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Polarographic determination of nitrate in vegetables

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Abstract

A polarographic method for the determination of nitrate in vegetables is presented. The method is based on the reduction of nitrate to nitric oxide which reacts in solution with colbalt (II) and thiocyanate ions forming an electroactive complex that is reduced at the dropping mercury electrode at -0.5 V (vs. SCE). The nitric oxide is generated outside the polarographic cell by addition of ferrous ammonium sulfate and ammonium molybdate in hydrochloric acid to the previously triturated vegetable matrices. The calibration graph was linear in the range of $2–12 \times 10^{-6}$ mol nitrate. The recovery of nitrate in vegetable matrices (broccoli, kale, lettuce, radish, red beet, spinach, turnip and watercress) varied from 85.4 to 107.4 % and the nitrate content, expressed as sodium nitrate, varied from 751 to 10 806 mg kg^{-1} of fresh vegetable. The relative standard deviation for the proposed method is lower than 7% and considering a sample of 5.0 g, the determination limit was 39 mg of nitrate per kg fresh vegetable weight. The precision and accuracy of the polarographic method were comparable to those of the reference spectrophotometric method (official AOAC reference method for the determination of nitrate in foodstuffs). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nitrate determination; Polarography; Nitric oxide; Vegetables

1. Introduction

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 at the curing process of certain meat products, due to their ability to inhibit the outgrowth spores of *Clostridium botulinum* and to impart characteristic color and flavor to this kind of foodstuffs [1]. However, vegetables constitute the major source of nitrate providing, approximately 80% of the average daily human dietary intakes [2].

The nitrate content in vegetable may be influenced by factors related to the plant (variety, specie and maturity) [3] and to the environment (temperature, light intensity and deficiency of some nutrients and use of fertilizers) [4].

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The significance of nitrate to human health is related to the fact that nitrate, after being metabolized or reduced to nitrite, can react with secondary or tertiary amines to form *N*-nitroso compounds, which are potent carcinogens. Nitrite can also interact with hemoglobin influencing the oxygen transport mechanism giving rise to methemoglobinemia [5].

A variety of analytical methods for the determination of nitrate and nitrite have been developed and applied to the analysis of food, water, biological material, soil, plants, animal feed, and other matrices. These include spectrophotometry [6], high performance liquid chromatography [7,8], gas chromatography [9], capillary electrophoresis [10], voltammetry [11], potentiometry with ion selective electrode [12] and chemioluminescence [13]. However, the most frequently employed methodology for the determination of nitrate and nitrite in food is still the spectrophotometric method where nitrite, and nitrate after reduction to nitrite, is reacted with a primary aromatic amine in acid solution to form a diazonium salt, which is coupled with an aromatic compound containing amino or hydroxyl substituents to form an azo dye [6]. Nonetheless, this methodology requires steps of extraction, clarification and reduction of nitrate to nitrite that turns it time consuming.

This paper describes the development and application of a polarographic method for the determination of nitrate in vegetables, without the need of previous extraction and clean-up procedures, recommended by the reference spectrophotometric method. In the proposed method, nitrate was determined through an electroactive complex formed in a cobalt (II)-thiocyanate-acid solution in the presence of nitric oxide generated by the reduction of nitrate outside the polarographic cell. It should be mentioned that under the experimental conditions employed, nitrite is also reduced to NO. Consequently, nitrite would contribute to the total nitrate level determined.

The presented methodology was applied to the determination of nitrate in vegetables. Since in fresh vegetables the nitrite level is extremely low (< 2 mg kg^{-1}) in comparison to nitrate [14], the contribution of nitrite to the total nitrate was not taken into account.

2. Experimental

2.1. Apparatus

A Metrohm Polarecord E 506 in connection with a EA 505 stand equipped with a three-electrode system and a 50 ml capacity Metrohm EA 875-20 cell was used for the polarographic determinations. The working electrode was a dropping mercury electrode (DME). As reference and counter-electrode a saturated calomel (SCE) and a platinum wire were used, respectively.

