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# Quantification of residual nitrite and nitrate in ham by reverse-phase high performance liquid chromatography/diode array detector

I.M.P.L.V.O. Ferreira *, S. Silva

*REQUIMTE- Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Rua Anibal Cunha 164, 4099-030 Porto, Portugal*

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**Abstract**

Nitrite and nitrate are used as additives in ham industry to provide colour, taste and protect against clostridia. The classical colorimetric methods widely used to determine nitrite and nitrate are laborious, suffer from matrix interferences and involve the use of toxic cadmium. The use of chromatography is potentially attractive since it is more rapid, sensitive, selective and provides reliable and accurate results. A rapid and cost-effective RP-HPLC method with diode array detector was optimized and validated for quantification of nitrites and nitrates in ham. The chromatographic separation was achieved using a HyPurity C18, 5 μm chromatographic column and gradient elution with 0.01 M *n*-octylamine and 5 mM tetrabutylammonium hydrogenosulphate to pH 6.5. The determinations were performed in the linear range of 0.0125–10.0 mg/L for nitrite and 0.0300–12.5 g/L for nitrate. The detection limits were 0.019 and 0.050 mg/kg, respectively. The reliability of the method in terms of precision and accuracy was evaluated. Coefficients of variation lower than 2.89% and 5.47% were obtained for nitrite and nitrate, respectively ($n = 6$). Recoveries of residual nitrite/nitrate ranged between 93.6% and 104.3%. Analysis of cooked and dried ham samples was performed, and the results obtained were in agreement with reference procedures.


*Keywords:* Nitrite; Nitrate; RP-HPLC; Ham; Validation

## 1. Introduction

Nitrite and nitrate salts of sodium and potassium ($E_{249}$, $E_{250}$, $E_{251}$, $E_{252}$) are permitted preservatives that may be added to cured meat products to protect these products against *Clostridium botulinum* and other *Clostridium* species [1]. Nitrite is a potent inhibitor of microorganisms, in particular clostridia. However, the contributions of nitrite to meat curing include not only antimicrobial effects but also characteristic colour development, oxidative stability of lipids, flavour production and texture improvement. The nitric oxide (NO) is the active specie and not its undissociated form. Nitrate is applied when a slow release of nitrite is wanted, nitrate diffuse in the product before it is reduced to nitrite [2,3].

Maximum levels of nitrate and nitrite that can be added to meat products and residual amounts are established in European Legislation [1]. The amount of nitrite necessary to inhibit *C. botulinum* differs from product to product, depending on the process, for example, for ham a maximum of 150 mg/kg of sodium nitrite can be added. Nitrite reacts with many components in the matrix and, for that reason, determination of nitrate and/or nitrite content does not reflect the amount of preservatives added. This particularly holds when ascorbic or erythorbic acid is added in order to enhance the release of nitric oxide from nitrite during heating. Most of the added nitrite is present as NO bound with myoglobin (5–15%), sulphydryl groups (5–15%), lipids (1–5%) and proteins (20–30%), partially is present as nitrate (<10%) and as nitrite (10–15%) [4,5]. Therefore, analytical methods usually determine the residual nitrite/nitrate.

The classic method for the determination of nitrite in food is based upon its ability to convert aromatic amines into diazonium ions, which, in turn, are coupled to another aromatic compound in order to produce an azo dye (the Griess-Romijn reaction) [6]. This is the base of many spectrophotometric methods. The most common arrangement utilizes sulphanilamide and *N*-(1-naphthyl)ethylenediamine as the target amine and coupler, respectively, with the product of the reaction detected at 540 nm. Nitrate is normally chemically reduced to nitrite and

* Corresponding author. Tel.: +351 2 22078929; fax: +351 2 22003977.
*E-mail address:* isabel.ferreira@ff.up.pt (I.M.P.L.V.O. Ferreira).

