A Simple and High Resolution Ion-Pair HPLC Method for Separation and Simultaneous Determination of Nitrate and Thiocyanate in Different Water Samples

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An ion-pair reversed-phase high-performance liquid chromatography method with isocratic elution and ultraviolet detection was developed and validated for the separation and simultaneous determination of nitrate and thiocyanate. The separation was performed on a C18 analytical column with mobile phase containing 0.08 mM hexadecyltrimethylammonium bromide as an ion-pair reagent, 40 mM of acetate buffer and 30% methanol at pH 3.2. The detection was monitored at 206 nm. The response was linear from 1 to 10 mM for nitrate, with a detection limit of 0.05 mM, and 1 to 10 mM for thiocyanate, with a detection limit of 0.31 mM. Calibration curves were found to be linear in these concentration ranges with correlation coefficient better than 0.99.

Introduction

Determination of common inorganic anions such as fluoride, phosphate, chloride, carbonate, sulfate, nitrite, nitrate and thiocyanate is a significant component in the characterization of the quality and extent of contamination in water. Water quality has received considerable attention over the last few decades due to serious surface and groundwater contamination by anthropogenic and natural contaminants. Anthropogenic activities like agriculture, sewage and industrial effluents have elevated toxic contaminants in surface water and shallow groundwater. Identification of the contaminant source can help in the determination of the potential impact of contaminants on water supplies and the development of management practices to preserve water quality and its decontamination (1).

The presence of high nitrate content in water reflects pollution from organic matter or synthetic fertilizers or manures (2). Fertilizers, animal husbandry and liquid and solid industrial wastes also increase soluble nitrogen compounds in water. Intense cultivation may lead to very high levels of nitrate in groundwater. Nitrate is also considered to be one of the important substances to be determined in water because its consumption through water or food results in health-related problems in humans. The permissible limit for nitrate in drinking water is 50 mg/L (3). Low levels of thiocyanate (SCN^-) normally present in human body fluids (e.g., serum, saliva and urine) are produced during the digestion of some vegetables (cabbage, turnip and kale) or by intake of thiocyanate-containing foods such as milk and cheese. Higher concentration of thiocyanate, which is a major metabolite of cyanide, in physiological fluids arises from exposure to cyanide, inhalation of fires or tobacco smoke. The presence of thiocyanate in body fluids may indicate cyanide exposure. Furthermore, thiocyanate content in human body fluid (especially saliva) is considered to be a biomarker for identification of nonsmokers and smokers (4). However, thiocyanate is toxic for humans and aquatic organisms (5).

Although a wide variety of methods have been proposed for the determination of anions in natural waters, only a few of these are rapid, sensitive, precise and relatively free of interferences, because most employ lengthy procedures (6, 8). Traditional methods used for the determination of anions in environmental samples are spectrophotometric and electrochemical methods (9, 10). Therefore, these methods are not able to simultaneously determine the concentration of anions (10). Thus, separation, monitoring and simultaneous determination of common inorganic anions in water samples are very important.

High-performance liquid chromatography (HPLC) has been primarily used as a technique for the separation of organic and inorganic solutes and its leading position in this role remains unchallenged. Some of the attractive features of this technique are fast and high-efficiency separation, sensitive detection, capability for simultaneous determination and low instrumentation and operating costs (11–13). Inorganic and organic anions, due to their high hydrophilic properties, cannot interact favorably with conventional octadecyl silica (ODS) stationary phases. The most frequently used methods for the simultaneous analysis of inorganic and organic anions include ion chromatography (IC) and ion-pair HPLC (14–16). In ion-pair HPLC methods, a commercial reversed stationary phase in conjunction with an aqueous-organic mixture containing ion-pairing agent as mobile phase are applied. A single stationary phase can be used for either anion or cation analysis (17, 18). The parameters such as stationary phase material, type and concentration of counter ion, pH and ionic strength of eluent, and organic modifier can be selected, and thus provide further advantages to increase the selectivity and resolution and to minimized possible interferences (19).

In the present study, the capability of an ion-pair HPLC method has been investigated for the separation and simultaneous determination of common inorganic anions such as nitrate and thiocyanate in different water samples.

Experimental

Chemicals

All analytical-grade sodium salts of the anions and HPLC grade methanol were obtained from Merck (Darmstadt, Germany).

Stock standard solutions of anions were prepared by dissolving a proper amount of the sodium salts in doubly distilled-deionized water and diluting to the mark in volumetric flasks. Standard anionic solution was prepared daily in doubly distilled-deionized water and then filtered on a 0.45-µm PTFE filter. All other reagents were analytical grade unless stated otherwise.

Equipment

All HPLC separations were performed using a Knauer-HPLC (Berlin, Germany) equipped with a K2500 ultraviolet (UV)-Visible detector and a Model 7125 Rheodyne injector with a 20-µL external injection loop. The column was a Vertex (Knauer) 250 × 4 mm i.d. packed with Eurospher-100 C18, 5 µm. The elution was performed under isocratic conditions at a flow rate of 1 mL/min. A B2000 pH meter equipped with a GCFC 11 combination glass electrode was applied for pH measurements. All glassware containers were carefully treated with 2.0M nitric acid (guaranteed reagent) and rinsed with HPLC-grade water.

