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A flow-injection spectrophotometric method for nitrate and nitrite determination through nitric oxide generation

Raquel Andrade[a], Claudia O. Viana[a], Silvane G. Guadagnin[b], Felix G.R. Reyes[b], Susanne Rath[a,*]

[a]*Department of Analytical Chemistry, Institute of Chemistry, State University of Campinas, PO Box 6154, 13083-970 Campinas, SP, Brazil*
[b]*Department of Food Science, Faculty of Food Engineering, State University of Campinas, PO Box 6121, 13083-970 Campinas, SP, Brazil*

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Abstract

A flow injection method with novel spectrophotometric detection for the determination of nitrite and nitrate in foodstuffs is presented. The method is based on the reduction of nitrite and nitrate to nitric oxide, with subsequent reaction with iron (II) and thiocyanate in an acid medium, forming $FeSCNNO^+$. The absorbance of the complex, with a maximum at 460 nm, is proportional to the nitrite and nitrate concentrations. The NO is generated in two stages: (1) reduction of nitrate to nitrite in a cadmium copper reductor column and (2) reduction of the nitrite to NO in a sulfuric acid medium. The influence of reagent concentrations and manifold parameters were evaluated. Nitrite and nitrate can be determined in the range of 0.30–3.00 and 1.00–10.00 mg l^{-1}, respectively. The sampling rate of analyses was 30–40 h^{-1} and, considering a sample of 5.0 g, the determination limit of the method was 20 and 13 mg kg^{-1} of nitrate and nitrite, respectively. Nitrite and nitrate were determined in vegetables and meat products by the proposed method. The precision and accuracy of the proposed method were comparable to those of the reference spectrophotometric method (official AOAC reference method for the determination of nitrate in foodstuffs).

Keywords: Nitrite; Nitrate; Meat products; Vegetables; FI analysis

1. Introduction

Nitrate and nitrite are commonly monitored for environmental protection purposes, in water, agriculture and food control. Nitrate is naturally present in vegetables and its concentration varies enormously, ranging from 1 to 10,000 mg kg^{-1} fresh weight. Furthermore, nitrate, as well as nitrite, have been added intentionally during the curing process of certain meat products, due to their ability to inhibit the growth of spores of *Clostridium botulinum* and to impart characteristic color and flavor to this kind of foodstuffs (Binkered & Kolari, 1975).

The toxicity of nitrite is primarily due to its interaction with blood pigment to produce methemoglobinemia. It has been also reported that nitrate can be reduced *in vivo* to nitrite which can react with secondary or tertiary amines to form N-nitroso compounds, some of which are known to be carcinogenic, teratogenic and mutagenic (MAFF, 1992).

Several methods have been reported for the quantitative determination of nitrate and nitrite, including kinetic methods (Koupparis, Walczak, & Malmstadt, 1982; Liang, Iwatsuki, & Fukasawa, 1994), chromatography (Butt, Riaz, & Iqbal, 2001; Siu & Henshall, 1998), potentiometry (Li, Wu, Yuan, Lin, & Yu, 1994; Schaller, Bakker, Spichiger, & Pretsch, 1994), amperometry (Bertotti & Pletcher, 1997), polarography (Ximenes, Rath, & Reyes, 2000), capillary electrophoresis (Öztekin, Nutku, & Erim, 2002) and spectrophotometry (AOAC, 1997; Kawakami & Igrashi, 1996). Among them, the spectrophotometric method, based on the reduction of nitrate to nitrite and subsequent colorimetric determination of nitrite with a diazo-coupling reaction (Griess reaction) has been adopted as an AOAC official method of analysis (1997). However, these methods have the disadvantage of the employment of large volumes of toxic reagents and time-consuming procedures.

* Corresponding author. Fax: +55-19-3788-3023.
E-mail address: raths@iqm.unicamp.br (S. Rath).

