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# Analysis of Nitrate Ion in Nettle (*Urtica dioica* L.) by Ion-Pair Chromatographic Method on a $C_{30}$ Stationary Phase

ZOLTAN SZABO,† KATALIN BÖDDI,‡ LASZLO MARK,† LASZLO G. SZABO,‡ AND ROBERT OHMACHT*,†

Department of Biochemistry and Medical Chemistry, Faculty of Medicine, and Department of Botany, Faculty of Science, University of Pecs, H-7624 Pecs, Hungary

Nitrate ion is a frequent pollutant not only in soil and natural water resources but in vegetables and foods as well. In our study we focused on nettle due to its increased ability to accumulate nitrate ions. A new, simple method for the separation and determination of nitrate ion based on reversed-phase ion-pair chromatography has been elaborated. A new four-step sample pretreatment method enables the precipitation of proteins and oxidative degradation of compounds that may disturb the identification of the nitrate ion: (1) extraction of the total nitrate content, (2) precipitation of proteins with acetonitrile, (3) oxidative degradation of the organic contaminants with $H_2O_2$, (4) evaporation of the solvent and taking up of the residue in water. The chromatographic separations were carried out on a high-density $C_{30}$ stationary phase under isocratic conditions. The optimal mobile-phase composition was 10% (v/v) acetonitrile and 90% (v/v) 20 mmol $L^{-1}$ phosphate buffer, containing 2 mmol of tetrabutylammonium hydroxide at pH 6.0. The method could also be used for the separation of $IO_3^-$, $SeO_3^{2-}$, $BrO_3^-$, $NO_2^-$, $Br^-$, $SeO_4^{2-}$, and $I^-$ ions. The validated method is sensitive (the detection limit is 0.18 ng of nitrate ion). The method is linear in a high concentration range (0.031−30.66 $\mu$g $mL^{-1}$). Recoveries varied between 98% and 103%. Reproducibility of the elaborated sample pretreatment method showed 1.54%. The method can be used for the determination of nitrate ion from different plants.

**KEYWORDS: Nitrate ion; nettle (*Urtica dioica* L.); reversed-phase ion-pair (RP-IP) chromatography, $C_{30}$ stationary phase**

## INTRODUCTION

Nettle (*Urtica dioica* L.) is a widespread plant frequently suggested for use in the treatment of rheumatism, arthritis, and inflammatory diseases. Several papers have recently been published concerning the antioxidant, antimicrobial, antiulcer, and analgesic properties of nettle (*1*−*7*). Nettle is frequently used in traditional medicine in several countries.

Nettle is mostly used in the form of a watery extract ("tea"). Nettle is able to accumulate and store nitrate ions from the environment in vacuoles, mostly from the soil (*3*). Nitrate belongs to the most frequent contaminants; according to the European Community the permitted maximum concentration of nitrate is 50 mg in 1 L of water. The high intake of nitrate in an organism can be a source of different carcinogenic and mutagenic dysfunctions (*1*). Methemoglobinemia is the direct consequence of the reduction of nitrate to nitrosamines generated by bacteria. (In methemoglobinemia, the iron content of hemoglobin is oxidized from II to III and the resulting methemoglobin is not able to carry oxygen any longer.)

Nitrate in plants is generally determined by the standard diazotization procedure. This method is generally applied after an enzymatic reduction of nitrate to nitrite as was first described by Lowe and Hamilton (*8*, *10*). Woolley et al. developed the so-called powder method (*9*) that can be regarded as a modification of the previous diazotization method. In this method zinc powder is used to reduce nitrate to nitrite quantitatively. Beda and Miranda elaborated a more selective method (*11*, *12*) which was capable of identifying nitrate and nitrite together without being separated. In these methods, the Griess reaction is applied and nitrate ions are reduced by vanadium(III) chloride. More recent applications enable the rapid measurement of nitrate ion by electrophoretic methods (*13*−*15*). Capillary electrophoresis can be a useful tool for the separation and determination of nitrate ion from different biological samples especially when isotachophoresis is used for effective sample stacking. This isotachophoretic "sample pretreatment" is followed by capillary zone electrophoresis to separate and determine anions (*16*−*19*).

