Sequential Injection Determination of Nitrate in Vegetables by Spectrophotometry with Inline Cadmium Reduction

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Abstract: A sequential injection system for the determination of nitrate (NO_3^-) in vegetables was developed to automate this determination, allowing for substantially reduced reagent consumption and generated waste using low-cost equipment. After extraction with water and filtration, the extracted nitrate is reduced inline to nitrite in a copperized cadmium (Cd) column and determined as nitrite. According to the Griess–Ilosvay reaction, nitrate is diazotized with sulfanilamide and coupled with N-(1-naphtyl)-ethylenediamine dihydrochloride to form a purple-red azo dye monitored at 538 nm.

Nitrate can be determined within a range of 1.35–50.0 mg L^{-1} of NO_3^- (corresponding to 0.270–10.0 g of NO_3^- per kg of vegetable), with a conversion rate of nitrate to nitrite of 99.1 $\pm$ 0.8%. The results obtained for 15 vegetable extracts compare well with those provided by the classical procedure, with a sampling throughput of 24 determinations per hour and relative standard deviations better than 1.2%.

Keywords: Copperized cadmium column, nitrate, sequential injection analysis, spectrophotometry, vegetables

INTRODUCTION

Plants contain a wide range of chemical substances that have significant effects on public health. It is therefore of extreme importance to assess

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plant composition to monitor harmful compounds that may cause any adverse effects. Nitrate (NO_3^-) is the primary nutrient form of nitrogen (N) that plants absorb from the soil to convert into proteins (Varennes 2003). Once ingested by animals or humans, this ion can be reduced to nitrite, which may lead to a disease known as methaemoglobinaemia.

In the plast few years, fresh and processed vegetables have often been reported as the major sources of dietary NO_3^- intake in several countries (Kenny and Walshe 1975; Lox and Okabe 1982; Bakr, El-Iraqui, and Huissen 1986; Lyons et al. 1991; Lyons et al. 1994; Forlani et al. 1997; Chung et al. 2003). For this reason, there has been an increase in interest concerning the factors that might influence nitrate accumulation in vegetables. Actually, the presence of high NO_3^- levels in several food products of conventional agriculture is related to the amount of commercial fertilizers applied (Lima, Rangel, and Souto 1995; Reeve 1996). Moreover, the leaching of those fertilizers causes the contamination by NO_3^- in potable water systems throughout the world.

The reference method (ISO 1984) for NO_3^- determination in vegetables is a colorimetric procedure based on the Griess–Ilosvay reaction. After prior reduction of NO_3^- to nitrite, this anion is diazotized and coupled with sulfanilamide and *N*-(1-naphtyl)-ethylenediamine dihydrochloride (N1NED) to produce a colored compound spectrometrically measured at 538 nm. This process is rather time consuming, requires highly skilled personnel, produces a large volume of effluents, and requires the use of considerable amounts of toxic reagents. To perform this determination automatically, segmented flow analysis (SFA) and flow injection analysis (FIA) systems have been described as advantageous automatic alternatives for the determination of NO_3^- in vegetables (Beljaars, VanDijk, and VanDerHorst 1994; Lima, Rangel, and Souto 1995; Andrade et al. 2003). Sequential injection analysis (SIA) systems, presented by Ruzicka and Marshall (1990), appeared as an evolution to SFA and FIA systems, presenting considerable advantages: higher degree of automation, minimization of reagent consumption, and considerable decrease of generated waste. This methodology is based on the sequential aspiration of well-defined sample and reagent zones through the selection valve into the holding coil, with subsequent flow reversal to propel the mixture to the detection system. In the literature, a few applications of this concept to the determination of nitrates in some food and water samples can be found (Oms, Cerdà, and Cerdà 1995; Cerdà et al. 1998; Lapa, Lima, and Pinto 2000a, 2000b; Galhardo and Masini 2001; Kazemzadeh and Ensafi 2001; Lednerová et al. 2002), but no application to vegetables was yet developed.