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This paper describes the development and application of a flow injection spectrophotometric method for the determination of nitrate and nitrite in foodstuffs (vegetables and meat products) based on the formation of $FeSCNNO^{+}$ in the flow injection system, whose transient absorbance signal, at 460 nm, is proportional to the nitrate, for example, nitrite concentrations in solution. The method was compared with the recommended AOAC method (AOAC, 1997) adapted to a flow injection system (Giné, Bergamin, Zagatto, & Reis, 1980).

2. Materials and methods

2.1. Apparatus

A diagram of the FI manifold employed is shown in Fig. 1. A four-channel Ismatec peristaltic pump fitted with Tygon tubing (1.2 mm i.d.) was used for the propulsion of fluids. Sample injecton was performed by a laboratory-constructed three section manual commutator made in acrylic, with two fixed side-bars and a sliding central bar, that moves for sampling and injection. The absorbance was measured at 460 nm with a FEMTO spectrophotometer (model 432) equipped with a glass flow cell with a 10 mm optical pathway. The transient absorbance signals were monitored by an Intralab two-channel strip-chart-recorder.

2.2. Reagents

All chemicals used were of analytical-reagent grade. Distilled water was used throughout.

2.3. Samples

Meat products (pork sausages and hot-dogs) and fresh vegetables (lettuce, watercress and arugula) were purchased from local markets (Campinas, SP, Brazil).

2.4. Method

2.4.1. Standards

A 100 mg l^{-1} standard solution of nitrite was prepared by dissolving 0.1500 g of sodium nitrite (Merck) in 1000 ml water. A 100 mg l^{-1} standard solution of nitrate was prepared by dissolving 0.1371 g of sodium nitrate (Merck) in 1000 mL water. Working standard solutions of nitrate and nitrite were prepared daily by dilution of the standard stock solutions.

2.5. Cadmium copper reductor column

The spongy cadmium was prepared as described elsewhere (Lara, Takahashi, & Silveira, 1978) and introduced into glass tube (7.5 cm × 3 mm i.d.), whose ends were closed with glass wool plugs. The cadmiun column was coated batchwise with copper by passing a solution containing 0.1% (m/v) copper sulphate in 0.1 mol l^{-1} EDTA (Van Staden, 1982). The column was activated by percolating 100 ml of R1-solution and 50 ml of 2 10^{-3} mol l^{-1} nitrate. The column was regenerated, when the reduction efficiency changed more than 5%, by percolating, for 5 min, at 1.2 ml min^{-1} the following solutions: (a) 0.1 mol l^{-1} HCl solution, (b) water and (c) R1-solution.

2.5.1. Reagents for the colorimetric reaction FI-system

The reagent solution (R1), pH = 8.5, was obtained by dissolving 100 g ammonium chloride, 20 g sodium tetraborate and 1 g Na_2EDTA in 1000 ml water. A 6 10^{-4} mol l^{-1} ferrous solution (R2) was obtained by dissolving ammonium ferrous sulfate in 0.06 mol l^{-1} sulfuric acid. The 0.4 mol l^{-1} thiocyanate solution (R3) was obtained by dissolving the salt in water.

2.5.2. Sample preparation

For meat products (pork sausages and hot-dogs), the sample (5.00 g) was triturated in a Waring blender. The

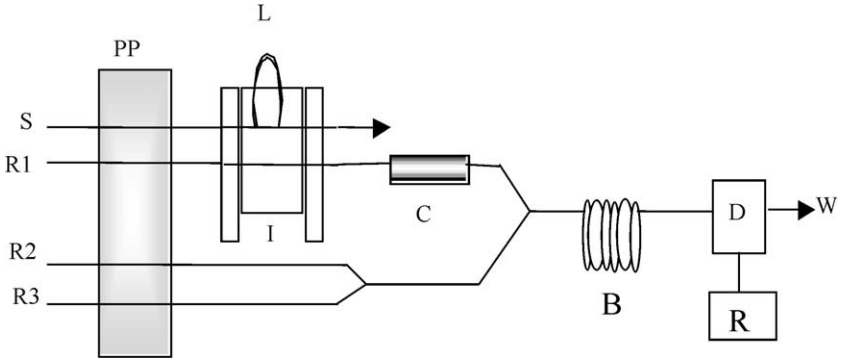

Fig. 1. Schematic diagram of the FI manifold for simultaneous determination of nitrate and nitrite: S: sample; R1: reagent solution; R2: ferrous solution; R3: potassium thiocyanate; PP: peristaltic pump; L: sample loop; I: proportional injector; C: copperized-cadmium reductor column; B: reaction coil; D: detector; R: recorder and W: waste.

