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# Determination of Nitrate in Leafy Vegetables by Flow Injection Analysis with Potentiometric Detection

MUBERRA ANDAC<sup>1\*</sup>, HILAL EREN<sup>1</sup> AND FATİH COLDUR<sup>2</sup>

<sup>1</sup>. Ondokuz Mayıs University, Faculty of Science, Department of Chemistry, Kurupelit-Samsun, Turkey

<sup>2</sup>. Department of Chemistry, Faculty of Science, Erzincan University, Erzincan, Turkey

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## ABSTRACT

A rapid, simple and inexpensive flow injection potentiometric method for the determination of nitrate in leafy vegetables is described. Six commonly consumed fresh leafy vegetables (mint, lettuce, savoy cabbage, stinging nettle, parsley and cress) were obtained from bazaar in Samsun. An all-solid-state miniaturized nitrate-selective electrode was used as a detector in a low-dead-volume flow through cell. Nitrate was determined in the linear range from 0.1 to 100 mmol/L with detection limit of 0.066 mmol/L. The calibration slope is  $52.96 \pm 1.02$  mV/decade in the linear range. The relative standard deviation (RSD) of the electrode response of 100, 10 and 1 mmol/L  $\text{NO}_3^-$  was 0.46, 0.49 and 0.98%, respectively. The flow injection analysis (FIA) throughput was 90 samples/h. The FIA results were compared with those obtained by spectrophotometric method. The results of the study show that nitrate contents in fresh leafy vegetables ranged from 97 to 1861 mg/kg.

Key words: nitrate in vegetables, leafy vegetables, flow injection potentiometry, nitrate-selective electrodes, solid-contact ion-selective electrodes

## INTRODUCTION

The determination of nitrate and nitrite in food, soil and natural waters assumed a vital importance in recent years, because these ions are intimately involved in the overall nitrogen cycle in soil and plants<sup>(1-3)</sup>. Human nitrate intake is mainly from vegetables, water supplies and from additives/preservatives used in meat and food<sup>(4,5)</sup>. It has been reported that, the green leafy vegetables (such as lettuce, spinach, beets, radishes, celery, etc.) are the major vehicles for the entry of nitrate into the human body. Nitrate content of vegetables may range from 1 to 10,000 mg/kg. About 87% of the total nitrate in normal diet is believed to be direct result of vegetable intake<sup>(6-8)</sup>.

Nitrate reduction to nitrite can cause methaemoglobinemia and has a harmful impact on human health due to its reaction with secondary amines to form carcinogenic N-nitrosamines<sup>(9)</sup>. Young babies may suffer from infantile methemoglobinemia due to excessive nitrates in their diet, where nitrite is substituted for oxygen in hemoglobin and

death may occur<sup>(10,11)</sup>. Also, vegetables are important part of babies' diets<sup>(8,12)</sup>. As a result, the determination of nitrate in various samples has been a subject of interest and discussion. Analytical techniques like spectrophotometry, potentiometry, ion chromatography, polarography, capillary electrophoresis as well as high-performance liquid chromatography have been used for quantification of nitrate in vegetables, food, water and other matrices<sup>(13,14)</sup>.

However, most of the developed methods are time consuming, require controlled reaction conditions, inapplicable to turbid and colored samples, suffer from severe interference by many common ions. Some quality-assurance determinations carried out in the food industry are still very complex and laborious; in particular, analysis for nitrate presents several inconveniences such as time consuming, expensive, several sample preparation steps, and requiring permanent trained laboratory assistance. There has been increasing demand for rapid analysis led to the development of automatic method for nitrate determination in various products<sup>(2)</sup>.

Advantages offered by using potentiometric sensors are ease of fabrication, simple monitoring instrumentation,

\* Author for correspondence. Tel : +90-362-3121919;  
Fax: +90-362-3126081; E-mail: mandac@omu.edu.tr

fast response, applicable to turbid or colored solutions, high sensitivity, wide linear dynamic range, low cost and possible interfacing with flow injection systems. The advantage of flow injection potentiometry (FIP) by ion selective electrodes, such as low cost, simple instrumentation, rapid response, high sampling rate, wide linear response and high selectivity have been well recognized over the last two decades<sup>(15-19)</sup>. Most of the potentiometric nitrate sensors described to date incorporate either true-liquid or liquid polymeric membranes consisting of nitrate ion-pairs of quaternary amines, quaternary phosphines and organometallic complexes<sup>(20-23)</sup>. The majority of these sensors are of the traditional bulky tubular design with internal reference electrode and internal reference electrolyte solutions. However, all-solid state PVC membrane potentiometric sensors have been suggested to offer several advantages over the conventional electrodes particularly the small size, simple design, low cost and mass production. These sensors do not incorporate internal reference electrode and filling solution<sup>(24,25)</sup>.

