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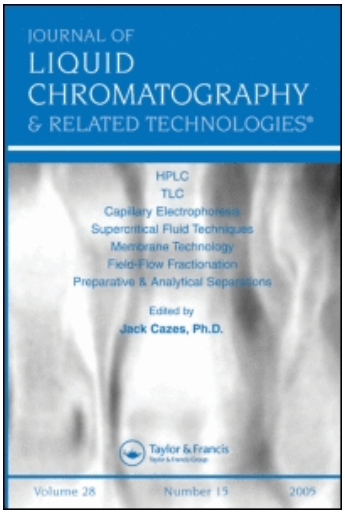

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DETERMINATION OF NITRATE, NITRITE, AND PHOSPHATE AT 214 NM BY REVERSE PHASE HPLC

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ABSTRACT

Nitrate and nitrite can be determined by reverse phase HPLC, using 1:1 methanol-water at pH 3.0 as the mobile phase and UV detection at 214 nm. Phosphate can be determined using 1:1:1 methanol-water-isopropyl alcohol at pH 3.0. At a flow rate of 1.0 mL/min, these anions elute within 5 min. A comparison of the mobile phases 1:1 methanol-water and Low UV PIC A reagent, indicates that 1:1 methanol-water yields 10 fold greater sensitivity to nitrate and 2 fold less to nitrite. The use of 1:1 methanol-water for the extraction of nitrate and nitrite from soil results in 19% higher recovery than in the case of water alone.

INTRODUCTION

The importance of determining nitrate and nitrite levels in agricultural soils is illustrated by the numerous methods of analysis for these ions (1). A technique not commonly employed for soil nitrate and nitrite analysis is high performance liquid chromatography (HPLC).

Ion chromatography HPLC for the analysis of inorganic ions was first reported by Small et al. (2). Both an analytical and a suppressor column

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were used and the concentrations of the ions were measured with a conductivity detector. Ion chromatography coupled with a conductivity detector has been used by numerous investigators (3,4,5). Typically, nitrate is eluted within 5 to 6 min, with a detection limit of 0.03-0.1 mg/L. Haddad and Heckenberg (6) used a low-capacity ion exchange column with an indirect refractive index detector. Retention times of 4.58 min for nitrite and 5.63 min for nitrate were obtained with detection limits of 0.08 and 0.12 mg/L.

Recently, reverse phase column packings have been used for the separation of inorganic ions. Molnar et al. (7) used a LiChrosorb RP-18 column with a mobile phase of 0.002 M tetrabutylammonium hydroxide plus 0.05 M phosphate buffer (pH 6.7) to separate various anions. For the separation of nitrate and nitrite in water samples, Kok et al. (8) used a Radial-Pak C_{18} uBondapak column, with PIC A UV grade reagent as the mobile phase. Vilsmeier (9) used 1 mM tetrabutylammonium hydrogen sulfate and 25 mM NaH_2PO_4 (pH 6.7) to separate nitrate, nitrite, and dicyandiamid.

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We have found that nitrate and nitrite may be separated by a RP-18 column using only acidified water as the mobile phase. This study examines the separation of nitrate, nitrite, and phosphate using acidified water and alcohols and compares these separations to those obtained with a paired ion regent, Low UV PIC A. The analysis of soil with added nitrate using water and 1:1 methanol-water as an extracting solution, is also examined and the results compared with the phenoldisulphonic acid method (10).

MATERIALS AND METHODS

HPLC analysis was performed using a Waters Scientific (Mississauga, Ont.) Model M45 pump, Model U6K injector, and Model 441 UV detector. The

detector was modified with the optional kit to allow detection at 214 nm. The output was monitored with an Omniscribe strip chart recorder. A 4 mm I.D. x 250 mm 10 micron LiChrosorb RP-18 column (Merck) was operated at ambient temperature (ca. 24°C).

