Determination of nitrate and nitrite in dairy samples by sequential injection using an in-line cadmium-reducing column

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Abstract

A sequential injection analysis (SIA) system for the spectrophotometric determination of nitrate in dairy samples was developed. A test portion was aspirated into a carrier solution containing ethylendiaminetetracetic acid (EDTA) and ammonium buffer, which flowed into a copperized cadmium reduction column installed in-line for the determination of the nitrate plus nitrite contents of samples. For the nitrite determination, another test portion of sample was aspirated and directly sent to the detector without reduction. Nitrate content was calculated from the difference between nitrate plus nitrite (expressed as nitrite) and nitrite content. The spectrophotometric determination is based on the Griess reaction. The proposed method was used to test several dairy samples (ultrapasteurized milk with 1.7% milk fat, whey, raw bovine milk and several cheese varieties). Results were statistically in good agreement with those provided by the reference procedure, with a detection limit of $0.15\,mg\,L^{-1}$. A sampling rate of 21 determinations per hour can be achieved with this procedure.

Introduction

The determination of nitrates in food, soils and natural waters assumed a vital importance in recent years, since these ions are intimately involved in the overall nitrogen cycle in soil and higher plants (Alberts et al., 2002). Nitrite is formed during the biodegradation of nitrate, and has a harmful impact on human health due to its reaction with secondary amines to form carcinogenic *N*-nitrosoamines (Hirata, Amma, Karthikeyan, & Toda, 2003). As a result of these considerations, the determination of nitrate in various samples has been a subject of interest and discussion.

Some quality-assurance determinations carried out in the dairy industry are still very complex and laborious; in particular, analysis for nitrate presents several inconveniences such as being time consuming (2 samples per hour), using large amounts of cadmium, which poses environmental problems, requiring several sample handling steps, and requiring permanent trained laboratory assistance. These considerations and the increasing demand for rapid analysis led to the development of automatic methods for nitrate determination in various products. Flow injection analysis (FIA), first described by Ruzicka and Hansen (1975), was used in the spectrophotometric determination of nitrate, with different colour reagents, in several biological samples (Higuchi & Motomizu, 1999), water (Ensafi & Kazemzadeh, 1999), soil (Brabcová, Rychlovsky, & Nemcová, 2003), meat, flour, or cheese (Ahmed, Stalikas, Tzouwara-Karayanni, & Karayannis, 1996). The use of detector systems such as tubular potentiometric (Lima, Rangel, & Souto, 1995) and biamperometric (Hulanicki, Matuszewski, & Trojanowicz, 1987) also was described for nitrate determination in vegetables and soil extracts, respectively.

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However, FIA manifolds still require frequent assistance and reagent consumption is relatively high in a continuous flow operation. To overcome these drawbacks, Ruzicka and Marshall (1990) introduced sequential injection analysis (SIA), where small volumes of samples and reagents are sequentially aspirated into a single channel, mixed by flow reversal and pumped to a detector. Since then, some SIA systems were developed for the spectrophotometric determination of nitrate in water: natural, surface and wastewater (Cerdà, Oms, Forteza, & Cerdà, 1998; Legnerová, Solich, Sklenárová, Satínsky, & Karlícek, 2002) and human serum (Pinto, Lima, & Saraiva, 2003), using the Griess reaction (Ivanov, 2004). Kazemzadeh and Ensafi (2001) determined nitrate in meat, flour or cheese samples using safranine O as colour developing reagent.

The aim of this work was to develop a sensitive, rapid and automatic technique that would allow the determination of nitrate in different kinds of dairy samples.

Materials and methods

Reagents and solutions

All chemicals were of analytical reagent grade, and water from a MilliQ plus system (Millipore, Billerica, MA, USA) was used throughout.

Standard solutions used to establish the sequential injection calibration graphs for nitrate (0.5–4.0 $mg\,L^{-1}$) and nitrite (0.5–2.0 $mg\,L^{-1}$) were prepared daily by dilution of a 1000 $mg\,L^{-1}$ standard solution. These solutions were prepared by precise weighing of previously dried (100 °C) sodium nitrate and sodium nitrite (Merck, Darmstadt, Germany). The chromogenic reagent consisted of 40 g sulphanilamide (Merck), 1 g *N*-(1-naphthyl) ethylenediamine dihydrochloride (NED) (Merck) and 67 mL concentrated HCl (37%, $d = 1.19\,g\,mL^{-1}$) per liter. This reagent was stored in a brown glass bottle in the refrigerator.