In this article, a sequential injection analysis system for the automatic determination of NO_3^- in vegetable extracts is described. The manifold was devised to decrease the consumption of reagents with subsequent production of effluents and to substantially decrease the overall analysis time using low cost equipment. Additionally, the working concentration range should

include the expected NO_3^- concentrations in vegetable extracts, so that no further sample treatment would be necessary.

MATERIALS AND METHODS

Reagents and Solutions

All solutions were prepared using Milli-Q water and analytical reagent-grade chemicals.

A 1.0 g L^{-1} NO_3^- stock solution was prepared from sodium nitrate, previously dried for 2 h at 105–110°C. Working standard solutions containing nitrate within a range of 1.35–50.0 mg L^{-1} were prepared daily by appropriate dilution of the stock solution with water.

To study the conversion rate, single analyte standards of nitrite and single analyte standards of nitrate, both in the range 1.35–50.0 mg L^{-1} (expressed as NO_3^-) and with equal concentrations were prepared.

The chromogenic reagent was prepared by dissolving 40 g of sulfanilamide and 1.0 g of N1NED in 100 mL of concentrated HCl (37%, $d = 1.19$ g mL^{-1}) and adjusting the volume to 1000 mL.

The carrier solution was prepared by dissolving 0.4 g of $Na_2H_2EDTA \cdot 2H_2O$ and 20 g of ammonium chloride in 1000 mL of water. The pH of this solution was adjusted to 9.0–9.5 with a few drops of ammonia solution.

Copperized cadmium column was prepared by adding 4 g of cadmium particles (diameter between 0.5 and 1 mm) to 50 mL of a copper (II) sulphate solution (5 g L^{-1}) and stirring for 2 min. A portion of these particles was packed using a syringe into a Teflon® tube (15 cm long with a 2.29-mm inner diameter). Ordinary dishwashing foam was placed at both ends of the minicolumn to entrap the particles, which were thereafter conditioned in the sequential injection system with 100 mL of 2 mol L^{-1} hydrochloric acid and 100 mL of a 10 g L^{-1} ammonium chloride solution, both at a flow rate of 1.0 mL min^{-1}.

Instrumentation

Solutions were propelled by a Gilson Minipuls 3 (Villiers-le-Bel, France) peristaltic pump with a Gilson pumping tube, connected to the central channel of a 10-port electrically actuated selection valve VICI (Houston, USA) (Figure 1). All tubing was made of PTFE Omnifit (Cambridge, UK), 0.8 mm id.

A Philips (Cambridge, UK) PU 8625 UV/VIS spectrophotometer set at 538 nm with a Hellma (Müllheim/Baden, Germany) flow cell (10-mm light path, 30-μL inner volume) was used as a detection system. Analytical signals were recorded using a Kipp & Zonen (Delft, Holland) BD 111 chart recorder.