The polarographic techniques of differential pulse-DP (pulse amplitude, − 50 mV; drop time, 1 s; scan rate, 10 mV s^{-1}), direct current-DC_{TAST} (drop time, 1 s; scan rate, 10 mV s^{-1}) and alternating current-AC ($\varphi \approx 90°$; frequency, 15 mV; drop time, 1 s; scan rate, 10 mV s^{-1}) were employed.

The spectrophotometric measurements were carried out on a Varian spectrophotometer, model 634, using a 1 cm quartz cell. The absorbance for the nitrate determination was measured at 474 nm against a blank reagent solution.

2.2. Reagents

All reagents employed were of analytical grade.

2.2.1. Nitrate standard solution

A nitrate standard stock solution was prepared by dissolving 1 g of KNO_3 (Merck) in 1 l distilled/deionized water.

2.2.2. Nitrite standard solution

A standard nitrite stock solution was prepared by dissolving 1 g of KNO_2 (Merck) in 1 l water. The solution was stored at the dark under refrigeration and was stable for at least a week. The working solutions was prepared daily by dilution of the standard stock solution.

2.2.3. Spectrophotometric reagents

The spongy cadmium was prepared according described in the AOAC Official Method [15]. *N*-naphthol and sulphanilic acid were prepared according Follett and Ratcliff [16].

2.2.4. Supporting electrolyte

In a volumetric flask 5 ml of 2×10^{-2} mol l^{-1} cobalt(II) chloride and 1 ml of 1 mol l^{-1} sodium thiocyanate were diluted with 0.2 mol l^{-1} hydrochloric acid to a final volume of 50 ml.

2.2.5. Reduction system

The reduction system was composed by 5 ml of 6 mol l^{-1} hydrochloric acid, 1 ml ferrous ammonium sulfate 8% (w/v) and 1 ml ammonium molybdate 4% (w/v).

3. Methods

3.1. Polarography

3.1.1. Sample preparation

Only the edible part of each vegetable (broccoli, kale, lettuce, radish, red beet, spinach, turnip and watercress), purchased at the local market in Brasília, DF, was analyzed. The vegetables were washed with distilled water and the retained water was absorbed using filter paper. The samples were homogenized with water 1:1 (w/w) in a Waring blender and portions of 5.0 g were stored at − 18°C.

3.1.2. Nitrate determination in samples

The homogenized sample (5.0 g) and 5 ml 6 mol l^{-1} hydrochloric acid were introduced into the reduction compartment (Fig. 1). A volume of 50 ml of the supporting electrolyte was added to the polarographic cell. The system was closed, allowing only the entrance of nitrogen into the reduction compartment and outlet of the gas at the polarographic cell. The system was deairated with nitrogen (flux of 1 ml s, during a period of 10 min) and the water bath, containing the reduction vessel, heated until boiling (96°C). The nitrogen was turned off and the DP polarogram recorded between − 200 and − 700 mV (blank). In the reduction compartment 1 ml of ferrous ammonium sulfate 8% (w/v) and 1 ml of ammonium molybdate 4% (w/v) were added. A nitrogen flux of 1 ml s^{-1} was passed through the system during a period of exactly 7.5 min. The nitrogen flux was interrupted and, after a reaction time of 15 min, the DP polarogram recorded. The peak current at − 500 mV was measured and the nitrate amount calculated from an external standard calibration graph.

3.1.3. Calibration curve

A calibration curve was obtained introducing different amounts of the nitrate standard solution, in the range of $2–12 \times 10^{-6}$ mol of nitrate, in the reduction compartment. The determination was performed according the procedure described for the nitrate determination in vegetables, where the sample was replaced by an aliquot of the standard nitrate solution.

