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DETERMINATION OF NITRATE AND NITRITE IN CURED MEATS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Several existing chromatographic techniques for the determination of nitrate and nitrite were evaluated and found to be unsuitable for the analysis of cured meats because of interferences present in these samples. An alternative procedure was developed, based on the use of a low capacity anion-exchange column with chloromethanesulphonate as eluent, and using ultraviolet absorbance at 210–214 nm as the detection method. This analytical method was shown to be rapid, sensitive and precise, and satisfactory recovery was observed when nitrate and nitrite were added to several food samples. A comparison of this method with a conventional spectrophotometric method showed poor agreement between the two techniques and is attributed to deficiencies in the spectrophotometric method. The proposed chromatographic procedure is shown to be applicable to the analysis of vegetables and cheese as well as cured meats.

INTRODUCTION

The determination of nitrate and nitrite in foodstuffs has become increasingly important because of concern over excessive human dietary intake of these species. The toxicity of nitrites, especially in relation to nitrosamine production, has been well established[1,2] and whilst nitrates are not very toxic, their ready conversion into nitrites means that levels of nitrate must be carefully monitored. Both nitrate and nitrite occur in a wide variety of natural and processed foods because of the general usage of nitrogenous fertilizers in the agricultural industry, and of nitrate and nitrite as curing salts in the meat industry.

The major methods developed for the determination of nitrate and nitrite have involved spectrophotometric procedures. These are generally based on the reduction of nitrate to nitrite, which is then used to convert sulphanilamide or sulphanilic acid into a diazonium salt, followed by coupling to a suitable aromatic amine[3,4]. Nitrite is determined by the same procedure, but with the omission of the reduction step. The spectrophotometric methods are time-consuming, very prone to interference[5] and can be unreliable for some samples (such as certain meats and cheeses) due to

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the difficulty in obtaining a clear solution for the final measurement. In addition, the sensitivity of these methods for nitrite is relatively poor and trace levels of nitrite in foods are often not detectable[6].

Some high-performance liquid chromatographic (HPLC) procedures for the determination of nitrate and nitrite have been reported and are generally based on pre-column derivatization methods[7–9], ion-exchange[10–14] or ion-interaction[15,16] techniques. Pre-column derivatization methods include the reaction of nitrite with diamines (such as 2,3-diaminonaphthalene) to form triazole derivatives which can be separated from the reaction mixture by reversed-phase chromatography[7]. Alternatively, the reaction of nitrite with hydralazine[8] or phenol[9] may be used. With these procedures, nitrate can be determined only after reduction to nitrite and this limitation represents a serious drawback to the use of pre-column derivatization.

Ion-exchange methods, including the suppressed ion chromatographic technique of Small *et al.*[17], have been applied to the determination of nitrate and nitrite using silica[10–12], resin[13] or cellulose-based[14] ion exchangers. Ion chromatography of nitrite is not completely reliable due to oxidation reactions occurring in the suppressor column[18], and other ion-exchange methods have been successfully applied only to simple samples such as waters and vegetable extracts. Ion-interaction methods for the determination of nitrate and nitrite in waters[15] and some foods[16] using C_{18} or polymeric supports have been described, however the separation achieved with the food samples was rather poor. An alternative approach using an amino column with phosphate as eluent has been reported[19] and was successfully applied to the determination of nitrate and bromide in cheese, whey, vegetables, flour and rice[20].

We have been interested in the simultaneous determination of nitrate and nitrite in cured meats using a procedure requiring minimal sample pre-treatment. The above-mentioned chromatographic procedures have been evaluated for this particular application and some alternative chromatographic methods were also investigated. Here, the results of this study are reported and a new procedure based on ion exchange with direct UV detection of nitrate and nitrite is described. The method has a wide application to other complex materials, such as foodstuffs in general.

EXPERIMENTAL

Instrumentation

The liquid chromatograph consisted of a M-6000 pump, U6K injector and Model 441 detector operated at 214 nm (Waters Assoc., Milford, MA, U.S.A.). Several other detectors were also used, including a Waters Assoc. Model 450 variable-wavelength detector, a Model 213A conductivity detector (Wescan Instruments, Santa Clara, CA, U.S.A.), a Model 7510 refractive index (RI) detector (Erma Optical Works, Tokyo, Japan) and a LC-3A amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) used in conjunction with the other detectors to confirm the retention time of nitrite ion. Chromatograms were recorded on a Omniscribe Model 5271 recorder (Houston Instruments, Austin, TX, U.S.A.) or a Waters Assoc. M730 data module. Columns were maintained at 30°C using a Bioanalytical Systems LC-234 column heater.