Recently, we have been involved in developing polyvinyl chloride (PVC) membrane nitrate selective electrode based on solid contact technology<sup>(26)</sup>. In the present study, an all-solid-state miniaturized nitrate-selective electrode was used as a detector in a low-dead-volume flow through cell for nitrate quantification. The electrode exhibits good performance characteristics for  $\text{NO}_3^-$ , requires small sample volume and easily accommodated in the flow injection system. The use of the prepared electrode for continuous monitoring of nitrates in samples of leafy vegetables gave results within  $\pm 9\%$  of those obtained by the standard spectrophotometric method.

## MATERIALS AND METHODS

### I. Fresh Plant Materials

Commonly consumed fresh leafy vegetables, mint, lettuce, savoy cabbage, stinging nettle, parsley and cress were obtained from bazaar in Samsun. Each type of vegetable was randomly bought from six different vendors. After sampling each vegetable was rinsed with water to remove soil or wind borne particles that may have been present on the vegetables.

### II. Reagents and Standards

All chemicals were of analytical reagent grade and deionized water was used throughout. Tetrahydrofuran (THF), high molecular weight polyvinyl chloride (PVC), dibutyl phthalate (DBP), tetradodecyl ammonium nitrate (nitrate ionophore) and graphite were purchased from Fluka (Bucks, Switzerland). Epoxy (Macroplast Su 2227) and hardener (Desmodur RFE), used in the preparation of conductive solid contact, were purchased from Henkel (Istanbul, Turkey) and Bayer AG (Darmstadt, Germany), respectively.

A  $1.0 \times 10^{-1}$  mol/L potassium nitrate stock solution was prepared and used for preparation of  $1.0 \times 10^{-2}$  to

$1.0 \times 10^{-6}$  mol/L solutions by serial dilution. A phosphate buffer solution ( $1.0 \times 10^{-1}$  mol/L) of pH 5.5 was prepared by dissolving 13.6 g of potassium monohydrogen phosphate in deionized water. The pH was adjusted to 5.5 using  $1.0 \times 10^{-1}$  mol/L potassium dihydrogen phosphate and the solution was made up to 1 L.  $0.025$  mol/L  $\text{Al}_2(\text{SO}_4)_3$  was prepared by dissolving aluminium sulfate in deionized water.

### III. Preparation of the Selective Membrane

A membrane cocktail consisting of 4% (w/w) nitrate ionophore, 30% (w/w) polyvinyl chloride (PVC), 66% (w/w) dibutyl phthalate (DBP) was prepared in 3 mL THF and thoroughly mixed to obtain a homogeneous transparent mixture.

### IV. Construction of Flow-Through Nitrate Sensor

The flow-through all-solid-state nitrate sensor was constructed as the similar procedure described in our previous works<sup>(24,25)</sup>. The electrode was prepared at two steps. At the first step, a mixture of conductive material consisting of 50% (w/w) graphite, 35% (w/w) epoxy and 15% (w/w) hardener was prepared by mixing in sufficient THF. This conductive material were filled into a 5 mm copper pipe with a diameter of 4 mm and kept at 30°C overnight. Then, an electric cable was soldered to the copper pipe for the electrical connection and a hole of 0.1 mm radius was opened in the centre of the conductive material. At the second step, the hole was covered with selective membrane cocktail. The coated surface was left to dry at laboratory overnight. The flow-through electrode was conditioned by soaking into 100 mmol/L  $\text{KNO}_3$  solution for at least 8 h before use. Then the electrode combined with a miniaturized salt bridge was directly attached to the end of the flow-line consisting PVC tubing (20 cm length and 0.1 mm i.d.) to minimize possible dilution of the sample in the carrier solution. When not in use, sensors were stored in the laboratory.