The carrier solution was prepared by dissolving 0.4 g of EDTA (Titriplex III) and 70 g of ammonium chloride (Merck) and making to 1 L with water and adjusting the pH to 9–9.5 with a few drops of NH_4OH solution.

The copperized cadmium column was prepared as described by Lapa, Lima, and Pinto (2000). Before use, evaluation of the reduction efficiency of each column was performed by comparing the signal for nitrate solutions to that of nitrite solution at the same N-concentration.

Instrumentation

A 386 personal computer (Samsung SD 700, Korea) equipped with an Advantec PCL 818 L (Taipei, Taiwan) interface card, running a homemade software written in QuickBasic 4.5 (Microsoft, Redmond, WA, USA) controlled the eight port selection valve position, the rotation sense, speed of the peristaltic pump P_1 and the timing course of the auxiliary peristaltic pump P_2. Two Gilson Minipuls 3 (Gilson, Villiers-le-Bel, France) peristaltic pumps, with 0.8 mm i.d. polytetrafluorethylene—PFTE (Cambridge, UK) tubing, propelled the solutions. One of the pumps was connected to the central channel of an eight-port electrically actuated selection valve (Valco VICI C15-3118E, Schenkon, CH). An ATI 5625 UV/VIS (Cambridge, UK) spectrophotometer equipped with a Hellma (178.712-QS, Mullheim/Baden, Germany) flow cell (inner optical volume 18 μL), with wavelength set to 538 nm, was connected to a Metrohm E586 Labograph (Herisau, Switzerland) chart recorder and used as the detection system.

Sample pre-treatment

Samples used for validation of results were dairy products of different types ultrapasteurized milk with 1.7% milk fat, whey from bovine milk, raw bovine milk and cheeses (Brie, Portuguese Flamengo, Mozzarella, Cream cheese, Roquefort, and Edam). Samples were treated as described in the reference procedures (AOAC, 1997 and ISO, 1985) for protein elimination. This was accomplished by precipitation with Carrez reagents (zinc sulphate and potassium hexacyanoferrate(II)) and subsequent filtration through a medium grade filter paper. Filtrate was collected and used for nitrate determination by both methodologies, SIA and reference procedure.

Reference method

Procedures suggested by the ISO (1985), for whey and milk, and by the AOAC (1997), official method for cheese were followed. The determination consists of the dilution of a weighed portion of milk and dried whey as well as cheese with warm water (55 °C), precipitation of proteins and filtration. In a portion of the filtrate, nitrate is reduced to nitrite by means of a copperized cadmium column. The eluate is used for nitrate plus nitrite determination. The other portion (unreduced) is used for nitrite determination. In both unreduced and reduced solutions, a red colour is developed by NED and sulphanilamide, and the absorbance is measured at 538 nm. Nitrate content is calculated from the difference between the two measurements.

Sequential injection procedure

The SIA system (Fig. 1) was developed in order to enable the sequential determination of the sum of nitrate and nitrite, followed by nitrite alone. The protocol sequence for these determinations is listed in Table 1.

Sample solution was aspirated (step 1) and propelled through the copperized cadmium column (step 2) where nitrate reduction occurred. For complete reduction, the flow was stopped for 30 s at this point (step 3). After reduction, the stream flowing from cadmium column joined the chromogenic reagent at confluence a and was sent to the detector (step 4) for nitrate plus nitrite determination. Then, another test portion of sample was

aspirated (step 6) and directed through port 4 to confluence a, where mixing with chromogenic reagent occurred without passing through cadmium reduction column. The mixture was directed to the detector (step 7) for nitrite determination. Nitrate content was calculated from the difference between nitrate plus nitrite (expressed as nitrite), and nitrite content. Two washing steps were included at the end of both determinations (steps 5 and 8) so that any remains present inside the reaction coil could be removed.