nitrate and nitrite were extracted from the samples with hot water (70 °C, 15 min) and clarification of the extract was conducted by addition of the Carrez reagent (0.1 mol l^{-1} potassium hexacyanoferrate (II) aqueous solution and 0.25 mol l^{-1} zinc acetate aqueous solution) followed by filtration through filter paper (Usher & Telling, 1975). The filtrate was further diluted to obtain the desired concentrations of nitrate and nitrite.

For the vegetables (watercress, arugula and lettuce) the edible part was washed with water and the retained water was removed using filter paper. The samples were homogenized with water 1:1 (m/m) in a Waring blender and 5.0-g portions were stored at −18 °C until analysis. The nitrate and nitrite were extracted from the sample with hot water, as described for the meat products. No clarification procedures were employed for these samples. The extracts were filtered through filter paper and the filtrate was diluted with water before injection in the FI system.

2.5.3. Calibration and quantification procedures

For the calibration graph, standard working solutions in the concentration range of 0.30–3.00 mg l^{-1} and 1.00–10.00 mg l^{-1} for nitrite and nitrate, respectively, were employed. The standard or the sample contained in the sample loop (L) was transported by the carrier R1. Only for the nitrate determination did the carrier pass through the reductor column (C). At the confluence point the pre-mixed reagents R2 and R3, were added to the sample zone (Fig. 1). The colorimetric reaction proceeds in the reaction coil and, finally, the chromophore was measured at 460 nm by the spectrophotometer.

3. Results and discussion

In a very strong acidic medium, nitrite is reduced to nitric oxide which, in the presence of thiocyanate and some divalent transition metals (M) forms, in aqueous solution, a ternary complex—$MSCNNO^+$. Cobalt (II) and ferrous ions have been employed for quantitative nitrite determination by polarography (Ximenes et al., 2000). In preliminary studies, it has been shown that the absorbance of $FeSCNNO^+$, which exhibits a maximum at 460 nm, is proportional to the nitrite concentration. For the development of the spectrophotometric flow injection method, based on the reaction of nitric oxide with ferrous and thiocyanate ions, several parameters were evaluated and optimized by the univariate method in order to achieve optimal conditions for the reduction of nitrate to nitrite and nitrite to nitric oxide and for the chemical reaction of complex formation.

3.1. Optimization of the FI-system

In order to optimize the FI-system (Fig. 1), the reagent concentrations of Fe(II), SCN^-, and H_2SO_4, as well as the cadmium copperized reductor column and the length of the reaction coil were evaluated.

Initially the concentration of iron (II) was varied from 1 10^{-4} mol l^{-1} to 12 10^{-4} mol l^{-1} and the sulfuric acid concentration was maintained 100 times more concentrated than the ferrous ion in solution. It was verified that the maximum $FeSCNNO^+$ formation was achieved with Fe^{2+} and H_2SO_4 at concentrations of 6 10^{-4} and 0.06 mol l^{-1}, respectively (Fig. 2). In order to evaluate the influence of sulfuric acid on the reduction of nitrite to nitric oxide, the ferrous solution (6 10^{-4} mol l^{-1}) was prepared in different concentrations of sulfuric acid in the range of 0.03–0.12 mol l^{-1}. At concentration lower than 0.06 mol l^{-1} the formation of NO was not quantitative. At concentrations from 0.06 to 0.12 mol l^{-1} no changes in the absorbance at 460 nm were observed.

The influence of thiocyanate ions on the complex formation is shown in Fig. 3. The maximum absorbance is reached with thiocyanate concentrations greater than 0.36 mol l^{-1}.

