression to identify quality-

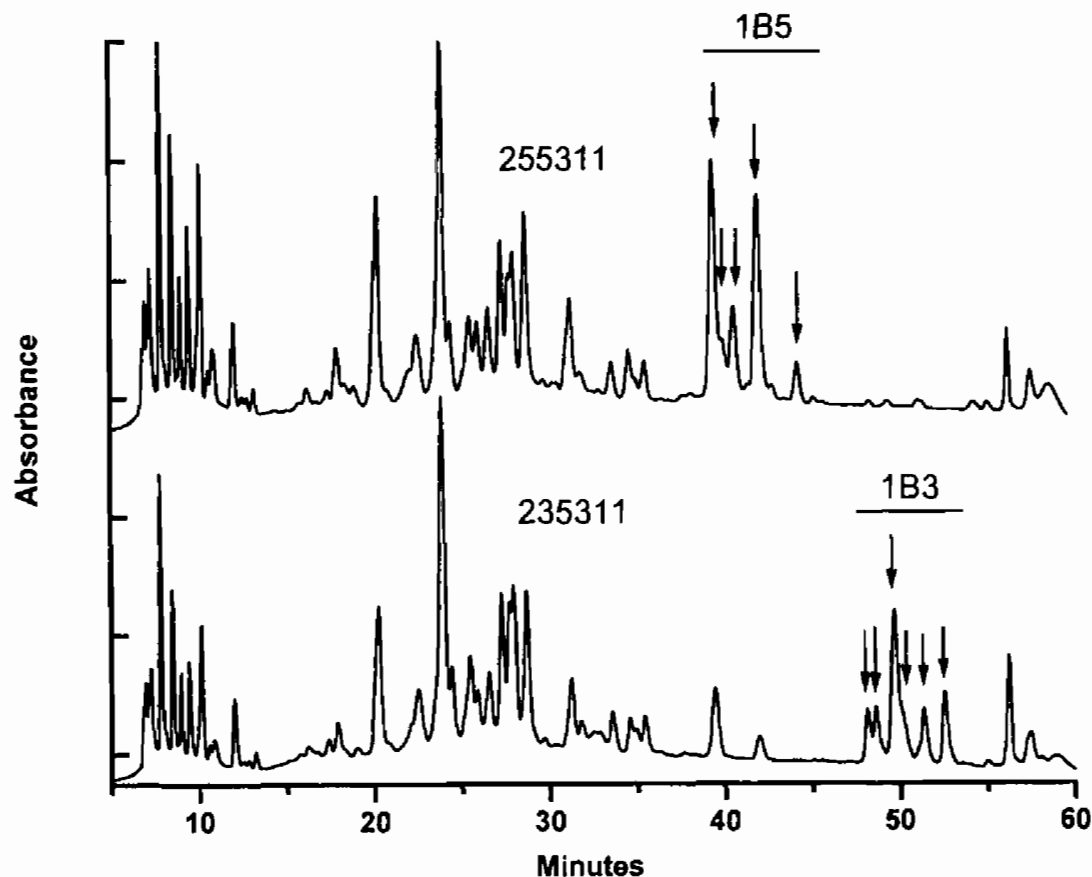


FIGURE 7 RP-HPLC of gliadins from hybrid genotypes selected from a cross of the wheat varieties Alcedo and Trakija differing only in gliadins coded by alleles 3 and 5 at the complex 1B loci. (From Ref. 84.)

related wheat proteins from electrophoresis data. These techniques were found equally valuable for complex HPLC data [86,87]. PLS and principal component analyses (PCAs) of nonintegrated RP-HPLC data (typically ~2000 paired time and voltage readings per chromatogram) were able to identify quality-related durum and bread wheat proteins and could differentiate winter from spring wheat varieties. An example (Fig. 8) shows that PCA and PLS can identify components contributing most to quality characteristics such as loaf volume; proteins eluting at times corresponding to greatest positive or negative PCA or PLS weights are most important. Similarly, statistical analysis of normalized RP-HPLC peak areas and elution times can reveal quality-related peaks [88] or classify varieties [89,90].

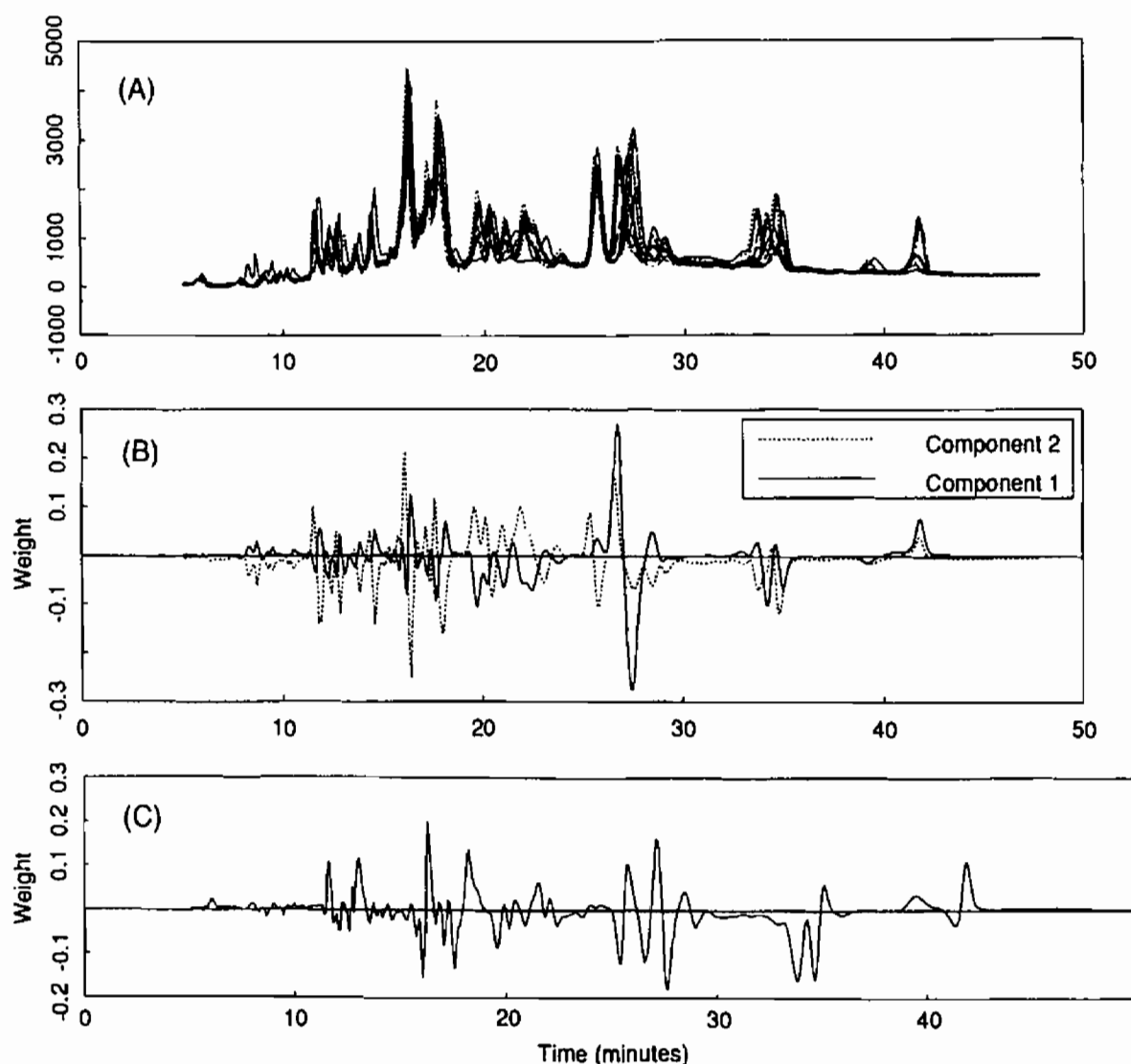


FIGURE 8 Statistical analysis of bread wheat RP-HPLC data: (A) chromatograms of 12 hard red spring wheats; (B) PCA weights for data from (A); and (C) PLS weights for loaf volume. (From Ref. 87.)

Procedures for statistical evaluation of analytical data from HPLC (and other chromatographic and electrophoretic methods) have been reviewed by Bietz and Simpson [91]. The reader is also referred to an excellent article by Scanlon [92] that discusses this topic in detail.

APPLICATIONS

Characterization of Proteins

HPLC has given investigators superb new tools, complementary and often superior to previous methods, to characterize complex cereal proteins. Hundreds of reports describe such studies, but these can only be summarized here, with references to more comprehensive reviews.

SE-HPLC was the first HPLC technique applied to cereal proteins [8]. It provided accurate MW values of wheat proteins plus MW distributions indicative of breadmaking quality. It is often used to characterize fractions from other preparative separations and to complement other methods for characterization of complex fractions. For example, Seilmeier et al. [69] separated glutenin subunits by SE-HPLC and RP-HPLC, revealing compositions of low-, medium-, and high-MW subunits in different varieties. Cornec et al. [35] extracted gluten with increasing HCl concentrations, characterized the fractions by SE-HPLC, and related MW distributions to rheological properties. Huebner and Bietz [22] used SE-HPLC and RP-HPLC to characterize ethanol-soluble glutenin oligomers and related composition to flour quality. An excellent article by Autran [58] reviews SE-HPLC of cereal proteins.

RP-HPLC also rapidly became important and widely used. Early studies showed resolution of wheat and maize proteins by RP-HPLC to be equal or superior to that of other methods [7]. For example, gliadin was resolved into more than 50 peaks and shoulders in 50 min on a C₁₈ column at 60°C [53]. RP-HPLC is also more rapid, sensitive, and reproducible than SE-HPLC; it gives good quantitation and recovery; and it is suitable for both preparative and analytical separations. Proteins resolve primarily on the basis of differences in surface hydrophobicity; therefore, RP-HPLC complements techniques such as gel electrophoresis based on size or charge [7,93,94].

RP-HPLC has been used to study accumulation of proteins during wheat development [95,96], and to examine effects of high-MW glutenin subunit alleles on protein polymerization [95]. Muller and Wieser [97] fractionated gliadins by RP-HPLC, digested them enzymatically, and characterized intramolecular disulfide cross-links by RP-HPLC before and after reduction. Popineau and Pincau [98] examined surface hydrophobicities of gliadins by RP-HPLC, hydrophobic interaction chromatography, and nonpolar ligand binding. The methods gave similar information, but RP-HPLC provided higher resolution. RP-HPLC can

relate wheat protein hydrophobicities to structures, aggregation tendencies, and functional properties, such as viscoelasticity, emulsification, and foaming [99]. Kruger et al. [27] used RP-HPLC to isolate and characterize sequentially extracted wheat proteins, after which they related the amounts to dough strength. Lookhart et al. [100] used RP-HPLC to compare gliadins from flour mill streams and fractions. Bean and Lookhart [101] combined RP-HPLC with capillary electrophoresis in a two-dimensional method and used it to characterize gliadins and glutenins. Surface contour plots constructed from these procedures show superior resolution, differentiate genotypes, and reveal quality-related proteins. Wieser et al. [102] reviewed preparative and analytical RP-HPLC of gluten subunits, showing how protein compositions are related to technological and rheological properties.

RP-HPLC is especially useful to characterize the numerous component polypeptides, or subunits, of polymeric wheat glutenin. In initial studies [19], 15–20 major subunits were resolved; early peaks contained less hydrophobic high-MW subunits, and later peaks contained lower MW subunits. Belitz, Wieser, Seilmeier, and Werbeck significantly improved glutenin RP-HPLC methods [32,33,69,70,103]. Glutenin was reduced, extracted with water–alcohol mixtures, and then fractions were characterized by RP-HPLC. Other studies recommended fractionation of glutenin by 70% aqueous ethanol under reducing conditions before RP-HPLC: low-MW glutenin subunits are ethanol soluble, and ethanol-insoluble subunits are primarily high MW. Typical examples are shown in Fig. 9. HPLC patterns of soluble subunits were unique for each variety, and high-MW subunit compositions were related to baking quality. Several solvent systems were developed to characterize glutenin subunits and used to relate qualitative and quantitative compositions to genotype and quality. RP-HPLC of glutenin fractions separated after stepwise reduction showed that both disulfide bonds and secondary forces aggregate glutenin subunits.

These and similar procedures have significantly enhanced our knowledge of the nature and role of glutenin's high- and low-MW subunits. High-MW subunits differentiate alleles associated with breadmaking quality. For example, Sutton [104] showed that RP-HPLC could differentiate alleles coding high-MW glutenin subunits in New Zealand cultivars (Fig. 10) and that their composition was related to breadmaking quality. Margiotta et al. [105] characterized allelic variation among high-MW durum glutenin subunits by RP-HPLC and showed the complementary nature of RP-HPLC to other techniques. RP-HPLC can also identify and characterize novel high-MW wheat glutenin subunits [106]. Lafian-dra et al. [107] used RP-HPLC to show that high-MW glutenin subunits from varieties differing in breadmaking quality may have the same SDS-PAGE mobilities but are not identical, emphasizing the importance of the use of multiple complementary analytical methods. Weegels et al. [108] used RP-HPLC to characterize glutenin isolated by various procedures and related the

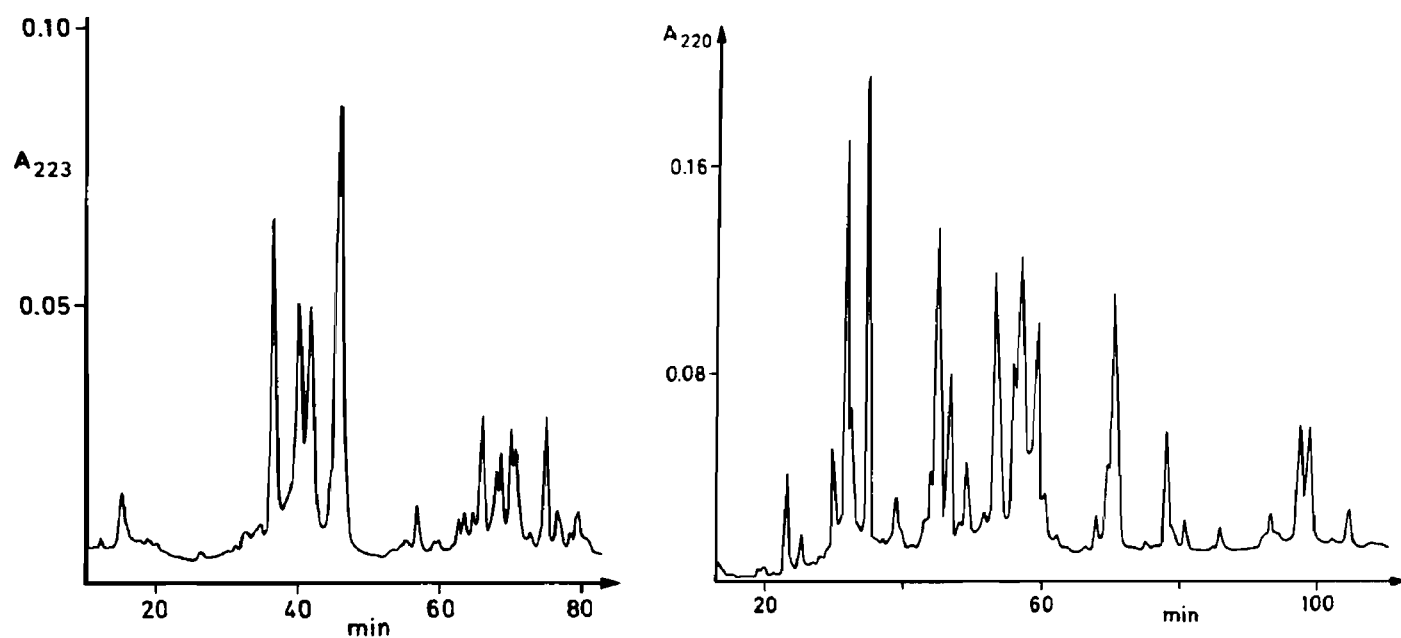


FIGURE 9 RP-HPLC separations of wheat glutenin subunits (cv. Apollo) insoluble (left) and soluble (right) in 70% ethanol-dithioerythritol. (From Ref. 32.)

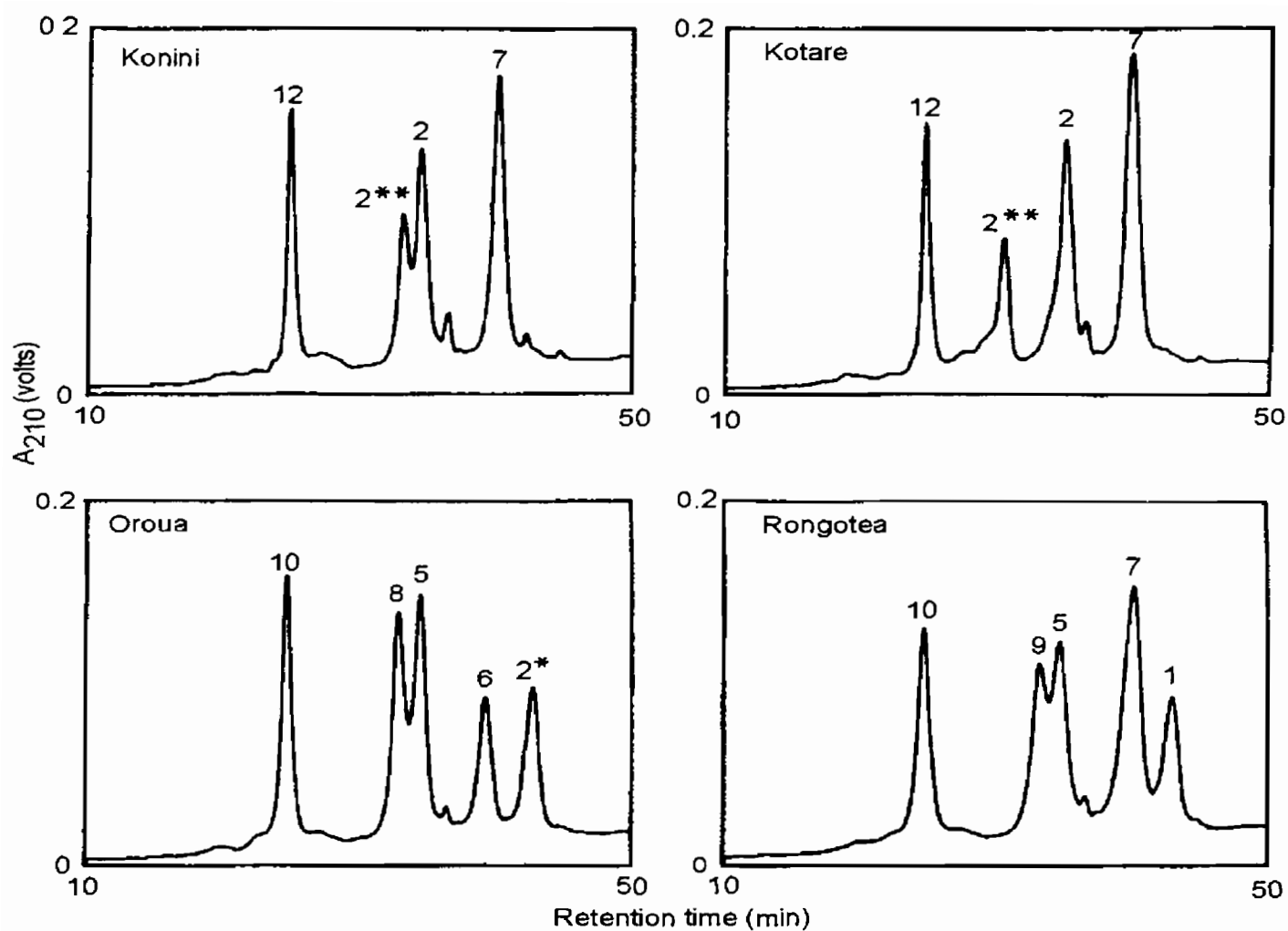


FIGURE 10 RP-HPLC chromatograms of alkylated high-MW glutenin subunits from the New Zealand wheat varieties Konini, Kotare, Oroua, and Rongotea. Peaks are labeled according to standard nomenclature. (From Ref. 104.)

results. Relative amounts and types of high-MW subunits differed between preparations, suggesting that x- and y-type subunits contribute differently to the size and composition of glutenin polymers.

Similarly, RP-HPLC is invaluable for studying glutenin's low-MW subunits. Gradients have been optimized to give excellent resolution of these proteins [32,33,69]. Huebner and Bietz [22] used SE- and RP-HPLC to characterize ethanol-soluble glutenin oligomers and to relate compositions to differences in quality among wheat varieties. Lew et al. [109] purified low-MW glutenin subunits by RP-HPLC and related their sequences to polymer structure. When Masci et al. [110] used RP-HPLC to isolate low-MW glutenin subunits from durum biotypes differing at the Gli-B1/Glu-B3 loci for sequence analyses, they found that differences in the structure and amount of these proteins affect glutenin's polymerization and its resulting quality. RP-HPLC is also valuable for analyzing minor wheat proteins, including purothionins [73] and lipid-binding proteins [111].

Similar methods to analyze storage proteins of all major cereals, including maize [42,94], barley [26,52,112], oats [43,113,114], sorghum [115–119], rye and triticale [120–122], and rice [44,123–127], have been developed and optimized. These procedures differ in extraction (e.g., solvent and presence or absence of reducing agent) and solvent gradient conditions. Nevertheless, HPLC methods for all cereal proteins are similar.

Varietal Identification

One of the most important applications of HPLC is varietal identification. This is possible because storage protein expression is nearly invariant within genotypes, and “fingerprints” vary little with environment [26,50,128–134] or even upon germination [135]. Such analyses may be important during marketing, for quality prediction, or to register varieties, especially as identity preservation becomes more common. In addition, selection of known and desired genotypes is increasingly important during breeding and in genetic studies. Although other procedures—especially gel electrophoresis—have been widely used for these purposes, the many advantages of RP-HPLC (especially speed, relative simplicity, automation, and quantification) can easily make it the method of choice, applicable to selection, identification, registration, and marketing of all plant genotypes. A review of this topic is provided by Bietz and Huebner [136].

Wheat

Wheat varietal identification is possible by IE-HPLC [61] and sometimes by SE-HPLC [8]. RP-HPLC of high- or low-MW glutenin subunits also differentiates wheat genotypes [19,32,70,137,138]. Most HPLC methods for wheat varietal identification, however, use RP-HPLC to analyze gliadins because these methods

are relatively mature and gliadins are easy to isolate and are, qualitatively, the most invariant wheat storage proteins.

Bietz et al. first developed and optimized procedures to identify wheat varieties by RP-HPLC of their gliadins [7,53,139,140]. Since then, many have used RP-HPLC of gliadins to differentiate cultivars [52,129,141,145]. These studies have been reviewed [146]. Procedures for computer-assisted data handling and statistical analyses have automated the method and improved its accuracy [78,80,81,147]. Earlier [53] and more recent [54] studies have shown that RP-HPLC of gliadins can differentiate and thereby identify wheat genotypes. Such analyses can be very rapid (Fig. 11), paving the way for inexpensive and routine RP-HPLC varietal identification and for related applications. RP-HPLC of gliadins also distinguishes biotypes within varieties [53,139,148]; knowledge of such genetic heterogeneity is essential for selection during breeding. Similarly, gliadin RP-HPLC can analyze the purity of hybrid wheats [149]. RP-HPLC can measure the amounts of wheat and rye in mixtures, based on quantification of late-eluting rye secalins [150]. Adulteration of durum wheat flour with common wheat can also be detected and quantified by RP-HPLC of gliadins or albumins

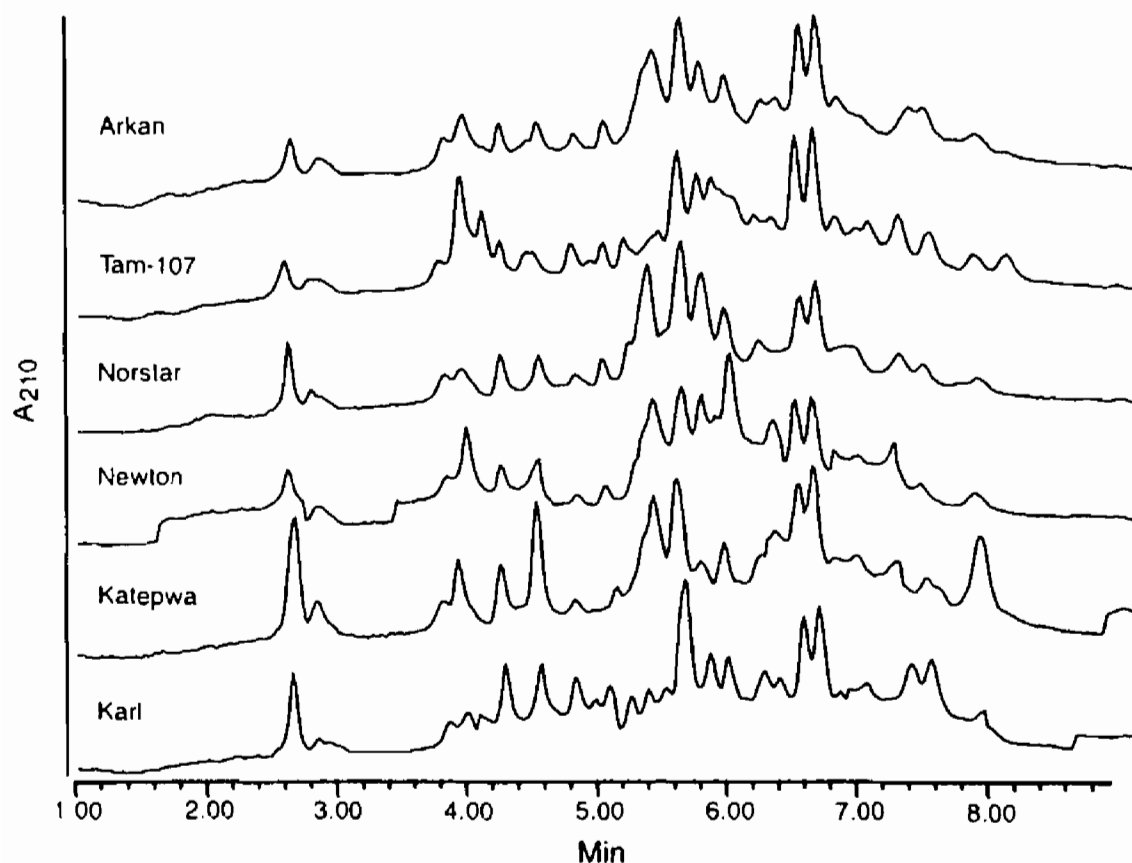


FIGURE 11 RP-HPLC separations of gliadins extracted from hard red winter wheat varieties. Analyses were performed on a 2.1-mm ID Vydac C4 column. (From Ref. 54.)

that consistently differentiate tetraploid and hexaploid genotypes [151–153]. Similarly, although SE-HPLC shows only subtle differences in MW distributions between varieties of hard red spring and winter wheat classes [154], RP-HPLC shows statistically significant differences [130,155], suggesting use of this method for wheat classification.

Maize

Similar techniques of varietal identification have been applied to maize [42,158]. Smith and Smith [131,157–161] used RP-HPLC of zeins and alcohol-soluble glutelins to identify and examine pedigrees of U.S. maize inbreds, hybrids, and F₂ populations. Advanced statistical programs used to analyze the data showed that most lines had unique patterns characteristic of genotype that varied little with environment, making RP-HPLC suitable for maize genotype identification and analysis. Paulis and Bietz [162] reviewed RP-HPLC varietal identification of maize genotypes, showing its usefulness for varietal identification, breeding, marketing, and research.

Rice

Several studies used RP-HPLC of rice prolamin and glutelin storage proteins for varietal identification [44,123–126]. Patterns are genotype specific and little influenced by environment. Long-, medium-, and short-grained cultivars are differentiated, as are closely related sister lines. RP-HPLC identification of rice varieties was reviewed by Lookhart and Juliano [127].

Sorghum

RP-HPLC identification of sorghum varieties and mutant genotypes was first demonstrated by Sastry and colleagues [115–117]. Smith and Smith [163] subsequently showed that RP-HPLC patterns of sorghum kafirins from different locations were stable and that variation was mostly genetic rather than environmental, making RP-HPLC suitable for inbred and hybrid identification. Sorghum varietal identification by RP-HPLC was reviewed by Smith [119].

Barley

Marchylo and Kruger [26,52] developed and optimized conditions to separate barley and malt prolamins (hordeins) by RP-HPLC, and used these methods for cultivar identification based on qualitative and quantitative differences. Elution profiles were largely independent of growth location, year, and protein content. Identification of barley cultivars by hordein IE-HPLC was also reported [164]. Barley HPLC varietal identification was reviewed by Marchylo [165].

Oats

Oat cultivars and species are differentiated by RP-HPLC patterns of extracted prolamins (avenins) [113,114]. This was reviewed by Lookhart and Peterson [166].

Rye and Triticale

Hay and Sutton [145] first used RP-HPLC of prolamins to identify rye cultivars. Kubiczek et al. [120] subsequently examined this problem, made more difficult by rye's outcrossing nature and the heterogeneity of many established cultivars. Procedures were optimized for isolation and RP-HPLC of rye prolamins (secalins), extended to the intergeneric wheat-rye cross *triticale*, and used for cultivar identification. These studies are reviewed by Kubiczek et al. [122].

Genetic Studies

RP-HPLC of wheat proteins can determine chromosomal locations of genes encoding gliadin [139] and glutenin [19] polypeptides. In later studies, Bietz and Burnouf [167] used RP-HPLC of aneuploids to show control of gliadins by the *Gli-1* and *Gli-2* loci on the short arms of group 1 and 6 chromosomes. Protein classes eluted as groups in order of increasing hydrophobicity. Similarly, Burnouf and Bietz [168] used RP-HPLC to show chromosomal control of glutenin subunits. Less hydrophobic high-MW subunits associated with breadmaking quality eluted first (see Figs. 1 and 9); more hydrophobic low-MW subunits followed. These studies showed that RP-HPLC is a powerful tool for selection during breeding and for genotype identification and analysis during genetic studies.

Subsequent genetic studies of wheat proteins have used both RP-HPLC and SE-HPLC. Khan et al. [169] used RP-HPLC to characterize proteins of high-protein hard red spring wheats derived from crosses with *Triticum dicoccoides*. Lookhart et al. [170] used prolamins RP-HPLC to identify 1BL/1RS wheat/rye translocation lines. Gupta and MacRitchie [171] used RP-HPLC and SE-HPLC to characterize amounts and types of gliadin and glutenin polypeptides coded by genes at major loci. Differences in resulting protein composition and size affected functional characteristics. Mackie et al. [172] differentiated tetraploid and hexaploid *Triticum* species based on high-MW glutenin subunit alleles with RP-HPLC. Bietz and Waga [84] used RP-HPLC to identify genotypes and characterize gliadin-coding blocks relating to quality. Lafandra et al. extensively used RP-HPLC for protein characterization in genetic and breeding studies, as described in an excellent review [173].

HPLC is also useful in genetic studies of other cereals. Devaux et al. [174] used RP-HPLC to analyze B- and C-hordeins from barley doubled haploid lines

produced by *Hordeum bulbosum* and anther culture techniques; lines having recombination between loci were identified.

RP-HPLC is especially valuable in genetic studies of maize. Paulis et al. [175] showed that prolamin RP-HPLC differentiates normal, *opaque-2*, and modified *opaque-2* genotypes differing in nutritional quality and endosperm texture. Modified *opaque-2* maize genotypes (“quality protein maize”) are enriched in high-proline alcohol-soluble glutelin subunits and contain less zein than normal maize.

Paulis et al. [176,177] showed the value of RP-HPLC as a quantitative genetics tool by analyzing alcohol-soluble proteins in single- and double-mutant maize genotypes. Synergism and epistasis of mutant genes significantly affected protein expression in double-mutant lines (Fig. 12). Bogyo et al. [178] further showed the value of RP-HPLC for quantitative genetics—quantifying zeins in crosses and backcrosses of two cultivars led to a new model for zein inheritance. RP-HPLC is thus useful during breeding to identify lines with improved nutritional quality and texture.

Preparative HPLC

From the outset, it was apparent that HPLC was a valuable preparative method for cereal proteins. Up to 5 mg of samples can be fractionated on analytical columns with good resolution [7], and this can be scaled up considerably. Electrophoresis of resulting fractions is a complementary technique that confirms the resolution of RP-HPLC and further fractionates by size or charge [7,94].

In early studies, Burnouf and Bietz [179] used preparative RP-HPLC to isolate durum gliadins associated with pasta quality. Preparative RP-HPLC also fractionated high-MW glutenin subunits related to breadmaking quality [168]. To isolate proteins from complex mixtures, however, it is often desirable to combine RP-HPLC with ion-exchange or size exclusion methods [66]. Wieser and Belitz [180], for example, described a preparative procedure combining RP-HPLC with gel permeation chromatography to isolate celiac-active gliadin peptides.

Others have since used preparative RP-HPLC to purify gliadins for characterization and structural studies and to relate them to rheological and breadmaking properties [181–183]. Similarly, high- and low-MW glutenin subunits have been fractionated by preparative or semipreparative RP-HPLC [108,109,184–186]. Wieser et al. [102] give an excellent overview of the use of RP-HPLC for preparative and analytical characterization of gluten subunits.

Paulis and Bietz [187] used preparative RP-HPLC to fractionate zein. Fraction homogeneity, MW, and charge were examined by electrophoresis; amino acid compositions related results of different techniques. Similarly, preparative RP-HPLC and IE-HPLC have fractionated oat prolamins for subsequent characterization [188–189].

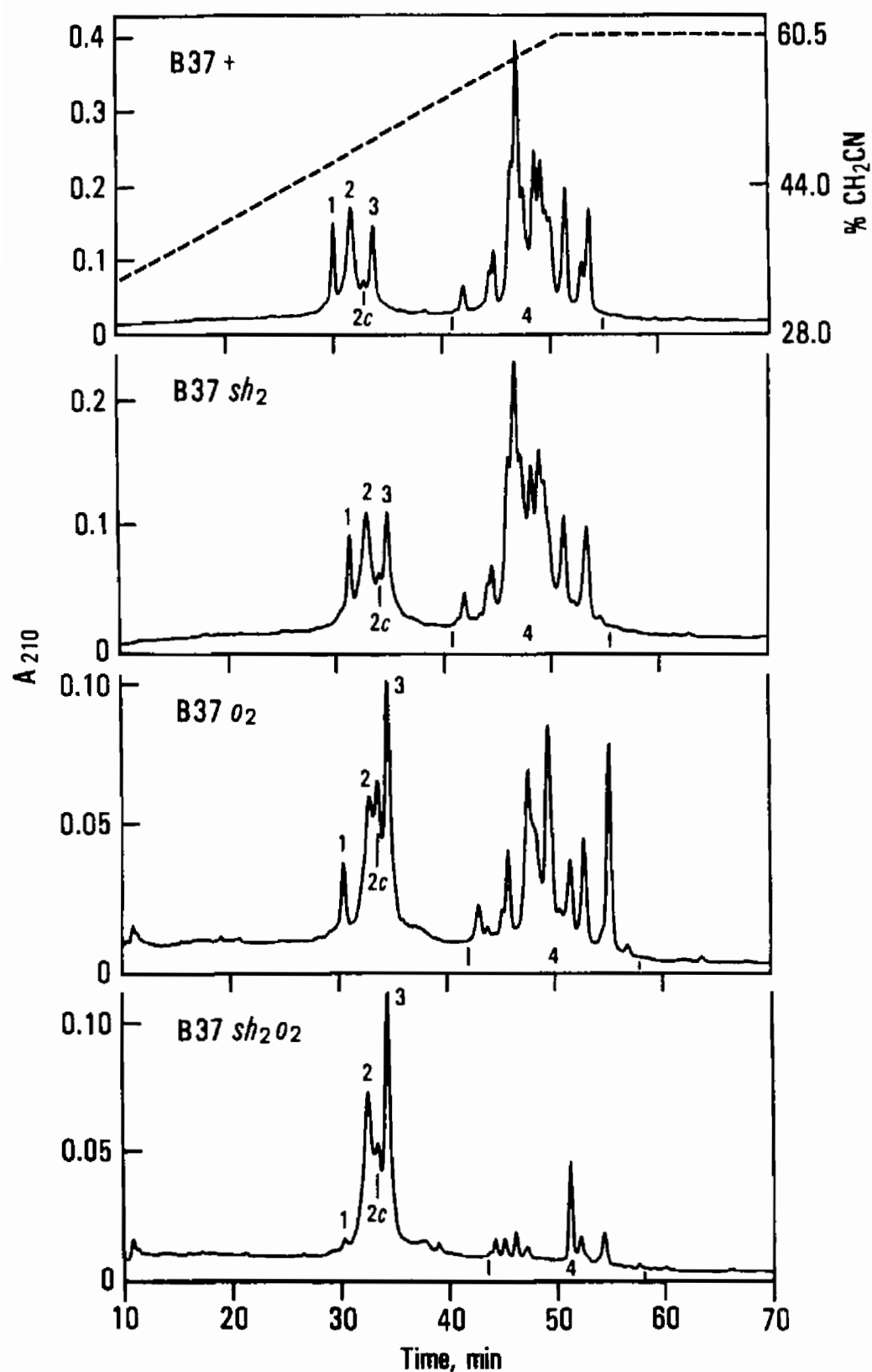


FIGURE 12 RP-HPLC of proteins extracted with 70% ethanol–0.5% sodium acetate–5% 2-mercaptoethanol from normal maize inbred B37, two single mutant (*sh*₂ and *o*₂) lines, and the double mutant *sh*₂*o*₂. (Data from Ref. 176.)

Prediction of Quality

One of the most important reasons to analyze cereal grains is to determine quality, i.e., functional properties that determine suitability for particular applications. Grain quality is often related closely to protein amount or type; thus, grain protein HPLC has rapidly achieved wide use for quality prediction.

SE-HPLC

MW distributions of native wheat proteins and their subunits are closely linked to breadmaking quality, mixing time, and strength [1]. This has long been estimated by gel filtration chromatography [190]. Bietz [8] subsequently used SE-HPLC to determine the MW values of unreduced wheat proteins; such analyses were rapid and sensitive, and the MW distribution could be correlated with mixing time or quality. Huebner and Bietz [57] confirmed these results but identified problems in protein extraction and stability. These problems were solved by Dachkevitch and Autran [39], who showed that MW distributions of unreduced proteins from SE-HPLC can predict baking quality during breeding. Quality can also be predicted by SE-HPLC of reduced proteins, especially glutenin's high- and low-MW subunits [57].

Many studies have since used SE-HPLC of native or reduced wheat proteins to analyze dough rheological properties or breadmaking quality [59,191–198]. Amounts of polymeric glutenins are related to quality in much the same way as Zeleny sedimentation and alveograph tests [196]. A general concept has developed that differences in aggregation of polymeric glutenin influence breadmaking quality [193]. SE-HPLC can also relate protein aggregation and MW distribution during kernel development to pasta or breadmaking quality [193,199] and can relate MW distributions to germination or sprouting [200]. SE-HPLC is especially valuable in showing how environment, location, and fertilizer affect protein composition and quality [201–206]. For example, SE-HPLC showed that heat stress during grain development affects quality by allowing synthesis of different amounts of total proteins or providing a different pattern of protein accumulation [207,208]. A detailed review of SE-HPLC for wheat quality prediction based on protein size distribution is given by Autran [58].

RP-HPLC

RP-HPLC is also an invaluable tool to estimate wheat quality. As already noted, this may be done by identifying genotypes [139]. RP-HPLC can also, however, detect specific proteins that serve as markers of quality. Bietz et al. [139] first identified durum γ -gliadin RP-HPLC peaks that correlated with pasta quality and

showed their correspondence to electrophoresis bands [179]. Early-generation samples could be rapidly screened for quality based on these RP-HPLC peaks. Many studies have since related the presence or amount of specific gliadins, individually or in combination, to quality [79,84,93,128,132,133,209–215]. Gliadin compositions vary quantitatively during kernel development and with kernel size and may thus affect quality [133,214]. Quantitative differences in gliadins may also reflect different environments or fertilization during growth [128,132]. It is especially interesting that RP-HPLC related amounts of hydrophobic, late-eluting gliadins to baking quality or to wheat class [209–212].

Similarly, RP-HPLC has related glutenin subunits to quality. As noted previously, high-MW subunits affecting breadmaking quality [216] resolve well upon RP-HPLC [19,168]; similarly, the ratio of high- to low-MW glutenin subunits is related to quality [57]. Additional studies have since used RP-HPLC to relate glutenin's subunit composition to quality [31,76,79,96,107,193, 214,215,217–221]. Both quantitative variation and allelic forms of high-MW subunits may influence wheat quality [96,219]. RP-HPLC showed that dough extensibility and bread and pastry water absorption relate closely to the amount of high-MW glutenin subunits [220] and that quality deteriorates during sprouting due to degradation of high-MW subunits [76]. RP-HPLC showed how quantitative variation in glutenin during wheat maturation may affect glutenin aggregation and quality [193,214], and that loaf volumes and bake scores can be predicted by quantifying RP-HPLC results [217]. The demonstration by Lafiandra et al. [106] using RP-HPLC that high-MW glutenin subunits from different varieties with the same numerical designations are sometimes structurally different is especially significant. Amounts of alcohol-soluble low-MW glutenin subunits have also been related to quality by RP-HPLC [79,215]. Computer analysis of RP-HPLC data is especially useful to identify proteins related to quality [21,83]. Melas and Autran [222] also fractionated glutenin subunits by IE-HPLC and related charge distributions to dough extensibility and baking strength. A detailed review of RP-HPLC prediction of breadmaking quality was presented by Huebner and Bietz [223].

RP-HPLC of storage proteins can also predict quality in other cereals. In barley, RP-HPLC of hordeins [112,224] and high-MW glutelins [225] can reveal malting quality. In maize, Paulis et al. [226] showed by RP-HPLC that there are more γ -zeins in quality protein maize than in normal maize; this relates to lysine content and nutritional quality. RP-HPLC can relate protein composition to kernel hardness, density, and vitreosity of both normal maize and quality protein maize genotypes [227–229]. Differences between alcohol-soluble proteins of hard and soft endosperm of normal and quality protein maize show that the zein distribution influences kernel texture and hardness [230–232].

Studies of Interactions and Processing

One of the most important applications of HPLC for cereal proteins may ultimately be the study of their interactions with each other and with other kernel constituents of stored grains or grain products. As noted, improved resolution of wheat and maize prolamins at increased temperature suggests that RP-HPLC can monitor disruption of hydrogen bond-mediated aggregation [53]. Other studies suggest that nonoptimal grain drying or storage is revealed by differing RP-HPLC resolution at varying temperatures. RP-HPLC may thus help optimize processing and storage conditions and maximize quality.

A few analyses of these types have been reported. Meier et al. [233] used RP-HPLC to analyze gliadin from untreated and heat-treated flours. For flours heated above 80–90°C, one peak was larger, indicating interaction or modification due to temperature. RP-HPLC has also characterized wheat proteins that interact with lipids [111]; such aggregates may form upon mixing, and their amounts correlate with bread loaf volume [234]. RP-HPLC has shown conformational changes upon disulfide cleavage or upon alkylation of cysteine residues [11,19]. SE-HPLC is also useful for studying protein reduction, reoxidation, and polymerization. Panozzo et al. [235] showed that bromate changes the MW distribution of wheat proteins, and Bekes et al. [236] used SE-HPLC to follow oxidation and repolymerization of gluten after partial reduction of its disulfides. Windemann et al. [237] found that RP-HPLC can detect gliadin in heated foods, permitting monitoring of wheat and rye in foods for individuals with celiac disease. RP-HPLC of wheat proteins during mixing, fermentation, and baking has also shown degradation or interaction of specific gliadins during baking; these modifications may relate to breadmaking potential [238–240]. Similarly, RP-HPLC can detect high-temperature drying of pasta by changes in retention times and areas of gliadin peaks [241]. SE-HPLC also reveals differences in protein composition and polymerization of heat-shocked wheat that may alter grain quality [207–208]. These examples emphasize the value of HPLC for monitoring changes in protein structures or interactions, related to processing or environment, that affect end-use properties and quality.

CONCLUSIONS

HPLC methods have, during the last 20 years, become widely used and are now methods of choice for many cereal protein analyses. RP-HPLC is especially useful because of its speed, sensitivity, high resolution, reproducibility, and quantifiability and because it complements other methods. SE-HPLC is also widely used to determine MW distributions quickly and accurately and relate them to processing or quality. IE-HPLC has developed more slowly but is becoming useful. This chapter has reviewed these methods, with special emphasis

on their use to characterize cereal proteins, to identify varieties, to act as preparative tools, to determine varietal purity, to analyze hybrids and mixtures, to be used as selection tools during breeding, to function as quantitative genetic tools, to predict quality, and to study interactions and conformations. HPLC methods are now essential tools for understanding, improving, and using the full potential of our most important agricultural commodities and the products derived from them.

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Analysis of Hemoglobin Variants and HbA_{1c}

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INTRODUCTION

Hemoglobin (Hb) is a tetrahedral iron-bearing protein responsible for oxygen transport in erythrocytes. It is composed of four polypeptide chains, two α and two non- α chains, each containing a heme group. The α chains are combined with various non- α (β , γ , δ , etc.) chains to form the different Hbs. Normal adult human HbA consists of two α and two β chains ($\alpha_2\beta_2$). The α chain contains 141 amino acids, the β chain 146 amino acids. A second adult Hb, HbA₂, a minor component, consists of two α and two δ chains ($\alpha_2\delta_2$) with the δ chain containing 146 amino acids and differing from the β chain at 10 positions. The newborn infant primarily produces fetal Hb (HbF), which is composed of two α and two γ chains ($\alpha_2\gamma_2$). Postnatally, HbA increases and HbF decreases and by the time an

infant is approximately 6 months old, HbF is present at only about 5%, but considerable fluctuations have been observed [1].

The identification of human Hb abnormalities is clinically important. They should be suspected in patients with anemia associated with abnormal red blood cell morphology, such as thalassemia and sickle cell anemia. Because the frequency of Hb disorders varies significantly in different populations, the family history and the ethnic background of the patient are essential in the diagnosis of thalassemia and other Hb disorders. Thalassemia-like disorders are characterized by typical red cell morphology with prominent microcytosis and hypochromia, target cells, spherocytes, fragmentation, and basophilic stripping. The morphology is dependent on the severity of the condition. The blood count is also typical: strongly decreased mean corpuscular volume (MCV) in relation to elevated red cell number is almost diagnostic for thalassemia. The red cell morphology resembles that in severe iron deficiency. However, in thalassemia the proportion of microcytic red cells (%micro) exceeds that of hypochromic red cells (%hypo). The ratio %micro/%hypo and other similarly calculated indices are of value in screening patients. Hb analysis is used for the diagnosis of both qualitative (variants) and quantitative disorders (thalassemias). In areas where thalassemia is endemic, isoelectric focusing or electrophoresis is used for screening to detect HbH, Hb Bart's, Hb Lepore, Hb Constant Spring, and other variants that may worsen the thalassemic condition. All clinically significant variants are identified by their characteristic appearance in isoelectric focusing or electrophoresis. In addition, these methods can be used in screening for elevated HbF and HbA₂. Chromatographic methods are used for confirmation and quantitative analysis. Elevated HbF and HbA₂ are typical findings in beta-thalassemia. In delta-beta-thalassemia the elevation of HbA₂ is not seen and in alpha-thalassemia HbF and HbA₂ are not elevated. DNA analysis to detect specific defects has become a valuable tool for genetic counseling purposes and antenatal diagnostics.

Some variants affect oxygen affinity. High-affinity variants are related to familial erythrocytosis and low-affinity variants to familial cyanosis. These variants are rare and occur in restricted geographical areas. Some rare variants cause instability of the affected globin chain leading to moderate hemolysis. The Hb abnormalities causing these disorders can mostly be detected by isoelectric focusing or electrophoresis.

In addition to the Hb variants giving rise to clinical symptoms, a large number of "silent" variants have been detected through the widespread use of cation-exchange chromatography for determination of glycated Hb, which is now a major method for control of diabetic patients.

By August 1997, a total of 731 Hb variants had been identified [2] in the almost 50 years after the variant HbS in sickle cell anemia was discovered in 1949. Most Hb variants are caused by a single point mutation resulting in the

replacement of one amino acid residue for another. For example, with a Glu → Val substitution at position 6 of the β chain a variant is known as sickle cell Hb (HbS). Other Hb variants may result from deletion of one or more amino acids, extension or addition of amino acids on either end of the chain, or fusion of two different chains through crossover during meiosis, as occurs in the Lepore Hbs ($\delta\beta$). Sometimes a double mutation appears in a polypeptide chain, such as in HbC-Harlem, which contains the $\beta 6$ Glu → Lys substitution, as well as the $\beta 73$ Asp → Asn substitution. The location and type of the substitution are of major importance because they can affect the physicochemical and functional properties of Hb quite differently. Some amino acid substitutions have drastic effects on the function but only minor effects on its net charge. Many Hb variants are not associated with any clinical abnormalities. However, approximately 25% of the abnormal Hbs discovered thus far do affect some hematological properties causing sickling, microcytosis, target cells, Heinz body formation, or erythrocytosis. This may be due to increased oxygen affinity, methemoglobin formation, etc. The thalassemias are genetic disorders characterized by deficient or absent synthesis of either polypeptide chain. The β -thalassemias are caused by diminished (β^+ or absent (β^0)) expression of the β chains, which usually leads to increased expression of δ chains and an increased proportion of HbA₂.

The separation of Hb variants has traditionally been carried out by electrophoresis on cellulose acetate at alkaline pH or on citrate agar at acidic pH [3] whereas quantitation of the “normal” Hb components, such as HbA₂, HbA₀, and HbA_{1c}, has most often been performed by macrocolumn and microcolumn chromatography [4], which provide satisfactory results but are lengthy and labor intensive. High-performance liquid chromatography (HPLC) allows separation and quantitation of numerous normal Hb components and abnormal human Hb variants in only minutes. HPLC has been found to be decidedly more sensitive and to give better resolution than routine electrophoretic methods. Several instruments that facilitate very rapid quantitation of specific Hb variants are now available [5]. When unexpected variants are detected in such a system, they must usually be identified by additional chromatographic or electrophoretic methods [6,7].

In chromatographic methods, abnormal Hb variants may cause erroneous HbA_{1c} results [8,9], but at the same time they permit identification of individuals with such variants. This information is of importance for the interpretation of the HbA_{1c} results. Therefore, it is an advantage if an HPLC method used for clinical purposes separates not only HbA_{1c} but also other Hb variants, permitting their identification. The good resolution of more than 35 frequently encountered human hemoglobins and HbA_{1c} on PolyCAT A [10] makes it a useful method for preliminary characterization of Hb variants.

This chapter describes aspects of the basic techniques used for analysis of suspected Hb variants and for determination of HbA_{1c}.

GENERAL PROCEDURES USED FOR THE IDENTIFICATION OF AN Hb VARIANT

Introduction

Analysis of hemoglobins is mainly performed for two different clinical purposes, i.e., identification of abnormal hemoglobins and determination of glycated hemoglobin, especially HbA_{1c}, for monitoring of long-term diabetic control. Chromatographic methods based on cation-exchange chromatography are now widely used to measure HbA_{1c} blood and this frequently leads to the identification of hemoglobin variants, which cause few or no clinical symptoms. However, once detected, it is customary to characterize these by the same techniques as used for characterization of variants causing hematological symptoms. The symptoms and diseases caused by various hemoglobin variants are quite variable and it is clinically important that the variants are properly identified.

Isoelectric Focusing

Polyacrylamide thin-layer isoelectric focusing is an efficient step in the screening of hemoglobinopathies. With this high-resolution system, many electrophoretically "silent" variants can be separated from the normal isoforms. For this purpose we use a 43 × 50 × 0.45 mm polyacrylamide gel at pH 5–8 (PhastGel, Pharmacia Biotech). Many samples can be processed simultaneously and compared side by side. Hb molecules with small differences in charge, such as Hb Malmö ($\beta 97$ His → Gln), not detected by conventional electrophoretic techniques, can be clearly separated from HbA [11]. It is principally possible to identify an Hb variant according to its isoelectric point. Thin-layer isoelectric focusing has a resolving power of at least 0.01 pH unit, which is far better than can be measured with the best contact electrodes on a small gel. Direct determination of the isoelectric point (pI) is therefore not precise enough to identify an abnormal Hb. Current procedure requires side-by-side comparison of the isoelectric position of an unknown Hb variant with that of known control variants. This requires use of a bank of Hb variants and a reference map. Such a map, comparing over 80 variants with over 40 distinct isoelectric points, has been published [12]. A limitation of this method is that it requires the use of control Hbs, which may not be available. On the other hand, the assay requires only a small amount of sample and control samples can be stored at -70°C for extended periods of time.

Analytical HPLC Cation-Exchange Chromatography

HPLC provides a rapid and sensitive means for the examination of abnormal hemoglobins. Methods with high sensitivity and specificity have been developed for screening and confirmation of hemoglobinopathies in newborns [13,14]. Most

methods have used Synchropak CM 300, a silica support with carboxylic acid residues, or PolyCAT A, porous silica coated with polyaspartic acid. The screening procedures are designed to detect the major variants rapidly. However, the resolution of these methods does not usually permit differentiation of some commonly encountered hemoglobin variants, such as HbE from HbA₂. The sensitivity for detection of hemoglobin variants occurring at low concentrations is also limited.

Ou and Rognerud [10] described a method for Hb variant analysis on the PolyCAT A column that permitted separation of more than 35 frequently encountered human Hbs. It is thus useful for preliminary identification of Hb variants. Earlier, PolyCAT A was used for screening of cord blood for hemoglobinopathies [15]. The column has the resolution necessary for both screening and confirmatory purposes. We have investigated the use of a 4.6×200 mm PolyCAT A column with 5- μ m particles for separation of Hb variants. A typical chromatographic separation of an Hb mixture containing A_{1c}, F, A₀, E, A₂, D, S, and C is shown in Fig. 1 using bis-tris buffers and a sodium acetate gradient. We have also separated other Hb variants by using bis-tris buffers and an NaCl gradient (Fig. 2a and b). The baseline resolution permits accurate quantitation of these hemoglobins, which is important for diagnosis of hemoglobinopathies. In addition, HbA₂ can be accurately measured. A slight adjustment of the gradient may be necessary for optimal separation of other variants.

A new automatic HPLC system using the Bio-Rad MA 7 polymeric cation-exchange material has been used to screen newborns for sickle cell anemia and other hemoglobinopathies [16]. The method is reported to resolve hemoglobins F, A, S, C, E, and D.

The Diamat system (Bio-Rad Laboratories, Hercules, CA) is also suitable for hemoglobinopathy screening. Although it was originally designed mainly for determinations of HbA_{1c} it can be used for detection of some Hb variants. It has been shown to separate seven variants, but many overlap partially with the HbA_{1c} peak [17]. Aberrant peaks detected with the Diamat method during routine assays for HbA_{1c} led us to the detection of two Hb variants, Hb Broussais and Hb Cemenelum [18]. With PolyCAT A, both variants could be totally separated (Fig. 3a and b). The elution pattern can be used for preliminary identification of these Hb variants, especially if standards are available.

SEPARATION AND CHARACTERIZATION OF GLOBIN CHAINS

Reversed-Phase Chromatography

Reversed-phase HPLC has been used successfully to separate and quantitate globin chains of Hb variants in both adults and newborns. Of the different

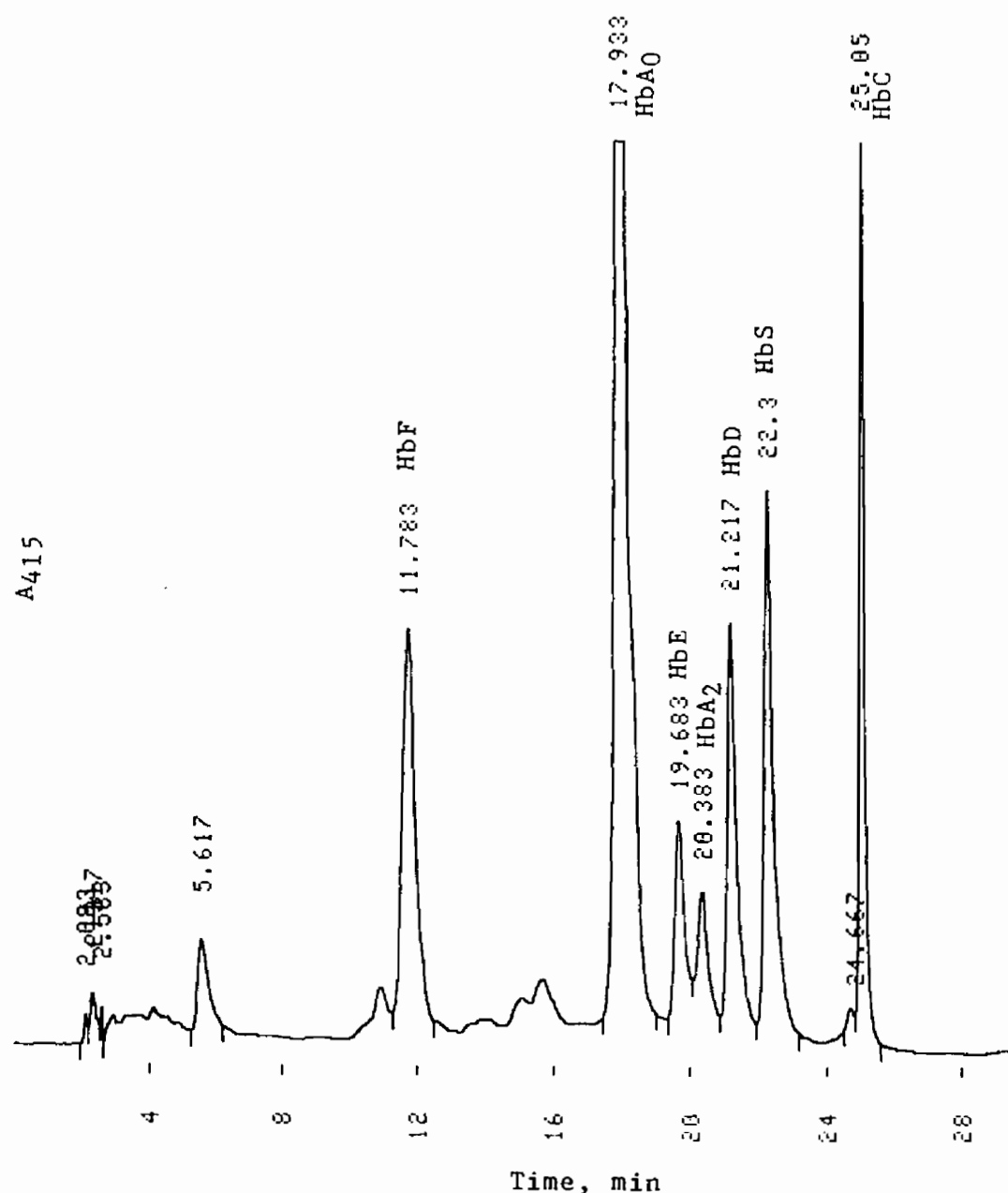


FIGURE 1 Cation-exchange chromatography of HbF, HbA₀, HbE, HbA₂, HbD, HbS, and HbC on PolyCAT A. Time of elution since sample injection is indicated at each peak. Detection at 415 nm. Ten microliters of the filtered hemolysate was injected onto the column of 200 × 4.6 mm. Elution was achieved with a linear gradient of 10 mM bis-tris, 1 mM KCN, pH 6.87 (buffer A) and buffer A plus 200 mM NaCl, pH 6.57 (buffer B) at a flow rate of 1.1 mL/min at room temperature. The gradient was: 0–2 min, 21% B; 16 min, 47% B; 22 min, 100% B; 24 min, 100% B; and 26 min, 21% B.

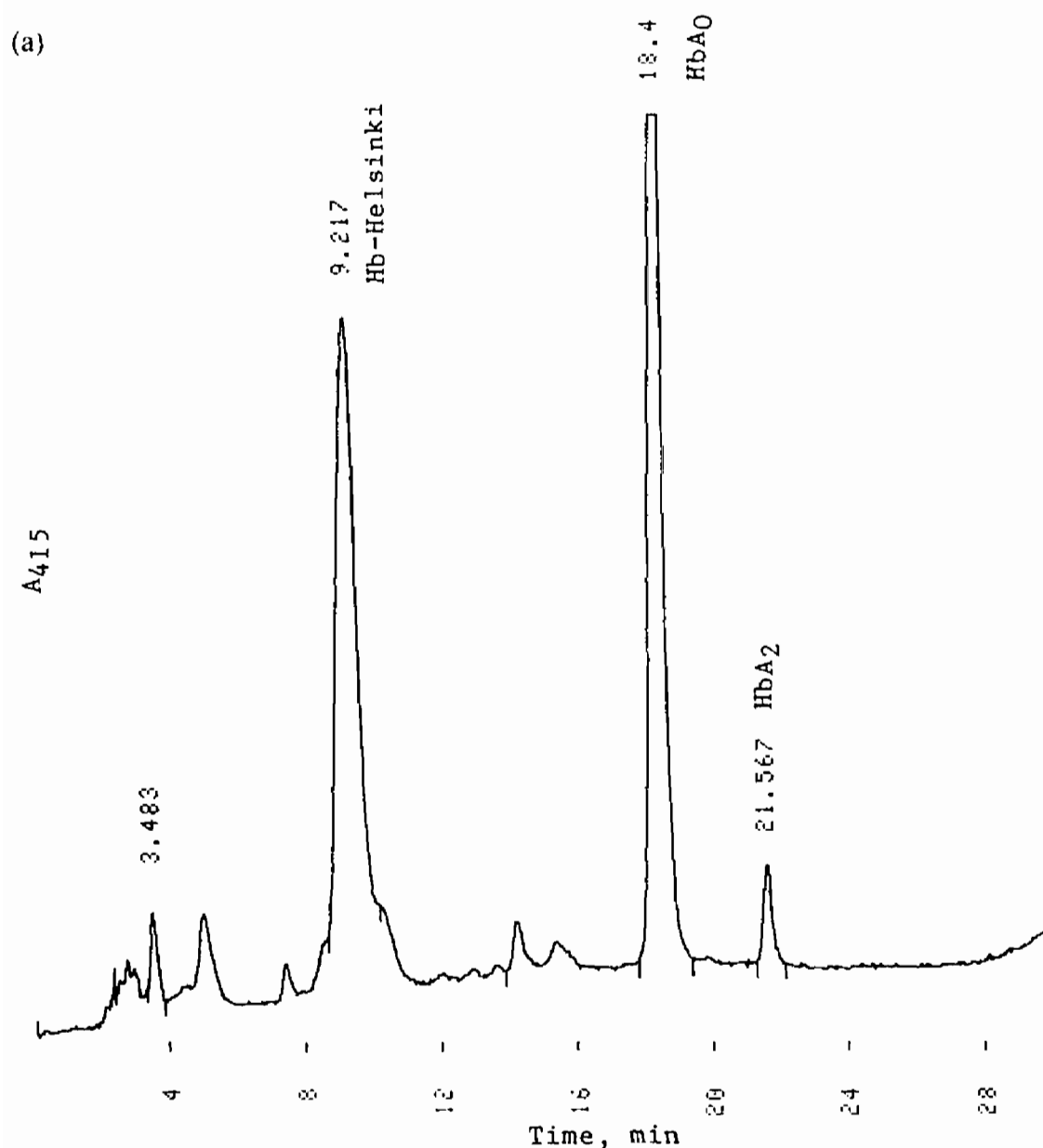


FIGURE 2 Cation-exchange chromatography on PolyCAT A of a red cell hemolysate with a β -chain variant of (a) Hb-Helsinki and (b) Hb-Vaasa. Time of elution since sample injection is indicated at each peak. Detection at 415 nm. Chromatographic conditions as in Fig. 1.

techniques developed, the one by Shelton et al. [19] has been found most useful. The chains are separated with an acetonitrile gradient in 0.1% trifluoroacetic acid in water and the effluent is monitored at 220 nm. Because of the acidity of the solutions, pH 2.0–2.2, the heme is removed from the globin and the chains are dissociated. Heme elutes first, usually in less than 10 min, followed by the β -chain, the δ chain, the α chain, and the γ chains. This technique has been especially important in detecting abnormalities with mutations that are undetect-

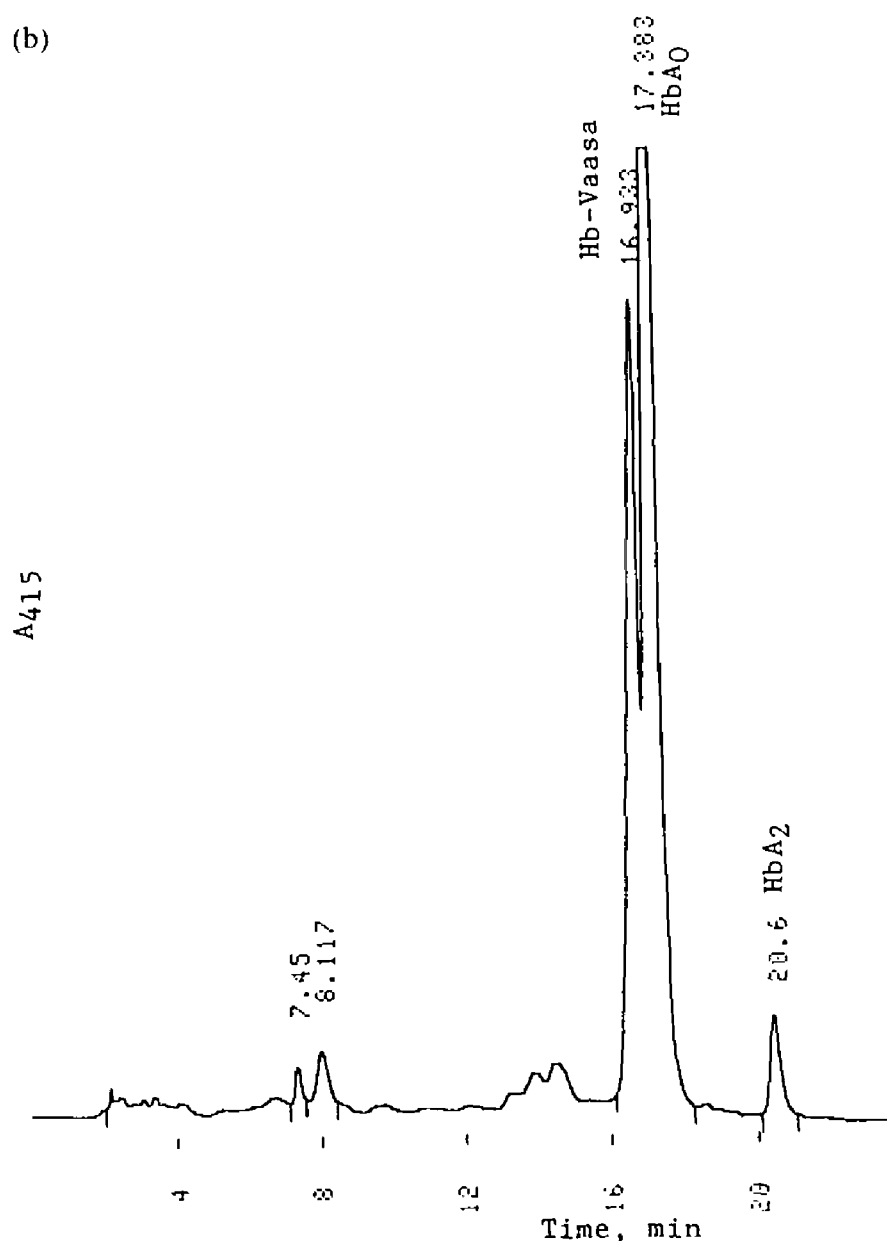


FIGURE 2 (continued)

able by electrophoretic and ion-exchange methods. One example is Hb-Linköping, which represents an “electrophoretically almost silent” mutant with a hydrophobic substitution $\beta 36 \text{ Pro} \rightarrow \text{Thr}$ [20]. Reversed-phase chromatography has also been widely used for the study of γ chain heterogeneity and it can also be used for quantitation of the δ chains in subjects with Hb E in association with thalassemia [21]. We routinely perform analytical separation of Hb chains on a $4.6 \times 250 \text{ mm}$ Vydac C4 column (The Separations Group, Hesperia, CA) according to the method of Shelton et al. [19]. We calculate the ratio between the α and the non- α chains based on the areas of the respective peaks. This ratio is

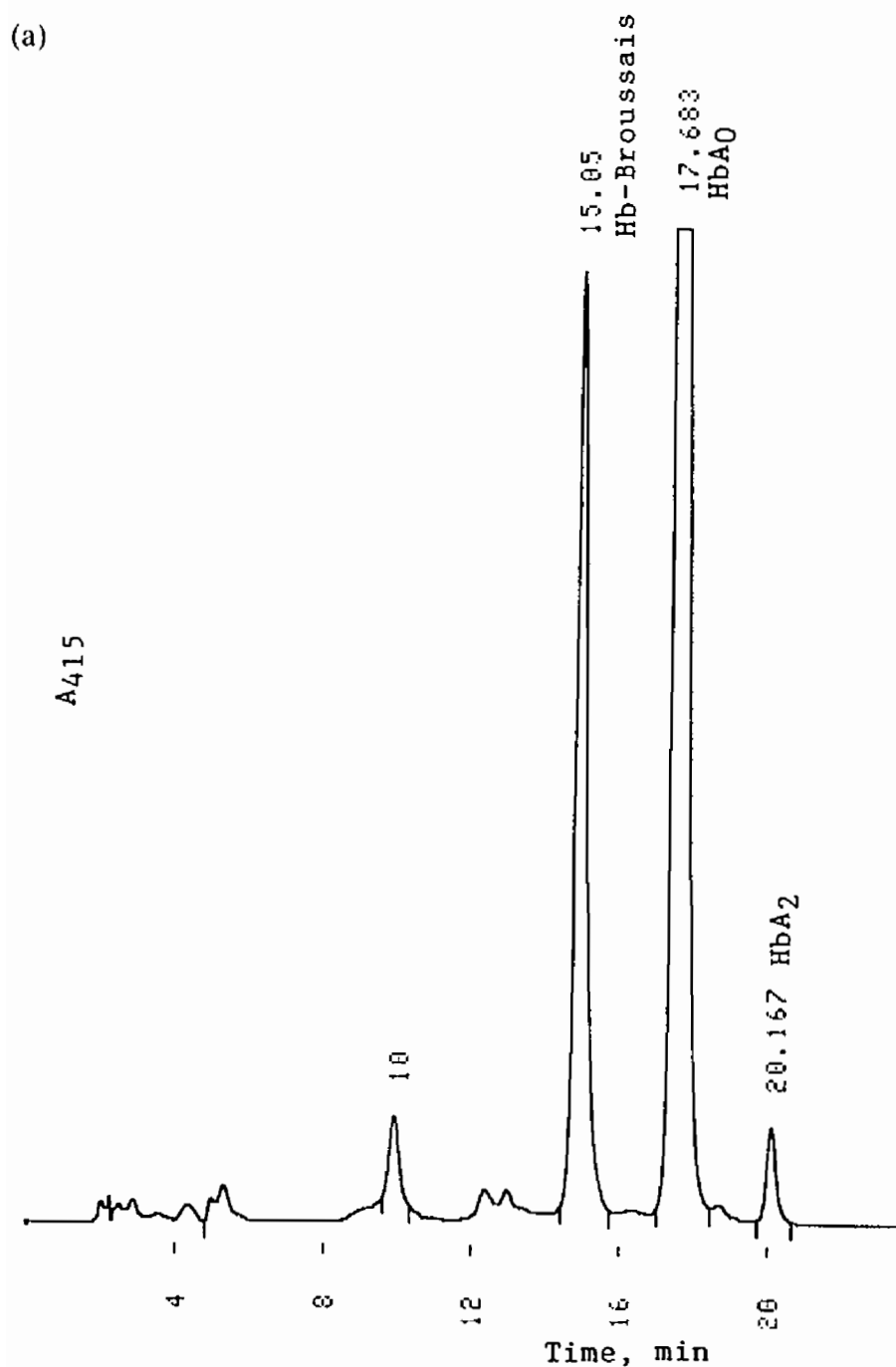


FIGURE 3 Cation-exchange chromatography on PolyCAT A of a red cell hemolysate with an α -chain variant of (a) Hb-Broussais and (b) Hb-Cemenelum. Time of elution since sample injection is indicated at each peak. Detection at 415 nm. Chromatographic conditions as in Fig. 1.

expected to approach one. Our results for the α/β ratio of 22 different control lysates is 1.05 with a coefficient of variation (CV) of 9%. Slight variations in the composition of solutions do not alter the elution pattern but alter the elution times. The steepness of the gradient can be modified to improve the separation of variant chains.

