Determination of nitrite levels in refrigerated and frozen spinach by ion chromatography

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Abstract

The determination of nitrites in samples of spinach after different times of refrigeration and freezing was studied. After cooking, the liquid and solid obtained were analysed. Ion chromatography with electrochemical detection, in which cell is composed of a single large porous graphite working electrode, was used as these solutions had, in many instances, concentrations of nitrites around 50 ng/ml; these levels are close to, or even below, the determination limits of other analytical methods usually employed. However, using this electrochemical detection, the determination of levels around 20 ng/ml was performed with good accuracy. The results obtained indicate that the freezing procedure does not modify the nitrite levels of the samples. In contrast, the refrigeration technique produces a considerable increase in their concentration 4–8 days later.

1. Introduction

In recent years there has been growing concern about the role of the nitrite ion in metahaemoglobin formation and as an important precursor in the formation of N-nitrosamines, many of which have been shown to be carcinogens. This gives rise to nitrite toxicosis, which may be a serious problem in babies and adults [1]. The occurrence of nitrite salts in the environment and accordingly in food and, in addition, their use as food preservatives is widespread [2–4].

It is therefore important that sensitive and accurate methods be available for the determination of nitrite ion. A large number of methods have been developed for the determination of this ion, based principally on spectrophotometric and fluorimetric techniques, but these have limited sensitivity and accuracy which depend on unstable colors [5–10]. More recently, several voltammetric and polarographic methods have been reported [11–18], some limited by poor sensitivity and others by long analysis times or the need to be adapted to flow systems. However, methods based on solid electrodes are more desirable for sensing, flow applications and short analysis times.

In the last decade, ion chromatography (IC) has undergone significant changes. Nowadays it has a much wider scope, which ranges from the determination of inorganic and organic cations to that of inorganic and organic anions [19,20]. Conductivity detection has been improved in several respects such as high suppression capacity, which not only represented a significant

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increase in sensitivity but also opened the door to gradient elution in IC using suppressed conductivity detection with corresponding optimization of the time and resolution of the chromatogram [21]. In spite of these improvements, conductivity detection has insufficient sensitivity for our aim of designing a procedure for detecting lower levels of nitrite in spinach.

Recently, various studies have been reported with some modification over traditional IC, using organic modifiers in the eluents [22], electrostatic separation [23], column switching [24], etc., with the aim of improving both the sensitivity and resolution in anion chromatographic separations. Most of these modifications require a special column treatment or equipment that is not always compatible with these determinations.

In this paper, a simple method based on the use of a very sensitive coulometric detector combined with a traditional separation exchanger for the determination of low concentrations of nitrites is discussed. After optimization of the method, it was tested with spinach samples in order to study the possible influence of the storage procedure and time on the nitrite content of this kind of vegetable. Spinach was chosen because it is one of the vegetables in which nitrites can most frequently be found. All the measurements were made simultaneously by the IC method and a reference spectrophotometric method and the results were compared.

2. Experimental

2.1. Chemicals

All chemicals were of analytical-reagent grade or HPLC grade (Merck, Darmstadt, Germany). Working standard solutions were prepared by appropriate dilutions of stock standard solutions. The pH was adjusted with aqueous sodium hydroxide. The eluents, standards and all solutions were prepared using ultra-pure 18 MΩ cm water obtained by passing doubly distilled water through a Milli-Q system (Millipore).

2.2. Apparatus

The components of the IC equipment were the following: a Kontron Model 320 high-pressure pump system, a Rheodyne injector with a 20-μl loop, a IC anion PRP-X100 column (5 μm particle size, 125 mm × 4 mm I.D.) (Hamilton) and an ESA Coulochem II coulometric detector equipped with a Model 5020 analytical cell. This cell contains two chambers in series; each chamber includes a porous graphite coulometric electrode (two high-surface-area electrodes), a double counter electrode and a double reference electrode in a stainless-steel body that is capable of withstanding pressures up to 600 psi.

All chromatograms were processed using the KONTRON called PC Integration Pack program.