The concentration and pH of the reagent R1, employed for the reduction of nitrate to nitrite on the copperized cadmium column, was also evaluated. The pH needs to be alkaline for the reduction step, however, for the second reduction, nitrite to nitric oxide, the medium needs to be acidic. Therefore, different pHs of the reagent R1 were prepared in the range of 7.5–9.5. The results presented in Fig. 4 indicate that the best pH value is about 8.5. At this pH, the maximum absorbance intensity from complex formation was observed with dilution of the reagent R1 1:100 v/v.

The optimum length of the cadmium copper reductor column was established by using a 3.0 mg l^{-1} nitrate solution. Lengths of 5.0, 7.5, 10.0 and 12.0 cm were evaluated. The highest efficiency was achieved with a length of 7.5 cm. At lower lengths the reduction was not complete and at higher values dispersion in the FI system becomes significant. The lifetime of the cadmium copper reductor column depends on the matrix complexity. Furthermore the column could be regenerated as previously described.

The peak height on the fiagram, which is proportional to the absorbance, depends on the residence time of the sample zone in the system, for example, on the total flow rate and the length of the reaction coil. The effect of the flow rate was checked over the range 0.6–2.4 ml min^{-1} for each channel. The transient absorbance signals decreased as the flow rate increased. This indicates that the overall reaction is not instantaneous. At higher flow rates the residence time of the reagent is decreased. The best analytical conditions were obtained with flow rates of 1.2 ml min^{-1} for each channel.

The influence of the reaction coil length on sensitivity was studied at a constant flow rate of 1.2 ml min^{-1} (Fig. 5). By increasing the length of the reaction coil

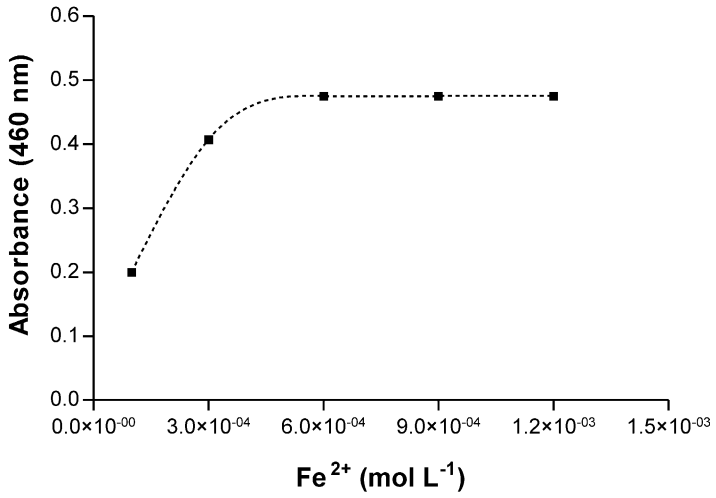

Fig. 2. Effect of Fe(II) concentration on $FeSCNNO^+$ formation. Conditions: SCN^- 0.24 mol l^{-1}; R1: 1:100 v/v (pH = 8.0) and NO_3^- : 3 mg l^{-1}.

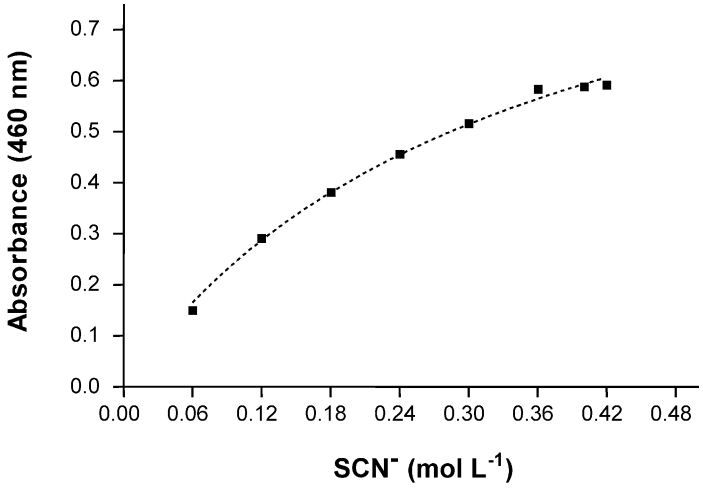

Fig. 3. Effect of thiocyanate concentration on $FeSCNNO^+$ formation. Conditions: Fe^{2+}: 6 10^{-4} mol l^{-1} in 0.06 mol l^{-1} H_2SO_4; NO_3^- : 3 mg l^{-1} and R1: 1:100 v/v (pH = 8.0).