Usually octadecyl phases ($C_{18}$) are used for the separation of ionic solutes by reversed-phase ion-pair chromatography. The use of a silica-based stationary phase with high hydrophobicity (longer than the usual alkyl chains and high surface coverage) results in increased adsorption of the solutes. Hiraki and co-

* To whom correspondence should be addressed. Phone: +36 72 536 223. Fax: +36 72 536 226. E-mail: robert.ohmacht@aok.pte.hu.
† Faculty of Medicine.
‡ Faculty of Science.

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workers made a successful attempt to separate some anions (including nitrate ion) by using a $C_{30}$ stationary phase (Develosil C30, YMC, Japan) (*20*). Recently Szabo and Ohmacht developed a new $C_{30}$ phase with very high surface coverage (*21*). In the present work this phase was used for the determination of nitrate ion from a plant extract. The advantages of $C_{30}$ phases in comparison with $C_{18}$ phases are the higher hydrophobicity and higher loading capacity. Therefore, a lower ion-pairing reagent concentration can be applied to achieve a given retention and a prolonged column lifetime can be observed.

In the present paper we suggest a new, simple method for the separation and determination of nitrate ion based on reversed-phase ion-pair chromatography with UV−vis detection.

## MATERIALS AND METHODS

**Plant Material**. The plant material (nettle, *U. dioica* sp.) used for the validation of our method was harvested on a nitrogen-poor soil and dried at room temperature. The air-dried leaves of each individual plant were removed and homogenized in a blend mixer.

**Instrumentation**. The measurements were carried out on a Dionex Summit 3000 LC system consisting of a P 580 NDG precision pump, a 340S diode array detector, and Chromeleon chromatography management software, version 4.12. (Dionex, Idstein, Germany). Samples were injected with a Rheodyne 8125 sample injector valve equipped with a 20 $\mu$L sample loop (Cotati, CA). A densely loaded $C_{30}$ stationary phase was synthesized in the laboratory on 5 $\mu$m Kovasil silica (Chemie Uetikon, Uetikon, Switzerland) and packed into 150 × 4.6 mm stainless steel columns. This new $C_{30}$ phase has been described in ref *21*.

**Chemicals**. Tetrabutylammonium hydroxide and potassium dihydrogen phosphate (analytical grade) were purchased from Fluka (Buchs, Switzerland). Chromatography-grade acetonitrile and Disodium hydrogen phosphate dihydrate were obtained from Merck (Darmstadt, Germany). The water used for the preparation of the mobile phase was bidistilled in our laboratory.

**Preparation of the Mobile Phase**. The phosphate buffer was prepared from $Na_2HPO_4$ and $KH_2PO_4$. After addition of the calculated amount of tetrabutylammonium hydroxide to the phosphate buffer, the pH of the solution was adjusted with phosphoric acid. The prepared solution was filtered through a 0.2 $\mu$m filter (Whatman, Maidstone, England). Acetonitrile was always freshly mixed with the buffer solution.

**Sample Pretreatment of Plant Material**. The sample pretreatment procedure consists of four steps: (1) treatment of the homogenized nettle leaf with boiling water for 15 min and filtering the mixture, (2) precipitation of the proteins with acetonitrile, (3) oxidative degradation of the organic contaminants with $H_2O_2$, (4) vacuum evaporation of the solvent. After evaporation the residue was taken up in bidistilled water and injected.

A 1.50 g sample of grounded nettle leaf was thoroughly mixed with 50 mL of bidistilled water, and the mixture was refluxed for 15 min. (In contrast to the literature (*9*, *10*) we recognized that incubation at 60 °C for 10 min is not enough for the extraction of the entire amount of the accumulated nitrate from the plant material.) The mixture was poured into a centrifuge tube, and the reflux flask was rinsed with distilled water (15 mL) two times. Then the mixture was centrifuged at 6000*g* for 30 min. The supernatant ("nettle tea") was removed, and the precipitate was resuspended twice with 30 mL of bidistilled water and centrifuged. The supernatants were collected (ca. 140 mL). The volume of the collected supernatants was filled up to 150 mL, and 0.5 mL was slowly poured into 4 mL of acetonitrile. Proteins present in the extract were precipitated, and the mixture was centrifuged at 6000*g* for 10 min. The supernatant was removed, and the precipitate was resuspended again in 4 mL of acetonitrile and centrifuged. The two supernatants were collected, and the solvent was evaporated. The residue was dissolved in 2 mL of bidistilled water, and 50 $\mu$L of 30% $H_2O_2$ solution was added to this solution to oxidize pyrogenic compounds which might disturb chromatographic separation. The $H_2O_2$-containing solution was incubated in boiling water for 30 min, and then it was evaporated to dryness. Finally, the residue was dissolved in 3 mL of bidistilled water and analyzed.