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determined by the same type of reaction. A variety of reducing agents have been investigated to facilitate this conversion and include amalgamated cadmium [7], copperized cadmium [8] and zinc [9], and more recently, photo-induced reduction [10]. Other current methods for the determination of nitrite and nitrate rely on segmented-flow or flow-injection analysis variants of the traditional colorimetric Griess diazotisation procedure [11–14].

Alternative methods for nitrate and nitrite determination in foodstuffs have been developed more recently, including spectroscopic determination after enzymatic reduction [15], differential pulse voltammetry [16] and capillary electrophoresis [17]. The use of chromatography is potentially attractive since it is more rapid, sensitive and selective than methods based on reduction/colorimetry [3,18,19]. A study was performed to obtain an alternative chromatographic method to determine residual nitrite and nitrate in foods. Limits of detection of 1 and 10 mg/kg, respectively, for nitrite and nitrate are reported [3]. Columns containing a strong anion exchanger packing material, such as, polymethacrylate resin with a quarternary ammonium functional group are necessary for assuring reliability, the inconvenient is that this type of columns are expensive.

Ion-chromatographic methods have also been widely studied for the separation of nitrite and nitrate and other ions in several matrices [20–22]. Ion-pair HPLC methods offer, with respect to ion chromatography, advantages of relatively lower cost in instrumentation and columns and can be advantageously employed in laboratories where only conventional HPLC systems are available. Additionally, the theoretical column efficiency of ion-pair HPLC is better than that of an IC column, assuming that a suitable ion-pair reagent is chosen. However, little information is available on the analysis of residual nitrite and nitrate in food samples using ion-pair HPLC.

The simplest and easiest sample preparation methods namely dilution or filtration, are not suitable with some samples, some authors describe a solid-phase extraction clean-up step, but it is difficult to achieve reproducible recoveries of each analyte [23]. In the case of nitrite, sample manipulations need to be kept to a minimum, due to the instability of this ion that oxidizes to nitrous acid and/or nitrate.

The objectives of this study were to develop and validate a sensitive, selective and faster reverse-phase chromatographic method based on UV absorption (RP-HPLC/diode array) for quantification of residual nitrites and nitrates in ham.

## 2. Experimental

### 2.1. Chemicals and samples

All reagents used were of analytical grade purity. Solvents for HPLC were filtered through 0.22 μm NL 17 filters and degassed under vacuum for at least 15 min before use. Sodium nitrate, sodium nitrite, L-ascorbate and tetrabutylammonium hydrogenosulphate were supplied by Sigma Chemicals Co. (St. Louis, MO, USA), acetonitrile Licrosolv and activated charcoal were from Merk (Darmstradt, Germany) and *n*-octylamine was supplied by Fluka. Sep-pak cartridges were from Waters. Standard solutions of sodium nitrite (1000 mg/L) and sodium nitrate (1000 mg/L) were prepared from $NaNO_3$ and $NaNO_2$ previously dried in an oven (100 °C during 1 h). More diluted standard solutions, used in the calibration curves, and were obtained from the concentrated solutions by dilution. The solutions were treated with some chloroform drops to prevent the development of microorganisms and were stored in a refrigerator.

### 2.2. Instrumentation

The chromatographic analysis was carried out in an analytical HPLC unit (Jasco) equipped with one Jasco PU-2080 HPLC pump, a MD-2010 Plus Multiwavelength detector and a type 7125 Rheodyne Injector with a 20 μL loop. The column was a reversed-phase HyPurity C18 from Thermo Electron Corporation (5 μm; 150 mm length; 3 mm internal diameter). The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used.

Fungilab Ultrasonic cleaner and a METROHM 632 pH-Meter were used for eluent preparation. A vortex Heidolph REAX 2000 was used for sample preparation.

### 2.3. Sample preparation

Seven ham samples purchased from a local supermarket were pre-treated by homogenization in a triturator and stored at 4 °C during the study. These included four samples of cooked pork ham (samples 1–4), two samples of cooked turkey ham (samples 5–6) and one sample of dried ham (sample 7). All samples were from different brands.