HPLC grade methanol, isopropyl alcohol, methylene chloride, and n-heptane were obtained from Caledon Laboratories (Georgetown, Ontario). Low UV PIC A (tetrabutylammonium hydrogen sulfate) (Waters Scientific) was prepared by dissolving the contents of the vial in one litre of distilled water. Glass distilled water was used for all preparations. All reagents were the highest purity available. For the preparation of the mobile phases or the standards, the solvents and water were filtered with a 0.45 micron Millipore filter and degassed. The pH was adjusted with 1 M H_2SO_4 unless otherwise noted. Potassium hydrogen phthalate buffer (pH 3.0) was made by adding 22.4 mL 0.1 M HCl to 50 mL 0.1 M potassium hydrogen phthalate.

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Almasippi soil, consisting of 89% sand, 5% silt, and 6% clay, was used to determine the recovery of added nitrate. Native nitrate and phosphate levels were 1.0 and 2.8 ppm, respectively. The soil pH was 7.9.

Nitrate recovery experiments were performed as follows: Five or 10 g of soil was added to 250 mL Erlenmyer flasks and nitrate was added as 5 or 10 mL of 50 mg/L nitrate (KNO_3), respectively. For one set of 10 g soil samples, the flasks were first silanized by rinsing the flask with a 15% solution of silanizing reagent (Surfasil, Pierce Chemical Co., Rockford, IL.), followed by rinsing with toluene and then acetone. The treated soil samples were shaken in a water bath (Fisher Model 129) at 25°C for 30 min and placed in a 95°C oven for 24 h to evaporate the water.

The 5 g of soil in one flask (Sample #1) was extracted with 25 mL of nitrate-extracting solution. This solution was prepared by mixing 20 mL of 1 M $CuSO_4$ and 100 mL of 0.6% Ag_2SO_4 and diluting to 1.00 L with distilled water. During the 30 min extraction procedure, 0.16 g of $Ca(OH)_2$ and 0.50 g of $MgCO_3$ were also added. The nitrate in this extract was then determined by a modified phenoldisulfonic acid method (Bremner 1965). For this procedure, 5.0 mL of the soil extract was pipetted into a 50 mL beaker and evaporated to dryness at 95°C. The concentration of the nitrate was determined with a Bausch and Lomb Spectronic 710 at 415 nm, using a 10 cm light path. We previously determined that this method of nitrate analysis in this soil resulted in 100% recovery for nitrate concentrations from 10 to 100 ppm (unpublished results).

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The flasks containing 10 g of soil were each divided into 1) a 5 g soil sample which was extracted with the nitrate extracting solution and analyzed by the phenoldisulphonic acid method (Sample #2), 2) a 2 g soil sample extracted with 4 mL of distilled water (#5), and 3) a 2 g soil sample extracted with 4 mL of 1:1 methanol-water, pH 2.9 (#7). The second set of corresponding 10 g soil samples, utilizing silanized flasks, were labelled numbers 3, 6, and 8.

The soil extracts of samples 4 through 8 were filtered with #5 Whatman filter paper. One millilitre of the filtered solution was then filtered with a 0.45 micron disposable syringe filter (Micron Separations Inc., N.Y.). One microlitre of this filtrate was directly injected into the HPLC for analysis at 214 nm.

RESULTS AND DISCUSSIONS

Separation of Nitrate, Nitrite, and Phosphate

Distilled water, at pH > 5.5 and flow rates of 0.5 to 1.0 mL/min, was ineffective at separating nitrate and nitrite. As the pH was lowered

to pH 2.9, the separation between nitrate and nitrite increased, with nitrate eluting first (Figure 1A). The choice of the acidifying agent, e.g., HCl, acetate, or H_2SO_4, was important in determining the elution characteristics. Acidification to pH 2.9 with HCl (Figure 1B) resulted in slightly longer retention of nitrate and nitrite, reduced sensitivity, and a noisier baseline, whereas the use of sulfuric acid produced a cleaner and more sensitive chromatographic response. However, in all cases, phosphate was found to have the same retention time as nitrate. Carbonate, phosphate, and hydrogen phthalate buffers, methanol, and isopropyl alcohol were also examined as mobile phases for the separation of nitrate, nitrite, and phosphate.