The statistical treatment of the data was conducted according to Miller and Miller [17]. The determination limit (DL) was calculated as $DL = (kS_{y/x})/m$, where $k = 10$; $S_{y/x}$, standard deviation and m = slope of the analytical regression line.

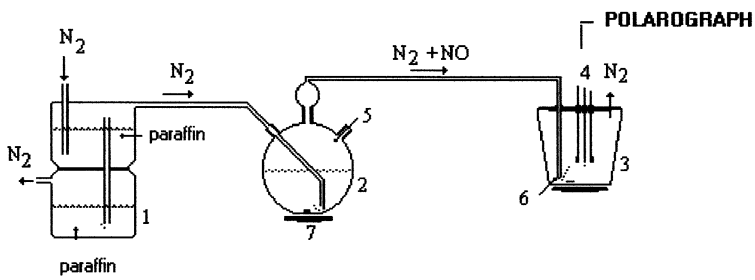

Fig. 1. Scheme of the reduction compartment and polarographic cell for the determination of nitrate, (1) pressure control system of nitrogen; (2) reduction vessel, volume of 50 ml; (3) polarographic cell with magnetic stirrer; (4) potentiostatic system with three electrodes; (5) septum for addition of reagents; (6) synterized outlet of gas; (7) water-bath and magnetic stirrer.

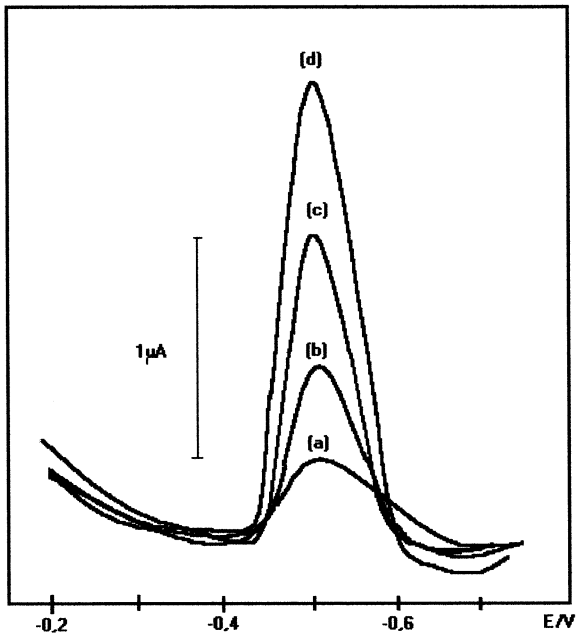

Fig. 2. Differential pulse polarograms, (a) 2×10^{-6} mol KNO_3; (b) 3×10^{-6} mol KNO_3; (c) 4×10^{-6} mol KNO_3; (d) 6×10^{-6} mol KNO_3. Reduction system: $KNO_3 + 2$ ml of Fe(II) 4% (w/v) + 2 ml of ammonium molybdate 2% (w/v). Supporting electrolyte, 0.2 mol l^{-1} HCl + 2×10^{-3} mol l^{-1} $CoCl_2$ + 2×10^{-2} mol l^{-1} KSCN.

3.2. Spectrophotometry

The calibration curve, sample preparation, extraction and clean-up procedures and the spectrophotometric determination of nitrate in vegetables were conducted according to the procedure recommended by Rath et al. [18] with the modification that the clean-up of the vegetable extract was done with potassium hexacyanoferrate (III) and zinc acetate [6].

The absorbance of the azo compound was read at 470 nm against a reagent blank.

4. Results and discussion

In acidic medium nitrite forms, in the presence of cobalt (II)-thiocyanate-ascorbic acid, a ternary complex ($CoSCNNO^+$) which is reduced at DME. It has been suggested that nitric oxide, which is formed by the reduction of nitrite with ascorbic acid, be involved in the electrochemical process. The ternary complex was already characterized and its reduction mechanism studied [19,20].