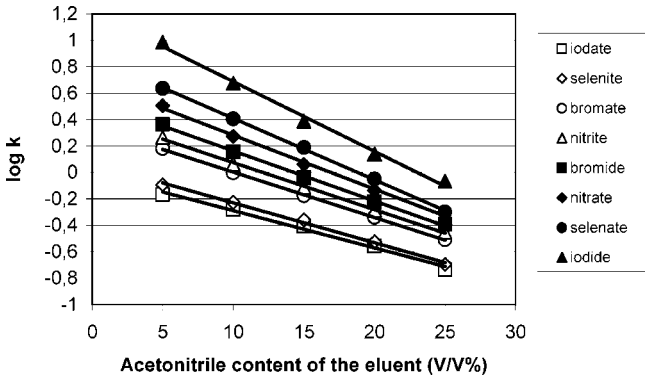


**Figure 1.** Logarithm of the capacity factor of the anions influenced by the amount of organic modifier. Mobile phase: 90% buffer (20 mmol $L^{-1}$, pH 6.0), 2 mmol $L^{-1}$ tetrabutylammonium hydroxide.

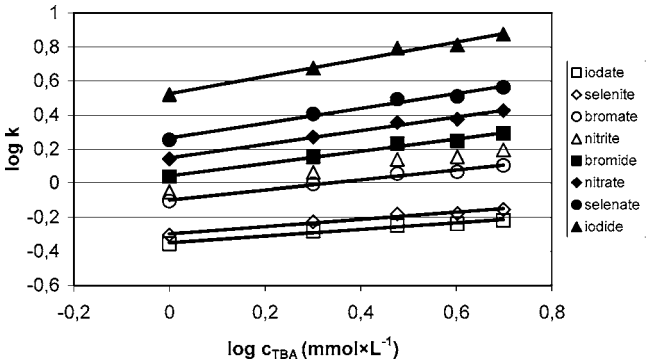


**Figure 2.** Influence of the concentration of the ion-pairing reagent on the retention of the anions. Mobile phase: 10% (v/v) acetonitrile, 90% buffer (20 mmol $L^{-1}$, pH 6.0).

## RESULTS AND DISCUSSION

**Optimization of the Mobile-Phase Composition**. An isocratic separation has been elaborated for eight common anions, including nitrate ion ($IO_3^-$, $SeO_3^{2-}$, $BrO_3^-$, $NO_2^-$, $Br^-$, $NO_3^-$, $SeO_4^{2-}$, $I^-$). The effect of the eluent composition, concentration of the ion-pairing reagent, pH of the mobile phase, and buffer concentration has been investigated. (Although in this paper we discuss the determination of nitrate ion, the determinations of other anions from plant materials are in progress.)

A 10% (v/v) organic modifier (acetonitrile, AcCN) in the mobile phase resulted in good separation and acceptable capacity factors for all anions. A lower amount of acetonitrile does not improve the selectivity of the separation, but the capacity factors—especially those of selenate and iodide ions—remarkably increase (**Figure 1**).

The high hydrophobicity of our stationary phase enabled us to use a low concentration of ion-pairing reagent. The relationship between the logarithm of the capacity factor and the logarithm of the ion-pairing reagent concentration ($c_{IP}$) has been found to be linear and parallel for the investigated anions (**Figure 2**). At a higher ion-pairing reagent concentration the electrostatic surface potential increases, resulting in increased retention of the solutes (*22*).