Columns

Ion-exchange, reversed-phase and amino columns were used in this study. The ion-exchange column was a Vydac 302 IC 4.6 anion exchanger, 250 × 4.6 mm I.D. (Separations Group, Hesperia, CA, U.S.A.). Reversed-phase columns were supplied by Waters Assoc. and consisted of a 10-μm C_{18} Radial-Pak (100 × 5.0 mm I.D.) or a 10-μm CN Radial-Pak (100 × 5.0 mm I.D.). Two amino columns were used: a Waters Assoc. 10-μm NH_2 Radial Pak (100 × 5.0 mm I.D.) and a laboratory-packed aminopropyl column (200 × 4.6 mm I.D.).

Mobile phases

The mobile phases described in the Results section were prepared using analytical grade reagents, chromatographic grade organic solvents and water purified on a Millipore Milli-Q system. All mobile phases were filtered through 0.45-μm filters and degassed in an ultrasonic bath before use.

Sample preparation

Samples of bacon, corned beef and salami sausage were purchased from a supermarket and, for comparative purposes, samples of spinach, lettuce, blue vein cheese and ham and cheese spread were also obtained. A 30–50 g sample was homogenized in a blender with 50 ml of hot water for 5 min and was then transferred to a Sonifier C-30 cell disruptor (Bransonic, Danbury, CO, U.S.A.) using a further 50 ml of hot water. The sample was further homogenized at 20 kHz for 15 min, after which the solution was cooled and made up to 250 ml in a volumetric flask. A 30-ml aliquot of this solution was centrifuged for 15 min at 2000 *g* and the supernatant solution decanted. A 5-ml portion of this solution was filtered through a 0.45-μm Millipore Millex filter and then passed through a Waters Assoc. C_{18} Sep-Pak cartridge which had previously been flushed with methanol. The first 3 ml of filtrate were discarded and the following 1 ml of filtrate was retained for direct injection onto the liquid chromatograph.

RESULTS AND DISCUSSION

Evaluation of existing chromatographic methods

Methods reported in the literature for the determination of nitrate and nitrite, as well as other inorganic anions, were evaluated for their suitability to the determination of nitrate and nitrite in cured meats. Standard solutions and meat samples prepared according to the procedure described under Experimental were used. In addition to the different separation methods, a number of alternative detection procedures were also studied, including conductivity, direct UV absorbance, indirect UV or RI methods and electrochemical detection.

The first of the possibilities investigated was the combination of anion exchange with conductivity or indirect UV or RI detection, using 2.5 m*M* potassium hydrogen phthalate (KHP) as eluent[21]. The operating principles of indirect UV and RI detection have been described elsewhere[21–23]. Using the Vydac anion-exchange column and KHP as eluent, excellent separation of the nitrate and nitrite standards was achieved and the detection sensitivity was best when indirect UV detection was used. When this approach was applied to the analysis of nitrate and nitrite in corned