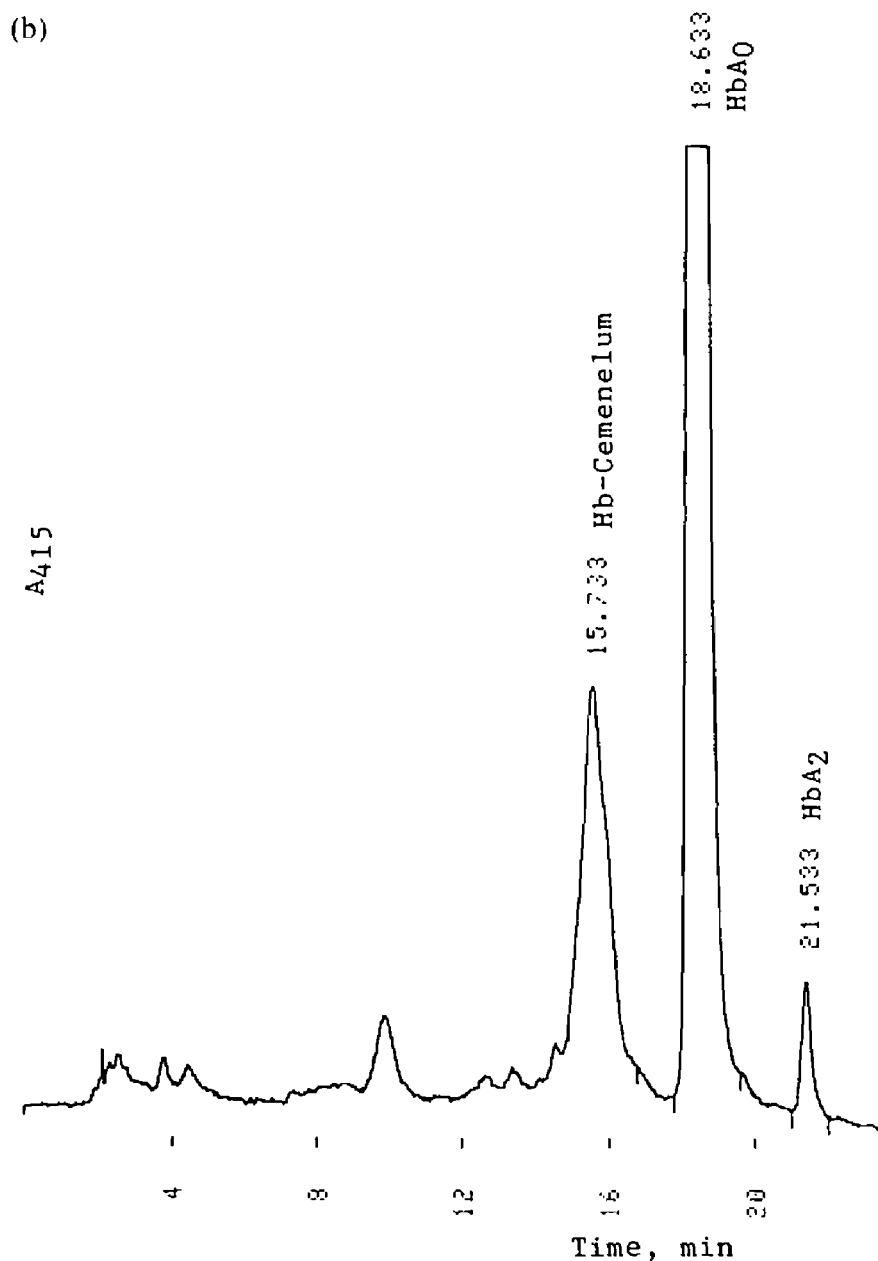


FIGURE 3 (continued)

Isolation and Characterization of Abnormal Hemoglobin Chains

For structural analysis of unknown Hb variants it is necessary to isolate the individual chains by a preparative procedure using a larger column, e.g., a 10 × 250 mm Vydac C4 column, at pH 2 and with detection at 220 or 280 nm as in analytical runs. This column can accept a load of several milligrams of Hb and we usually apply about 1.5 mg to the column. The aberrant chain is collected and evaporated to dryness under nitrogen.

Tryptic peptides are prepared by incubating about 500 µg of the isolated chain with 5–10 µg of porcine trypsin overnight in 130 mmol/L NH_4HCO_3 at

room temperature. After incubation, the solution is evaporated to dryness under nitrogen and the peptides are redissolved in dilute acetic acid. To separate the tryptic peptides, we use a Rexchrom Prep-5/300 C₈, 2.1 × 150 mm column (Regis Chemical Co., Morton Grove, IL). We inject about 8 µg of tryptic peptides and elute them with a linear gradient of acetonitrile in trifluoroacetic acid (0.1%) at a flow rate of 150 µL/min. Peptide peaks are automatically collected using a collector with slope detection. Aberrant peptides can usually be identified on the basis of their elution pattern [22].

Aberrant peptides are further characterized by amino acid sequencing. The sequence provides positive identification of the variant.

Genomic Analysis of Hemoglobin Variants

The development of polymerase chain reaction (PCR) and sequencing techniques based on the procedure developed by Sanger et al. [23] have changed the characterization of Hb variants. DNA is isolated from 5–10 mL of freshly collected blood. On the basis of protein analysis, the Hb chain containing an amino acid replacement can be tentatively identified. The corresponding exon is amplified by PCR using appropriate primers and sequenced. This approach has led to the discovery of some unexpected amino acid replacements that are electrophoretically silent but greatly affect physicochemical and functional properties [2].

Analysis of HbA_{1c}

Determination of HbA_{1c} by ion-exchange HPLC is widely used for monitoring of glycemic control in diabetic patients. The percentage of HbA_{1c} reflects blood glucose concentrations of the previous 2–3 months. It is therefore considered the most reliable indicator of long-term diabetic control [24].

The classical method for rapid determination of HbA_{1c} by chromatography on the weak cation-exchange resin Bio Rex 70 was introduced by Trivelli et al. in 1971 [25] based on the method of Allen et al. using Amberlite IRC-50 [26]. Several approaches, including minicolumns [27] and HPLC [28], have been used to make ion-exchange chromatography acceptable for clinical laboratories. Several methods using ion-exchange resins for HPLC, such as Synchropak CM 300 [29], silica-based carboxymethylpolyamide [30], Mono S [28,31] and the Glycopak resin of the Diamat analyzer (Bio-Rad), have been developed. They differ in their sensitivity to interferences by adducts formed between Hb and urea, aldehydes or acetyl salicylic acid, and other hemoglobin variants [32,33], which tend to cause overestimation of HbA_{1c}. In spite of these limitations, automated HPLC methods have become the de facto standard against which other methods

for determination of HbA_{1c} are calibrated [34]. With these methods, the reference interval is in the range of 4–6% of total Hb.

Glycohemoglobin can also be assayed by affinity chromatography using boronate agarose columns [35]. The boronate affinity chromatography methods are not affected by Hb variants, slight temperature changes, carbamylated Hb, and the labile glycated fraction [36]. In spite of this, the reference values are similar to those obtained with rapid chromatographic methods based on ion-exchange chromatography. This is because Hb fractions other than A_{1c} are to some extent glycated and the HbA_{1c} fraction obtained by ion-exchange chromatography contains a similar amount of nonglycated Hb. An automatic affinity HPLC system is now available and has been evaluated for the monitoring of glycohemoglobin [37].

HPLC METHODS FOR DETERMINATION OF HbA_{1c} BY CATION-EXCHANGE CHROMATOGRAPHY

Mono S

This system was introduced by Stenman et al. [28] and Jeppsson et al. [31]. The Mono S method has been widely used in clinical laboratories and is precise, accurate, and easily operated [31]. The acetaldehyde adduct of Hb in alcoholics may interfere with HbA_{1c} determinations because it coelutes with HbA_{1c}. In uremic patients, carbamylated Hb may increase the HbA_{1c} values by as much as 1% (e.g., from 5% to 6%). We have extensively used a slightly modified variant of the original Mono S method [28] by using phosphate buffers with 10% acetonitrile, a pH of 6.85, and a column temperature of 30°C. These changes improved both separation and column life, which increased up to several thousand samples with constant resolution. The reference range for HbA_{1c} is 4.2–6.6% (Table 1). At pH 6.85, HbF elutes after HbA_{1c}, whereas with malonate buffers at pH 5.7, HbF elutes before HbA_{1c}. A slight modification of the original

TABLE 1 Reference Ranges of HbA_{1c} with Various Methods

Method	HbA _{1c} (%)	Reference
Mono S	4.2–6.6	28
Diamat	4.5–5.8	42
PolyCAT A	2.5–4.4	42
PolyCAT A	2.8–5.5	43
PolyCAT A	3.9–5.7	41
Affinity chromatography	5.0–7.6	54
Affinity chromatography	4.8–6.4	55

malonate buffer gradient, where lithium has been used as an elution cation, has been used for quantitation of HbF [38].

PolyCAT A

PolyCAT A, a weak cation exchanger with polyaspartic acid linked to silica [39,40], has been used for the determination of HbA_{1c} [41,42] and for identification of several minor hemoglobin variants [10,43]. The separation of HbA_{1c} from other Hb components such as acetylated Hb [33] and HbF depends critically on buffer composition, particularly on the pH and salt gradient. It is often necessary to make minor modifications in the steepness of the salt gradient to obtain good separation.

PolyCAT A with bis-tris buffers and sodium acetate gradients has been used to separate and quantitate many normal and abnormal Hb components [42,43].

Minor fetal Hb forms such as HbF₁, HbF_{1b2}, and HbF_{1c2} can be separated from HbA_{1c} and simultaneously quantitated. We have compared the performance of PolyCAT A chromatography with a boronate affinity method and the automated Diamat system for determination of HbA_{1c} [42]. The two cation-exchange methods, PolyCAT A and Diamat, showed acceptable correlation ($r = 0.90$) but the regression equation (PolyCAT A = $1.06 \times \text{Diamat} - 3.06$) showed that much lower results were obtained by the PolyCAT A method. This suggests that the HbA_{1c} value measured by the Diamat assay includes a background of 2–3% of the total Hb. The negative bias is present at all levels of HbA_{1c}. This is probably due to the fact that the Diamat method also includes in the HbA_{1c} value carbamylated and acetylated forms of Hb [32] and possibly some other Hb derivatives formed in blood during storage, whereas these components can be separated from the HbA_{1c} peak by using high-resolution methods. However, the difference between the Diamat method and PolyCAT A cannot be explained only by carbamylated and acetylated Hbs, for which levels below 0.4% of total Hb have been reported [32]. Because the slope of the regression line is close to unity (1.06), the PolyCAT A method reflects differences in glycosylated Hb in the same way as the Diamat method; however, because the variable nonspecific background is eliminated in the PolyCAT A method, it may be expected to reflect glycemic control more accurately than the routine methods.

Diamat and Variant Analyzers

The Diamat analyzer of Bio-Rad Laboratories is an automated HPLC instrument using a weak cation-exchange column. The support material is silica with carboxymethyl functional groups. The analyzer forms a stepwise gradient composed of three phosphate buffers of increasing ionic strength. The system has been optimized for the quantitation of HbA_{1c}, while HbA₂ coelutes with

HbA₀. Quantitation is based on comparison of the absorbance values at 415 and 690 nm. Before analysis, the samples are incubated at 37°C for 30 min to remove the labile HbA_{1c} fraction.

The Variant analyzer of Bio-Rad Laboratories offers a broader selection of methods than the Diamat system. Although the separation principle is the same, with various programs it is possible to separate and quantitate HbF and HbA₂ in addition to HbA_{1c}. The method has been calibrated against the Diamat method. In practice, however, there seems to be a small difference between these systems, the Variant giving slightly higher results than the Diamat [44].

REFERENCE METHODS FOR HbA_{1c}

The methods currently used for measurement of HbA_{1c} in clinical chemistry laboratories show large differences between reported values, and comparison of results from different laboratories is difficult [45]. Because of this, it is important to determine method-specific reference ranges. With the PolyCAT A method, the reference values of HbA_{1c} are lower than with the Diamat method, apparently because of better separation of HbA_{1c} from nonglycated coeluting forms of Hb (Table 1). The values for the PolyCAT A method correspond to about 60–70% of those obtained by boronate affinity chromatography. This suggests that the PolyCAT A method fairly specifically measures the glycated component of the HbA_{1c} peak.

Lack of standardization of glycohemoglobin measurements is a major source of interlaboratory variation. To achieve international standardization, the International Federation of Clinical Chemistry (IFCC) has established a working group for HbA_{1c} standardization with the aim of developing a reference method [46]. The group has defined HbA_{1c} as Hb that is irreversibly glycated at one or both N-terminal valines of the β chains, including Hb that is also glycated at any lysine residue in the β chain. Hb that is solely glycated at a lysine residue is not considered to be HbA_{1c}.

A reference method that measures HbA_{1c} specifically according to the molecular definition has been published [47]. The method is based on HPLC-electrospray ionization-mass spectrometry (ESIMS) and measures the ratio of the N-terminal hexapeptides of HbA_{1c} and HbA obtained by endoproteinase Glu-C cleavage. The potential of ESIMS as a reference but also as a routine method for the quantification of glycohemoglobin has recently been evaluated [48]. An HPLC-capillary electrophoresis method has also been suggested as a reference method. These methods will be evaluated further in an international network of reference laboratories established by the IFCC Working Group on HbA_{1c} standardization [47].

IMMUNOCHEMICAL METHODS FOR THE DETERMINATION HbA_{1c}

The need for more rapid assays for glycated Hb has led to development of immunological methods based on mono- and polyclonal antibodies. Polyclonal antibodies against glycated Hb became available in 1978 [49], and monoclonal antibodies soon after [50]. Antibodies have primarily been used for development of immunoturbidimetric assays [51].

Several commercial immunological assays are available. Novolone HbA_{1c} (Dako) is an enzyme-linked immunoassay (ELISA) kit based on a monoclonal antibody specific to the N-terminal of the β chain with glucose attached in its stable ketoamine form. Tina-quant HbA_{1c} (Boehringer Mannheim) is an immunoturbidimetric inhibition assay, utilizing an antibody specific for the glycated amino terminus of β chain. When the Tina-quant method was first compared with the Diamat method, a negative bias and a positive slope were obtained [34]. Because the Diamat method is widely used, the manufacturer decided to calibrate the new assay according to the Diamat method. The comparison between the Novoclone assay (y) and Diamat (x) also gave a negative bias and a positive slope: $y = 1.27x - 3.88$ [52]. The Unimate HbA_{1c} method is a latex-enhanced competitive turbidimetric immunoassay (Roche Diagnostic Systems) using monoclonal antibodies. When it was first compared with an HPLC cation-exchange method, the correlation was: Unimate = 1.20 HPLC - 2.65 [53]. Recently, the manufacturer has introduced a conversion factor, which can be changed by the user to make the results comparable to those obtained by HPLC.

The use of a fairly nonspecific chromatographic method for calibration of other assays is based on the wish to maintain the results on the level familiar to the clinician. However, from a theoretical and principal point of view this approach is unacceptable [42].

CONCLUSIONS

HPLC methods have become a useful addition to routine electrophoretic techniques in identifying and quantifying Hb variants and it is the dominant technique for determination of HbA_{1c}, although recently developed immunoassays are competing with HPLC. Because of its good precision, ion-exchange HPLC has been recommended as the "gold standard" for measurement of glycohemoglobin. However, the ongoing standardization of the HbA_{1c} assay methods initiated by the International Federation of Clinical Chemistry aims at developing a new reference method.

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Antibodies

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INTRODUCTION

The application of antibodies in biochemistry, medicine, and other fields including agriculture, forensics, and environmental monitoring has become so important that it will remain a vital force in the continuing growth of the industry. Since the introduction of the hybridoma technology more than 20 years ago by Koehler and Milstein [1], the monoclonal antibody has revolutionized biomedicine. As diagnostic agents in many diseases, especially in neoplastic diseases, monoclonal antibodies have proved to be extremely useful. Developments in cancer therapy has resulted in Food and Drug Administration (FDA) approvals of several monoclonal antibodies as the new class of biotherapeutic cancer drugs. Currently, therapeutic monoclonal antibodies represent more than 30% of the biotechnology products in U.S. clinical development. The need to develop effective, economical, and rapid purification methods for antibodies from a variety of biological fluids becomes imperative for in vitro or in vivo application.

Antibody purification can be divided into two main groups: precipitation methods and chromatographic methods. The latter are grouped further according to the operational characteristics into nonaffinity and affinity chromatography.

Depending on the application of the antibody, purity and recovery are likely to determine the strategy of purification. In this chapter, common chromatographic methods for antibody purification will be described. In general, multiple-step purification and combination of methods are required to obtain sufficient purity of antibodies. Because every antibody is unique and each has a distinctive distribution of hydrophobic and positive and negative charge characteristics, modification and optimization of purification procedures might be necessary [2].

Antibodies are globulin proteins (immunoglobulins) that react specifically with the antigen that stimulated their production. Based on the electrophoretic migration rate, there are three types of globulins: alpha, beta, and gamma. Antibodies are gamma globulins. There are five classes of antibodies: Immunoglobulin G (IgG), IgM, IgA, IgD, and IgE. Within these classes, subclasses also exist. IgG and IgM are antibody classes involved in the primary and secondary immune responses that can be found in the sera in relatively high concentrations. Only the purification of these two classes will be discussed in detail.

IMMUNOGLOBULIN STRUCTURE

Immunoglobulins are glycoproteins made up of light (L) and heavy (H) polypeptide chains as based on their respective molecular weights. Light chains have a molecular weight (MW) of ~25 kDa, whereas heavy chains have a molecular weight of 50–70 kDa. The simplest molecule has a Y shape and consists of four peptide chains: two H and two L (Fig. 1). The four chains are linked by disulfide bonds. An individual antibody molecule always consists of identical H chains and identical L chains.

The L and H chains are subdivided into variable and constant regions. The regions are composed of three-dimensionally folded, repeating segments called domains. An L chain consists of one variable (VL) and one constant (CL) domain. Most H chains consist of one variable (VH) and three constant (CH) domains. Each domain is approximately 110 amino acids long. The variable regions are responsible for antigen binding, whereas the constant regions are responsible for various biological functions, e.g., complement activation and binding to cell surface receptors. The remarkable specificity of antibodies is due to the variable antigen binding regions [3].

The light chains belong to one of two types, κ (kappa) or λ (lambda), on the basis of amino acid differences in their constant regions. The regulation within B cells ensures the synthesis of either kappa or lambda light chains, but not both, in any immunoglobulin molecule. The heavy chains are distinct for each of the five immunoglobulin classes and are designated γ chains in IgG and μ chains in IgM. Each IgG molecule consists of two L chains and two H chains linked by disulfide bonds (molecular formula H_2L_2 , MW = 150 kDa). Because IgG has two identical antigen binding sites, it is said to be divalent. There are four subclasses,

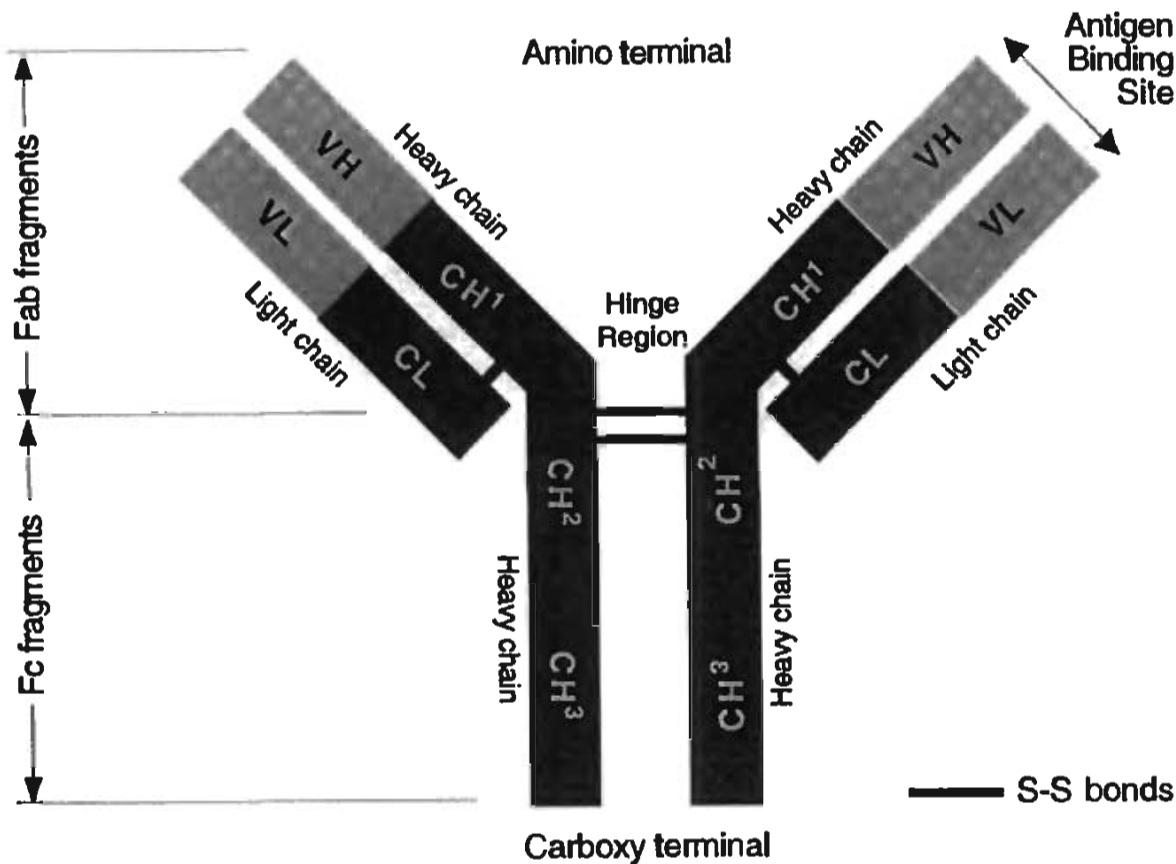
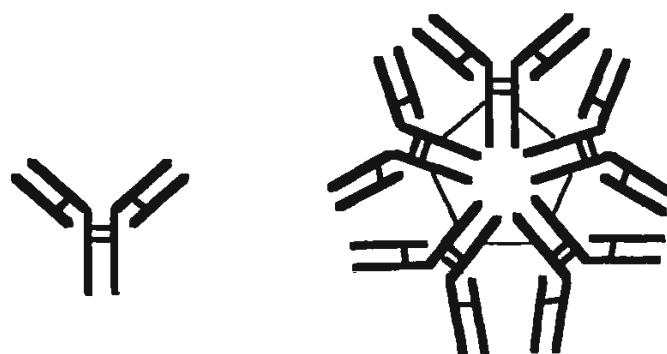


FIGURE 1 Structure of an antibody molecule. The Y-shaped IgG consists of two light chains and two heavy chains. Each heavy chain consists of a variable region and a constant region that is divided into three domains: CH¹, CH², CH³. The CH² domain contains the complement binding site and the CH³ domain is the attachment site of IgH to receptors on neutrophils and macrophages. The antigen binding site is formed by the variable regions of both the light and heavy chains.

IgG1–IgG4, based on antigenic differences in the H chains and on the number and location of disulfide bonds. IgM is the main immunoglobulin produced early in the primary response [4]. It is present as a monomer on the surface of virtually all B cells. In serum, it is a pentamer composed of five H₂L₂ units with an addition of one molecule of J (joining) chain. The pentamer of 900 kDa has a total of 10 antigen binding sites (Fig. 2).

Limited proteolytic cleavage with enzymes such as papain, pepsin, and ficin produces well-defined fragments of antibody molecules containing different combinations of domains. Two antigen-binding fragments (Fab) of ~50 kDa and the crystallizable fraction Fc are produced by the digestion of IgG with papain [5,6]. Pepsin or ficin digestion of IgG results in a dimer, F(ab')₂, of ~110 kDa [7,8]. The Fc fragments are fully or partially digested under these conditions (Fig. 3).



Ig Classes:	IgG	IgM
Heavy chain:	γ	μ
Light chain:	κ or λ	κ or λ
Antigen binding site:	2	10
Molecular weight (Da):	150,000	900,000

FIGURE 2 IgM is a pentamer composed of five IgG-like molecules and coupled by a J (joining) chain. It has a total of 10 antigen binding sites and a molecular mass of 900,000 Da.

Fragmentation of IgM can be obtained by similar enzymes with less well defined fragments than is the case for IgG molecules [9,10]. Trypsin and pepsin as well as 2-mercaptoethylamine-HCl (2-MEA-HCl) generate a variety of IgM fragments (Fig. 4). Developments in recombinant phage technology enable researchers to design and produce a repertoire of single-chain variable fragment genes in which the variable regions of the antibody heavy (VH) and light (VL) chain genes are joined by a flexible linker [11,12]. The soluble phage-displayed recombinant antibody fragments are called ScFv and are functional and useful as

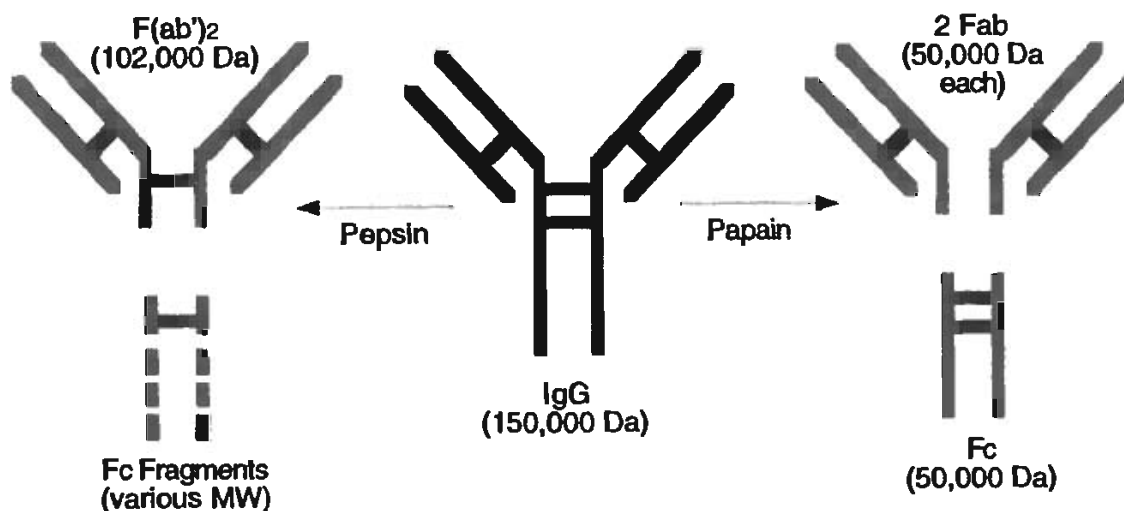


FIGURE 3 Proteolytic cleavage of antibodies.

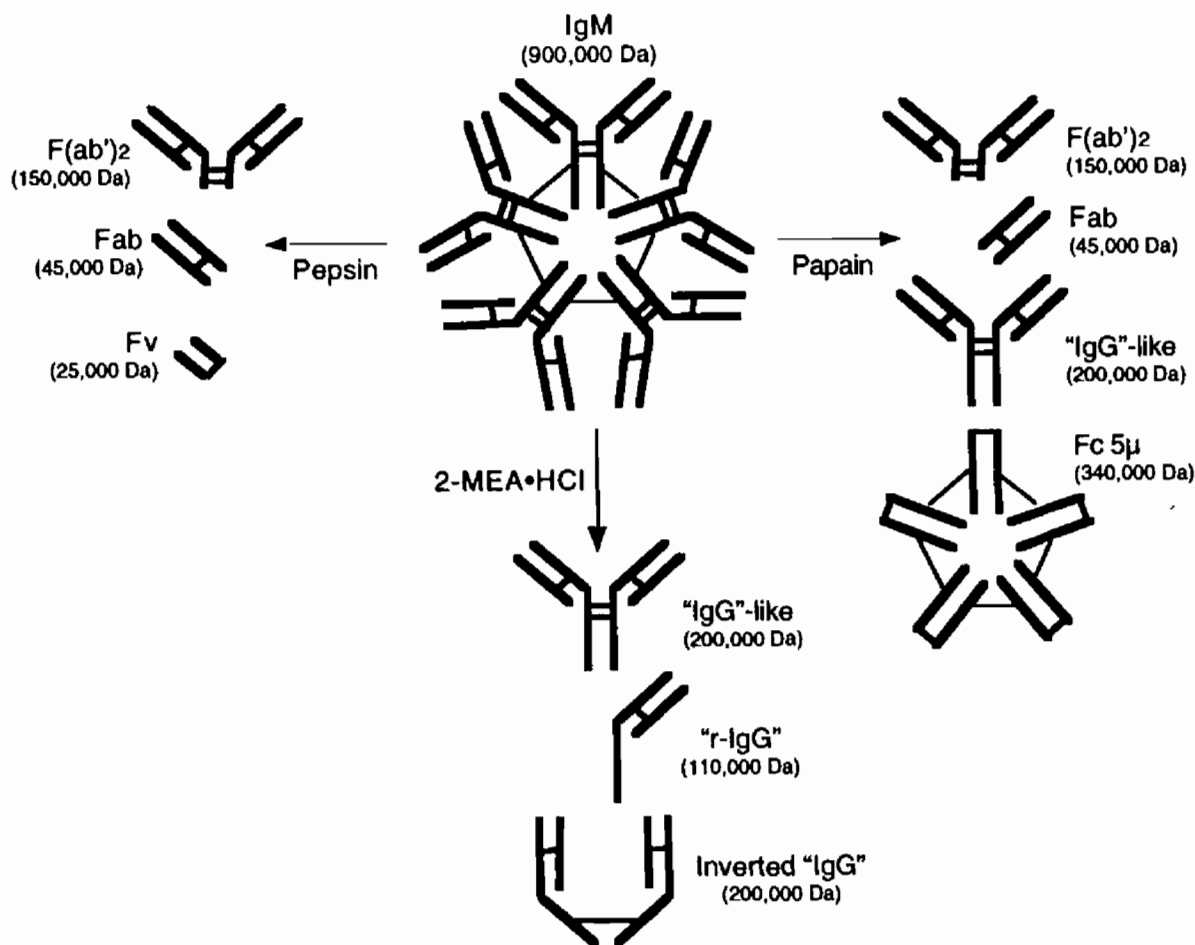


FIGURE 4 Fragmentation of IgM.

immunological reagents (Fig 5). One of many advantages of this technology is that a peptide tag such as polyhistidine located at the carboxyl terminus of ScFv recombinant antibodies can be integrated and expressed. Peptide tags on the ScFv molecule facilitate an easy affinity purification strategy by anti-peptide tag antibody affinity chromatography or by immobilized metal-ion affinity chromatography [13,14].

POLYCLONAL AND MONOCLONAL ANTIBODIES

There are two different approaches to generating antibodies: polyclonal and monoclonal methods. Polyclonal antibodies are often raised by hyperimmunizing animals with antigens. The hyperimmune animal serum contains a mixed population of antibodies against an entire range of epitopes or determinants present on the antigens. Hyperimmune sera often have higher levels of IgG—up to 10% of the total IgG content is specific antibody. The use of these mixed populations of antibodies sometimes creates problems in immunochemical

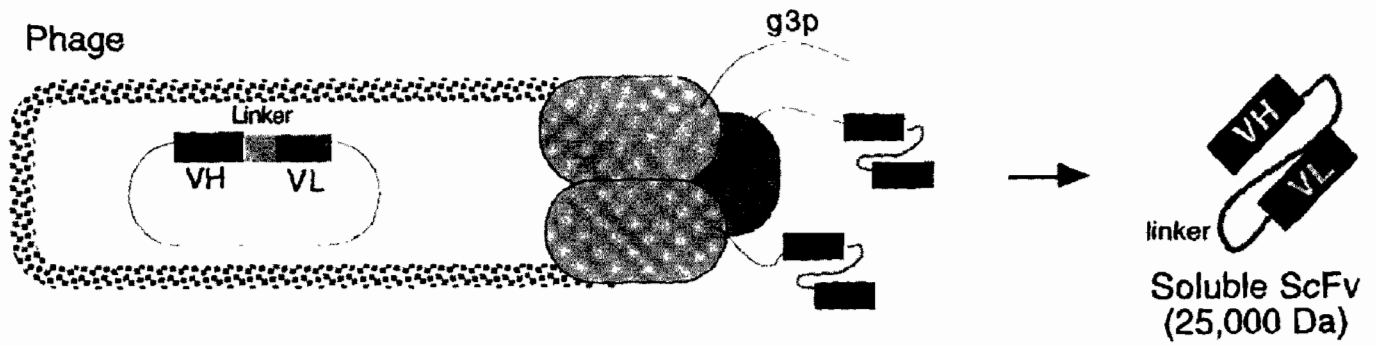


FIGURE 5 Single-chain variable fragment (ScFv). The variable regions of the antibody heavy (VH) and light (VL) chain genes are joined by a flexible linker and cloned in recombinant phage. The phage display fully functional antibody fragments on their surface. Following affinity selection, positive recombinant phage can be used to infect *E. coli* to produce soluble ScFv.

techniques. Therefore, the preparation of homogeneous antibodies with a defined specificity is desirable.

One of the most important scientific advances of the 20th century was the hybridoma technology introduced by Koehler and Milstein in 1975 [1]. This technique allows the growth of clonal populations of cells secreting antibodies with a defined specificity, e.g., reactive against a single epitope. An antibody-secreting cell is fused with a myeloma cell to create a hybrid cell or hybridoma that secretes a single molecular species of immunoglobulin. These immunoglobulins, which are known as monoclonal antibodies, are identical because they are produced by descendants of one hybridoma cell. The monoclonal antibodies offer many advantages because of their specificity of binding, homogeneity, and ability to be produced in unlimited quantities. The monoclonal antibody can be produced *in vitro* (cell culture, bioreactors) or *in vivo* as ascites fluid in mice or rats. Although monoclonal antibodies produced by hybridomas are homogeneous in nature, variable posttranslational glycosylation is possible that can cause differences in isoelectric point (pI).

Chimeric or humanized monoclonal antibodies consisting of mouse variable regions and human constant regions are being constructed for use in treating a broad range of human diseases [15,16]. The advantages of the chimeric antibodies include complement activation and less immunogenicity for subsequent administration [17].

GENERAL CONSIDERATIONS

The most common sources of specific antibodies are serum, ascites fluid, and cell culture supernatant. The major contaminants present are albumin, transferrin, and nonspecific endogenous antibodies, which are either produced by the host animal or added as supplements to the media (fetal calf serum or horse serum). Unlike other contaminants, endogenous antibodies are more difficult to separate from the specific antibodies. Affinity purification with immobilized reactive antigens can result in a highly purified specific antibody preparation if a sufficient amount of reactive antigen is available. If the antibody-producing hybridoma cells could be grown in the culture or bioreactors in serum-free media, the purification of the antibody would be much simpler. Chromatographic separation of IgG and IgM based on differences, in size, charge, hydrophobicity, and affinity or a combination of several separation modes will be described in the remainder of this chapter.

Because biological fluids and crude cell supernatant or feed streams are the starting materials of the antibody to be purified, precautions should be taken to prevent column fouling by floating fats and other lipids, cell debris, and mucinous impurities. Filtering raw samples through 0.2- μm membranes prevents fouling by gross cell debris. Because most foulants are negatively charged and most

antibodies are strongly electropositive, foulant removal can be achieved by anion exchange at physiological pH and ionic strength. The simplest removal can be obtained by adding microgranular cellulosic anion-exchange media directly to the raw material. A simple clarification agent and nonionic lipid absorbent such as Cleanascite (CPG, Inc., Lincoln Park, NJ) can also be used to bind impurities effectively. Another approach is to use an in-line prefilter macroporous-based anion exchanger as a guard column. Column cleaning as suggested by the packing material manufacturer should be routinely performed to keep the column performance intact and avoid microbial contamination [18,19].

CLASSICAL PRECIPITATION METHODS

Fractionation of antibodies by precipitation has been in practice for many years [20]. By the time monoclonal antibodies emerged, a variety of precipitation mechanisms were already in use with polyclonal antibodies. Precipitation methods alter the properties of the solvent to cause selective precipitation of antibodies using salts such as ammonium sulfate or organic polymers such as polyethylene glycol (PEG). Alternatively, precipitation of the contaminants can be achieved by reducing the solubility of contaminant proteins using organic complexants such as short-chain fatty acids or organic bases. In addition to their unique selectivities, each method offers a different mix of practical advantages and limitations. In general, poor reproducibility of precipitation processes and offset between purity and recovery curves are characteristic of precipitation methods in purifying antibodies. Nevertheless, precipitation methods make an effective partnership with chromatographic methods. Salt precipitation followed by ion-exchange HPLC is the classical IgG purification method. In IgM purification, euglobulin precipitation followed by size exclusion HPLC or hydroxyapatite HPLC offers an effective purification method [21]. Precipitation by electrolyte depletion is traditionally referred to as euglobulin precipitation [22]. The major class of antibodies precipitating in water are IgM and IgG3 [23].

NONAFFINITY CHROMATOGRAPHIC METHODS

Nonaffinity chromatographic methods of purifying antibodies from a protein mixture are based on differences in size, charge, hydrophobicity, and interaction with specific media such as hydroxyapatite.

Size Exclusion Chromatography

Size exclusion chromatography (SEC) has been popular for laboratory-scale purification of antibodies because of its simplicity. Because the separation is determined predominantly by the gel matrix, method development is minimal.

Due to the large size of the molecule, SEC is useful in purifying antibodies of the IgM isotype. The major limitations of SEC that restrict the wide use of this method for antibody purification, especially for commercial use, are low resolution, low capacity, and its general slowness. Because SEC allows only a limited volume of sample to be injected into the column, ascites fluid with a high IgM concentration is more suitable for this method than cell culture supernatant. Due to the gentle separation conditions, antibodies separated by this method typically retain their bioactivity, in contrast to those produced by other methods that require extreme exposure to harsh pH or a high concentration of salts or chaotropic agents. Reasonable purities of 50–80% for IgG and 80–90% for IgMs are typically expected from SEC. However, the separation cannot discriminate between the specific target monoclonal antibodies and nonspecific antibody contaminants of the same class. Major contaminating proteins in IgG separations are complements C3 and C4, haptoglobulin, β_1 -haptoglobulin, ceruloplasmin, and inter-alpha trypsin inhibitor. Typical contaminants found in IgM fractions are α_2 -macroglobulin and high- and low-density lipoproteins (HDL and LDL) [24].

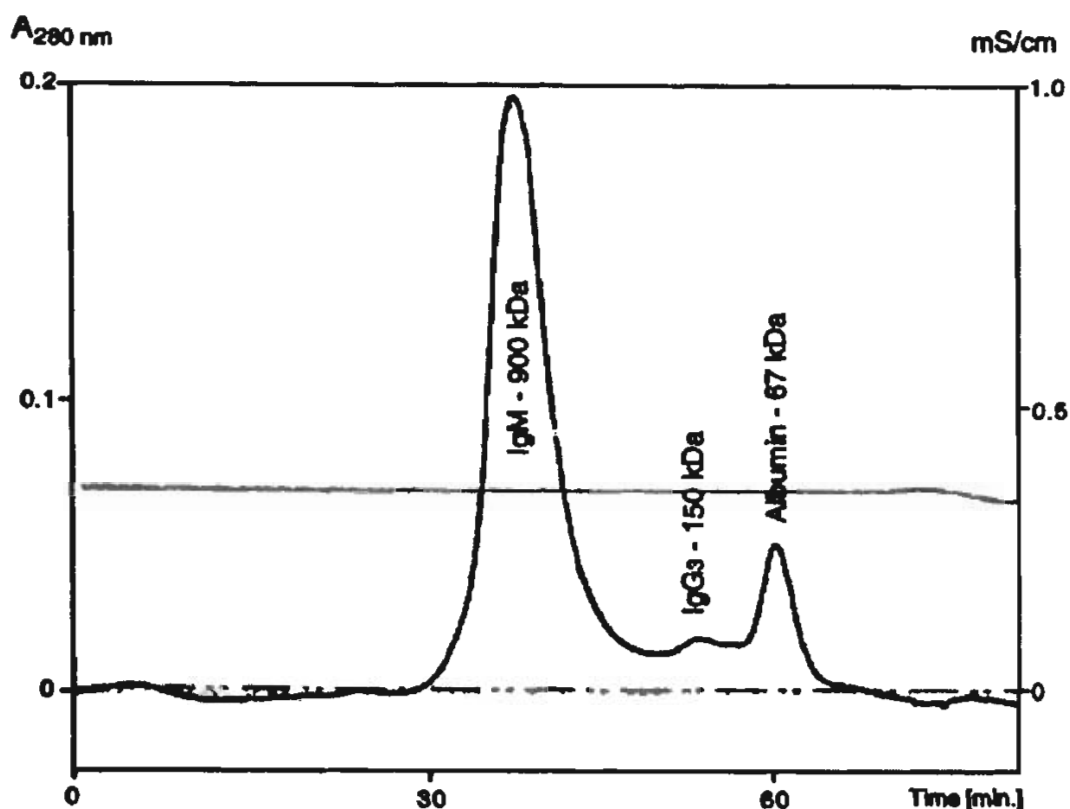
It is important that the antibody be fully soluble under the buffer conditions. A buffer of moderate salt concentration is preferable because IgGs and especially IgMs are poorly soluble in a low ionic strength buffer. High concentrations of salts or organic additives such as polyethylene glycol in the running buffer may also alter SEC separation performance. Like salt, glycine has a solubilizing effect on antibodies, especially IgM [25], and it is a recommended additive in the running buffer for SEC.

It is highly desirable to choose an inert SEC medium to avoid charge interactions, hydrophobic interactions, hydrogen bonding, and biospecific interactions between antibody molecules and the medium that can cause elution interference for antibodies [26,27]. Agarose-based media often bear residual sulfates, dextrans carry residual carboxyls, and polyacrylamides carry primary amino groups that can potentially cause charge interactions. These interactions can be reduced by adding 0.2–0.5 M sodium chloride to the buffer [28]. Most of the SEC media are rich in hydroxy groups as potential hydrogen donors, which contribute to nonspecific binding of antibodies. The addition of urea or sucrose as a hydrogen donor or acceptor in the buffer can reduce these effects [29].

The most powerful use of SEC is in combination with other chromatographic steps following precipitation, such as hydrophobic interaction or ion-exchange HPLC. An example of the purification of a monoclonal antibody MAb 46.3 (IgM isotype) by SEC following euglobulin precipitation is shown in Fig. 6.

Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) has been a platform for antibody purification for many years [30]. IEC separates proteins on the basis of differences in the



Sample: 0.25 mL Euglobulin precipitated Ascites MAb 46.3
 Column: Superose 6 (Pharmacia Biotech)
 Buffer A: Phosphate Buffered Saline, pH 7.4
 Buffer B: -
 Flow rate: 0.25 mL/min
 Gradient: -

FIGURE 6 Purification of IgM by size exclusion chromatography following euglobulin precipitation. Monoclonal antibody of IgM isotype (MAb 46.3) was purified from ascites by euglobulin precipitation. Precipitated IgM was contaminated by IgG3 and albumin, which were separated further by size exclusion chromatography on a Superose-6 column.

surface charge of the molecules. Because antibody molecules have a more basic isoelectric point than the majority of other serum or contaminating proteins, IEC is useful in purifying antibodies regardless of isotype [31–33]. The general strategy is to keep the pH below the isoelectric point for antibodies so that they will not bind to an anion exchanger such as diethylaminoethyl (DEAE)-modified resin or, alternatively, to raise the pH above the pI where the antibodies will bind to the DEAE groups. The opposite strategy works for cation exchangers. The bound antibodies are commonly eluted with a salt or pH gradient. Under these

conditions, most eluted antibodies are expected to maintain their biological activity [34].

Because every antibody is unique and its pI can vary, binding to an IEC resin needs to be explored and determined experimentally on an individual basis. The contaminant spectrum with which an antibody is associated on a cation exchanger is usually different from the spectrum on an anion exchanger. Even on the same exchanger, altering the pH may significantly change the profile of contaminants coeluting with the antibody [35]. Therefore, it is recommended that the appropriate pH for optimal separation be determined. Effects on the retention of IgG on an anion-exchange resin (Poros 20 HQ) under various pH conditions are shown in Fig. 7.

Likewise, the elution conditions of IEC can be optimized. Elution can be conducted in either step or linear gradient formats. In general, a linear gradient offers better reproducibility with a disadvantage of dilution effects.

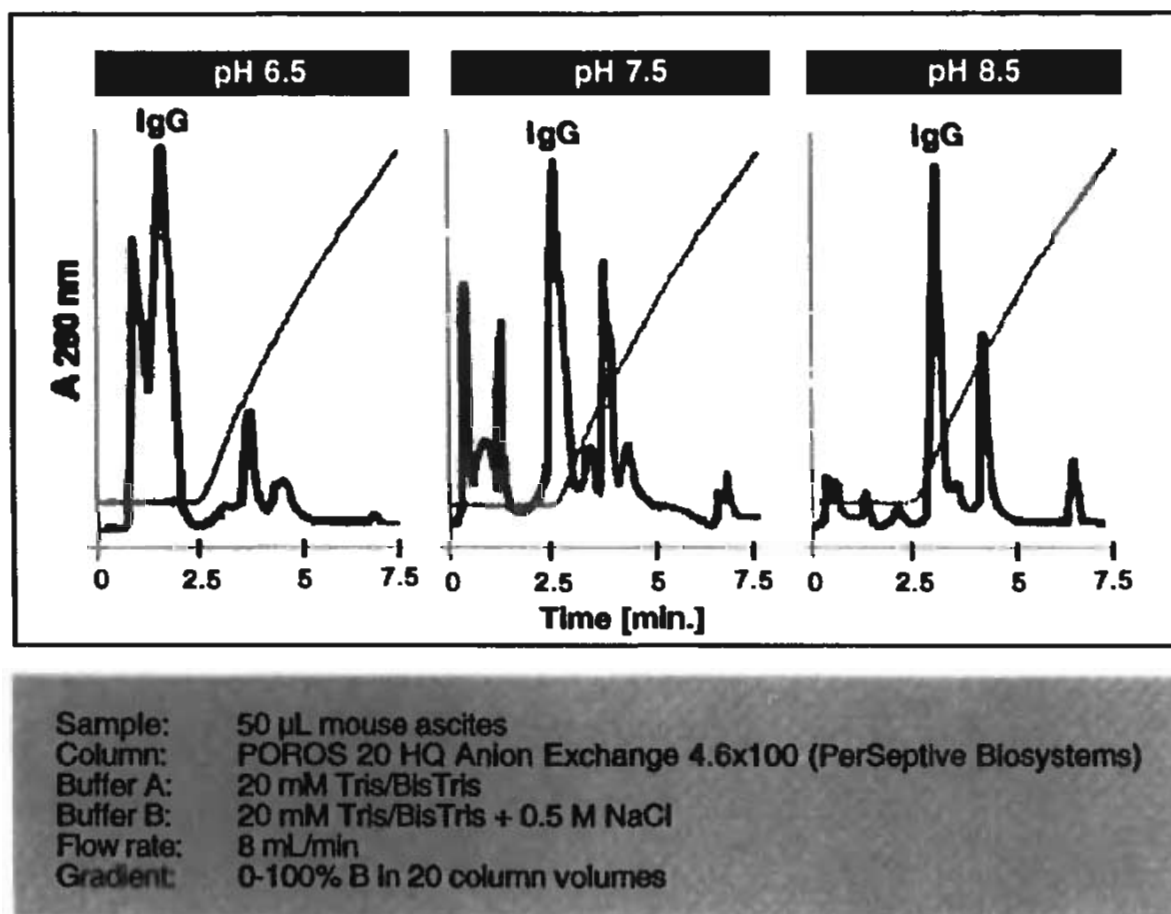
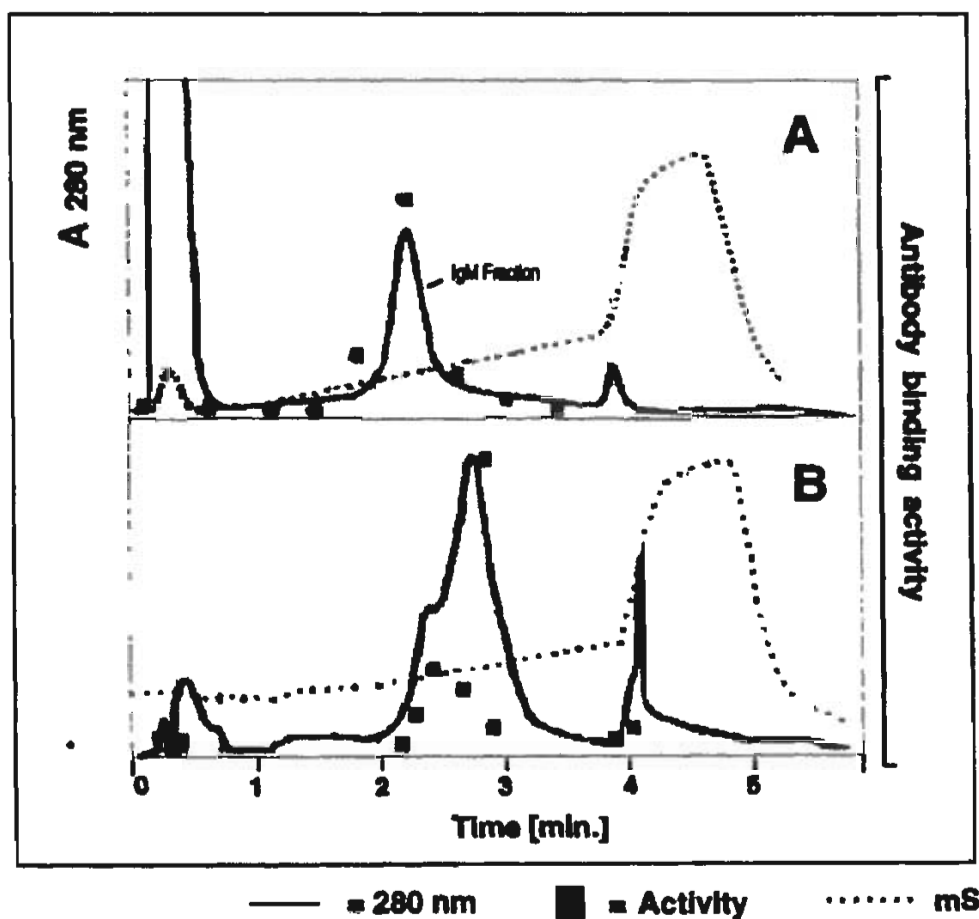


FIGURE 7 pH map on an anion-exchange resin. The appropriate pH for optimal separation of IgG from crude ascites on POROS HQ anion exchange resin was determined by pH mapping experiments. High purity and high yield of IgG were achieved by pH 8.5.



Sample: 2 mL crude murine ascites of MA6 46.3 (IgM)
Column: POROS 20 HS Cation Exchange (4.6x100)
Buffer A: 50 mM sodium phosphate, pH 7.2
Buffer B: Buffer A + 1.0 M NaCl, pH 7.2
Flow rate: 8.5 mL/min
Gradient: 0- 35%B linear gradient in 20 CV

Sample: 2 mL of IgM Fraction from A
Column: POROS 20 HQ Anion Exchange (4.6x100)
Buffer A: 30 mM sodium phosphate, pH 7.2
Buffer B: Buffer A + 1.0 M NaCl, pH 7.2
Flow rate: 10 mL/min
Gradient: 15 to 20%B step gradient held for 2 CV;
 20 - 35%B linear gradient in 15 CV

IEC resins are available in two charge modes (anion and cation exchangers) with several ionic forms (strong and weak exchangers). It makes this technique broadly applicable in providing different selectivities ideal for initial capture or intermediate purification of antibodies. Moreover, IEC is capable of separating a target monoclonal antibody from a host or media supplement – derived polyclonal antibody.

Although it is not impossible to obtain a single-step purification of antibody by IEC, such an effect requires rigorous and systematic optimization of separation [36,37]. Furthermore, high single-step purity requires narrow peak cutting that can reduce recovery significantly. A combination of both anion and cation exchangers offers a powerful method for antibody purification, such as that of IgM (Fig. 8). Alternatively, a combination of IEC with a non-charge-based separation method such as SEC to purify IgM or with hydrophobic interaction HPLC for IgG purification is common [31,34,38].

New IEC media introduced by several manufacturers support rapid separation of complex proteins including antibodies. Perfusive or convective media offer high-speed processing that encourages more thorough optimization (pH, gradient, and surface chemistry) and ultimately results in better purification products. The high flow rates might minimize the risk of aggregation and help preserve biological activity of labile antibody molecules, especially of the IgM subclass [39].

Further progress has been achieved with media that are capable of direct capture of biomolecules from crude feed streams without preliminary clarification and concentration of cell culture supernatants using fluidized bed technology or oversized beads (CellThru BigBeads).

Hydroxyapatite Chromatography

Hydroxyapatite chromatography (HAC) has gained wider popularity as a purification tool for antibodies because of the development of uniform ceramic media that provide excellent physical strength, high flow properties, and scale-up

FIGURE 8 IgM purification by a combination of cation- and anion-exchange chromatography. (A) The separation of crude ascites of monoclonal antibody IgM 46.3 (diluted 1 : 5 in PBS) on a cation-exchange column. The IgM peak was pooled and a 2-mL aliquot of this pool was injected onto the anion-exchange column. The resulting chromatogram is shown in B. In each chromatogram, the activity of collected fractions as measured by ELISA is shown. High purity of IgM was achieved by this combination method. The rapid separation with high flow rates minimized the risk of aggregation and helped preserve the biological activity of the labile IgM.

capabilities [40]. Hydroxyapatite, which has the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, acts as both the ligand and the matrix. The interactions of antibodies with hydroxyapatite are complex and mostly based on attraction of the amino and carboxyl groups of the molecules to the phosphate and calcium sites of hydroxyapatite.

The samples are usually applied in 5–50 mM phosphate buffer at pH 6.8 and the antibodies are eluted from the hydroxyapatite by a gradient elution of phosphate buffer up to 0.5 M at pH 6.8. Solubility problems can occur for many IgM species and some IgG molecules under the low phosphate loading conditions. Aggregation that can lead to peak broadening and smearing can reduce the recovery significantly. Addition of 0.1 M NaCl to the loading buffer improves the solubility, especially for IgMs, without affecting the binding efficiency of the antibodies [19]. Moreover, the addition of NaCl to the loading and elution buffers improves binding selectivity, which can result in higher purity and yield of antibodies (Fig. 9).

HAC is useful for fractionating antibodies regardless of species, class, or subclass. Purity up to 90% from serum or ascites in a single step is possible. Low levels of albumin, transferrin, and β_1 -haptoglobin are found as contaminants [19,41]. A combination of HAC with SEC is especially effective for purifying IgM.

It has been reported that monoclonal antibodies are better separated at a lower pH than the conventional elution using pH 6.8 [42]. However precautions should be taken because the crystalline structure of hydroxyapatite degrades rapidly below pH 5. Matrix degradation can also occur due to acid secretion by microbial contaminations following poor cleaning and sanitization [19]. Loss of separation performance, capacity, and reproducibility and short column lifetimes as low as 24 hr can be prevented only by rigorous cleaning and appropriate storage of the column, e.g., in 60% methanol [19].

Hydrophobic Interaction Chromatography

Separation by hydrophobic interaction chromatography (HIC) is based on adsorption of biomolecules to lipophilic groups such as phenyl or butyl that are covalently bound on the hydrophilic HIC support. Because antibodies have stronger hydrophobicity than the majority of their contaminants [43], HIC is an effective technique for purification. The adsorption of antibodies to the HIC matrix is enhanced by the presence of high salt [44], the choice of which has effects on binding selectivity. Salt concentration should be optimized individually for each antibody depending on its hydrophobicity. Bound antibodies are eluted by decreasing the ionic strength and/or adding hydrophobic competitors, such as organic solvents, in linear gradient modes.

Because of the denaturing risks associated with a combination of strong organic solvents with excessively hydrophobic ligands, the medium should be

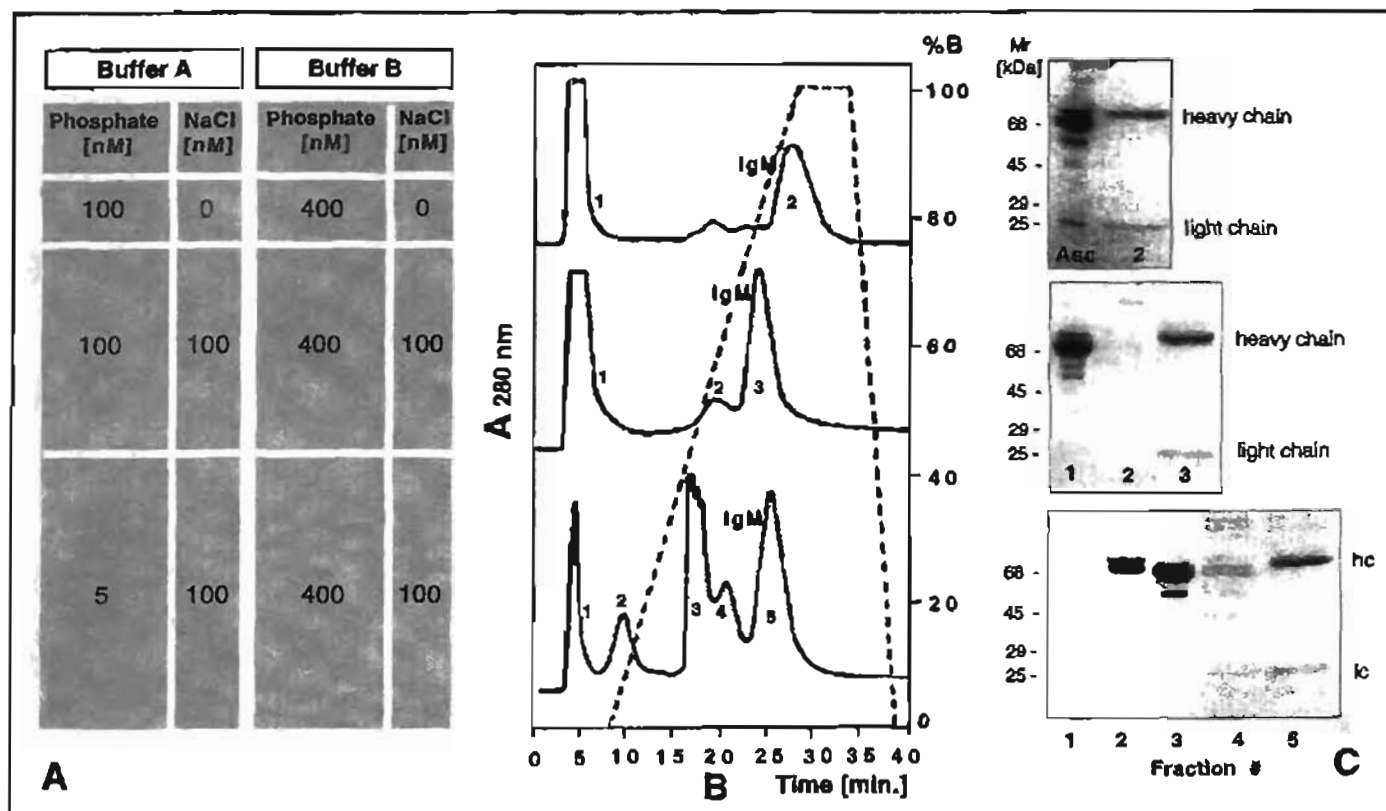


FIGURE 9 Separation of mouse IgM from crude ascites by hydroxyapatite chromatography under various buffer conditions (A). The effect of solubilizing sodium chloride in the buffer is demonstrated by a sharper IgM peak (B). The SDS-PAGE analyses of the fractions are shown in C. Starting material (mouse ascites) is shown in lane 1 on the top panel (Asc). Mass recovery of IgM can be improved from 75% to >90% by simple inclusion of NaCl in the buffer.

carefully selected to obtain high recovery of bioactive antibodies. Generally, a strongly hydrophobic medium for antibody separation is not recommended. Phenyl ligands have been successfully reported for monoclonal antibody purification because the required salt concentration is often beneath the level at which antibody precipitation begins to occur [45]. Strong organic solvents in the mobile phase may improve recovery; however, they also increase the probability of antibody denaturation. Addition of 25–50% ethylene glycol in the elution buffer is generally sufficient for obtaining high purity and recovery of antibodies.

Similarly to other techniques, HIC requires routine method development for optimal sample application conditions. Screening of conditions of mobile phase pH that allow the most favorable binding selectivity of the antibody should be carried out. The hydration of residues within the hydrophobic sites on antibody molecules is known to be pH dependent, and consequently pH has a significant impact on selectivity [46]. Buffers containing potassium or sodium phosphate with ammonium sulfate at pH 8.5, 7.0 and 5.5 should be screened for optimal selectivity.