The conditions for analysis were as follows: eluent, 2 mM phthalic acid–10% acetone (pH 5.0); flow-rate, 1.0 mL/min; injection volume, 20 μl; and oxidation potential, 700 mV. All measurements were carried out at room temperature. Under these conditions, the net retention time was 3.65 ± 0.5 min.

2.3. Sample preparation

Eight packages of each of three brands of frozen spinach were bought and kept at $-18°C$ until their study. These samples were analysed during 4 months. At the beginning of each month we proceeded as follows.

The total contents of two packages of each brand of spinach were defrosted and their nitrite content was measured (DF). The spinach was then boiled together with 1 l of tap water to minimize errors in analysis due to different amounts of nitrites in different packets. The tap water used in the sample pretreatment was previously analysed and found to be free from nitrite. The time of treatment was about 5 min from the time it started to boil; the spinach was then cooled immediately and drained. The nitrite level was determined in both the cooking liquid (CL) and the cooked spinach (CS) and the remainder was reserved for subsequent treat-
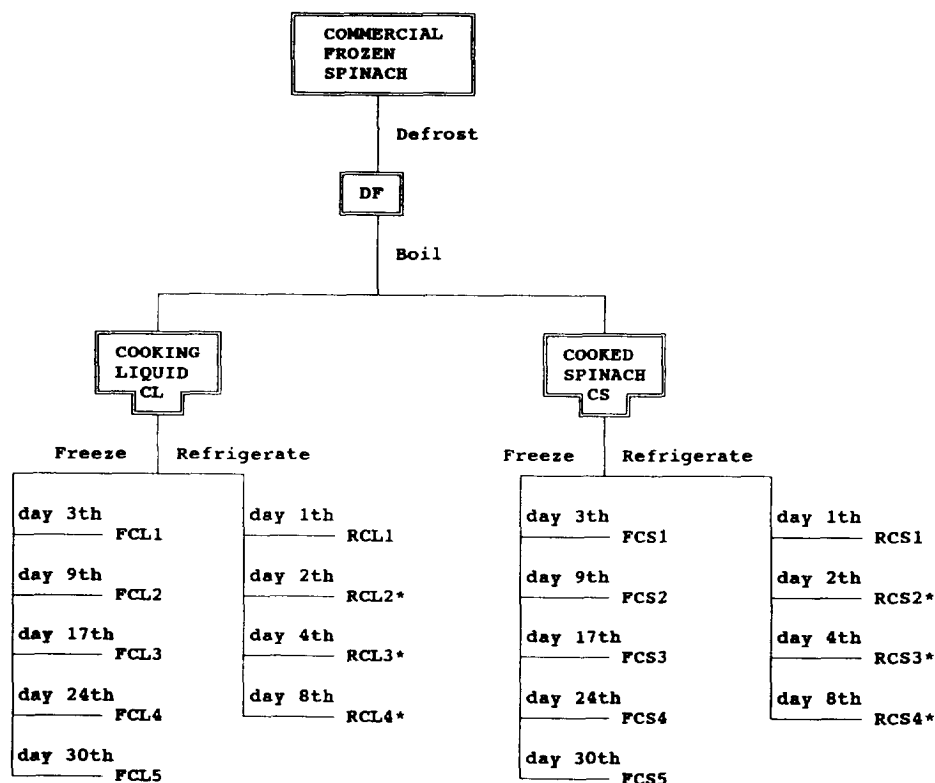
Fig. 1. Schematic diagram showing the treatment of the samples, the storage procedure and the day of analysis. * = sample whose nitrite level has been determined by spectrophotometric and ion chromatographic methods.

ment. One part, liquid and solids separated, was frozen (−18°C) and the other was refrigerated (2–5°C). Samples for measuring nitrite concentration were taken during 1 month as shown in Fig. 1.

In all instances, after trituration of the cooked spinach, three samples were taken from the homogenate for nitrite determination. Three aliquots of the cooking liquid were also analysed.