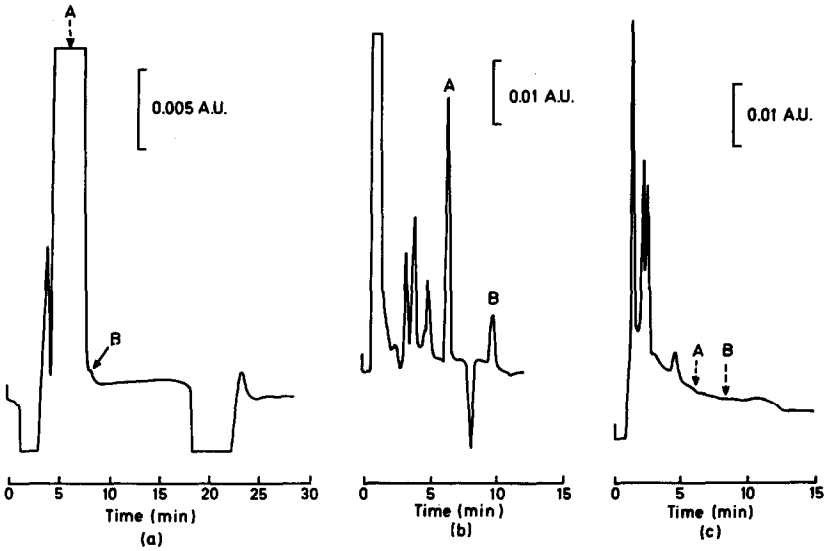

Fig. 1. Results obtained with existing chromatographic procedures for the determination of nitrite (A) and nitrate (B) in corned beef. The procedures used were ion exchange with indirect UV absorbance detection (a), ion interaction with direct UV absorbance detection (b) and use of an amino column with direct UV absorbance detection (c). Dashed arrows indicate the retention times of nitrite and nitrate standards. Conditions: (a) Vydac 302 IC column with 2.5 m*M* phthalate eluent at pH 4.2, detection at 265 nm; (b) CN Radial Pak column with methanol–0.1 *M* KH_2PO_4 eluent (35:65, v/v) containing 1% (w/v) cetrimide; (c) aminopropyl column with 16.0 m*M* KH_2PO_4 eluent (at pH 3.0).

beef extract the chromatogram shown in Fig. 1a was obtained. Neither nitrate nor nitrite could be quantitated due to the large excess of chloride in the sample. The resulting chloride peak completely masked the nitrate and nitrite peaks, regardless of the mobile phase conditions used.

The second approach studied was the use of ion-interaction chromatography coupled with direct UV-absorbance detection. This detection method has obvious potential for the meat samples used because both nitrate and nitrite have appreciable absorptivities at 214 nm (9000 and 5000 l mol^{-1} cm^{-1}, respectively), whereas chloride has no significant absorbance at this wavelength. Ion-interaction methods have been reported using C_{18}[15], PRP-1[16] or CN[24,25] columns. Since the PRP-1 column has already been shown to give inadequate separation of food samples[16], no further studies were made with this column. The C_{18} column was used with 5 m*M* tetramethylammonium phosphate (pH 7.0) eluent and the CN column was used with methanol–0.1 *M* KH_2PO_4 (35:65, v/v) (pH 5.0) eluent, containing 1% (w/v) cetrimide as the ion-interaction reagent. With both methods, excellent resolution of standard solutions was obtained, with the CN column giving superior results. When this latter method was applied to the corned beef sample, the chromatogram shown in Fig. 1b was obtained. Nitrate and nitrite were clearly resolved, however the retention times obtained were not reproducible with repeated injection of the same sample and also showed variation when the type of sample was changed. This factor, together with the observation that calibration plots were non-linear due to the presence of spurious peaks, suggested that the ion-interaction method was not optimal for the samples studied.

The third method evaluated was the use of amino columns with direct UV absorbance detection. This approach has been reported for the separation of a number of inorganic anions[19] and has been applied to the determination of bromide and nitrate in foodstuffs[20]. Cured meats have not previously been analysed by this procedure. Using an amino column and 16.0 m*M* KH_2PO_4 at pH 3.0 as eluent, nitrate and nitrite were adequately resolved in standard mixtures, however poor results were obtained with the corned beef sample (Fig. 1c).

The conclusion reached from this evaluation of existing chromatographic procedures for nitrate and nitrite determination was that no method could be considered satisfactory for application to the cured meat samples, all of which were tested with each separation method. The direct UV absorbance method was optimal for detection since this allowed selective detection of nitrate and nitrite in the presence of excess of chloride. In view of this, a method was sought to combine direct UV detection with separation on a high efficiency anion exchanger.