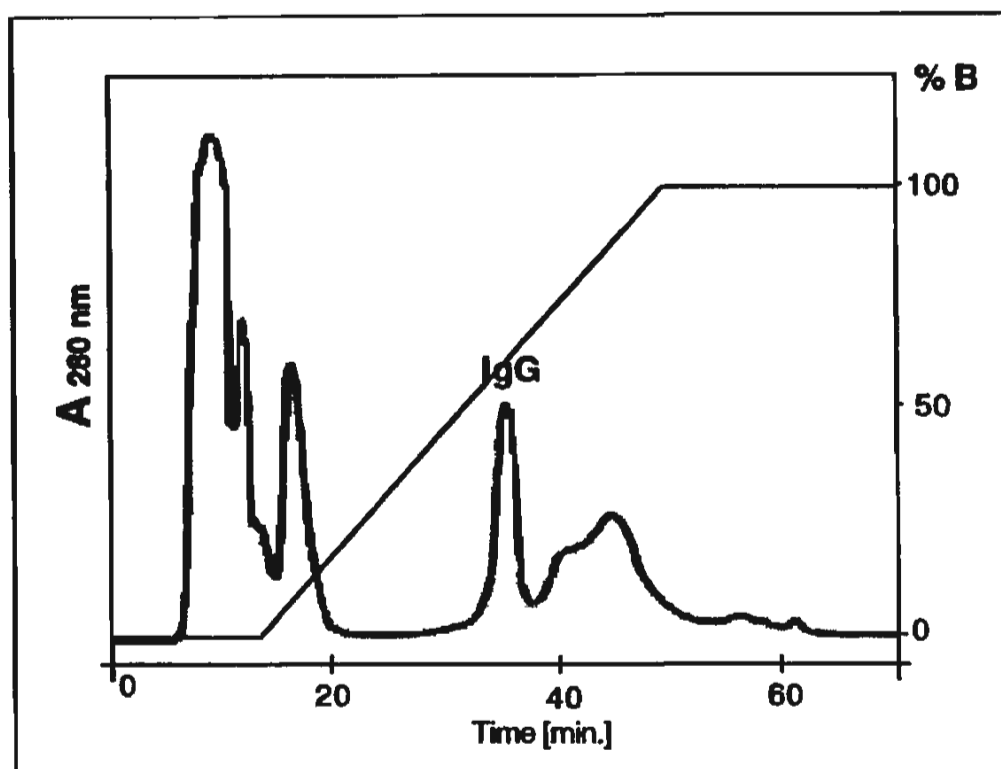
In general, HIC is very useful in purifying antibodies of the classes and subclasses from all species. It is especially attractive because it easily removes the contaminants that are most difficult for the other HPLC techniques such as SEC, IEC, and HAC. Because albumin and transferrin are usually unretained under antibody binding conditions, HIC is often a suitable first purification step. Furthermore, HIC partially removes up to 50–75% of nonspecific antibodies present in the starting material. In combination with IEC, complete removal of the nonspecific antibodies can be achieved. Figure 10 illustrates the separation of IgG from ascites fluid by HIC.

AFFINITY-BASED SEPARATION

Protein A

After the introduction of protein A for the affinity purification of IgG in 1972 by Hjelm et al. [47] and Kronvall [48], this method for antibody purification became so popular that it currently ranks with ion-exchange chromatography.

Protein A (Cowan strain I) is a wall component of *Staphylococcus aureus* that binds the Fc portion of IgG specifically. Variations in protein A affinity for IgGs from different species and subclasses are well known (Table 1). The most significant feature of protein A affinity chromatography is its simplicity—it can be performed quickly and easily with or without sophisticated instrumentation. Most mammalian and mouse or rat IgGs bind well to Protein A, achieving efficient purification under physiological conditions [49,50]. Bound antibodies are most commonly eluted by a low-pH buffer, such as 0.1 M glycine-HCl, pH 3.0 or 0.1 M citric acid, pH 2.4 using a step gradient. Neutralization of the



Sample: Cell culture supernatant of murine hybridoma
Column: Merck Fractogel EMD Propyl 650 (S)
Buffer A: 20 mM sodium phosphate + 1.0 M ammonium sulfate, pH 7.0
Buffer B: 20 mM sodium phosphate, pH 7.0
Flow rate: 1 mL/min
Gradient: 0-100 %B in 20 column volumes

FIGURE 10 Separation of a monoclonal antibody by hydrophobic interaction chromatography. IgG is eluted during the decreasing gradient at a salt concentration of about 500 mM ammonium sulfate.

solution immediately after the elution preserves the biological activity of the purified antibodies.

Protein A affinity chromatography is capable of providing total IgG purity as high as 95% in a single step; however, nonspecific antibodies from the host or serum supplements, which also bind to protein A, coelute with the target IgG and contaminate the preparation. The contamination by nonspecific antibodies can be reduced with linear gradient pH elution, but it cannot be eliminated completely (Fig. 11). An additional ion-exchange chromatography step can be utilized to remove the nonspecific Ig. Antibody aggregates bind to protein A with higher affinity than monomeric antibody; therefore, linear gradient pH elution can partly resolve antibody aggregates from the monomeric antibody (Fig. 11). α_2 -Macroglobulin and kinogen are also known to bind to protein A and might be found as contaminants in the antibody preparation [51].

TABLE 1 Binding Affinities of Immunoglobulin from Different Species and Subclasses

Antibody:		Protein A	Protein G	Protein L
Human	IgG1	+++	+++	+++
	IgG2	+++	+++	+++
	IgG3	+	+++	+++
	IgG4	+++	+++	+++
	IgM	+	-	+++
Mouse	IgG1	+	++	+++
	IgG2a	+++	+++	+++
	IgG2b	+++	+++	+++
	IgG3	+++	+++	+++
	IgM	-	-	+++
Rat	IgG1	+	++	n/a
	IgG2a	-	+++	n/a
	IgG2b	-	+	n/a
	IgG2c	+++	+++	n/a
Bovine	IgG1	+	+++	-
	IgG2	+++	+++	-
Sheep	IgG1	+	+++	-
	IgG2	+++	+++	-
Goat	IgG1	+	+++	-
	IgG2	+++	+++	-
Horse	IgG(ab)	+	-	n/a
	IgG(c)	+	-	n/a
	IgG(T)	-	+++	n/a
Rabbit	IgG	+++	+++	+
Cow	IgG	+	+++	-
Guinea Pig	IgG	+++	+	n/a
Hamster	IgG	++	n/a	n/a
Pig	IgG	+++	+	+++
Horse	IgG	+	+++	n/a
Donkey	IgG	++	+++	n/a
Dog	IgG	+++	+	n/a
Cat	IgG	+++	+	n/a
Monkey	IgG	+++	+++	n/a
Chicken	IgG	-	-	-

+++ = strong binding; ++ = medium binding; + = weak binding;
 - = no binding; n/a = information not available.

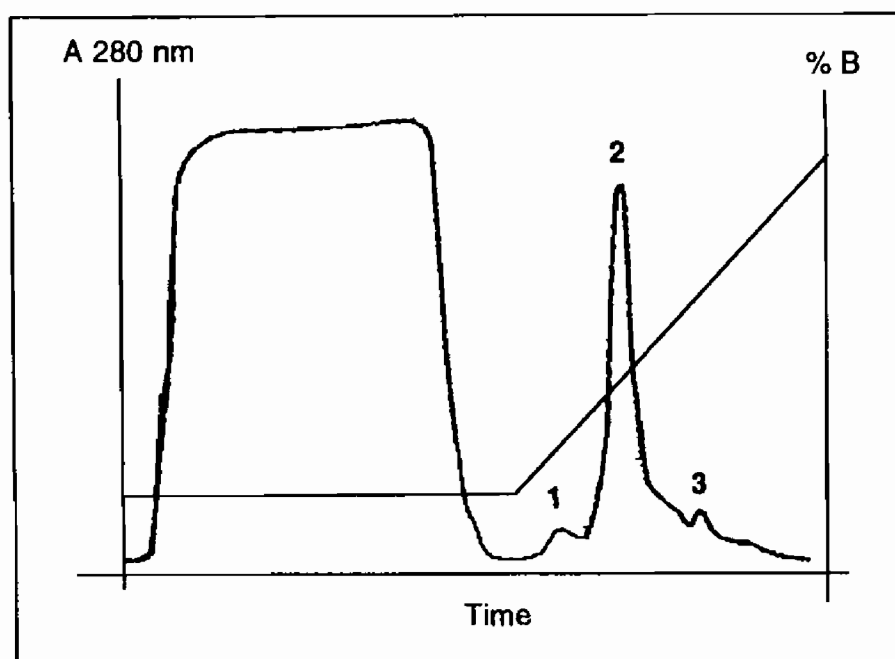


FIGURE 11 Separation of IgG by linear pH gradient elution. Cell culture supernatant of murine hybridoma (IgG2a) was fractionated on a protein A Sepharose column. Running buffer: phosphate-buffered saline, pH 7.4. Bound proteins were eluted by 10 column volumes of a linear gradient to 0.1 M citric acid, pH 2.2. Bovine IgG (peak 1) and IgG aggregates (peak 3) were partially separated from the main elution peak of murine IgG2a (peak 2).

The most common concern about protein A purification is antibody denaturation that can be manifest as aggregation, fragmentation, and loss of biological activity due to harsh elution conditions [52,53]. Some antibodies undergo permanent conformational alterations at extremely low pH [54]. In such cases, minimum elution conditions should be determined. Elution above pH 4.5 rarely results in antibody denaturation. Elution with chaotropic salts such as urea, sodium isothiocyanate, and potassium iodide is also possible [50]. Another problem with protein A is leaching from the column. This is potentially a concern for in vivo antibody use because protein A is a potent immunomodulator and has proved toxic in clinical trials [55,56]. Recombinant protein A (r-protein A), a genetically engineered form of protein A that is produced in a nonpathogenic form of *Bacillus*, is currently available. It is derived from secreted extracellular variants that lack the cell wall-associated region [57]. Compared with Cowan strain I, there are minor differences in the amino acid sequences of the IgG binding domains, but the antibody binding property is indistinguishable. The main advantage of r-protein A is that toxic contaminants from a pathological bacterial source can be eliminated.

Protein G

An alternative to protein A, protein G, which is derived from group C and G streptococci, offers similar applications for purification of antibodies [58]. There are some advantages in the binding selectivity of mouse and rat IgG for protein G over protein A (see Table 1). Wild-type protein G contains albumin-binding domains as well as IgG-Fc and Fab binding domains [59]. Currently, recombinant protein G without an albumin binding property is available. Purification performance varies significantly among commercial protein G products [60] and binding capacities may vary by a factor of 2–3. This partly reflects variations in ligand density, spacer characteristics, and the variations in protein G itself.

Although protein G offers some advantages over protein A, the concerns associated with the use of protein A such as nonspecific antibody contamination and ligand leaching are shared by protein G. Moreover, protein G is more expensive than protein A. Alternatively, to obtain a broader range of antibody binding, a hybrid of recombinant protein A/G may be useful [61].

Protein L

Another microbial protein that binds mammalian immunoglobulin has been made commercially available. The protein was originally derived from the cell wall of the bacterium *Peptostreptococcus magnus* and is termed protein L [62]. The protein has the unique ability to bind immunoglobulins through the kappa light chains without interfering with the antigen binding site and allows a wider range of antibody classes and subclasses than protein A or protein C [63]. Protein L binds IgM most strongly, apparently as a result of the pentameric nature of IgM, which allow five times as many binding sites [62]. Furthermore, protein L is useful for purifying single-chain variable fragments (ScFv) and Fab fragments of immunoglobulins [64]. A comparison of binding capabilities of bacterial immunoglobulin binding proteins is shown in Table 1.

Biomimetic Ligand Affinity Chromatography

Developments in techniques such as rational design and combinatorial chemistry have allowed the generation of a vast array of new synthetic ligand structures that might be useful for purification of biomolecules including antibodies. These techniques have been successfully used to identify low-molecular-weight synthetic molecules that mimic the immunoglobulin binding activity of native biological macromolecules such as *Staphylococcus aureus* protein A [65]. This biomimetic ligand has been reported to bind IgG competitively with protein A in solution, and when immobilized on agarose beads, it displays an affinity constant of 10^5 – 10^6 M⁻¹ [65]. The immobilized ligand (Mimetic A, ProMetic Bio-

Sciences, Inc.) has been used on affinity adsorbents to purify polyclonal IgG from diverse sources effectively. A purity of 80–99% has been reported for human IgG purification from diluted human plasma in a single step [66,67].

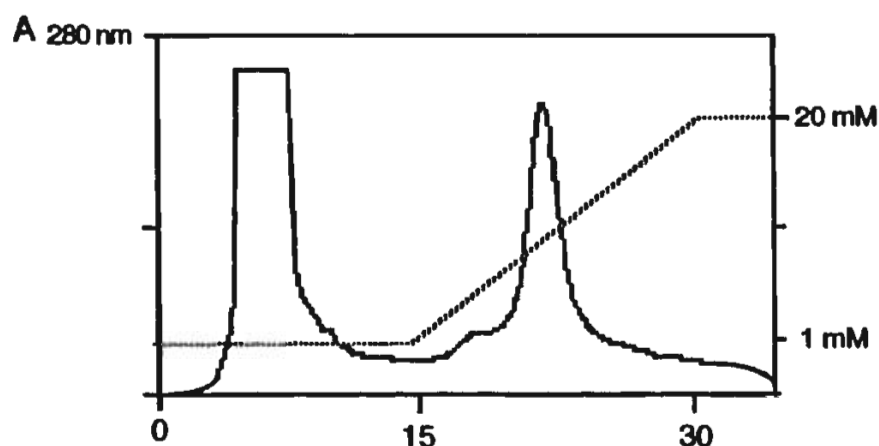
The combination of fast and effective affinity chromatographic techniques and the synthetic nature of biomimetic ligands offer many advantages over conventional antibody purification methods, especially for downstream processing of *in vivo* products. A mimetic ligand might suspend all concerns related to biological origin and toxic contaminants from a pathological bacterial source [68]. In addition, the robustness of the ligand enables harsh cleaning, sterilization, and depyrogenation, which ensures long operational lifetimes. The high binding selectivity of the mimetic ligand to human IgG might be useful to purify the fast-growing products of therapeutic human IgGs or chimeras with human Fc regions.

Immobilized Metal Affinity Chromatography

Immobilized metal affinity chromatography (IMAC) has been used for protein purification since 1975 [69] but was not widely utilized for antibody purification, despite its extraordinary capabilities, until later [70,71]. IMAC is a powerful technique for purifying IgG molecules due to the affinity binding of histidyl residues in the antibody molecules to a chelated nickel support. Mammalian IgGs have a highly conserved histidyl cluster at the junctures of the CH² and CH³ domains [52]. Under alkaline conditions, nickel-loaded iminodiacetic acid (Ni-IDA) captured IgG through the histidyl residues selectively and effectively [70,71]. The binding specificity may be increased by the presence of 0.5 mM imidazole in the loading buffer [72]. The bound antibodies are eluted by titrating the histidyl residues at low pH [73]. Alternatively, a competitive elution with imidazole or its analogues, histidine and histamine, achieves similar results. A typical chromatogram is shown in Fig. 12.

IMAC is particularly suitable for the separation of biomolecules such as antibodies because it can be performed under very mild, nondenaturing conditions. Single-step purification is often possible for antibodies produced in cell culture supernatants. However, monoclonal antibodies produced in ascites fluid often need an additional step to remove nonspecific IgG and other contaminants. A combination of IMAC with hydrophobic interaction or ion-exchange chromatography would provide an excellent method for antibody purification [71].

The advances in the production of recombinant single-chain Fv antibody fragments (scFv) in bacteria permit rapid access to genetically engineered molecules with antigen-binding properties and clinically useful applications for either *in vivo* diagnosis or therapy. Metal chelate chromatography has been widely utilized in purifying expressed scFv from bacterial supernatant [74,75]. Simple insertion of a hexahistidine tail fused at the C terminus can provide an



Sample: 0.25 mL mouse ascites
 Column: TSKgel Chelate-5PW (TosoHaas)
 Metal Ion: Ni^{2+} , saturated
 Buffer A: 1 mM Imidazole in 20 mM HEPES-NaOH buffer, pH 8 + 0.5 M NaCl
 Buffer B: 20 mM Imidazole in 20 mM HEPES-NaOH buffer, pH 8 + 0.5 M NaCl
 Flow rate: 0.8 mL/min
 Gradient: 30 min. linear gradient from 1 mM to 20 mM Imidazole

FIGURE 12 Purification of IgG by immobilized metal affinity chromatography (IMAC). The low concentration of imidazole in the loading buffer (buffer A) should enhance the binding specificity and addition of 0.5 M NaCl in the buffers may improve the purity of the antibody. Elution can be achieved by reducing pH or by competitive elution with imidazole.

affinity tag that selectively binds to metal ions immobilized on an iminodiacetic acid-derivitized solid phase matrix.

Immobilized Cu^{2+} , Ni^{2+} , and Zn^{2+} ions have been shown to be effective in retaining the His-tagged antibody fragments with up to 90% purity on elution [75].

Immobilized Antigen Affinity Chromatography

The classical immunoaffinity format utilizes antigens that are immobilized to chromatographic supports. This effective technique, however, is rarely used for purification of monoclonal antibodies primarily due to lack of sufficient amounts of antigen and labor-intensive preparation of the purified antigens, which are prohibitive to this approach. Other possible factors that might have negative impacts on the antibody binding are secondary steric modifications of the antigens induced by immobilization that might alter the capture efficiency of the target antibody. Binding and elution may also alter the antigen binding site of

the antibody. Moreover, leached antigens will interfere with the intended application of the antibody. Despite these limitations, this technique is attractive for purification of monospecific antibodies from a mixture of polyclonal antibodies [76]. Figure 13 demonstrates the purity of the monospecific antibody against annexin 6 (CBP 65/67) separated by immobilized annexin 6 affinity porous disks.

Antigens that can be expressed as recombinant proteins and engineered to bear multiple histidyl residues at their N or C terminus would facilitate a simple immobilization procedure in preparing immunoaffinity absorbents on metal affinity resins. This technique has been used successfully in purification of antibody or antibody fragments [77,78]. By taking advantage of biospecific interactions of antigen and antibody molecules, separation of bioactive forms of single-chain antibody fragments (scFv) from inactive scFv molecules can be achieved in a single step by using immobilized antigen affinity chromatography [78].

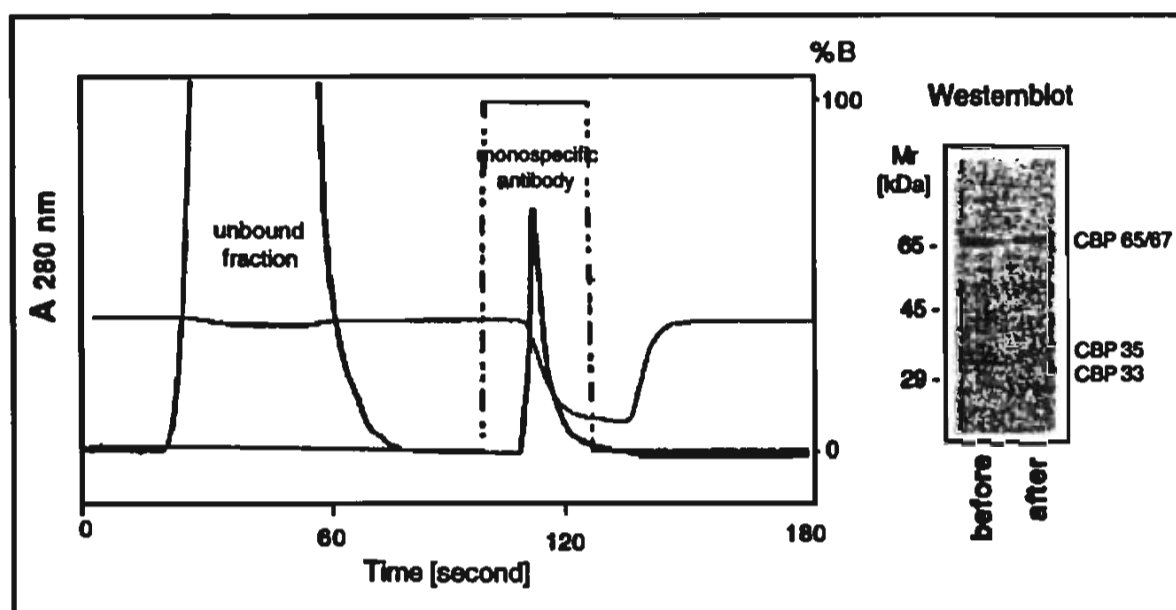


FIGURE 13 Purification of monospecific rabbit antibody against annexin VI. The rabbit polyclonal antibody against rat annexin Iv (CCBP 65/67) reacts against other annexins (CBP 35 and CBP 33). Monospecific polyclonal antibodies against annexin VI were prepared by immobilized antigen affinity chromatography. Purified annexin VI is immobilized on an epoxy activated CIM disk (BIA Separations) and 1 mL of antiserum is diluted 1:5 in PBS and applied to the disk (flow rate 3 mL/min). Bound antibodies were eluted by 0.1 M glycine HCl, pH 3.0, in a step gradient. The Western blot analysis of EDTA extracts of Morris hepatoma rat plasma membranes with the antiserum before and after the affinity purification is shown.

CONCLUSION AND OUTLOOK

Antibodies, especially monoclonal antibodies, play an important role in many research, diagnostic and therapeutic applications. HPLC has proved to be a powerful technique for the analysis, separation, and purification of antibodies. A variety of chromatographic methods based on physicochemical separation mechanisms, biological affinity, and mixed-mode mechanisms are available to achieve successful antibody purification. Each method and each combination of methods has its particular strengths and weaknesses.

Defining a broadly applicable strategy in purifying monoclonal antibodies is challenging because of the chemical diversity of antibodies. A successful purification strategy needs to be developed systematically and tested individually for each antibody. Antibody purification for research and in vitro applications can usually be accomplished in a single step or two fractionation steps; antibody purification for in vivo applications requires additional steps because of the stringent purity standards. The powerful combination of affinity chromatography and synthetic biomimetic ligands that can selectively bind antibodies offers a promising approach to purification, especially of in vivo products.

Advances in high-throughput HPLC media such as perfusive and convective media enable purification of antibodies in a much shorter time than with the conventional media. Not only will this encourage a more systematic method development and optimization process that can lead to a better product, it will probably also have a positive economic impact in downstream processing.

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Glycoproteins

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INTRODUCTION

Glycoproteins, i.e., proteins with covalently bound sugars, are ubiquitous in nature and are found in almost all living organisms. Glycoproteins occur in cells in soluble and membrane-bound forms as well as in the intercellular matrix and extracellular fluids. Human serum is a particularly rich source of glycoproteins. The serum proteins constitute the most extensively characterized group of glycoproteins with respect to the number, location, structure, and function of their oligosaccharide moieties [1]. Two different kinds of linkage between carbohydrate moieties and serum proteins have been recognized: first, *N*-glycosidic linkage of glucosamine, where asparagine in the obligatory signal sequence,

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Asn-X-Thr/Ser, is involved in the carbohydrate-peptide bond, and second, O-glycosidic linkage of galactosamine, where serine and/or threonine, as well as hydroxylysine in no apparent signal sequence, are involved in the carbohydrate-peptide bond. The constituents of carbohydrate chains include galactose, mannose, fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid.

The unique structural feature of glycoproteins in terms of chromatographic behavior is the heterogeneous character of the oligosaccharides present at a given glycosylation site. This heterogeneity can take a number of different forms. Even worse is the fact that in glycoproteins with multiple carbohydrate chains, there may be additional heterogeneities in the number and location of attachment sites for the oligosaccharides. If the combination of these heterogeneities is taken into consideration, any particular glycoprotein may have a large number of multiple forms due to the microheterogeneities of the oligosaccharide chains attached. For this reason, when dealing with glycoproteins using HPLC we are often put into a dilemma—the more the separation power of the column is increased, the more the separation becomes ambiguous. This, of course, may be solved in the future by the development of a column that enables complete resolution of such a complex mixture.

There have been great advances in the methods for identifying proteins by mass spectrometry (MS) coupled with HPLC since this chapter was first written almost 10 years ago. These methods are now being applied to the separation of glycoproteins with enormous heterogeneity due to the carbohydrate chains attached. In some cases, a single glycoprotein with only a single glycosylation site could be separated completely into a number of molecular species that differ in one sugar component in the carbohydrate structure. Although it is still difficult to resolve even a purified glycoprotein with multiple glycosylation sites into its individual molecular species, these advanced methods do facilitate identification of the attachment site of carbohydrate chains and determination of their structures. Nonetheless, there is no single method to obtain complete resolution of a complex mixture of glycoproteins. Thus, to purify glycoproteins it is still very important to select a column that can minimize the effects of microheterogeneities of the carbohydrate moieties and can still fit the purpose of the separation.

APPLICABILITY OF DIFFERENT SEPARATION MODES

There is no single method applicable to isolation of all glycoproteins because glycoproteins are individually characteristic in their amino acid sequences, carbohydrate compositions and structures, and the number and location of attachment sites for the oligosaccharide. Therefore, the applicability of methods with different separation modes is examined here. In order to facilitate understanding the structural features of glycoproteins, the linear structural models of the glycoproteins used for these studies are illustrated in Fig. 1.

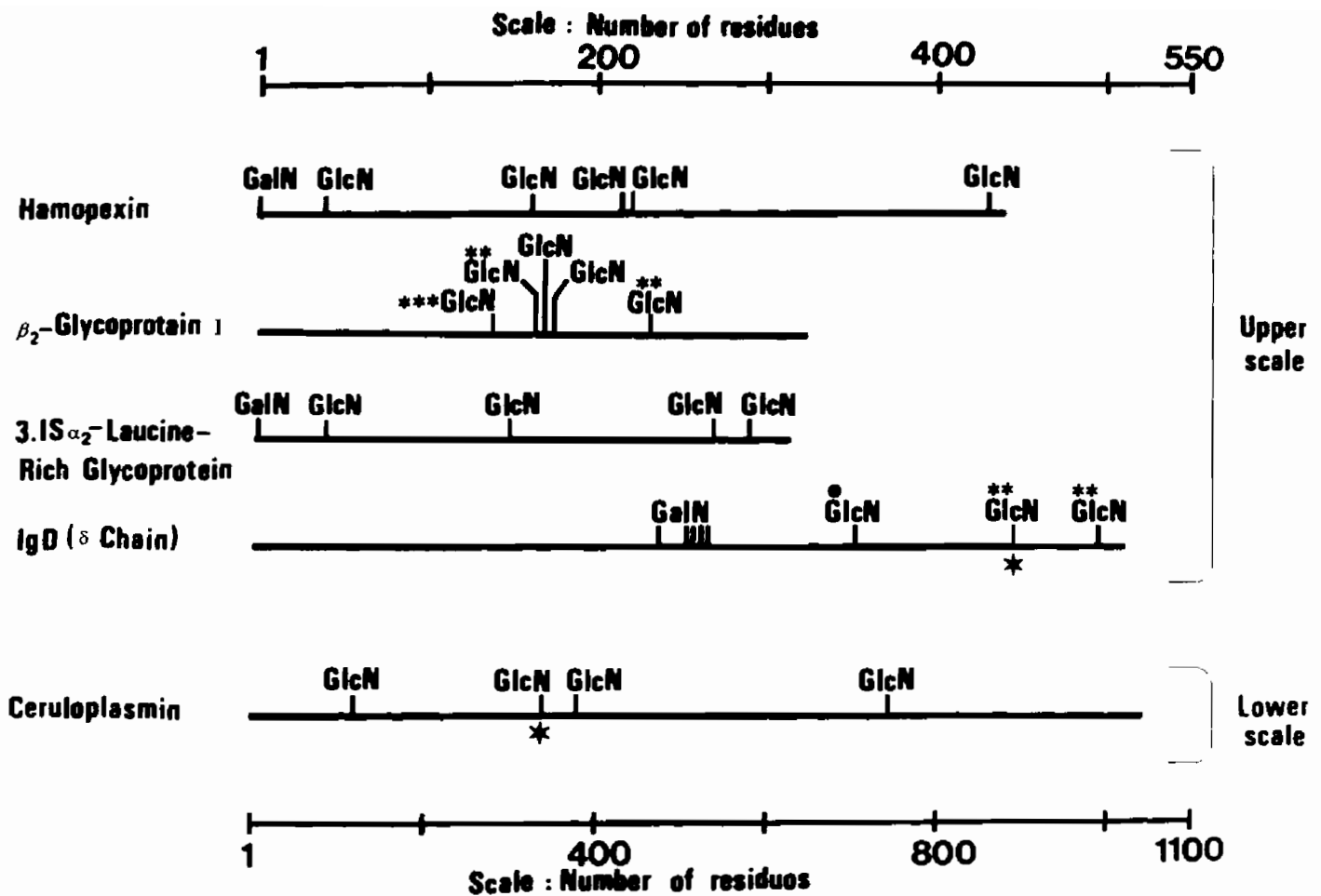


FIGURE 1 Linear structural models of human plasma glycoproteins [14]. The attachment sites for galactosamine and glucosamine oligosaccharides are indicated by GalN and GlcN, respectively. The oligosaccharides for which the structures are known are also distinguished by ** for diantennary, *** for triantennary, and • for a high-mannose type of GlcN oligosaccharide. If the carbohydrate is missing in some molecules, the site is marked by *. The upper scale for the number of amino acid residues is used for the upper four proteins, and the lower scale is used for ceruloplasmin.

Ion-Exchange Column

Heterogeneity of oligosaccharide chains due to variations in the number of terminal sialic acid residues is very common. Glycoproteins, therefore, are often split into multiple bands or obtained as broad or smeared bands by separation methods based on charge differences, such as isoelectric focusing, disk electrophoresis [2,3], and ion-exchange chromatography. In ion-exchange chromatography, glycoproteins are frequently eluted as multiple peaks or a broad smear of peaks, especially if a glycoprotein is glycosylated at multiple sites (Fig. 2). This is a common phenomenon observed in ion-exchange chromatography of serum proteins glycosylated at multiple sites. Human immunoglobulin D (IgD), which is a predominant surface immunoglobulin on most B lymphocytes, is a good example. IgD has three glucosamine and at least four galactosamine oligosaccharide chains [4], displaying extensive microheterogeneity at each location. At least eight species are known for the high mannose-type oligosaccharides at Asn-354, and there are at least 10 species of the dibranched complex type oligosaccharides at Asn-445 and Asn-496, respectively [5]. In addition, at least four sites in the hinge region bear galactosamine oligosaccharides. Approximately 50% of the galactosamine chains have the structure $\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}$, and the remainder contain one or two residues of *N*-acetylneuraminic acid [6]. Therefore, at each galactosamine oligosaccharide attachment site, there are at least three species sialylated at different positions. Taken together, there are probably more than 1000 species of IgD.

IgD is split into 21 bands by isoelectrophoresis [7] and elutes in a broad band in ion-exchange HPLC, as shown in Fig. 2. The poor performance of ion exchange is due to two phenomena. One is that the column efficiency is not sufficient to resolve components with such complex charge heterogeneity. The other is that terminal microheterogeneities of the neutral sugar also contribute to the interaction with the column, even though the separation principle is based mainly on charge.

Other examples of proteins exhibiting charge heterogeneity, such as ceruloplasmin, β_2 -glycoprotein 1, hemopexin, and leucine-rich glycoprotein (LRG), are also shown in Fig. 2. Ceruloplasmin and β_2 -glycoprotein I have four and five glucosamine oligosaccharide chains, respectively [8,9]. Hemopexin has one galactosamine and five glucosamine oligosaccharides [10], and leucine-rich glycoprotein has one galactosamine and four glucosamine oligosaccharide chains [11] (Fig. 1). These proteins are also eluted as broad peaks from an ion-exchange column (Fig. 2). In the case of leucine-rich glycoprotein, the protein was separated as three broad peaks, even though the protein is eluted as a single peak by hydrophobic interaction and reversed-phase chromatography, as described later (see Fig. 6). Thus, when ion-exchange HPLC is applied to analyze glycoproteins, the separation may not be as effective as that expected from the column efficiency with nonglycoproteins, glycoproteins with fewer oligosaccharide chains, or glycoproteins with larger charge differences.

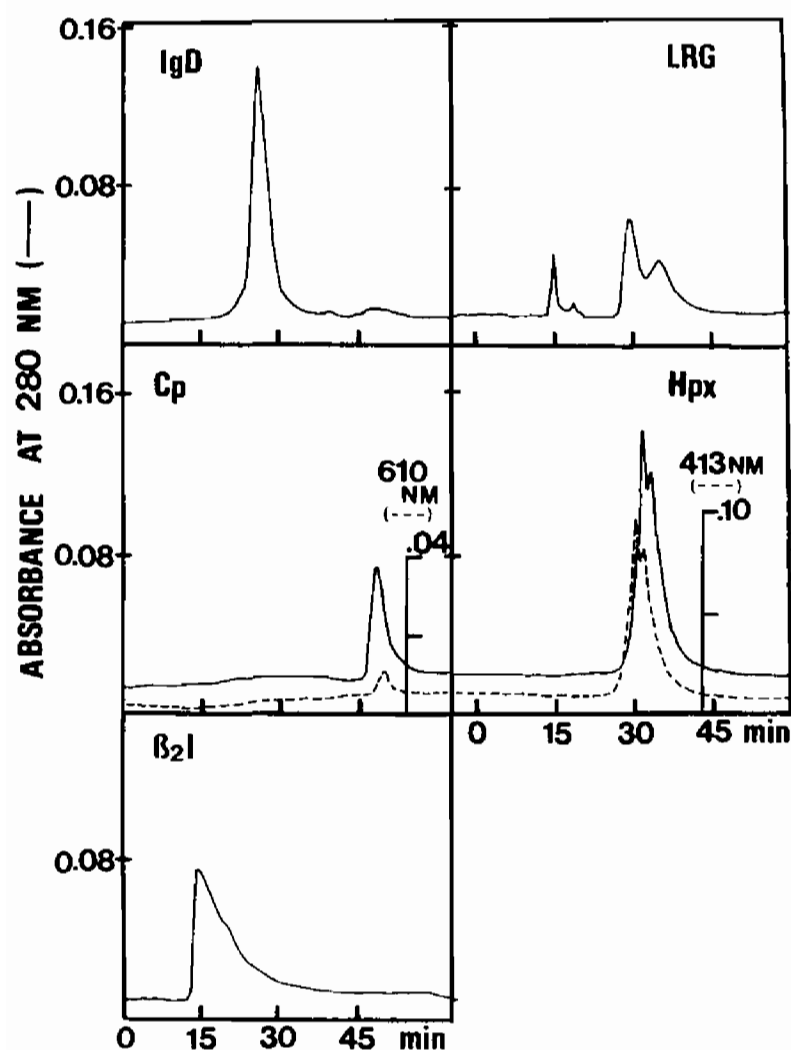


FIGURE 2 Anion-exchange HPLC of serum glycoproteins on a SynChropak AX-300 column (0.4×25 cm). The column was eluted at a flow rate of 1.0 mL/min with a linear gradient from 0.02 M Tris-acetic acid buffer, pH 8.0 to 0.02 M Tris-acetic acid buffer, pH 8.0, containing 1.0 M sodium acetate. The eluent was monitored at an absorbance of 280 nm. The absorbance at 610 nm was also used to monitor the blue copper of ceruloplasmin (Cp), and 413 nm was used for the heme of hemopexin (Hpx). The other abbreviations used are IgD, immunoglobulin D; β_2 I, β -2-glycoprotein I; LRG, leucine-rich glycoprotein. The sample amount applied to the column is about 800 μ g for each chromatogram. The capacity of the column can be about 5–10 mg before excessive peak spreading occurs.

Hydroxyapatite Columns

Hydroxyapatite columns have been shown to be helpful for the purification of proteins, especially when other types of chromatographic columns were not successful [12]. Although the separation mechanism of hydroxyapatite chromatography is not understood, it is known that it is not an ion-exchange mechanism. Applications of hydroxyapatite HPLC to the analysis of glycoproteins including

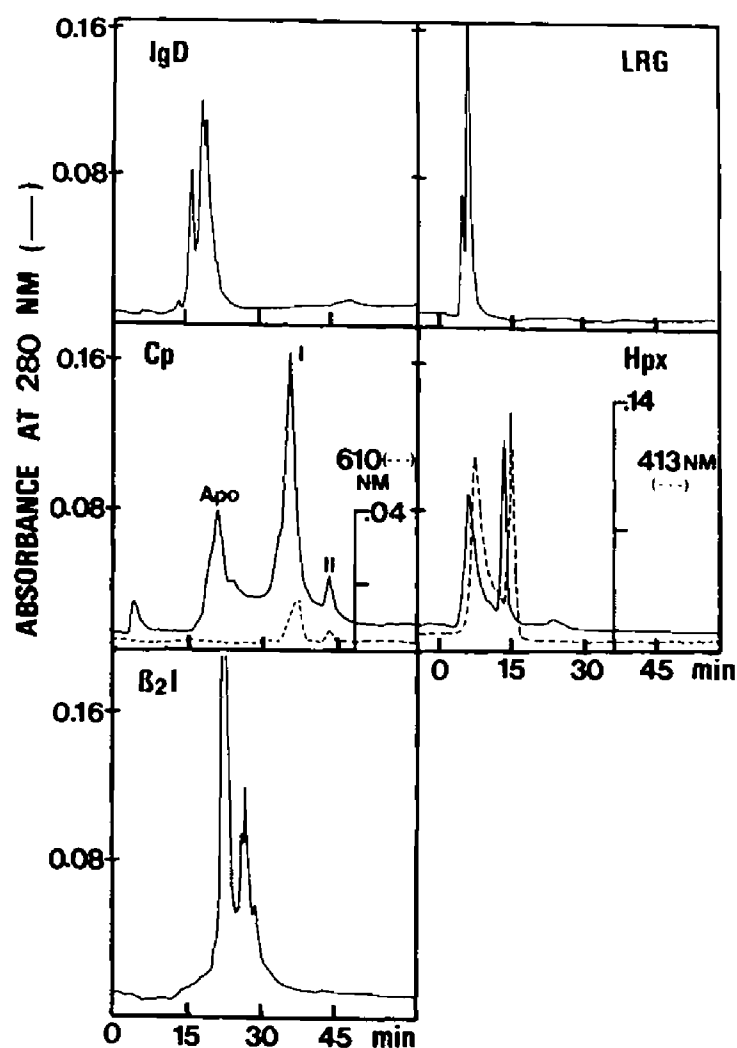


FIGURE 3 Hydroxyapatite chromatography of serum glycoproteins on a spherical hydroxyapatite column (0.75×10 cm, Toa Nenryo K.K., Tokyo), with guard column. The column was eluted at a flow rate of 0.8 mL/min for 60 min with a linear gradient from 0.01 M sodium phosphate buffer, pH 6.8, containing 0.3 mM CaCl_2 to 0.5 M sodium phosphate buffer, pH 6.8, containing 0.01 mM CaCl_2 . The abbreviations in Fig. 2 are used. The following abbreviations are used in the chromatogram of ceruloplasmin: Apo, apoceruloplasmin; I, carbohydrate variant type I; II, carbohydrate variant type II. A sample up to 20 mg could be applied without any loss of resolution.

IgD, ceruloplasmin, β_2 -glycoprotein I, hemopexin, and leucine-rich glycoprotein are shown in Fig. 3. Ceruloplasmin, which is a multicopper oxidase whose amino acid sequence is known to be homologous to that of coagulation factor VIII, was separated into three major peaks by hydroxyapatite HPLC. The first peak does not have absorbance at 610 nm ; therefore, it contains apoceruloplasmin that does not have enzyme activity. The second peak (type I) contains four glucosamine

oligosaccharide chains, while the third peak (type II) contains only three of the four oligosaccharide chains in type I [13,14]. Although the ceruloplasmin molecule was not separated into several components by ion-exchange chromatography, the two carbohydrate variants, types I and II, as well as the apoform of the protein were effectively resolved by hydroxyapatite HPLC. The separation of ceruloplasmin by hydroxyapatite HPLC seems to be based on conformational differences of the molecular forms. The presence of one carbohydrate chain on holoceruloplasmin obviously affected the separation of the protein.

Likewise, IgD myeloma protein was not separated by ion-exchange chromatography (Fig. 2) but was resolved into three major peaks by a hydroxyapatite column, as shown in Fig. 3. IgD was also found to have several carbohydrate variants—one with three glucosamine oligosaccharide chains in the Fc region and the other with only two of those chains. In addition, the hinge region of IgD is known to have some heterogeneity in the number and location of attachment sites for galactosamine oligosaccharides [14]. Although there are no reports of β -2-glycoprotein I, hemopexin, and leucine-rich glycoprotein carbohydrate variants, these proteins were also split into several peaks by hydroxyapatite HPLC (Fig. 3). In the case of hemopexin, this may represent the separation of the oxidized and reduced forms of heme because the second peak gradually shifted to the first peak with time. The separation of oxidized and reduced forms of cytochrome *c* by hydroxyapatite chromatography has been observed [15]. Thus, the separation on a hydroxyapatite column seems to be affected not by the charge heterogeneity on the carbohydrate or the other structural microheterogeneities, such as terminal branches of neutral sugars, but by the gross conformational differences, such as lack of an entire carbohydrate chain, binding of metal, or denaturation.

Reversed-Phase Columns

There has been little research on the comparative separation of glycopeptides and nonglycopeptides having the same amino acid sequence. In order to simplify the effect of the carbohydrate chains in the chromatography, glycopeptides with one or two oligosaccharide chains have been isolated from an enzymatic digest of glycoproteins [14] and their behavior examined in reversed-phase chromatography. Glycopeptide analytes with defined attachment sites included (1) glycopeptides with a single attachment site for GlcN or GalN oligosaccharide, (2) glycopeptides glycosylated at multiple sites with either GlcN or GalN oligosaccharides, (3) glycopeptides with extreme microheterogeneity of carbohydrate structures, and (4) glycopeptides with high-mannose or complex-type structures of GlcN oligosaccharide. Peptides differing in the presence or absence of carbohydrate were also isolated to examine the effect of the carbohydrate chain in reversed-phase HPLC.

In Fig. 4B, the retention times in reversed-phase chromatography of the glycopeptides just described are plotted against $\ln(1 + H)$, in which H indicates the hydrophobicity of the peptide, calculated by the method of Sasagawa et al. [16] using their list of nonweighted retention constants. For reference purposes, the retention times of nonglycosylated peptides are also plotted against $\ln(1 + H)$ in Fig. 4A. The retention times of both the nonglycopeptides and the glycopeptides are linearly related to the natural logarithm of the sum of the retention constants of Sasagawa et al. for the constituent amino acids. The behavior of the glycopeptides in reversed-phase chromatography is very similar to that of nonglycosylated peptides regardless of the type of carbohydrate attached; however, the glycopeptides with multiple attachment sites tend to be eluted earlier than expected from the hydrophobicity estimated only from their constituent amino acids (Fig. 4B) [14]. A glycopeptide of IgD offers a good example for comparison of the behavior of glycopeptides in reversed-phase HPLC. Although the glycopeptide was eluted 2.5 min earlier than the nonglycopeptide with the same amino acid sequence, the difference in the elution positions is far less than that expected from the large size of the carbohydrate structure, whose

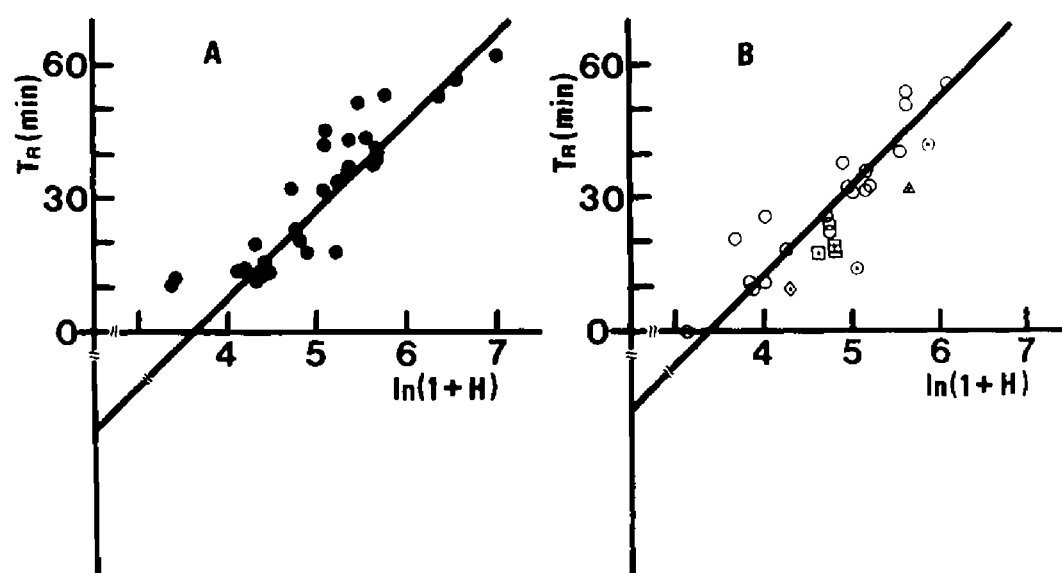


FIGURE 4 Relationship between retention time (T_R) and $\ln(1 + H)$ [14]. The observed retention times are plotted against $\ln(1 + H)$, where H indicates the hydrophobicity of the peptide calculated by the method of Sasagawa et al. using their list of nonweighted retention constants [16]. (A) Data for nonglycopeptides. (B) Data for glycopeptides. (○) glycopeptides with a single GlcN oligosaccharide; (□) glycopeptides with a single GalN oligosaccharide; (⊙) glycopeptides with two GlcN oligosaccharides; (△) glycopeptides with a single GalN and a single GlcN oligosaccharide; (◇) glycopeptide glycosylated at multiple sites for GalN oligosaccharide.

contribution to the molecular weight is about 50% of the total for the glycopeptide [14]. The heterogeneities of oligosaccharides, regardless of the type of the oligosaccharides—complex, high-mannose, or highly microheterogeneous—seem to have no distinguishable effects on the chromatography. In general, even though the effect of the carbohydrate is considerably smaller than expected, it seems to increase the hydrophilicity. Thus, if carbohydrate is attached, the glycopeptide tends to be eluted on a reversed-phase column slightly earlier than the nonglycosylated peptide.

When reversed-phase HPLC is applied to glycoproteins with multiple attachment sites, the glycoproteins are eluted within a relatively narrow range despite the heterogeneity of the carbohydrate chains. This is because the effect of carbohydrate on the chromatography is relatively small, as described previously. However, the peak is not a symmetrical Gaussian curve. Instead, it has an irregular shape, indicating multiple components. Heterogeneities due to the carbohydrate chains contribute to some extent to the separation by reversed-phase HPLC (Fig. 5). The retention times of polypeptides are linearly related to the natural logarithm of the sum of the hydrophobicity for the constituent amino acids; therefore, the longer the polypeptide chain, the smaller the relative contribution of the change of hydrophilicity to the retention time. Thus, for larger glycoproteins, the effect of the carbohydrate on reversed-phase HPLC appears to be relatively less significant.

The advantage of this diminishing effect of carbohydrate on a reversed-phase column is that the separation is about as efficient as that expected from the column efficiency obtained for a nonglycoprotein with an identical amino acid sequence. However, for some proteins, reversed-phase chromatography is not suitable because they precipitate on the column. This occurs because the elution is performed with a gradient of organic solvent, such as acetonitrile. Some proteins are easily denatured and precipitated in such organic solvents. The chromatograms of ceruloplasmin and IgD, which show irregularity and long tailing, are typical. In the case of ceruloplasmin, the peak at 610 nm was not observed, indicating that the blue copper was lost during the chromatography. Meanwhile, hemopexin, which is the heme-binding protein in serum, could still bind heme in this condition and was obtained as a very sharp peak (Fig. 5).

Hydrophobic Interaction Columns

Hydrophobic interaction columns separate proteins using hydrophobicity, similar to that of reversed-phase HPLC. However, this method provides an alternative approach with little or no denaturation of proteins because organic solvents are not required for the elution. In fact, ceruloplasmin, which was denatured during reversed-phase chromatography could be eluted from a hydrophobic interaction column without denaturation (Fig. 6). β_2 -Glycoprotein I, which has been

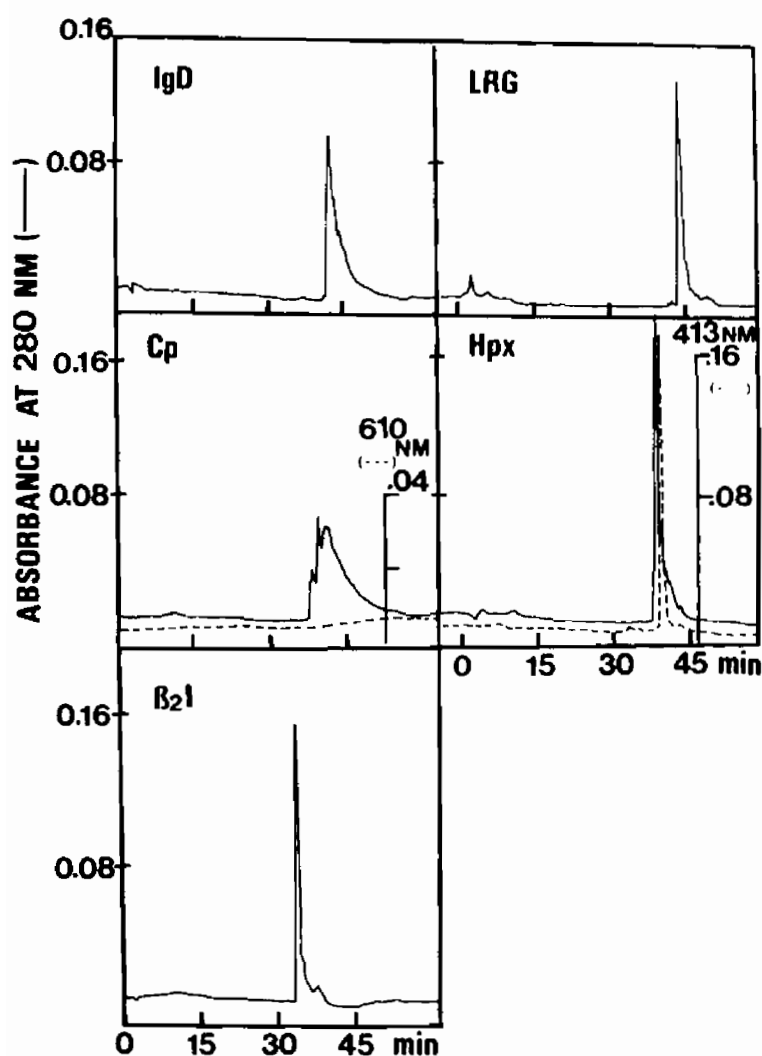


FIGURE 5 Reversed-phase HPLC of serum glycoproteins on a SynChropak RP-P column (0.4×25 cm). The column was eluted at a flow rate of 1.0 mL/min with a linear gradient from 0.1% trifluoroacetic acid (TFA) to 60% acetonitrile containing 0.1% trifluoroacetic acid during 60 min. The abbreviations used are the same as in Fig. 2.

identified as a constituent of chylomicrons, very low density lipoproteins, and high-density lipoproteins [17] and whose amino acid sequence is homologous to the regulator proteins in the alternative pathway of the complement system and to interleukin-2 receptor, is another example that shows the advantage of hydrophobic interaction chromatography. This protein was obtained as a single peak by reversed-phase HPLC; however, it was separated into two peaks by hydrophobic interaction chromatography. The heterogeneity of the carbohydrate chains is not likely to be the cause because the protein was not separated by reversed-phase HPLC (Fig. 5). Rather, the explanation is that some of the molecules bound a

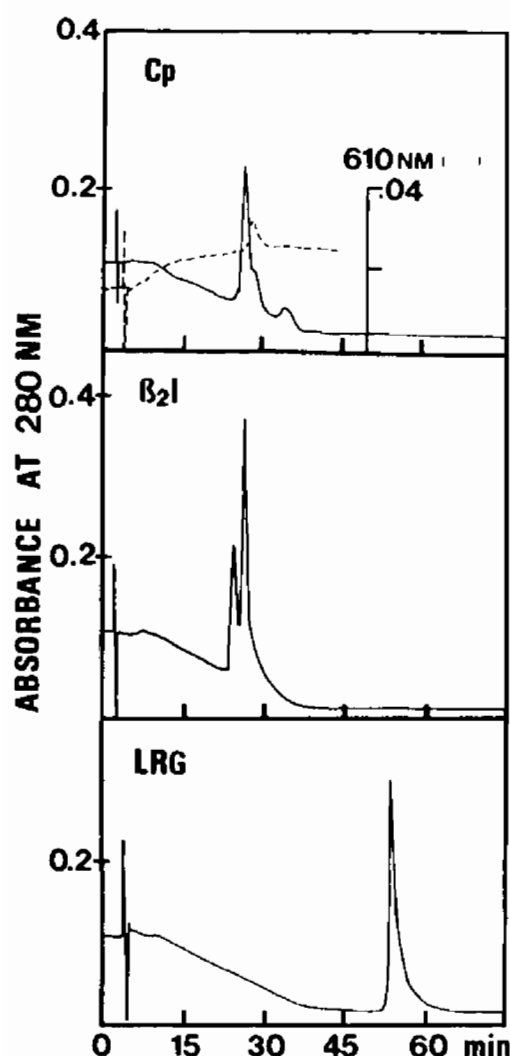


FIGURE 6 Hydrophobic interaction chromatography of serum glycoproteins on a TSK Phenyl 5-PW column (0.75 × 7.5 cm). The proteins are eluted at a flow rate of 1.0 mL/min with a gradient of decreasing salt concentration, from 1.7 M ammonium sulfate in 0.1 M sodium phosphate buffer, pH 7.0 to 0.01 M sodium phosphate buffer, pH 7.0 during 30 min. In the case of LRG, water was used for elution of the protein after 40 min. Because of the low grade of ammonium sulfate used, the baseline decreased gradually with the decrease of the salt concentration. The ceruloplasmin peak observed at 610 nm indicates that the protein can retain blue copper during the chromatography.

kind of lipid because the protein is a constituent of lipoprotein. The lipid could be removed with the organic solvent; thus the protein was eluted as a single peak by reversed-phase chromatography. On the other hand, under the gentle conditions of hydrophobic interaction chromatography, two forms of β_2 -glycoprotein I—one with and the other without lipid—were separated and obtained as double peaks because no organic solvent was used. Thus, the further separation of β_2 -glycoprotein I could be achieved by hydrophobic interaction HPLC without the interference of the heterogeneous carbohydrate chains.

The amino acid sequence of leucine-rich glycoprotein, which is a trace serum protein with unknown function, shows a 24-residue tandem repetition with a periodicity of leucine. From this structural characteristic, it can be predicted that the protein is a membrane-derived or membrane-associated protein [11]. Subsequently, the amino acid sequence of adenylate cyclase from yeast was shown to have homology to that of leucine-rich glycoprotein [18]. Adenylate cyclase is known to be buried inside the membrane; thus leucine-rich glycoprotein is probably a membrane protein. However, the LRG protein is highly soluble and is resistant to heat coagulation. These properties were attributed to the combination of a high carbohydrate content, which increases the hydrophilicity of the protein surface, and to the clustering of the hydrophobic leucine residues, which might be expected to be internalized in the protein [11]. If the clustered leucine residues are internalized, the surface of the protein has to be relatively hydrophilic; therefore, the protein would be expected to behave as an ordinary protein during reversed-phase chromatography. To the contrary, the protein behaved as one of the most hydrophobic proteins during reversed-phase HPLC (Fig. 5). This hydrophobic behavior was also observed in hydrophobic interaction chromatography, indicating that the behavior in reversed-phase chromatography cannot be attributed to denaturation with organic solvent. This suggests that the clustering of the hydrophobic leucine residues is not internalized in the protein but is probably exposed outside the molecule. The high solubility of the protein can thus be attributed to the carbohydrate attached; however, the carbohydrate did not have much effect on the interaction between the protein and the column.

Advances in the Analysis of Glycoproteins by Mass Spectrometry

Because of the advances in the gas-phase ionization of biomacromolecules, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), mass spectrometry (MS) has become a powerful tool for detection, identification, and structural analysis of proteins, peptides, and polynucleotides. The molecules ionized in a gas phase by these methods are subsequently analyzed by sector, quadrupole, ion-trap, or time-of-flight mass spectrometers. In particular, the MS systems consisting of ESI and triple-stage quadrupole (ESI/TSQ) or ion-trap (IT) mass spectrometry and MALDI time-of-flight (MALDI/TOF) mass spectrometry have been most widely applied to the field of protein chemistry for the accurate determination of molecular mass of proteins and peptides, determination of amino acid sequence, identification of proteins by "peptide mass databases," and analysis of posttranslational modifications such as phosphorylation and glycosylation. In general, current techniques allow determi-

nation of the molecular mass of proteins and peptides with an accuracy of 0.01–0.03% using subpicomole quantities of samples.

Figure 7 shows a mass spectrum of bovine brain calreticulin (CalR) obtained by capillary column (0.25 mm ID) reversed-phase high-performance liquid chromatography (HPLC) connected online to ESI/TSQMS. Calreticulin is an acidic calcium binding protein found in the lumen of endoplasmic reticulum as well as in the nucleoplasm, and it participates in the regulation of protein folding, assembly, and steroid-mediated transcription [19,20]. The previous sequence analyses suggested that the bovine CalR was posttranslationally modified, presumably by glycosylation at 162Asn [21]. The spectrum (Fig. 7) shows that the CalR preparation has a major molecular species with a mass value of 47,610 Da, which differs by approximately 1,229 amu from that estimated from the amino acid sequence (46,381 Da). This analysis also identified a series of subspecies with an approximate mass difference of a single hexose unit (162 amu) which could arise from the heterogeneity of the oligosaccharide chain.

To characterize CalR further, it was digested with lysyl-C endopeptidase after the protein was *S*-pyridylethylated, and the digest was analyzed by capillary HPLC-ESI/TSQMS. The method yielded a high-resolution peptide map of CalR and identified a single glycopeptide, L16, as a series of heterogeneous ions, all of which had larger mass values than those expected from its polypeptide sequence by 162 mass units (Fig. 8). The detailed analysis of the ESI/S spectrum of Li 6, as well as the ESI/MS/MS of this glycopeptide, proved that the oligosaccharide moiety is heterogeneous and has the compositions, GlcNAc₂Man ($n = 5-9$), in

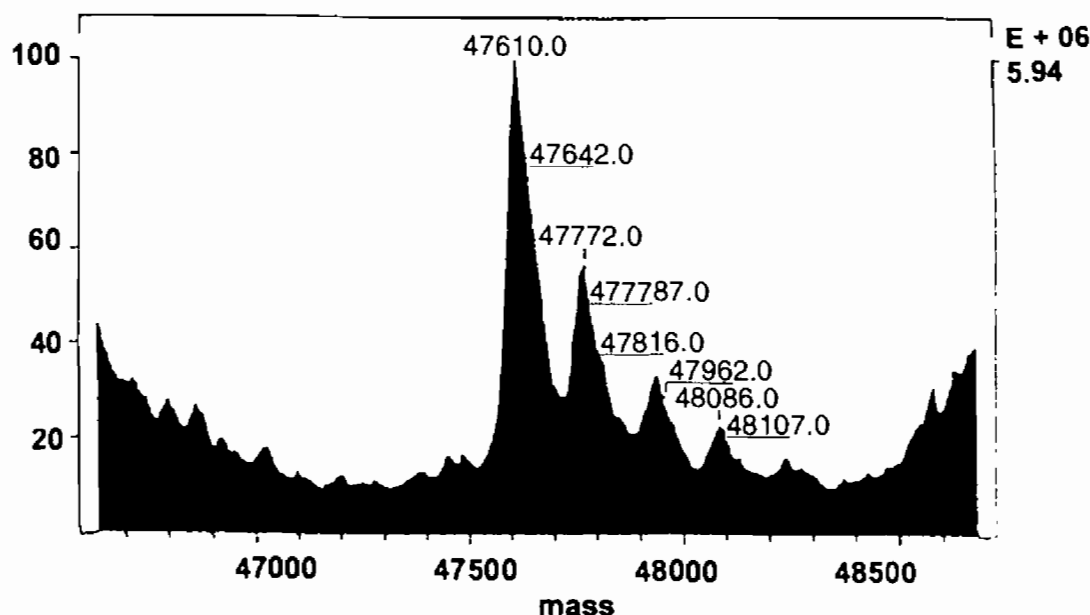


FIGURE 7 A mass spectrum of bovine brain calreticulin obtained by capillary column (0.25 mm ID) reversed-phase high-performance liquid chromatography (HPLC) connected online to ESI/TSQMS.

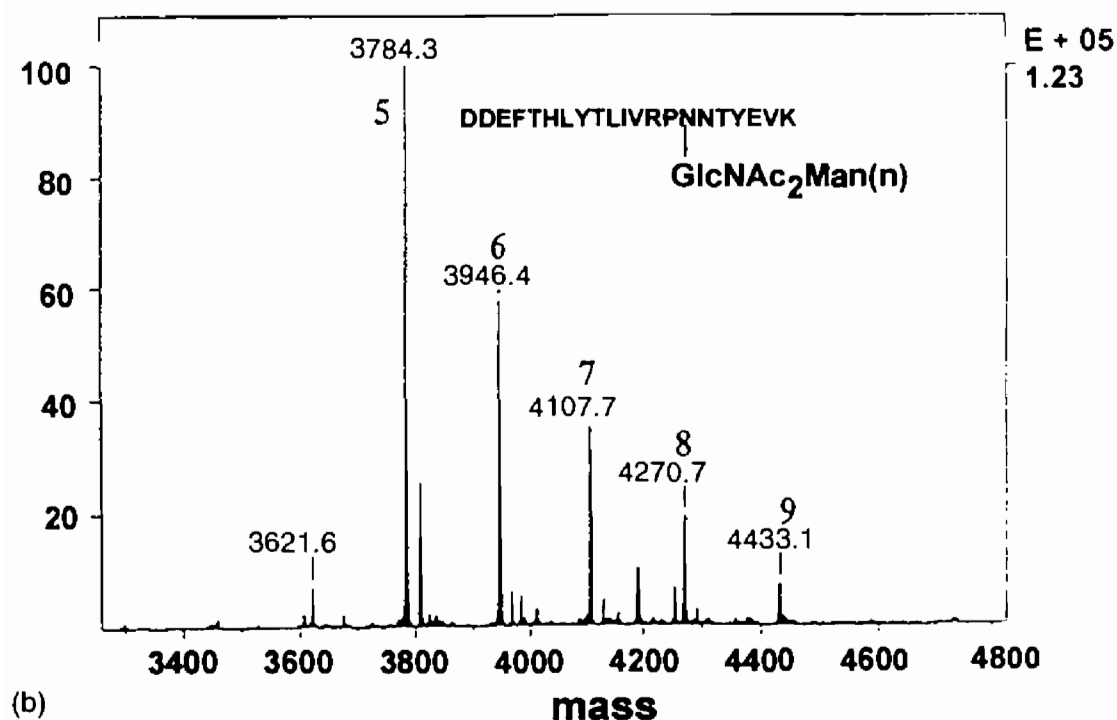
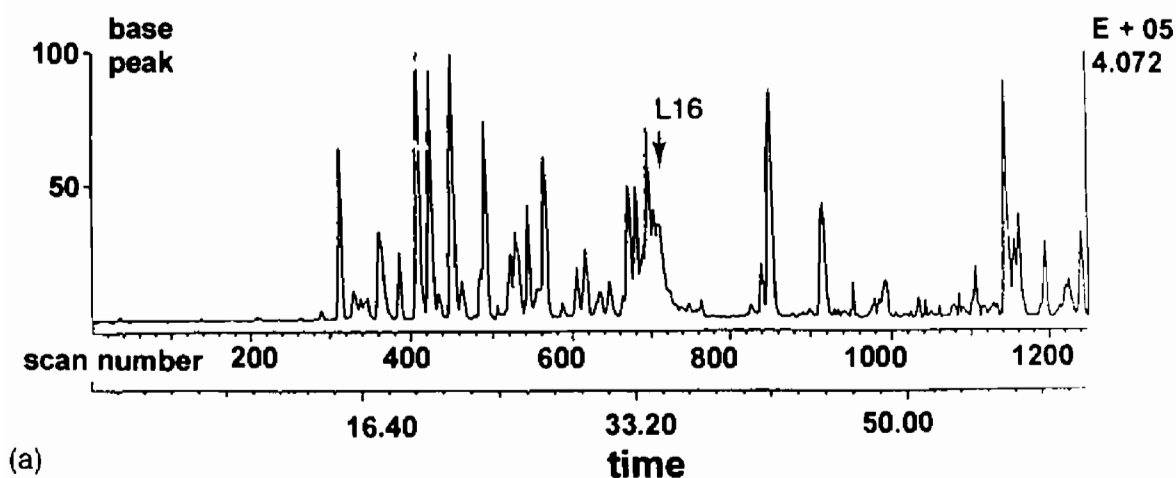


FIGURE 8 (A) Separation of the lysyl-C endopeptidase digest of calreticulin by packed capillary HPLC and detection of its eluate with ESI/TSQMS. The pyridyl-ethylated calreticulin was digested with lysyl-C endopeptidase and applied to a capillary column (1.0 mm ID $\times$ 10 cm) packed with CapcellPak C18 (3 μ m). The peptides are eluted at a flow rate of 50 μ L/min with a linear gradient of increasing concentration from 0 to 100% of acetonitrile containing 0.1% TFA. The eluent from the capillary column was detected with ESI/TSQMS. (B) Heterogeneity in molecular weight of the L-16 glycopeptide derived from calreticulin observed by the packed capillary HPLC-ESI/TSQMS analysis. The spectrum shown is one deconvoluted from the original mass spectrum.

which GlcNAc₂Man₅ is the most abundant component. Therefore, the MS analyses established that bovine brain CalR is a glycoprotein with heterogeneous, *N*-linked high-mannose type oligosaccharides attached to a single site of the polypeptide chain, 162Asn. At the same time, these results proved that calreticulin was resolved into a series of single molecular species by capillary HPLC-ESI/TSQMS, the species differing one from another by one mannose molecule in the carbohydrate structure. These are shown in Fig. 7 as a series of ion species with an approximate mass difference of 162 amu.

Figure 9 shows a mass spectrum of the same lysyl-C endopeptidase digest of bovine CalR, analyzed by MALDI/TOFMS. In this case, a portion of the digest was mixed with a matrix (dihydroxybenzene), a reagent to facilitate ionization of peptides and proteins upon laser irradiation, and the mixture was directly analyzed by a TOFMS system. In the resulting peptide map (Fig. 9A), most of the peptides expected from the amino acid sequence of CalR were identified on the basis of the mass value of each peptide. However, a peptide fragment with a mass of 2568 amu, corresponding to a polypeptide portion of L16, was not detected and, instead, a series of glycosylated forms of L16 was identified (Fig. 9B).

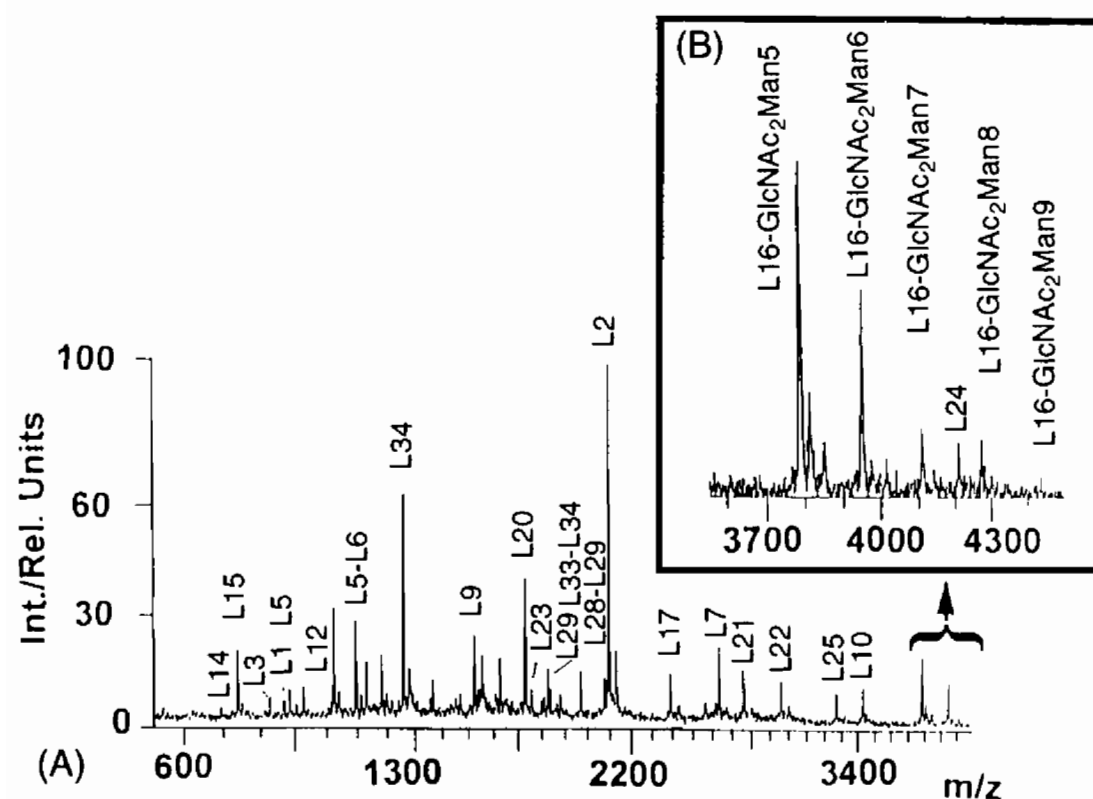


FIGURE 9 A MALDI/TOF-MS spectrum of a digest of the pyridylethylated calreticulin. (A) Mass range (m/z) from 500 to 4500. (B) Expanded spectrum of a part of the spectrum A.