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We found that potassium hydrogen phthalate buffer, pH 3.0, was effective at separating nitrate and nitrite, but peak shape deteriorated (Figure 1C). Nitrate, nitrite, and phosphate can be more effectively separated with 4 mM potassium hydrogen phthalate, pH 4.0, using a Vydac anion column (6). Although nitrate and nitrite have been shown to be separated by 45-50 mM phosphate buffer, pH 3.0, using a Partisil-10 SAX (strong anion exchange) column (11) we found potassium dihydrogen phosphate (0.005 M), pH 3.2, was not effective at separating phosphate and nitrate. In contrast to the separation of nitrate, nitrite, and phosphate possible with Dionex low-capacity anion resin using 3.0 mM $NaHCO_3$ + 2.4 mM Na_2CO_3 (5), the use of 0.1 N $NaHCO_3$, pH 7.6, produced poor separation on our LiChrosorb RP-18 column.

Isopropyl alcohol-water mixtures were found to result in a dramatic reduction in nitrite sensitivity. However, a 3:1 isopropyl alcohol-water mixture was effective at slightly separating phosphate from nitrate (Figure 1D).

Figure 1: HPLC chromatograms of 25 mg/L nitrate, 25 mg/L nitrite, and 400 mg/L phosphate with mobile phases of a) water acidified to pH 3.0 with H_2SO_4, b) water acidified to pH 3.0 with HCl, c) potassium hydrogen phthalate buffer pH 3.0, d) 3:1 isopropyl-water pH 3.0, and e) 1:1 methanol-water pH 3.0. Peaks: 1 = nitrate, 2 = nitrite, and 3 = phosphate. Conditions: Lichrosorb RP-18 column at 25°C, 1.0 mL/min flow rate, 0.1 in/min chart speed. Arrow indicates point of injection.

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A 1:1 mixture of methanol and water (pH 2.9) was found to result in the best separation of phosphate from nitrate (Figure 1E). The high concentration of phosphate needed to elicit a detector response indicates that phosphate would be essentially non-detectable at concentrations normally found in our soils (< 100 ppm). A concentration of 2000 mg/L was required to confidently determine the retention time for phosphate. At this high concentration, it was found that column performance deteriorated with phosphate use. Column efficiency could be restored by eluting the column with 100 mL methanol, 100 mL methylene chloride, 100 mL n-heptane and then methylene chloride and methanol again. The column was then washed with 1000 mL of distilled water. Flow rates were approximately 2.5 mL/min. Successive analysis of 2 mg/L nitrate, 100 mg/L nitrite, and 300 mg/L phosphate were also found to result in temporary deterioration of the nitrite peak. Peak width was broadened slightly and, more significantly, there was excessive noise at maximum absorbance making peak height determination difficult. However, it appeared that the levels of phosphate normally found in our soils did not interfere with nitrate and nitrite analysis.

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The separation of phosphate from nitrate is enhanced by use of the methanol in the mobile phase and may be due to a moderating effect of the methanol on the column (12). The methanol may interact with the bonded phase allowing the ionic species to interact with the bonded alcohol. Rudzinski et al. (12) found that a Partisil 5-ODS column with 1:3 methanol-water as the mobile phase resulted in essentially "unretained" nitrate. Van der Houwen et al. (13) speculated that very polar uncharged substances could interact with residual silanol groups and an increase in an organic modifier would increase this interaction. No retention was speculated for charged substances unless a hydrophobic portion was

present. We thereforespeculated that the addition of an alcohol with a larger carbon number would have a more modifying effect.

Various proportions of methanol-water-isopropyl alcohol were tried to optimize the retention times and sensitivities of the three ions. It was found that a 1:1:1 ratio resulted in adequate separation of nitrate, nitrite, and phosphate (Figure 2). As the ratio of methanol declined, the retention time of phosphate decreased and approached that of nitrate. Conversely, as the isopropyl alcohol ratio increased, the phosphate retention increased.