Chromatographic conditions and measurement procedure

A solution containing 0.08 mM of hexadecyltrimethylammonium bromide (HTAB) as an ion-pair reagent in methanol–water (30:70) was used as a mobile phase. The pH was adjusted to 3.2 using 40 mM of a sodium acetate–acetic acid buffer. It was filtered through a 0.45-µm PTFE filter and degassed under vacuum before use. Twenty microliters of water samples or standard solutions were directly injected into the HPLC system. The other parameters were as follows: flow rate, 1.0 mL/min; column temperature, 25°C; wavelength of UV detection, 206 nm; analysis time, less than 20 min. The capacity factors were calculated using $k = (t_r - t_0)/t_0$, where t_r is retention time of the anion, averaged over repeated measurements at the peak of the elution curve and t_0 is the elution time of a non-retained substance. In the present work, fluoride was used to determine t_0. Determination of anions in different water samples were performed by driving suitable calibration curves based on peak height of the anion versus concentration of the anion.

Result and Discussion

Chromatographic behavior of anions

Charged surfactants have been widely used as mobile phase modifiers to improve the partitioning characteristics of charged solutes in reversed-phase HPLC. Various studies have been made to identify the interactions that occur between charged surfactants and ionic solutes, and two retention mechanisms have been proposed. The first mechanism assumes ion-pair formation in the mobile phase before its adsorption on to the non-polar stationary phase, while the second mechanism assumes that the hydrophobic surfactant ion is held on the surface of the hydrophobic stationary phase, and the ionic solute is retained by electrostatic attraction to the charged hydrophobic surfactants [19].

In this work, HTAB was selected to form hydrophobic ion-pairs with analyte anions to be retained and separated on the
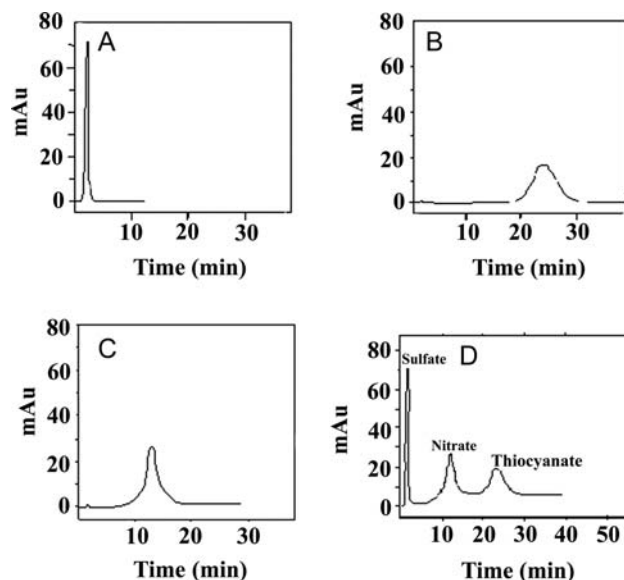

Figure 1. Chromatograms of: SO_4^{2-}, CO_3^{2-}, F^-, Cl^- and PO_4^{3-} (A); SCN^- (B); NO_3^- (C); mixture of anions (D). Conditions: sample volume 20 mL; concentration of SO_4^{2-}, NO_3^- and SCN^-, 7, 1 and 2 mM, respectively.

reversed-phase C18 column, and the separated anions were determined by direct photometric method.

Figure 1 shows a typical chromatogram of standard solutions of SO_4^{2-}, CO_3^{2-}, F^-, Cl^-, PO_4^{3-}, NO_3^-, SCN^- and a mixture of anions in the presence of HTAB by UV detection at 206 nm. As shown in the chromatogram, three peaks appear for non-retained anions, NO_3^- and SCN^-, respectively. NO_3^- and SCN^- anions form hydrophobic ion-pair complexes with different polarities, but the other anions cannot form ion-pair complexes or form hydrophilic ion-pair complexes, so the behavior of these are alike and appear in t_0 (the peak height of each peak was directly proportional to the concentration of its anion).

Effect of HTAB concentration

HTAB is a long chain surfactant with very little critical micelle concentration (CMC). The CMC of HTAB is 328 mg/L or ~0.9 mM [20]. Our literature review did not show any uses of HTAB in HPLC of inorganic anions. The dependence of resolution on the concentration of HTAB was investigated by using mobile phases containing 0.02–0.16 mM of HTAB, 40 mM acetate buffer and 30% methanol at pH 3.2. Some preliminary experiments showed that in the absence of HTAB, the C18 column cannot separate anions and all anions appeared in t_0. At the presence of 0.08 mM of HTAB, the resolution, analysis time and peak widths are optimum. By increasing HTAB concentration (>0.08 mM), an analysis time of longer than 30 min was obtained and sensitivity of the method decreased because of high matrix absorbance. On the other hand, lower concentrations of HTAB caused a decrease in the resolution (Figure 2).