This procedure was applied in order to investigate the changes in nitrite content during storage. This study was another of the main objectives of this work.

The method used in nitrite extraction is described in the NORME AFNOR (FR-AFNOR-NF.V.04.409.1974) [25], which uses borax to reach a pH of about 8.6 to avoid nitrite destruction. Hot water extraction of this ion from

samples was followed by cleaning, filtration and nitrite assay by the IC procedure.

3. Results and discussion

3.1. Spectrophotometric method

The most common procedure to determine nitrite ion is that in which, in the presence of acid buffer, nitrite is converted into nitrous acid, which diazotizes sulfanilamide. The diazotized product couples with N-(1-napthyl)ethyl-enediamine to produce a red-violet azo dye. The limit of detection for this method is 0.1 μg/ml in the final extract.

Only the nitrite level of the samples kept

under refrigeration for at least 4 days of storage could be determined by this method.

We observed a high dispersion of the nitrite content of these samples, which was in the range 12.18–177 mg NaNO$_2$/kg. As expected, the longer the storage time, the higher was the concentration of nitrites. In addition, higher levels of nitrites in the samples agree with a significant decrease in nitrate levels, which were also determined.

3.2. Chromatographic method

Preliminary experiments

We started this work with a traditional IC detection system, namely a conductimetric detector. The limit of determination for nitrites was found to be around 0.1 μg/ml with this kind of detector, but we expected to find lower concentrations in spinach samples, especially in those taken before storage. In addition, as the samples were boiled using the usual culinary procedure, we found a large peak corresponding to chlorides from sodium chloride whose retention time is lower than that of nitrites, making detection of the nitrite peak impossible. Because of these two problems we investigated other types of detection, and finally we chose electrochemical detection.

Factors affecting the detection system

In an electrochemical detector, for a given sample, the potentiostat applies a voltage to a working electrode. The potential is the driving force that causes an oxidation or reduction reaction to take place at the surface of the working electrode. Determination of sample concentration is based on the measurement of the current generated, which is proportional to sample concentration. Measurement of the current resulting from oxidation or reduction of a species at the surface of a working electrode is called amperometry. In the amperometric detector some fraction (usually 5–15%) of the species is oxidized (or reduced). In contrast, in a coulometric detector essentially 100% of the species is oxidized (or reduced). Since a coulometric detector has a very high conversion

efficiency, it can provide an enhanced signal compared with an amperometric detector.

As the Coulochem II system contains two working electrodes, we first studied the influence of the working electrodes' potentials both on the oxidation current of the nitrites and on the elimination of possible interferences. After several experiments we found the optimum response when the potentials were +0.200 V for the first electrode and +0.700 V for the second whose current was measured and registered. Under these experimental conditions, Pearson's correlation coefficient for a concentration range of 20–100 ng/mL was 0.9968.

Results

In contrast with the reference methods, IC presents two advantages: a short analysis time, which was always around 5 min, and most important a suitable sensitivity and accuracy, as proof that the concentration of nitrites has been determined in all of the samples studied. Table 1 shows the results for the spinach just after defrosting, without cooking (DF) and just before cooking, liquid (CL) and cooked spinach (CS) before storage. The results are the means of three determinations. The relative standard deviations (R.S.D.s) were generally in the range

Table 1
Concentration of nitrites (mg NaNO$_2$/kg spinach) in spinach samples before starting the storage procedure during the 4 months after the purchase

Brand	Month	DF	CL	CS
A	1	1.82	N.D.[a]	1.32
	2	3.25	2.30	3.70
	3	N.D.	3.40	4.82
	4	2.67	1.80	4.30
B	1	2.20	1.44	1.53
	2	2.16	1.26	3.40
	3	1.83	4.80	1.84
	4	2.75	2.00	2.48
C	1	N.D.	1.40	1.80
	2	1.87	1.50	2.17
	3	1.64	1.50	2.20
	4	2.20	1.54	1.80