Low UV PIC A was effective at separating nitrate and nitrite (Figure 3A). In contrast to the other mobile phases studied, Low UV PIC A eluted nitrite first. Phosphate concentrations of up to 2000 mg/L were not detectable. Table 1 shows the retention times of nitrate, nitrite, and phosphate for PIC A and 1:1 methanol-water.

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Response for Nitrate and Nitrite

An absorbance of 0.001 of was obtained for approximately 0.05 mg/L nitrate and 2.5 mg/L nitrite, utilizing 1:1 methanol-water (pH 2.9).

TABLE 1

Retention Times (min) of Nitrate, Nitrite, and Phosphate with 1:1 Methanol-Water and Low UV PIC A.

Mobile Phase	Retention Times For Nitrate	Nitrite	Phosphate
1:1 MeOH-H_2O	1.62	4.90	2.57
Low UV PIC A	5.08	2.92	n.d

n.d - not detectable

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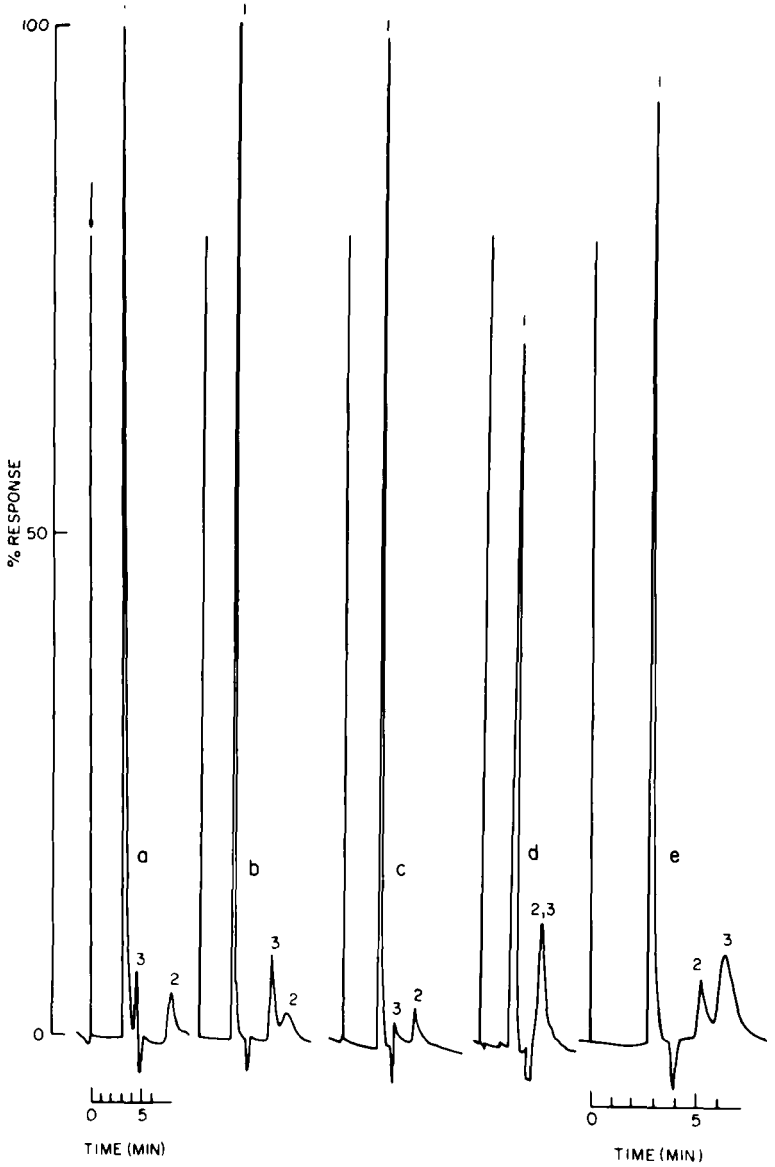

Figure 2: HPLC chromatograms of 25 mg/L nitrate, 25 mg/L nitrite, and 400 mg/L phosphate with ratios of a) 2:1:1, b) 1:1:1, c) 1:14:1:1, d) 0.86:1:1, and e) 0.67:1:1 of methanol-water-isopropyl alcohol. Peaks and conditions as for Figure 1 except chart speed for e was 0.2 in/min.