A remarkable change of the selectivity between the multiply charged selenate ion and singly charged ions was observed depending on the buffer concentration. The increase of the capacity factor of the selenate ion as a function of buffer concentration is more expressed than the change of the capacity factor of singly charged anions (**Figure 3**). An increasing eluent salt concentration increases the shielding of the surface charges and therefore lowers the electrostatic surface potential. Accord-

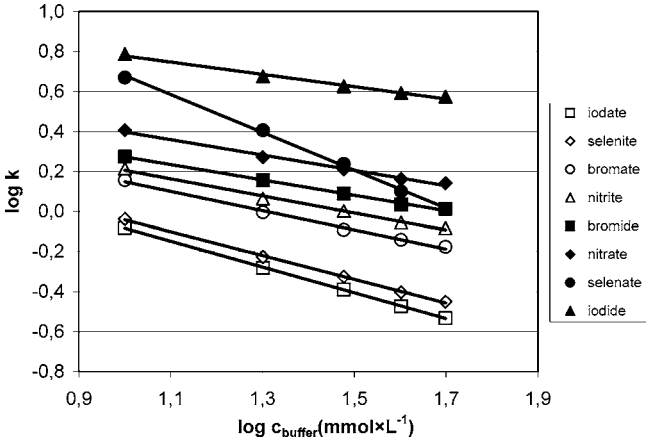


**Figure 3.** Effect of the change of the buffer concentration on the retention. Mobile phase: 10% (v/v) acetonitrile, 90% buffer (2 mmol $L^{-1}$, pH 6.0).

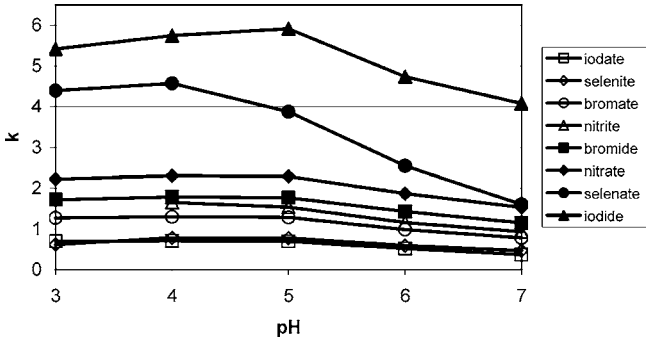


**Figure 4.** Influence of the pH on the retention of the anions. Mobile phase: 10% (v/v) acetonitrile, 90% phosphate buffer (20 mmol $L^{-1}$), 2 mmol $L^{-1}$ tetrabutylammonium hydroxide.

ingly, the retention of anions will be lower with increasing salt concentration (*22*).

The pH of the mobile phase also has a significant effect on the retention of our investigated anions. At higher pH values the retention of the anions is lower than under acidic conditions (**Figure 4**). Variation of the eluent pH can introduce large changes in the degree of ionization of both solutes and ion-pairing reagent. Consequently, the capacity factor of the solutes varies in a wide range. In our case the typical effect is the decrease of the retention with increasing pH, due to the decrease of the ionized fraction of ion-pairing reagent (*22*).

Optimal separation has been achieved using 10% (v/v) acetonitrile, 20 mmol $L^{-1}$ phosphate buffer at pH 6.0, and 2.0 mmol $L^{-1}$ tetrabutylammonium hydroxide as the mobile phase (**Figure 5**). At this setting, the overall retention time is reasonable and a satisfactory separation of the low retention solutes can be achieved.

The results show that the optimized parameters of the mobile phase including the pH and concentration of the buffer, ion-pairing reagent, and organic compound of the eluent are similar to those obtained by ref *20*.

**Sample Pretreatment**. The crude extract obtained from nettle after the treatment with boiling water contains several compounds among other proteins that can disturb the identification of nitrate ion (**Figure 6**). These compounds not only coelute with nitrate but reduce the lifetime of the column. After the precipitation of proteins with acetonitrile the peak of nitrate ion on the chromatogram becomes narrower, which indicates the successful removal of some disturbing compounds, mostly proteins (**Figure 7**). In the final step (oxidative degradation of pyrogens with $H_2O_2$), the quantitative analysis could be well accomplished as is demonstrated in **Figure 8**.

**Validation of the Nitrate Determination**. **Limit of Detection (LOD), Limit of Quantification (LOQ), and Linearity**. The detection limit of the new method (defined as the amount of nitrate ion giving a peak height 3 times higher than the noise level) was low: 2.9 pmol (0.18 ng) of nitrate ion. The quantification limit of the method (defined as the amount of nitrate ion giving a peak height 5 times higher than the noise level) was low: 4.8 pmol (0.30 ng) of nitrate ion. As a result of the effective sample cleanup procedure an interference of accompanying substances with the analyte was not observed.