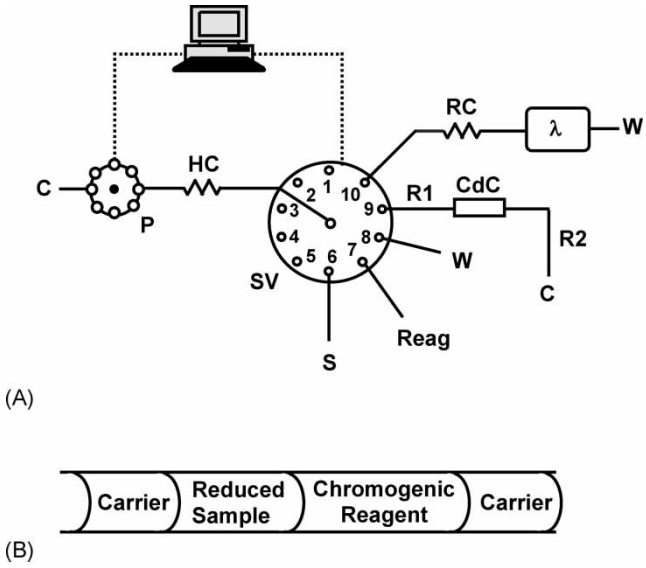

Figure 1. (A) Sequential injection analysis (SIA) manifold used in the determination of nitrate in vegetable extracts: S, sample or standard; C, carrier; Reag, chromogenic reagent; W, waste; P, peristaltic pump; SV, 10-port selection valve; HC, holding coil (160 cm); Ri, reactors: R1 = 1 cm; R2 = 90 cm; CdC, cadmium column; RC, knitted reaction coil (120 cm); λ, UV/VIS spectrophotometer (538 nm). (B) Sequence of the solutions in the holding coil before the colorimetric measurement.

A Samsung (Korea) SD700 386 personal computer, equipped with a PCL818L interface card, running homemade software written in QuickBasic 4.5, controlled the selection valve, the pump rotation, and the flow direction.

Sequential Injection Procedure

Nitrate is monitored by the use of a copperized cadmium minicolumn, incorporated in one of the channels of the selection valve, to reduce nitrate to nitrite, followed by the color development reaction and spectrophotometric detection at 538 nm. Copperized cadmium (Cd) was chosen to perform the reduction step because, according to others (Nydahl 1976; Margeson, Suggs, and Midgett 1980; Moorcroft, Davis, and Compton 2001), more promising results have been obtained by using this material as the reductant. The mechanism of reduction of NO_3^- to NO_2^- by cadmium seems to be (Margeson, Suggs, and Midgett 1980)

$$NO_3^- + H_2O + Na_2H_2EDTA + Cd^0 \leftrightarrow NO_2^- + 2OH^- + Na_2EDTA \cdot CdH_2(EDTA).$$

The column was kept alkaline during reduction to stabilize the formed nitirite (NO_2^-), and EDTA was added to chelate Cd^{2+}. Without EDTA, cadmium hydroxide [$Cd(OH)_2$] would precipitate on the column and impair the sample flow.

After setting the manifold configuration to accommodate these reactions, optimization procedures were carried out, regarding both linear range and conversion rate of nitrate to nitrite.

The sample was aspirated and propelled to the reducing Cd column through port 9 (steps A and B, Table 1). While the holding coil was being washed (to ensure that none of the sample volume remained on it), the sample was in contact with cadmium particles for 10 s (step C). Then, the reduced NO_3^- was aspirated from the reducing column, chromogenic reagent was aspirated, and the mixture was sent to the detector (steps D to F).

The last two steps washed and regenerated the reducing column as carrier solution was aspirated through port 9 and propelled to waste (steps G and H).

The flow rate for all the passages through cadmium column was fixed at 1.0 mL min^{-1}.

To estimate the exact NO_3^- concentration in the samples, a preliminary study of the nitrite content in the vegetable extracts was done. None of the samples contained nitrite above 0.1 mg L^{-1}, which was an insignificant value compared to NO_3^- concentrations, resulting in no contribution to the measured nitrate concentration.

Nitrate concentration was determined by interpolation of the sample signal on the analytical curve absorbance vs. NO_2^-, followed by the application of the conversion factor $[NO_2^-] \times [MW(NO_3^-)/MW(NO_2^-)]$ (mg L^{-1}).

The final concentration of NO_3^- in the vegetables was expressed in mg of NO_3^- per kg of vegetable.

Table 1. Sequential injection protocol for the determination of nitrate in vegetables

Step	Valve position	Operation time (s)	Volume (μL)	Description
A	6	3	50	Aspirate sample or standard
B	9	18	300	Propel toward the reducing column
C	8	10	333	Wash the holding coil
D	9	18	300	Aspirate from cadmium column
E	7	6	250	Aspirate chromogenic reagent
F	10	65	3250	Propel towards the detector; signal registration
G	9	20	333	Aspirate carrier solution
H	8	10	667	Propel toward the waste

Statistical Analysis

To evaluate the accuracy of the results obtained by the developed SIA methodology, NO_3^- content was determined by SIA and by the reference method (ISO 1984) in 15 vegetable extracts. Statistical treatment of the results was established (Miller and Miller 1993) by the following relation: $C_S = C_0 + SC_r$, where C_S represents the sequential injection results; C_r the results provided by reference method; S, the obtained slope; and C_0, the intercept with a 95% significance level for 13 degrees of freedom (15 samples).