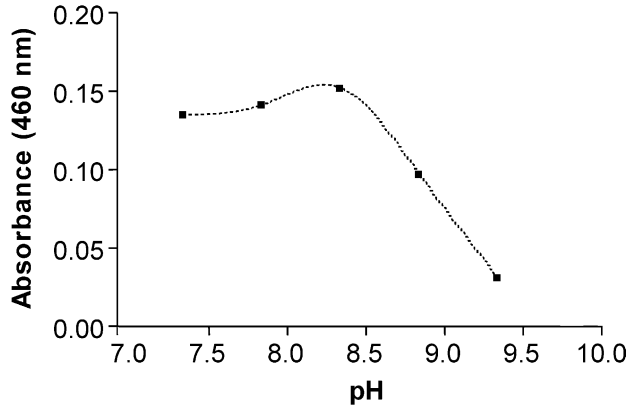

Fig. 4. Effect of pH of R1 on $FeSCNNO^+$ formation. Conditions: Fe^{2+}: 6 10^{-4} mol l^{-1} in 0.06 mol l^{-1} H_2SO_4; SCN^- : 0.36 mol l^{-1} and NO_3^- : 3 mg l^{-1}.

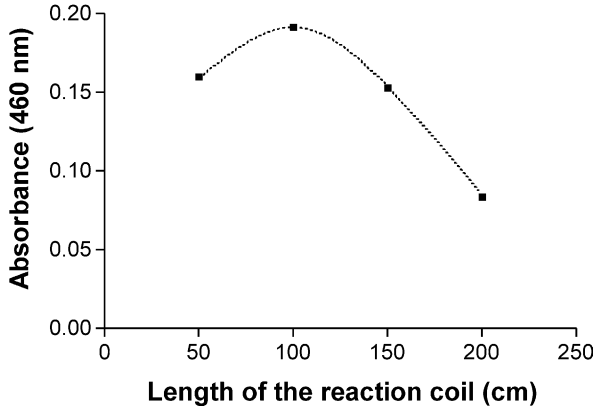

Fig. 5. Effect of the length of the reaction coil of the $FeSCNNO^+$ formation. Conditions: Fe^{2+}: 6 10^{-4} mol l^{-1} in 0.06 mol l^{-1} H_2SO_4; SCN^-: 0.36 mol l^{-1}; NO_3^-: 3 mg l^{-1}, R1: 1:100 v/v (pH = 8.5).

from 50 to 100 cm an increase in the transient absorbance signal was observed due to the longer residence time for the reaction mixture. Furthermore, a reaction coil longer than 100 cm decreases the absorbance at 460 nm and peak broadening was observed. Thus a 100 cm reaction coil was selected for the method.

Thus, the optimum FI-conditions were attained with the following parameters: sample loop: 300 μl, length/diameter of the cadmium copper reductor column: 7.5/0.3 cm; reaction coil: 100 cm; R1: $NH_4Cl + Na_2B_4O_7 + Na_2EDTA$, pH 8.5 (flow rate 1.2 ml min^{-1}); R2: Fe^{2+} / H_2SO_4 : 6 10^{-4} / 0.06 mol l^{-1} (flow rate 1.2 ml min^{-1}); R3: SCN^- 0.4 mol l^{-1} (flow rate 1.2 ml min^{-1}).

Under these conditions, calibration graphs linear over the concentration range of 1–10 and 0.30–3 mg l^{-1} for nitrate and nitrite, respectively, were obtained. The typical transient absorbance signals are illustrated in Fig. 6.

The sampling rate comprises 30 and 40 h^{-1} for nitrate and nitrite, respectively. The repeatability ($n=6$), expressed by *within-run-precision*, were 1.2 and 1.0% for 3 nitrate and 1.5 mg kg^{-1} nitrite, respectively.