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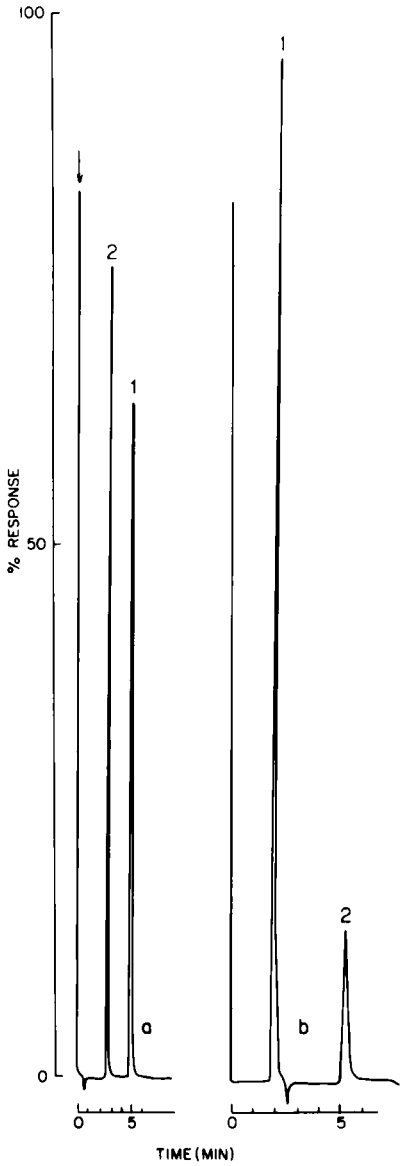

Figure 3: Comparison of chromatograms produced by 50 mg/L nitrate, 50 mg/L nitrite, and 400 mg/L phosphate with a) Low UV PIC A, and b) 1:1 methanol-water pH 3.0. Peaks and conditions as for Figure 1. Flow rate for PIC A was 2.5 mL/min. Chart speed was 0.1 in/min for a and 0.2 in/min for b.

With PIC A, approximately 1.0 mg/L nitrate and 0.6 mg/L nitrite were required to produce absorbances of 0.001. The nitrate absorbances with 1:1 methanol-water tended to exhibit more variability. At the 95% confidence level, 5 injections of 1 mg/L produced absorbances varying by 10.0% and at 100 mg/L, by 1.7%. With PIC A, the absorbance varied by < 1.2% at all concentrations. The nitrite absorbance values varied within 3.6% for both 1:1 methanol-water and PIC A.

The nitrate standard curve produced with 1:1 methanol-water is linear from 0.1 to 80 mg/L, while the nitrite standard curve is linear from 2.5 to 400 mg/L. Nitrate concentrations greater than 80 mg/L result in non-linear responses. Nitrite concentrations > 400 mg/L were not analyzed. With PIC A, the nitrate standard curves were linear from 0.75 to 100 mg/L (Figure 4) and the nitrite curves were linear from 0.75 to 1.75 mg/L (Figure 5). The 1:1 methanol-water system provides an approximate 10 times greater sensitivity for nitrate than the PIC A. In contrast, the PIC A is twice as sensitive to nitrite. The 1:1 methanol-water system gives better overall sensitivity than the PIC A reagent, although at a reduction in confidence. The 1:1 methanol-water mixture is less expensive and works at a lower flow rate, viz., 1.0 vs 2.5 mL/min, resulting in an overall savings in mobile phase expense.

Using the same type of column with PIC A reagent and UV detection, Kok et al. (8) calculated the detection limit to be 0.1 mg/L for nitrate and nitrite.