Based on this reaction, a polarographic procedure for the determination of nitrate, and its applicability to quantification of nitrate in vegetables was studied. Nitric oxide was generated in a reaction vessel outside the polarographic cell by reduction of nitrate with ferrous ammonium sulfate and ammonium molybdate as reducing agent and catalyst, respectively, and transported by nitrogen flux to the polarographic cell containing the supporting electrolyte solution of cobalt (II)-thiocyanate-hydrocloric acid (Fig. 1). In solution NO reacts with Co(II) and thiocyanate ions forming the electroactive complex $CoSCNNO^+$.

The reduction of the ternary complex at DME proceeds in three peaks at − 0.5 V, − 0.9 V and − 1.1 V. Nevertheless, only the peak current at − 0.5 V was proportional to the amount of nitrate added to the reduction compartment, and consequently it was the only one suitable for analytical purposes (Fig. 2). The peak current at − 0.5 V was dependent on the pH-value of the supporting electrolyte. In addition, it was verified that the cathodic current at − 0.5 V decreases as a function of time, consequently the electroactive complex is not stable in the supporting electrolyte. The complex was identical to that formed by the reduction of nitrite with ascorbic acid in solution within the polarographic cell, characterized by AC, DC and DP polarography.

The parameters related to the reduction of the electroactive complex at the DME, generation of NO by reduction of nitrate and formation and stability of the electroactive complex in solution was evaluated.

4.1. Reduction of $CoSCNNO^+$ at DME

4.1.1. Supporting electrolyte

Perchloric acid, hydrochloric acid and acetate buffer, at concentrations of 0.1 mol l^{-1} in a solution containing 2×10^{-3} mol l^{-1} $CoCl_2$ and 5×10^{-3} mol l^{-1} KSCN were studied. Initially, it was confirmed that the peak current is independent of the ionic strength, adjusted by addition of

KCl at values lower than 0.6 and, consequently, further adjustment of the ionic strength for the different supporting electrolyte was not performed.

No peak current was observed when the acetate buffer pH 4.2 was employed. This result indicate that the protonation of the electroactive specie is the rate determining step of the subsequent reduction and corroborate the mechanism proposed by Li et al. [20], which suggested that three protons and three electrons are involved in the reduction of $CoSCNNO^+$, with the formation of $CoSCN^+$ and hydroxylamine.

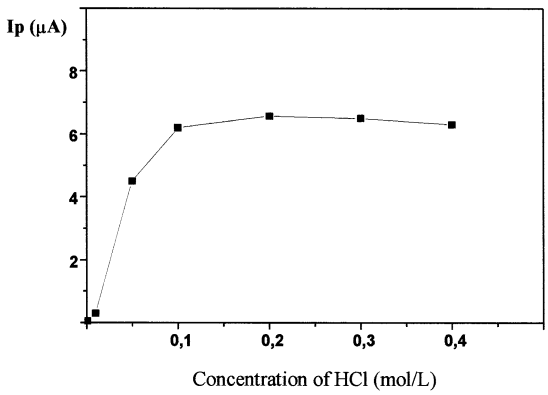

Fig. 3. Effect of HCl concentration on the peak current at -0.5 V. Reduction system, 8.4×10^{-5} mol KNO_3; 2 ml of Fe(II) 4% (w/v) and 2 ml of ammonium molybdate 2% (w/v). Supporting electrolyte, x mol/l HCl; 2×10^{-3} mol/l $CoCl_2$; and 5×10^{-3} mol/l KSCN.

Table 1
Peak current intensity in function of the molar ratio CO^{2+}/SCN^- in 0.2 mol l^{-1} HCl and 0.1 mol l^{-1} KCl

Molar ratio co^{2+}/SCN^-	Concentration (mol/l)		I_p (μA)[a]
	CO^{2+}	SCN^-	
25	0.05	0.002	*
1	0.002	0.002	4.81
0.4	0.002	0.005	7.60
0.2	0.002	0.01	11.30
0.1	0.002	0.02	15.56
0.07	0.002	0.03	15.33
0.04	0.002	0.05	*

[a] Average value ($n = 5$).
* Non reproducible values.