These approaches could be applicable to the analyses of more complex glycoproteins, such as serum proteins. Figure 10 shows the results of one such analysis. LRG, which has one galactosamine and four glucosamine oligosaccharide chains, was digested with trypsin and subjected to packed capillary reversed-phase HPLC-ESI/TSQMS. Each peak in Fig. 10 was analyzed similarly to calreticulin. The peaks containing glycopeptides are indicated with the sugar structures under the chromatogram in Fig. 10. Thus, HPLC-MS in combination with proteolytic digestion was useful for analyzing the carbohydrate structure and identifying the number and location of attachment sites for the carbohydrate chain, even if the glycoprotein itself could not be separated into all the individual molecular species having a single carbohydrate structure.

These examples illustrate that the current mass spectrometry technology in ESI/TSQMS, as well as MALDI/TOFMS, has sufficient resolution to detect and analyze glycoproteins and glycosylated peptides. When a protein is glycosylated at a single site, as in the preceding example, the direct MS analysis gives valuable information on the oligosaccharides. However, in many instances including most of the serum proteins, complex glycosylation occurs at multiple sites. Because of the limitation of the resolving power of currently available MS, characterization of oligosaccharides in these proteins would require proteolytic cleavage and subsequent identification of each glycopeptide. However, because advances in mass spectrometry are occurring rapidly, this problem will probably be resolved to a significant extent in the near future. Fourier-transformed ion cyclotron MS may provide a solution.

CONCLUSIONS

The unique structural feature of glycoproteins is the heterogeneous character of the oligosaccharide chains. The heterogeneities of glycoproteins are individually characteristic and depend on their amino acid sequences, carbohydrate composition and structures, and the number and location of attachment sites for oligosaccharides. Therefore, the HPLC method selected should depend on the purpose of the analysis. Although all the HPLC methods described here have both advantages and disadvantages, reversed-phase and hydrophobic interaction chromatography seem to be the most suitable methods to minimize the effect of carbohydrate microheterogeneities. For the separation of carbohydrate variants that differ only by the presence of a certain carbohydrate chain, hydroxyapatite chromatography seems to be most favorable. It is still difficult to resolve a purified glycoprotein with multiple glycosylation sites into all of its individual molecular species, even when using advanced methods such as mass spectrometry combined with HPLC. Nonetheless, these techniques have greatly facilitated identification of the attachment site of carbohydrate and the determination of the carbohydrate structures bound to a glycoprotein at multiple sites.

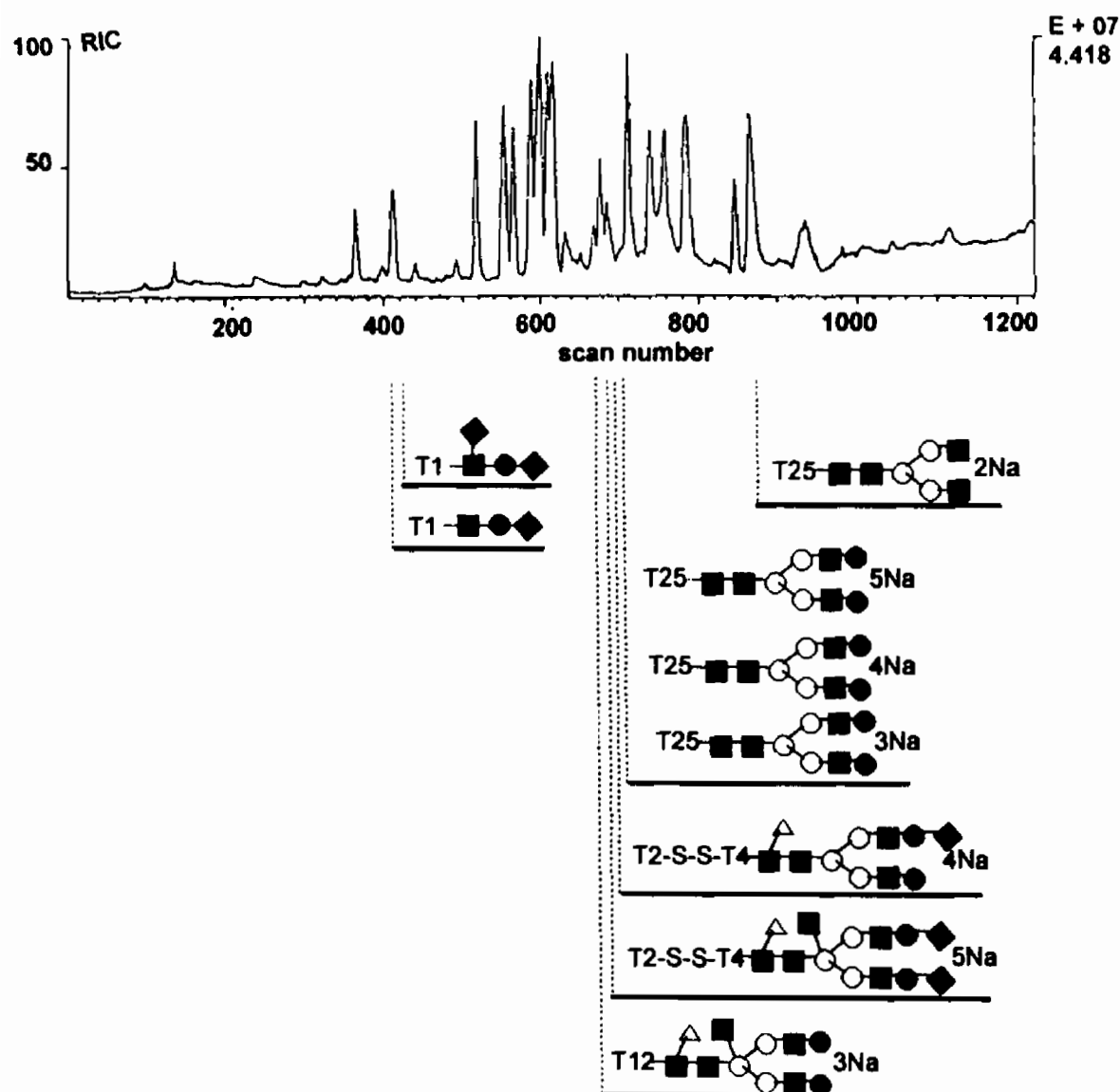


FIGURE 10 Identification of peptides of LRG that bind carbohydrate chains and structural determination by a packed capillary HPLC-ESI/TSQMS combined with MS/MS analysis. The intact LRG was digested with trypsin and applied to a capillary column (2.1 mm ID×10 cm) packed with CapcellPak C18. The peptides were eluted at a flow rate of 200 μ L/min with a linear gradient of increasing concentration from 0 to 100% of acetonitrile containing 0.1% TFA at an increasing rate of 1% acetonitrile concentration/min. The eluent from the capillary column was detected with ESI/TSQMS. The peak containing carbohydrate chain was further analyzed by collision-induced decomposition with MS/MS. The structure of carbohydrate chain is shown under the corresponding peak. Square, GlcNAc; open circle, mannose; closed circle, Gal; diamond, sialic acid; and triangle, Fuc.

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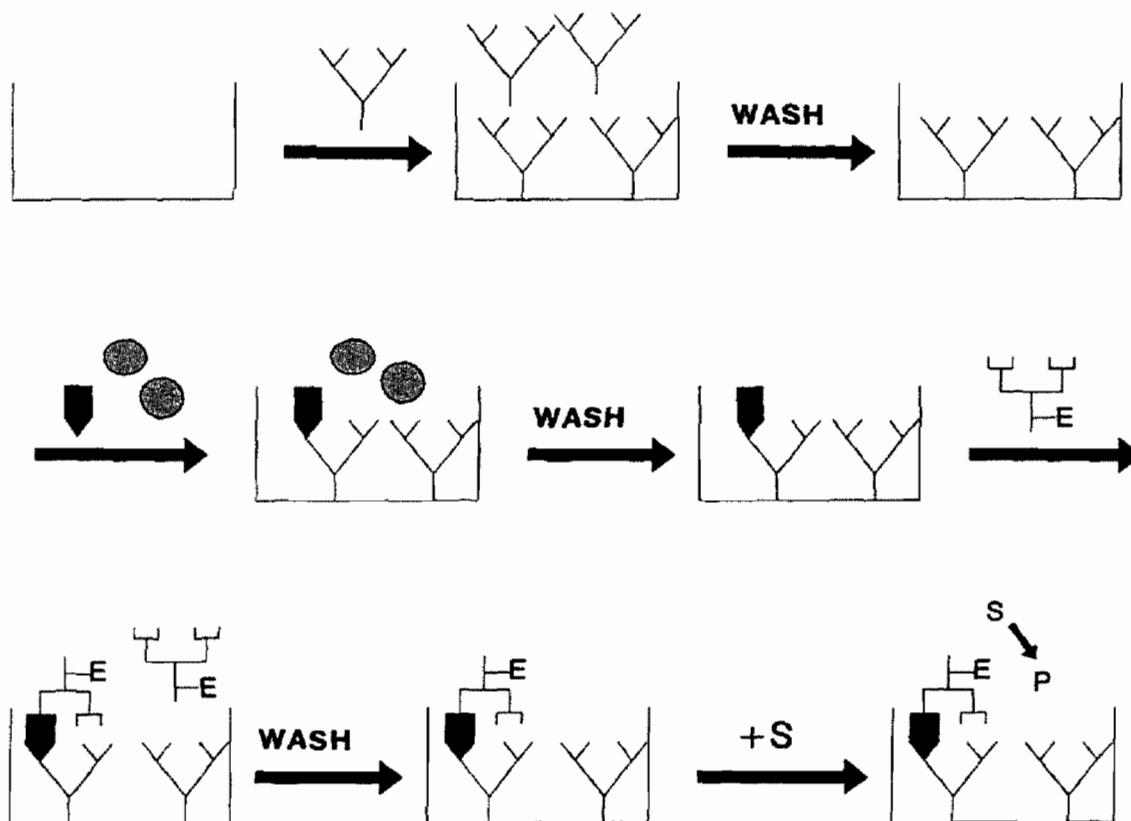
Immunodetection of Proteins in High-Resolution Separation Systems

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Angel Puerta**

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INTRODUCTION

Antibody-antigen (Ab-Ag) interactions provide a high degree of selectivity to analytical methods based on this type of molecular recognition. Traditionally, these immunoassays have been performed in a batch format in tubes or plates made of polystyrene or similar materials. Different ways of carrying out these assays have led to classifying them in several formats [1]. As an example of the steps involved in these assays, an enzyme-linked immunosorbent assay (ELISA) in a sandwich format is depicted in Fig. 1 [2]. In this format antibodies are bound to the wells of the plate. An aliquot of the sample containing the antigen is added to the well and incubated. After a washing step to eliminate the nonantigenic components of the sample, a second antibody labeled with the enzyme is added and incubated. The excess of labeled antibody is eliminated by a washing step. Substrate for the enzyme is added, and the amount of product formed during the enzymatic reaction, which is proportional to the amount of antigen in the sample, is measured.



SYMBOLS

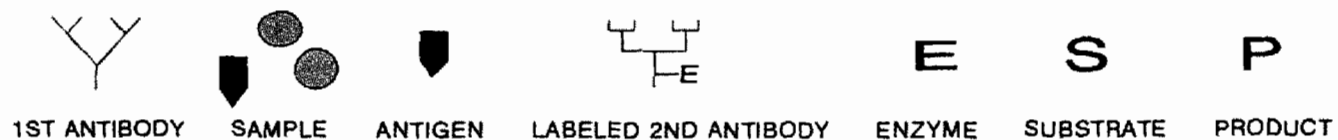


FIGURE 1 A general scheme depicting an ELISA in sandwich format. (Adapted from Ref. 2.)

Although the utility of classical immunoassays as an analytical tool has been shown for many types of biomolecules, the technique has some drawbacks. One is poor reproducibility. Another is sample volume limitation. Yet another is the laborious, time-consuming washing and incubation steps required to execute an assay [2]. One way to avoid these disadvantages is to perform the assays in a flow system such as a high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) instrument. An attractive feature of this format is that separate washing and addition steps are avoided due to the inherent continuous flow of liquid through the system. The fact that assays are carried out in a well-controlled flow regime allows greater control of the reactions. This allows incubation time to be reduced by making measurements before the system reaches equilibrium. Furthermore, large sample volumes may be used and the analyte concentrated to accommodate samples of low analyte concentration [3]. Sample dilution is avoided in concentrated samples by using a small volume.

Several reviews devoted to or including immunoassays performed in HPLC or CE systems have been published [4–12]. These methods have been used for analysis of small and large molecules. Besides the application to detect the presence and concentration of an analyte in a sample, immunochromatographic and immunocapillary electrophoretic systems have a wide application in the determination of antibody-antigen binding constants.

This chapter is focused on methods that use antibody-antigen interactions in HPLC or CE systems for the detection of proteins. Although immunoassays performed in LC and CE systems are similar, they vary in many practical aspects. For this reason, immunochromatographic and immunocapillary electrophoretic detection will be described separately.

IMMUNOCHROMATOGRAPHIC DETECTION

Most assays using the chromatographic format are performed on immunoaffinity columns in which antibodies are attached to the chromatographic support (Fig. 2a). Antigen is captured and enriched by the immobilized antibody as samples are eluted through the column while nonantigenic components are eluted unretained. Quantitation is achieved by releasing the antigen with a desorption buffer and transporting it to a detector placed at the column outlet. In this way, the same column can be reused for many analyses. Similarly, antibodies can be analyzed in biological samples by using a column with antigen attached to the support. Protocols other than the one described have been adopted to improve selectivity, sensitivity, and speed even more.

Several parameters enhance the performance of immunochromatographic assays, such as (1) the class of antibody and its binding to the chromatographic support, (2) the nature of the stationary and mobile phases, and (3) different approaches to increase immuno-recognition [2]. Berry et al. [13–15] have shown

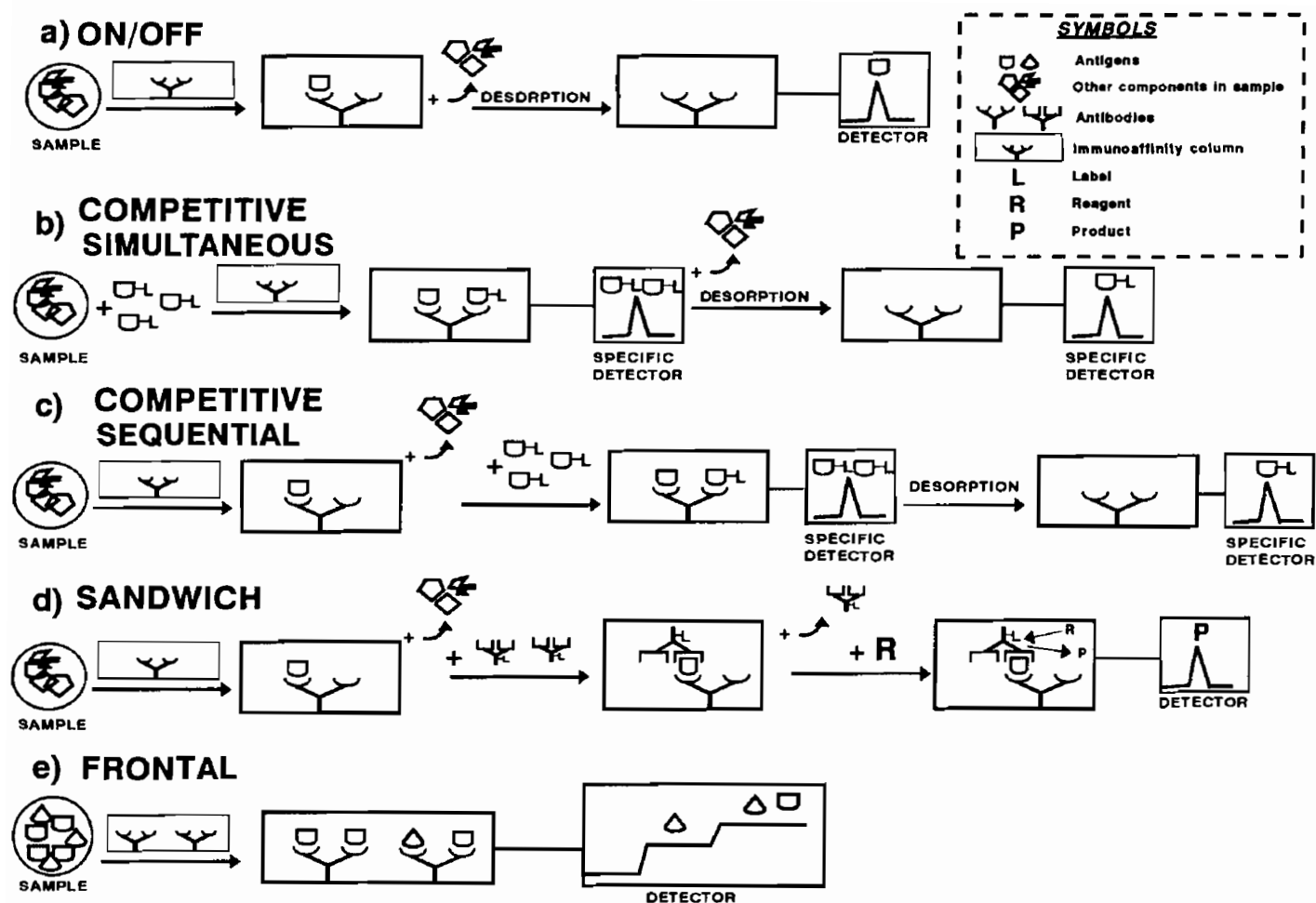


FIGURE 2 Schemes for different kinds of immunochromatographic assays.

that Fv antibody fragments immobilized on silica have a higher capacity for antigen than whole antibodies and that the use of single-chain Fv fragments does not have a marked effect on the immunosorbent stability. Another variable is the origin of the antibody. Comparison of antibodies elicited in both the primary and following immunizations showed that antibodies raised after the primary antigenic challenge are more sensitive to pH and ionic strength and allow better control of the binding and dissociation steps [16]. Monoclonal antibodies (MAbs) ensure higher homogeneity than polyclonal antibodies (PABs), but in some instances PABs are preferred as they can recognize a wider range of epitopes (specific zones of the Ag being recognized by the Ab) of the target molecule [17]; this fact is of interest when a group of cross-reacting antigens are being determined.

Immobilization strategies also play an important role in activity. Although antibodies may be immobilized on a support in any of a variety of physical adsorption or covalent bonding strategies, retention of antigen-binding capacity is affected by the immobilization technique. Practical ways to perform binding of ligand to the chromatographic support can be found in general papers dedicated to affinity and immunoaffinity chromatography [18,19]. It was noted in a review that the immobilization process should maximize the possibility that paratopes (the specific zones of antibodies that recognize epitopes of the antigen) oriented away from the surface have the maximum possibility of binding antigen [20]. This can be achieved by attachment of the antibodies through their oxidized carbohydrate groups. The influence of several factors on oxidation of antibodies and methods for coupling them to hydrazide-activated supports for immunoaffinity chromatography (IAC) have been studied [21]. In the immobilization of proteins on chromatographic supports, the use of spacer arms has been found to influence the capacity for biological recognition. Although in some instances the spacer is not required, probably because the functional group formed during the binding plays the role of spacer [22], in other cases a well-controlled length of the spacer is needed as too short or too long spacer arms promote steric hindrances [23]. A widely used and attractive approach to immobilize antibodies is to attach the Ab to protein A or protein G bound to the chromatographic support. These proteins recognize the Fc region of antibodies and do not interact with the paratopes. In spite of the random orientation promoted by general immobilization methods, such as binding through amino groups, they are still widely used to prepare immunosorbents. In these cases, advantage is taken of the fact that the amount of antibody bound to the column generally widely exceeds the amount of antigen in the sample. Even though a substantial amount of immobilized antibody is useless, there is enough to perform the assay.

The distribution of Ab can also enhance the antigen-binding efficiency of the immunosorbent [24]. Microencapsulation of Abs inside polymeric microparticles is a method that provides high adsorption capacity and stability of

immunosorbents over a wide range of chemical and biochemical conditions [25]. The support material is also an important consideration, particularly in regard to nonspecific interactions of the antigen with the matrix [26–28]. Silica and polymeric materials, including those working in perfusion mode, are among the most widely used supports. The main drawback of silica is the restricted pH range in which it is stable. For polymeric materials attention should be paid to the treatment necessary to decrease unspecific hydrophobic interactions. Beads are generally the preferred geometry for the support, although direct binding of the ligand to silica of capillary columns [29] and to porous frits contained in pipettor tips [30] and columns packed with pieces of membranes to which Abs have been bound [31,32] have also been used. Computational studies have shown that, depending on the operational mode, capillary solid supports can perform better than beads and membranes [33].

Studies including the effect of several of the mentioned factors, such as Ab (or Ag) immobilization method, length of the spacer arm, or characteristics of the IAC support, on the extent of Ab (or Ag) immobilization and column capacity have been carried out [34,35]. Knowledge of variables that affect antigen-antibody binding equilibria and the binding constant allows selection of the most appropriate mobile phases for binding and dissociation under some given experimental conditions [36].

As with all immunological assay formats, antigen diffusion is a major issue in the speed of immunochromatographic assays. This limitation has been attacked in several ways. One is to incubate antigen and antibody prior to injection into the column [29,37]. Another is to incorporate a stop-flow step in the method to allow incubation in the column [29]. The use of microcolumns made of perfusive packing materials, with small diffusion distances and high surface areas, is still another way to overcome these difficulties [38].

The final phase of optimizing a flow assay is selection of appropriate conditions for antigen-antibody dissociation. If the mobile phase fails to dissociate the complex completely, quantitation errors will occur in the present and subsequent assays. Different dissociating agents have been tried and compared [29,39–44]. In columns in which antibodies are captured by peptide antigens bound to the matrix, it has been shown that the concentration of chaotropic agent needed to desorb the Ab from the column is related to the affinity between the Ab and the epitopes in the different peptides immobilized in the column [42]. Among dissociating agents, acidic mobile phases are the most widely used. However, this is not always the best choice, as the recovery of protein can be low [41]. Comparison of different dissociating agents is usually performed using the immunochromatographic column itself. Other approaches, which have the advantage of avoiding spoiling of the columns, consist of making comparisons using traditional ELISA [43] or immunodotting methods [44]. In addition to dissociation efficacy, the effect of dissociating agents on column lifetime has to

be considered. Extremely acidic or basic conditions may negatively affect the support itself, alter the binding of the antibody to the chromatographic support, or irreversibly denature the antibody.

Schemes for Performing Immunochromatographic Assays

Reference has already been made (Fig. 2a) to the so-called *on/off* or *capture/elution* mode, in which the antigen in the sample is captured by the antibody attached to the immunochromatographic column, released using a desorption buffer, and monitored at the detector. A modified version of this *noncompetitive* format consists of injecting the previously mixed antibody and antigen onto a protein G perfusion column [37]. An attractive approach of the *on/off* mode is the so-called recycling immunochromatography [17,45,46]. This procedure allows the simultaneous measurement of several analytes in a single sample. It is based on the passage of a sample through a battery of different immunoaffinity columns. Each of the columns has antibodies against one of the antigens of interest that capture each target analyte. A system of valves allows sequential desorption of the battery of columns. The earliest analyses carried out in the *on/off* mode gave rise to broad peaks that were difficult to quantitate because of slow Ag-Ab desorption kinetics. The desorption agent can also cause interferences in the chromatogram. Several strategies, including stepwise or linear gradient elution steps that lead to narrow peaks, have been proposed to enhance detection [47].

An easy way to avoid difficulties at the desorption step is to quantitate the antigen before the dissociation. This can be achieved with assays performed in several formats, ranging from the competitive or the noncompetitive to a frontal elution approach. In the *competitive* methods, the antigen in the sample and an analogue of the antigen compete for binding to a limited amount of antibody in the column (Fig. 2b and c). The amount of antigen in the sample is inversely proportional to the amount of analogue retained by the column and directly proportional to the amount of analogue eluted unretained. The sample antigen and its analogue can be injected onto the column simultaneously (Fig. 2b) or sequentially (Fig. 2c). Theoretical studies of competitive immunochromatographic assays in sequential [48] and simultaneous [49] formats have been carried out. Monitoring unretained antigen analogue has been used to quantitate antigen in the sample by sequential injection of sample and labeled or nonlabeled analogue onto the column [48,50] and by simultaneous injection of sample and labeled analogue. In most of the cases, the analogue of the antigen is labeled because, as shown later, sensitivity can be enhanced in both simultaneous and sequential injection assays.

Besides these methods, competitive ones in which quantitation is performed by eluting and measuring the retained labeled antigen analogue have also been applied [51,52].

A noncompetitive method in which the Ag-Ab-label is detected in the immunochromatographic system without performing any desorption step has been carried out in a microchip format [53]. The Ag to be determined is adsorbed onto beads that are introduced into the microchannels, followed by Ab labeled with colloidal gold. Detection of the Ag-Ab-label takes place inside the channel.

Noncompetitive methods based on reacting the Ag to be determined with labeled Ab, capturing the excess labeled Ab on an Ag column, and measuring the Ag-Ab-label complex eluted unretained from it represent a different way to avoid problems caused by the desorption agent [35,54].

Methods very similar to classical immunoassays in the *sandwich* format are easily implemented in flow systems (Fig. 2d). In this type of noncompetitive assays, again, antigen is captured and concentrated from an appropriate volume of sample on an immunosorbent ($-Ab_1$) column while nonantigenic components are eluted. Subsequent to the capture step, labeled second antibody (Ab_2 -label) is introduced into the mobile phase and swept into the column, where it binds to the $-Ab_1$ -Ag complex to form $-Ab_1$ -Ag- Ab_2 -label. Unbound Ab_2 -label is swept from the column, and when the label is an enzyme, antigen is quantitated indirectly by conducting an enzyme assay in the column. After substrate incubation, the reaction product is transported to a detector at the column terminus. Ag and Ab_2 -label can be introduced in the column sequentially or simultaneously. In some instances both modes led to similar sensitivity [55], and in other cases simultaneous injection produced a greater response than sequential injection [56]. The term sandwich has also been applied to the procedure carried out to quantitate Ab by capturing a complex Ab-Ag-label onto a protein G capillary column [57]. In this case detection is performed after elution.

The detection limit is usually lower in noncompetitive than in competitive methods, although similar sensitivities have been achieved for both kinds of methods in some instances [58,59]. The two antibodies involved in sandwich methods give these methods higher specificity than that achieved with methods in which only one antibody is used. The main drawback of sandwich methods does not apply to proteins, the target of this chapter. For small molecules, steric hindrance and the nonavailability of two separate epitopes prevents the use of sandwich procedures for their analysis.

All the assays just described fail to discriminate between cross-reacting antigenic species, i.e., all the species having epitopes that are recognized by the immunosorbent column. One way to distinguish between crossreacting species with an immunoaffinity column is to introduce the sample in the *frontal* mode [60] (Fig. 2e). In this method, polyclonal antibodies are bound to a protein A column through which a large volume of sample is introduced. Differences in the

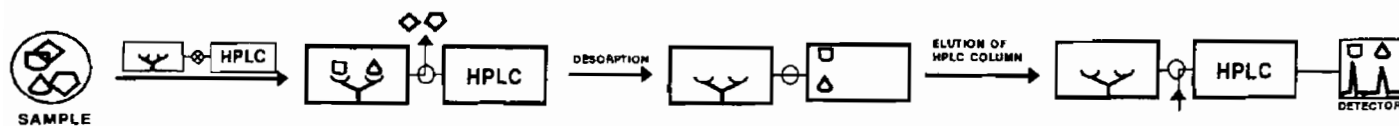
epitope composition cause antigens with epitope deletions or modifications that reduce binding affinity to elute before antigens with the full complement of unmodified epitopes. In this way, conformational variants of bovine serum albumin produced by reduction can be distinguished from the native protein.

More general approaches to discriminate between proteins with shared epitopes consist of using two or more separation columns of which one is an immunosorbent [61]. These *tandem immunoaffinity chromatography-chromatography* methods can be divided into two groups: those in which immunorecognition can be carried out either before (Fig. 3a and b) or after (Fig. 3c) separation in the chromatographic mode.

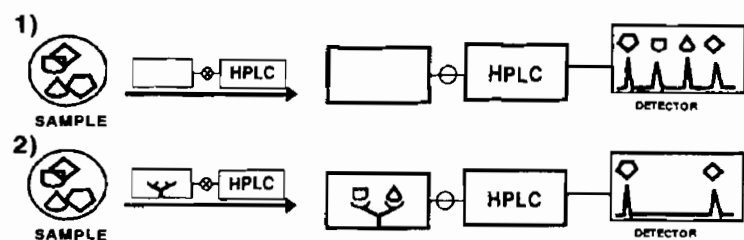
The most widely used way to perform tandem assays is to employ an immunoaffinity chromatography (IAC) column first to capture all the cross-reacting proteins and eliminate nonantigenic components of the sample. In a second step, the retained proteins are released from the immunoaffinity column onto another HPLC column where they are separated from each other (Fig. 3a). Several chromatographic modes have been used as the second HPLC dimension. Reversed phase (RP) has been shown to discriminate between antigens that differ more than 40% in amino acid composition [62,63]. The concentration of recombinant proteins in a yeast cell lysate has been analyzed with an IAC-RP dual system similar to that in the scheme of Fig. 3a [64]. The combination IAC-RP has also been used to obtain pharmacokinetic profiles of a glycoprotein in animals and humans and for process monitoring of the same glycoprotein in a cell culture fluid [65]. Introduction of more elements, namely ion exchange and trypsin columns, between the IAC and the RP columns and the addition of a mass spectrometer have allowed automated discrimination between protein variants at the amino acid sequence level [66]. Figure 4 shows the tryptic peptides of normal hemoglobin (HbAo) and sickle cell hemoglobin (HbS) analyzed by MS after this multidimensional HPLC system. MS-MS showed a substitution of valine for glutamic acid at position 122 in HbS. Ion exchange (IEX) has also been found to be an effective column as the second HPLC dimension (Fig. 3a) in differentiating between protein variants [63]. The amino acid composition in this case differed in up to 20 amino acids.

In some instances the conditions needed to release the proteins from the IAC column are so drastic that the proteins are irreversibly denatured. This can be a problem in cases in which the second chromatographic dimension separates only native proteins. The problem may be circumvented by using a subtraction mode in which the sample is run twice on the HPLC columns, once through the IAC column and the other time without passage through the immunosorbent. In one application of this method the first chromatogram is obtained by passing the sample through a tandem protein G-size exclusion chromatography (SEC) column set. To obtain the second chromatogram, antibodies against the antigen of interest are adsorbed onto the protein G column and the sample passed through

a) IAC-HPLC



b) SUBTRACTIVE IAC-HPLC



CHROMATOGRAM 1 - CHROMATOGRAM 2



c) SEC-IAC

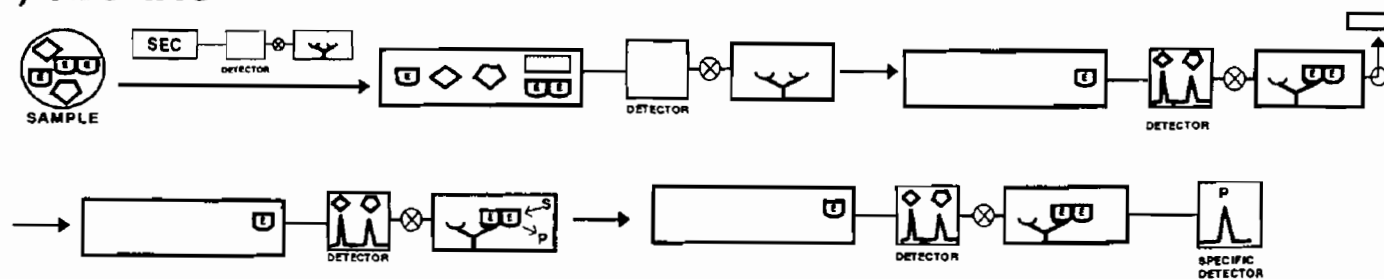


FIGURE 3 Schemes for coupling immunoaffinity chromatography with other HPLC separation steps. Symbols as in Fig. 2.

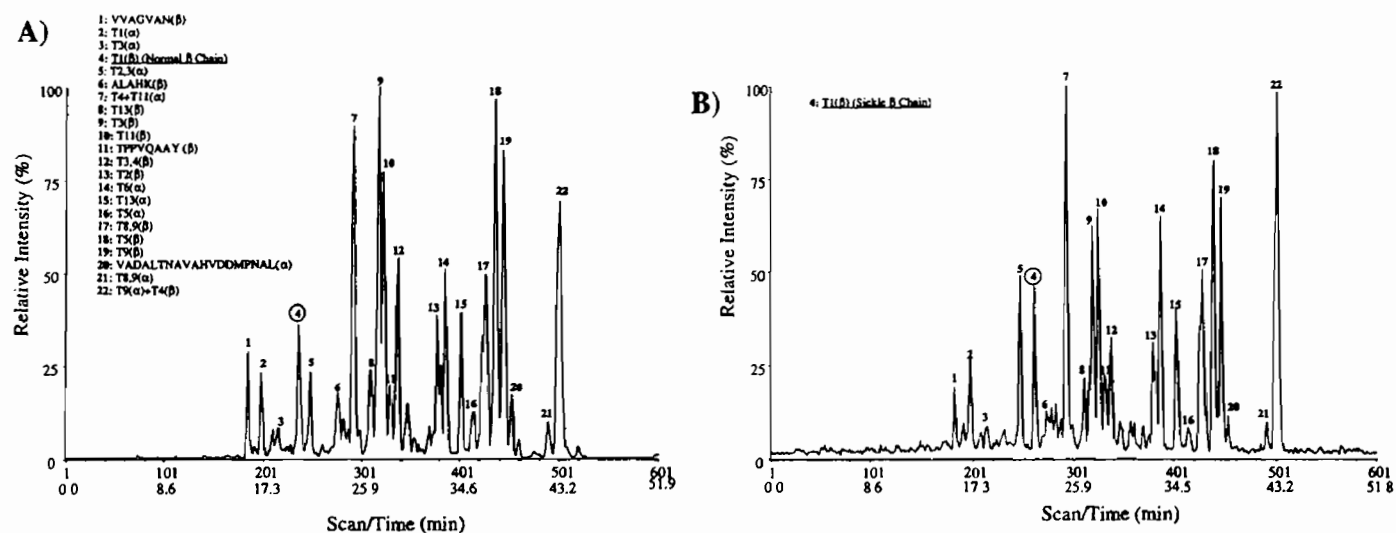


FIGURE 4 Total ion chromatograms for (A) normal cell hemoglobin (HbAo) and (B) sickle cell hemoglobin (HbS) produced from a five-column chromatography mass spectrometric analysis system. The peak numbers correspond to the peptide sequences listed in the upper left of A. Peptides identified that correspond to expected tryptic peptides are labeled T#, with the number corresponding to the sequential position of the tryptic peptide in the protein sequence. The α and β refer to the α and β chains of hemoglobin. The only peak differing in retention time and mass of the observed peptide is peak 4 in chromatogram B. (From Ref. 66.)

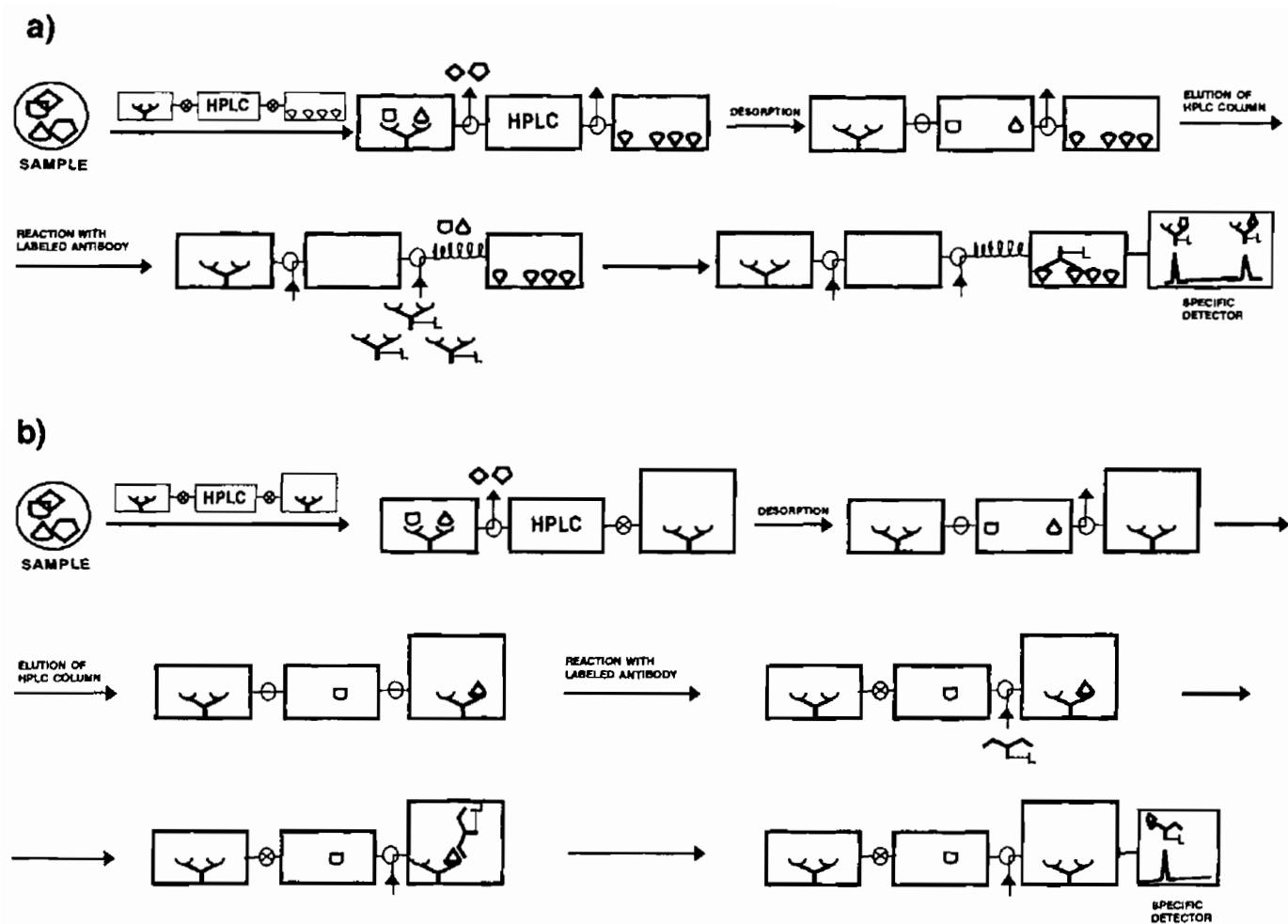


FIGURE 5 Scheme for performing immunochromatographic analysis in three-column systems including two immunorecognition steps. Symbols as in Fig. 2.

the tandem columns. The difference between these two chromatograms corresponds to the antigens retained by the immunoaffinity column [67] (Fig. 3b). IEX in the second HPLC dimension in subtractive detection has allowed monitoring the production of recombinant γ -interferon in a mammalian cell culture [68].

Another way to avoid the denaturation problem is to invert the tandem column sequence; i.e., IAC is the second step (Fig. 3c). Evidently, the chromatographic conditions used in the first dimension must not alter the epitopes in the antigen. This would preclude it from being recognized by the antibody in the second column. In the SEC-IAC tandem set, the first dimension allowed the separation of different enzyme aggregates, and the second dimension discriminated between enzymes of similar size and substrate (S) specificity [69].

Triple-column systems with immunorecognition at the first and third steps and RP or IEX at the second HPLC step (Fig. 5) have been employed to show, with model systems of proteins and tryptic peptides, that recombinant proteins and their metabolites may be determined in complex matrices [70,71]. The first IAC column prevents overloading the RP or IEX column (indicated as the HPLC column in Fig. 5). Experimental conditions for interfacing columns in such a way as to favor retention on the second column and still obtain antibody-antigen recognition in the third step have been studied. Two ways to perform the third step, that is, immunodetection, have been devised. The first (Fig. 5a) is based on a protocol previously used for immunodetection of small molecules [72]. Antigenic proteins in the sample are captured by the IAC column, released and separated by RP or IEX, and then reacted with soluble antibodies to form immunocomplexes. Unreacted antibodies are removed prior to detection by capturing them in an immobilized antigen column [70]. Figure 6 shows the configuration of the

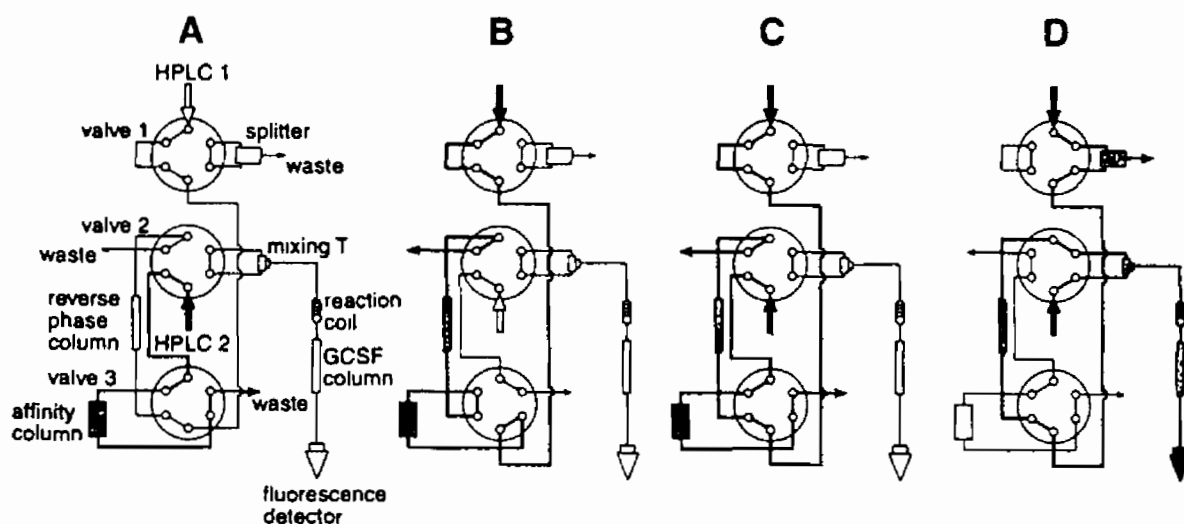


FIGURE 6 Diagram of the instrument used to perform immunodetection in the way indicated in Fig. 5a. (A) loading step; (B) transfer step; (C) equilibration step; (D) separation and detection step. (From Ref. 70.)

instrument employed at each step of the analysis. The second approach (Fig. 5b) includes capture of antigens at an IAC column, desorption onto an RP column, and then onto another antibody column. Once the antigen from the sample has been captured by the third column, Ab is introduced onto it. The retained Ab is finally released from column and measured [71].

IMMUNOCAPILLARY ELECTROPHORETIC DETECTION

These methods are based on the separation of an antibody-antigen complex from the individual antibody and antigen due to their different electrophoretic mobilities. In the electropherogram a new peak corresponding to the complex, with a different migration time than Ab and Ag, is observed as long as the dissociation kinetics of the complex is slow. Dissociation of the immunocomplex during the analysis should be avoided by having antibody-antigen complexes that are kinetically stable during the separation time [73]. In instances in which the electrophoretic mobilities of antibody and antigen-antibody complex are very similar, the difference can be increased by modification of the charge of the antibody with charge-bearing molecules, such as succinic anhydride, as shown in Fig. 7 [74].

Antibody and antigen can also be mixed prior to analysis and simultaneously injected, or be sequentially injected as long as the sequence of introduction and the corresponding electrophoretic mobilities allow their mixing in the capillary column. With the immuno-CE methods, it is possible to distinguish between cross-reacting antigens, binding to and desorption from the column are not required, the choice of an appropriate desorption solution is avoided, and capillary columns are easily reused for several analysis. Difficulties in performing CE immunodetection can arise from the heterogeneity of labeled antibodies, which can give rise to multiple or broad peaks for the complexes corresponding to a single Ag [75].

Schemes for Performing Immuno-CE Assays

Immunodetection of proteins by CE in diverse modes has been achieved. Figure 8a depicts a general scheme for *noncompetitive* CE immunoassays to determine antigens in a sample. Following a similar scheme, variants of a recombinant protein have been detected simultaneously by *capillary isoelectric focusing* (CIEF) in a noncompetitive format [73]. Prior to the introduction into the capillary, the antibody was incubated with a mixture of protein variants with different pI values. The CIEF method allowed detection of the variant proteins as completely separated immunocomplexes. In this case, to prevent the heterogeneity in the pI of antibody originating from variation in the number of sialic acids attached to the Fc region of IgG, the Fab' fraction was used instead of the

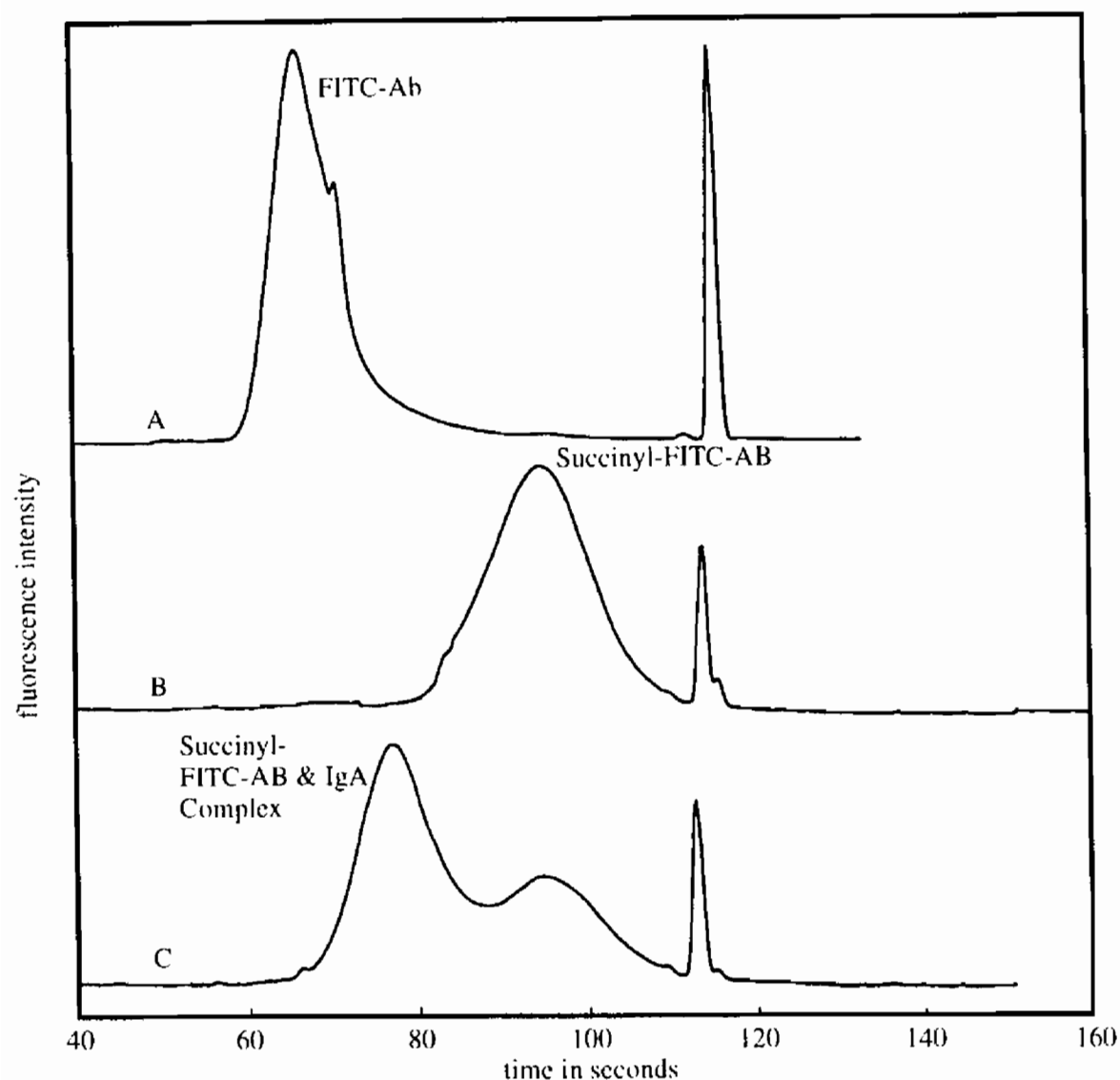


FIGURE 7 (A) Electropherogram of FITC-antibody (fluorescein isothiocyanate-antibody) to human IgA, (B) electropherogram of succinyl FITC-antibody to human IgA, and (C) electropherogram of reaction mixture of human IgA with succinyl FITC-antibody to human IgA. (Adapted from Ref. 74.)

whole antibody. Besides the separation efficiency of the method, CIEF provides a way of increasing the sensitivity by concentrating the separated species in narrow bands. The authors estimated that the detection limit was 100-fold lower by CIEF than by capillary zone electrophoresis (CZE).

Separation of antibody-antigen complex from unreacted Ab and Ag has been achieved with a *micellar electrokinetic capillary chromatography* (MECC) method using cholate micelles and the appropriate buffer pH, temperature, voltage, and electrolyte and modifier concentrations [76]. Mixing of antibody and antigen before their injection into the capillary showed that the immuno-complex has a longer retention time than antibody alone.

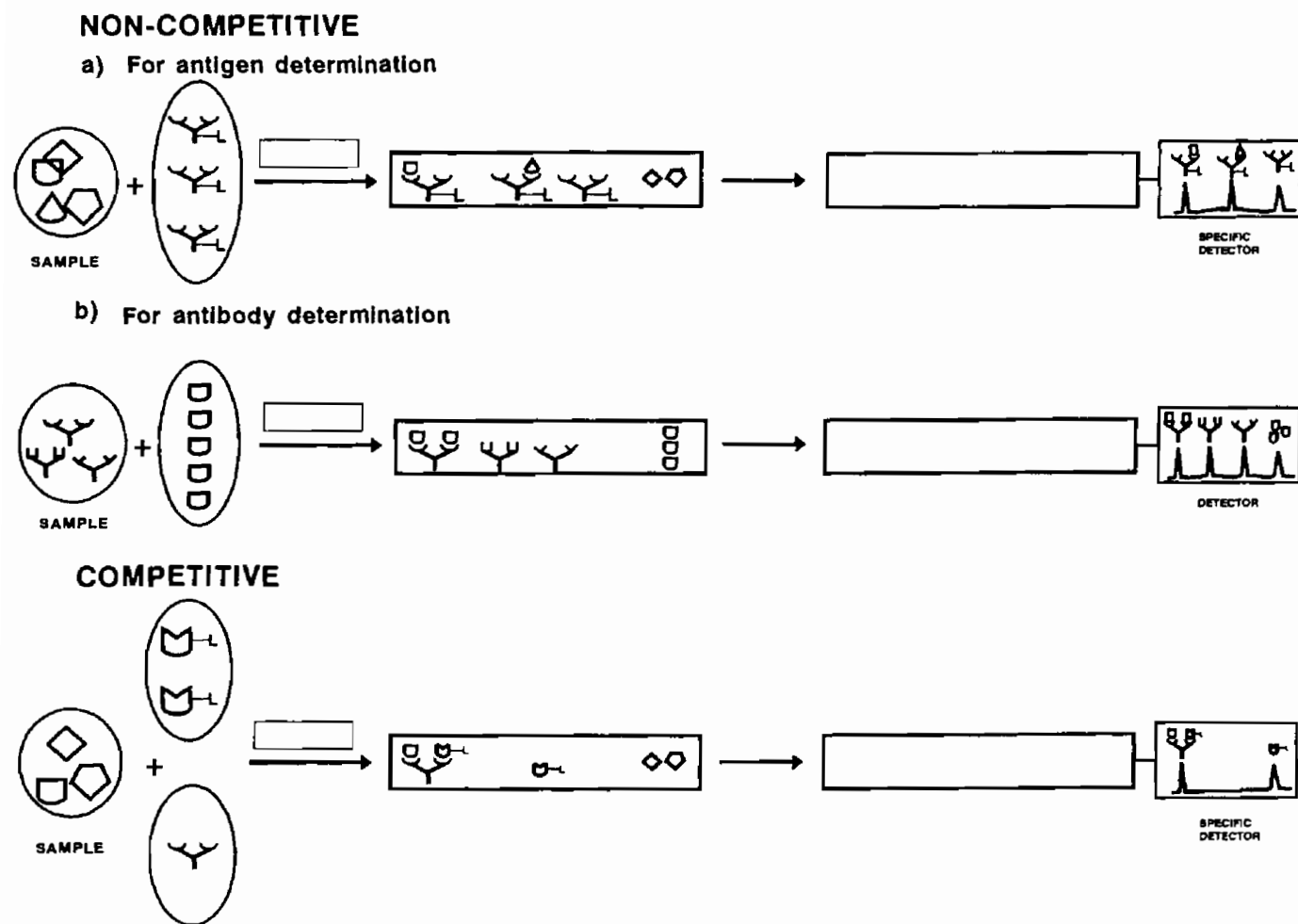


FIGURE 8 Scheme of different ways to perform capillary electrophoretic immunodetection. Symbols as in Fig. 2 except for the rectangle, which represents the CE column.

Separations based on the different molecular size of the Ab-Ag complex compared with each of the separate components of the complex have been performed by a non-competitive immunoassay in *sodium dodecyl sulfate-capillary gel electrophoresis* (SDS-CGE) [77]. In this mode of analysis, separation should be carried out under nondenaturing conditions, as the presence of 2-mercaptoethanol would separate heavy and light chains of antibodies. Although this method is claimed to have the potential of being of general use for large molecules, speed should be increased to achieve larger applicability. In some instances disruption of the oligomeric Ag-Ab complex needs to be carried out as its large size prevents detection [78].

Capillary zone electrophoresis (CZE), the most widely used CE mode, is also the mode most frequently used for performing CE immunodetection. The first assay of immunoaffinity capillary electrophoresis described was carried out with CZE in noncompetitive format (Fig. 8a) [79,80]. This format has been used to determine a protein in a matrix as complex as human serum by incubating the sample with specific antibody for 1 hr before the introduction onto the CE column [81]. The size of the immunocomplex peak was seen to increase with incubation time. One drawback of this method is the long incubation time needed because of the slow reaction kinetics of formation of Ab-Ag complex. To prevent noticeable complex dissociation during the analysis, conditions to achieve a separation time shorter than 3 min were chosen.

The *competitive* assay format [82–84] (Fig. 8) performed better than noncompetitive assays [85] for the determination of prion protein (PrP) in brain samples. In the competitive assay, antigen in the sample competes in solution, prior to injection onto the CE column, with a labeled peptide of the PrP to bind to an antibody directed against this peptide. The presence of antigen in the sample increased the size of the CZE peak corresponding to free labeled peptide and decreased that of peptide bound to antibody. Probably, in this case, the higher performance of the competitive mode was not related to the format of the assay but to the successive improvements in sample and antibody purification, in the process of peptide labeling, and in the careful choice of the separation buffer carried out in this format.

Noncompetitive and competitive formats have proved valid to determine both antibodies and antigens, respectively [86]. In the first case, sample incubated with antigen for some minutes is introduced onto the CE column where the Ab-Ag complex and excess antigen are separated (Fig. 8b). As in any noncompetitive format, the larger the concentration of analyte in the sample, the larger the peak of the immunocomplex and the smaller the excess reagent peak. The area of either peak can be used to determine the antibody concentration in the sample. In a competitive assay for antigen, the sample was preincubated for some minutes with known amounts of antibody and labeled antigen (Fig. 8) prior to analysis. The separation efficiency of this procedure was enhanced by using capillaries

coated with a neutral hydrophilic polymer. Quantitation was enhanced by determining the peak size of free labeled antigen, which elutes earlier than the immunocomplex, relative to an internal standard, and the method was used to determine a protein in a complex biological matrix [87]. A further modification of this competitive procedure included on-line mixing of sample and reagents and allowed monitoring of antigens in flowing systems [88]. Sample, labeled antigen, and antibody were pumped from three channels into a capillary reactor that was interfaced with the CE separation column, where separation of immunocomplex from unreacted labeled antigen took place in 3 sec. Besides the size of peaks corresponding to free and bound labeled antigen, the ratio of these two peaks was used to quantitate antigen in the sample. A minor modification of this instrument allowed determining the antigen in biological fluids, namely insulin in single islets of Langerhans, in about 1 sec [89]. A similar method has been developed by the same authors for glucagon, making possible simultaneous determination of both antigens in model systems [90].

The influence of several parameters, such as amounts of tracer and antibody [91], coated versus uncoated capillaries [92], whole Ab versus Fab fragment [92], and the presence of additives in the running buffer [93], on the performance of competitive CZE assays has been studied. As mentioned for immunochromatographic assays, the competitive format has some drawbacks compared with the noncompetitive format. However, the competitive format avoids some of the problems found in noncompetitive immunocapillary electrophoresis methods [94]. One of the main advantages of the competitive assay is the possibility of performing quantitation of the antigen by measuring the amount of labeled tracer not bound to the antibody. In this way, the problems mentioned before arising from antibody heterogeneity are avoided.

A direct CZE immunoassay for determination of monoclonal antibodies in ascites fluid has been developed using an attractive system in which fused silica capillary columns are substituted by small channels (240 μm wide) microlithographically engraved on square glass chips (4 $\times$ 4 in.) [95]. The separation scheme is similar to that shown in Fig. 8b. Samples of ascites fluid were incubated with labeled antigen for 15 min and an aliquot was introduced into the on-chip sample reservoir. The applied voltage caused the sample components to be separated, making it possible to differentiate the peaks of free labeled antigen and labeled antigen-antibody complex. The electropherogram in Fig. 9 illustrates an assay of anti-bovine serum albumin (anti-BSA) in mouse ascites fluid using fluorescein isothiocyanate (FITC)-labeled BSA. The increase in area and height of the peak at 27 sec, which corresponds to the immunocomplex, and the decrease of BSA-labeled peak at 36 sec were proportional to the amount of specific antibody in the sample. The speed and success of this design, and a similar one shown for small molecules [96], suggest that very high throughput could be achieved in parallel channel chip systems. Some advantages of chips are

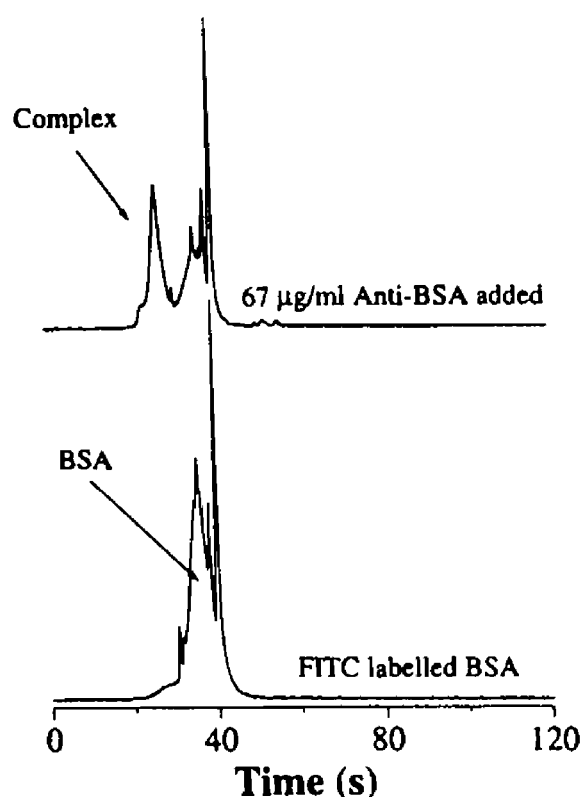


FIGURE 9 On-chip electropherograms of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-labeled BSA) (bottom) and of a mixture containing FITC-labeled BSA and monoclonal anti-BSA in diluted mouse ascites fluid. (From Ref. 95.)

added to this one, such as lower reagent and sample consumption, shorter analysis time, and the possibility of performing on-chip aliquoting, diluting, mixing, incubating, and labeling [97,98]. One drawback of microchips made of glass, quartz, or silicon arises from the manufacturing process itself. The high temperature needed for sealing the cover of the chip precludes binding of biomolecules to the channels previous to sealing. Use of polymeric chips that require low temperature for sealing avoids this problem [99].

Some classical immunoassays are based on agglutination of antibody-coated particles in the presence of an antigen. This principle has been used in the CE format to determine antigens in single human cells [100]. The cell was introduced onto the CE column, where it was lysed due to the low ionic strength of the buffer. Voltage was interrupted to allow the incubation of the antigen from the sample with the antibody-coated particles in the buffer. After incubation, voltage was again applied to move the aggregated particles to the detection window. Discrimination is based on the differential increase in light scattering by the larger aggregated particles formed when antigen cross-links antibody-coated particles. Determination of antigen does not require separation of the immuno-complex from the unreacted antibody.

Mixed methods combining the specificity of immunoaffinity chromatography and the separation efficiency of capillary electrophoresis (IAC-CE) have been developed. In one of these methods the immunoaffinity column consisted of a capillary packed with protein G bound to a perfusive support [101]. Antibodies were loaded onto the column prior to sample injection. Antigens and antibodies were desorbed from the IAC column and introduced into the CE column. The off-line combination consisted of injecting fractions collected from the IAC column onto the CE column. On-line coupling was achieved with a flow-gated interface (see Fig. 10). Recombination of desorbed antibodies and antigens was prevented during CE by using the IAC desorption buffer in the CE column. As can be seen in Fig. 11, the IAC step provides selective preconcentration of analyte and avoids introduction of a large number of compounds into the second column while the CE column performs the separation of antibodies and antigens and avoids the loss of efficiency due to slow desorption kinetics. The increase in sensitivity provided by the preconcentration during the IAC step has led to the development of several kinds of concentrators, as will be mentioned subsequently. The use of the method of recycling immunoaffinity chromatography coupled off line with CE separations [17] allows the analysis of multiple antigens. A different approach for the analysis of multiple antigens consists of binding of several Abs to the first part of the capillary. This method allows capturing on it different antigens from body fluids, which after desorption are separated by capillary electrophoresis [102,103].

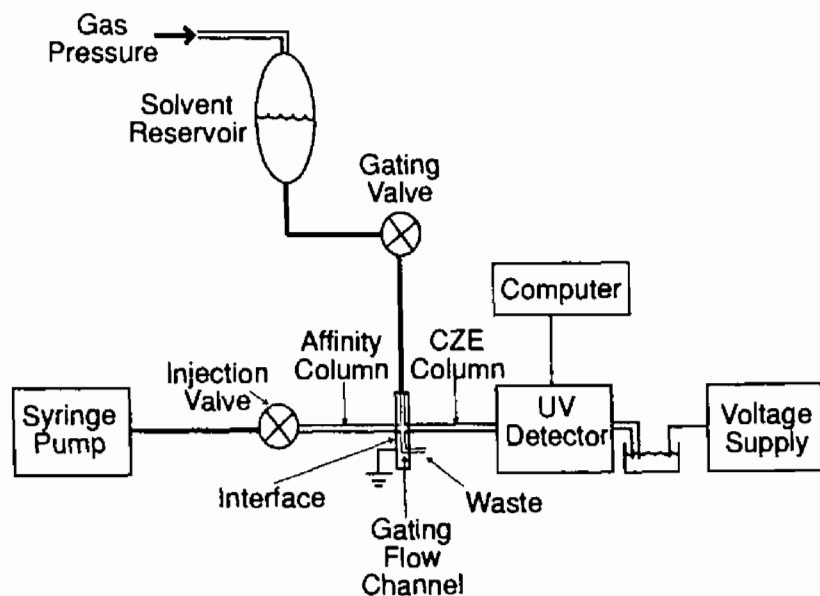


FIGURE 10 Schematic diagram of a system used to perform on-line IAC preconcentration of analyte onto the capillary electrophoresis column. (From Ref. 101.)

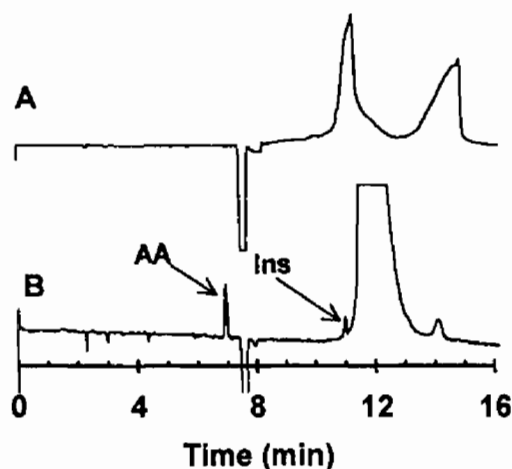


FIGURE 11 (A) Electropherogram of bovine serum sample spiked with insulin injected directly onto CZE. (B) Electropherogram of the same sample after preconcentration with the affinity column. AA indicates the ascorbic acid internal standard and Ins indicates the insulin zone. The large zone to the right of the insulin zone is the desorbed antibody. A 10-fold higher gain was used in the preconcentrated electropherogram. (From Ref. 101.)

METHODS FOR ENHANCING SENSITIVITY

In a high percentage of HPLC and CE separations, monitoring is performed with ultraviolet detectors. However, many immunoassays are aimed at the determination of analytes that are present at concentrations below the UV detection limit. Furthermore, as indicated before, desorption buffers can decrease the sensitivity of immunochromatographic assays. Approaches used to enhance sensitivity in conventional immunoassay formats do the same in immuno-HPLC and immuno-CE detection and for this reason are considered together under this subheading.

One difference between immunochromatographic systems and immuno-capillary electrophoretic systems that is inherent in the methods themselves and affects sensitivity is the concentration that takes place in the first group of methods. As an example, in the determination of anti-BSA using labeled BSA, the chromatographic method provided concentration sensitivity about 100-fold compared with the electrophoretic one; the mass sensitivity is similar for both methods [104].