To assess the repeatability of the SIA procedure, relative standard deviation (RSD) was calculated from 10 consecutive injections of sample solutions.

The detection limit was calculated as the concentration that gives a signal three times the standard deviation of 10 consecutive injections of the blank (IUPAC 1976). The quantification limit was calculated as the concentration that gives a signal 10 times the standard deviation of 10 consecutive injections of the blank (Long and Winefordner 1983).

Sample Treatment

Vegetable extracts were prepared as described in the reference method (ISO 1984). A representative amount (ca. 1 g) of the sample, previously homogenized in a mechanical grinding mill, was weighed and introduced into a 250-mL Erlenmeyer flask, where 5 mL of a 5% disodium tetraborate decahydrate aqueous solution and about 100 mL of deionized water, at a temperature greater than 70°C, were added. The resulting suspension was maintained warm (60–70°C) and stirred for 15 min, after which the suspension was cooled to room temperature and 2 mL of each *Carrez* reagent (a 15% potassium ferrocyanide and a 30% zinc acetate aqueous solution) were added. The suspension was stirred and transferred to a 200-mL volumetric flask, adjusting the volume with water. Then, the suspension was filtered, resulting in a clear solution.

RESULTS AND DISCUSSION

SIA Optimization

Carrier Stream

A solution containing EDTA and NH_4Cl was used as carrier solution. The influence of the NH_4Cl concentration was studied in the range 10.0 to 80.0 g L^{-1}. With ammonium chloride concentration lower than 20.0 g L^{-1},

the repeatability decreased. A conversion rate of 99.1 $\pm$ 0.8% ($n = 5$) was obtained using 20.0 g L^{-1} of NH_4Cl. Above this value, the conversion rate of nitrate to nitrite was maintained, but the sensitivity decreased 7% for the 40.0 g L^{-1}, 12% for 60.0 g L^{-1}, and 17% for 80.0 g L^{-1} of NH_4Cl. Thus, the concentration of 20.0 g L^{-1} ammonium chloride was chosen. The EDTA concentration was set to 0.4 g L^{-1} and the pH adjusted to 9.0–9.5, as previously described (Lapa, Lima, and Pinto 2000a).

Chromogenic Reagent

According to a previous work (Lapa, Lima, and Pinto 2000a), better results were obtained when the mixture of sulfanilamide and N1NED was used instead of separated solutions, so a mixture was used in this work. The concentration of each component of the chromogenic reagent was varied, whereas the others were kept.

Different concentrations of sulfanilamide, ranging from 30 to 50 g L^{-1}, were tested. The sensitivity increased 5% up to 40 g L^{-1} and decreased for upper values, so a sulfanilamide concentration of 40 g L^{-1} was chosen.

Different concentrations of N1NED were tested in the range 0.5–2.0 g L^{-1}. The sensitivity increased up to 1.5 g L^{-1}, but better linearity and repeatability were attained using N1NED in a concentration of 1.0 g L^{-1}, so this concentration was chosen for further work.

The concentration of HCl was varied from 0.6 to 2.4 mol L^{-1}, showing an increase of the sensitivity within this range, but the absorbance values were too high, which compromised the working linear range. The chosen concentration of HCl in the chromogenic reagent was of 1.2 mol L^{-1}, which was a compromise between sensitivity and linear range.

Reactor Length

Reactors 100, 110, 120, and 140 cm in length were tested. Better sensitivity was achieved using the reactor 120 cm long, so this was the chosen length.