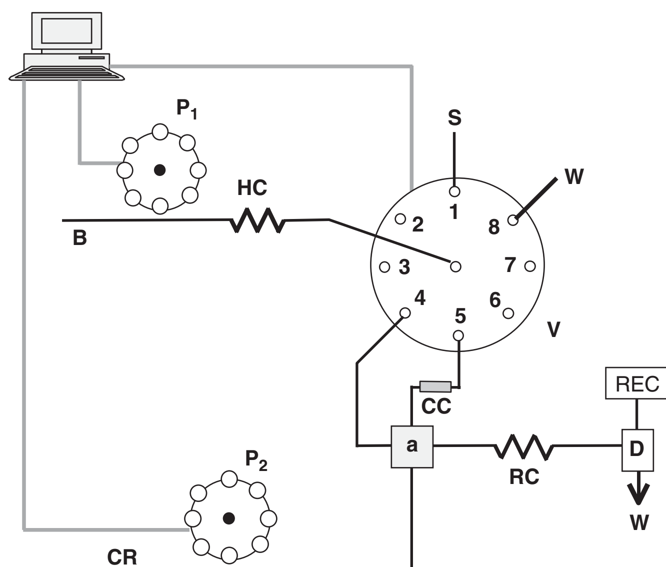

Fig. 1. SIA manifold for the determination of nitrate and nitrite in dairy products: B—buffer (pH 9.0–9.5); P_1 and P_2—peristaltic pumps; HC—holding coil; W—waste; S—standards or dairy samples; CC—cadmium column; a—confluence point; CR—chromogenic reagent; V—eight port selection valve; RC—reaction coil (60 cm); D—UV/VIS spectrophotometer; REC—recorder output.

Statistical analysis

To evaluate the accuracy of the results obtained by the developed SIA methodology, nitrate content was determined by SIA and by the reference procedure in four different types of dairy products (ultrapasteurized bovine milk, raw bovine milk, whey from bovine milk and cheese). As the nitrate content of almost all analyzed samples was below the detection limit (calculated according to IUPAC, 1976) for this determination, samples were spiked with nitrate before analysis. Statistical treatment of the results was established (Miller & Miller, 1993) by the following relation:

$$C_{\mathrm{SIA}} = C_0 + SC_{\mathrm{ref}}, \tag{1}$$

where C_{SIA} represents the sequential injection results; C_{ref} the results provided by reference method; S, the obtained slope; and C_0, the intercept with a 95% significance level for 16 degrees of freedom (18 samples). Additionally, results were validated by recovery studies; t-test was performed and the t-value obtained was compared with $t_{\mathrm{critical}} = 2.45$, to indicate if there is a difference between the mean recovery percentage and 100%.

To assess the repeatability of the SIA procedure, relative standard deviation (RSD) was calculated from 10 consecutive determinations in UHT milk samples.

Results and discussion

Optimization of these quential injection system

In order to optimize the proposed SIA manifold, the influence of hydrodynamic and chemical parameters on the magnitude of peak height, repeatability and accuracy of results were studied. In this system, nitrate is reduced

Table 1
Sequential injection sequence for the determination of nitrate in dairy products

Step	Description	Port[a]	Time (s)	Pumping flow rate (μL s^{-1})	Auxiliary pump	Flow direction	Volume (μL)
1	Test portion aspiration	3	8	16	Stopped	Reverse	128
2	Propelling towards the column	5	8	16	Stopped	Forward	128
3	Reduction in the column	5	30	0	Stopped	Stopped	—
4	Reduced test portion meets chromogenic reagent and is propelled to the detector	5	20	16	Forward[b]	Forward	320
5	Washing the cadmium column	5	35	64.7	Stopped	Forward	2265
6	Test portion aspiration	1	15	16	Stopped	Reverse	240
7	Propelling towards the detector	4	22.5	64.7	Forward[c]	Forward	360
8	Washing the reactor coil	4	40	64.7	Stopped	Forward	2590

[a] Fig. 1.
[b] Simultaneously, 146 μL of chromogenic reagent are introduced by the auxiliary pump.
[c] Simultaneously, 166 μL of chromogenic reagent are introduced by the auxiliary pump.

in-line to nitrite by passing through a copperized cadmium column, and nitrite is determined spectrophotometrically after Griess reaction. This colour development methodology consists of the reaction of nitrite with sulphanilamide to form a diazonium salt, which is coupled to NED, resulting in a highly absorbing purple azo dye.