Five different sample preparation procedures were tried. One set of samples was extracted with hot water (50–60 °C) and clarified by filter through filter paper and membrane filter. Two sets of samples were extracted with hot water (50–60 °C) and deproteinized, one with acetonitrile and the other with Carrez reagents and filtered. One set of samples was dried for 1 h in an oven, extracted with hot water (50–60 °C), added of 1 g activated charcoal, deproteinized with acetonitrile and filtered. Another set of samples was extracted with hot water (50–60 °C), deproteinized with acetonitrile and purified with sep-pak C18. A summarized description of the analytical methods assessed is presented in Table 1.

### 2.4. Method validation

Analyses were performed over a period of 2 weeks. Each batch consisted of replicate analyses of blanks (limit of detection), standard solutions (sensitivity and linear range) and both spiked and unspiked samples (recovery and precision).

#### 2.4.1. Linearity and range

Linearity was addressed by preparing eight standard solutions of sodium nitrite and sodium nitrate ranging between 0.0125–10.0 and 0.025–15.0 mg/L. A linear regression analysis of analyte concentration versus peak response was performed.

Table 1
Summarized description of the analytical methods assessed

| Method | Extraction | Clarification |
|---|---|---|
| 1 | 10 g sample homogenized in 15 mL hot water (50–60°), placed in a water bath 15 min | Filter through filter paper and membrane filter |
| 2 | 10 g sample homogenized in 10 mL hot water (50–60°), placed in a water bath 15 min | 5 mL Carrez I and Carrez II. Filter through filter paper and membrane filter |
| 3 | 10 g sample homogenized in 10 mL hot water (50–60°), placed in a water bath 15 min | 5 mL of acetonitrile. Filter through filter paper and membrane filter |
| 4 | 10 g sample homogenized and dried in an oven + 1 g activated charcoal in 20 mL hot water (50–60°), placed in a water bath 15 min | 5 mL of acetonitrile. Filter through filter paper and membrane filter |
| 5 | 10 g sample homogenized in 10 mL hot water (50–60°), placed in a water bath 15 min | 5 mL of acetonitrile. purified with sep-pak C18 (2 mL methanol and 4 mL water, load 1 mL sample, wash with 3 mL × 1 mL water, elute with 2 mL 0.5 M NaCl solution |

#### 2.4.2. Detection limits

The detection limits were calculated as the concentration corresponding to three times the background noise of the blank.

#### 2.4.3. Quantification limits

The quantification limits were calculated as the concentration corresponding to ten times the background noise of the blank.

#### 2.4.4. Recoveries

The experimental recovery was obtained from difference between two measurements (sample and spiked samples), according to the following relationship:

$$\text{Recovery, \%} = (\text{total analyte found} - \text{analyte originally present}) \times \frac{100}{\text{analyte spike}}$$

The recovery ranges for nitrite and nitrate estimated immediately after sample preparation.

#### 2.4.5. Precision

A total of three analyses were performed for one ham sample to evaluate the relative standard deviation (RSD%) of the method.

#### 2.4.6. Reference methods

As no certified reference materials were available and in order to evaluate the accuracy of the results obtained by HPLC, the reference methods of the International Standards Organization ISO 3091–1975 and ISO 2919–1976 [24,25] were used for the determination of nitrite and nitrate levels, respectively in meat products.

## 3. Results and discussion

### 3.1. Method optimization

The optimization of the RP-HPLC/diode array procedure was focused on the chromatographic separation as well as on the sample preparation procedure. The stationary phase selected required the use of counter ions for peak separation. Different mobile phases and preparation procedures were assayed at a flow rate of 1 mL/min, with the aim of assess which mobile phase and sample preparation give better performance. An injection volume of 20 μL was chosen since it is usually described in literature [3].