Sample Volume

The sample aspirated to the system was sent to the reducing column in a way that ensured that all the volume passed through the column. Sample volumes from 20 to 100 μL were studied. Using volumes of less than 50 μL, both precision and sensitivity were poor, and at volumes greater than this, the absorbance values were too high, which compromised the aimed working range. Thus, a sample volume of 50 μL was chosen.

Reagent Volume

Experimental runs were carried out to define the optimum reagent volume to be used in this determination. Different reagent volumes within 100 and

350 μL were aspirated. The repeatability and sensitivity increased up to 250 μL of aspirated reagent, decreasing for upper values. Hence, a reagent volume of 250 μL was selected.

Column Length

The effect of the length of the reducing column (2.29 mm id) was examined with 4.5 cm, 7.5 cm, and 15 cm lengths. With a 15-cm column length, the conversion of nitrate to nitrite was complete and the desirable linear range and sensitivity were already attained. In this way, no longer lengths were tested.

Conversion Rate

The conversion rate of nitrate to nitrite was evaluated by comparing the signal for nitrate solutions to that of nitrite solutions at equivalent concentrations and in reducing conditions.

The sample volume propelled to the reducing column and the volume of the sample aspirated from the column, after reduction (and used in the spectrophotometric reaction), were two considerably relevant factors that affected the conversion rate as well as the precision. It was noted that better precision was observed when the sample volume sent to the reducing column was 300 μL and equal to the collected sample volume after reduction.

Different sample volumes, from 167 to 333 μL, were tested. Using 167 μL of sample, the repeatability was low. With sample volumes of 233 and 267 μL, a conversion rate greater than 100% was obtained. With 333 μL of sample, the linear range became narrower because of the elevated absorbance values. More precise results and a conversion rate close to 100% were attained using 300 μL of sample, so this volume was selected as the optimum reduction sample volume as well as the spectrophotometric reaction volume.

The conversion rate obtained with these conditions in the range 1.35–50.0 mg L^{-1} of NO_3^- was 99.1 $\pm$ 0.8% ($n = 5$).

Evaluation of the Method and its Application to Vegetable Samples

The performance of the proposed system for the determination of nitrate in vegetable extracts was evaluated regarding the application range, detection and quantification limits, accuracy, precision, and sampling frequency.

A linear correlation (recorder output presented in Figure 2) between 1.35 and 50.0 mg L^{-1} was obtained, and the typical calibration curve was as follows: $A = 0.0317\ (\pm 0.0022)\ |NO_2^-| + 0.161\ (\pm 0.030)$, $R = 0.9998$.

The detection limit was 0.95 mg L^{-1} (190 mg of NO_3^- per kg of vegetable), and the quantification limit was 1.35 mg L^{-1} of NO_3^- (270 mg of NO_3^- per kg of vegetable).

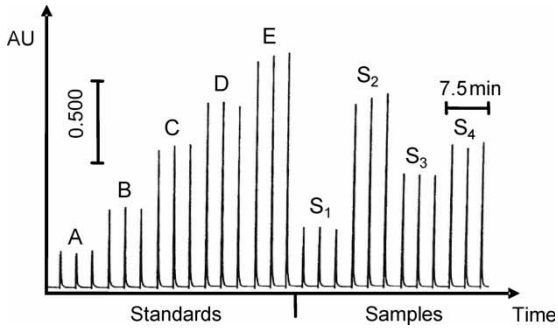

Figure 2. Recorder output obtained in the spectrophotometric determination of nitrates in vegetables, corresponding to the injection of a set of standards (A = 1.60 mg L^{-1}, B = 12.0 mg L^{-1}, C = 26.8 mg L^{-1}, D = 37.6 mg L^{-1}, E = 50.0 mg NO_3^- L^{-1}) and four different samples.