DF = defrosted sample; CL = cooking liquid; CS = cooked spinach.
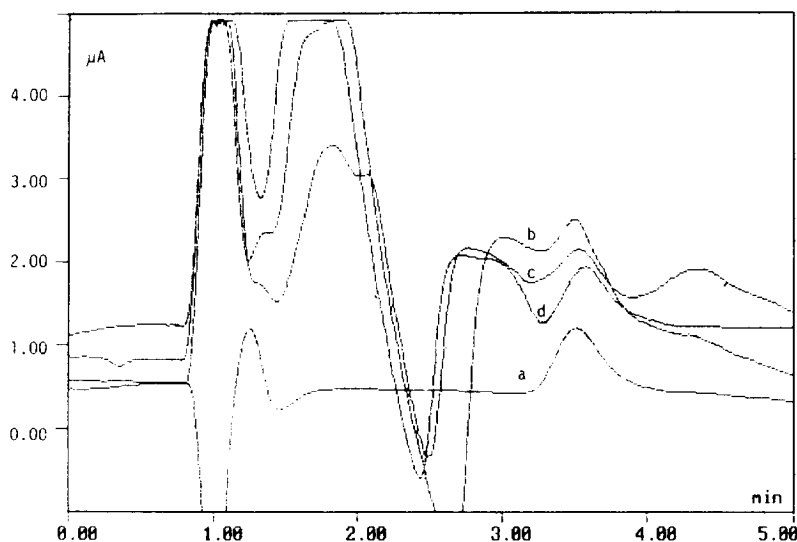[a] N.D. = not detected.

Fig. 2. Chromatograms of (a) standard solution of nitrite (80 ng/ml), (b) defrosted sample (DF), (c) cooked spinach (CS) and (d) cooking liquid (CL) of brand B analysed 4 months after purchase. Chromatographic conditions: IC anion PRP-X100 column, 5 μm particle size (125 mm × 4 mm I.D.); mobile phase, 2 mM phtalic acid–10% acetone (pH at 5.0); flow-rate, 1.0 ml/min; injection volume, 20 μl, electrochemical detection at +700 mV.

2–16%, being higher only in four instances. This study was carried out using three different brands and, as Table 1 shows, the levels of nitrites before storage were very similar, irrespective of the brand and the time for which they were stored before analysis. These levels were,

Table 2
Concentrations of nitrites (mg $NaNO_2$/kg spinach) during different times of both freezing and refrigeration storage procedures, expressed as the mean and relative standard deviation of twelve analyses from 4 months after the purchase

Brand	Freezing			Refrigeration		
	Day	Solid : FCS	Liquid : FCL	Day	Solid : RCS	Liquid : RCL
A	3	8.29 ± 5.02	5.13 ± 4.22			
	9	3.48 ± 1.05	2.34 ± 0.24	1	2.29 ± 0.77	2.14 ± 0.56
	17	3.23 ± 1.27	2.05 ± 0.70	2	3.77 ± 0.63	1.00 ± 0.11
	24	2.95 ± 0.90	1.52 ± 0.40	4	9.10 ± 10.2	7.16 ± 9.36
	30	5.60 ± 1.56	3.50 ± 0.44	8	[a]	[a]
B	3	3.94 ± 2.32	1.99 ± 0.92			
	9	3.90 ± 2.95	1.53 ± 1.19	1	3.71 ± 3.74	1.73 ± 0.97
	17	4.90 ± 2.98	2.71 ± 1.80	2	7.12 ± 7.36	2.00 ± 1.10
	24	4.36 ± 1.27	2.71 ± 1.55	4	15.5 ± 8.69	1.67 ± 0.53
	30	3.71 ± 2.36	1.82 ± 0.84	8	[a]	[a]
C	3	8.32 ± 6.32	4.50 ± 4.64			
	9	3.80 ± 1.28	3.20 ± 2.66	1	2.02 ± 0.36	1.82 ± 0.40
	17	3.40 ± 2.01	1.30 ± 0.30	2	4.77 ± 0.25	1.57 ± 0.27
	24	2.00 ± 0.75	1.49 ± 0.44	4	5.93 ± 3.49	6.35 ± 5.48
	30	2.80 ± 1.45	3.77 ± 3.20	8	[a]	[a]

[a] Samples also analysed by the spectrophotometric method.

fortunately and as expected, very low. The mean of all samples was 2.32 mg $NaNO_2$/kg spinach and the R.S.D. was 41%, but for brand C the mean was 1.78 mg $NaNO_2$/kg spinach and the R.S.D. was 16%.