### V. Apparatus and FIA System

Flow injection potentiometric measurements were carried out at room temperature ( $20 \pm 2^\circ\text{C}$ ) using a laboratory-made computer-controlled high-input impedance multi-channel potentiometric measurement system with an all solid state nitrate sensor in conjunction with a saturated calomel electrode (Gamry). The potentiometric system was controlled by home-made software program. Figure 1 indicates the schematic diagram of the FIA system. The home-made flow cell was used. The FIA system consists of a carrier solution ( $1.0 \times 10^{-2}$  mol/L  $\text{CuSO}_4$  and  $5.0 \times 10^{-3}$  mol/L phosphate buffer of pH 5.5) propelled by means of a P680 HPLC pump (Dionex), through PVC tubing (20 cm length and 0.1 mm i.d.) at a flow rate of 1.0 mL/min. Samples and standards were injected into the carrier solution using a two-position injection unit (Vici) fitted with 20  $\mu\text{L}$  sample loop. At least three signals for each sample were recorded and their average potential value at the maximum height was

measured. A calibration graph (signal potential versus  $\log [\text{NO}_3^-]$ ) was obtained using  $1.0 \times 10^{-6}$  to  $1.0 \times 10^{-1}$  mol/L of standard nitrate solutions.

Spectrophotometric measurements were made with Shimadzu spectrophotometer (model UV-1601) using 1.00 cm quartz cuvettes.

The pH of the buffer solutions was adjusted by using a glass pH electrode (Schott) with a Jenway 3040 model Ion Analyzer. Solutions at required concentrations were homogenized using Ultrasonic LC30 (Germany) stirrer.

## VI. Extraction and Analysis

Baker and Thompson method<sup>(27)</sup> with minor modifications was used for the extraction of nitrate. Homogenized sample (10 g) was accurately weighed and blended for 5 min with 20 mL of water. Then 30 mL of 0.025 M  $\text{Al}_2(\text{SO}_4)_3$  was added and shaken for 15 min. The solution was filtered through a 0.45  $\mu\text{m}$  membrane filter and collected in an erlenmeyer flask. The solutions were kept in refrigerator (4°C) until analysis.

A 20  $\mu\text{L}$  aliquot of the filtered solutions was injected, in triplicates, in the flow system. The potential was recorded and compared with the calibration graph.

## VII. Measurement of Sensor Selectivity

Potentiometric selectivity coefficients of the PVC membrane sensor were evaluated using the separate solutions method<sup>(28)</sup>. The  $\log C$  versus  $E$  relations of the sensor for  $\text{NO}_3^-$  and interfering ions were obtained independently. Then the concentrations that correspond to the same potential value were used to determine the  $K_{A,B}^{\text{Pot}}$  value according to the equation:

$$K_{A,B}^{\text{Pot}} = C_A / (C_B)^{Z_A/Z_B}$$

where  $C_A$  and  $C_B$  are concentrations, and,  $Z_A/Z_B$  are charges of nitrate and interferent ions, respectively.

## RESULTS AND DISCUSSION

### I. Potentiometric Performance of the Sensor and Optimization of the FIA Method

The constructed solid state membrane sensor based on tetradodecyl ammonium nitrate salt was successfully used as a suitable indicator electrode in the flow injection system shown in Figure 1. In order to achieve the best FIP response, several flow injection parameters including tubing length, flow rate, sample volume, composition of carrier solution and sampling rate were thoroughly investigated. The length of tubing from injection valve port to cell was made as small as practical to minimize dispersion and dilution. Thus 10 cm was selected for tubing length with respect peak heights. The dependency of the peak heights and peak width

with flow rate was studied using the electrode response to a  $1.0 \times 10^{-3}$  mol/L solution of nitrate ion. As the flow rate increased, the peaks became narrower and increased in height to near plateau at a flow rate of 1 mL/min. As can be seen in Figure 2, at flow-rates above the 1.0 mL/min, no significant decreases were seen in peak widths. Thus, a flow rate of 1 mL/min was selected as an optimum value for further studies. The high flow rate increases sampling rate and decreases the total time of analysis. In general, the peak height increased with the increasing sample volume, although the effect was less marked at higher concentrations. In the work, different sample volumes from 20 to 100  $\mu\text{L}$  were studied; the peak height reached nearly steady-state at 20  $\mu\text{L}$  injected. Thus, this sample volume was selected as an optimum amount.

It is well-known that, in FIP, the composition of the carrier solution also affects the response behavior of ion-selective electrode in terms of the base line stability<sup>(17,29)</sup>. In the proposed flow system, a  $1.0 \times 10^{-2}$  mol/L  $\text{CuSO}_4$  and  $5.0 \times 10^{-3}$  mol/L phosphate buffer of pH 5.5 was used as carrier, which resulted in stable base line, when samples were injected in the concentration range of  $1.0 \times 10^{-5}$  to  $1.0 \times 10^{-1}$  mol/L.

In the analytical flow system, the sampling rate (sample throughput) is important. The proposed potentiometric FIA system revealed sampling rates of 90 injections per hour.