Tandem IAC-RP (Fig. 3a) can be used to enhance detectability of some compounds by (1) concentrating the peak of antigen eluted from the IA column in a narrow band in the second column prior to its elution and (2) avoiding interferences from coeluting compounds if IAC or RP were used alone. For example, the RP step separates antibody and antigen peaks from the UV disturbance caused by the change in the refraction index during desorption from protein G columns. This enhances antigen quantitation in the characterization of antisera [39]. The IAC step also allows the capture of a component that is

present in high concentration and would obscure detection of minor components in the subsequent RP separation [105]. Miniaturized versions (250 μm ID columns) of IAC-RP coupling have also been used for the dual goals of stripping and concentrating antigen(s) prior to RP [106]. Examples of coupled IAC-RP systems are shown in Figs. 12 and 13. Figure 12 shows the possibility of analyzing minor components in human plasma after an anti-human albumin (hAlb) column has stripped the very abundant hAlb from the sample. Figure 13 corresponds to the RP analysis of human α_1 -antitrypsin (h α_1 AT) after capture and desorption from the IAC (anti-h α_1 AT) column.

The increase in sensitivity provided by the IAC step is clearly shown for IAC-CZE tandem systems. Different ways of building the immunoaffinity concentrators and of connecting them with the capillary used for electrophoretic separations have been published. The first systems were made by packing antibody-bound beads onto a capillary tubing. Beads were held in the capillary by two sintered frits. Captured antigens were eluted, collected, and introduced in the CE system [107]. Several modifications aimed to avoid the drawbacks of the off-line connection include the use of sleeves to connect the capillary micro-concentrator to the CE capillary [108] and the use of a single capillary the first part of which contains Abs bound to beads that are held between frits [109]. The design consisting of particles held between frits has some problems, such as laboriousness of construction, tendency of beads to collapse, and bubble production during analysis. Several alternative procedures have been proposed as microconcentrators, including a bundle of multiple capillaries with Abs bound to the wall [108], binding of Abs to the through-holes made in a glass rod [108], binding of Abs directly to the wall of a capillary tubing [102,110], and the use of magnetic particles to which Abs are bound [111]. Characteristics of these types of microconcentrators have been reviewed [94,112].

One example of the effect of microconcentrators on sensitivity can be seen in Fig. 11 [101]. In this case, bovine insulin was concentrated up to 1000-fold in a protein G-anti-insulin capillary column. A detection limit (DL) of 9 nM was achieved with off-line coupling compared with a DL of 2 μM for the direct CZE separation. Detection limits in the range 10^{-18} M can be obtained by laser-induced fluorescence detection in the analysis of multiple cytokines using a capillary tubing with different antibodies bound to its first part [102]. An additional increase in the enhancement in sensitivity provided by the micro-concentrator can be obtained by performing an isotachophoretic focusing step of the antigen eluted from the concentrator prior to the CE separation [111].

Detection of chemiluminescent, fluorescent, and electrochemically active compounds allows higher sensitivity than that achieved by UV detection. These detection methods are easy to achieve if the protein analyte has functionality amenable to one of these detection methods. Proteins, as in the case of albumin, have been quantitated in serum after an IAC-RP separation (scheme similar to

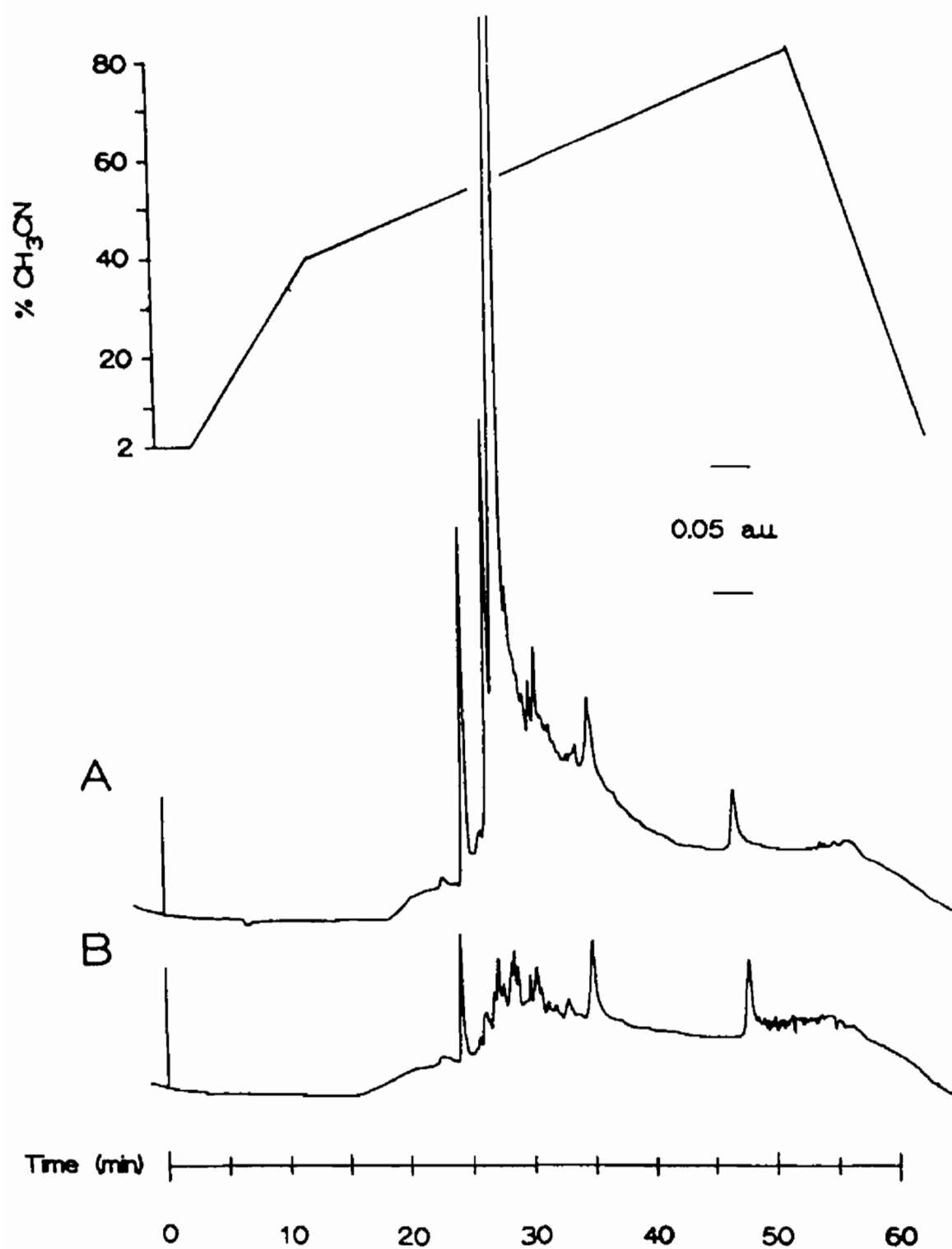


FIGURE 12 RP-HPLC profiles of (A) 600 nL of plasma and (B) 400 nL of plasma after elution through an anti-human albumin (anti-hAlb) IAC column. (From Ref. 106.)

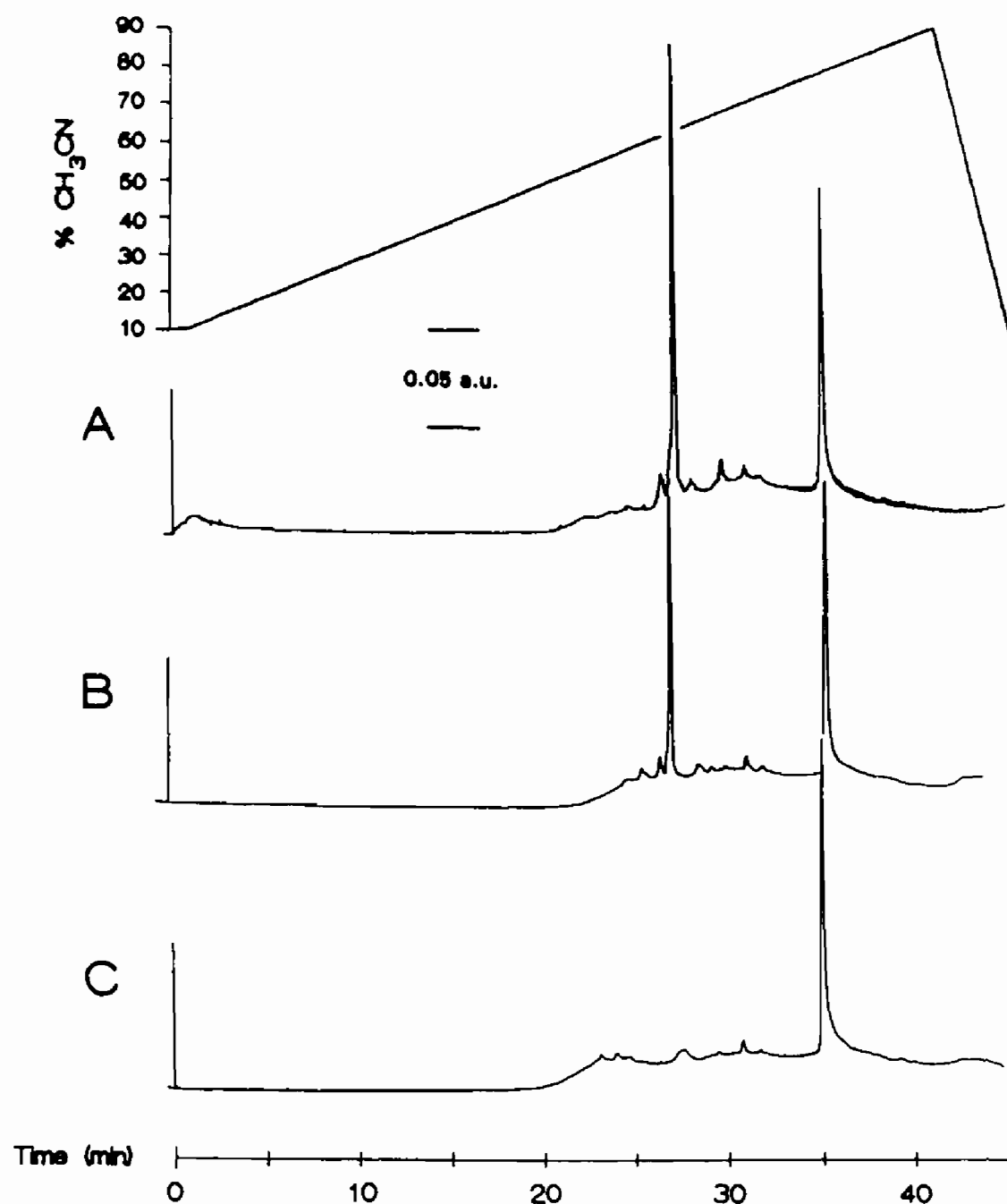


FIGURE 13 RP-HPLC analysis of eluent from anti-human α_1 -antitrypsin (anti-h α_1 AT) after rinsing the IAC column with 0.1% TFA. (A) plasma, (B) h α_1 AT standard solution, and (C) blank. (From Ref. 106.)

Fig. 3a) by measuring their native fluorescence [105]. In CE systems, the native fluorescence of green fluorescence protein (GFP) was used to study the effect of several parameters on sensitivity and resolution of the complex GFP-anti-GFP from the corresponding Ab and Ag [93]. The analysis of enzymes that produce products with functionalities amenable to high-sensitivity detection is another

case. After capture of the enzyme by the antienzyme column, substrate was introduced into this IAC column and the flow was stopped to allow product accumulation; after this stop-flow incubation, the product was then convectively transported to the column outlet and quantitated [69]. The scheme for this assay is similar to the on/off example in Fig. 2a, but instead of antigen elution, the product of the enzymatic reaction was eluted.

In those cases in which the analyte itself is not able to provide sensitivity enhancement through chemiluminescence, fluorescence, electrochemical activity, or the production of a detectable product, labeling is another avenue to enhance detection. This can be done in the case of immunological assays by labeling a competing antigen (to be used in competitive assays) or an antibody (usually employed in noncompetitive assays) with a thermo-optically active, chemiluminescent, fluorescent, or enzymatic probe. When this approach is used, it should be taken into consideration that the biological activity, and hence the sensitivity in the immunoassays, of labeled antigens or antibodies can be affected by the position at which the label is bound, as shown for FITC-labeled insulin [113]. On the contrary, colloidal gold does not interfere with the immunological activity of the conjugated antibody. Photothermal spectrometry of anti-IgA labeled with colloidal gold was used to detect IgA in an IAC microchip system [53]. Beads on which the sample had been adsorbed were introduced into the microchannel followed by gold-labeled anti-IgA. Thus, 7 nM IgA could be detected by measuring the retained Ab-gold by laser-induced thermal lens microscopy.

Immunoaffinity chromatography performed in sandwich format and chemiluminescent detection provided by labeled antibodies have been shown to provide a sensitive method to quantitate parathyroid hormone (PTH) in plasma [56]. Acridinium ester-labeled antibodies and plasma were injected into a column containing antiparathyroid antibodies, after which antigen and the associated labeled antibody were eluted from the IAC column and reacted with a postcolumn reagent. The resulting light production was measured with a chemiluminescence detector. Careful optimization of several parameters including choice of antibodies with preselected elution conditions, additives to decrease nonspecific interactions, simultaneous injection, and rate of light production allowed a PTH detection limit in plasma of 16 amol (2.4×10^{-13} M).

Fluorescent probes (tags) are another option to increase sensitivity in immunodetection systems that have been used in one-, two-, and three-column chromatography strategies. Texas Red (TR)-labeled human growth hormone (hGH) has been used to quantitate anti-human growth hormone (anti-hGH) antibodies in serum [114]. Immunocomplex was captured on a protein G column and then released into a downstream fluorescence detector. TR was the probe of choice because the fluorescence was not quenched at the low pH required for desorption from the protein G column. Labeling hGH with TR enhanced detection of anti-hGH antibodies by at least 10^3 -fold [115] with a minimum

detectable concentration (MDC) of antibodies of 250 ng/mL (66 pmol, 1.7×10^{-9} M) [114]. Sensitivity can be further increased (down to an MDC of 50 ng/mL) with tandem IAC-RP by separating the serum background fluorescence from the fluorescence of TR-hGH using a reversed-phase column at the outlet of the protein G column [115].

The use of antibodies labeled with fluorescein isothiocyanate (FITC) in immunochromatographic assays in sandwich format increases the assay sensitivity compared with the on/off detection method [55,116]. The effect of Ab-Ag binding constants on sensitivity was shown by the higher peak size obtained for insulin B chain than for insulin in their sandwich immunochromatographic analysis using FITC-labeled antipeptides antibodies with a higher affinity constant for the first protein [55]. No differences in sensitivity were observed between sequential and simultaneous procedures.

FITC has been used as a fluorescence probe in immunodetection assays shown in Fig. 5. This approach includes two immunorecognition steps as mentioned before. To separate and determine recombinant human methionyl granulocyte colony-stimulating factor (GCSF) and GCSF modified with polyethylene glycol (PEG-GCSF), the setup shown in Fig. 6 was used. The antigenic analytes captured at the anti-GCSF column and separated by RP or IEX microcolumns were allowed to react with fluorescently labeled antibodies to form fluorescent immunocomplexes in concentrations proportional to analyte concentration. Subsequently, the unreacted, labeled antibodies were removed prior to fluorescence detection by capturing them in a GCSF column (Fig. 5a). Figure 14 shows the chromatogram for GCSF and PEG-GCSF standards and the corresponding serum sample from rats in which PEG-GCSF had been injected 4 hr before. A detection limit of 12.5 ng/mL (6.7×10^{-10} M) for GCSF was achieved by this method [70]. FITC has also been used as a tag in a three-column system to detect an analogue of bovine growth hormone releasing factor (GHRF) with a detection limit of 1 ng/5 mL (5.7×10^{-11} M). Trypsin digestion products of GHRF were employed to approximate metabolic products. The assay included capture of antigens at an anti-GHRF column, desorption onto an RP column, and from this one onto another anti-GHRF column. After antigen(s) was captured from the sample, FITC-Ab was introduced onto the column. Retained FITC-Ab was released from the column and its fluorescence measured (Fig. 5b) [71].

The fluorescence of FITC-Ag after being captured and desorbed from the column has also been used to quantitate bovine serum albumin (BSA) in a simple competitive assay performed in capillary chromatography [51]. A detection limit of 1.2 nM was achieved.

The utility of FITC-labeled insulin has been proved in noncompetitive and competitive CZE assays with laser-induced fluorescence (LIF) detection (scheme shown in Fig. 8). The DL for Fab anti-insulin in the noncompetitive format was 2 nM (280 zmol) [86]. A similar DL (3 nM) was achieved in competitive assays

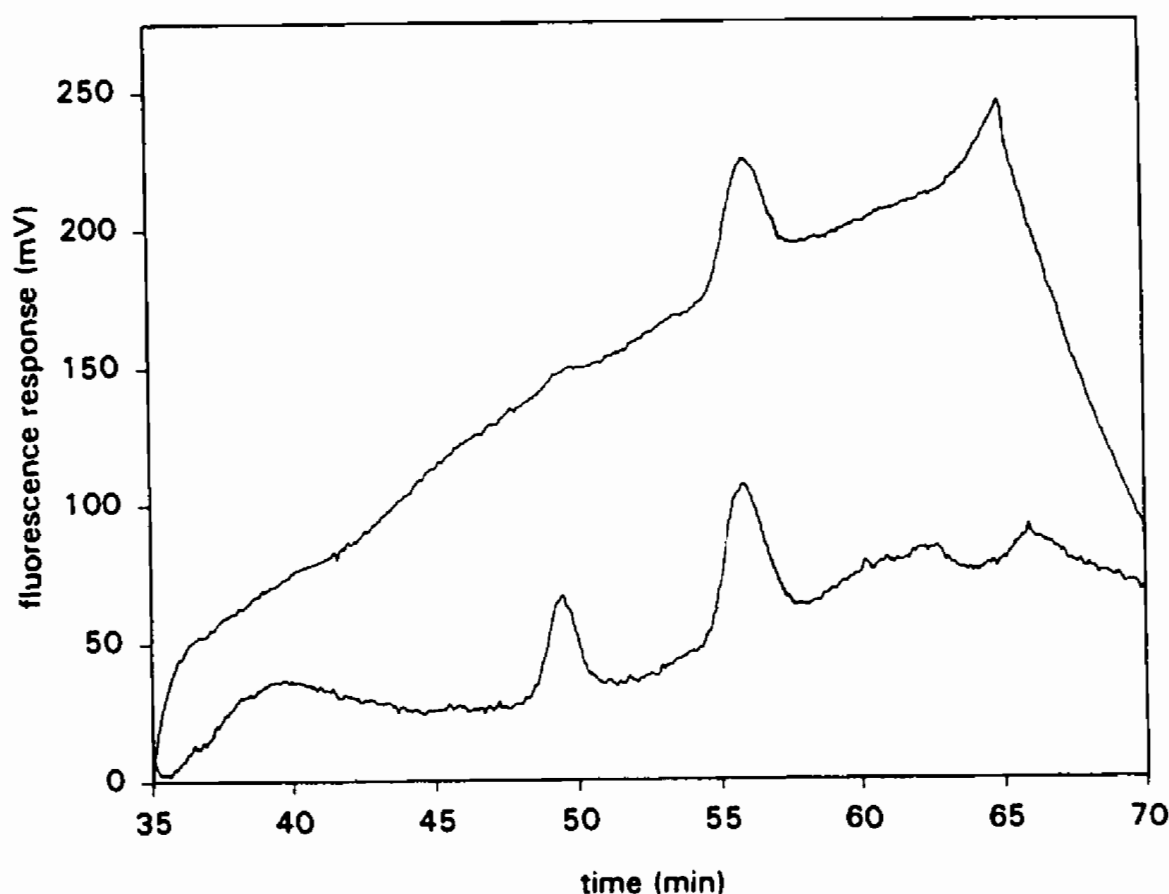


FIGURE 14 Upper chromatogram: serum of rats that had been injected with PEG-GCSF (granulocyte colony-stimulating factor modified with polyethylene glycol) 4 hr before; lower chromatogram: standard containing GCSF and PEG-GCSF. (From Ref. 70.)

for bovine insulin in pharmaceutical preparations [86] and islets of Langerhans [87]. The use of an antibody with a high binding constant led to a DL of 0.3 nM for insulin in competitive assays performed completely online to monitor analyte in a flowing stream [88]. A similar assay for glucagon allowed its quantitation with a DL of 0.76 nM [90]. The combination of both methods allowed simultaneous determination of standards of glucagon and insulin with DL values of 4.3 nM and 5.5 nM, respectively. Simultaneous determination was not possible in islets of Langerhans due to the overlapping by the large insulin Ab-labeled peak as islets contain much more insulin than glucagon.

IgA can be determined with a DL of 6.6×10^{-10} M by CZE with LIF detection employing succinyl-FITC-F(ab')₂ anti-IgA [74]. The charge modification achieved by reaction of FITC-F(ab')₂ with succinic anhydride allowed quantitation of IgA whose immunocomplex would otherwise coelute with the unbound labeled reagent (Fig. 7).

The use of FITC-labeled antirabbit IgG made it easy to identify prion protein (PrP) in samples of brain from sheep with some transmissible spongiform

encephalopathies. PrP, which is not present in normal sheep brain, is a good marker for these diseases. However, a capillary electropherogram with UV detection of the complex PrP–anti-PrP was not clearly distinguished from that of brain samples taken from normal sheep. Recognition of anti-PrP raised in rabbits by an antirabbit IgG labeled with FITC allowed LIF detection of PrP through the complex FITC–antirabbit IgG–anti-PrP (raised in rabbit)–PrP, which was separated by CZE from the excess of reagents [85].

An approach to a universal FITC-labeled reagent for immunoanalysis is to use FITC–protein G. This reagent can be used to tag antibodies and the complex formed can be employed to quantitate antigens. Although long incubation times are required to accommodate the slow reaction kinetics, a CZE method has been successfully used to quantitate human IgG1 in serum using anti-human IgG1 [81].

CE analysis of PrP in sheep brain affected by transmissible spongiform encephalopathies has been achieved by competition of PrP and a fluorescently tagged synthetic peptide of PrP for binding to an antibody directed against the peptide (Fig. 8). Fluorescein iodoacetamide was preferred to fluorescein isothiocyanate for labeling the peptide due to the lysine residues at different positions [82]. Labeling of the peptide during its synthesis provided 100-fold enhancement in sensitivity compared with labeling by traditional reactions [84].

Using near-infrared fluorescence detection in biological fluids is sometimes preferred to visible fluorescence detection. The lower price of lasers used in that spectral region and the small number of compounds exhibiting fluorescence in it have prompted the use of Cy5 and similar dyes. A detection limit of 0.2 nM for anti-BSA was obtained by measuring Cy5-BSA in a capillary IAC sandwich system [57].

Labeling of standards and samples with the fluorochrome Cy5 and the use of a mirror applied to the back of the capillary LIF detector cell made it possible to determine recombinant cytokines in biological fluids at the pg/ μ L level. Antigens separated by CE had been previously captured from the sample by MAbs bound to the first part of the column [103].

Comparison of Cy5.5 and FLUOS [5(6)-carboxyfluorescein-*N*-hydroxy-succinimide ester] showed slightly better sensitivity for interleukin-8 (IL8) using anti-IL8–FLUOS (DL 0.1 nM) than using anti-IL8–Cy5.5 (DL 0.5 nM). This fact could be related to the higher affinity for IL8 in solution shown for anti-IL8–FLUOS than for anti-IL8–Cy5.5 [54]. Off-line incubation of IL10 with anti-IL10–FLUOS led to better sensitivity for IL10 (DL 0.4 nM) than on-line incubation (DL 2.5 nM) in a noncompetitive IAC system similar to that used for IL8 [35]. In both IL8 and IL10 assays, IL was reacted with labeled Ab. The excess of labeled Ab was captured by an IAC antigen column and fluorescence of nonretained Ag-labeled Ab was measured.

By using two fluorophores that can be resolved spectroscopically, simultaneous analysis of two antigens has been performed. Albumin labeled with

Cascade blue and transferrin labeled with FITC have been used as analogues to the corresponding proteins in a competitive chromatographic assay [52]. Tiophilic adsorption gels (T-gels) were used to avoid the detrimental effect that acidic solutions used to elute protein A/G columns could have on most fluorescence labels. T-gels bind antibodies at high ionic strength and near-neutral pH values, and reduction of ionic strength without a pH change elutes them. A mixture of samples, labeled antigens, and antibodies was injected onto the T-gel column. Synchronous scanning fluorimetry of the labeled Ag-Ab complexes eluted from the column showed the feasibility of performing dual fluorescence immunochromatographic detection.

A different approach to avoid the effect of pH was followed in CIEF using a tetramethylrhodamine fluorescent tag. This tag was chosen because it is pH insensitive in the whole range of pH considered for separation [73]. Labeling the Fab' fragment of monoclonal anti-human growth hormone allowed LIF determination of methionyl recombinant human growth hormone with DL 5×10^{-12} M (0.1 ng/mL).

Labeling with enzymes has been the most widely employed approach to increase sensitivity in chromatographic immunodetection, probably because of broad experience with the very well known enzyme-linked immunoassays. Enzyme tags have been used to form electroactive, fluorescent, and colored products. Assays developed by de Alwis and Wilson are landmarks in the field of immunodetection. Electrochemical detection of hydrogen peroxide formed by the reaction of a glucose oxidase tag in both sandwich [58] and competitive [59] formats allowed detection of subpicomoles of bovine and human IgG in serum.

Chemiluminescence detection of an immunocapillary electrophoretic assay performed on chip has been carried out by determining mouse IgG with horseradish peroxidase (HRP)-labeled antimouse IgG [117]. The device integrates an injector, a separator, and a postseparation reactor. Optimized factors included reagent concentration, pH, size of the sample plug, design of the injector, and depth of the channels. The assay was carried out by mixing off chip the antigen with an excess of the enzyme-labeled antibody. Quantitation was performed by measuring the labeled antibody not bound to the antigen after the reaction.

The use of antibodies labeled with alkaline phosphatase and a substrate that yields a fluorescent product provided a marked increase in sensitivity over the 1 μ g absorbance detection limit in immunochromatographic UV detection of an analogue of bovine growth hormone releasing factor (GHRF). After reduction of background signal and stop-flow incubation on column, the detection limit for a sandwich ELISA, in which an Ab column was used, was reduced to 10 ng (1.4×10^{-7} M). A further enhancement in sensitivity, with a detection limit of 0.6 ng (8.6×10^{-9} M), was achieved in a different noncompetitive format after optimization of pH, substrate concentration, and incubation time [118]. In this

case, sample and labeled Ab were incubated prior to injection into an Ag column, where an excess of labeled Ab was retained. In the sandwich format, simultaneous injection of Ag and enzyme-labeled Ab onto the Ab column provided better results than sequential injection.

A similar strategy of premixing antibody and enzyme-labeled antigen prior to the injection into the immunochromatographic column has been used in a competitive assay performed to quantitate insulin by photometric detection using a horseradish peroxidase (HRP)-labeled insulin. Antiantibody immobilized on the chromatographic support was used to capture the Ab-Ag-label complex. In this way 1.7×10^{-7} M insulin can be detected [119].

Increased sensitivity and reduced detection time have been achieved by conducting immunodetection assays in a microvessel (HPLC capillary columns of 100 μ m ID) [29]. Comparison of simultaneous and sequential injection of antigen and antibody indicated that for sandwich ELISA in HPLC capillary columns, performing the initial immunorecognition of Ab and Ag in the liquid phase provided superior results. Choosing the right buffer additives to reduce background, operating at the pH optimum of the enzyme, adding the appropriate excess of labeled Ab to avoid a high background, using stop-flow incubation, and simultaneous injection of Ag and labeled Ab provided high sensitivity. With a 500-nL sample volume the lower detection limit of IgG was 300 zmol (6×10^{-13} M) using absorbance at 405 nm to monitor the product formed by alkaline phosphatase (ALP) (Fig. 2d).

Simultaneous injection of premixed antigen and enzyme-labeled antibody into the column has also been employed in studies where HPLC microcolumns (1 mm ID) were packed with perfusable packing materials. This approach provided short diffusion distances and a high surface area/volume ratio in the immobilized antibody bed. Using a horseradish peroxidase label and absorbance detection at 450 nm, the detection limits for thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG) in the sandwich format were 4.3×10^{-13} and 5×10^{-12} M, respectively [38].

Light scattering from particle aggregates is another method of monitoring immunocomplex formation [100], as noted previously. A detection limit of 1 zmol (620 molecules) of glucose-6-phosphate dehydrogenase (G6PDH) was achieved by measuring the agglutination of anti-G6PDH-coated latex particles in the presence of G6PDH in a CE column.

RELATED DETECTION SYSTEMS

Several immunochromatographic and immunoelectrophoretic methods that have been used for small (hapten) molecules, to the authors' knowledge, have not been

used for proteins. One such approach consists of using electrochromatography instead of chromatography to preconcentrate the target analyte [120]. This immunoaffinity capillary electrochromatography–capillary electrophoresis coupling was carried out using a capillary column with its first millimeters packed with the immunoaffinity support. The absence of a device to obtain pressure-driven flow may facilitate design and automation of separations.

Hollow fibers have proved to be useful to separate labeled hapten from the complex labeled hapten-Ab after a competitive on-line HPLC-immunochromatographic detection method [121]. First, hapten eluting from the HPLC column was made to react with Ab. Then, labeled hapten was added to react with the excess of Ab. Separation of free labeled hapten from labeled hapten-Ab was performed using a hollow-fiber module. The cross-flow membrane of this module allowed separation based on size, as opposed to a restricted-access media (RAM) column in which size and hydrophobicity account for separation [122]. No restriction on polarity and size of the label and no need for regeneration of the separation device are characteristic of the use of hollow fibers. Although the method has been used for analysis of haptens, it could be used to analyze proteins as it has been shown that proteins of 40 kDa can be separated from the corresponding protein-Ab complex if the appropriate hollow fiber is chosen.

Competitive flow immunoassay using RAM columns has been used to separate immunocomplexes from free haptens [123]. The system used was valid for antigens of molecular mass lower than 3000 Da.

Laser-induced fluorescence polarization (LIFP) has been used as a detection technique in immunocapillary electrophoresis analysis of haptens. Polarization of fluorescence depends on the molecular size of the molecule. Small molecules exhibit low fluorescence polarization. LIFP was used to distinguish the peak of the fluorescent hapten-Ab complex from the peak of the excess of free fluorescent hapten in competitive assays [124]. Besides, the dependence of LIFP on molecular size has made it possible to perform quantitation without separating free from bound labeled hapten [125]. An attempt to use LIFP to quantitate staphylococcal enterotoxin A (28 kDa) did not show an increase of polarization upon formation of the Ag-Ab complex due to the high molecular weight of the Ag [126]. Formation of the complex could be identified by measuring the LIFP peak while varying the Ag/Ab ratio.

Antibody-antigen interactions are actually interactions between paratopes and epitopes. Besides the use of immunodetection systems shown in this chapter for analysis of proteins as a whole, immunocapillary electrophoresis has proved to be useful in the study of epitopes on proteins recognized by Abs of clinical interest [127].

Some other methods related to immunodetection in chromatographic and capillary electrophoretic systems have been carried out for analyzing proteins.

A combination of capillary electrophoresis and off-line immunological recognition carried out in a multicapillary instrument combined with a robotic station for sample handling has been used for automated serum protein immunotyping. The procedure is based on the immunosubtraction principle. After obtaining the CE profile, samples were added to wells containing beads coupled to Abs against different immunoglobulins. Immunoglobulins captured by specific Abs led to the loss of specific peaks in the subsequent electropherogram, allowing identification of abnormal peaks corresponding to different gammopathies [128].

The borderline between immunoassays performed in the flow injection analysis format and those achieved in the immunochromatographic format is sometimes difficult to establish. Flow injection renewable surface immunoassay (FIRSI) avoids the desorption step of immunochromatographic assays by using a layer of antibody-coated beads held in a jet ring cell instead of the chromatographic column. The coated beads are discarded after each analysis. To ensure assay reproducibility, antibody-coated beads with constant antigen capacity should be obtained [129,130].

A flow immunoelectrode based on highly dispersed carbon to which Abs are bound has been used for IgG and IgM determination. In this approach, immunoreaction and electrochemical determination took place at the same immunocolumn [131].

Optical chromatography, a separation technique involving the use of a radiation force and a medium flow, has been used to carry out immunoassays. The radiation force induced on a particle when irradiated by a laser beam and the force induced by the medium flow are related to the diameter of the particle. Optical chromatography allows separation of free Ab-particles without Ag from the Ab particles bound to Ag taking into account that particles coated with Ab agglutinate in the presence of Ag. The concentration of Ag can be calculated through the ratio of bound to free particles [132].

Accumulation of Ag on protein A-coated crystals to which Ab has been bound has been used to quantitate glutathione-S-transferase (GST) [133]. The piezoelectric (PZ) biosensor detects a decrease in the resonant frequency of the PZ oscillating crystal when antigens are adsorbed on it.

Immunoaffinity separations performed on a matrix-assisted laser desorption ionization-mass spectrometer (MALDI-MS) probe tip have given rise to the term PAMS (probe affinity MS) [134]. Antibiotin covalently bound to the surface of the probe allowed MS discrimination of bovine insulin with different degrees of biotinylation. The method has also been used to detect lysozyme in human tears by binding antilysozyme to the MS probe. Based on the same PAMS principle, modifications aimed to obtain faster and improved retention of Abs have been carried out by immobilizing the Abs on the probe via a nitrocellulose film instead of binding them directly to the MALDI probe surface [135]. The procedure allowed determination of a therapeutic peptide and one metabolite.

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Characterization of Proteins, Peptides, and Polynucleotides by Mass Spectrometry

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INTRODUCTION

The combination of efficient separation methods such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) with detection methods that provide structural or functional information is a powerful tool for the analysis of biological macromolecules. In particular, characterization of complex molecules such as proteins, peptides, and oligonucleotides necessitates the use of complementary analytical methods to assign molecular structures to the peaks occurring during chromatography or electrophoresis. Although a wide range of analytical methods can be “hyphenated” with HPLC, there are only a few that function on line with the separation system and that are sufficiently rapid to provide on-line information. Among those, mass spectrometry is certainly one of the most powerful detection systems.

The major hurdle standing in the way of directly coupling HPLC with mass spectrometry was that, until recently, ion sources needed a high vacuum. This was difficult to reconcile with the introduction of large volumes of solution. The discovery of electrospray ionization (ESI) provided a means to ionize fragile

nonvolatile biomolecules at atmospheric pressure. ESI is the basis for the successful coupling of HPLC with mass spectrometry, commonly referred to as LC-MS [1]. Mass spectrometry stands out as a technique that provides a wealth of information on a very limited amount of sample in a time span that is compatible with on-line detection. The potential for automation is another asset of this approach to combining separation and detection techniques. LC-MS is now widely used in pharmacokinetic studies and drug screening applications in addition to having expanding significance as a general analytical tool. This chapter will give examples of the use of LC-MS for protein, peptide, and oligonucleotide analysis, showing the potential of this technology to aid in their characterization.

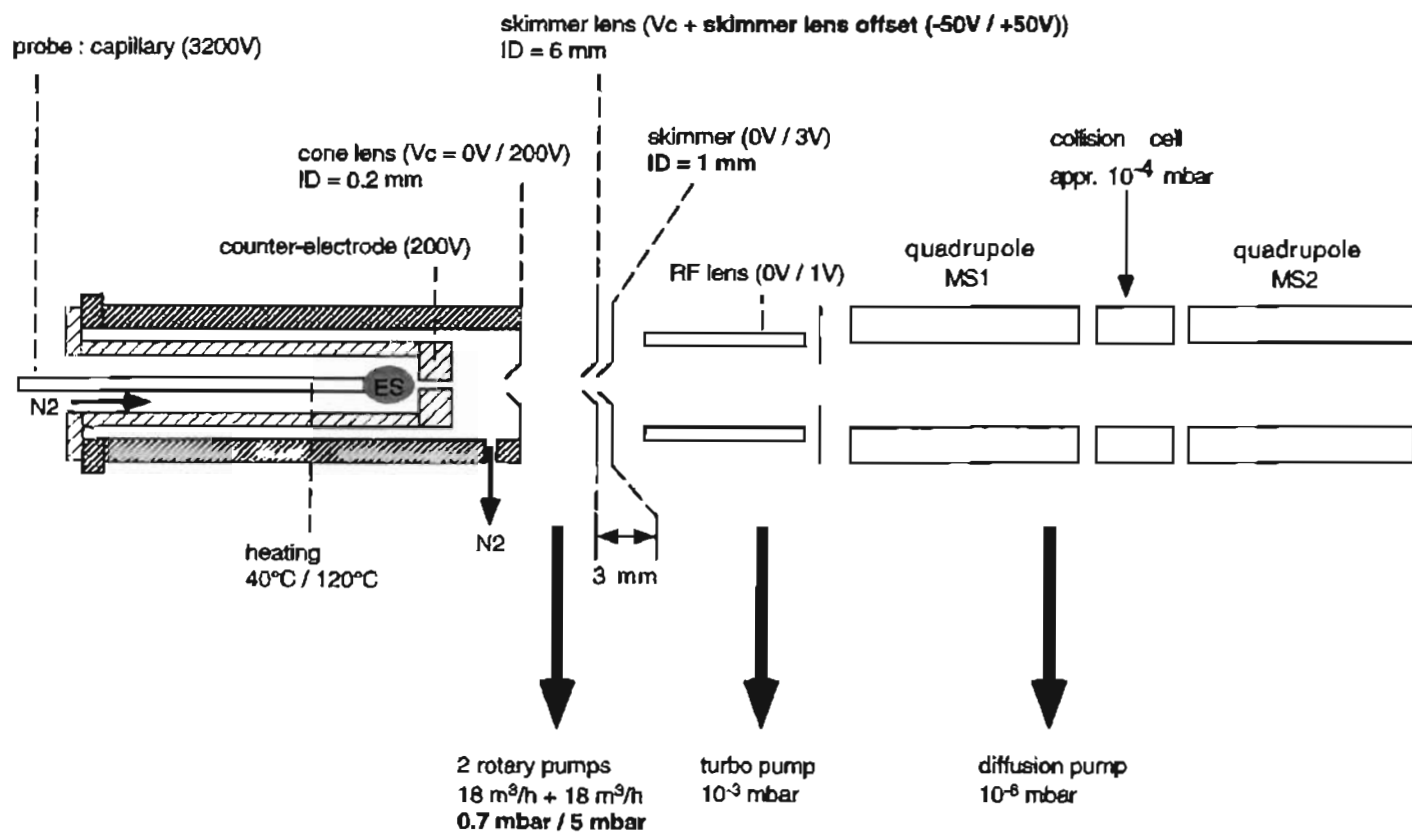
Although LC-MS is widely used in the development of novel pharmaceuticals, such as recombinant proteins, fraction collection followed by subsequent "off-line" analyses may offer additional flexibility and is still a common approach in the research setting. Localization of proteins or peptides following their separation is often based on measurements that are difficult to perform on line; bioassays and immunological tests are two such cases. Modern robotic systems allow automation of more complex operations, but most analyses in laboratories dealing with limited numbers of research samples are performed manually. Off-line analyses of an aliquot by mass spectrometry allow the major part of the sample to be available for complementary studies. Furthermore, a wider range of ionization methods can be used that may either be more adapted to the sample or more sensitive. Mass spectrometric techniques that rely on surface ionization, such as matrix-assisted laser desorption/ionization (MALDI), are not easily coupled to HPLC or CE, thus necessitating fraction collection and off-line sample preparation. More recent developments in the areas of nanotechnology may, however, make it possible to apply the eluent from a capillary HPLC system directly to a matrix-coated sample plate [2]. Performing MS/MS studies on line is also not an easy task because of the limited time that is available to select the parent ion, submit it to collision-induced dissociation (CID), and analyze the generated fragment ions (see "Fragmentation Studies and Structural Information" for more details on MS/MS). However, modern instruments have been improved in this respect, allowing MS/MS to be performed more rapidly using specially designed software for optimization.

An important feature of mass spectrometry is its inherent sensitivity, which is unequalled by most other techniques. Mass spectrometry has therefore opened the possibility of rapidly identifying proteins and peptides at the subpicomole level and is widely applied to a new field of research that was recently called "proteomics" [3–5]. Proteomics research has the goal of bridging the gap between the immense amount of DNA sequence information and the lack of data on the nature and function of the corresponding gene products. The most widely applied separation technique in this field is undoubtedly two-dimensional

gel electrophoresis (2D electrophoresis), which allows separation of a wide range of proteins with high resolution, a capacity that is presently not equaled by chromatography. Combining slab gel electrophoresis with mass spectrometry is necessarily an off-line technique although certain steps such as washing of the gel slice may be automated with robotic systems. MALDI has been most successfully combined with gel electrophoresis because of its high sensitivity and its relative insensitivity to buffer ions and other gel-derived contaminants. A discussion of the possibilities for identifying proteins separated by gel electrophoresis based on the mass of a number of proteolytic fragments followed by database searches will be given later in this chapter.

The application of mass spectrometry to the field of biological macromolecules started with the development of “soft” ionization techniques such as fast atom bombardment (FAB) or plasma desorption (PD) 20 to 25 years ago [6,7]. Major breakthroughs in this area were the development of ESI and MALDI, which successfully generate molecular ions of biomolecules beyond 100 kDa molecular mass [1,8]. These ionization techniques differ significantly from each other in that ESI is performed on molecules in solution whereas MALDI requires the analytes to be cocrystallized with a light-absorbing matrix. Furthermore, ESI is most commonly combined with quadrupole mass analyzers and MALDI lends itself more to time-of-flight (TOF) mass analyzers, although systems that combine ESI with a TOF analyzer have been developed and are now commercially available. In particular, the introduction of quadrupole–TOF hybrid mass analyzers has had a major impact on protein analysis, especially when it comes to high-sensitivity tandem MS [9–12]. ESI is more amenable to direct coupling with separation techniques such as HPLC or CE because it allows introduction of solutions into the ion source. On the other hand, it is more sensitive to the presence of ions and surfactants in the sample than MALDI, making ESI an ideal choice for coupling to reversed-phase HPLC (RPC). Numerous examples of the use of such LC-MS systems can be found in the literature, in particular for peptide mapping and the identification of posttranslational modifications in proteins [13–16]. More recently, oligonucleotide analysis has also been described using LC-MS [17]. Two developments have further improved the capacities of ESI and MALDI: the introduction of a miniaturized ESI inlet (nanoelectrospray) that allows analyses to be performed at nL/min flow rates instead of the customary $\mu\text{L}/\text{min}$ [18] and the addition of delayed extraction (DE) to the MALDI source to reduce the energy dispersion of the initially generated ion cloud and thus improve resolution and mass accuracy [19].

This chapter will (1) outline the basic principles of ESI and MALDI, (2) give examples of applications in protein, peptide, and oligonucleotide analysis with particular emphasis on LC-MS coupling; and (3) describe the analysis of proteins following gel electrophoresis. These examples will illustrate the possibilities and limitations of the methodology and allow the reader to evaluate the



applicability to his or her particular problem. Some consideration will be given to sample preparation, which is critical when using highly sensitive detection methods. Although this chapter focuses on mass spectrometry as an analytical tool to characterize biological macromolecules, it should not be forgotten that a number of alternative and complementary methods are generally necessary to characterize these complex molecules fully and that mass spectrometry needs to be integrated into an analytical strategy.

ELECTROSPRAY IONIZATION (ESI)

ESI has a number of features that make it particularly suitable for coupling with liquid separation systems such as HPLC or CE. First, it allows ion generation at atmospheric pressure from a solution containing the analyte(s). Second, it can generate molecular ions of high-molecular-weight nonvolatile molecules. Third, ESI produces multiply charged (generally protonated) molecular ions that allow utilization of mass analyzers working at mass-to-charge (m/z) ratios up to 2000, such as standard quadrupole analyzers. A limitation of ESI is its sensitivity to buffer ions and other contaminants such as surfactants that may be present in biological samples. RPC is therefore an ideal technique to separate components prior to ESI-MS as it eliminates such contaminants and generally yields mass spectra of high quality.

Application of a strong positive or negative electrostatic field gradient (~ 3 kV/cm) to a solution of charged molecules leaving a capillary results in the generation of a spray containing highly charged droplets (so-called electrospray). The average initial size of the droplets depends on the inner diameter of the capillary from which the spray is generated and reaches $1\text{ }\mu\text{m}$ for standard capillaries of $100\text{ }\mu\text{m}$ inner diameter. Droplet size diminishes rapidly due to solvent evaporation, which is generally accelerated by a flow of a preheated inert gas such as nitrogen through the ion source. A coaxial gas flow (generally nitrogen) assists further in solvent evaporation and the formation of the spray, as shown in Fig. 1. As ions within the droplets become more and more concentrated

FIGURE 1 Schematic drawing of an electrospray ionization (ESI) source and a triple-quadrupole mass analyzer. Ions are generated from the spray in the electrostatic field between the capillary and the counterelectrode. They are subsequently transmitted through a number of electrostatic lenses into the high-vacuum region of the first quadrupole mass analyzer (MS1). Optionally, ions selected by MS1 can be fragmented by collision-induced dissociation (CID) with gas (e.g., argon) in the collision cell and fragment ions analyzed in the second quadrupole mass analyzer (MS2). Representative residual pressures are indicated for the different regions of the instrument.

by solvent evaporation, charge repulsion leads to an explosion of the primary droplet to generate a multitude of smaller droplets finally leading to ion evaporation [20]. A few studies have shed some light on the conditions that prevail within the microenvironment of the evaporating droplet. It was found that the pH in a droplet may differ significantly from that in the bulk solution [21]. It is thus not certain that the physicochemical characteristics of the bulk solution are representative of the conditions during ionization.

Proteins and peptides are generally analyzed in the positive ionization mode (ESI^+), which selects positively charged ions; in contrast, oligonucleotides are analyzed in the negative mode (ESI^-). ESI^+ leads to a mixture of multiply charged molecular ions due to protonations (adducts with alkali metal ions such as sodium or potassium may also be observed). More recently, TOF analyzers have been used with ESI, leading to greatly enhanced sensitivity [9,22]. Studies of noncovalent interactions necessitate that ESI be performed under conditions that preserve the native structure of the protein, often requiring the use of mass analyzers with an extended m/z range as native structures are generally more compact and do not provide as many ionizable groups on the surface [23]. Upgraded quadrupole analyzers with a range up to 8000 and TOF analyzers with a range well above 100,000 are adequate for this important field that is finding use in studying protein-drug interactions.

Most research-grade ESI mass spectrometers are equipped with three quadrupole analyzers in series (so-called triple-quadrupole instruments). With this configuration, structural analyses can be performed by collision-induced dissociation (CID) within the second quadrupole, which serves as a collision cell. CID is of particular interest for peptides because it provides additional sequence information or may indicate the location of posttranslational modifications. An example of the application of MS/MS to sequencing peptides and to characterizing posttranslational modifications will be given later in this chapter. Fragmentation can also occur in the ion source itself due to collisions with residual gas molecules. In-source CID depends on the cone voltage (V_c), which determines the kinetic energy of the ions in the source before they enter the high-vacuum portion of the instrument (see Fig. 1). In-source fragmentation can be used to generate characteristic ions that can be monitored to detect specific posttranslational modifications such as glycosylation or phosphorylation [24]. Selective monitoring of such fragment ions can be performed during on-line LC-MS, allowing the detection of posttranslationally modified peptides [25]. In favorable cases, in-source CID may also generate some sequence information on peptides that may help in their identification [26]. However, sequencing of peptides by mass spectrometry is more often done by CID in the specifically designed collision cell of a triple-quadrupole analyzer. In this case, the first quadrupole serves to select the parent ion and to transmit it into the gas-filled collision cell, which is a quadrupole working in the radio-frequency (RF) mode that keeps the ions

focused. Fragments that are generated in the collision cell are subsequently analyzed in the third quadrupole. Fragmentation patterns of peptides have been extensively studied and can provide partial sequence information (see Fig. 8B and Ref. 27 for nomenclature of fragment ions). Software available with most mass spectrometers has features to help in the identification of the commonly observed fragment ions.

Calculation of the molecular mass of an analyte observed by ESI-MS is based on the presence of a mixture of multiply charged ions (Fig. 2) and on the assumption that adjacent signals differ by one charge which is carried by a proton or, less commonly, an alkali metal ion (according to Ref. 28). At least two consecutive charge states are needed to perform the calculation. The following equation describes the case of a multiply charged ion of mass m carrying z_1 or z_2 protons:

$$m/z_1 * z_1 = M_r + M_H * z_1 \quad (1)$$

where

z_1 = number of charges of the ion

M_r = average molecular weight of the analyte

M_H = molecular weight of H (1.0079)

An adjacent signal ($z_2 = z_1 - 1$) can be described by an analogous equation:

$$(m/z_1 - 1) * (z_1 - 1) = M_r + M_H * (z_1 - 1) \quad (2)$$

Equations (1) and (2) can be combined to calculate the number of charges z_1 and thus the average molecular weight M_r .

$$z_1 = (m/z_2 - 1.0079)/(m/z_2 - m/z_1) \quad (3)$$

$$M_r = m/z_1 * z_1 - 1.0079 * z_1 \quad (4)$$

Similar calculations can be performed for analytes that are charged due to alkali metal ions. The software of commercially available instruments performs such calculations automatically and some software packages contain more sophisticated algorithms that are based on the maximal entropy approach. Such advanced algorithms should, however, be used with caution because the results depend very much on user-defined parameters. Because calculation of the molecular weight of a protein is based on a large number of multiply charged ions, there is a redundancy of information that the software uses to calculate an average value, followed by a standard deviation. The standard deviation is a measure of the reliability of the calculated mass and reflects the quality of the spectrum. Standard deviations of 0.01% (100 ppm) of the measured mass are routinely obtainable with pure, salt-free protein samples.

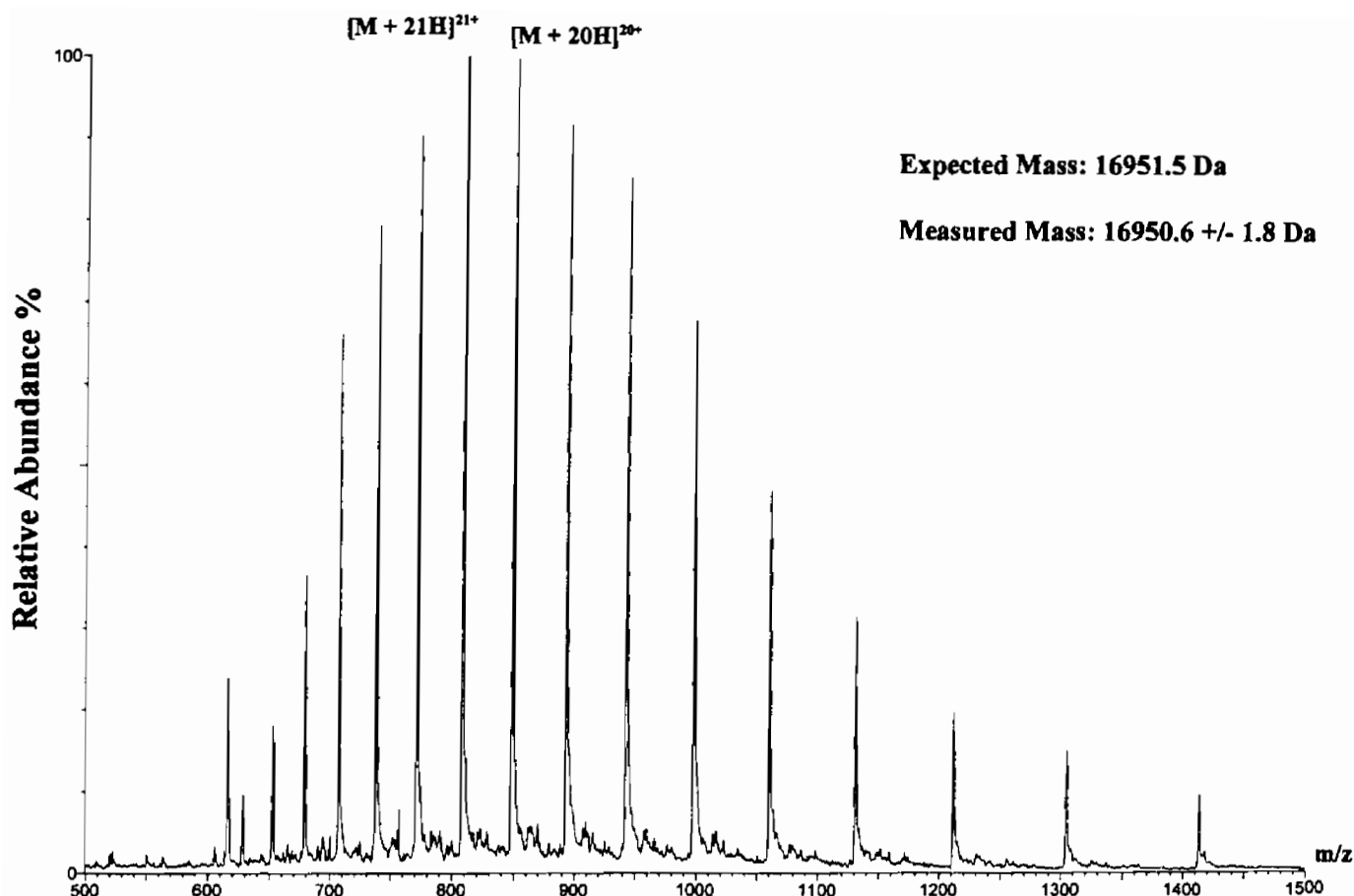


FIGURE 2 Mass spectrum of horse heart myoglobin obtained by ESI-MS. Sample introduction was performed via a syringe pump at 6 $\mu\text{L}/\text{min}$ at a concentration of 2 $\text{pmol}/\mu\text{L}$ ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$; 1 : 1). The two most abundant multiply charged molecular ions are shown and the measured mass is given as compared with the calculated molecular weight. The standard deviation of ~ 100 ppm (1.8 Da) results from the calculation of the average mass based on all of the multiply charged molecular ions (see text for details of the calculation).

Resolution is generally limited by the width of the isotopic envelope that can no longer be resolved into individual isotope peaks by the quadrupole analyzer for higher charge states as well as by possible adduct formation [29]. A resolution of 1000, which is easily achievable in quadrupoles, allows the measurement of the monoisotopic mass for smaller peptides ($m < 1000$ Da) based on their singly charged molecular ion. However, ESI of proteins with quadrupole instruments generally yields the average mass based on the envelope over all the isotopic species. Calibration in ESI-MS is usually done externally by introduction of a calibration standard, such as horse heart myoglobin or a mixture of polyethylene glycols of different molecular weight distributions, and repeated at regular intervals depending on the stability of the analyzer. It is evident that the calibration standard should cover the m/z range that is used to analyze the sample, but this is not always easy in practice, particularly if the spectrum covers a wide range of m/z values (e.g., the spectra from an LC-MS run).

COUPLING HPLC WITH ESI-MASS SPECTROMETRY (LC-MS)

Introduction of samples into an ESI source is performed through a capillary as shown in Fig. 1. Standard sources use flow rates of 1–10 $\mu\text{L}/\text{min}$ that are delivered either by an injection pump (flow-injection analysis) or by an HPLC system (LC-MS). Standard HPLC equipment can be adapted to produce such low flow rates by integrating a stream-splitting device either after a conventional column (postcolumn splitting) or prior to a capillary HPLC column (precolumn splitting) (Fig. 3). Dedicated miniaturized equipment using either stream-splitting or syringe pumps is also available for capillary HPLC and will probably be used more often in the future, especially when sample amounts are limited. Capillary electrophoresis (CE) has been adapted to ESI-MS, albeit with greater difficulty [30]. On-line coupling requires electrical insulation between the high-voltage power supplies of the CE instrument and the ion source, as well as a sheath liquid to augment the flowrate to produce a spray (31). A further difficulty is that CE uses buffer salts that may interfere with the spray and lead to adduct formation, thus reducing sensitivity and resolution. Compared with LC-MS, there are fewer applications of CE-MS at present. A number of applications of LC-MS for the analysis of biomolecules will be given in this chapter.

MATRIX-ASSISTED LASER DESORPTION/IONIZATION (MALDI)

MALDI is a surface ionization technique that is based on energy transfer from the absorbed laser light to the sample leading to simultaneous desorption and ionization. Energy transfer is mediated by a so-called matrix, which absorbs in the wavelength region of laser emission. Figure 4 illustrates several compounds

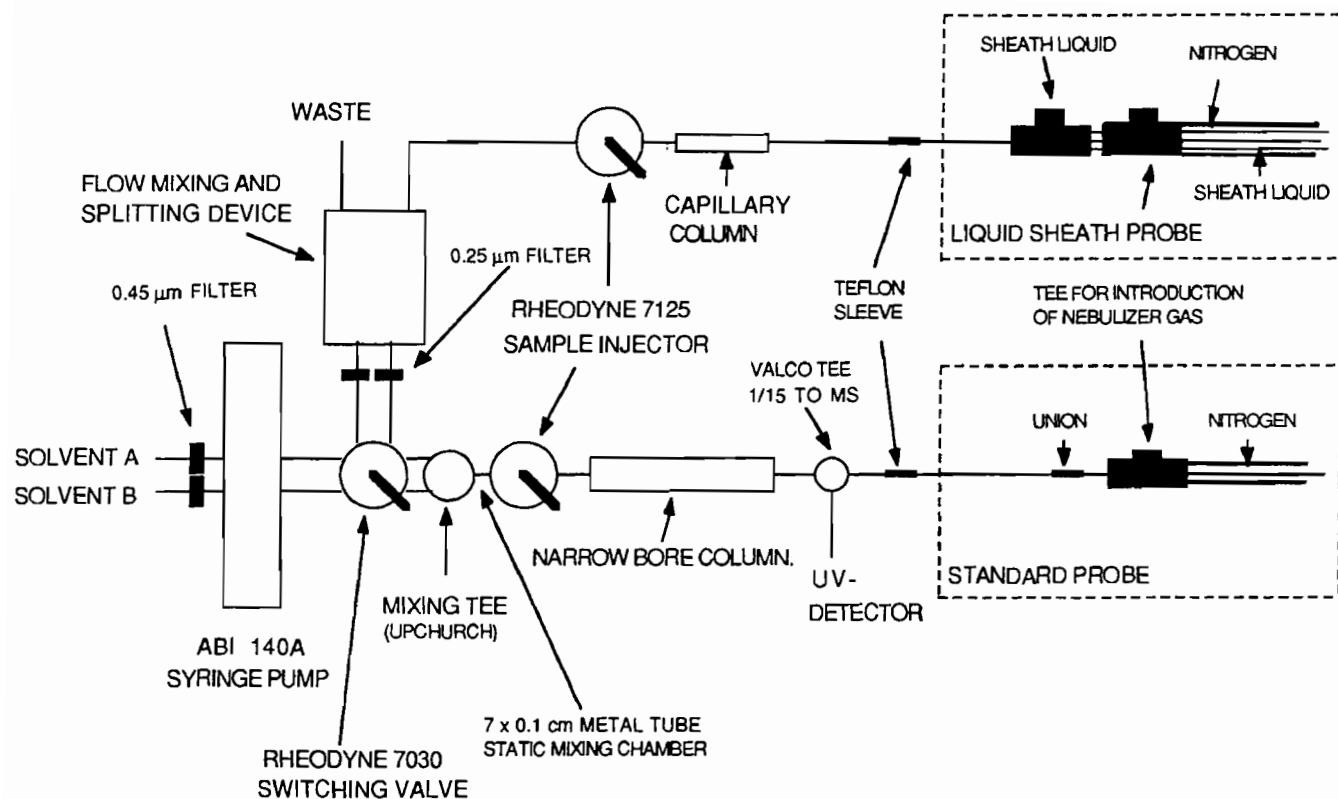


FIGURE 3 Schematic drawing of the interface between HPLC and ESI-MS (LC-MS). The system can be modulated to use either capillary (about 0.1–0.5 mm inner diameter) or narrow-bore columns (about 1.6 mm inner diameter) by performing precolumn or postcolumn stream splitting. This setup was used to obtain the peptide maps shown in Figs. 11 and 13. (From Ref. 16.)

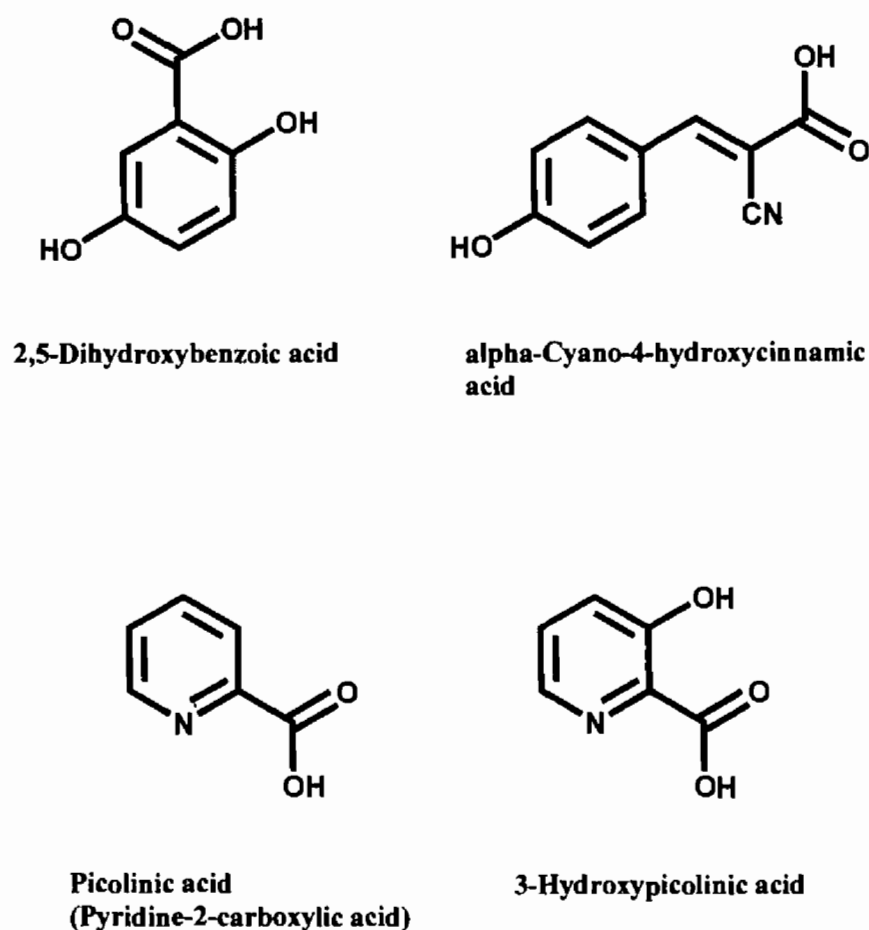


FIGURE 4 Representation of the structures of some commonly used matrices for ultraviolet matrix-assisted-laser desorption/ionization (MALDI). These matrices are used with nitrogen lasers emitting at 337 nm. α -Cyano-4-hydroxycinnamic acid is widely used for the analysis of peptides; the picolinic acid derivatives are more suitable for oligonucleotides.

used as the matrix. MALDI primarily generates singly charged, protonated molecular ions of peptides and proteins that are analyzed in the positive mode. Oligonucleotides, on the contrary, produce negatively charged ions more efficiently due to their polyanionic nature. The choice of the matrix and optimization of the crystallization procedure are critical parameters in MALDI and numerous protocols have been published [32,33]. Because the sample must be mixed with a large excess of matrix prior to analysis, MALDI is not easily adapted to on-line coupling with either HPLC or CE, although some attempts have been made [34,35]. Although most currently available MALDI mass spectrometers are equipped with a nitrogen laser emitting at 337 nm as a standard, it is possible to install a CO₂ laser emitting in the infrared region (10.6 μ m). The CO₂ laser has the potential advantage of transferring energy through the rotational and vibrational bands of water to the sample, thus eliminating the need for a separate

matrix. This may allow easier direct analyses of proteins separated by polyacrylamide gel electrophoresis or in ice crystals [36,37].

MALDI is most often combined with a TOF mass analyzer, which allows very sensitive detection of ions (Fig. 5). This renders MALDI-TOF considerably more sensitive than ESI, which is generally combined with scanning quadrupole mass analyzers. Instruments combining ESI with TOF analyzers have indeed shown a significant increase in sensitivity and speed of spectrum acquisition [9,22]. The combination of a continuous ion source such as ESI with TOF has been rendered feasible by delayed-extraction (DE) technology, which delays application of the accelerating voltage for a short time after the laser pulse. The compatibility of ESI with separation techniques such as HPLC and the sensitivity of the TOF analyzer make these instruments particularly suitable for LC-MS, and it is to be expected that numerous applications will be developed in the future. In view of their price and increased flexibility, it is to be expected that TOF analyzers will become more widely used with different ion sources including the combinations of MALDI with a hybrid mass analyser or an ion trap [10–12].

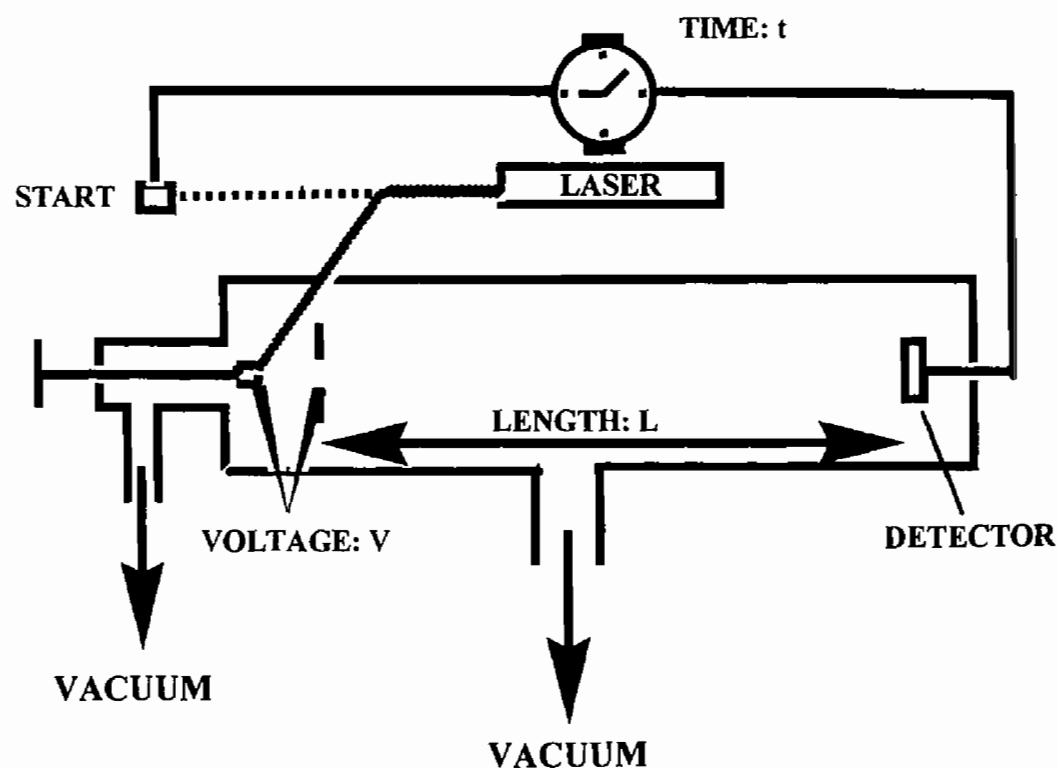


FIGURE 5 Schematic drawing of a MALDI ion source connected to a time-of-flight (TOF) mass analyzer. Ions are generated by the impact of the laser on the target followed by acceleration in an electrostatic field (V). The flight time (t) of all of the ions to cover length (L) is measured and is proportional to $(m/z)^{1/2}$ (see text).