#### 3.1.1. Evaluation of mobile phase

A standard solution containing 1.25 mg/L of sodium nitrite and 2.50 mg/L of sodium nitrate was separated on a C18 column by using five different mobile phases: 0.01 M tetrabutylammonium hydrogenosulphate, pH 6.5; 0.01 M tetrabutylammonium hydrogenosulphate, pH 6.5 in 20% methanol; 0.01 M tetrabutylammonium hydrogenosulphate, pH 6.5 in 20% acetonitrile; 0.01 M *n*-octylamine, pH 4 and 0.01 M *n*-octylamine/5 mM tetrabutylammonium hydrogenosulphate, pH 6.5.

Isocratic elution with a mobile phase containing 0.01 M *n*-octylamine/5 mM tetrabutylammonium hydrogenosulphate, pH 6.5 enables nitrite and nitrate ion-pair chromatographic separation. The use of two different ion-pair compounds as eluents allows for adjustment of coeluting peaks and ensures appropriate peak resolution (Fig. 1). Additionally, the eluent should not give rise to high background UV absorbance.

Nitrite and nitrate spectrums were inserted in the software spectrum library. When studying the interference of L-ascorbate a usual ham additive, it reported a distinct retention time and spectrum.

#### 3.1.2. Evaluation of sample extraction procedure

One of the problems in measuring nitrites and nitrates in food samples are the matrix interferences that may hamper the determination of the substances of interest, for example, the liquid chromatographic techniques generally utilized the inherent low-wavelength UV absorption of nitrate and nitrite anions (205–215 nm) and spectral interferences from closely eluting compounds can occur [26]. Thus, appropriate sample treatment is necessary.

The extraction of nitrite and nitrate from ham using only hot water (see Table 1, Method 1) was not appropriate, since interfering compounds were also extracted. Neither the deproteinization

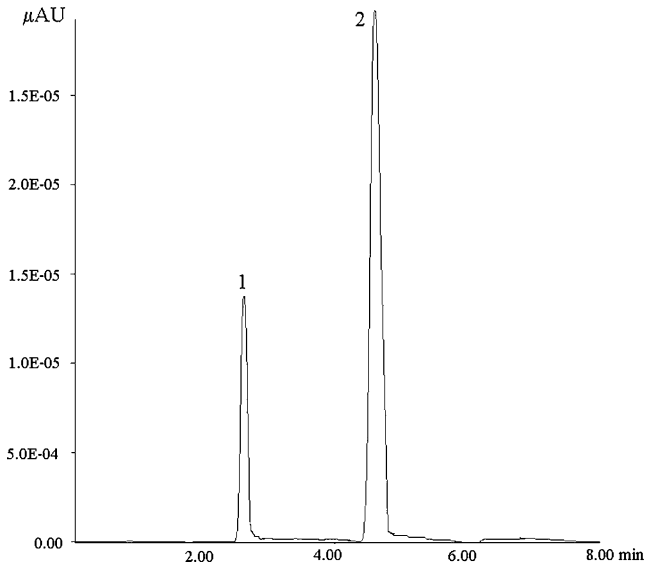


Fig. 1. Chromatogram obtained with the C18 column for an aqueous standard solution containing 1.25 mg/L of sodium nitrite and 2.50 mg/L of sodium nitrate.

with acetonitrile (Method 2) nor with Carrez reagents (Method 3) removed the interferences that co-elute with nitrite peak, and remain even after purification with Sep-Pak C18 (Method 5). Spectrum is a useful tool to determine peak purity and to establish correlations between the spectrum of sample and standard peaks with similar retention times using Borwin PDA controller software.

Ham samples extracted with hot water (50–60 °C), added of 1 g activated charcoal, deproteinized with acetonitrile (Method 4) and filtered gave a clean and interference-free baseline. Thus, this extraction procedure was chosen for further analyses. No peaks were observed in sample extracts after elution of nitrate peak, thus chromatographic analysis of 8 min could be performed (Fig. 2).