The composition and concentration of the carrier solution was based on literature, as this parameter has been fully studied by other authors (Lapa et al., 2000). A solution of 0.4 g of EDTA and 70 g of ammonium chloride diluted to 1 L with water was used. The pH of this solution was varied from 4.0 to 10.0. At pH 9.5 the most reduction of nitrate led to better sensitivity. The presence of EDTA in the carrier avoided precipitation of $Cd(OH)_2$ and other divalent cations.

Regarding the chromogenic reagent, Lapa et al. (2000) mentioned that use of two reagents together led to better results than when used separately. Consequently, a combined solution containing sulphanilamide and NED was used. This technique presented the additional advantage of making the determination faster, with less dispersion (higher sensitivity). The NED concentration was studied from 0.25 to 1.5 $g\,L^{-1}$. Sensitivity of determination strongly increased up to 1.0 $g\,L^{-1}$ and slightly beyond this concentration. Therefore, the 1.0 $g\,L^{-1}$ was chosen. Concerning the sulphanilamide concentration, it was evaluated between 10 and 45 $g\,L^{-1}$. As there was no improvement in sensitivity above 40 $g\,L^{-1}$, this concentration was selected. The hydrochloride concentration present in the chromogenic reagent was studied in the concentration range from 0.6 to 1.8 $mol\,L^{-1}$. The maximum sensitivity was obtained with 0.8 $mol\,L^{-1}$. Concentrations above 1.2 $mol\,L^{-1}$ decreased this parameter, probably because the pH of the solution changed from the optimum pH for the colour development reaction.

Sample volume introduced in the system had a marked influence on the analytical signal on both determinations. For nitrate plus nitrite determination, this volume was varied from 80.0 to 160 μL. Optimum results were obtained with 128 μL, with maximum sensitivity. For larger sample volumes, the sensitivity decreased, probably due to the fact that the selected volume (128 μL) corresponded to the entire filling of the reducing column. For nitrite determination, this volume was modified from 160 to 273 μL. The higher the sample volume used, the higher the sensitivity achieved. However, the chosen volume was 240 μL, since beyond this value the increase in sensitivity was not significant (about 5%) and would consume more chromogenic reagent.

The stopped flow period for reduction of nitrate to nitrite in the cadmium column was studied in the range of 0 to 40 s. A time of 30 s was selected for use in further experiments since it enabled the highest sensitivity. Longer times decreased sensitivity probably because of extended residence time in the column, increasing dispersion.

The chromogenic reagent is introduced in the system by means of an auxiliary pump P_2 (Fig. 1). As the computer only controls its timing course, the rotation speed was fixed before starting the determinations. Moreover, this reagent was introduced in the system during steps 4 and 7, respectively, for nitrate plus nitrite determination and for nitrite determination, for colour development during transport to the detector. Therefore, the procedure for the optimization of the volume of chromogenic reagent introduced in the system was to fix the timing courses of the pump to the ones presented in Table 1 for steps 4 and 7, while manually varying the rotation speed of the pump before each determination. The rotation speeds studied corresponded to introduced volumes ranging from 130 to 273 μL for the nitrate plus nitrite determination and from 146 to 308 μL for the nitrite determination. When investigating the effect of different reagent volumes on sensitivity, decreasing volumes led to better sensitivity. However, for those volumes corresponding to low rotation velocity in the pump, repeatability was compromised. Hence, volumes of 146 and 166 μL were selected for nitrate plus nitrite and nitrite determinations, respectively.

The length (50–100 cm) of the reactor before the detector was studied. A 60 cm coil was used for further work since there was no evidence of difference between 60 cm and greater lengths.

Since literature provided several batch methods for milk protein precipitation, its applicability in the developed SIA system was studied to evaluate possible interference of the precipitating solutions on nitrate and nitrite determination. This was accomplished doing recovery studies. The tested methods applied to several milk samples were warm (ISO, 1985) and cold (ISO, 2000) with precipitation by Carrez reagents, 24% trichloroacetic acid (MSDA, 1973), and HCl 6 $mol\,L^{-1}$ (Fox, 1989). The results obtained are presented in Table 2 and demonstrate that better recovery percentages in milk were achieved by using warm precipitation and Carrez reagents. This was the same methodology used in the reference procedure.