Because a TOF analyzer measures the flight time of an ion, which is subsequently converted into an m/z scale via an external or internal calibration standard of known mass, any factor that affects the precision of time measurement will affect resolution (peak width) and mass accuracy. The basic relationship between time (t) and the mass of an analyte (m) can be deduced from two fundamental equations that describe the kinetic energy of an ion of z elemental charges accelerated in an electric field of V volts: $zeV = (1/2)mv^2$, with e being the elemental charge and v the velocity. The time (t) an ion travels with velocity (v) is determined by the flight path L : $t = L/v$ (see Fig. 5). Taken together, this results in an equation that establishes the relationship between the mass of the analyte and the measured flight time:

$$m = (2zeV/L^2)t^2 \quad (5)$$

or

$$t = L(m/2zeV)^{1/2} = k_1(m/z)^{1/2} \quad (6)$$

This simple equation, which applies only to linear TOF analyzers, shows that the measured time is proportional to the length of the flight path (longer flight paths lead to increased flight times and thus to easier time measurements), the square root of the mass-to-charge ratio (m/z) (heavier ions of the same charge travel more slowly), and the inverse of the square root of the applied voltage (higher extraction voltage leads to a higher kinetic energy and consequently to a shorter flight time). In reality, a second constant (k_2) is needed, which depends on the time difference between ion formation and the start of data acquisition.

It is of interest to know the time scale involved in TOF mass analysis. For example, a monocharged ion of m/z 3494.6 takes 201.8 μsec to reach the detector in an instrument with a flight path of 6.6 m at 20 kV extraction voltage [38]. If monoisotopic resolution is required, time differences on the order of 30 nsec have to be accurately measured. Practical limitations to such accurate time measurements are related to the homogeneity of the sample surface, the energy dispersion of the generated ions due to the impact of the laser, adduct formation (especially for proteins of higher molecular weight), and inherent instrument factors such as drift of the high-voltage power supply, slight differences between the laser shot and the start of data acquisition, and, finally, the response time of the detector (1–10 nsec).

The performance of TOF mass analyzers in terms of resolution and mass accuracy depends upon the configuration of the analyzer. The simplest design consists of a linear flight tube with a detector at the end (linear TOF). Such detectors were, until recently, limited to a resolution of about 200–500 with a mass accuracy of 0.01% (100 ppm) in optimal cases using internal calibration standards and peak centroiding. Including a reflectron in the flight path, as shown in Fig. 6, increases resolution to above 1000, which is equivalent to that of the

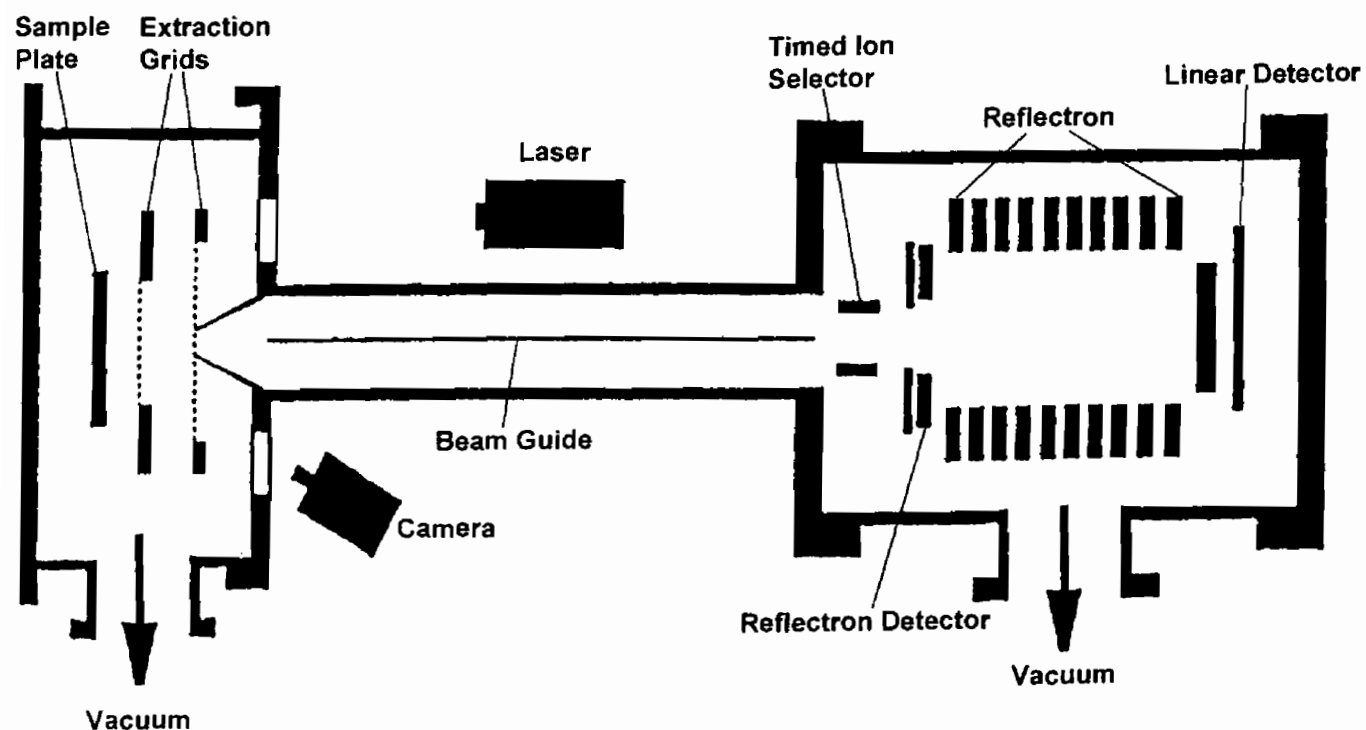


FIGURE 6 Schematic drawing of a MALDI-TOF instrument that is equipped with a reflectron mass analyzer. The extraction grids allow one to diminish the initial width of the ion cloud by delayed extraction (DE) and thus to achieve higher mass resolution (see also Fig. 10 for fragment ion analysis in a reflectron). (From Ref. 19.)

most commonly used quadrupole analyzers. A reflectron is an electrostatic mirror (electric field of slightly lower strength and opposite direction to the initial accelerating field) that returns and focuses the ions into a detector that is situated at a fixed angle.

Energy dispersion of the initially generated ions is a major limiting factor to achievement of higher resolution. Ions that are generated in the plasma after the laser impact can be refocused by “time-lag focusing,” a technique that was initially described in the 1950’s [39]. More recently, this technique was perfected and is now available in commercial instruments and referred to as “delayed extraction” [19]. Delayed extraction generates the ion cloud at a low extraction voltage, followed by a rapid increase to the full extraction voltage in about 20 nsec (Fig. 6). The optimum lag time can be predicted by theory and depends on a number of parameters such as the molecular mass (m), the charge of the ion (z), the extraction voltage (V_x), the initial kinetic energy (T_0), and a parameter (w) that is dependent on the specifications of the ion source and the TOF analyzer. DE has been shown to improve resolution in both linear and reflectron instruments, reaching up to 10,000 in the latter. Furthermore, DE reduces background noise and the dependence of m/z measurements on laser power. Resolution up to 10,000 and mass accuracy of 2 ppm have been reached by combining a reflectron TOF analyzer (6.6 m flight path) with delayed ion extraction using internal calibration standards, although this is not routine [38]. The possibility of determining the monoisotopic mass of peptides with high accuracy of ~ 30 ppm is of importance for identifying proteins isolated by gel electrophoresis based on the mass of a few peptides following proteolytic digestion [40]. An example of the application of this technique will be presented in this chapter. Dispersion of the initially generated ion cloud has been greatly reduced by DE, but adduct formation remains a significant limiting factor for higher mass analytes such as proteins and nucleic acids.

Another source of variations in flight time is the surface at which ionization occurs. Inhomogeneities of, for example, 10 μm in thickness can lead to time differences of 1 nsec, representing 5 ppm in the case of a singly charged ion of m/z 3494.6 [38]. Since MALDI-TOF mass spectra are generally obtained by averaging 50–100 individual spectra, each obtained after a laser shot that may be directed at different regions of the target, it is critical that the surface be as smooth and homogeneous as possible. The highest mass accuracy can be reached when using internal calibrants that are cocrystallized with the sample. However, suppression of ionization of the sample may occur when very low amounts are present. External calibrations that are performed under rigorously identical conditions also lead to satisfactory results, but care must be taken to verify the calibration regularly if high mass accuracy is required.

It is not possible to cover even superficially the wide range of other mass analyzers and combinations that are presently available and that are undergoing

rapid development such as the quadrupole ion trap. Readers wanting to learn more about their possibilities and technical details are referred to some excellent overviews [41–43].

FRAGMENTATION STUDIES AND STRUCTURAL INFORMATION

The molecular mass of an analyte, whether a biological macromolecule or a chemically synthesized compound, is often not sufficient for its identification. This has led to the widespread application of fragmentation studies, often referred to as MS/MS, tandem mass spectrometry, or even MSⁿ in the case of ion trap mass analyzers. Fragmentation is a common phenomenon during ion formation and can often lead to undesirable effects, such as loss of sensitivity for the analyte of interest or the generation of overly complex spectra. To extract useful structural information, it is necessary to control the fragmentation conditions and to be able to interpret the resulting fragment ion spectra. Fortunately, numerous studies have elucidated certain fragmentation patterns, in particular for peptides, and software delivered with modern mass spectrometers greatly facilitates the interpretation of spectra. Nevertheless, the analysis of fragment ion spectra can be tedious if unexpected fragments occur or if complementary structural information is not available.

Even the so-called soft ionization techniques, such as ESI or MALDI, lead to some fragmentation, especially in the case of high-molecular-weight biomolecules such as peptides, proteins, or polynucleotides. In ESI, fragmentation can occur within the region between the cone and the skimmer lenses, where residual pressure is approximately 1 mbar (see Fig. 1). Collision of the generated ions with gas molecules can lead to fragmentation (in-source collision-induced dissociation or in-source CID); the extent of fragmentation depends on the electrostatic potential (V_c) between the lenses and the residual pressure (see Fig. 1). Adjustment of V_c can be used to generate fragment ions that are characteristic for certain posttranslational modifications, such as glycosylation or phosphorylation [24,25]. Combining LC-MS with in-source CID and selective monitoring of the fragment ions is a powerful approach for on-line detection of posttranslationally modified peptides in a peptide map without the need for radioactive labeling (Fig. 7). In-source CID may also provide partial amino acid sequence information (so-called sequence tags), which is important for the identification of proteins from databases by comparing the measured mass values of proteolytic peptide fragments with those predicted from known sequences. An example of this approach will be given later. Figure 8A shows how in-source CID can be provoked by increasing V_c to 120 volts. An internal sequence of five amino acids was identified based on a series of B ions (see Fig. 8B for fragmentation pattern and nomenclature according to ref. 27). A disadvantage of in-source CID is that there is no possibility of selecting the ion to be fragmented (the so-called parent or precursor ion). In-source fragmenta-

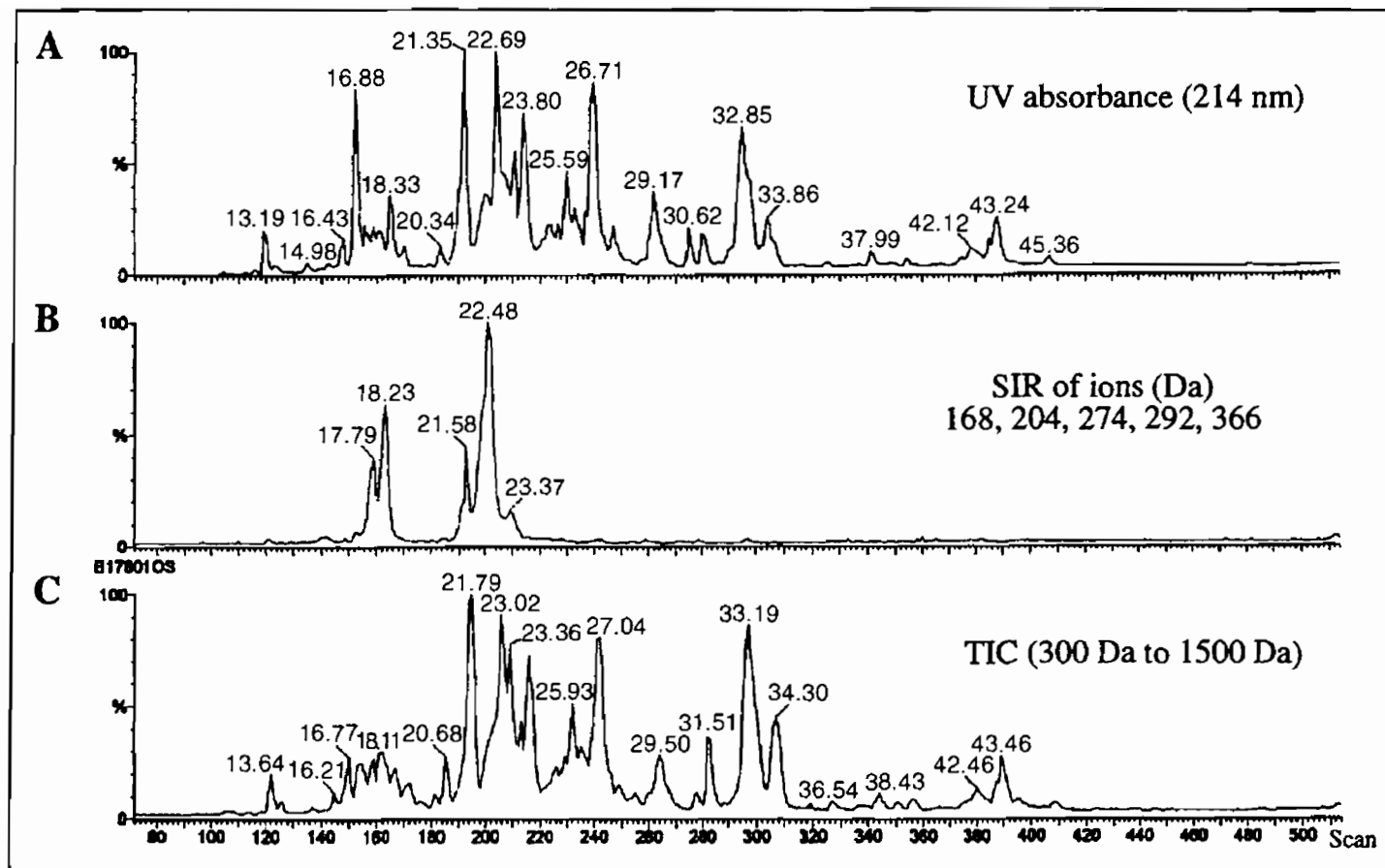


FIGURE 7 Specific detection of glycopeptides in a Lys-C digest of bovine chromogranin A by LC-MS based on selective ion recording (SIR). (A) The trace at 214 nm and (B) detection of the glycopeptides based on characteristic fragment ions that were generated by in-source CID. (C) The total ion current (TIC) of all the ions generated in the ion source. (From Ref. 25.)

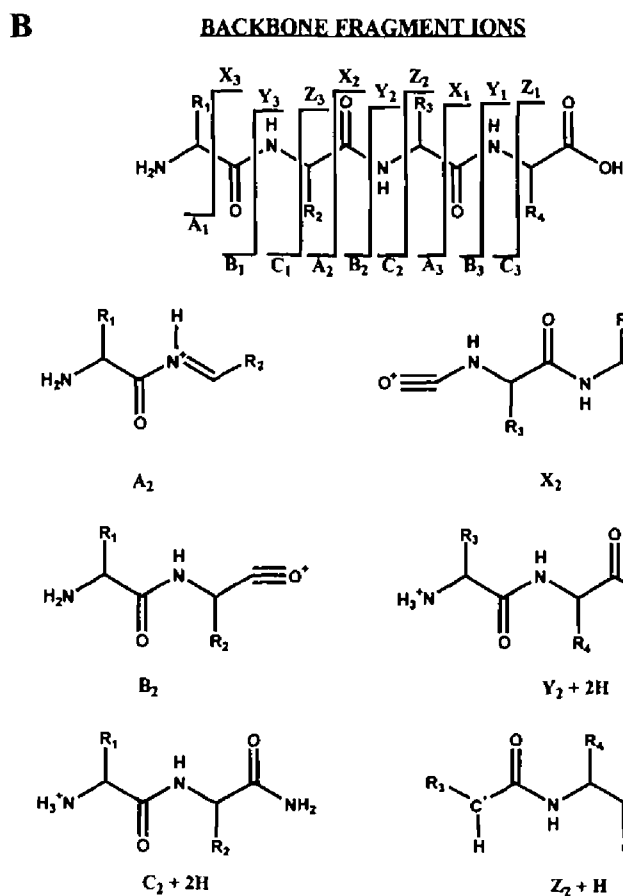
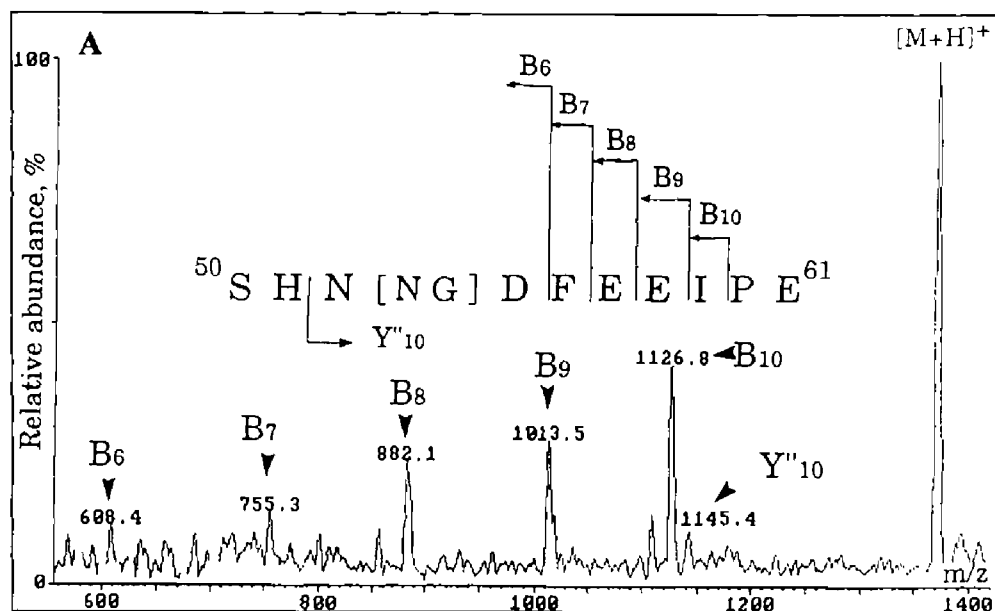


FIGURE 8 (A) In-source CID following ESI of a peptide fragment of recombinant hirudin at a cone voltage V_c of 120 volts. Part of the B-ion series can be observed, which allows reading an internal sequence of five amino acids (isoleucine [I] and leucine [L] cannot be discriminated in this analysis because they have identical molecular weights). Both the parent ion $[M + H]^+$ and a $Y + 2H$ (Y'') fragment are present. (From Ref. 26.) (B) Schematic representation of the major fragmentation pathways for peptides (nomenclature according to Ref. 27). Note that CID leads to pairs of fragment ions with charge retention in either the N-terminal (A, B, or C fragments) or the C-terminal part (X, Y, or Z fragments). Depending on the charge distribution within the peptide, some fragment ions will be favored.

tion therefore requires that the analyte be of high purity when introduced into the ion source to avoid fragment ions being derived from multiple parent ions, which makes interpretation very difficult, if not impossible. This condition may be fulfilled for certain peptides following LC-MS.

A more widely used approach to generate fragment ions and to gain structural information from a given analyte is to induce fragmentation in a dedicated collision cell using a triple-quadrupole analyzer (see Fig. 1). Most contemporary triple-quadrupole instruments are equipped with an ESI source. As shown in Fig. 1, the analyzer consists of a first quadrupole (MS1) that allows selection of the parent ion from a mixture of ions that have been generated in the source. This capacity is very important when the analyte is not pure (mixture analysis), for example, the tryptic digest of a protein. Selection of a particular parent ion in MS1 will allow MS/MS studies on only this ion, independent of the presence of other ions. An example is given in Fig. 9, where MS1 was used to select the doubly or singly charged molecular ions of the hexapeptide AGEHIK, which were subsequently fragmented with argon (1.9×10^{-3} mbar) in the collision cell.

Scanning of MS2 gave the fragment ion spectra shown in Fig. 9A and B, respectively. The spectra were obtained using a modification of the nanoelectrospray ion source [18,44]. This allows optimization of critical parameters such as the energy (in eV) with which the parent ion enters into collisions with the gas atoms and the pressure of the collision gas (in the range of 10^{-3} to 10^{-4} mbar), which determines the average number of collisions due to very low flow rates (~ 20 nL/min) and thus increased measuring time. In this case, fragmentation of the doubly charged molecular ion allowed reading of the complete sequence of the hexapeptide based on the presence of a complete series of Y'' ions (see Fig. 8B and Ref. 27 for nomenclature of fragment ions). On the contrary, less structural information was derived from fragmentation of the monocharged parent ion despite the fact that identical conditions were used. This illustrates the importance of charge distribution within the ion to be fragmented with respect to the number and the type of fragment ions that are generated. It is also of interest that most of the fragment ions resulting from the doubly charged molecular ion are singly charged with charge localization on either the N terminal (A and B ions) or the C-terminal part (Y''-ions). Indeed, most fragment ion spectra contain redundant information, which allows confirming part of the sequence based on different ion series (see, for example, the presence of A, B, and Y''-ions in Fig. 9A). It is also noteworthy that amino acid-specific fragments, such as the observed immonium ions of histidine (i_4) and isoleucine (i_5), may be obtained. Generating fragment ion spectra of high quality is not always an easy task because conditions need to be optimized for each analyte. In particular, peptides of different size and amino acid sequence require different conditions, and some of them may not give any usable sequence information at all.

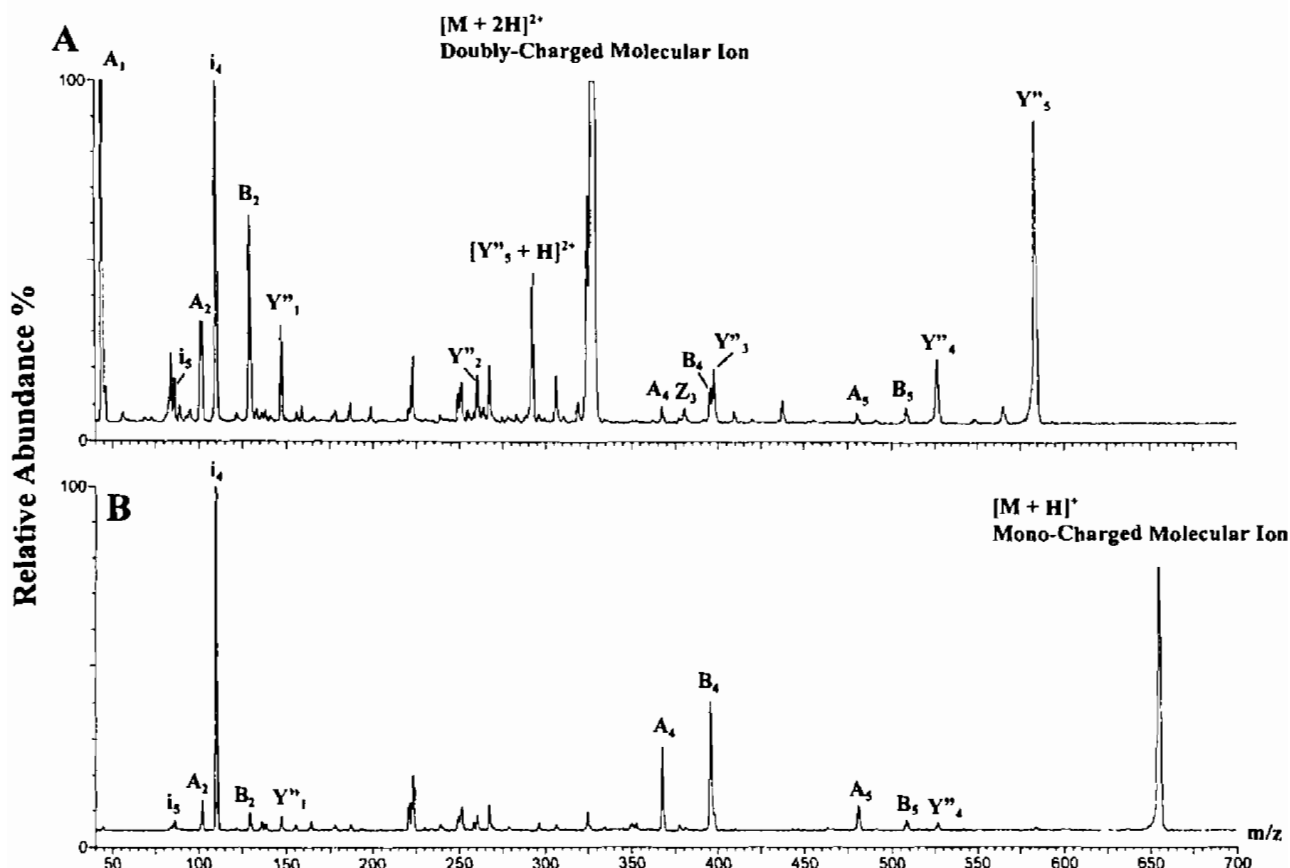


FIGURE 9 Tandem mass spectrometry of the N-terminal hexapeptide of rSmp28 (AGEHIK) using the nanoelectrospray technique [18]. Fragmentation was performed by collision with argon at 1.9×10^{-3} mbar and a collision energy of 60 eV. (A) The daughter ion spectrum (MS2, see Fig. 1) of the doubly charged molecular ion $[M + 2H]^+$ ($m/z = 328$) and (B) that of the monocharged molecular ion $[M + H]^+$ ($m/z = 655$). Note that the doubly charged parent ion gives rise mainly to singly charged fragment ions except for Y''_5 . Comparison between the daughter ion spectra shows that the fragmentation patterns are quite different and that the doubly charged parent ion provides more information because of a wider range of fragment ions. It is of interest that the Y'' ion series is essentially absent in the MS/MS spectrum of the monocharged molecular ion.

Although many different configurations of mass analyzers can be used to obtain MS/MS spectra, it is the improved quadrupole ion trap that holds great promise as an affordable mass analyzer allowing performance of MS^n studies. Combining this analyzer with advanced ionization methods such as nanoelectrospray will give a wider range of users access to biomolecule analyses by mass spectrometry because of its sensitivity and the capacity to obtain detailed structural information on analytes by selectively fragmenting a parent ion followed by further fragmentation of selected daughter ions. Combination with HPLC or CE further widens the scope of the ion trap in modern analytical biochemistry. The reader is referred to two reviews as an introduction to this technique [42,43].

Reflectron TOF analyzers also allow the analysis of fragment ions that form in the field-free region of the flight tube (so-called post source decay or PSD [45]). An inconvenience of PSD is that the collision energy is not independent of the ionization conditions but rather determined by the extraction voltage that is used to accelerate the ions. A principal difference between CID within the ESI source and PSD is the collision energy, which is in the eV range for ESI (low-energy CID) and in the keV range in the case of PSD (high-energy CID). High-energy collisions may add information to the fragment ion spectra, such as side-chain fragments of certain amino acids, but may also complicate the interpretation. The analysis of fragment ions generated by PSD in a TOF analyzer is based on their separation in the discontinuous electric field of the reflectron (Fig. 10). As all ions generated from a precursor ion within the flight tube have been accelerated in the same electrostatic field and have thus acquired the same velocity (v), heavier ions possess a higher kinetic energy ($\frac{1}{2}mv^2$) and will thus penetrate deeper into the oppositely charged electric field of the reflectron before being returned to the detector (see Fig. 10). By stepping up the reflectron field strength, it is therefore possible to return fragment ions of increasing m/z in the direction of the detector and thus to measure their m/z ratio.

PSD of a mixture of compounds is subject to the same limitations described for in-source CID, namely that fragment ion spectra originating from a mixture of analytes will be difficult, if not impossible, to interpret. Some instruments offer the possibility of selecting ions by a process termed "ion gating." Ion gating consists of deviating the generated ions with an additional electric field that is perpendicular to the flight path (see Fig. 10). Switching this field off for a given time will allow an ion package traveling with a specified velocity to pass and be analyzed. This configuration puts high demands on the electronic components, which have to function precisely in the nsec range. Resolution for ion gating is therefore limited to about 50, so that ions with closely spaced m/z values will be difficult to analyze separately. Nevertheless, ion gating adds important potential to the TOF analyzer with respect to fragment ion studies and further improvements in the electronic components will most likely improve the precision of ion gating. PSD is not as widely used as CID because optimization of the collision conditions is more difficult and selection of the precursor ion is less precise. In particular, the introduction of an instrument that combines CID in a quadrupole collision cell with fragment ion analysis by TOF measurements promises to be superior to PSD analysis [9].

APPLICATIONS

It is obviously impossible to cover all of the applications of mass spectrometry in the biosciences that have been published in recent years (see ref. 46 for a review). The purpose of the following examples is rather to highlight some of the potential of mass spectrometry including peptide mapping of recombinant proteins by LC-

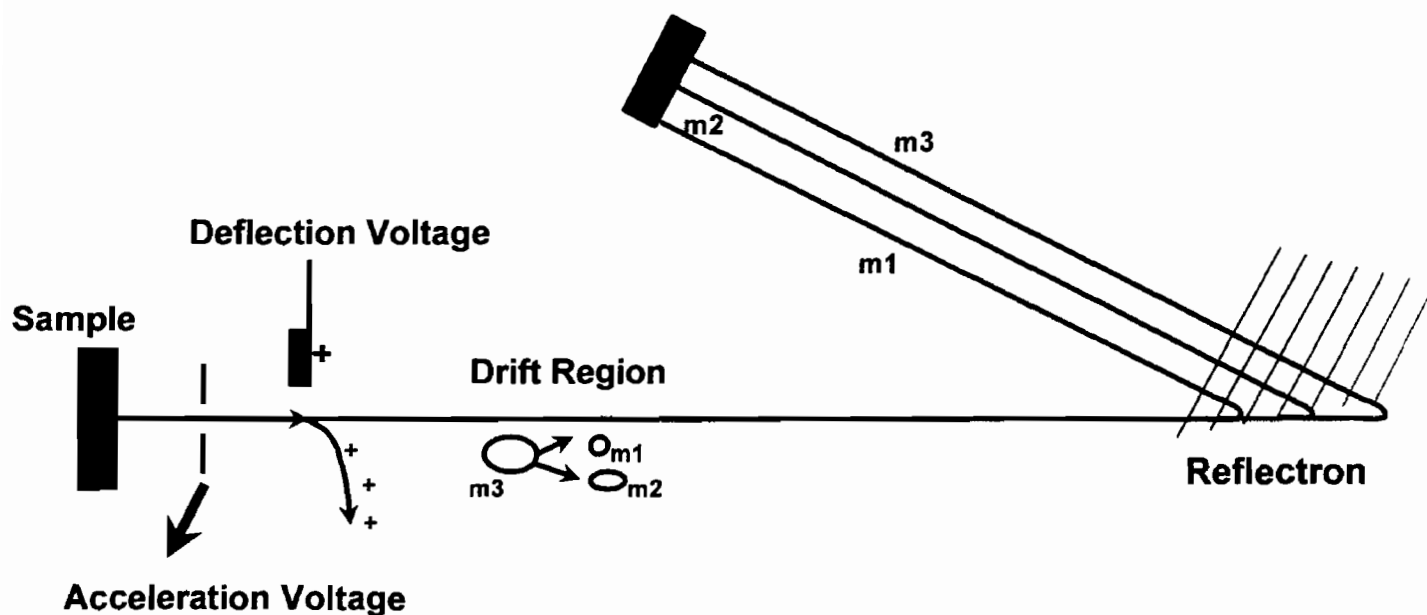


FIGURE 10 Schematic drawing of a MALDI-TOF system with a reflectron indicating fragment ion generation within the field-free region (so-called post source decay or PSD [45]). Fragment ions of different m/z ratios can be separated in the electrostatic field of the reflectron due to their different kinetic energies (they have the same velocity but different mass). Stepwise increases in the field strength of the reflectron result in deviation of ions of increasing m/z into the detector. An additional deflection voltage that can be switched off for a defined time window (nsec range) allows selection of an ion package of a given m/z range for PSD analysis (so-called ion gating).

MS, the highly sensitive analysis of naturally occurring peptides either extracted from MHC class I or II proteins or present in the hemofiltrate of patients with kidney disease, the identification of proteins purified by gel electrophoresis through matching of the mass of peptide fragments with entries in the ever growing databases, and finally, the use of mass spectrometry to analyze oligonucleotides and DNA fragments. This selection of examples from different areas of the life sciences will provide insight not only into the wide-ranging applications but also the limitations of present-day mass spectrometry. The continuing rapid development of technology, however, will push these limits further and further.

Analysis of Recombinant Proteins by LC-MS

Recombinant proteins are gaining increasing importance as pharmaceuticals with annual sales of some \$9 billion (U.S.) in 1994 [47,48]. Therapeutic applications of these biopharmaceuticals include stimulation of cell growth, regulation of the immune system, or fibrinolysis, to name just a few. Putting recombinant proteins on the market was possible because of the development of efficient and reproducible production and purification systems that allow manufacture of these complex molecules in large amounts with consistent quality suitable for human use. Control of the quality of biopharmaceuticals is crucial not only at the stage of large-scale manufacturing but also during the research and development phase, as impurities and contaminants must be characterized and reduced to acceptable levels.

The complete analysis of a recombinant protein necessitates the combined use of separation techniques, such as HPLC, with methods that allow the characterization of the separated molecular species, such as protein sequencing, amino acid analysis, and mass spectrometry. LC-MS is clearly one of the most powerful approaches to obtaining detailed information in a rapid and automatable fashion suitable for pharmaceutical development. The power of analyzing separated components by mass spectrometry results from the fact that the molecular mass is an inherent property of a given compound. In the case of recombinant proteins, where the amino acid sequence is generally known, it is possible to compare the calculated mass with the measured mass and thus to confirm the expected structure or to detect modifications. More detailed information can be obtained when analyzing defined proteolytic digests of a protein (peptide mapping). Agreement between the measured and the calculated mass of the individual peptides is an important criterium showing conformity between the expected and determined primary sequences without having to resort to lengthy amino acid sequencing in each case. Such analyses also allow the detection of possible modifications in a protein, as well as localizing them to specific peptide

fragments. Certain modifications, such as glycosylations, may be expected, whereas others may be related to the production process.

Analysis of the primary structure of a 210-amino-acid protein (Smp28) isolated from the parasite *Schistosoma mansoni* which is being evaluated as a vaccine candidate for schistosomiasis, a disease affecting about 200 million people worldwide, shows how LC-MS accelerates the analysis of such complex biological macromolecules. Tryptic digestion of the recombinant protein produced in *Saccharomyces cerevisiae* generated 28 peptides that were separated by RPC using a syringe pump system to generate a flow rate of 100 $\mu\text{L}/\text{min}$ [16]. For LC-MS, the flow was split either before or after the column (precolumn or postcolumn stream splitting) as shown in Fig. 3.

Postcolumn splitting allows recovery of $\sim 95\%$ of the separated components for complementary analyses while 5% enter the ion source of the mass spectrometer for on-line mass determination. If a UV detector is placed between the stream splitter and the fraction collector, two traces are obtained simultaneously—one corresponding to the standard chromatogram (UV absorbance) and one to the recording of the total ion current (TIC) measured by the mass spectrometer (Fig. 11). In order to establish the identity of the separated peptides, it is indispensable to perform complementary analyses because the molecular mass alone is not sufficient for unambiguous identification. Once, however, a peptide map has been established and verified by a method, such as sequencing by Edman degradation and quantitative amino acid analysis, LC-MS can be performed on a routine basis with complementary analyses on peptides that have unexpected mass values or elute at unexpected positions. Such analyses revealed, for example, that peptide T19 was present in three different forms due to modification of the single cysteine with glutathione (T19A) or to disulfide bond formation (T19C) (Table 1).

Precolumn stream splitting or a dedicated capillary HPLC system may be employed to reduce the amount of sample, as well as solvent consumption (Fig. 12) [16,49]. Successful applications of such miniaturized equipment have been shown with recombinant proteins, but it should be noted that capillary HPLC columns are more delicate to handle. In order to have a flexible system, it may be of interest to use modules that can be switched from postcolumn to precolumn stream splitting (see Fig. 3). In cases in which sufficient sample is available, it is easier and more robust to use narrow-bore HPLC columns with postcolumn stream splitting although capillary LC-MS may be indispensable in cases where the sample amount is limiting (see example in the section "Analysis of Peptides from Natural Sources"). On the other hand, some ESI mass spectrometers have pumping systems efficient enough to allow introduction of the full flow rate from narrow-bore HPLC columns allowing on-line coupling with standard HPLC equipment.

LC-MS is an efficient method to compare different forms of the same protein that may have resulted from posttranslational modifications (expected or

TABLE 1 Analysis of rSmp28 by LC-MS After Tryptic Digestion (see Fig. 11)

No.	Sequences	Position	Expected (Da) ^a	Measured (Da)
T1	AGEHIK (N-term.)	1–6	653.7	652.9
T2	VIYFDGR	7–13	869.0	868.8±0.3 ^a
T3	GR	14–15	231.3	Not found ^b
T4	AESIR	16–20	574.6	574.5
T5	MTLVAAGVDYEDER	21–34	1568.7	1568.2±0.6
T6	ISFQDWPK	35–42	1020.1	1020.0±0.8
T7	IKPTIPGGR	43–51	938.1	937.7±0.5
T8	LPAVK	52–56	526.7	526.4
T9	VTDNHGHVK	57–65	1006.1	1005.7±0.7
T10	WMLESLAIAR	66–75	1189.4	1189.0±0.1
T11	YMAK	76–79	511.6	511.3
T12	K	80	146.2	Not found ^b
T12–	KHHMMGETD	80–96	2113.3	2112.6±0.3
T13	EEYYSVEK			
T13	HHMMGETD	81–96	1985.1	1984.4±0.2
	EEYYSVEK			
T14	LIGQAEDVEHEYHK	97–110	1667.8	1667.5±0.4
T15	TLMKPQEEK	111–119	1103.3	1103.2
T16	EK	120–121	275.3	Not found ^b
T17	ITK	122–124	360.5	360.4
T18	EILNGK	125–130	672.8	672.6±0.4
T19	VPVLFNMICESLK	131–143	1492.8	1492.4
T19A	VPVLFNMICESLK (+glutathione)	131–143	1798.1	1797.7
T19C	VPVLFNMICESLK (dimerized)	131–143	2983.6	2982.8±0.4
T20	GSTGK	144–148	448.5	Not found
T21	LAVGDK	149–154	601.7	601.6±0.1
T22	VTLADLVLIAVIDHV	155–174	2163.5	2162.6±0.2
	TDLDK			
T23	GFLTGK	175–180	621.7	621.5±0.4
T24	YPEIHK	181–186	785.9	785.5±0.5
T25	HR	187–188	311.3	Not found ^b
T25–T26	HRENLLASSPR	187–197	1279.4	1279.6±0.3
T26	ENLLASSPR	189–197	986.1	985.6±0.2
T27	LAK	198–200	330.4	330.3
T28	YLSNRPATPF (C-term.)	201–210	1165.3	1165.0±0.2

^aStandard deviation is based on at least two protonated forms of the peptide.^bScanning started at $m/z = 320$.

Source: Adapted from Ref. 16.

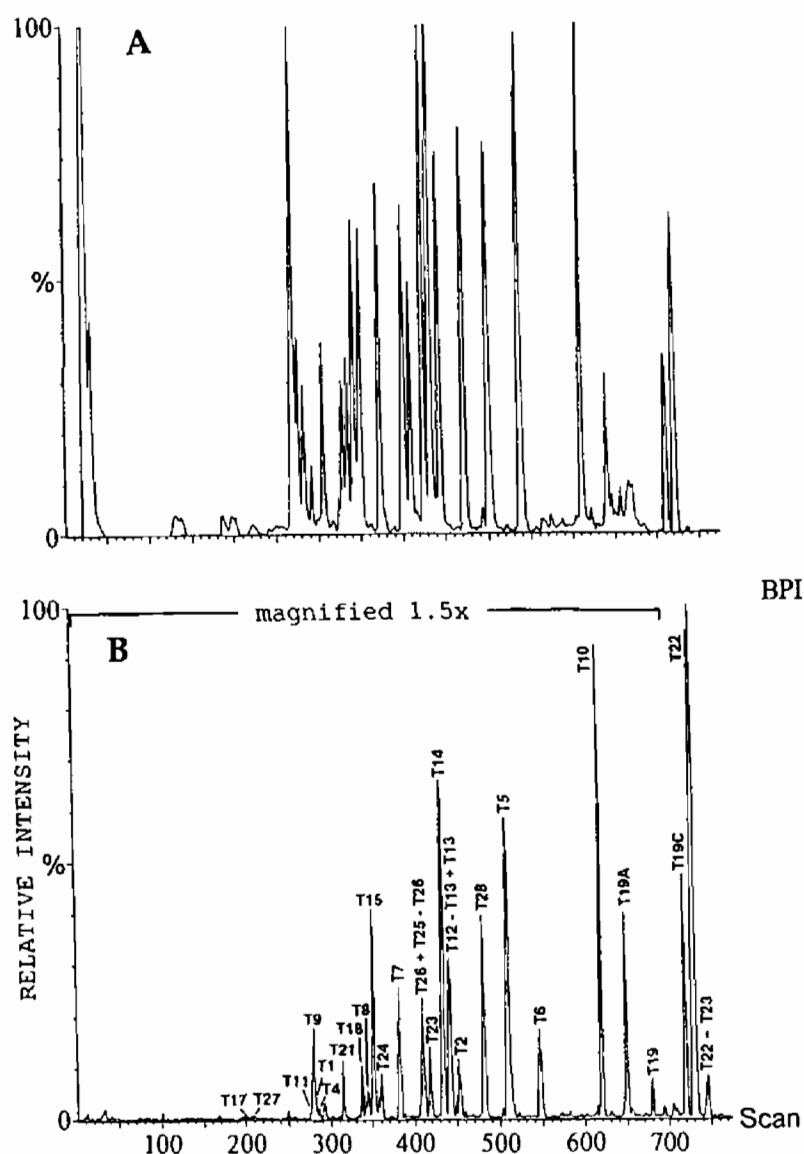


FIGURE 11 Peptide mapping of recombinant Smp28 by LC-MS using postcolumn streamsplitting as shown in Fig. 3. The upper trace (A) shows the UV absorbance at 205 nm, and a representation of the base peak corrected TIC is given in the lower trace (B). The analysis was performed at a flow rate of 100 μ L/min with 1 nmol rSmp28 on a 1.6×150 mm C_{18} column. Approximately 5% of the eluate was directed toward the mass spectrometer and the rest collected for further analyses. Peptides are labeled according to Table 1. (From Ref. 16.)

unexpected). Detection of modified peptides may be done on line using ion-selective monitoring if the type of modification is known and specific fragment ions can be obtained (see Fig. 7) or modified peptides may be analyzed off line following fraction collection. The example of an artifactual modification in rSmp28 during production in *S. cerevisiae* illustrates the possibilities offered

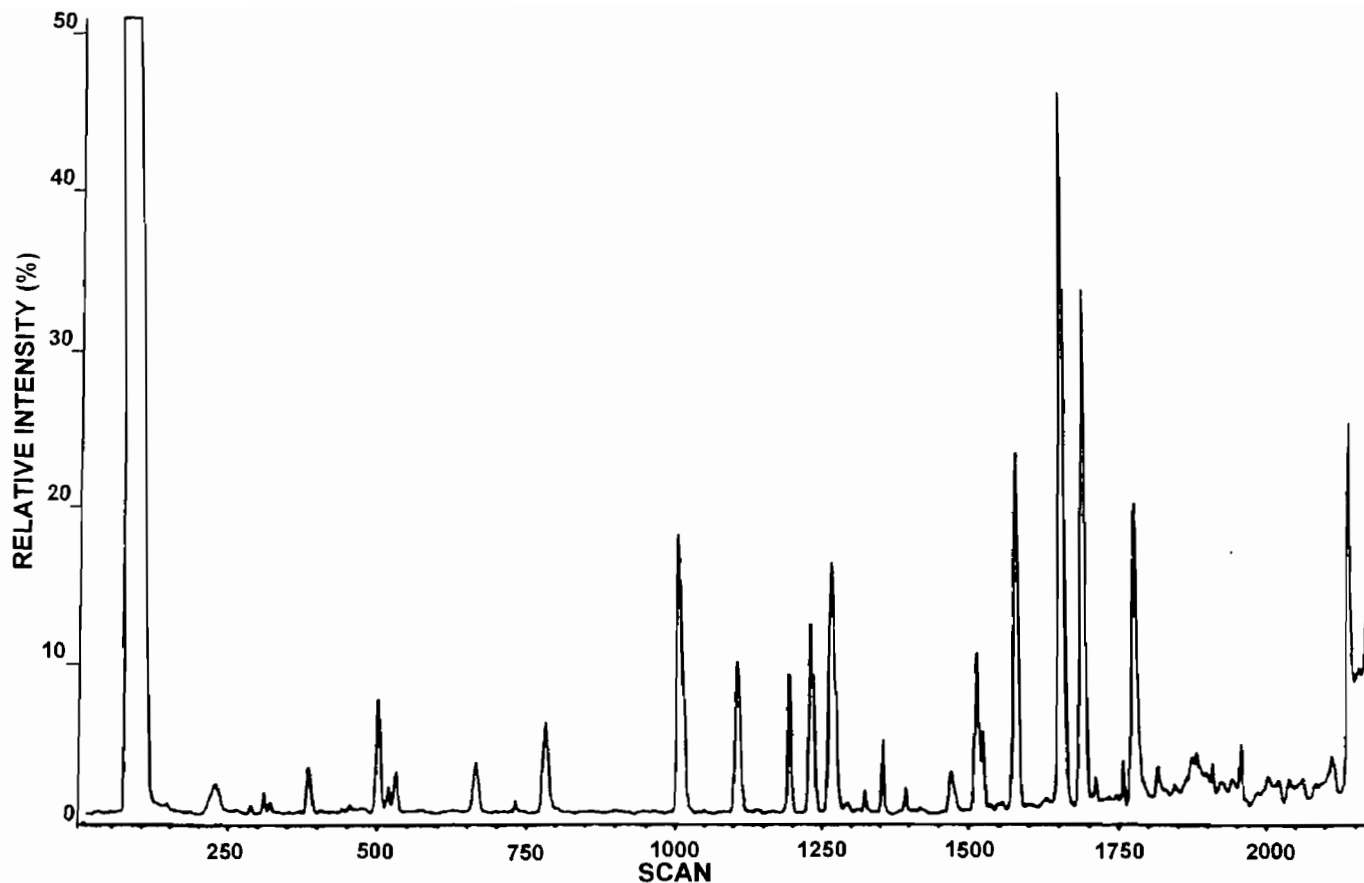


FIGURE 12 Peptide mapping of recombinant human growth hormone (90 ng, 4.1 pmol) by capillary LC-MS using precolumn streamsplitting (200–400 $\mu\text{L}/\text{min}$ initial flow rate) to generate a flow rate of 3.5 $\mu\text{L}/\text{min}$. The column (0.32 $\times$ 150 mm) was packed with a C_{18} stationary phase. The scan time is ~ 3.7 sec. (From Ref. 49.)

by mass spectrometry [44]. Following the detection of a modification by isoelectric focusing and isolation of the protein by anion-exchange HPLC, LC-MS of a tryptic digest of the modified and the nonmodified forms detected a mass difference in a peptide fragment corresponding to the N-terminus of the protein (Fig. 13). Further analyses by tandem mass spectrometry using a triple-quadrupole mass spectrometer with an ESI source adapted to the nanospray technology [18] allowed localizing the modified amino acids within this peptide and elucidating their structures (Fig. 14). The result of these analyses was that acetaldehyde, produced by anaerobic glucose metabolism (fermentation) at

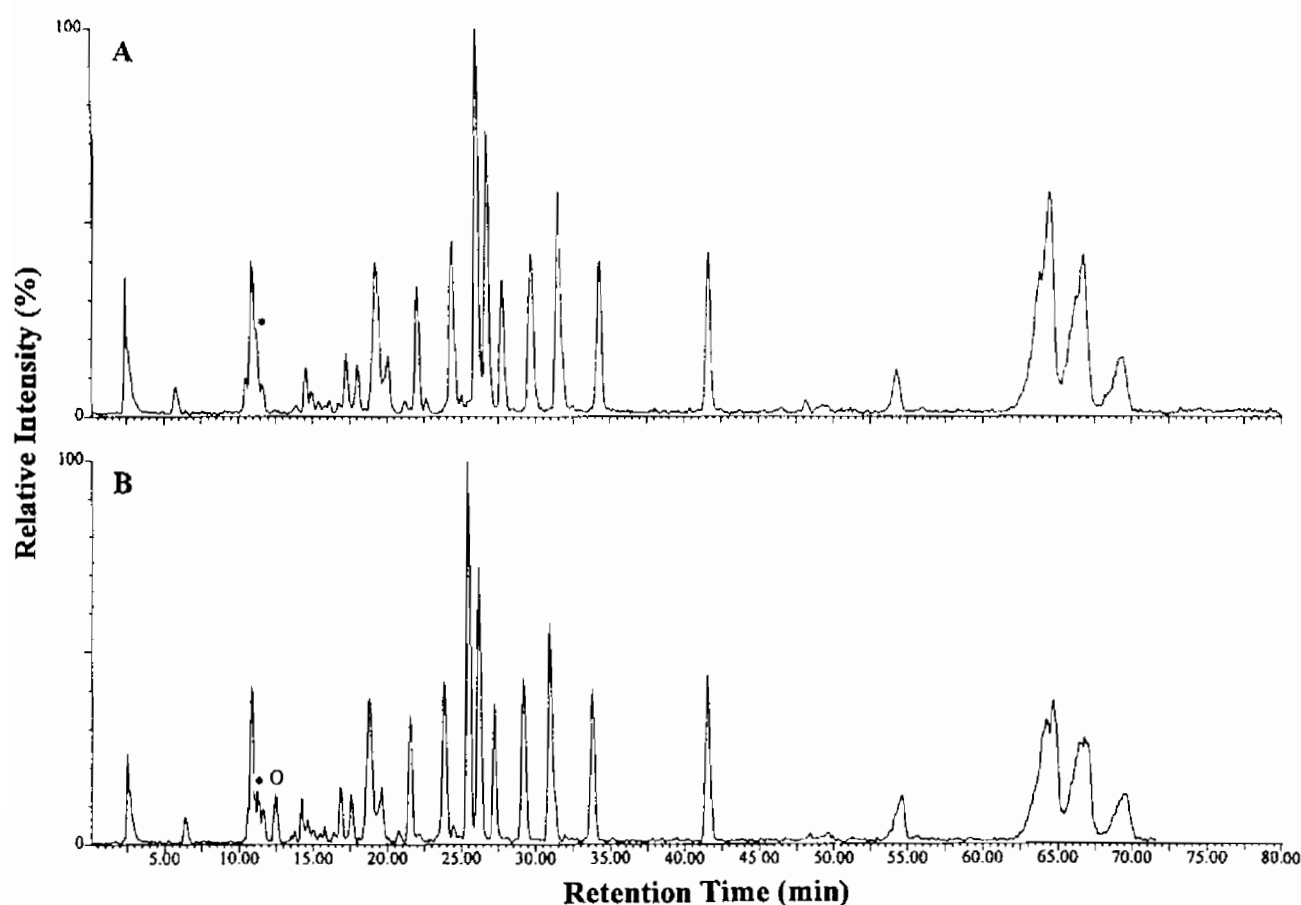


FIGURE 13 Comparative peptide mapping of two forms of rSmp28 by LC-MS (postcolumn stream splitting; see Fig. 3). (A) Analysis of the nonmodified and (B) of the modified protein (* labels the nonmodified and O the modified N-terminal peptide). The on-line mass spectra of the nonmodified (C) and the modified (D) N-terminal peptides indicate a difference of 26.1 Da, and amino acid composition analysis of the collected peptide fractions gave identical results (expected sequence: AGEHIK). The modified peptide was inaccessible to Edman degradation, lacked two deuterium-exchangeable hydrogen atoms, and could be separated into two isomers. Detailed analysis by tandem mass spectrometry showed that it was modified due to addition of acetaldehyde to A¹, H⁴, and K⁶ (see Fig. 14 and Ref. 44). (From Ref. 44.)

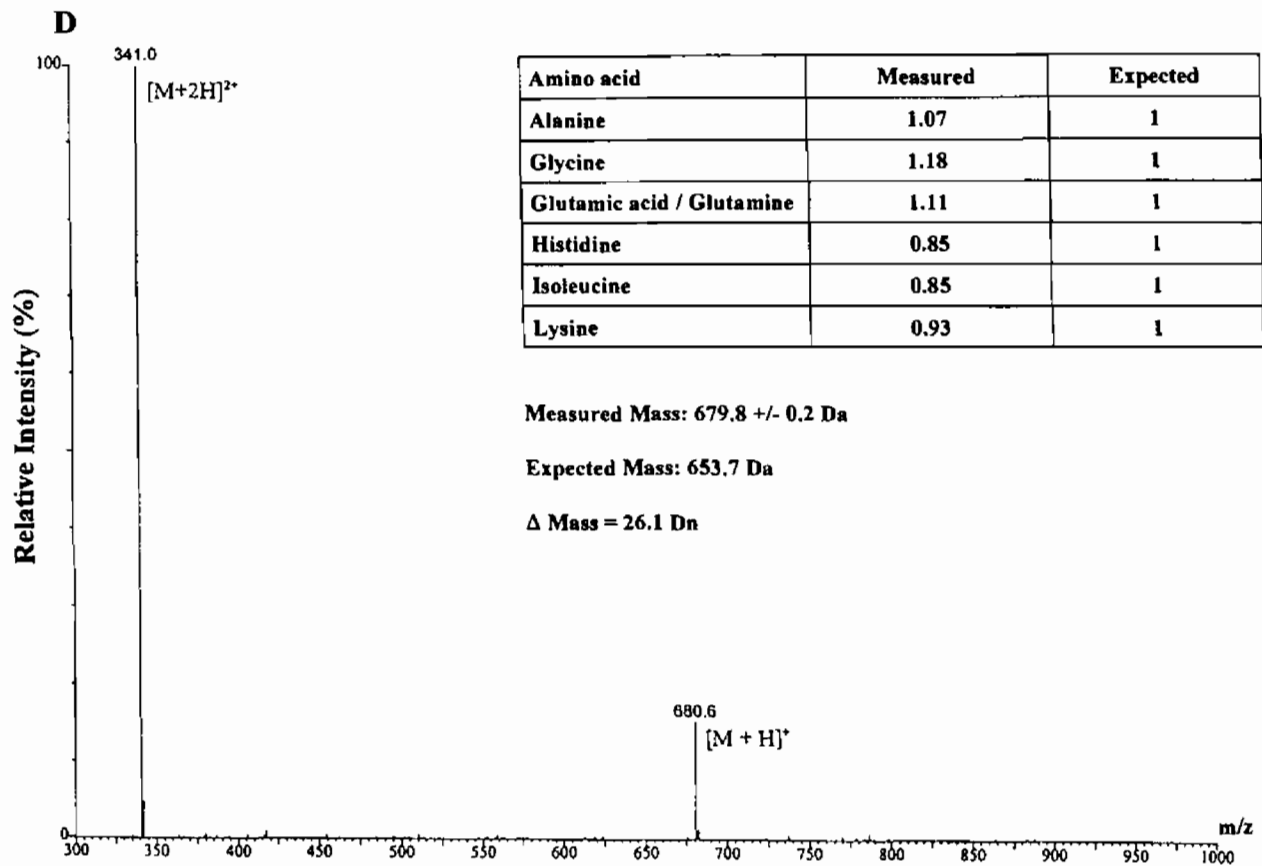
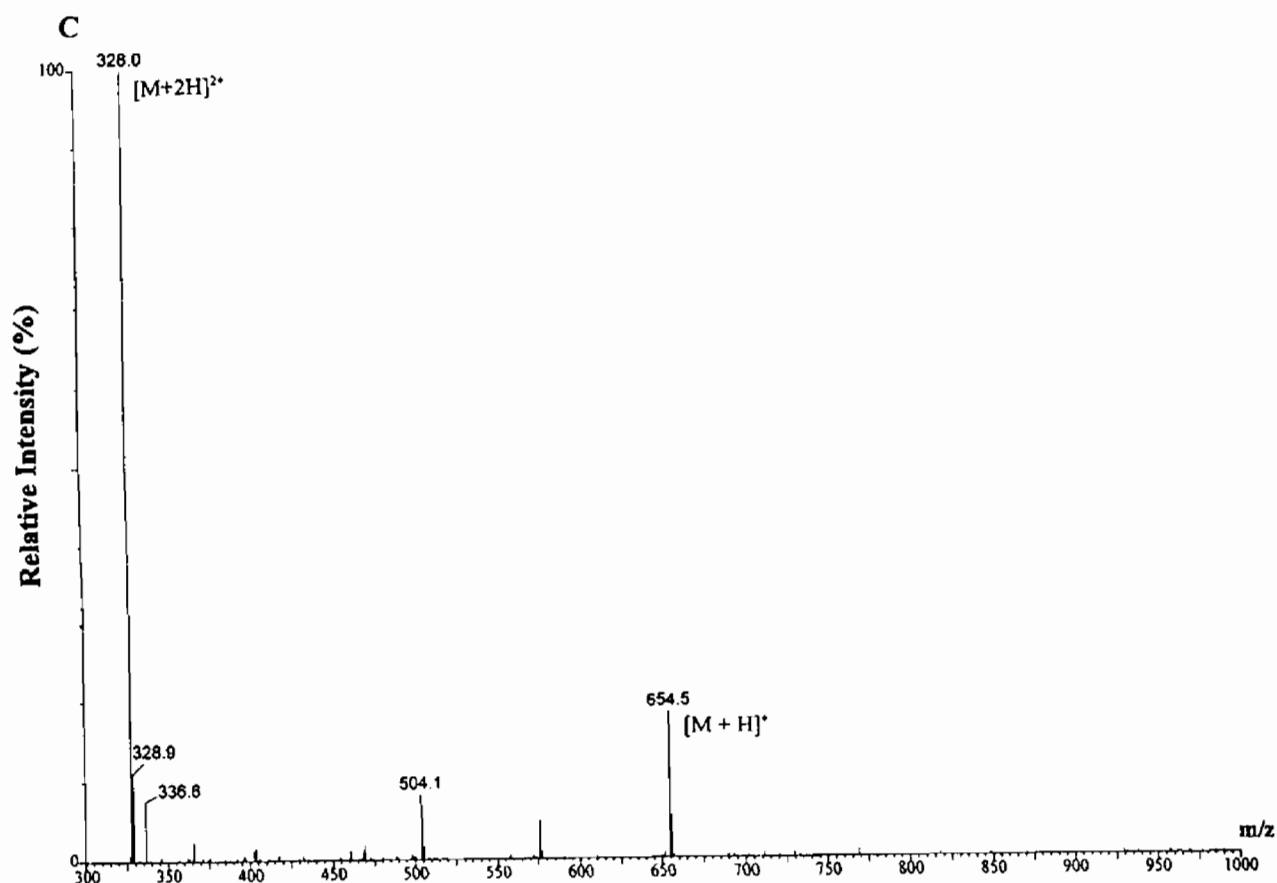
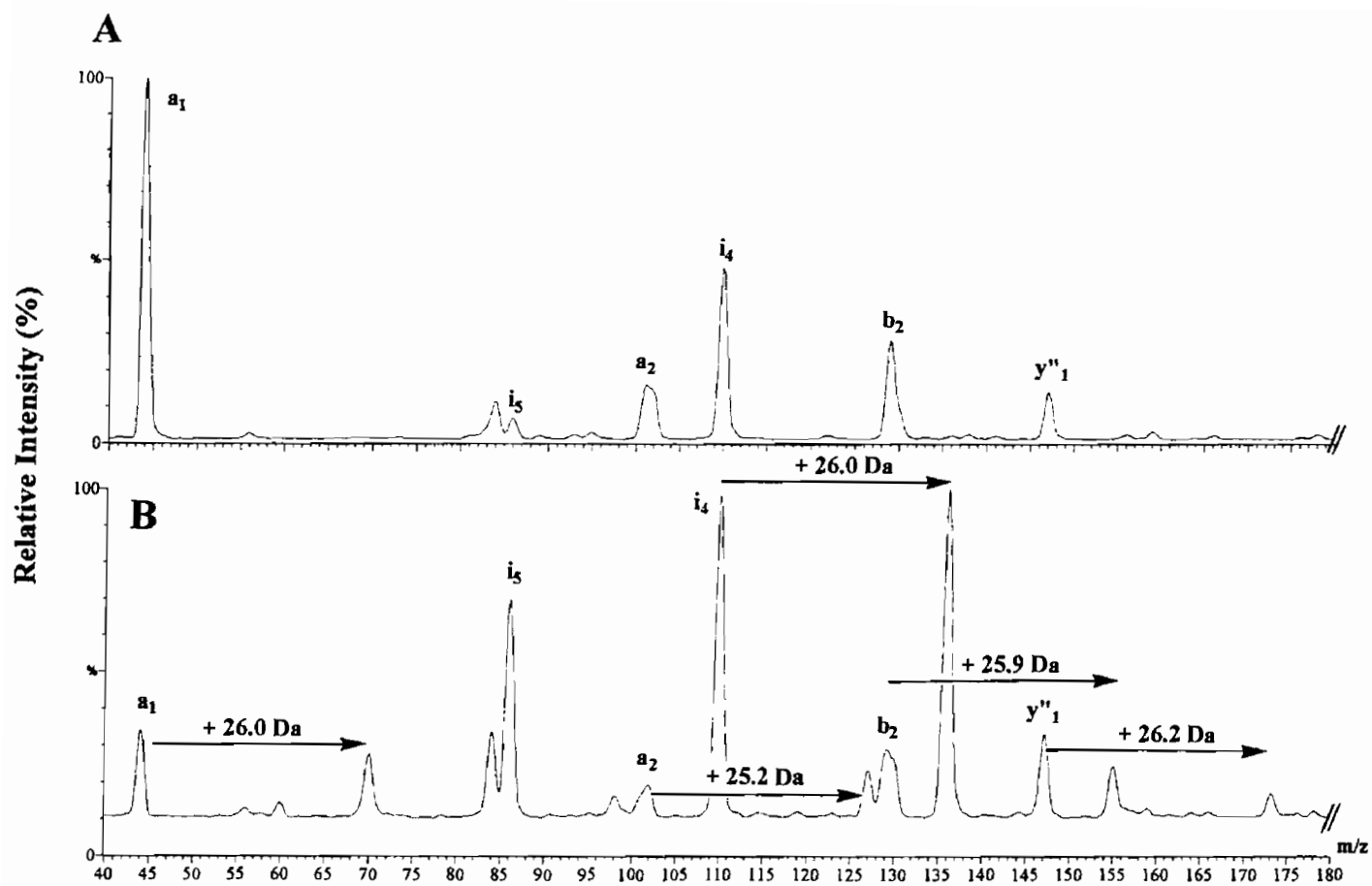


FIGURE 13 (continued)



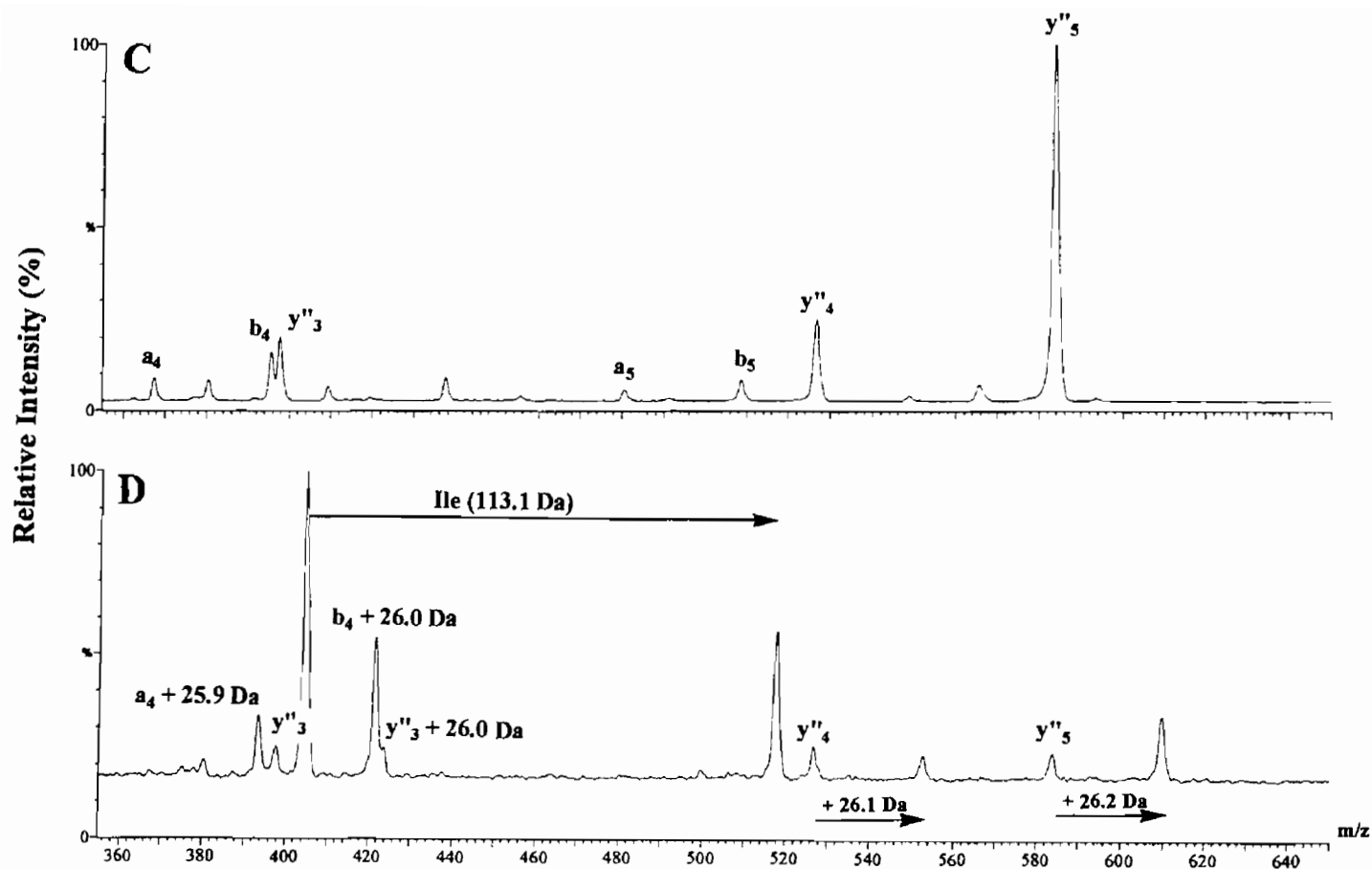


FIGURE 14 Tandem mass spectrometry of the nonmodified (A and C) and the acetaldehyde-modified N-terminal peptide (B and D) fragment derived by tryptic digestion of rSmp28. Fragment ion spectra were obtained from the doubly charged molecular ions at m/z 328 and 341 at 50 and 60 eV, respectively (argon pressure: 1.5×10^{-3} mbar). Fragment ions of the a and y'' series allow reading the complete sequence of the hexapeptides AGEHIK. Modified fragment ions with increased masses of ~ 26 Da allow localization of the acetaldehyde modification to amino acids Ala¹, His⁴, and Lys⁵ (see fragment ions a_1 , y''_1 , and i_4). (From Ref. 44.)

high biomass, was responsible for the modification of the N-terminus of rSmp28. This example of a recombinant protein that was modified because of the production process illustrates the impact of advances in mass spectrometry on the characterization of biopharmaceuticals, although other methods such as amino acid analysis and sequencing by Edman degradation remain indispensable tools.