Analysis for nitrate in soil

Extracted soil samples, analyzed with 1:1 methanol-water, revealed only two major peaks (Figure 6). The first and largest peak was the nitrate peak. The second smaller peak is currently unknown. The resolution of the peaks was better using 1:1 methanol-water (Figure 6A)

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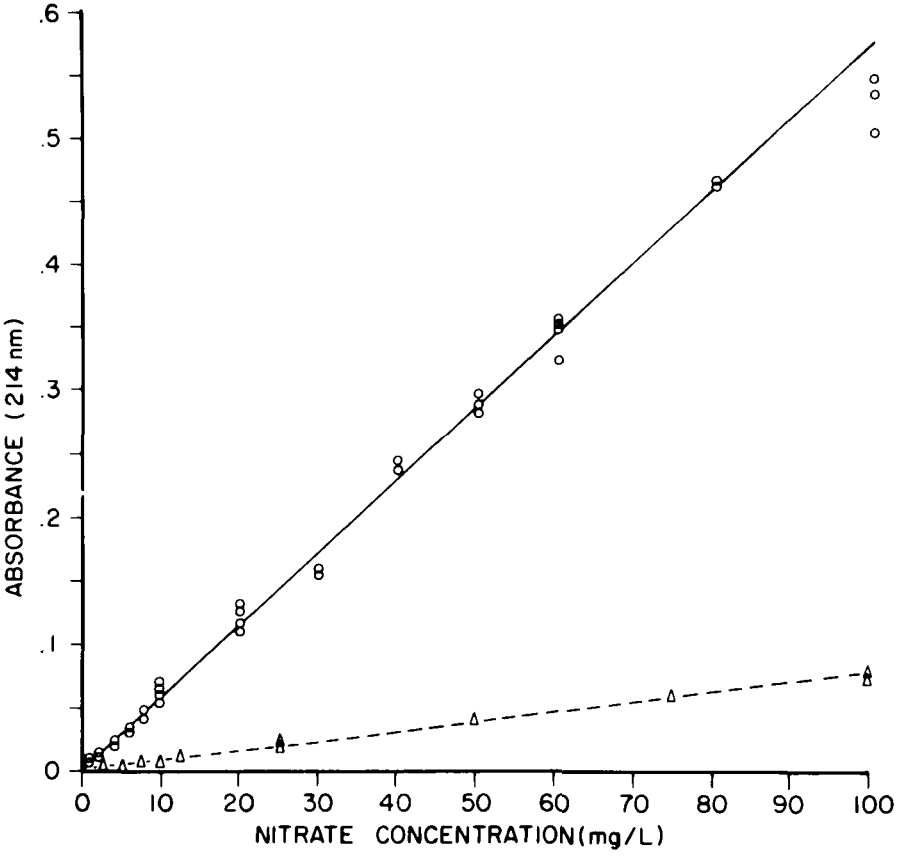

Figure 4: Standard curves for nitrate generated with 1:1 methanol-water (O-----O) and Low UV PIC A (Δ- - -Δ). A minimum of 3 assays for the 1:1 methanol-water and 2 for the Low UV PIC A were performed at each indicated concentration. The 100 mg/L analyses for 1:1 methanol-water were not included in the statistical analysis.

rather than water (Figure 6B) as a soil extracting solution. When PIC A was used as the mobile phase (Figure 6C) only a nitrate peak appeared, although a noisier baseline was evident.