3.2. Validation

The proposed method was validated by comparison with the AOAC recommended method, employing the Griess reaction (AOAC, 1997) in a FI system (Viana, Migotto, Reyes, & Rath, 2000) by the quantification of nitrate in different vegetable matrices (lettuce, arugula and watercress) and nitrate and nitrite determination in cured meats (pork sausage and hot-dogs). The results are presented in Table 1.

For the different matrices of fresh vegetables the nitrate content varied from 1619 to 8778 mg kg^{-1}. For the cured meat products the levels determined were in the range of 39–86 mg kg^{-1} and lower than 4–77 mg

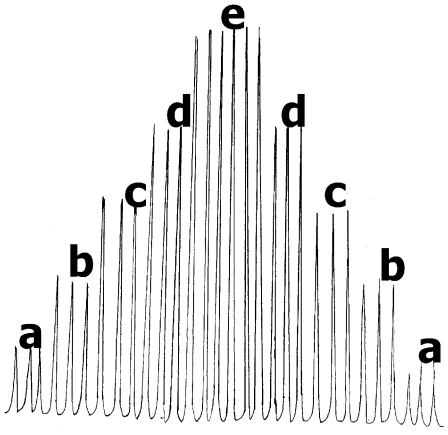

Fig. 6. Transient absorbance signals obtained for reference solutions. Nitrate concentration (mg l^{-1}): (a) 1.00; (b) 2.00; (c) 3.00; (d) 4.00 and (e) 5.00. Conditions: Fe^{2+}: 6 10^{-4} mol l^{-1} in 0.06 mol l^{-1} H_2SO_4; SCN^-: 0.36 mol l^{-1} and R1: 1:100 v/v (pH = 8.5).

Table 1
Results for the determination of nitrite and nitrate in food samples[a]

Samples	Content of nitrite $\pm ts_{x0}$ (mg kg^{-1})		Content of nitrate $\pm ts_{x0}$ (mg kg^{-1})	
	Proposed method	Griess method	Proposed method	Griess method
Pork sausage				
1	n.d.	n.d.	86±6	83±4
2	39±3	41±3	59±3	59±5
3	n.d.	n.d.	84±2	84±3
Hot-dog sausage				
4	26±5	28±5	58±6	56±4
5	77±5	76±3	39±5	38±3
6	27±3	28±3	40±5	43±3
Lettuce				
7	n.d.	n.d.	1978±180	1977±139
8	n.d.	n.d.	2044±181	2066±141
9	n.d.	n.d.	1619±183	1706±140
Watercress				
10	n.d.	n.d.	4802±47	4827±220
Arugula				
11	n.d.	n.d.	8740±79	8778±370

[a] S_{x0} = standard deviation of the mean value (5 determinations) of the concentration of nitrate and nitrite. t = t-distribution for confidence interval of 95% with ($n-2$) degrees of freedom. n.d.: not detected (lower than 4 mg kg^{-1}).

kg^{-1} for nitrate and nitrite, respectively (Table 1). Those levels determined in cured meat products were in accordance with Brazilian food laws (MS, 1988) Independent of the matrix, the mean values obtained by the proposed method and the AOAC recommended method did not differ significantly ($P<0.05$). The recovery of nitrate in the matrices varied from 93 to 110% and nitrite from 88 to 97% for the proposed method. The *between-run-precision* over 5 days was 2.3 and 5.5% for 242 nitrate and 19.7 mg kg^{-1} nitrite, respectively. Considering a sample of 5.0 g, the determination limit was 20.0 and 13.0 mg kg^{-1} of nitrate and nitrite, respectively.

4. Conclusion

The proposed flow injection spectrophotometric methodology was shown to be suitable for food quality control for simultaneous nitrate and nitrite determination in vegetables and meat products. The method offers advantage of being simple with a high analytical sampling rate, requiring low cost equipment and reagents and dispenses the need for carcinogenic reagents, which are employed by the recommended AOAC methodology (Griess reaction).

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