The peak current obtained with perchloric acid was half the value obtained when hydrochloric acid, at the same concentration, was used. At hydrochloric acid concentrations greater than 0.2 mol l^{-1} the peak current is independent of the proton concentration (Fig. 3).

The peak current at -0.5 V was dependent on the molar ratio of cobalt (II) and thiocyanate ions in solution (Table 1). The optimum molar ratio was Co^{2+}, SCN-1/10, with a cobalt concentration of 0.002 mol l^{-1}.

In relation to the temperature, between 20 and 30°C, no change in the intensity of peak current was observed.

4.2. Generation of NO by reduction of nitrate

According to Cox [13], the optimum condition for NO generation from nitrate was achieved by using ferrous ammonium sulfate and ammonium molybdate 1:2 (w/w) in the presence of a strong acid. Sulfuric acid [13], phosphoric acid [21] and hydrochloric acid [22] were recommended.

According to our results, the optimum conditions for the polarographic nitrate determination was achieved by using ferrous ammonium sulfate and ammonium molybdate in a concentration range of 1.3–28.0 and 0.7–14.0 mg l^{-1}, respectively (Table 2). At concentrations of ferrous ammonium sulfate greater than 28 mg l^{-1} and lower than 1.3 mg l^{-1} the peak current decreases significantly. In addition, hydrochloric acid showed a better response than sulfuric and phosphoric acids. Nevertheless, the concentration of hydrochloric acid influences the efficacy of the reduction process. Concentrations between 2.5 and 8.3 mg l^{-1}, enhances the intensity of the peak current at -0.5 V and at concentrations lower than 2.5 mg l^{-1} the reduction process was not quantitative. The efficacy of the reduction was improved by heating the system up to 96°C. At 80°C only 64 % of the peak current intensity obtained at 96°C was obtained. At temperatures lower than 60°C no current was recorded.

Table 2
Peak current in function of the concentrations of ferrrous ammonium sulfate and ammonium molybdate in 6 mol l^{-1} HCl in the reduction compartment containing 5×10^{-6} mol of nitrate[a]

Concentration (mg ml^{-1})		I_p (μA)	S_R (%)	n
Ferrous ammonium sulfate	Ammonium molybdate			
0.6	0.3	0.53	18.8	6
1.3	0.7	1.38	13.8	5
2.7	1.3	1,.9	9.3	5
5.5	2.7	1.36	3.7	5
11.0	5.5	1.44	13.2	5
17.0	8.5	1.49	12.8	5
28.0	14.0	1.44	6.9	5
69.0	34.5	1.35	7.8	5
165.0	83.0	0.58	12.4	3

[a] Temperature, 96°C; nitrogen flux, 1 ml s^{-1}; NO transport time, 7.5 min; and reaction time, 12.5 minutes. I_p, Medium value of the peak currenta of 'n' determinations; S_R, relative standard deviation.

4.3. Formation and stability of the electroactive complex

The formation of the electroactive complex $CoSCNNO^+$ as a function of NO carrying time, at 1 ml s^{-1} nitrogen flux, was evaluated. Under the established conditions the peak current increases during the first 10 min and decreases rapidly after 15 min of NO transport (Fig. 4). The decrease in the peak current after 15 min indicates that the concentration of the electroactive complex in the solution decreased, which could be due to instability of the electroative complex in the supporting electrolyte or to the dissociation of the electroactive complex formed in the solution, as a consequence of the continuous passage of the nitrogen gas, carrying NO, throughout the polarographic cell. To study this hypothesis the nitrogen flux was interrupted after 10 min of the beginning of the reduction of nitrate to NO and the peak current intensity monitored as a function of time. An increase in the current intensity during the first 15 min after the interruption of the nitrogen flux was observed, which remained stable for at least 20 more min (Fig. 5). This result indicates that the electroactive complex is stable in the supporting electrolyte and that the decrease in current intensity observed in Fig. 4 could be due to the dissociation of the electroactive complex, with formation of NO which is removed from the polarographic cell by the continuous flux of nitrogen. The increase in current intensity during the first 15 min indicate that the chemical reaction of the complex formation in the supporting electrolyte is rate determining.