Calibration was carried out in the concentration range of 0.031−30.66 $\mu$g $mL^{-1}$ nitrate ion. The relation between the peak height and concentrations in 4 orders of magnitude was linear with a regression coefficient ($R^2$) of 0.9999. The slope and *y*-intercept of the linear regression line for the peak area versus concentration plot are 1.052 and 0.0541, respectively. All injections were repeated three times.

**Stationary-Phase Effect**. It has been found that the new type of stationary phase (highly loaded $C_{30}$ phase on 15 nm pore size silica) has some advantages compared to commercial $C_{30}$ packings. Due to the high separation capacity of the phase a low separation time (8 min) for the separation of the eight anions has been achieved.

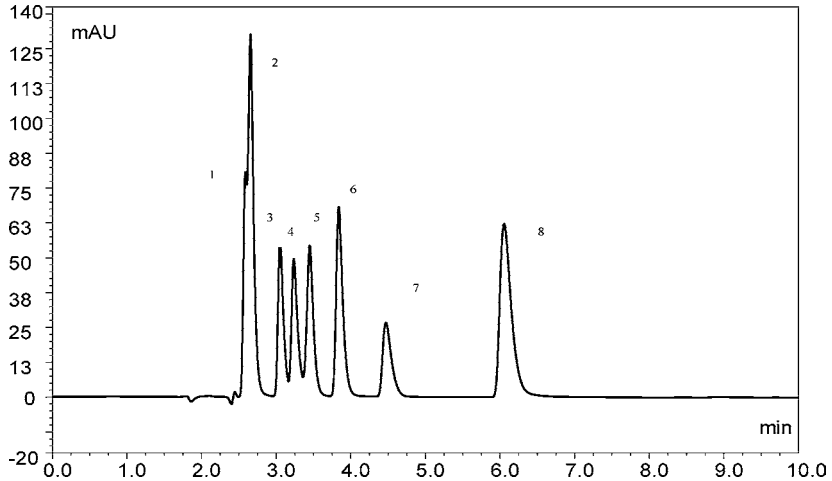


**Figure 5.** Separation of anions. Stationary phase: 5 $\mu$m $C_{30}$ RP packing; column dimensions 150 × 4.6 mm. Mobile phase: 10% (v/v) acetonitrile, 90% phosphate buffer (20 mmol $L^{-1}$, pH 6.0), 2 mmol $L^{-1}$ tetrabutylammonium hydroxide. Key: 1, $IO_3^-$; 2, $SeO_3^{2-}$; 3, $BrO_3^-$; 4, $NO_2^-$; 5, $Br^-$; 6, $NO_3^-$; 7, $SeO_4^{2-}$; 8, $I^-$. Detection at 210 nm. Flow rate: 1.2 mL $min^{-1}$.