Analysis of dairy samples

The performance of the optimized SIA system for nitrate determination in dairy samples is depicted in Table 3.

The results obtained for the spiked dairy samples are presented in Table 4. The corresponding parameters

Table 2
Comparison of nitrate recovery[a] percentages following four methods of milk protein precipitation

Methods	Recovery (%)
Cold Carrez reagents	111.1 ± 0.5
Warm Carrez reagents	107.2 ± 0.6
24% trichloroacetic acid	63.2 ± 1.2
HCl 6 $mol\,L^{-1}$	37.1 ± 0.2

[a]These recoveries were obtained for additions of 5 $mg\,L^{-1}$ of nitrate. Presented values are means (± standard deviation) of three replicate experiments.

Table 3
Sequential injection performance for nitrate determination in dairy products

Parameter	Value
Detection limit	0.15 mg L^{-1}
RSD[a]	3.4% (4.84 mg L^{-1})
Sample consumption[b]	128 μL
Carrier consumption[b]	0.27 mg EDTA
	47 mg NH_4Cl
Chromogenic reagent consumption[b]	5.8 mg sulphanilamide
	146 μg NED and 64 mg HCl

[a]The RSD (relative standard deviation) is calculated from 10 consecutive sample determinations.
[b]Per determination.

Table 4
Nitrate in several dairy products and nitrite in cheese by sequential injection analysis, reference procedure and respective relative deviation (RD)[a]

Determination	Sample[b]		SIA (mg L^{-1})	Ref. (mg L^{-1})	RD (%)
Nitrate	Whey	1	2.74 ± 0.06	2.85 ± 0.00	−3.9
		2	1.53 ± 0.05	1.52 ± 0.13	0.7
		3	3.27 ± 0.09	3.05 ± 0.02	7.2
		4	4.76 ± 0.06	4.84 ± 0.00	−1.7
		5	0.91 ± 0.02	1.00 ± 0.03	−9.0
	Raw Milk	1	2.51 ± 0.01	2.33 ± 0.01	7.7
		2	2.88 ± 0.03	3.00 ± 0.00	−4.0
		3	3.37 ± 0.09	3.21 ± 0.01	5.0
	Milk	1	1.50 ± 0.01	1.53 ± 0.02	−2.0
		2	4.46 ± 0.13	4.59 ± 0.01	−2.8
		3	0.77 ± 0.03	0.71 ± 0.02	8.5
	Cheese	1	1.56 ± 0.06	1.63 ± 0.05	−4.3
		2	1.29 ± 0.06	1.27 ± 0.02	1.6
		3	1.25 ± 0.04	1.21 ± 0.01	3.3
		4	0.23 ± 0.03	0.22 ± 0.02	4.5
		5	3.23 ± 0.08	3.46 ± 0.03	−6.6
		6	2.98 ± 0.13	3.08 ± 0.02	−3.2
		7	1.47 ± 0.01	1.48 ± 0.01	−0.7
Nitrite	Cheese	8	0.725 ± 0.015	0.743 ± 0.012	−2.4
		9	1.06 ± 0.01	1.02 ± 0.13	3.9

[a]These results correspond to spiked samples, except for cheeses 6 and 7. Presented values are means (± standard deviation) of three replicate experiments.
[b]Samples are ultrapasteurized milk with 1.7% milk fat, whey from bovine milk, raw bovine milk. Cheese varieties are: Brie (samples 1 and 7); Portuguese Flamengo (samples 2, 4 and 5); Mozzarella (sample 6); Cream cheese (sample 8); Roquefort (sample 9); and Edam (sample 3).

of Eq. (1) were: $C_0 = 0.039$ (±0.111), $S = 0.977$ (±0.043) and $r = 0.9960$, showing no evidence of systematic differences between the SIA and the reference methodologies. Additionally, results were validated by recovery studies (Table 5); a t-value (experimental) of 1.77 was obtained in comparison with $t_{critical} = 2.45$, indicating that there is no significant difference between the mean recovery percentage and 100%.