In similar conditions for the production of NO from nitrate, Awad and Hassan [22] verified that 5 min is the time required for the quantitative reduction. In order to evaluate the time necessary for carrying the NO from the reduction vessel to

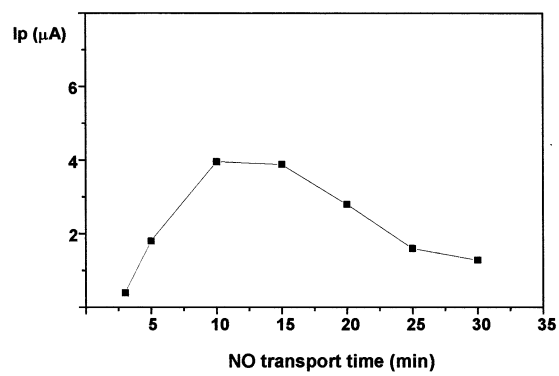

Fig. 4. Formation and stability of the electroactive complex. Reduction system, 1×10^{-5} mol $KNO_3 + 5$ ml of 6 mol l^{-1} HCl + 1 ml of Fe(II) 8% (w/v) + 1 ml of ammonium molibdate 4% (w/v); nitrogen flux, 1 ml s^{-1}; temperature, 96°C. Supporting electrolyte, 2×10^{-3} mol l^{-1} $CoCl_2 + 2 \times 10^{-2}$ mol l^{-1} KSCN + 0.2 mol l^{-1} HCl ($n = 3$). In all of the period the nitrogen gas was only interrupted when recording the polarogramm.

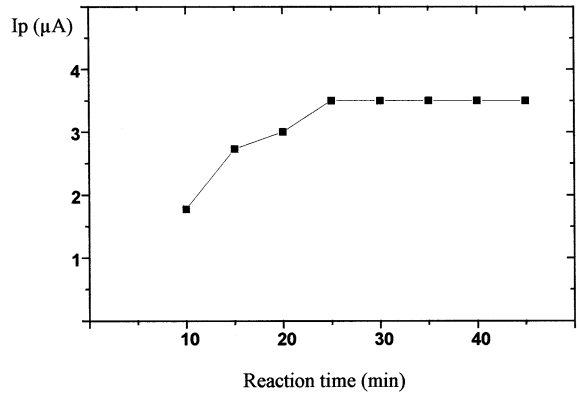

Fig. 5. Formation and stability of the complex $(CoSCNNO)^+$. Reduction system, 1×10^{-5} mol $KNO_3 + 5$ ml of 6 mol l^{-1} HCl + 1 ml of Fe(II) 8% (w/v) + 1 ml of ammonium molibdate 4% (w/v); nitrogen flux, 1 ml s^{-1}; temperature, 96°C. Supporting electrolyte, 2×10^{-3} mol l^{-1} $CoCl_2 + 2 \times 10^{-2}$ mol l^{-1} KSCN + 0.2 mol/l HCl ($n = 3$). The nitrogen flux was interrupted after 10 min at the beginning of the reduction of nitrate.