Analysis of Peptides from Natural Sources

Mass spectrometry with its high sensitivity is used in numerous applications dealing with the characterization of peptides or proteins that can be obtained only in limited amounts. Cells expressing new antigens related to a viral infection or tumorigenic transformation can be detected by cytotoxic lymphocytes ($CD8^+$) of the immune system due to presentation of peptides by proteins of the class I major histocompatibility complex (MHC-I) [50,51]. Analysis of the presented peptides can help identify the antigens from which they are derived and thus define new targets for antiviral and antitumor therapy. Until recently, it was not possible to determine the primary structure of individual peptides because of their low abundance (fmol range) and the large number of very similar peptides that need to be characterized. While amino acid sequencing of pools of peptides allowed the definition of certain sequence motifs [52], mass spectrometry and, in particular, capillary LC-MS/MS provided further information allowing identification of a number of antigens by comparing partial sequence data with entries in databases (Fig. 15) [53]. More extensive analyses have also been performed on peptides derived from extracellular antigens that stimulate another subset of lymphocytes ($CD4^+$) when presented by MHC-II proteins [54]. A combination of Edman degradation and mass spectrometry (ESI and MALDI-TOF) was used to characterize more than 200 peptides from five different types of MHC-II proteins, illustrating the high throughput and the wealth of information that can be obtained.

LC-MS has also been successfully applied to the analysis of peptides and small proteins or protein fragments from the hemofiltrate of patients with renal disease [55]. Because of the multitude of peptides that are present in this complex biological fluid and the large volumes that need to be processed (2500 liters per batch), it is necessary to have efficient upstream fractionation methods as well as high-throughput analytical tools. Table 2 gives an overview of the peptides and proteins that were identified in a single HPLC fraction based on partial amino acid sequence data obtained by Edman degradation and mass measurements. It is foreseeable that on-line LC-MS/MS and comparison with the ever growing sequence databases will further accelerate identification of peptides in human body fluids and lead to a comprehensive database very much like those on

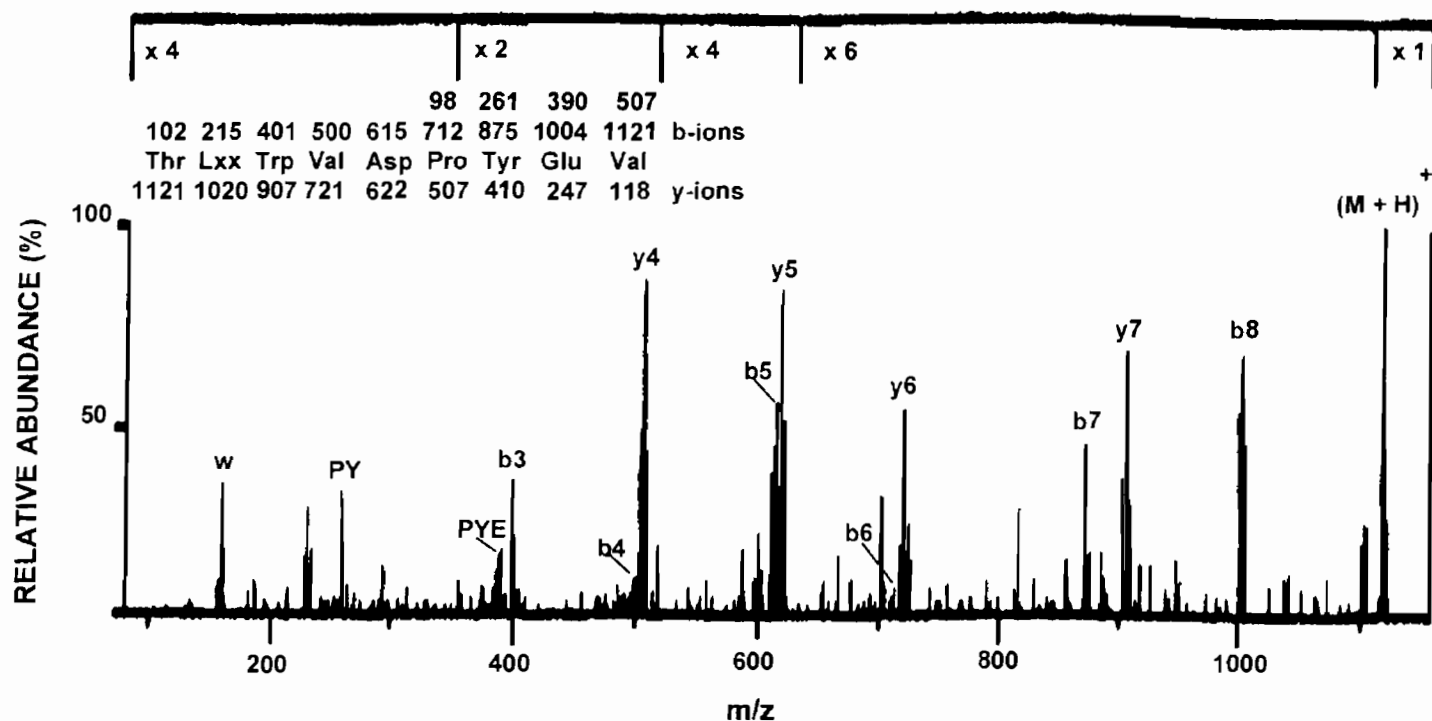


FIGURE 15 MS/MS of a peptide extracted from the MHC-I protein HLA-A2.1 (m/z 1121) following on-line capillary HPLC (estimated amount: 0.1–0.3 pmol). The fragment ion spectrum obtained (b- and y-ion series) allows reading the sequence T(L/I)WVDPYEV, which matches a sequence of TIS21 (TLWVDPYEV), a member of the primary response group of genes induced by growth factors. The identity of the peptide was subsequently confirmed by chemical synthesis and comparative analyses. (From Ref. 53.)

TABLE 2 Identification of Peptides in Human Hemofiltrate Following Fractionation by Cation-Exchange HPLC (Example of One Fraction)

Mass (Da)	Sequence (Edman)	Substance
1572	VVRAR.....AGVNY	Cystatin C (75–88)
2354	GTLSG.....DEAAF	Fibrinogen A α (479–500)
2428	DAHKS.....FKALV	Albumin (25–47)
2672	SYKMA.....KRGHA	Fibrinogen A α (581–605)
2862	MADEA.....KSRPV	Fibrinogen A α (584–610)
2898	Not investigated	—
2906	Not investigated	—
3019	ESKSY.....KRGHA	Fibrinogen A α (582–605)
3188	Not investigated	—
3402	EEVSG.....DKELR	Fibrinogen A α (395–424)
3708	AVGEY.....AGVNY	Cystatin C (56–88)
3841	Not investigated	—
3926	DHYNC.....AKCCK	h β -Defensin 1 (new)
4251	AEIEK.....QAGES	Thymosin β 4 (7–43)
4574	FTSST.....KRGHA	Fibrinogen A α (564–605)
5095	Not investigated	—
5425	FRDPC.....HGSVL	hMeprin α (New)
6257	Not investigated	—
6803	Not investigated	—
7637	Not investigated	—
7664	Unknown	New
7879	Not investigated	—
8178	MQIFV.....HLVLR	Ubiquitin (1–72)
8292	MQIFV.....LVLRL	Ubiquitin (1–73)
8564	MQIFV.....RLRGG	Ubiquitin (1–76)
9072	Not investigated	—
12917	Unknown	New

Source: Adapted from Ref. 55.

expressed proteins identified after separation by two-dimensional gel electrophoresis.

Analysis of Proteins after Gel Electrophoresis

The analysis of complex protein mixtures from tissue, cells in culture, or body fluids is a demanding task because of the wide range of physicochemical properties such as solubility, isoelectric point, and molecular weight; the possibility of protein-protein or protein-nucleic acid interactions; the chemical lability of certain amino acids; and, finally, the presence of membrane proteins

that necessitate optimal conditions for solubilization. Two-dimensional polyacrylamide gel electrophoresis is the most widely used method to separate such highly complex mixtures. Optimization and rigorous standardization of the operating conditions have opened the possibility of generating gels of high reproducibility that can serve as comparative standards and may allow the identification of proteins based on their position [4,57]. Reference gels from various tissues, cell types, or organisms have been generated and are gaining increasing importance in areas related to elucidating disease mechanisms, the discovery of new drug targets, and the study of the mechanism of action of therapeutic compounds at the molecular level. There is also interest in finding disease markers of diagnostic significance and predicting toxic side effects of drug candidates.

At the same time, rapid progress in DNA sequencing technology has led to some genomes being completely sequenced and others undergoing sequencing at a breathtaking pace. This opens new ways of deciphering disease-related mechanisms leading to the discovery of proteins that may be implicated in a disease and thus of interest for therapeutic intervention. While the analysis at the DNA or messenger RNA (mRNA) level has led to a number of important advances in the discovery of disease-related genes, it is of great interest to understand the function and regulation of the corresponding gene products. Following in the footsteps of the growing field of “genomics” in which analyses are performed at the mRNA level is therefore a new discipline called “proteomics” that attempts to correlate the observed changes with those at the protein level. Indeed, there is important information that can be obtained only at the protein level, such as the regulation of biological activity through post-translational modifications.

Analysis of proteins following gel electrophoresis is complicated by the relatively low amounts (low pmol to fmol range), the possibility of artifactual modifications, and the large number of samples to be processed. Furthermore, sample preparation is necessarily an off-line technique accompanied by the possibilities of sample loss and contamination [58]. The development of robotic systems for sample preparation may alleviate some of these limitations but will not overcome all of the problems [59].

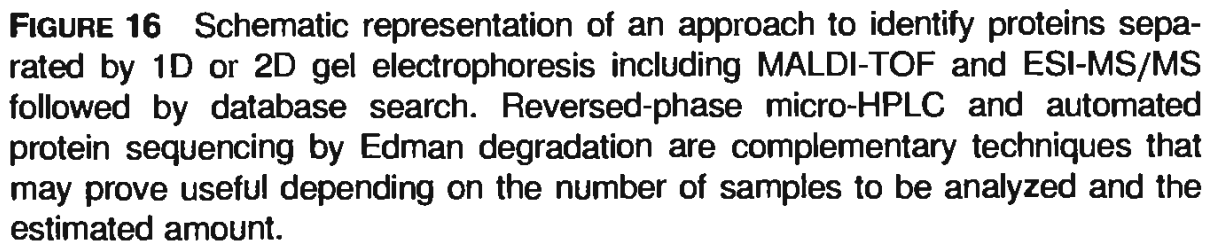
Protein identification following gel electrophoresis may be achieved in various ways depending on the amount of knowledge that is available concerning the protein(s) of interest. If a known protein needs to be detected and if specific antibodies are available, it is often easiest and least expensive to perform an immunoblot [60]. The success of this approach is, however, dependent on the primary antibody in terms of specificity (not specific enough: detection of unrelated proteins; too specific: missing of protein variants or modified forms of the protein of interest). The immunological approach, although very powerful and easy, does not allow absolute identification unless other techniques are available.

Protein sequencing by Edman degradation of gel-separated proteins has a long tradition and has contributed to the discovery of numerous unknown proteins of great biological significance. Proteins can be sequenced directly after transfer to an inert support such as polyvinylidene difluoride (PVDF) or they can be proteolytically digested on the membrane as well as in the gel to obtain peptide fragments for subsequent sequence analysis [61–64]. Proteolytic digestion is generally preferred because a large percentage of proteins, in particular those of mammalian origin, are N-terminally modified and thus not accessible to Edman degradation. Protein sequencing by Edman degradation necessitates thorough purification of the sample to avoid reading of multiple sequences, which will make interpretation of the results impossible. It is therefore imperative to employ efficient separation techniques to resolve the peptide mixture. RPC is certainly the most widely used method, and miniaturization has led to sensitivities in the pmol range.

There are two major reasons that limit the use of Edman degradation in proteomics: (1) the rather low sample throughput and (2) the limited sensitivity. The most advanced automated protein sequencers allow cycle times of about 30 min and experienced laboratories reach sensitivities of some 20 pmol of protein deposited on a gel followed by in-gel digestion, RPC, and Edman degradation. Mass spectrometry has overcome some of these limitations, but Edman sequencing remains a mainstay technology for protein identification and is often preferable when only a few samples need to be analyzed and the amount is sufficient. The great advantage of mass spectrometry lies in its greater sensitivity and its capacity to obtain sequence information from nonhomogeneous samples such as a protein digest due to the possibility of precursor ion selection. The continuing progress in mass spectrometric equipment is now being applied to the identification of proteins from 2D gels on a scale that was previously considered impossible.

Figure 16 gives a schematic representation of how mass spectrometry can be used to identify proteins that have been separated by either 1D or 2D gel electrophoresis. Sample preparation is in itself an important operation and greatly affects the outcome of the analysis. Complex mixtures of proteins are most often obtained from cell culture or tissue samples that need to be solubilized in buffers containing detergents and denaturants. The quality of the electrophoretic separation depends very much on this step, which serves to maintain all of the proteins in solution throughout the separation [65]. Indeed, it has been noted that membrane proteins are particularly difficult to recover in good yields, and this remains a challenge even for 2D gels.

Following separation, proteins need to be visualized, and not all staining methods are compatible with subsequent recovery of peptide fragments after proteolytic digestion. Furthermore, to make full use of the high sensitivity of mass spectrometry, staining needs to reach detection levels in the low- to subpmol



range. The most widely used technique is silver staining, which is generally applied following covalent fixing of proteins [66]. Covalent fixing, however, is not compatible with efficient recovery of peptides from gel-separated proteins. Modifications that avoid the covalent fixing step have been described and applied to protein analyses by mass spectrometry [67]. The active search for other sensitive staining techniques that are compatible with mass spectrometry of the recovered peptides has found a number of alternatives such as a reverse stain with Zn^{2+} or Cu^{2+} ions or noncovalent fluorescent stains [68,69]. It is likely that there is no one universal staining technique that works well at this level for all proteins. Generally speaking, highly glycosylated proteins are more difficult to stain and proteins below 15 kDa may be partially lost from the gel during the washing procedures unless the fixing step is adapted. Furthermore, some proteins may be quite resistant to proteolytic digestion even under denaturing conditions

because of their compact structure, the maintenance of internal disulfide bonds, and/or their highly glycosylated nature. Reduction and alkylation in the gel may be a way to overcome such problems. Extraction of the peptides from the gel is another step that may lead to important losses and is often rather work intensive. The goal is to reduce the volume in which peptides are recovered in order to increase the concentration and also to reduce the amount of gel-related contaminants, which may interfere with mass spectrometry. It has been elegantly shown that sonication of the gel slice is sufficient to extrude a small volume of liquid for subsequent analysis by MALDI-TOF [70]. Enrichment and cleanup of digests by solid-phase extraction can also enhance sensitivity by MALDI-TOF considerably [71].

There are a number of options for analyzing a peptide mixture extracted from a gel by mass spectrometry. The most widely used techniques are MALDI-TOF, preferably with delayed extraction, and ESI with a triple-quadrupole analyzer or a combination of quadrupole and TOF analyzers. The combination of ESI with an ion trap is also of great interest due to its sensitivity and the possibility of performing structural studies by MSⁿ. Downsizing of the electrospray capillary such as in the nanospray technique results in another gain in sensitivity and an extension of the measuring time, which is important to optimize fragmentation conditions.

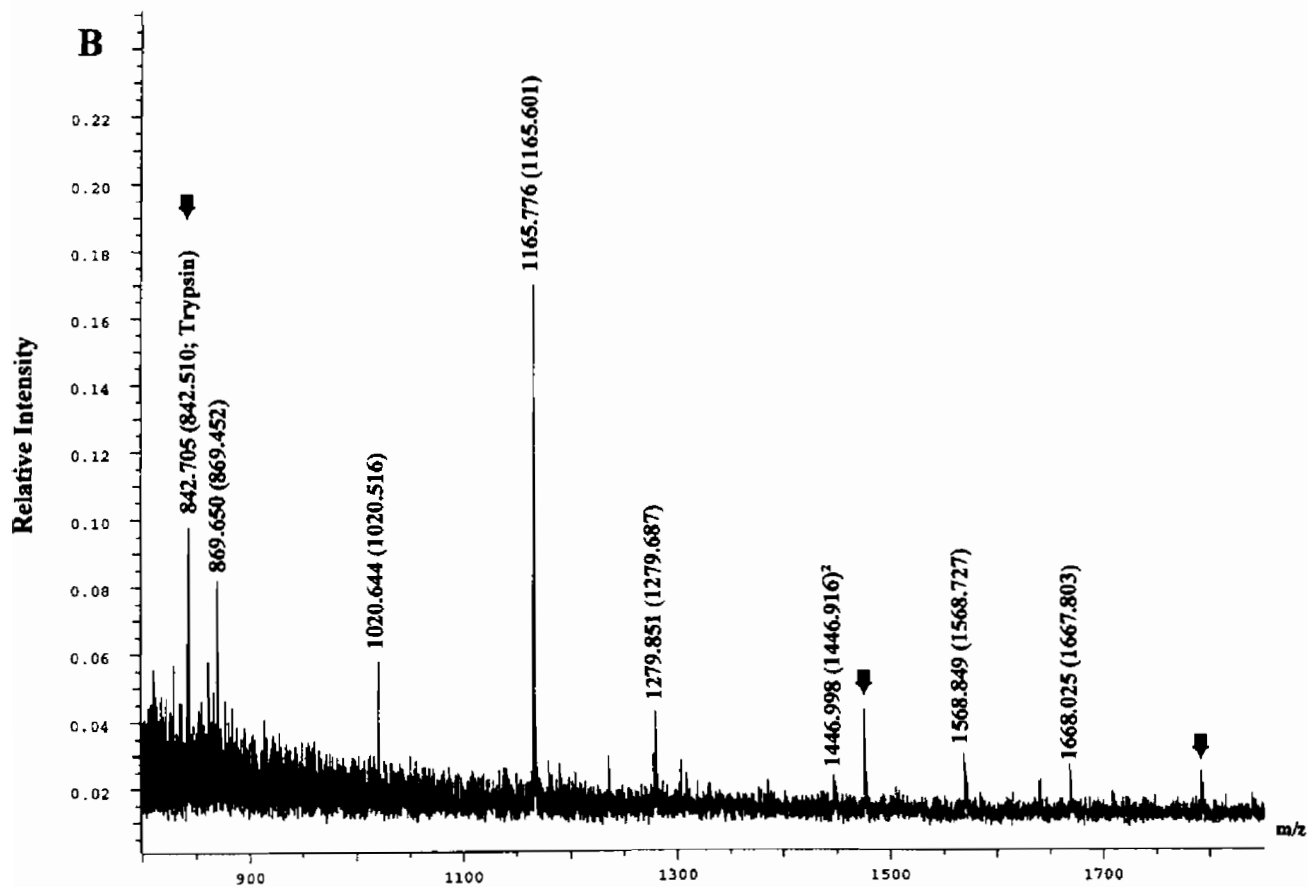
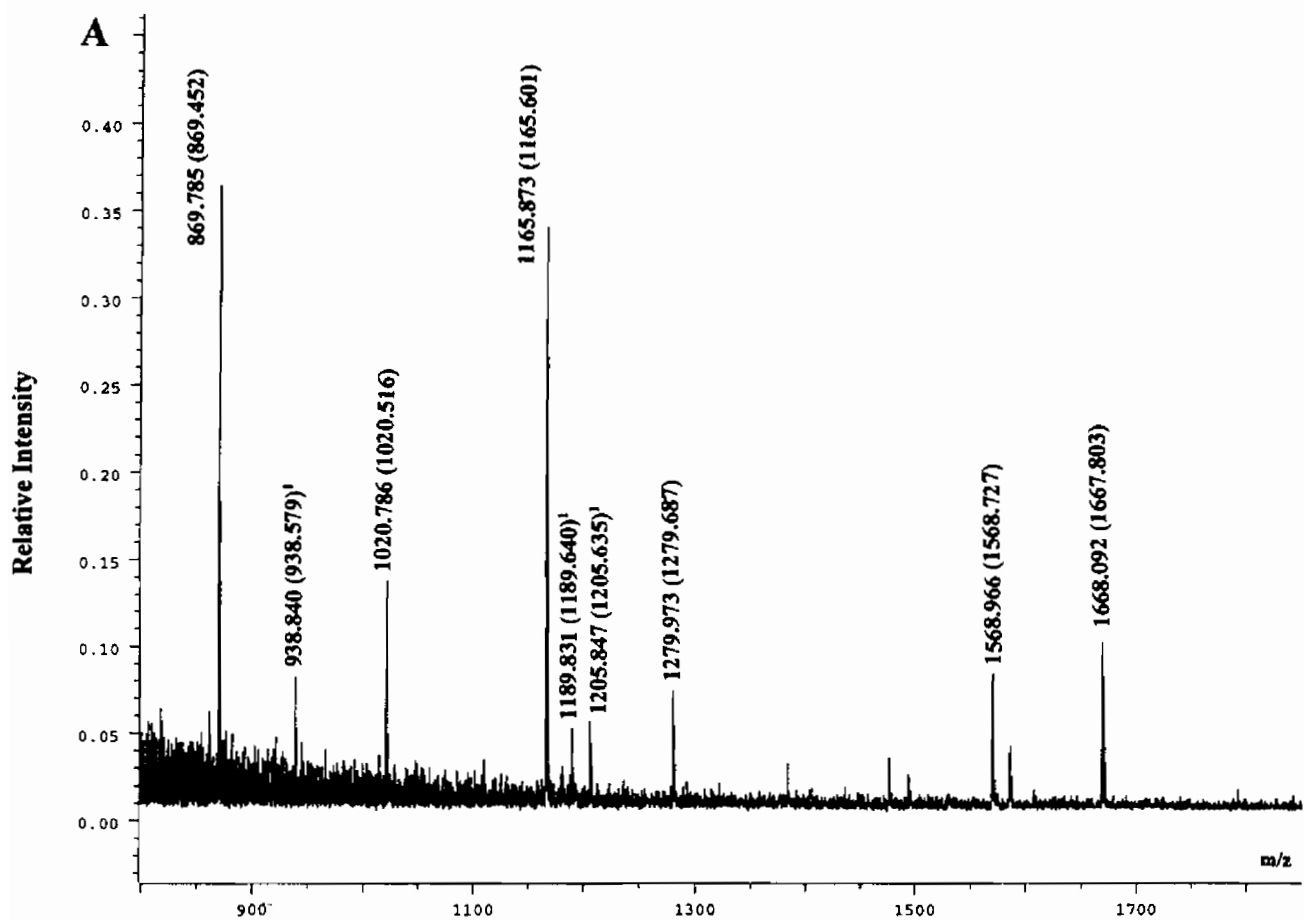
The following example shows that proteins can be identified on the basis of precise mass measurements on peptides obtained after in-gel proteolytic digestion with as little as 2 pmol deposited on the gel. Fifty nanograms of rSmp28 (23 kDa) was digested with trypsin after reverse staining with Zn²⁺ ions. Peptides were extracted by sonication [70] and their monoisotopic masses determined by DE-MALDI-TOF. Mass matching with calculated molecular masses of peptides from sequence databases was performed using an on-line search algorithm (<http://prospector.ucsf.edu/htmlucsf/msfit.htm>). The comparison of 2 pmol of rSmp28 digested in solution and in a gel following 1D electrophoresis (shown in Fig. 17) shows that gel electrophoresis and staining did not lead to extensive protein modifications or interfere with proteolysis. It is, however, evident that the signals were considerably lower after gel electrophoresis, probably because of protein loss during electrophoresis, incomplete recovery of peptides from the gel slice, and possibly some suppression of ionization due to gel-related contaminants. Nevertheless, even such low amounts allow unambiguous assignment of the protein to Smp28 based on a match between seven peptide fragments using a mass window of ± 0.5 Da. The higher signal-to-noise ratio of the spectrum from rSmp28 digested in solution allowed matching nine peptides, which is sufficient to identify the protein with confidence. It should not be overlooked that not all of the major peaks could be assigned to peptide fragments from rSmp28. The analysis of the in-gel digestion, in particular, resulted in a number of signals that did not correspond to expected mass values (see arrows in Fig. 17B). One of

those signals was derived from trypsin due to autodigestion, although a modified enzyme was used that should have reduced this phenomenon. Such peptide fragments may, in fact, serve as internal calibration standards, thus improving mass accuracy. Submitting three unknown m/z values (labeled with arrows in Fig. 17B) to a database search using the same criteria resulted in a match with an irrelevant protein, indicating the limitations of this approach. Higher mass accuracy and thus more stringent search criteria might have avoided such a false positive.

Mass matching alone may not be sufficient to identify a protein for a number of possible reasons: (1) the protein sequence is not represented in the database; (2) the protein is posttranslationally modified, thus reducing the number of matches, as the databases generally do not contain sufficient information on such modifications; (3) the protein was chemically modified during gel electrophoresis (some search algorithms allow inclusion of commonly observed modifications such as oxidations of methionine or the addition of SH groups to acrylamide); or (4) the number of matched peptides may not be sufficient to discriminate between different candidates depending on the accuracy of the mass measurements [40]. At any rate, some doubt as to the correct identification may persist, and it is of great importance to obtain sequence information from the peptides to increase confidence. Partial sequence information, also referred to as "sequence tags," can be obtained by MS/MS or MSⁿ using, for example, a triple-quadrupole analyzer, a combination of a quadrupole with a TOF analyser, or an ion trap. Database searches with some sequence information provide much higher stringency and often allow identification of proteins unambiguously [72,73].

Multidimensional chromatographic systems may represent an alternative to 2D gel electrophoresis for the separation of complex protein or peptide mixtures. Indeed, multidimensional chromatography is the most widely used methodology for the purification of biomolecules; however, such separations may take days and need to be optimized for each individual molecule. In addition, they often require considerable amounts of starting material depending on the initial abundance of the protein in question and the losses that occur at each purification step. Purification of a given biomolecule is often followed by a specific bio- or immunoassay and enrichment is evaluated by gel electrophoresis or specific activity. Although this approach can be accelerated by using stationary phases with improved mass transfer properties, the principal problem of having to optimize separation conditions individually remains. Furthermore, chromatography has been less successful for membrane proteins that need surfactants for solubilization.

Despite these difficulties, there are a number of approaches to automate multidimensional chromatographic separations and to couple them to mass spectrometry. Column switching that combines, for example, cation exchange with reversed-phase HPLC, followed by on-line ESI mass spectrometry, has been



successfully applied to soluble *Escherichia coli* proteins [74]. Such systems have the potential to be fully automated, thus improving sample throughput and reproducibility, criteria that are of great importance in the context of proteomics, where reliable comparative analyses are critical. Although coupled column chromatography systems have not found the general applicability of 2D gel electrophoresis, they may find more widespread use in the case of soluble proteins from expressed complementary DNA (cDNA) libraries or in body fluids [55,56]. Future improvements in separation technology and work on interfacing different methods such as capillary electrophoresis with HPLC, in combination with miniaturization and highly sensitive mass spectrometry, may well hold promise for the efficient analysis of biomolecules in complex biological matrices [75,76].

Oligonucleotides and Nucleic Acids

The analysis of chemically synthesized oligonucleotides and nucleic acid fragments by mass spectrometry has seen major advances. Again, new ionization techniques and improved equipment have opened possibilities for precise mass measurements of these polyanionic macromolecules that allow confirming their primary structure after nucleic acid sequencing by the Sanger method or analysis

FIGURE 17 DE-MALDI-TOF analysis of tryptic peptide digests of 50 ng rSmp28 (~2 pmol) in solution (A) or after 1D gel electrophoresis (B). The solution digestion was performed with 25 ng of modified trypsin (Promega) and the in-gel digestion with 100 ng trypsin. Proteins were revealed by negative staining using Zn^{2+} [68]. Samples were deposited on the target according to Kussmann et al. [33] using α -cyano-4-hydroxycinnamic acid (see Fig. 4). An external calibration was used and the given values correspond to the monoisotopic mass. The expected mass values are given in parentheses. Mass matching with entries in the SwissProt database release 34 (<http://prospector.ucsf.edu/htmlucsf/msfit.htm>) using a mass window of ± 0.5 Da and a molecular mass window of 10–50 kDa results in 9/9 matches with Smp28 for the solution digest (39% coverage of the sequence; the peptide at m/z 1189.831 is matched when considering one oxidized methionine), while 7/7 matches are obtained for the in-gel digest using the same search criteria (36% coverage of the sequence). When considering the m/z values labeled with arrows as unknowns and searching with the same criteria, 3/3 matches were obtained with human neutrophil cytosol factor 2, which is an erroneous result. It is also noteworthy that the measured values are 0.1–0.3 Da higher than the expected mass, indicating a systematic error of about 150 ppm in mass accuracy possibly related to the fact that an external calibration standard was used. Peaks labeled with ¹ in (A) correspond to peptide fragments that were observed only in the solution digest while the peak labeled with ² in (B) was observed only in the in-gel digest.

of double-stranded DNA fragments generated by PCR [77–85]. A number of developments in the biomedical field have also contributed to these advances, in particular the use of oligonucleotides as potential therapeutic agents [86,87] and the need for the rapid analysis of DNA fragments in large-scale DNA sequencing projects, genomics, and diagnostic genetic analyses [88–92].

The development of efficient mass spectrometric methods for oligonucleotides has been more difficult than for peptides because of their polyanionic nature and the ensuing strong affinity for metal ions such as sodium, potassium, or magnesium. This adduct formation resulted in large signals and low resolution as well as in low mass accuracy. Sample pretreatment with ammonium acetate followed by ethanol precipitation greatly improved the quality of spectra obtained by ESI, providing confirmatory data on chemically synthesized oligonucleotides up to at least 100-mers (~ 33 kDA) [79] (Fig. 18). Mass spectrometry is one of the few techniques that also allows confirmation of the incorporation of modified nucleotides that are of importance in most antisense applications. Adduct formation also proved to be a problem for MALDI of oligonucleotides. Again, replacement of nonvolatile cations with ammonium ions improved spectral quality significantly [93]. Furthermore, the systematic optimization of numerous matrices has led to improvements that allow the routine analysis of double-stranded DNA fragments, for example, to map single-nucleotide polymorphisms (SNPs). Picolinic acid and 3-hydroxypicolinic acid are two of the preferred matrices for the analysis of nucleic acids by MALDI-TOF (see Fig. 4) [94].

Mass spectrometry by either ESI or MALDI is widely used in the field of antisense and ribozyme therapeutics. It is pivotal for every molecule that is destined for human use to be able to ensure that the product corresponds to the expected structure and that contaminants are absent above a defined level. Furthermore, if contaminants are present, they need to be characterized. These requirements are met by using highly efficient separation methods such as CE and HPLC in combination with techniques that provide the necessary structural information. Mass spectrometry is a cornerstone technique in this respect. It has been shown that oligonucleotides separated by RPC can be analyzed by ESI-MS online [17] (Fig. 19). More detailed structural information can be obtained when combining enzymatic reactions, such as phosphodiesterase digestion from the 3' or 5' end with MALDI-TOF analysis (95). Such analyses permit sequencing the chemically synthesized oligonucleotide, an otherwise rather arduous and lengthy procedure.

DNA fragment analysis is widely performed by polyacrylamide or agarose slab gel electrophoresis. In particular, DNA sequencing by the Sanger method relies on the precise migration and high resolution of DNA fragments in thin polyacrylamide gels and labeling of each of the four dideoxynucleotides. Today, most of the high-throughput sequencing projects employ fluorescent labeling and laser-induced detection of the emission from the different fluorophores following

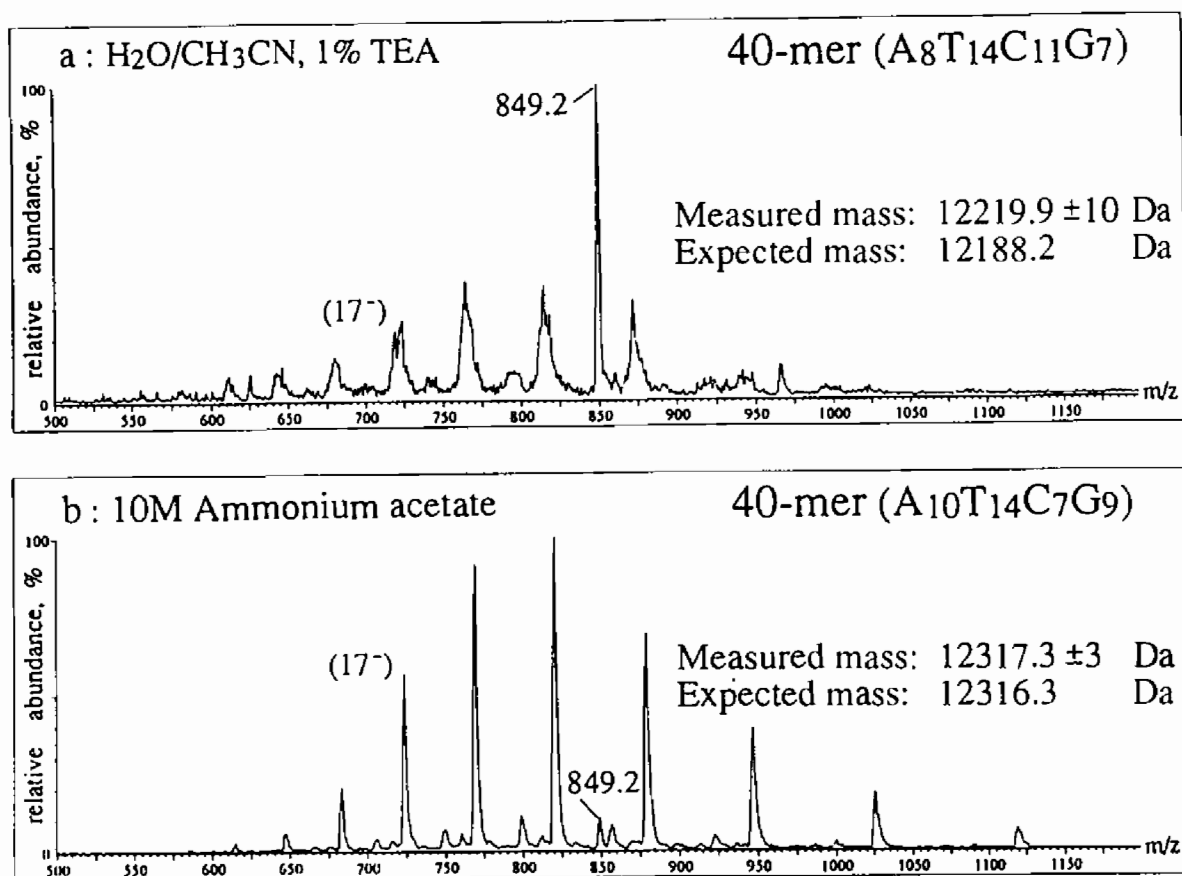


FIGURE 18 Negative ESI mass spectrometry of two 40-mer oligonucleotides without (a) and with prior treatment with ammonium acetate to replace alkali metal ions (b). Replacement of metal cations with ammonium ions results in a significant improvement in both mass accuracy and resolution. The signal at m/z 849.2 corresponds to the internal standard that was infused simultaneously. (From Ref. 79.)

electrophoretic separation of end-labeled fragments. In order to be applicable to DNA sequencing, mass spectrometry needs to assign a difference between the four nucleobases adenosine, guanosine, thymidine and cytosine. Gel electrophoresis allows sequencing 20,000–30,000 bases per gel, but separation is rather slow. Throughput has been greatly increased by using CE in a multiarray format culminating in the recently completed draft of the human genome [96], (<http://genome.ucsc.edu/>).

Mass spectrometry, particularly MALDI-TOF, is finding more widespread applications in the fields of SNP mapping and pharmacogenomics in general [97,98]. The challenge for mass spectrometry is to ionize large DNA fragments while maintaining a resolution that permits discrimination between the n and $n + 1$ fragments. Analysis of shorter fragments may prove to be a viable alternative, because mass spectrometry is a much faster technique than gel

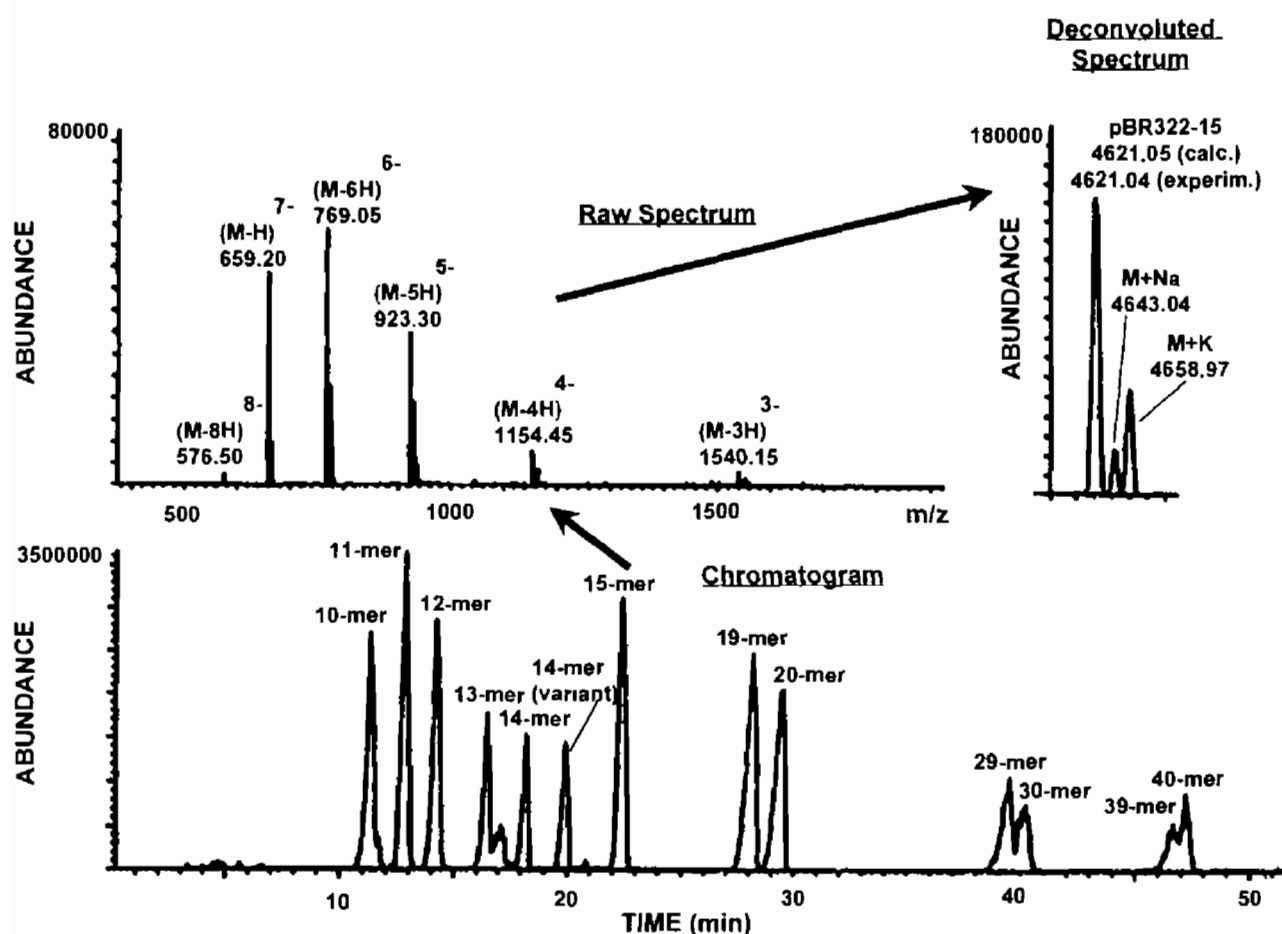


FIGURE 19 Analysis of oligonucleotides by LC-MS using a C_{18} reversed-phase stationary phase and a gradient from 100 mM triethylammonium acetate, pH 7/ acetonitrile to 400 mM hexafluoro-2-propanol/methanol at 0.2 mL/min (column: 2.1×250 mm). The upper panel shows an ESI spectrum acquired on line of a 15-mer, and the lower trace represents the chromatogram (TIC) acquired during the LC-MS run. Deconvolution of the spectrum allows discrimination between the free 15-mer and its sodium and potassium adducts. (From Ref. 17.)

electrophoresis. Some results have shown that this may be achievable and that such an approach may be amenable to automation, an important feature when considering high sample throughput [82,83] (Fig. 20). Requirements on resolution are less demanding when analyzing DNA fragments obtained by PCR amplification. Because the reaction itself provides a high degree of specificity due to hybridization between the oligonucleotide primers and the target sequence, it is often sufficient to estimate the size of the obtained fragment by agarose gel electrophoresis in comparison with standards of known size. Sequencing of the DNA fragment and hybridization with a labeled probe by Southern blotting are further possibilities to increase the level of confidence. Although of very high

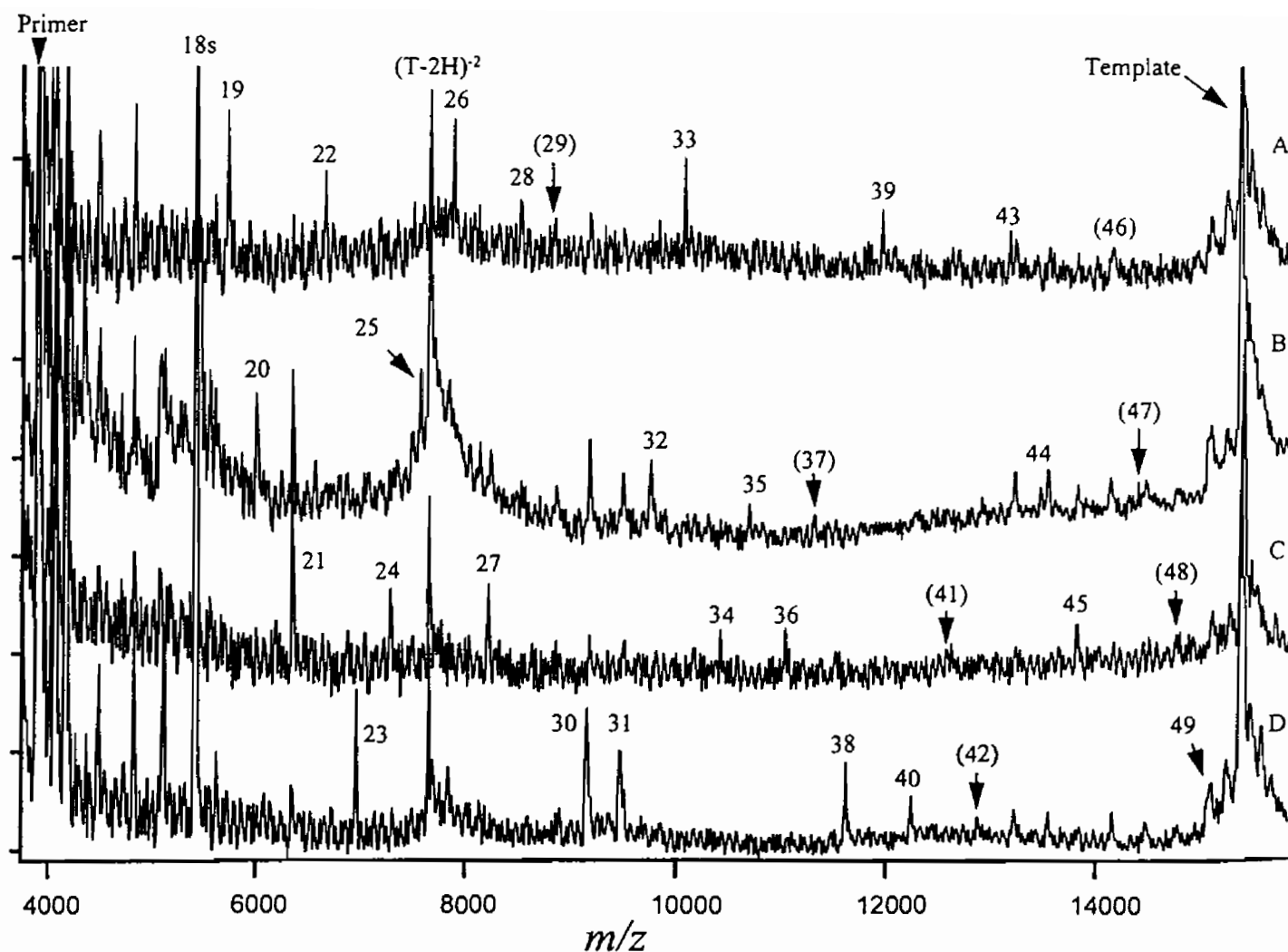


FIGURE 20 DE-MALDI-TOF spectrum of a mixture of Sanger dideoxy DNA sequencing reactions containing about 0.1 pmol of each termination fragment generated by primer extension on a 50-base-pair template. The matrix used was a mixture of 3-hydroxypicolinic acid and picolinic acid including diammonium citrate to complex metal ions (prepared according to Wu et al. [98]). The four panels correspond to termination with (A) ddA, (B) ddC, (C) ddG, and (D) DDT. (From Ref. 82.)

discriminatory power, these methods are slow and labor intensive and thus very costly in view of routine applications in genetic diagnostics. Mass spectrometry with its high sample throughput and the rapid generation of data might be a viable alternative, in particular when combined with sample preparation by laboratory robots. The improvements in mass accuracy and resolution due to delayed extraction have already given some promising results [84], and it will be interesting to follow the future of mass spectrometry in this burgeoning field.

CONCLUSIONS AND FUTURE PERSPECTIVES

This chapter has highlighted a few applications of mass spectrometry in the characterization of proteins, peptides, and polynucleotides. The field is so large and the number of published reports so vast that total coverage is impossible in this short review. The goal of this chapter was thus to show with selected examples the possibilities of mass spectrometry in combination with separation methods, notably HPLC, and to refer the reader to more specialized reviews or publications for further information. Clearly, the biotechnology industry would not be where it is today without the use of mass spectrometry, and research into the discovery of new targets for therapeutic intervention relies more and more on this technique which has seen tremendous growth. Indeed, the future looks bright for mass spectrometry in life sciences, as a number of developments such as delayed-extraction MALDI, the combination of quadrupole or magnetic sector with TOF mass analysers, and the ion trap are only beginning to penetrate into the various research laboratories. It is to be expected that the intricate combination of highly efficient separation tools with mass spectrometry integrated into an environment of growing knowledge from databases and the use of bioinformatics will lead to a better understanding of biological processes and possibly to the development of better drugs for the treatment of disease. It is because of such highly efficient methods that we can now study complex biological systems at the molecular level in a realistic time frame.

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Detection and Analysis of Proteins by High-Performance Liquid Chromatography with Photodiode Array Detection

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INTRODUCTION

Spectroscopic analysis of proteins is nearly as old as spectrophotometers themselves. In the 1930s the first generation of laboratory-built ultraviolet-visible (UV-Vis) spectrophotometers were used to study proteins even before spectrophotometers were commercialized. In the earliest work, strong absorbance at 280 nm, due to the aromatic content of proteins, was the basis of detection in many protein-containing samples. The commercial availability of scanning spectrophotometers in the early 1950s provided an important tool for fundamental studies of protein structure and function. Quantitation of protein aromatic content via spectrophotometry was first proposed by Goodwin and Morton [1]). Beaven [2,3], Wetlaufer [4], and Donovan [5] have written comprehensive reviews of the early spectroscopic studies of peptides and proteins.

Two fundamental contributing structures determine protein spectra. The amide (peptide) bonds that compose the linear head-to-tail arrangement of the polypeptide chain have strong absorbance in the deep UV with an absorbance

maximum near 190 nm. The other major contribution stems from the individual absorbances of the amino acid side chains. The three aromatic side-chain amino acids, Tyr, Trp, and Phe, provide the vast majority of protein absorbance above 240 nm, with only a minor contribution from Cys-Cys disulfide bonds. Trp also significantly augments absorbance in the far UV (200–230 nm), with smaller contributions from the side chains of His, Met, Tyr, Phe, and Cys. There is a rich history of protein research based on protein absorbance in the aromatic region, and studies with photodiode array (PDA) detectors are often extensions of classical studies in static systems to separation-based HPLC experiments.

In the 1960s changes in the UV spectrum as a function of protein structure came under intense study. Characteristic alterations in the aromatic region were used to monitor protein denaturation. Both kinetic and thermodynamic parameters for the unfolding process can be studied via spectrophotometry. The basis for the spectral changes has been attributed to the influence of the environment in the vicinity of Tyr and Trp on their absorbance. In the native state, these relatively hydrophobic amino acids are most often buried inside the hydrophobic protein core, but upon denaturation, they become exposed to the aqueous buffer environment. Model studies with aromatic amino acids, their analogs, or small peptides containing Phe, Tyr, and Trp demonstrated the environmental effects of a hydrophilic versus hydrophobic solvent system.

Derivative spectroscopy was introduced half a century ago [6] as a method for resolving two overlapping absorption bands. It was most commonly applied to gas chromatographic analysis in the 1960s and early 1970s [7], and Ichikawa and Terada [8,9] reported the first studies using second-derivative spectra to resolve the overlapping bands of the aromatic amino acids in proteins. Although this study explored only the ability to quantitate Phe in proteins containing all three aromatic amino acids, one important contribution was the use of the acetylated amino acid ethyl esters of Tyr, Trp, and Phe as model compounds to provide a detailed analysis of the second-derivative spectra. Of particular interest in later studies was the observation that Trp and Tyr have characteristic minima in their derivative spectra at 289 and 283 nm, respectively.

Balestrieri et al. [10] expanded on this earlier work by developing a method for the full analysis of all the aromatic residues using the second-derivative spectrum. Significantly, they noted the effect of the Trp/Tyr ratio on the magnitude of various minima in the second-derivative spectrum. This phenomenon has been extensively exploited in HPLC-based studies described later in this chapter.

These classical-type cuvette-based studies are still in widespread use today. Spectrophotometers are both common in the biochemical laboratories and easy to use. Spectral resolution is excellent (typically <1 nm) and the data easily collected and analyzed with modern computer-based systems. One major drawback of these instruments is that the observed spectrum is a weighted average of all

species present. Thus, samples with impurities, absorbing buffer constituents, mixtures of proteins and peptides, and samples containing multiple related structures cannot be fully characterized by homogeneous analysis. The past 15 years have seen a rapid increase in the use of separation-based systems using HPLC with PDA detection for such complex samples. Often, HPLC can resolve the components in a heterogeneous system and modern PDA detectors can provide significantly increased sensitivity for low-concentration samples.

THE DESIGN OF PDA DETECTORS FOR HPLC

HPLC systems employ UV-Vis absorption detection more than any other technique. Most molecules, including peptides and proteins, absorb at wavelengths above the atmospheric oxygen cut-off (185 nm). Common mobile phases, water, acetonitrile, and methanol are transparent above 220 nm. Moreover, the technique is sensitive, requiring no more than a picomole in the cell to quantitate a moderately strong absorber.

Individual compounds absorb UV, and less commonly visible light, at characteristic wavelengths. The power of a PDA detector is to record analyte spectra during the elution of a peak. The raw measurement is the optical power transmitted through the flow cell as a function of wavelength. This is a strongly nonlinear function of concentration. To be useful to an analyst, the detector's output is presented in dimensionless absorbance units, equal to the common logarithm of the ratio of transmitted light with solvent only in the cell to sample *plus* solvent in the cell. Absorbance is proportional to the product of sample concentration and path length. A good absorbance detector is linear over five decades of concentration.

In HPLC analyses, spectra are most commonly used to assess peak purity and for qualitative identification by searching against stored libraries. Spectra are also useful for qualitatively categorizing a component based on its characteristic spectrum. This is a common approach with peptides and proteins that exhibit strong absorbance in the 200–230 nm range and, if they contain aromatic amino acids, have a smaller peak typically with a maximum around 280 nm. Absorbance at a specific wavelength is used for quantitation. The PDA detector has become the workhorse detector for analytical problem solving and methods development and is growing in acceptance for quality control. Even when a mass spectrometer is the primary detector for identification, a PDA detector is indispensable for quantitation and system diagnostics.

During the 1970s and early 1980s the need for full spectrum detection was clear, but the technology was still being developed. Today, high-quality diode arrays are available from several vendors; fast, efficient, flat-field concave holographic gratings exist; and adequate computing power is available off the shelf. Thus, PDA detectors are now mature products.

Basic Design Principles: Optical Layout

In this section, we concentrate on the underlying principles that lead to optimal design, commenting on some of the alternative approaches taken by different manufacturers. Comparative specifications are given in Table 1. First we review the generic layout of a PDA detector. Figure 1 is a schematic to show the principal components and the order in which they are placed. A deuterium arc lamp is the universal choice for the UV light source because of its high, spectrally smooth output and low noise. A number of manufacturers add a tungsten halogen lamp to boost signal in the visible and near infrared (NIR), where D₂ lamp output is low. In some detectors a small amount of light is split off ahead of the flow cell to monitor lamp output. This reference signal is used to reduce the effect of lamp fluctuations in the diode array signals, improving baseline noise and drift.

Another method, employed in the Agilent series 1100 diode array detector, makes use of a user-selected portion of the array as a reference, typically at wavelengths beyond the absorbing region of the analytes. This has the advantage of reducing pump or refractive index artifacts in the flow cell but errors result if material that absorbs at the reference wavelengths elutes unexpectedly. In a well-defined application, the user can choose the reference wavelength to suppress the absorbance of some selected peak.

Spectrograph Design

The most common spectrograph design consists of a single optical element, a concave holographic diffraction grating. It both images the entrance slit onto the plane of the diode array and disperses the wavelengths across the array. The grating images the slit onto the array with roughly unit magnification so that spectral resolution is nominally the slit width in diodes times the number of nanometers per diode. The latter number is simply the wavelength range of the detector divided by the number of diodes in the array. For example, an array of 512 diodes, each 50 μm wide, covering the range 190 to 800 nm with a 100- μm -wide slit, has a nominal resolution given in Eq. (1):

$$(100/50) \times (610/512) = 2.4 \text{ nm} \quad (1)$$

For the example given, the resolution is twice the “diode resolution” of 1.2 nm. Generally, the focal plane of the grating is a gentle curve rather than flat, so that the slit can be in perfect focus at only a couple of points in the spectrum. At other wavelengths, the resolution can be up to a nanometer worse than the nominal resolution.

Most PDA detectors have a short-wavelength limit of 190 nm. Some extend just through the UV to around 350 nm, but most go further to 600, 800, or 950 nm (see Table 1). The majority of analytes absorb in the range 190 to 350 nm and are

TABLE 1 Manufacturers and Specifications of Current Photodiode Array Detectors

Manufacturer and model	Light source	No. of diodes	Wavelength range (nm)	Wavelength accuracy (nm)	Optical resolution (nm)	Noise ×	Drift	Linearity	Standard Flow cell (Pathlength, volume)
Agilent 1100	Deuterium and tungsten	1024	190–950	±1	1.0	±1.0 × 10 ⁻⁵ AU at 254 nm	2 × 10 ⁻³ AU/hr at 254 nm		10 mm, 8 µL
Beckman/Coulter 168	Deuterium	512	190–600	±1	< 2	±1.0 × 10 ⁻⁴ AU	2 × 10 ⁻³ AU/hr	1% to 1.5 AU with acetone at 256 nm > 2 AU	10 mm, 11 µL
Dionex PDA-100	Deuterium and tungsten	1024	190–800	±1	1.0	1.0 × 10 ⁻⁵ AU at 254 nm, flowing water	5 × 10 ⁻⁴ AU/hr		9 mm, 10 µL
Groton Technology 20/20	Deuterium and tungsten-halogen	512	190–700	±1	4	±2.0 × 10 ⁻⁵ AU peak-to-peak at 254 nm	0.5 × 10 ⁻³ AU/hr at 254 nm		10 mm, 12 µL
Hitachi L-7455	Deuterium	512	190–800	±1	1	±1.5 × 10 ⁻⁵ AU at 240–260 nm	1 × 10 ⁻³ AU/hr at 240–260 nm		10 mm, 18 µL
Jasco MD-1515	Deuterium and halogen	512	200–900			±0.7 × 10 ⁻⁵ AU at 250 nm	1 × 10 ⁻³ AU at 250 nm	Up to 2.56 AU	10 mm
PerkinElmer Series 200	Deuterium and tungsten	512	190–700	±1	4	±1 × 10 ⁻⁵ AU at 254 nm	1 × 10 ⁻⁴ AU/hr at 254 nm	1.5 AU at 254 nm	10 mm, 12 µL
Shimadzu SPD-M10A VP	Deuterium and tungsten	512	190–800	±1	1.2	±0.8 × 10 ⁻⁵ AU/hr at 250, 600 nm	< 1 × 10 ⁻³ AU/hr at 250, 600 nm		10 mm
ThermoQuest UV6000LP	Deuterium and tungsten-halogen	512	190–800	±1	1.2	±0.3 × 10 ⁻⁵ AU at 254 nm	1 × 10 ⁻³ AU/hr at 254 nm	2 AU at 254 nm, < 5%	50 mm, 10 µL
Varian ProStar 330	Deuterium	512	190–800	±1 at 253.7 nm	1	±1.0 × 10 ⁻⁵ AU at 250 nm	1 × 10 ⁻³ AU	1.6 AU at 260 nm	10 mm, 18 µL
Waters 996	Deuterium	512	190–800	±1	1.2	±1.5 × 10 ⁻⁵ AU, peak-to-peak, dry flow cell	1 × 10 ⁻³ AU/hr at 254 nm	5% over –0.1 to 2.0 AU	10 mm, 8 µL
Waters CapLC	Deuterium	512	190–800	±1	2.0	±1.0 × 10 ⁻⁴ AU, peak-to-peak, flowing methanol		1.0 AU	5 mm, 0.25 µL

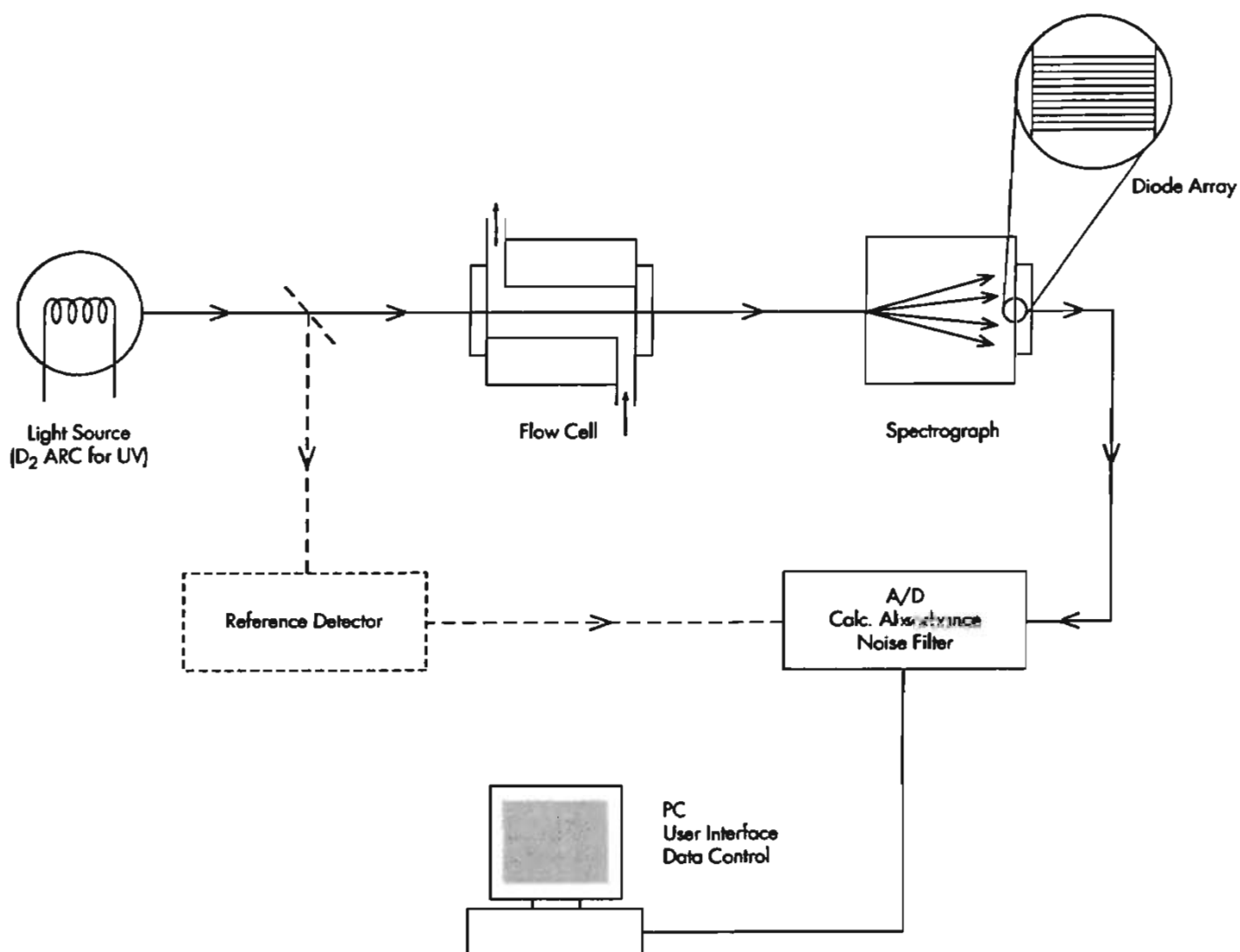


FIGURE 1 Schematic of the principal components of a photodiode array detector. This diagram shows the normal order for the optical components in most PDA detector designs.

transparent in the visible (400 to 700 nm) and NIR. It is instructive to note that according to Eq. (1), users who work exclusively in the UV, but nevertheless demand these wide wavelength ranges, do so at the expense of performance in the UV.

Note that the flow cell is invariably placed ahead of the spectrograph so that it can be located where the beam is compact. This is the opposite of the arrangement in tunable UV-Vis (TUV) detectors, where the monochromator precedes the flow cell. Because TUVs predated PDA detectors, the PDA order of components is frequently referred to as “reverse optics.”

Flow Cell Design

A logical starting point for the designer is the flow cell. It is the focus of decisions that affect both chromatographic resolution and detector sensitivity. For high-performance LC detection, desirable features are:

1. A long path length for high sample absorbance—but only when mobile phase absorption is low
2. High optical throughput for low noise
3. A small volume for low band spreading or dispersion
4. Low sensitivity to sample or mobile phase refractive index change

Clearly, these factors are not all independent.

Optical Throughput and Baseline Noise

Diode array pixel signals are proportional to optical throughput, which is a purely geometric property of an optical system. It is measured as the cross-sectional area of a limiting aperture, such as the flow cell, times the solid angle of rays passing through that aperture. We count only rays that pass through the whole system to be measured by the photodetector. For a design with the best combination of the preceding factors, the cell is the optical bottleneck, and we ensure, as far as possible, that optical throughput is not limited by any other component of the PDA detector.

High optical throughput is clearly in direct conflict with small volume and long path length. How high should the throughput be? In the following we show how a designer can start with a baseline noise target and optimize the cell parameters for a given application.

Any flow of discrete entities, for example, light (photons) or an electric current (electrons), is never completely uniform. Statistical probability dictates a level of fluctuation about the average flow. These inherent fluctuations are called shot noise and are proportional to the square root of the average flow. It is not within the scope of this chapter to discuss all the sources of noise that may contribute to a detector's baseline. Suffice it to say that shot noise is inescapable,

but other sources of noise (fluidic, lamp instability, amplifier noise) can, in principle, be controlled by good engineering so that their effect on the baseline is less than that of the shot noise over most of the working range of the instrument. Fluctuations in an electrical current, irrespective of their source, can be reduced by filtering. Filtering attenuates the high-frequency components, but narrow chromatographic peaks are flattened and distorted if the time constant is too long. Baseline noise in an absorbance detector is proportional to the noise-to-signal ratio of the current generated by the photodiodes. Consequently, in a well-engineered detector where shot noise dominates, the baseline noise is inversely proportional to the square root of the photodiode signal.

Flow Cell Parameters for a Target Baseline Noise

Because shot noise is directly calculable from the magnitude of the diode array pixel photocurrent, the design engineer can work backward and determine the photocurrent needed to achieve a target baseline noise. In turn, because the properties of key detector components are well characterized (light source spectral radiance, grating efficiency, detector array quantum efficiency, etc.), it is possible to determine the optical throughput needed to achieve a given photocurrent. Etendue is another name for optical throughput. Suitable units are $\text{mm}^2 \text{ sr}$.

If sensitivity (a low concentration limit of detection) is most important, we initially specify the path length, and this in combination with the etendue determines the cell cross section and volume needed to achieve a certain baseline noise. Or if low band spreading is essential, we start with the maximum tolerable cell volume, which fixes the path length.

In this way, the designer can choose the flow cell geometry and predict with confidence a yet-to-be-built detector's baseline noise. For this strategy to work, images of the light source must be propagated through the system with minimal aberrations, and rays that pass through the cell must not be lost elsewhere in the optical system. It is therefore crucial to match the optical characteristics of the three main components—the light source, flow cell, and spectrograph.

Optical Matching Strategies in a PDA

Returning to the flow cell, the starting point for PDA design, Fig. 2 shows how to squeeze the most light through what has been characterized as the optical bottleneck of the system. The light source is focused on one end of the cell, which makes the other end the limiting aperture of the optical system. Every point on the flow cell entrance cross section will then be connected by a ray to every point on the exit cross section. Note that the beam envelope expands beyond the cell at either end.