### 3.2. Method validation

Linearity within the concentration range of 0.0125–10.0 mg/L for nitrite and 0.0300–12.5 mg/L for nitrate was checked through the calibration curves, and obtained by linear regression of peak area versus concentration. The coefficients of determination ($r^2$) were 0.99981 and 0.99999, respectively. The detection limits of sodium nitrite and sodium nitrate were 0.0126 and 0.0318 mg/L, respectively. The quantification limits of sodium nitrite and sodium nitrate were 0.134 and 0.341 mg/L, respectively.

Three different extractions, as described above, were made for each ham sample and subsequently injected twice. The RSD ($n = 6$) reported for sample concentration was less than 2.89% and 5.47% for nitrite and nitrate, respectively.

The reliability of the method was confirmed by two recovery experiments. Two levels of nitrites (0.00075 mg and 0.0015 mg/kg) and nitrates (0.0008 and 0.0012 mg) were tested. Recoveries varied between 93.6% and 97% for nitrite and 99.5% and 104.3% for nitrate.

Similar qualitative chromatographic profiles were obtained for the three types of ham assayed (cooked pork ham, cooked turkey ham and dried pork ham), however, quantitative differences were obtained (Fig. 2). Table 2 presents results obtained from the analysis of seven ham samples. Nitrite and nitrate levels found in ham samples were in the same order of magnitude of those quantified in Portuguese ham (during its shelf-life) by automated flow injection analysis [27]. To confirm the reliability and accuracy of the method, part of the samples were analysed by the reference methods of the International Standards Organiza-

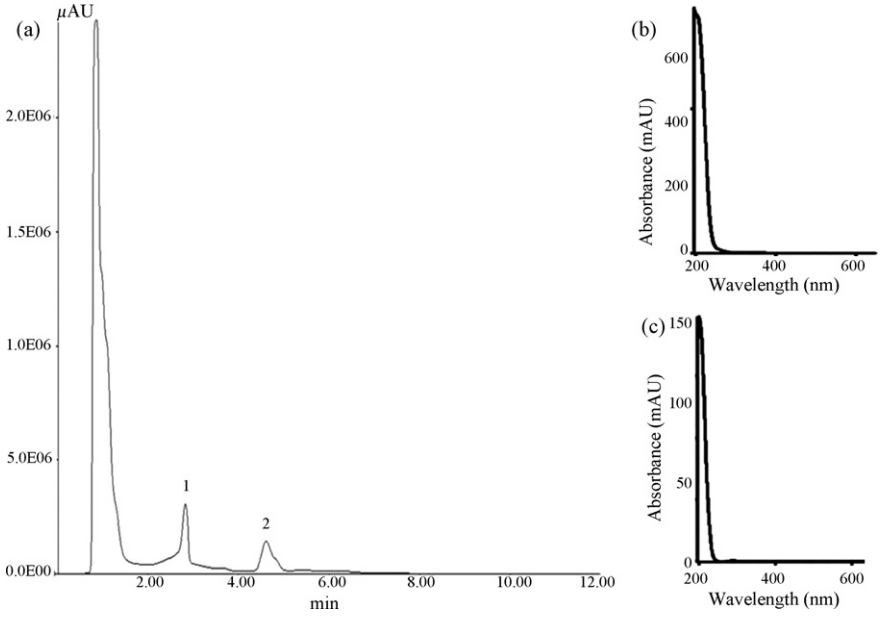


Fig. 2. (a) Chromatogram obtained for a ham sample extracted with hot water (50–60 °C), added of 1 g activated charcoal, deproteinized with acetonitrile and filtered. Peak 1 was identified as nitrite and peak 2 was identified as nitrate. (b) Spectrum of peak with a retention time of 2.6 min taken from 4a chromatogram. (c) Spectrum of peak with a retention time of 4.7 min taken from 4a chromatogram.