The final concentration of nitrites is given in mg $NaNO_2$/kg spinach but the nitrite levels of the sample injected into the chromatograph were about 20–50 ng/ml. These results show that IC is a selective and accurate method even when the concentration of nitrite is very low.

The chromatograms in Fig. 2 correspond to a standard solution of 80 ng/ml of nitrite and to the defrosted (DF), cooking liquid (CL) and cooked spinach (CS) of brand B analysed 4 months after purchase.
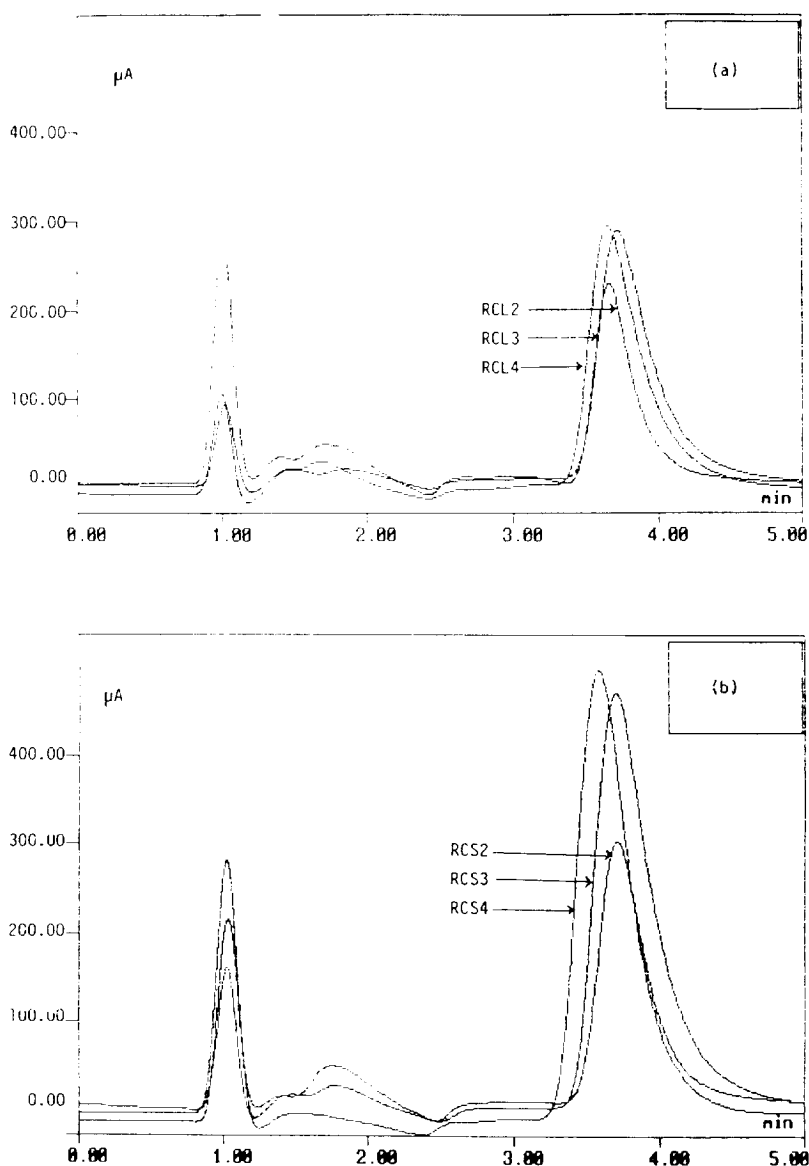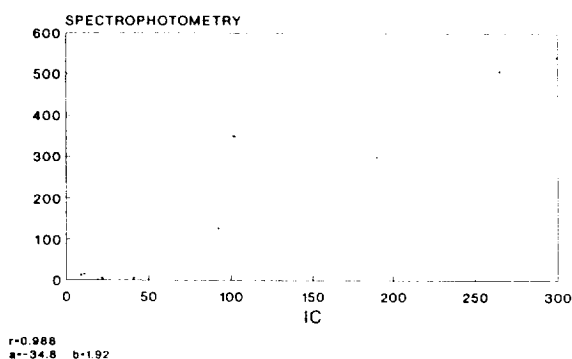