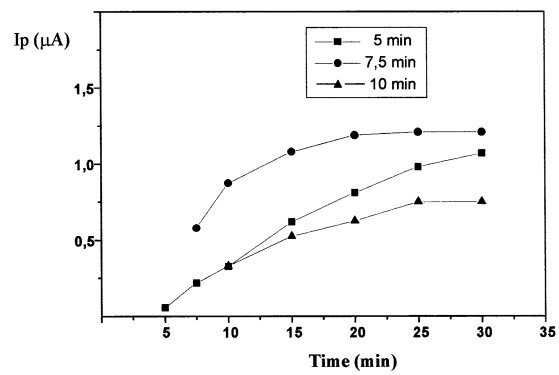

Fig. 6. Stability of the complex $(CoSCNNO)^+$ formed in solution after 5, 7.5 and 10 min of NO transport to the cell under a nitrogen flux of 1 ml s^{-1}. Reduction system, 5×10^{-5} mol KNO_3; 5 ml of 6 mol l^{-1} HCl + 1 ml of Fe(II) 8% (w/v) + 1 ml of ammonium molibdate 4% (w/v); temperature, 96°C. Supporting electrolyte, 2×10^{-3} mol l^{-1} $CoCl_2 + 2 \times 10^{-2}$ mol l^{-1} KSCN + 0.2 mol l^{-1} HCl ($n = 3$).

the polarographic cell NO was carried during 5, 7.5 or 10 min. In all cases the nitrogen flux was interrupted after the established period of time and the formation of the electroactive complex evaluated (Fig. 6). Carrying the NO during 7.5 min with an additional reaction time between of 15 min, for the maximum development of the electroactive complex (without nitrogen flux at the polarographic cell), showed to be the optimum experimental conditions for the determination of nitrate.

A calibration graph was plotted and the peak current intensity was proportional to the nitrate content in the reduction system in the range of $2–12 \times 10^{-6}$ mol. The regression for the calibration graph was $y = 0.501 \times 10^6 x - 0.667$, where y, intensity of peak current in μA and x, mol of nitrate; with a correlation coefficient of 0.9998.

4.4. Validation

The polarographic method developed was validated by comparison with the spectrophotometric method in the quantification of nitrate in several vegetables (broccoli, kale, lettuce, radish, red beet, spinach, turnip and watercress). The mean values of nitrate in the vegetables obtained by the polarographic and spetrophotometric methodologies did not differ significantly ($P < 0.05$). The nitrate content, expressed as sodium nitrate, varied from 751 to 10 806 mg kg^{-1} of fresh vegetable, for the different matrices (Table 3). The recovery of nitrate in the matrices varied from 94.2 to 102.5% and from 85.4 to 107.4% for the spectrophotometric and polarographic method, respectively. The relative standard deviation for the proposed method is lower than 7% and, considering a sample of 5.0 g, the determination limit was 39 mg of nitrate kg^{-1}.

In relation to the spetrophotometric method, the polarographic methodology developed for the determination of nitrate in vegetable allowed a higher number of analyses per time and it is free from the procedures of extraction and clarification. The reduction compartment to generate nitric oxide is easily adapted to the polarographic system and of simple maintenance and, consequently, recommended for routine analysis in the monitoring of nitrate in vegetables.

Acknowledgements

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Table 3
Determination of nitrate in different vegetables by spectrophotometry and polarography[a]

Spectrophotometry					Polarography			
Matrices	Mean value of sodium nitrate[a] (mg kg^{-1})	$ts/(n)^{1/2}$	n	S_R (%)	Mean value of sodium nitrate[a] (mg kg^{-1})	$ts/(n)^{1/2}$	n	S_R (%)
Broccoli	775	12	6	1.5	751	31	4	2.2
Kale	2511	5	6	0.2	2515	88	4	2.2
Lettuce	1945	20	6	1.0	1936	260	4	6.5
Radish	2003	16	6	0.7	2.241	94	5	1.7
Red beet	10233	488	4	3.0	10806	628	4	3.7
Spinach	723	20	6	2.7	762	31	4	2.6
Turnip	2874	11	6	0.3	3.164	233	5	5.9
Watercress	1871	21	6	1.0	1797	50	5	2.3

[a] The results were corrected in function of the recovery. n, Number of determinations; t, t-distribution for a confidence interval of 95%; s, standard deviation; S_R, relative standard deviation.

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