To evaluate the accuracy of the proposed method, 15 vegetable extracts were analyzed by the developed sequential injection system and by the reference method (ISO 1984). The paired results, together with corresponding relative deviations, are presented in Table 2. The results (C_s), expressed in mg of NO_3^- kg^{-1} of vegetable, were compared with those furnished by the recommended procedure (C_r) and the equation parameters, and 95%

Table 2. Determination of nitrates in vegetables by sequential injection analysis (SIA) methodology and reference method and corresponding relative deviations (RD)

Samples		Ref. method[a]	SIA[a]	RD (%)
Number	Type	(mg NO_3^-/kg)	(mg NO_3^-/kg)	(mg NO_3^-/kg)
1	Lettuce	3046 ± 10	3019 ± 7	−0.89
2	Lettuce	2367 ± 3	2292 ± 25	−3.17
3	Lettuce	2098 ± 16	2170 ± 4	+3.43
4	Lettuce	1709 ± 20	1700 ± 19	−0.53
5	Lettuce	2184 ± 5	2316 ± 33	+6.04
6	Lettuce	3528 ± 6	3311 ± 36	−6.15
7	Spinach	4195 ± 3	4206 ± 37	+0.26
8	Spinach	2439 ± 2	2400 ± 29	−1.60
9	Spinach	3481 ± 15	3609 ± 4	+3.68
10	Spinach	1507 ± 4	1544 ± 3	+2.46
11	Broccoli	279 ± 5	276 ± 4	−1.08
12	Broccoli	282 ± 6	297 ± 4	+5.32
13	Coriander	3935 ± 9	3860 ± 6	−1.91
14	Coriander	4273 ± 5	3977 ± 5	−6.93
15	Parsley	1056 ± 10	1068 ± 7	+1.14

[a]Concentration ± standard deviation ($n = 3$).

confidence limits (Miller and Miller 1993) were the following: $C_s = 0.963$ (± 0.071) $\times$ $C_r + 66$ (± 129), $R = 0.9967$. These values indicate that, for a 95% confidence level, there is no statistical difference between the two sets of results.

The results (mg L^{-1} of NO_3^-) for assessing repeatability were as follows: 7.54 $\pm$ 0.09, 28.4 $\pm$ 0.3, and 38.9 $\pm$ 0.4. The corresponding relative standard deviations were 1.2, 1.0, and 1.1%, respectively.

The sampling frequency was of 24 determinations per hour, because it took 150 seconds to complete an analytical cycle.

CONCLUSIONS

The sequential system with spectrophotometric detection proposed for the determination of nitrate in vegetables is a reliable alternative to more costly and extensive methodologies such as FIA and classical procedures.

The simplicity and low cost of the developed manifold allows its routine implementation in plant-analysis laboratories. Besides that, this sequential injection methodology presents a higher degree of automation than FIA procedures already described, allowing runs for extended periods without the need for the analyst to be present, because all operations are controlled by computer. The permanent inline reconditioning of the reducing column maintains maximum conversion efficiency, ensuring accuracy and precision of the system. The proposed methodology does not require offline concentration adjustments for the determination of nitrate in the range of interest for vegetable products. Furthermore, if necessary, nitrate can be determined in lower concentrations just by increasing the sample volume without any further changes.

The developed system is less time consuming than the reference method, allowing 24 determinations per hour compared with 2 determinations per hour typical of the reference procedure. Moreover, low reagent consumption, the small amount of cadmium particles, and the low volume of effluents produced by the proposed SIA methodology are important aspects for waste reduction (Table 3).

Table 3. Reagent consumption and effluent volume produced per determination by SIA methodology and reference method

Characteristic	Ref. method	SIA
N1NED (mg)	1.00	0.25
Sulfanilamide (mg)	10.0	10.0
12 mol L^{-1} HCl (μL)	1835	25
NH_4Cl (mg)	187	91
Effluent (mL)	140	4.25

The proposed method provides accuracy and precision (RSD lower than 1.2 %, $n = 10$), and the results obtained using this methodology, applied to the determination of nitrate in vegetable samples, showed good agreement with those obtained by the reference method.

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