The peak heights recorded under the optimized conditions demonstrate fast response, high sensitivity, and good

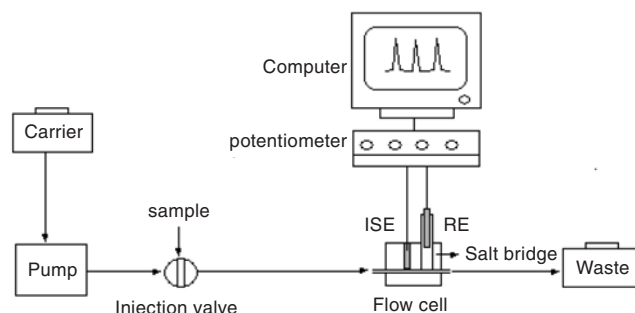


Figure 1. Schematic representation of the potentiometric FIA system.

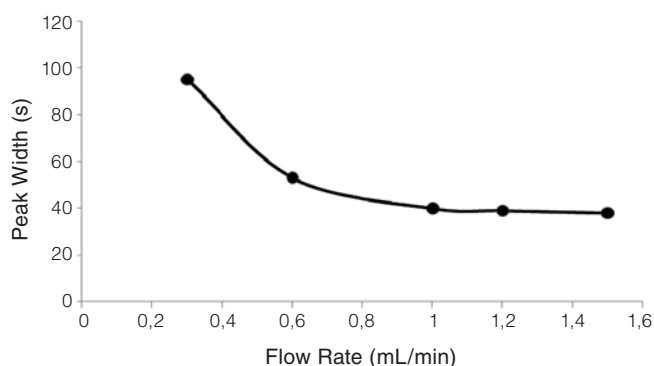
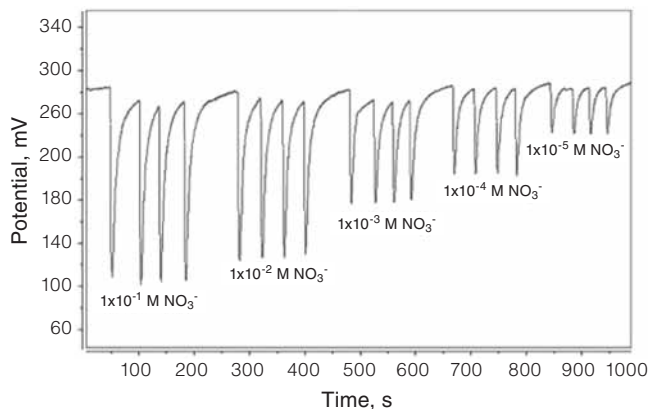


Figure 2. Peak widths for  $1 \times 10^{-3}$  M  $\text{NO}_3^-$  solutions at different flow rates.

stability. Figure 3 shows the triplicate peaks from the proposed FIP system obtained under the optimal experimental conditions for varying concentrations of  $\text{NO}_3^-$  solutions in the range of  $1.0 \times 10^{-5}$  to  $1.0 \times 10^{-1}$  mol/L. The average potential response was plotted versus concentration. Linear regression relation between potential response and nitrate concentration is;



**Figure 3.** Peaks with the injection of  $\text{NO}_3^-$  standard solutions: (1)  $10^{-1}$  mol/L, (2)  $10^{-2}$  mol/L, (3)  $10^{-3}$  mol/L, (4)  $10^{-4}$  mol/L, (5)  $10^{-5}$  mol/L.

**Table 1.** Potentiometric selectivity coefficients of the  $\text{NO}_3^-$  selective electrode calculated according to SSM in FIP modes

Interfering Ion	Log $k^{\text{pot}}_{\text{NO}_3, \text{A}}$
$\text{Br}^-$	-2.29
$\text{SCN}^-$	0.20
$\text{NO}_2^-$	-2.24
$\text{I}^-$	-0.94
$\text{PO}_4^{3-}$	-2.73
$\text{Cl}^-$	-3.18
$\text{F}^-$	-3.59
$\text{SO}_4^{2-}$	-3.47
$\text{CO}_3^{2-}$	-3.08

$$\Delta E = (230.63 \pm 2.96) - (52.96 \pm 1.02) \log [\text{NO}_3^-]$$

( $n = 5$ , correlation coefficient ( $r^2$ ) = 0.9967)

The electrode showed non Nernstian behavior with a slope of 52.96 mV in the range of linear response. The relative standard deviations (RSD) of the electrode responses of 100, 10 and 1 mmol/L  $\text{NO}_3^-$  were 0.46, 0.49 and 0.98%, respectively. Limit of detection (LOD) and limit of quantification (LOQ) of the electrode were calculated as 0.066 mmol/L and 0.18 mmol/L respectively.