Of the 50 mg/L nitrate added to the soil, the amount recovered ranged from a low of 46.0 ppm to a high of 59.0 ppm (Table 2). The data

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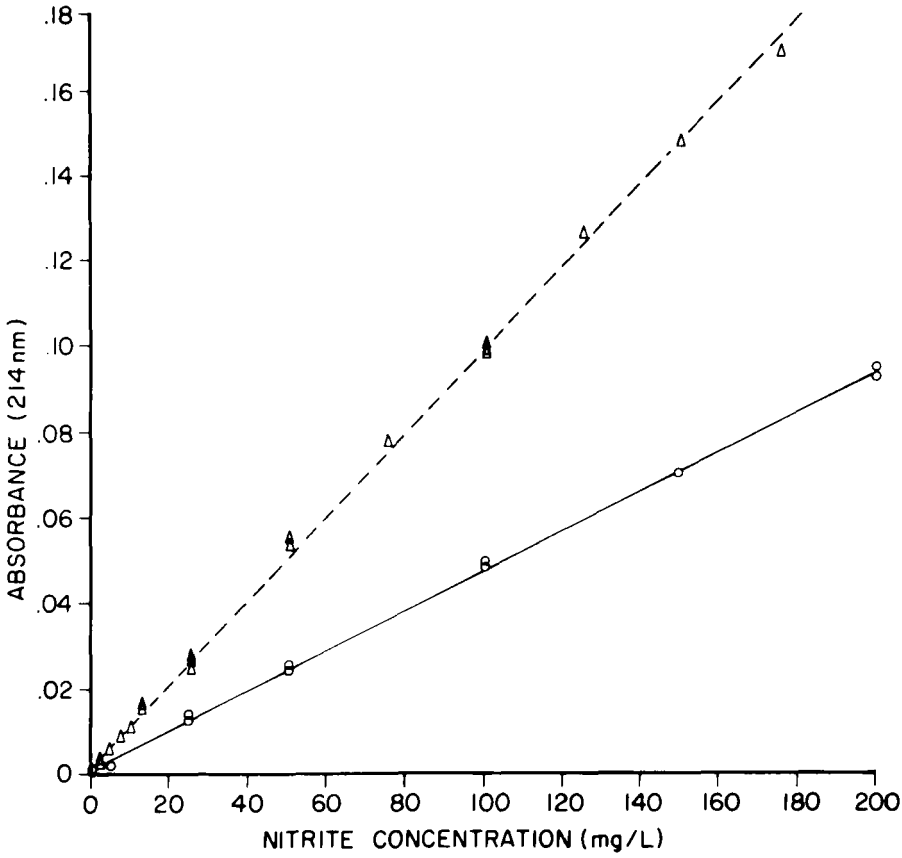

Figure 5: Standard curves for nitrite generated with 1:1 methanol-water (O-----O) and Low UV PIC A (Δ- - -Δ). Each concentration assayed represents a minimum of 2 analyses.

for flasks from which the soil had been removed (sample #'s 2, 3, 5-8) indicates that the amount of nitrate recovered from the soil in the silanized flasks (#'s 3, 6, and 8) was 3.1 to 14.6% higher, than that recovered from the unsilanized flasks (#'s 2, 5, and 7). This may indicate that wet soils dried in glass containers could loose up to 15% of the nitrate present through adsorption to the glass surface.

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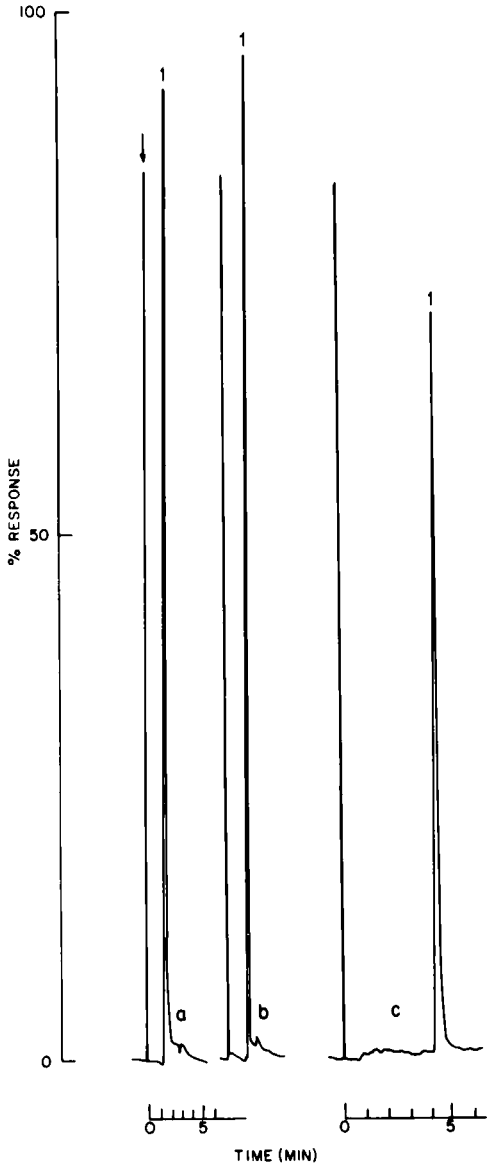