Fig. 3. Chromatograms of refrigerated samples for different times: (a) cooking liquid (RCL) of the samples and (b) cooked spinach (RCS). These determinations were started 4 months after purchase. Chromatographic conditions as in Fig. 2.

Table 2 gives the concentration of nitrites in the samples kept in storage, which could be determined only by IC because the limit of detection of the spectrophotometric method was above these levels. The dispersion of these results is due to the fact that the samples were analysed 4 months after purchase and the results, unlike those in Table 1, are assembled according to the days of storage but not to the months after purchase. However, these results show that the freezing procedure does not affect the nitrite levels. Storage for 2 days under refrigeration does affect the nitrite levels. These levels become significant after 4 days under refrigeration.

We were able to arrive at this conclusion because of the higher sensitivity of IC compared with reference methods.

The remainder of the samples were analysed by both methods. Fig. 3 shows the chromatograms for the refrigerated samples. It is possible to see the evolution of the nitrite levels with the time of refrigeration, both in cooking liquid (RCL) and in cooked spinach (RCL). In these samples the concentration of nitrites increased significantly up to 700 mg $NaNO_2$/kg spinach.

Fig. 4 shows the correlation between the results obtained by the two methods studied when the concentration of nitrites was higher than the limit of detection of the spectrophotometric method. We can deduce that there is a good correlation between both methods except for brand B. Brands A and C show a good correlation coefficient but in both instances the slope was higher than unity, meaning that the results had the same tendency but the spectrophotometric values were always higher than chromatographic values. We can explain this as a result of the difference between the times of analysis: spectrophotometric analyses were made on the day of the extraction but the chromatographic analyses at least 2 days later. This time appears to be sufficient to cause oxidation of nitrite to nitrate.

BRAND C
SPECTROPHOTOMETRY-IC

r-0.988
a--34.8   b-1.92

BRANDS A, B and C
SPECTROPHOTOMETRY-IC
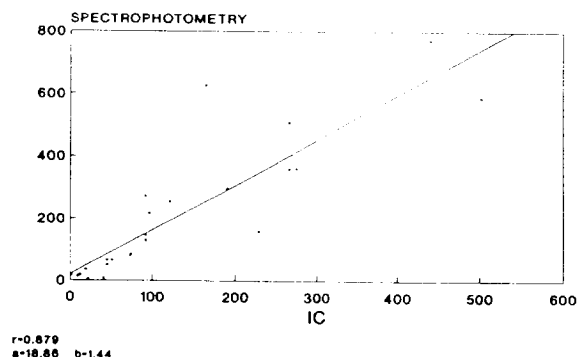

r-0.879
a-18.86   b-1.44

Fig. 4. Correlation graphs between the nitrite concentrations (mg $NaNO_2$/kg spinach) obtained by the spectrophotometric and chromatographic methods.

4. Conclusion

Ion chromatography with electrochemical detection could be considered as a suitable method for the determination of nitrite in vegetable samples because of its detection limit, which is significantly lower than those of reference methods.

Concerning the other aim of our study, when the spinach is kept frozen, the nitrite levels remain unchanged, but the refrigeration procedure seriously affects the nitrite levels and these become dangerously high.

From these results we recommend that cooked vegetables should never be kept under refrigeration for more than 2 days.

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