The selectivity coefficients of the  $\text{NO}_3^-$  selective electrode obtained in the FIP mode are listed in Table 1. The separate solutions method was used with a  $1.0 \times 10^{-2}$  mol/L of both nitrate and diverse ions. Sensor selectivity coefficients for carbonate, sulfate, fluoride and chloride ions are of the order  $10^{-3}$  indicating negligible effect due to these ions. Bromide, nitrite and phosphate have influence on the sensor and exhibit selectivity coefficient values of the range  $10^{-2}$  to  $10^{-3}$ . Thiocyanate and iodide, however, have more influence on the sensor and exhibit selectivity coefficient values of the range  $10^{-1}$  to  $10^{-2}$ . The selectivity coefficients are dependent on the diffusion rate and exchange reaction of the interferents.

## II. Determination of Nitrate in Leafy Vegetables

Nitrate contents in six commonly consumed fresh vegetables, produced in Samsun, were determined using developed FIA method. AOAC spectrophotometric method 993.03 was used as the reference method. In this method, nitrate was reduced to nitrite using spongy cadmium then nitrite reacted with Griess reagent to produce a dye that was detected at 525 nm<sup>(30)</sup>.

The data obtained from both methods (for  $N=3$ , at 95% confidence level) are shown in Table 2. With the use of the FIP, the nitrate content in the vegetables obtained from triplicate measurements was found to be in satisfactory agreement with spectrophotometry. The results obtained (Table 2) by both methods are within  $\pm 8.9\%$  difference. The calculated one-tailed critical  $t_c$  values for precision test were found to be in the range of 2.13 - 2.91 which are lower than the critical

**Table 2.** Nitrate content (mg/kg) in leafy vegetables

Sample	Potentiometry <sup>a</sup>	Spectrophotometry <sup>a</sup>	$E_r^b$	$t$ value <sup>c</sup>	$R^d$
Mint	1861.47 $\pm$ 14.68	1891.56 $\pm$ 20.85	-1.59	2.35	
Lettuce	203.08 $\pm$ 10.93	221.54 $\pm$ 15.96	-8.33	2.91	101.3
Savoy cabbage	867.09 $\pm$ 20.07	894.94 $\pm$ 23.61	-3.11	2.13	
Stinging nettle	184.12 $\pm$ 9.91	197.53 $\pm$ 15.04	-6.79	2.35	101.8
Parsley	96.72 $\pm$ 8.59	89.23 $\pm$ 10.15	+8.39	2.13	102.4
Cress	728.67 $\pm$ 7.25	743.25 $\pm$ 11.32	-1.96	2.35	

Five samples ( $n = 5$ ) for each type of vegetable were analyzed in triplicate.

<sup>a</sup>Average of three determinations  $\pm$  SD.

<sup>b</sup>Relative error for the potentiometry versus spectrophotometry.

<sup>c</sup>One-tailed critical  $t$  value for precision comparison of mean.

<sup>d</sup>Mean spike recovery (%).



value ( $t_c(0.05) = 3.18$  at 95% confidence level). This means that the proposed method is of comparable precision to the spectrophotometry.

The precision of the method was evaluated by the analysis of ten replicates of sample. The relative standard deviation ( $n = 10$ ) was 0.92% for a sample containing 62 mg/kg nitrate. Standard nitrate additions were used to evaluate the accuracy of the method and the average percentage of spike recovery, for three different additions, was calculated. The results obtained (Table 2) are within the acceptance range. The accuracy of the method was 101.8%.

The nitrate content of the six leafy vegetables ranged from 97 to 1861 mg/kg. Mint had the highest nitrate content within the studied vegetables followed by cress, savoy cabbage, lettuce and stinging nettle. The nitrate values reported are lower than those published in literature<sup>(31,32)</sup>. There was no data available on savoy cabbage. The FIA methodology described affords an accurate, rapid and precise measurement of nitrate in vegetables.

## CONCLUSIONS

The use of the potentiometric sensor as a detector in a low-dead-volume flow through cell enables measurements of nitrate in leafy vegetables was demonstrated. A comparison of the proposed method for the determination of nitrate with other FIA procedures<sup>(13,14)</sup> shows that proposed FIA procedure has higher sample throughput. The system is reasonably cost effective providing a good sample frequency of 90 injections per hour. Also it is a simple and inexpensive method. Successful application of the FIP methodology for nitrate determination in leafy vegetables was demonstrated, as an alternative to the conventional laborious and time consuming classical procedures. The FIA system presents a high degree of automation and substantial reduction of reagent consumption and waste disposal.

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