Figure 6. Chromatograms of soil extracts: a and c from soil extracted with water, and b from soil extracted with 1:1 methanol-water. A and b analyzed with 1:1 methanol-water as the mobile phase with conditions as for Figure 1. C was analyzed with Low UV PIC A as the mobile phase with conditions as for Figure 1 except the flow rate was 2.5 mL/min.

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TABLE 2

The Concentrations (ppm) of Nitrate Recovered from Sandy Soil 50 mg/L of nitrate, as KNO_3, was added. Numbers in parenthesis for samples 2 and 3 indicate the percentages recovered in relation to samples 1 and those for samples 5 to 8 in relation to sample 4.

SAMPLE NO.	HPLC ANALYSIS WITH MOBILE PHASE OF 1:1 METHANOL-WATER TRIAL 1	1:1 METHANOL-WATER TRIAL 2	LOW UV PIC A TRIAL 1	LOW UV PIC A TRIAL 2	PHENOLDISUFONIC ACID METHOD TRIAL 1	PHENOLDISUFONIC ACID METHOD TRIAL 2
1	55.6 (100.0)	46.6 (100.0)	51.5 (100.0)	45.0 (100.0)	46.0 (100.0)	48.0 (100.0)
2	48.9 (87.9)	46.6 (100.0)	45.0 (87.3)	45.0 (100.0)	46.0 (100.0)	46.0 (100.0)
3	50.7 (91.2)	46.6 (100.0)	48.9 (95.0)	45.0 (100.0)	51.2 (111.3)	49.3 (102.7)
4	54.1 (100.0)	50.5 (100.0)	53.4 (100.0)	49.0 (100.0)		
5	43.1 (79.9)	44.8 (88.7)	42.2 (81.9)	43.1 (88.0)		
6	45.3 (87.3)	47.3 (93.7)	42.6 (82.7)	45.5 (92.9)		
7	54.4 (100.6)	53.1 (105.1)	51.1 (99.2)	51.5 (105.0)		
8	59.0 (109.1)	60.1 (119.8)	56.5 (109.7)	58.3 (119.0)		

Sample No.:
1. Nitrate extracting solution, same flask analysis.
2. Nitrate extracting solution.
3. Nitrate extracting solution, silanized flask.
4. Water extraction, same flask analysis.
5. Water extraction.
6. Water extraction, silanized flask.
7. 1:1 methanol-water extraction, silanized flask.

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An approximate 20-30% increase in recovery was obtained by using 1:1 methanol-water as an extractant compared to that with water. No substantial difference in recoveries was noted between the use of 1:1 methanol-water or PIC A as the HPLC mobile phase. With PIC A, the use of 1:1 methanol-water as a soil extractant resulted in another small peak eluting at approximately the void volume and was attributed to the methanol in the extractant.

The results indicate that 1:1 methanol-water is an effective eluant for the analysis of nitrate and nitrite by RP-HPLC. The use of 1:1 methanol-water at a flow rate of 1.0 mL/min would result in a substantial savings in the cost of the mobile phase without deterioration in HPLC performance. For phosphate analysis, 1:1:1 methanol-wter-isopropyl alcohol at pH 3.0 can be used. Nitrate may also be effectively analyzed in sandy soil, with maximum extraction being obtained through the use of 1:1 methanol-water.

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