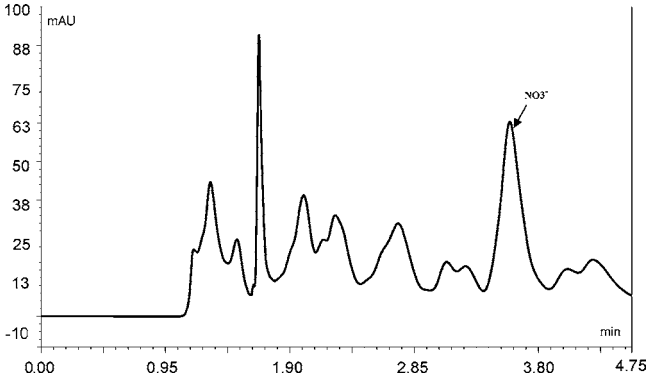


**Figure 6.** Separation of a crude nettle leaf extract. For experimental conditions see **Figure 5**.

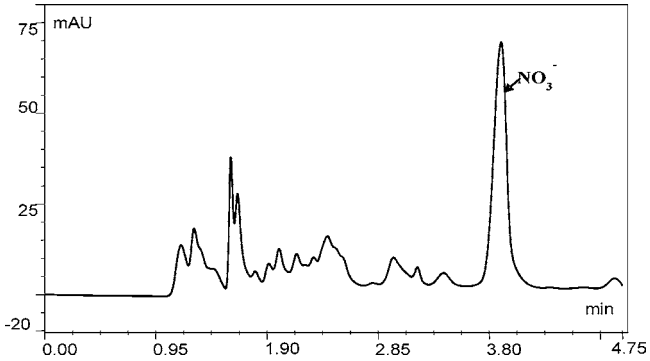


**Figure 7.** Separation of an extract after precipitation of proteins. For experimental conditions see **Figure 5**.

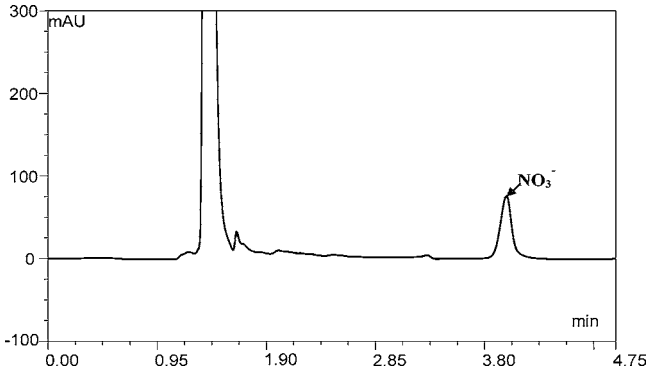


**Figure 8.** Final determination of nitrate from nettle leaf extract after precipitation and oxidative degradation of impurities. For experimental conditions see **Figure 5**.

**Reproducibility of the Method**. The reproducibility of the method has been controlled in two different processes each by a 5-fold assay: (process 1) the complete procedure was repeated five times; (process 2) the cleanup procedure from one leaf extract was repeated five times. The relative standard deviation proved the accuracy and reproducibility to be very satisfactory. It also demonstrated the importance of careful laboratory work at the extraction step (treatment of the nettle leaf with boiling water). The reproducibility of retention times was also satisfactory (**Table 1**).

**Recovery**. The recovery of the method was controlled by the "addition method". Instead of water the extraction was carried out with $KNO_3$ solutions at three different concentrations. At each concentration the extractions were 5-fold repeated. A total of 98−103% of the added amount of nitrate has been recuperated. The standard deviation (%) was never higher than 1.9% at all applied concentrations (**Table 2**). According to the results our new analytical method enables processing of our sample well without loss of nitrate.

**Table 1.** Reproducibility of the Method for Nitrate Ion Determination[a]

| | amt (mg) of $NO_3^-$ in 1 g of nettle leaf | rel std dev (%) | av retention time (min) | rel std dev (%) |
|---|---|---|---|---|
| process 1 | 2.59 | 1.54 | 3.90 | 1.00 |
| process 2 | 2.56 | 0.97 | 3.89 | 0.89 |

[a] Data are means from five determinations each.

**Table 2.** Recovery of the Method[a]

| | recovery (%) | rel std dev (%) |
|---|---|---|
| 6 $\mu$g/mL | 98.04 | 1.32 |
| 30 $\mu$g/mL | 103.34 | 1.90 |
| 150 $\mu$g/mL | 103.40 | 1.89 |

[a] Data are means from five determinations each.

**Table 3.** Nitrate Content of Commercial Nettle Tea[a]

| | amt (mg) of $NO_3^-$ in 1 g of nettle leaf | | amt (mg) of $NO_3^-$ in 1 g nettle leaf |
|---|---|---|---|
| bio nettle leaf (Melius) | 3.6 | nettle leaf (Herbaria) | 7.9 |
| nettle leaf (Melius) | 4.7 | nettle leaf (Naturland) | 7.2 |

[a] Data are means from five determinations each.

**Determination of the Nitrate Content of Commercial Nettle Teas**. The presented results (**Table 3**) demonstrate that the "bio nettle tea" contains a significantly lower amount of nitrate than other commercially available nettle teas. It became obvious that all commercial teas (inclusive "bio" product) contain more nitrate than the nettle we used for the elaboration of the new analysis method (this nettle was grown on a special nitrate-poor soil).

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