Table 2
Reported values of nitrite and nitrate in ham samples (cooked and dried) from the market, values expressed as mg of sodium nitrite/kg ham and sodium nitrate/kg ham

| Sample | $NaNO_2$ (mg/kg) | | $NaNO_3$ (mg/kg) | |
|---|---|---|---|---|
| | HPLC method | Reference | HPLC method | Reference |
| 1 | 0.354 ± 0.012 | – | 0.810 ± 0.023 | – |
| 2 | 0.113 ± 0.006 | – | 0.663 ± 0.028 | – |
| 3 | 0.895 ± 0.022 | – | 0.791 ± 0.019 | – |
| 4 | 0.934 ± 0.022 | 0.986 ± 0.032 | 0.701 ± 0.020 | 0.789 ± 0.043 |
| 5 | 1.155 ± 0.024 | 1.201 ± 0.085 | 0.987 ± 0.019 | 1.003 ± 0.022 |
| 6 | 1.253 ± 0.021 | 1.307 ± 0.061 | 0.999 ± 0.041 | 1.121 ± 0.045 |
| 7 | 0.682 ± 0.022 | 0.701 ± 0.055 | 0.872 ± 0.031 | 0.901 ± 0.034 |

Part of the samples were analysed by the reference methods of the International Standards Organization ISO 3091-1975 and ISO 2919-1976 [24,25].

Table 3
Comparison between the optimized RP-HPLC method and NMKL method [3]

| Methodologies | RP-HPLC/diode array | | Method no. 165 2000 NMKL | |
|---|---|---|---|---|
| Parameters | $NO_2^-$ | $NO_3^-$ | $NO_2^-$ | $NO_3^-$ |
| Detection limit (mg/kg) | 0.0189 | 0.0477 | 1 | 10 |
| Correlation coefficient | 0.9998 | 1.0000 | 0.9999 | 0.9997 |
| Recovery (%) | 93.7–97 | 99.5–104 | 96–108 | 96–107 |
| Linearity (mg/L) | 0.0126–10 | | 0.5–20 | |
| Running time (min) | 8 | | ≫8 | |

tion ISO 3091-1975 and ISO 2919-1976 [24,25]. No significant statistical difference between the two methods was observed, at the 1% significance level, from the analysis of variance, however, it should be pointed out that the reference method gave higher levels than the HPLC method. It is well known that classical colorimetric methods suffer from matrix interferences, caused primarily by added reducing compounds, such as ascorbate, that are not completely eliminated and will interfere with colour formation during the diazotisation method for nitrite analysis. A number of reviews have covered the effects of such interferences within food and environmental matrices [13,14] and justify our results.

Finally, comparison was performed between the results obtained by the validated methodology and the results described in the literature for NMKL Collaborative study [3] using a quaternary ammonium column and European standard ENV 12014-4. Weak anionic exchange chromatography with aminopropyl bonded-phase column and strong anionic exchange chromatography with two different eluents (5 mM $K_2HPO_4$) in 15% acetonitrile, pH 8.6 and buffer (boric acid, gluconic acid, lithium hydroxide and glycerol) in 12.5% acetonitrile, pH 6.5 were tested. The strong anionic exchange chromatography method with buffer in 12.5% acetonitrile as mobile was preferred and validated in a collaborative study. The authors, reported similar recoveries of nitrite and nitrate from meat products, and higher detection limits for nitrite and nitrate ions (Table 3). However, chromatographic column is much more expensive and eluent preparation much more laborious.

## 4. Conclusions

In conclusion, the described extraction procedure and RP-HPLC method seems to fulfil the criteria of selectivity, sensitivity, reproducibility and convenience for analysing the nitrite and nitrate content in ham. It is less laborious than reference procedures and strong anionic exchange chromatography. Thus, it is suitable for routine assays. HPLC analysis time is lower than 8 min and extraction procedure is rapid and cost effective. Chromatograms with a clean and interference-free baseline are obtained.

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