Development of an alternative separation method

The eluents used with low capacity silica-based anion-exchange columns (such as Vydac 302 IC) have been selected with a view to their suitability for conductivity detection. For this reason, only eluents of low equivalent conductivity have been used, particularly aromatic acid anions[26,27]. These eluents are unsuitable for direct UV absorbance detection because of their high molar absorptivities in the UV region. We have therefore used chloromethanesulphonic acid as eluent because it has very little UV absorbance, has sufficient ion-exchange displacing ability to elute nitrate

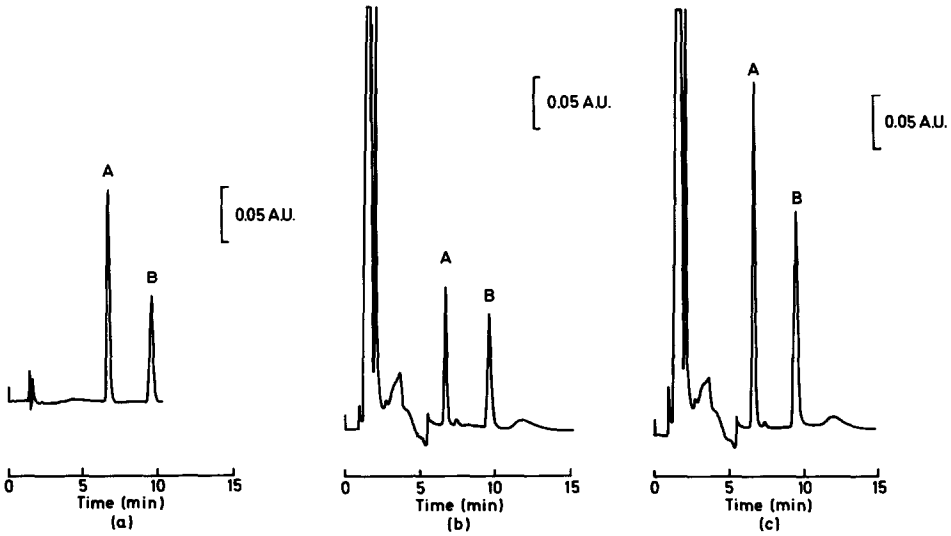

Fig. 2. Separation of nitrite (A) and nitrate (B) in standards (a), bacon sample (b) and spiked bacon sample (c), using the proposed chromatographic procedure. The sample used for (c) was spiked with 0.5 μg of nitrate and nitrite in a injection of 25 μl. Conditions: Vydac 302 IC column with 11.0 m*M* chloromethanesulphonic acid (pH 5.0) eluent. The flow-rate was 2.0 ml/min and the detector was operated at 210 nm using a sensitivity of 0.4 a.u.f.s. For chromatogram (a), 10 μl of 50 ppm nitrate and nitrite were injected onto the column.

and nitrite and its degree of ionization is not affected by pH over the working pH range of the column. A similar eluent has recently been reported for ion chromatography with UV or conductimetric detection[28].

When 11.0 m*M* chloromethanesulphonate at pH 5.0 was used as eluent with the Vydac column an excellent separation of nitrate and nitrite in standard mixtures was observed (Fig. 2a). Linear calibration plots were obtained for nitrate and nitrite over the range 0–250 μg of injected solute, the precision of replicate injections falling within the range 1–4% relative standard deviation, depending on the amount of sample injected. Detection limits were 2.3 and 2.6 ng for nitrite and nitrate, respectively. The application of the method to cured meat samples also gave excellent results, as indicated by the chromatogram obtained for the bacon sample shown in Fig. 2b. Spiking of this sample with a standard mixture of nitrate and nitrite gave the chromatogram shown in Fig. 2c, thereby confirming the peak identities.

Recovery studies and comparison with spectrophotometric analysis

Recovery studies were performed on each of the cured meat samples by adding known quantities of nitrate and nitrite to the sample solution prior to the initial homogenization step. In this way, potential losses occurring during the sample treatment procedure were assessed, together with interferences occurring during chromatographic separation. Other sample types, including vegetables and cheese, were also used. The results are given in Table I which indicates that satisfactory recoveries were achieved for the samples tested. Some typical chromatograms obtained with samples of corned beef and lettuce are presented in Fig. 3. Comparison of Fig. 3a with Fig. 1 clearly illustrates the superiority of the developed chromatographic system over existing methods.