Table 5
Results obtained for nitrate recovery studies in different dairy samples[a]

Sample	Added nitrate (mg L^{-1})	Recovered nitrate (mg L^{-1})	Recovery (%)
Whey	1.50	1.66 ± 0.07	110.7 ± 2.3
Whey	3.00	3.14 ± 0.03	104.7 ± 2.4
Whey	4.00	4.13 ± 0.09	103.3 ± 1.9
Raw milk	4.00	4.13 ± 0.05	103.3 ± 1.4
Milk	2.50	2.51 ± 0.08	100.4 ± 2.3
Milk	3.00	2.87 ± 0.07	95.7 ± 3.3
Milk	4.00	4.13 ± 0.14	103.3 ± 3.6

[a]Samples are ultrapasteurized milk with 1.7% milk fat, whey from bovine milk and raw bovine milk. Presented values are means (± standard deviation) of three replicate experiments.

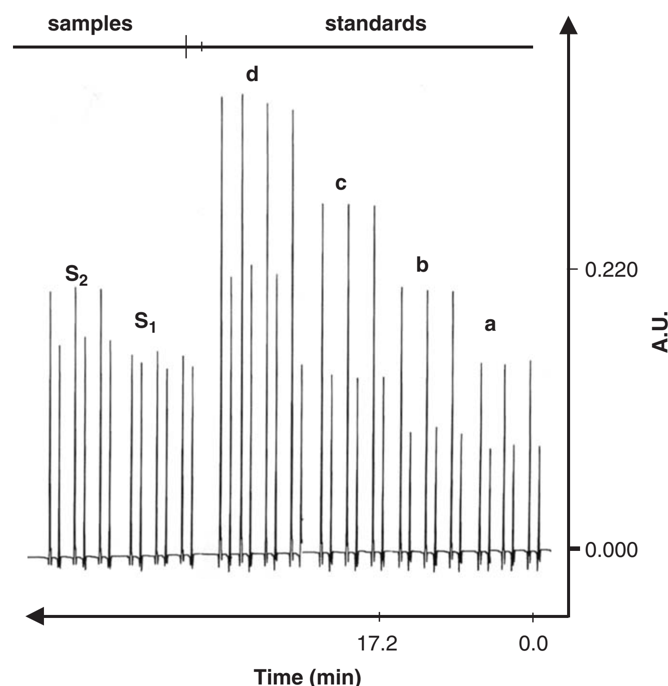

Fig. 2. Recorder output for the sequential determination of nitrate plus nitrite (mg L^{-1}) followed by the nitrite determination (mg L^{-1}). In each pair of consecutive peaks, the first one corresponds to nitrate plus nitrite, and the second one to nitrite; a–d are mixed standards containing: a—0.5, 0.5; b—1.0, 1.0; c—2.0, 1.5; d—4.0, 2.0 mg L^{-1} of nitrate and nitrite, respectively; S_1 and S_2—cheese samples.

The repeatability (expressed as RSD) of the developed SIA procedure was: 1.22%, 1.51% and 3.4% for nitrate concentrations of 1.66, 2.48 and 4.84 mg L^{-1}, respectively.

The signals obtained for the determination of nitrate in two cheese samples by the sequential injection methodology is shown in Fig. 2.

Conclusions

A successful application of SIA methodology to perform the nitrate determination in dairy products was

demonstrated, as an alternative to the conventional laborious and time consuming classical procedures. The devised system presents a high degree of automation and substantial reduction of reagent consumption and waste disposal. The introduction of a cadmium column in the SIA system proved successful to achieve total reduction of nitrate to nitrite when using a stopped flow period of 30 s.

Since this methodology for nitrate determination implies a previous determination of nitrite content in the samples, this ion can easily be detected and quantified whenever present, without any reconfiguration of the sequential injection manifold.

Response time, compared with the standard methodology, is short and the results show good agreement with that obtained by the reference procedure, demonstrating an interesting feature of applicability for routine quality control of dairy samples, using higher throughput (21 samples per hour) than that obtained with the reference procedure (2 per hour). These possibilities offer new perspectives for future developments of methodologies applied to dairy industry that can run for extended periods without the necessity of continuous assistance from the operator.

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