The first optical matching step is the source to the flow cell. A typical analytical flow cell with a 1 mm diameter is larger than the 0.5-mm-diameter

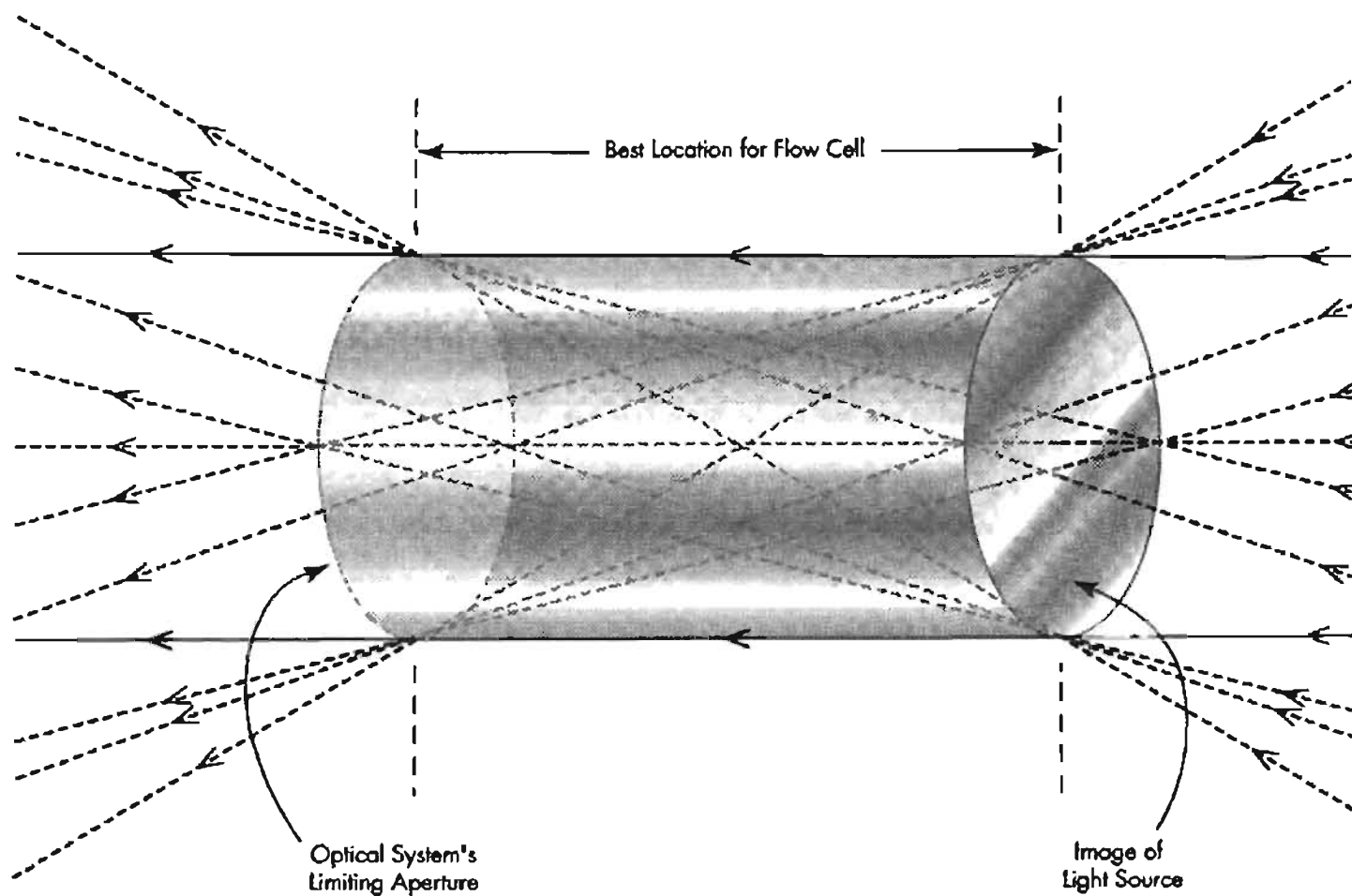


FIGURE 2 Optimal location of flow cell for maximum etendue showing the combination of long path length, small cell volume, and high optical throughput. The light paths are shown by the dotted lines.

emitting aperture of a high-radiance D_2 lamp. Best efficiency results if a magnified image of the arc is focused on the flow cell entrance. In the Waters model 996 PDA, a 2.5-fold magnification is provided by an off-axis ellipsoidal mirror to overfill the flow cell entrance. Mirrors are superior to lenses because all wavelengths focus at the same place. It is not practical to make truly achromatic lenses that span the UV, visible, and NIR ranges. The use of mirrors allows reasonable performance through the visible without recourse to a second light source.

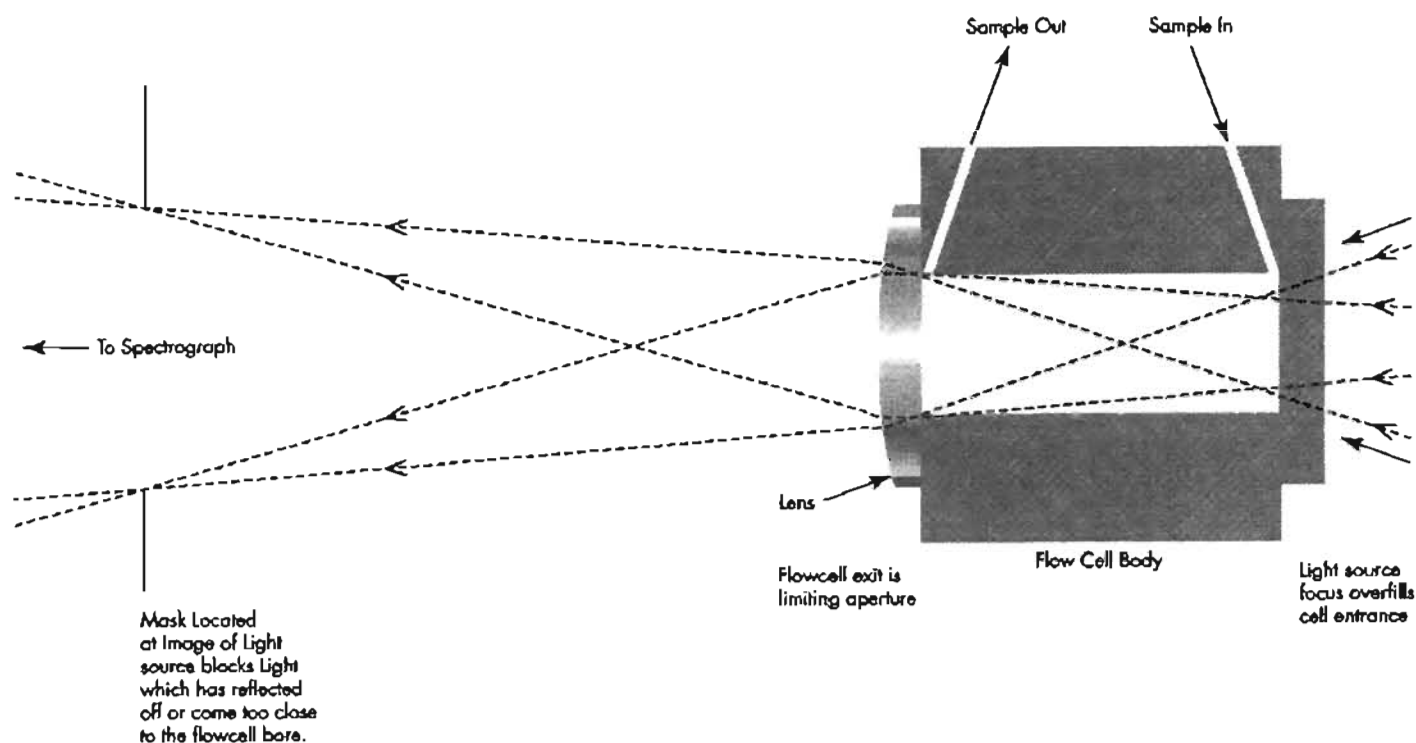
Sensitivity to mobile phase composition changes and pump pulsations is often a reflection of the detector's sensitivity to mobile phase refractive index (RI). RI sensitivity is a function of flow cell optical design. As a rule, both density and RI change with mobile phase composition. Stratification develops in the flow cell creating a liquid prism, steering rays to touch or not touch the inner cell bore. Rays that touch the cell bore are partially absorbed and interpreted (erroneously) by the detector as a change in sample absorbance.

It is therefore good practice to prevent light rays from striking the inner wall of the flow cell or, if they do, to prevent them from reaching the photodetector. Figure 3 shows a lens as the flow cell exit window and a mask placed further on in the light path. This combination allows rays that pass through the cell, well clear of the walls, to proceed to the spectrograph. Rays too close to the cell bore or reflecting off the bore are stopped by the mask. Some loss of throughput is the price paid for low RI sensitivity [11].

The second, more challenging, optical matching step is the flow cell to the spectrograph. For the conventional type of flow cell described here, the numerical aperture (NA) of the flow-cell (defined in Fig. 3) and that of the spectrograph are drastically different, as is the shape of the slit compared with the circular cross section of the cell. If these mismatches are not addressed in the design, optical efficiency is poor.

The spectrograph slit must be narrow to preserve spectral resolution, and the preceding throughput calculations must be adjusted to allow for the light lost on the sides of the slit. The loss is minimized if we match the numerical aperture of the cell and spectrograph.

The cell shown in Fig. 3 has a very small NA, about 0.04, whereas a typical grating spectrograph can accept a ray bundle with a considerably larger NA. Simply placing the exit of the flow cell at the entrance to the spectrograph means placing a narrow slit, needed for good spectral resolution, on the exit of the flow cell. With a 50- μm slit (equal to the width of one diode on a typical 512-diode array) and a flow cell 1,000 μm (1 mm) in diameter, a discouragingly small fraction, about 6%, of the light exiting the flow cell will pass through the slit. Because of the flow cell's small NA, only the center portion of the grating will be illuminated. Increasing the slit width improves the efficiency but at the cost of



Numerical Aperture $NA = \sin \alpha$

FIGURE 3 Flow cell optics design to minimize refractive index effects.

spectral resolution and linearity. Spectra do not match well with standards recorded at a different slit width.

Aperture matching boosts this fraction to about 25% in the Waters model 996 PDA without widening the slit. An ellipsoidal mirror positioned after the flow-cell focuses a fourfold diminished image of the flow cell exit on the spectrograph slit. This enables a larger fraction of the light to pass through (Fig. 4). This fraction can be doubled with no increase in cell volume using a flow-cell with a rectangular or slit-shaped exit cross section [12]. The entrance cross section remains circular to match the shape of the light source and the spectrograph grating.

ThermoQuest has a different strategy to match the shape of the flow cell to the spectrograph slit in the UV6000. A circular bundle of fibers collects light exiting the flow cell, and the fibers are rearranged in a line at the slit. A similar approach is used in the Waters CapLCTM PDA [13]. Efficiency is improved but by less than might be expected because of dead spaces between the fibers and absorptive loss in the UV. This “light funnel” is an old trick used by astronomers to couple light from the circular image of a star into a narrow spectrograph slit [14].

Light-Guiding Flow Cells

There is an alternative to the cell design just described. If the flow cell walls are a transparent material with a lower index of refraction than the mobile phase, light rays that strike the cell walls at a sufficiently oblique angle undergo total internal reflection. For example, the fluoropolymer Teflon AF has these properties, with a lower refractive index than most common HPLC solvents. Light is guided through the liquid in the cell in the same way that light is guided through an optical fiber. The result is a flow cell with a much larger NA. Moreover, the NA is independent of flow cell path length. If the optics of this flow cell are matched to the rest of the system, this strategy can be used to increase path length for a given cell volume (TSP 6000, 50 mm path, 10 μ L cell volume) or to reduce cell volume while retaining a reasonable path length (Waters CapLC, 5 mm path, 250 nL volume).

In summary, the optics of PDA detectors are well understood, and the theoretically achievable specifications of instruments intended for different applications can be predicted with confidence. The best designs are approaching these physical limits of sensitivity, within the boundaries set by a user's requirements—flow cell geometry, wavelength range, and spectral resolution. When these limits are reached, significant additional improvements will require a higher radiance light source or a higher quantum efficiency array.

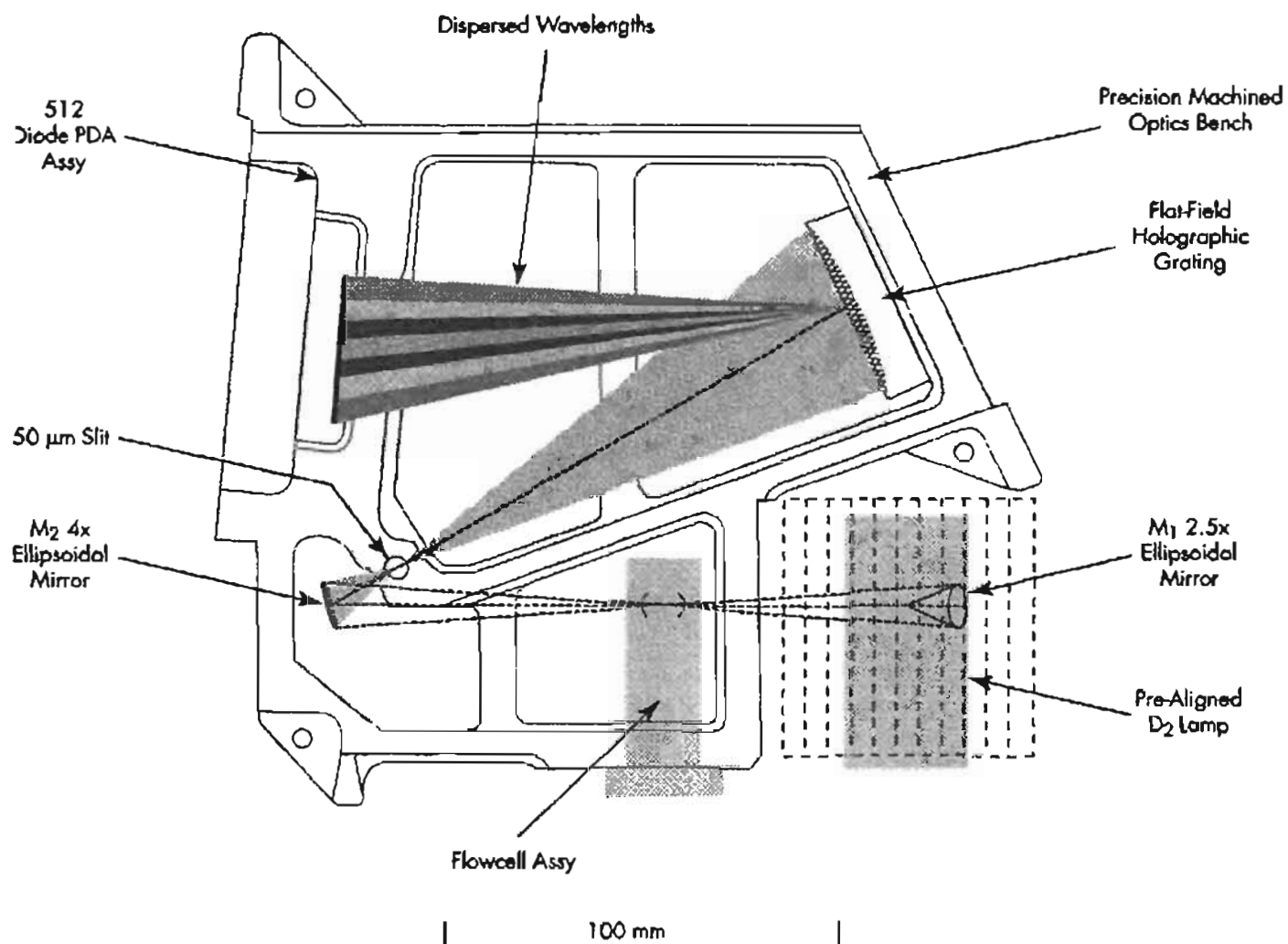


FIGURE 4 Design of optics bench for Waters model 996 PDA.

APPLICATIONS OF PDA DETECTION IN PROTEIN LIQUID CHROMATOGRAPHY

Dual and Multiwavelength Detection

In its simplest mode, a PDA detector is easily utilized as a single-wavelength or multiwavelength detector. Protein and peptide separations can be monitored at 214 nm, which provides a general detection mode for all peptidic components, and at 280 nm, which selectively detects only the components containing aromatic amino acids. For peptides, this dual-wavelength mode is useful in distinguishing peptides with Tyr, Trp, or Phe from those without, but proteins always contain one or more of the aromatic amino acids. The benefit of simultaneous detection at 214 and 280 nm for proteins lies in the improved sensitivity at the lower wavelength while the absorbance at 280 nm provides evidence that the UV spectrum of the detected species is consistent with that of a protein.

Two-wavelength detection has also found application in quantifying protein solution concentration. Using methods developed for homogeneous cuvette-based assays, Iersel et al. [15] have compared several of these procedures as applied to HPLC separations with PDA detection. Most of these strategies use one wavelength in the far UV range (205–230 nm) and a second wavelength in the aromatic region to calculate a protein extinction coefficient. Approximations of total protein concentration can be calculated from simple algebraic functions of the two absorbances. The advantage of an HPLC analysis over solution techniques is the separation of the proteins of interest from other proteins and the removal of potential interference from buffer constituents, detergents, or stabilizers. These authors reported that using Eq. (2) gave results superior to those from other methods:

$$A_{280}^{0.1\%} = 34.14 \times (A_{280}/A_{205}) - 0.02 \quad (2)$$

Protein absorbances were readily determined using a PDA detector and size exclusion chromatography. Unfortunately, this mode of concentration determination has not found widespread use, possibly due to an inability to apply this to a wide variety of protein systems and unknown samples.

Monitoring wavelengths >300 nm finds applications with proteins containing prosthetic groups such as heme or flavin-containing proteins. Heme and other porphyrin-containing proteins, usually forming intensely red-colored solutions, have spectra with significant absorbance in the 380–550 nm region. The actual peak maximum will depend on the specific heme structure, the oxidation state, and the ligands bound to the prosthetic group. Various forms of iodinated Tyr and derivatives such as thyroxine feature peak maxima ranging up to 310 nm. These modified Tyr species are found in the physiologically important hormonal protein thyroglobulin and peptides derived therefrom.

Chromophores can also be synthetically attached to specific protein groups, either to assist identification or incidental to blocking reactive functionalities. One common chromophore is the pyridylethyl group added to cysteine using 4-vinylpyridine after disulfide bond reduction but prior to enzymatic digestion. The pyridylethyl cysteine has a strong absorbance maximum near 254 nm that permits easy on-line identification of Cys-containing peptides [16].

Simultaneous two-wavelength detection is also easily used for calculating wavelength ratios. Most PDA detectors have software that makes it routine to calculate automatically the 280/214 nm absorbance ratio. This ratio will be characteristic for any single, homogeneous component and a resulting constant ratio across a peak is the one of the simplest approaches to demonstrating peak purity. For example, the use of absorbance ratios to monitor protein conformational changes, discussed later, is readily adapted to on-line HPLC analysis by PDA detection. Of course, a PDA detector allows much more sophisticated routines that use part of or the entire component spectrum for purity determinations. These are also discussed later in this chapter.

Characteristic absorbance ratios have been shown useful for several other applications. For example, various heme structures can be distinguished by specific spectral elements in the Soret band (400–450 nm) region. Two separate groups of researchers have shown that Trp and Trp-related structures can also be differentiated by characteristic wavelength ratios at 255 and 270 nm [17,18]. However, as pointed out by Rozing [19], these methods have serious limitations, particularly when more than one aromatic residue occurs in a peptide.

Full Spectrum Analysis for Aromatic Amino Acid Characterization

PDA detection, however, can provide much more information than simple multiwavelength analysis. Despite the lack of distinguishing minima and maxima, protein UV spectra are more information rich than is apparent to the eye. Characteristic spectra in the 250–300 nm range have been useful in numerous studies of protein aromatic content.

Many aromatic amino acid content studies by HPLC-PDA have focused on analysis with derivative spectroscopy. These studies followed directly from work of several groups with benchtop UV-Vis spectrophotometers. In particular, Balestrieri and co-workers have demonstrated that the second derivative in the 250–300 nm range can be used to identify Trp-containing proteins and distinguish proteins containing Trp from those with only Tyr [10,20,21]. The advantage of derivative spectroscopy is that shoulders and inflection points are converted to minima and maxima (e.g., in Fig. 5). Even derivatives (e.g., second and fourth) have the added advantage that the maxima in the zeroth spectra are at the same wavelength of a minimum in the derivative spectra. Other minima are at the wavelengths of inflection points in the zeroth spectrum.

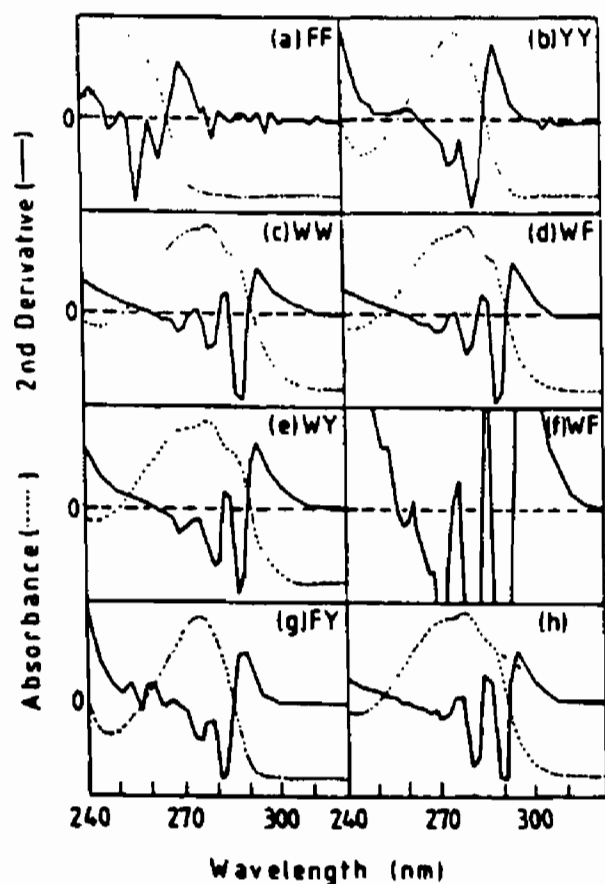


FIGURE 5 Absorbance and second-derivative spectra for model synthetic peptides containing Trp, Tyr, and Phe. Peptides were analyzed by HPLC in a gradient system with mobile phase A = 0.1% trifluoroacetic acid (TFA) in water and solvent B = 0.1% TFA in acetonitrile-water (60:40). Both the UV absorbance (dotted lines) and the second-derivative spectra (solid lines) have been plotted at 100% full scale except for panel (f), in which the sensitivity has been increased 20-fold over panel (d). The peptide in (h) was YVMGHFRWDRFG. Single-letter codes are used for the amino acids. (From Ref. 23.)

Most procedures using derivative spectra are based on the occurrence of two prominent minima in the second-derivative spectra of Trp-containing compounds at approximately 282 and 291 nm. The ratio of these two minima varies as a function of the Trp/Tyr ratio. In peptides and proteins with no Tyr, this ratio is approximately 0.5, increasing to 8 with the Tyr content as high as 9:1 over Trp. With no Trp present, in the 280–300 nm range there is only a minimum at 285 nm.

In HPLC-PDA studies, Grego et al. first reported on the use of derivative spectroscopy for the analysis of aromatic amino acids in peptides and proteins [23], extending the static studies of Balestrieri et al. [21] to separations-based analyses of mixtures. First, derivative spectra for a set of model peptides and

amino acids were derived from reversed-phase separations of the models (Fig. 5). The models included peptides with a single aromatic residue as well as ones with mixed aromatic composition. Subsequent experiments compared the derivative spectra of the model compounds with peptide spectra in tryptic map analysis. Using minima analysis similar to the procedures described by Balestrieri, they obtained very good agreement between the predicted and actual content of aromatic amino acids.

Palladino and Cohen [24] also used HPLC-PDA detection and derivative spectroscopy for the characterization of the aromatic amino acids for a number of proteins. This work is noteworthy because it showed reasonable correlation between the second derivative spectra and the relative Tyr and Trp content in large proteins up to molecular weight (MW) 50,000, not simply peptides, where the analysis is greatly simplified. As with the peptide and small protein examples reported earlier by Grego et al., these authors demonstrated the practicality of extending derivative analysis to much larger molecules.

CONFORMATIONAL STUDIES VIA PDA DETECTORS

A number of studies in the 1950s and 1960s firmly established that the conformation of a protein has a significant impact on its UV spectrum, particularly in the aromatic region from 250 to 300 nm where absorbance is a function of the protein's Phe, Tyr, and Trp content [25]. The explanation for this effect became apparent using model compounds such as the free aromatic amino acids and peptide-like analogs such as the *N*-acetylated amino acid ethyl esters [26]. Comparisons of spectra for Tyr and Trp (or their analogs) in aqueous buffer solution with those obtained in organic solvent, or mixtures of organic solvent and water, show significant blue shift in the absorbance maximum for the organic-containing samples.

Analogous shifts in protein spectra have been observed as a result of conformational changes associated with denaturation. Initial reports on this phenomenon (see Ref. 24) attributed the spectral difference to the transfer of the aromatic amino acids from the hydrophobic interior of the protein to the more aqueous surface environment as a result of the conformational change. Spectral changes for several proteins correlated well with independent measurements of denaturation such as intrinsic viscosity, circular dichroic spectra, and heat capacity measurements. For example, Edelhoch [26] compared the ribonuclease UV spectrum in buffer with that obtained in 6 M guanidine hydrochloride (GuHCl).

Derivative spectroscopy has also found application in protein unfolding studies. Ragone et al. [27] followed up on their work describing derivative spectroscopy for proteins with studies showing how the technique could be used to follow aromatic amino acid exposure and hence unfolding. Up until the mid-

1980s these spectroscopic studies were limited to bulk solution systems. These static measurements are simple to make but can suffer from the presence of other UV interfering components and necessitate the use of purified protein samples. In addition, limited detection sensitivity requires sample quantities of at least 10 μg to achieve an acceptable signal-to-noise ratio.

The effect of conformation on protein retention in HPLC became the focus of several groups in the early 1980s. Experiments employing size exclusion chromatography (SEC) were logical extensions of similar work performed on low-pressure media (e.g., dextrans) correlating reduced retention with protein unfolding. The increase in solute hydrodynamic volume as a result of the denaturation process can be easily monitored. Protein structure can be manipulated by varying the chromatographic conditions to promote unfolding. Column temperature, denaturants such as urea and GuHCl, and buffer pH are common conformational disrupters. A good example of this type of study is the work of Stellwagon and co-workers [28], who explored the reversible denaturation of ribonuclease in GuHCl.

One potential pitfall in using SEC with single-wavelength detection is the potential for retention time changes due to processes not associated with denaturation. For example, increased retention may be caused by secondary interactions with the column stationary phase. These may be ionic or hydrophobic in nature. Protein aggregation or other protein-protein associations result in decreased retention in SEC, neither of these phenomena being the result of unfolding. The different effects can be distinguished through on-line spectral analysis with a PDA detector. Thus, aggregation and nonspecific retention should not have any effects on a protein spectrum, whereas spectral changes accompanying denaturation can be significant.

Spectral analysis can also provide information on conformation in interactive chromatographic separations such as reversed-phase, ion-exchange, and hydrophobic interaction chromatography. Karger and co-workers [29–31] first demonstrated the influence of hydrophobic stationary phases on protein structure and chromatographic retention with various proteins such as ribonuclease, β -lactoglobulin, and papain. Although these initial studies provided good evidence for the influence of conformation on retention, the correlations of retention with other analytical assessments of denaturation were indirect comparisons with off-line measurements.

A series of papers in 1986 from the Karger group that followed up their earlier work [32–34] illustrated the power of PDA detection for protein conformation studies. In the first of these publications, the behavior of β -lactoglobulin A and lysozyme during chromatographic separation on hydrophobic interaction chromatography (HIC) columns was described. They used two methods for spectroscopic analysis of eluted proteins, absorbance ratioing and second-derivative spectroscopy. Analysis focused on the aromatic region (240–310 nm),

where the local environmental influence on Trp and Tyr spectra was most significant. To assess Trp solvent exposure, they monitored the 292/254 nm ratio where an increase in the ratio was indicative of increased Trp exposure. Tyr exposure was followed by measuring the increase in the 272/292 nm ratio along with a decrease in the 292/254 nm ratio.

Although absorbance ratio measurements allowed the exposure of Tyr and Trp to be followed independently, strong Trp absorbance can interfere with Tyr determinations. This was overcome using second derivative spectroscopy following the general procedure developed for benchtop spectrophotometers [27]. This method calculates the ratio of the peak-to-peak magnitude from the local minima at approximately 282 and 290 nm to the maximum between the two minima. An increase in the a/b ratio (see Fig. 6) is indicative of increased Tyr exposure, even in the presence of excess Trp. The extent of conformational changes during HIC as a function of temperature could then be monitored using Tyr exposure as the indicator. This worked well for β -lactoglobulin, but with lysozyme in which the Trp/Tyr ratio is high (six Trp vs. three Tyr residues), the sensitivity of derivative spectroscopy to conformational changes is somewhat limited.

PDA analysis was also applied to the study of ribonuclease A in reversed-phase chromatography [33]. Ribonuclease A conformation has been the focus of numerous studies, and its denaturation is an excellent example of a reversible unfolding process. Cohen et al. [31] first reported the influence of a hydrophobic stationary phase on ribonuclease denaturation. In this work, the change in the 254/280 nm absorbance ratio was followed using multiple injections of the same sample. The increase in the ratio correlated extremely well with a number of other physical measurements of structural changes such as circular dichroism and intrinsic viscosity.

Using single-channel detectors for monitoring absorbance changes, however, was very cumbersome, and a more detailed analysis of the denaturation process was accomplished via PDA detection. With a PDA detector, simultaneous monitoring of multiple wavelengths is much simpler, and, unlike experiments with two detectors in series or with repeat injections under ostensibly well-controlled conditions, the entire spectrum is recorded under identical conditions and the ratios calculated are subject to fewer errors. Lu et al. [33] verified that the denaturation is consistent with a two-step process and were able to determine the apparent rate constant for refolding in the mobile phase. In addition, they showed that the rate constant varied with the gradient steepness, column temperature, and the mobile phase composition.

A more recent publication demonstrating the power of PDA analysis for conformational studies focused on the stability of closely related proteins [35]. Using lysozyme as a model protein for showing the influence of protein conformation on the experimentally determined r value (Fig. 6), the second-derivative spectroscopy then provided a straightforward means of comparing the

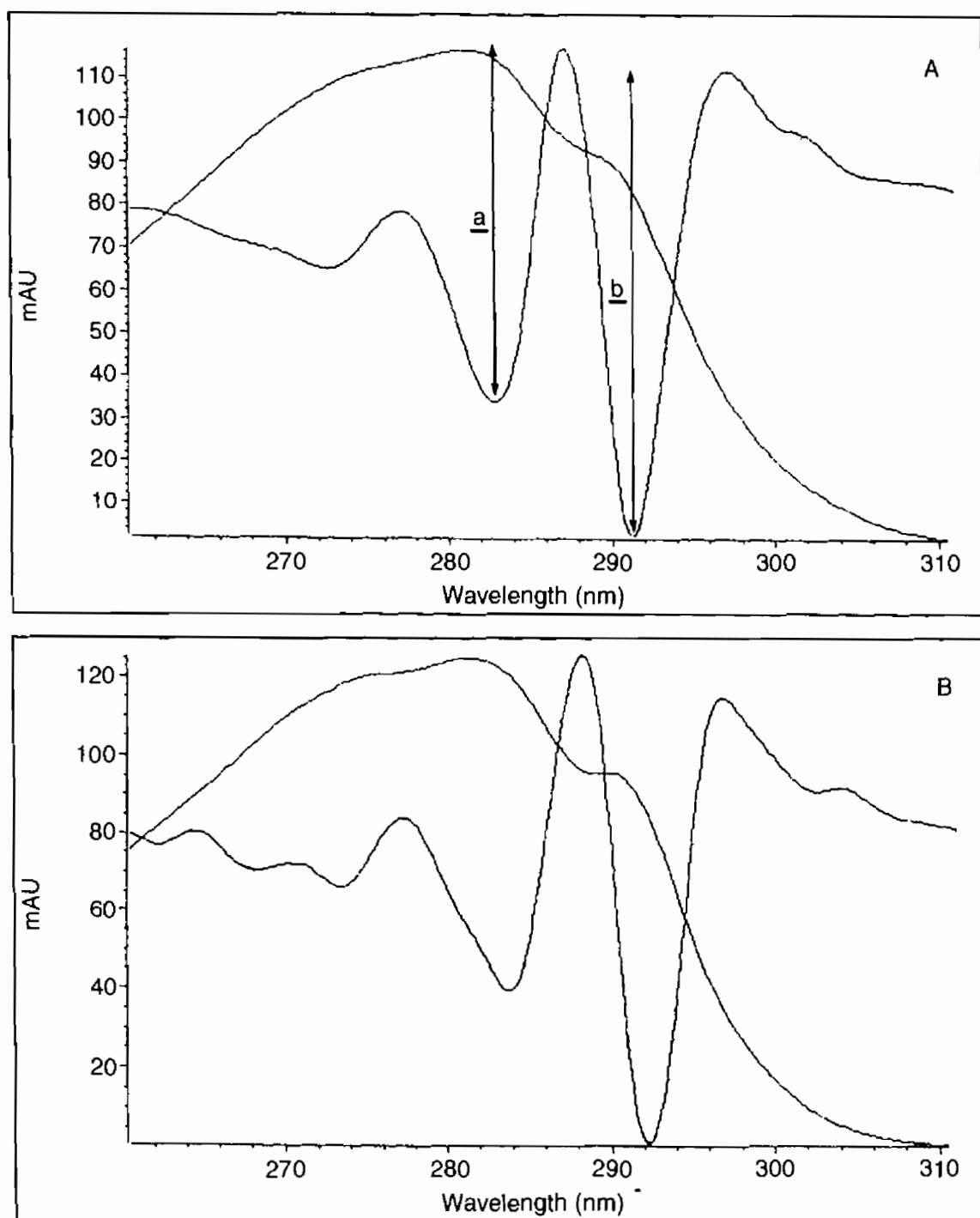


FIGURE 6 Absorbance and second-derivative spectra for (A) denatured (in 6 M GuHCl in 50 mM sodium phosphate, pH 7.0, 150 mM KCl) and (B) native lysozyme (in 50 mM sodium phosphate, pH 7.0, 150 mM KCl). The denatured spectrum was obtained via flow injection; the native spectrum was obtained from the peak eluting from a size exclusion analysis. The r ratio is obtained as shown from the ratio a/b . (From Ref. 35.)

resistance to denaturation of a series of closely related interleukin-2 mutants produced by site-directed mutagenesis. The second-derivative spectra were evaluated according to the method of Ragone et al. [27] in which the increase in the r value was used to monitor Tyr exposure and hence provide a measure of denaturation. Although the spectral analysis indicated that the degree of Trp exposure was altered in some of the mutants, the derivative spectra verified that none of the mutants exhibited any significant denaturation under normal environmental conditions. The magnitude of the minimum at 290 nm did indicate that the Trp environment was modified in some of the mutants. Using size exclusion chromatography for the analysis permitted the simultaneous determination of conformation and aggregation information in a single analysis.

Whole Spectrum Analysis

One of the difficulties with the derivative and absorbance ratio methods is the lack of automation in the numerical calculations. All of the cited references used a manual calculation for the parameter of interest. This is especially laborious for the derivative calculations. Most modern PDA software will automatically generate the derivative spectra, but calculating the peak-to-peak or peak-to-valley quantities (i.e., a and b) and thus the r values is a manual process. Conformational changes contribute to further difficulties in that the exact wavelengths of the minima vary as a function of the degree of denaturation. This prevents the use of routine data processing methods with fixed wavelengths for calculating r . Moreover, derivative spectroscopy, while visually clarifying spectral features such as shoulders and bumps, actually reduces the measurement sensitivity and decreases the signal-to-noise ratio. Common smoothing procedures used in generating the derivative spectra also decrease spectral resolution [36].

In our laboratory, we have applied full spectral analysis of the zeroth derivative (absorbance) spectrum to the conformational analysis of ribonuclease undergoing size exclusion chromatography. The basis of this quantitative analysis is a comparative procedure termed "spectral contrast" that has been described in detail in several papers [37,38]. Briefly, in this method, a spectral curve is converted to a vector in n dimensions where n is the number of data points (wavelengths) in the spectrum. The magnitude of the vector in each dimension is the absorbance for that wavelength. Calculating the dihedral angle between two such spectral vectors, the "spectral contrast angle," then provides a quantitative comparison between any two spectra. In this manner, the information from the entire spectrum, or any portion of the entire spectrum, can be used for monitoring spectral changes as a function of protein structural perturbations.

One study focused on the thermally induced denaturation of ribonuclease in an acidic environment. Ribonuclease was analyzed using a Protein-Pak 300SW

size exclusion column with a dilute acetic acid mobile phase at pH 2.85. The column temperature was varied between 30 and 65°C. As the temperature increased, retention in SEC decreased, as expected for the increase in hydrodynamic volume associated with unfolding. In Fig. 7 the analyses of ribonuclease at temperatures ranging from 25 to 60°C are overlaid. A clear trend is the decrease in retention as the column temperature is increased. For selected temperatures, the UV spectra from 250 to 300 nm are plotted for the peak maxima (Fig. 8). A trend is also evident in these data as the peak maximum for ribonuclease is shifted to a lower wavelength at the higher temperatures.

Using the spectrum at 30°C as the reference spectrum, ribonuclease spectra at higher temperatures were compared with the reference conditions using the spectral contrast algorithm. Figure 9 shows a plot of the experimentally determined spectral contrast angles as a function of temperature. Figure 9 also shows the retention time for the peak. It is noteworthy that the shape of both curves is that of a classic ribonuclease denaturation curve. One interesting observation is that although the plot shape is very similar to the retention time versus temperature plot, the two curves do not overlap exactly. This difference is may be due to errors in one or both of the measurements but could also be the result of measuring different phenomena in the two plots. Specifically, the spectral contrast angle measures changes in aromatic amino acid environment, largely due to Tyr exposure (ribonuclease does not contain Trp). In contrast, the retention time data are a measurement of the molecular size.

As it is a much-studied protein, there is a wealth of information on the denaturation of ribonuclease. For example, Pace [39] studied ribonuclease thermal denaturation in a static system with conditions of pH and ionic strength very similar to those we have employed. Data from this study are plotted in Fig. 10 along with the spectral contrast data and correlate extremely well with the dynamic SEC system, indicating similar ribonuclease stability in the two systems. The spectrum at 60°C showed a distinct blue shift of approximately 3 nm in the peak maximum, in agreement with previous reports for ribonuclease denaturation. As pointed out earlier, this is consistent with increased exposure of Tyr to the aqueous solvent environment (ribonuclease has no Trp). Throughout the entire temperature range, the ribonuclease peak shape varied only slightly, indicating rapid equilibration between the native and denatured states.

Denaturation of ribonuclease in GuHCl has several significant differences from the thermal denaturation described previously. As the concentration of GuHCl is increased from 0.5 to 6 M, ribonuclease retention decreases as it does with increasing temperature. However, in the midpoint of the transition region, approximately 2–4 M, the peak shape is very broad, with ribonuclease almost resolving into two distinct peaks. Results from these experiments are shown in Fig. 11. In contrast, ribonuclease peak shape does not degrade in a similar fashion during thermal denaturation. In the previous work by Stellwagon and co-workers

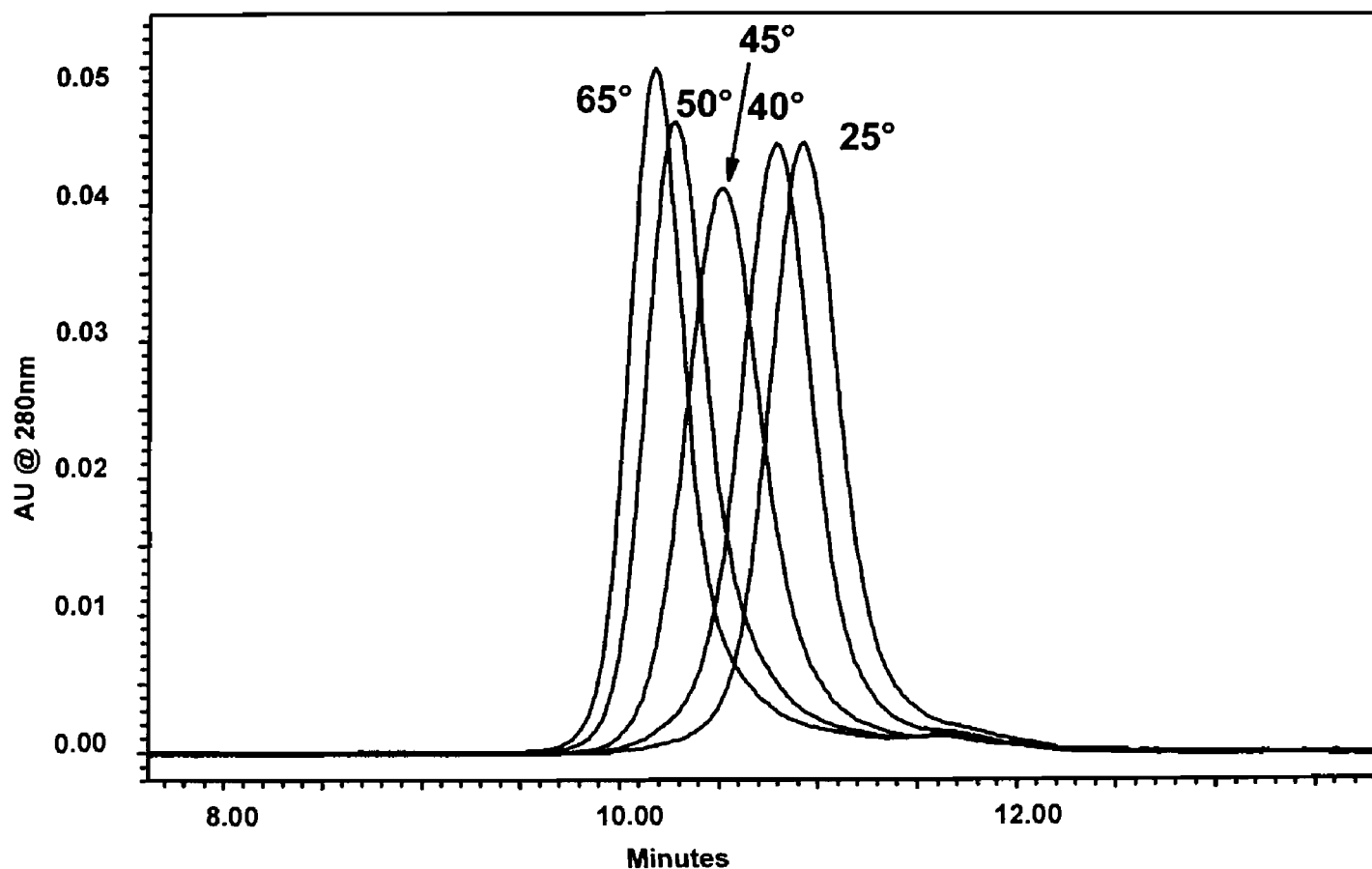


FIGURE 7 Size exclusion chromatography of ribonuclease as a function of temperature. Samples were separated on a Protein-Pak 300 SW column (7.8 × 300 mm). The eluent was 50 mM sodium phosphate, pH 2.85 containing 150 mM NaCl. The flow rate was 1.0 mL/min. Spectral data were collected from 250 to 300 nm.

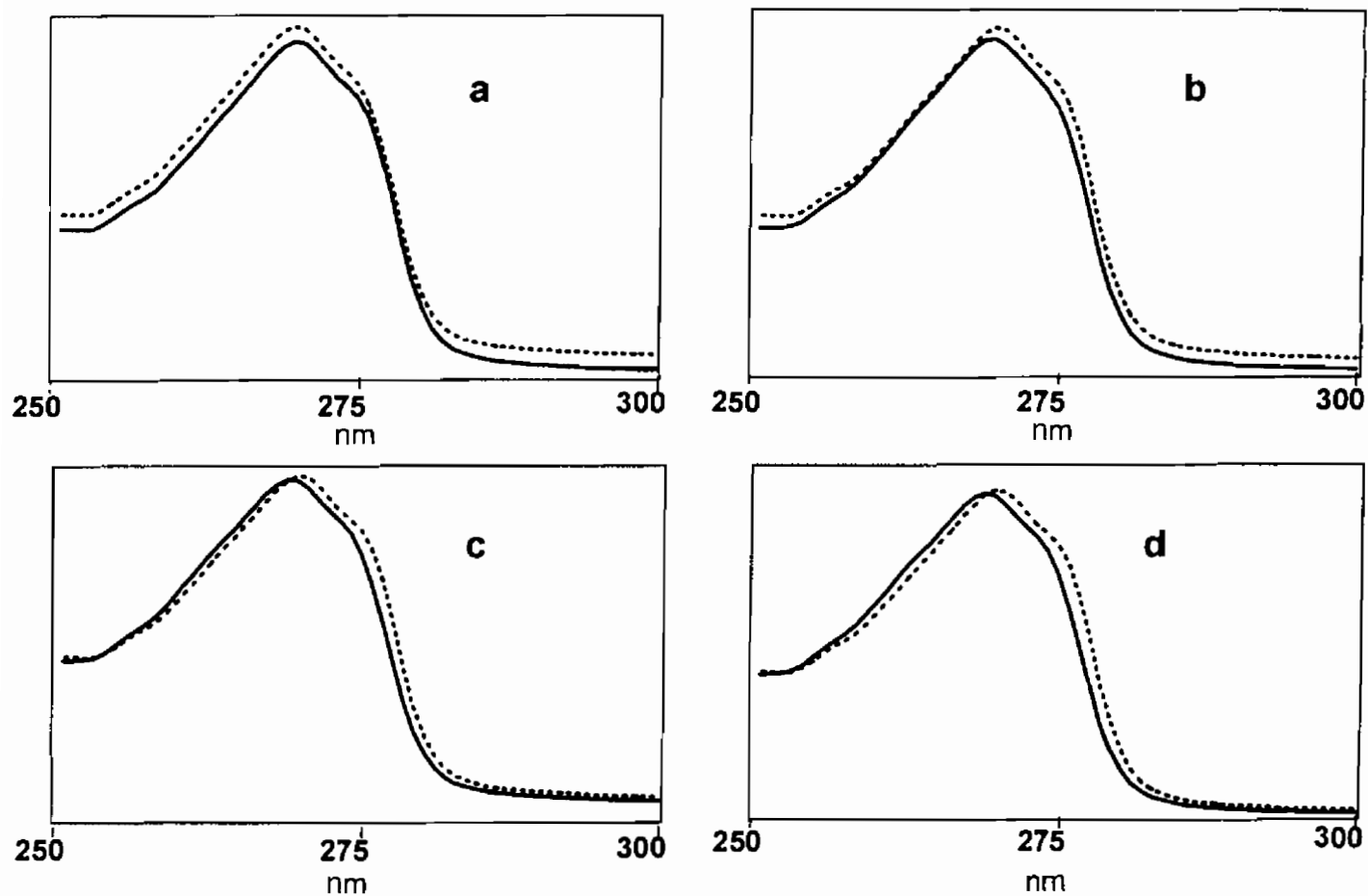


FIGURE 8 Spectra of ribonuclease as a function of temperature. Separations were performed as described in the legend to Fig. 7. The spectra at the temperatures for a–d were 35, 50, 55, and 65°C, respectively, and are drawn with a solid line. For reference, the spectrum at 25°C is shown in each panel with a dotted line.

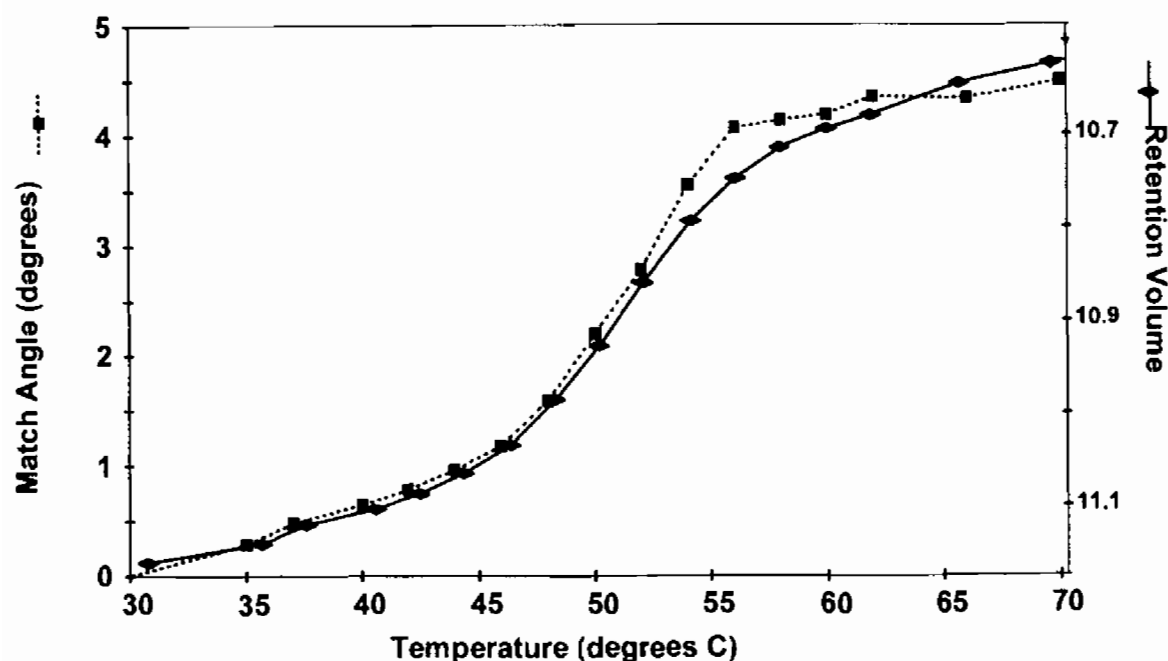


FIGURE 9 Comparison of match angle and retention data for ribonuclease as a function of temperature. The retention times are for the peak maxima for each temperature. The match angle data were calculated using the spectral contrast software package.

[28], this broad elution profile in 3 M GuHCl was attributed to a slow equilibrium between the native protein and one or more partially unfolded conformations. This work used single-wavelength detection but allowed a consistent explanation for the GuHCl-mediated denaturation. With full spectral analysis of ribonuclease

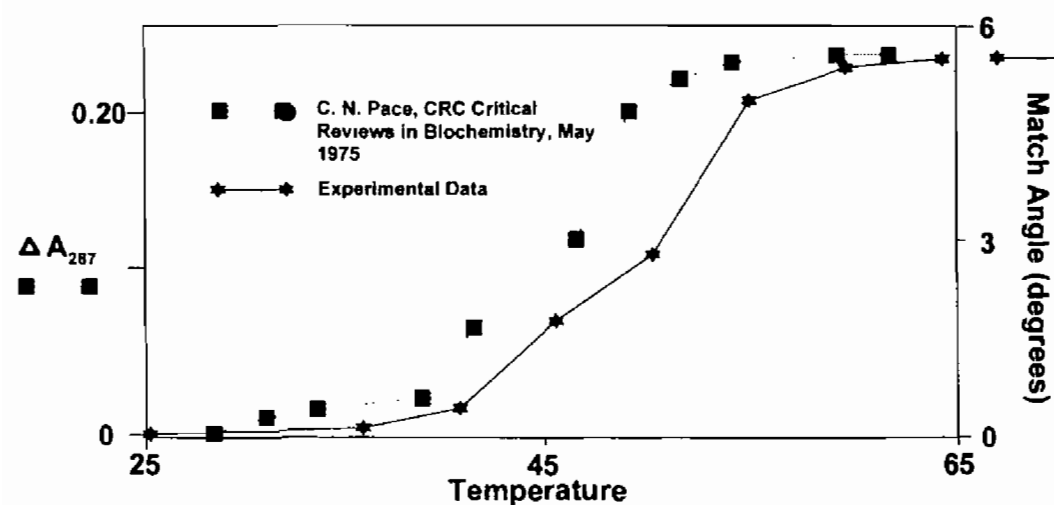


FIGURE 10 Unfolding curves for ribonuclease: comparison of match angle and difference spectroscopy plots. The match angle data are the same as plotted in Fig. 9. Difference spectroscopy data were generated from data published in Ref. 39.

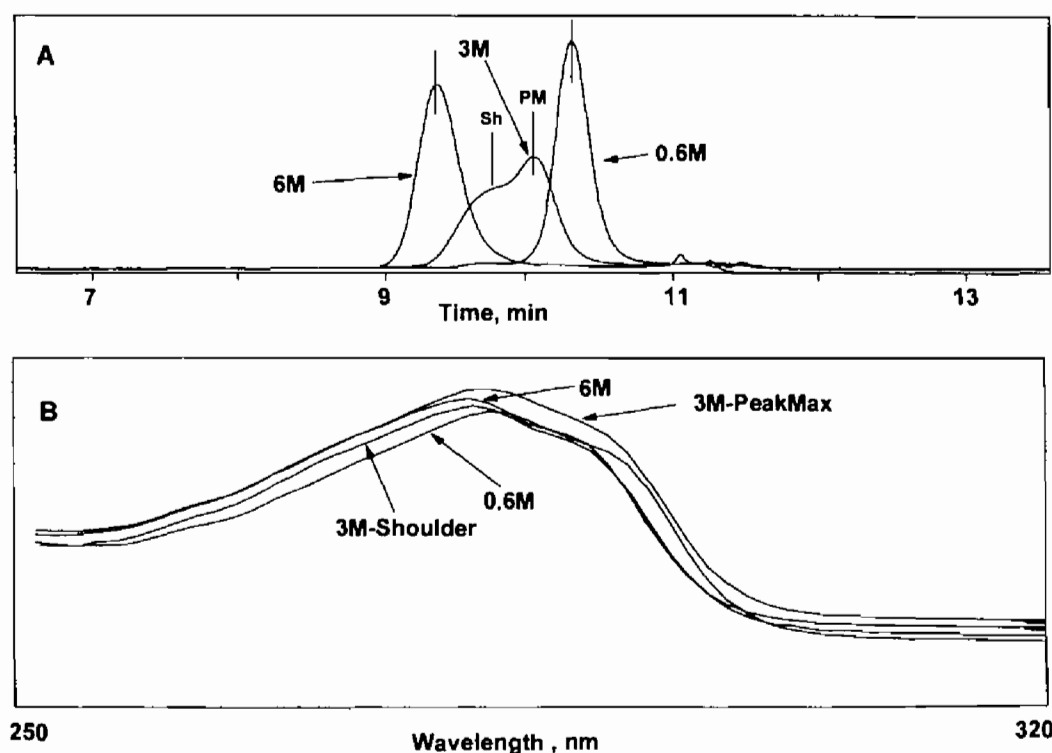


FIGURE 11 Size exclusion chromatography of ribonuclease as a function of GuHCl concentration. Proteins were analyzed in eluents with varying amounts of GuCl in the range of 0.6–6 M. A Protein-Pak 300 SW column (7.8×300 mm) was used. The eluent was 0.1 M sodium acetate, pH 6.80 with 0.53 M NaCl, containing the desired amount of GuHCl. The column temperature was 25°C. The flow rate was 1.0 mL/min with spectra collected from 250 to 300 nm. (A) The chromatographic curves are plotted for three selected concentrations. (B) Four spectra from the three separations shown in A are plotted. Two spectra from the analysis in 3 M GuHCl are plotted as indicated by the arrows.

we were able to provide additional support for this slow mechanism. First, the plot of the spectral contrast angles calculated for ribonuclease as a function of GuHCl concentration, where the spectrum in 0.5 M GuHCl was the reference spectrum, shows a curve shape similar to the retention time plot. Second, the ribonuclease spectrum shows the distinct blue shift with increasing GuHCl concentration. The key result supporting the slow unfolding hypothesis, however, is the spectral data collected at different points in the chromatographic profile at 3 M GuHCl. These show significant variation across the peak, with the spectrum of the earlier elution portion matching well with the spectrum in 6 M GuHCl, and the spectrum for the longest retention part matching well with the reference spectrum in 0.5 M GuHCl.

The spectral contrast method also permits peak purity measurements by comparing all spectra in a peak with the spectrum at the peak apex. Purity calculations at the extreme concentrations of GuHCl indicate spectrally homo-

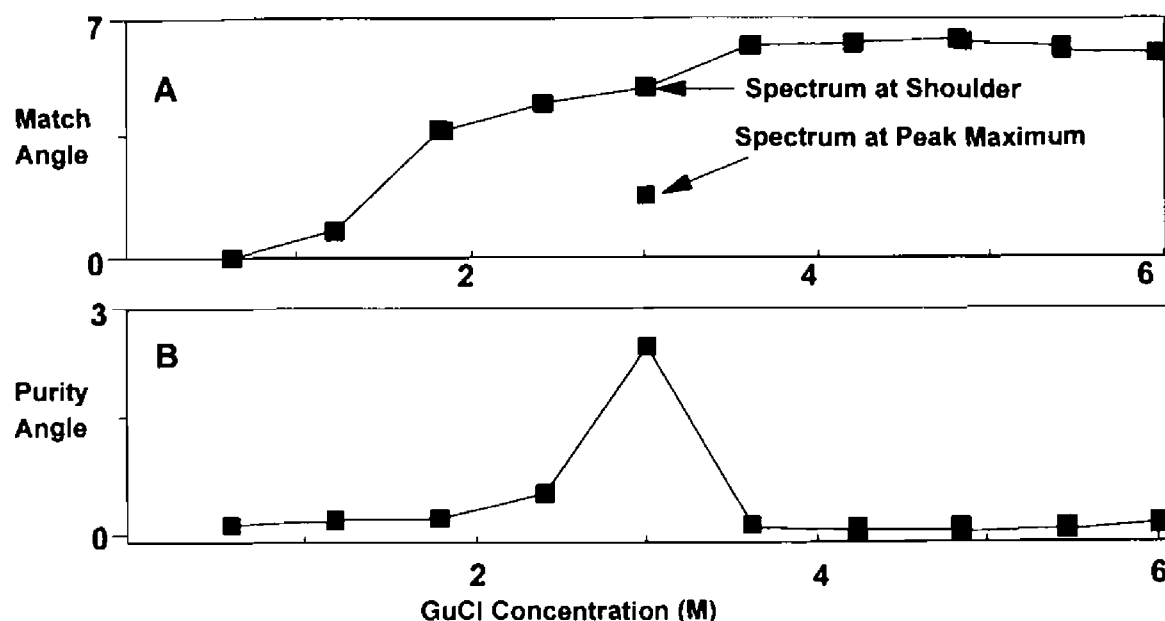


FIGURE 12 Spectral contrast analysis of ribonuclease in GuHCl-containing buffers. Match angles were calculated for ribonuclease by comparing the spectrum at each concentration with a reference spectrum generated in 0.6 M GuHCl. The purity angles were calculated for the main peak in each analysis as a measurement of the peak conformational homogeneity.

geneous peaks (Fig. 12). In contrast, in the range 2–4 M GuHCl, the peaks are distinctly inhomogeneous. The most striking differences are observed for the spectra in the 3 M GuHCl experiment. This spectral change across the peak clearly supports Stellwagon's earlier work in which the broad or even split peaks observed in 3 M GuHCl are explained by a slow equilibration process between native and denatured ribonuclease.

Using PDA Spectroscopy for Evaluating Eluent and Stationary Phase Effects on Protein Structure

Karger and co-workers were the first group to demonstrate convincingly the strong influence of reversed-phase packings on protein structure [e.g. 29–31]. His group's work on HIC phases hinted at the possibility of characterizing chromatographic stationary phases by comparative analysis of protein denaturation on different columns [32,34]. For example, the systematic study of monitoring β -lactoglobulin unfolding by second-derivative spectroscopy showed that the HIC columns, where the ligand was an ether-bonded phase, had significantly less impact on protein denaturation than alkyl-bonded phases such as C4-silica. In general, they concluded that at a given column temperature the more hydrophobic the stationary phase, the more likely one would observe phase influence on protein structure.

When a protein is adsorbed to a hydrophobic surface, denaturation is often not an instantaneous phenomenon. Thus, the extent of unfolding will increase with increasing time of adsorption. Both Karger and Hearn et al. [40] have monitored this residency effect on reversed-phase and hydrophobic interaction columns using a combination of techniques including derivative spectroscopy. The Hearn group also studied denaturation kinetics in size exclusion chromatography in the presence of the powerful denaturant urea.

Karger's group determined that the extent of denaturation was influenced by the length of exposure to a hydrophobic surface such as a reversed-phase column. Hearn's group studied the effect of reversed-phase columns on the conformation of a series of growth hormones. The extent of denaturation was assayed by following the r value calculated from the second-derivative spectrum of the eluted proteins [27]. By varying the amount of time between sample loading and the initiation of the gradient, they demonstrated that the protein denaturation increased with longer exposure time to the hydrophobic packing surface. Although it was not an unexpected result, the direct measurement of this influence was simplified via PDA detection. Similarly, elution with steeper gradients reduced the amount of unfolding observed due to decreased residence time on the column.

Regnier's group has studied the effect of acid mobile phases on protein retention [41]. In systems using formic acid in both the aqueous and organic eluents, they observed significant increases in retention up to 30–50% acid, depending on the protein. When the acid was present only in the aqueous eluent, maximum retention was at approximately 30% acid. Similar results were obtained with trifluoroacetic acid. By correlating spectroscopic changes in the protein spectra with the acid concentration, they concluded that the initial increased retention was largely due to conformational changes in protein structure. For ribonuclease, which lacks Trp, the decrease in the A_{274}/A_{254} ratio indicated an increase in Phe exposure at the surface. In proteins with Trp, interpretation was more difficult, but increased exposure of Tyr was also indicated. Higher acid concentrations also altered the Tyr exposure, thus affecting retention, but the decreased retention was also a result of the higher solvophilic power of the eluent.

More recently, Poppe and co-workers [42] studied the influence of various chromatographic parameters on protein structure. As others had previously noted, these authors commented that strong retention of proteins on reversed-phase materials can cause denaturation. In order to compare various stationary phase retention characteristics, proteins eluted from a series of mildly hydrophobic phases were spectrophotometrically monitored and transition curves for protein unfolding generated from data collected over the temperature range 20–80°C. The extent of the conformational change in lysozyme was assessed via absorbance difference spectroscopy at 292 or 297 nm. Myoglobin conformational changes were measured by difference spectroscopy at 410 nm. A shift in the

transition curve to lower or higher temperature could be used to indicate the influence of either mobile phase or stationary phase on the transition temperature. For example, an increase in mobile phase organic content resulted in lower transition temperatures, as did a decrease in mobile phase pH in the range of 0.7–2.5. The same group also found that protein residence time influenced the transition, thus supporting the earlier work of both Karger and Hearn.

Using difference spectroscopy as a rapid assay for assessing protein structure upon elution, they calculated the transition point for the denaturation of lysozyme and myoglobin at various concentrations of NaCl. They then compared the effect of the salt with that of the column stationary phase and concluded that with the weak hydrophobic epoxy-glucose-coated phase they used, the influences of the two parameters were of the same order of magnitude. As noted before in the work by Karger, the unfolded forms of the proteins exhibited increased retention compared with the retention of native proteins on weak hydrophobic phases.

MISCELLANEOUS APPLICATIONS

In addition to the normal amino acid chromophores of the aromatic amino acids, some proteins exhibit UV-Vis absorbance due to modified amino acids or covalently or noncovalently linked non-amino acid structures. Examples include heme proteins such as hemoglobin or myoglobin, flavin-containing proteins, pyrrole-linked proteins, and other so-called prosthetic groups. Although PDA detection can be extremely useful in the study of these proteins, examples of its use are scarce in the literature. In one such report, Swanson and Glazer [43] analyzed phycobiliproteins from cyanobacteria and red algae, proteins that contain covalently attached open-chain pyrrole (bilin) groups. These chromophores are somewhat unusual in that they have significant absorbance between 400 and 700 nm, thus requiring a visible light source for complete spectral analysis. Separation of different bilin protein species was accomplished on C4 columns with TFA/acetonitrile gradients. The spectral characterization of different bilin protein subunits allowed the authors to assign specific structures to resolved species. In addition, by using the known extinction coefficients of two different bilins in acid solution, they were able to calculate the ratio of two subunit types in a cyanobacterium phycobiliprotein.

Another interesting study by Simat and Steinhart [44] focused on the oxidation of native Trp in peptides and proteins. Trp is one of the most readily oxidized residues in proteins and monitoring this degradation is important in a number of areas including stability of protein-based pharmaceuticals and other samples where long-term protein stability is of interest. Trp oxidation products may include kynurenine, *N*-formylkynurenine, oxindolylalanine (Oia), and 5-hydroxytryptophan. Identification of these breakdown products in peptides and

proteins can be facilitated through a combination of UV and fluorescence characterization. A combination of these detection techniques was used to monitor the spectra of peptides isolated after enzymatic digestion of hydrogen peroxide-treated proteins. The nature of Trp degradation products was determined by a comparison of the reversed-phase separated products with genuine standards of the known oxidation products. They were also able to differentiate patterns of degradation in free Trp versus peptide- and protein-bound Trp and proposed a mechanism for this difference that invokes the participation of the free α -amino group in the formation of the iminoacid 3 α -hydroxy-1,2,3,3 a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid. This mechanism is, of course, not available to the bound forms of Trp. It was also interesting to note that Trp oxidation in a protein (lysozyme) was considerably slower than that observed in peptides or free Trp. Another finding was that Oia was one of the major products formed from Trp during a standard hydrochloric acid hydrolysis protocol.

PDA detection of sarcoplasmic fish proteins by reversed phase has been used as a rapid means to identify the source fish [45]. This was similar to earlier reports using a single wavelength (280 nm) for species identification, but PDA detection provided additional information, particularly with closely related fish species. The identification was made by combining the information from the chromatographic patterns obtained at several wavelengths.

During separation development or compound purification, it is often useful to use on-line detection schemes to monitor the elution of a particular analyte and not rely on an off-line assay. Using PDA detection for such peak tracking is a routine procedure in small molecule analyses but is underutilized in studies of peptides and proteins. Hearn et al. [46] have shown how powerful this can be with peptides, even with the very similar spectra one observes with many peptides. They developed an algorithm that compared the zero-, first- and second-derivative spectra of peaks in a sample with those derivative spectra in standards. Match factors were calculated for each derivative spectrum, and an overall match factor was generated from the three spectral matches. Their results showed that only the second-derivative comparison was sufficiently sensitive to distinguish between very similar spectra. Given sufficient sensitivity and spectral resolution, however, the zeroth-order spectrum contains all of the information required for peak matching peptides. This was demonstrated in a study that incorporated the spectral contrast techniques for analyzing data from structurally similar proteins [38].

Unfortunately, there have apparently been no descriptions in the literature of similar peak tracking efforts applied to protein separations. In our laboratory we have applied this to an ion-exchange separation of a protein mixture and followed the change in elution via the spectral contrast technique (B. Warren, private communication). Although protein spectra are visually quite similar in appearance, the technique was able to distinguish all the proteins in a mixture of

myoglobin, ribonuclease A, chymotrypsinogen A, cytochrome *c*, and lysozyme. Myoglobin and cytochrome *c* contain heme but the remaining three proteins do not. During separation optimization, in which the eluent pH was varied between 5.0 and 8.0, there were several instances of peak order reversal that could be identified with PDA detection. Only when the pH change also resulted in a significant alteration in the spectrum did the tracking fail.

SUMMARY

PDA detection is now a mature technique, with nearly 20 years of practical application in the laboratory. The analysis and quantitation of aromatic amino acids in peptides and proteins are probably one of the most widely reported uses of PDA for these molecules. Conformational and stability analyses of proteins are another significant application. Also useful, although less frequently appearing in the literature, is PDA detection of a variety of naturally occurring chromophores and detection of chemical modifications such as oxidation that result in spectral changes. In all likelihood, chromophore analysis is more widespread than the literature indicates and is just routinely applied with little fanfare.

In our opinion, the widespread use of PDA detectors for aromatic amino acid studies is probably in decline today because of the rapidly increasing use of liquid chromatography—mass spectrometry in the analysis of peptides. This is especially true for characterizing aromatic content, in large part because identification and sequence analysis are both faster and more sensitive with MS and MS/MS techniques, which are rapidly replacing other analytical methods. Partial sequence data combined with genomic or protein database searching easily confirm the presence of particular amino acids such as Trp. Conformational studies, however, cannot be easily approached via MS, and PDA analysis of proteins will continue to find important applications in protein structure analysis. Protein activity is dependent on the fidelity of the native structure, and analytical tools for readily assessing the conformational status of a protein are indeed valuable to the analyst.

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