Table II shows a comparison of results obtained for the determination of nitrate and nitrite in selected foods using the proposed chromatographic method and a standard spectrophotometric procedure[4]. The agreement between the two methods was poor, especially for nitrite determination, and this situation persisted when the analysis was repeated several times. The recovery data given in Table I suggest that

TABLE I

AMOUNTS OF NITRITE AND NITRATE PRESENT AND RECOVERIES FOR SAMPLES

Recoveries were determined in triplicate.

Sample	*Nitrite*				*Nitrate*			
	Amount present (ppm)	*Amount added (ppm)*	*Amount recovered (ppm)*	*% Recovered*	*Amount present (ppm)*	*Amount added (ppm)*	*Amount recovered (ppm)*	*% Recovere*
Cheese spread	2.8	2.5	5.2	96	12.9	10.0	21.7	88
Corned beef	38.4	35.0	72.0	96	120	100	209	89
Bacon	78.5	75.0	153.5	100	94.0	75.0	164	93
Blue vein cheese	10.0	7.5	17.3	97	0.8	1.0	1.7	89
Salami	7.6	7.0	14.5	98	455	450	932	106
Spinach	2.0	2.0	3.9	96	2728	2500	5252	101
Lettuce	4.6	4.0	8.4	96	1725	1500	3255	102

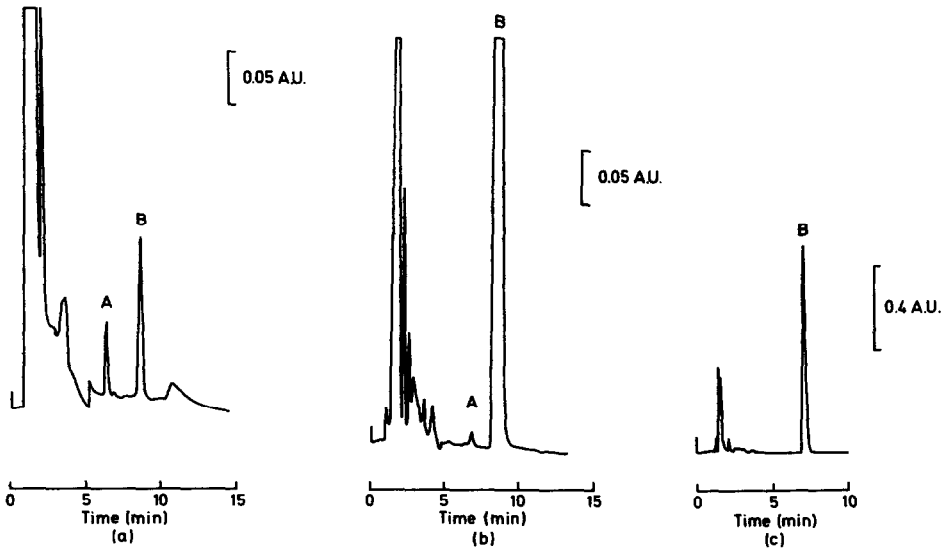

Fig. 3. Typical chromatograms obtained with samples of corned beef (a) and lettuce (b, c) using the proposed procedure. Conditions: As for Fig. 2, except that the detector was operated at 2.0 a.u.f.s. in (c) to enable quantitation of the nitrate ion.

TABLE II

COMPARISON OF CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF NITRITE AND NITRATE IN SAMPLES

Sample	*Chromatographic method*		*Spectrophotometric method*	
	Nitrite (ppm)	*Nitrate (ppm)*	*Nitrite (ppm)*	*Nitrate (ppm)*
Bacon	78.5	94.0	76.5	75.0
Spinach	2.0	2728	1.4	2766
Lettuce	4.6	1725	3.8	1618

the lack of agreement between the two analytical procedures may be attributed to deficiencies in the spectrophotometric method. These deficiencies include such factors as a small linear range of concentrations, poor sensitivity, high blank corrections required for coloured or turbid sample extracts and the requirement that nitrate be reduced to nitrite prior to the determination. In comparison, the chromatographic method is rapid, sensitive and precise, and the small sample volume required for analysis enables efficient small-scale sample clarification techniques to be used.

CONCLUSIONS

This study has indicated that the determination of nitrate and nitrite in cured meat cannot be reliably performed using existing chromatographic methods, due to interferences present in these samples. An alternative method based on ion-exchange

separation and UV absorbance detection has been developed and successfully applied to these samples.

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