Effect of pH and concentration of buffer

Some preliminary experiments showed that acetate buffer is suitable for the formation of an ion-pair between anions and HTAB. Thus, the influence of pH on the separation and
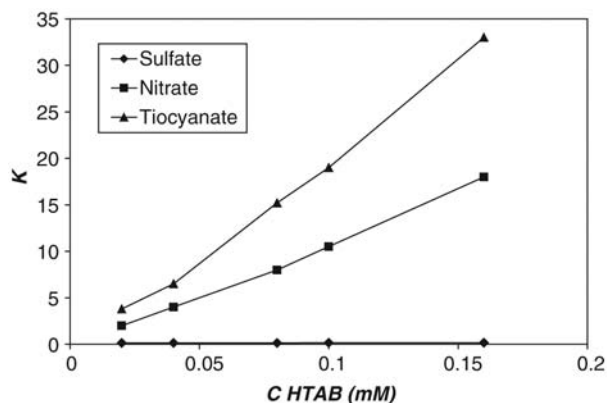
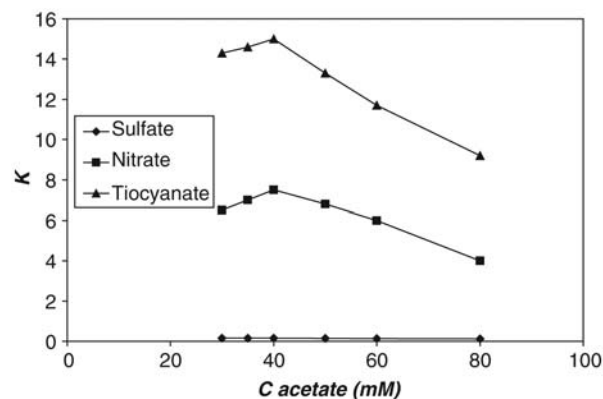**Figure 2.** Dependence of capacity factor of anions on HTAB concentration.

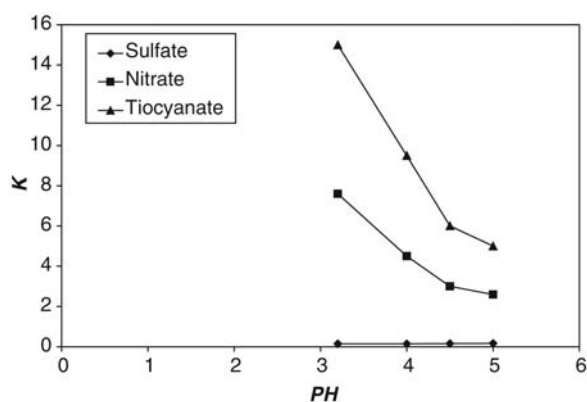**Figure 4.** Dependence of capacity factor of anions on acetate buffer concentration.

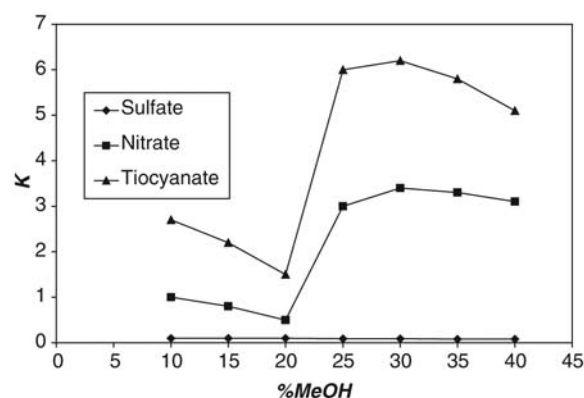**Figure 3.** Dependence of capacity factors of anions on the pH of mobile phase.

Figure 5. Dependence of capacity factor of anions on MeOH%.

determination of anions was studied in the range of 3.2–5.0, using acetate buffer solution (40 mM) and HCl (1M) or NaOH (1M) for pH adjustment. We did not use strongly acidic or basic mobile phases, because under strongly acidic conditions, the bonded phase becomes hydrolyzed, which also happens to the silica backbone at high pH. The effect of pH on retention is related to the degree of ionization of the anions and their ability to form ion-pairs with HTAB (21). Figure 3 shows dependencies of the K values of the anions on pH of the mobile phase in the presence of an ion-pair agent (0.08 mM). An increase in pH of the eluent from 3.2–5.0 dramatically decreased the resolution of anions. Because under acidic conditions, the silanol groups on the C18 stationary phase are protonated, hydrophobic interactions between reverse stationary phase and anion-HTAB ion pairs can be possible. However, with increases in the pH, the silanol groups deprotonated, hydrophobic interactions decreased and a mixed mechanism of interaction was performed, because anionic deporotonated sinanol groups are a exclusion stationary phase for anions. In strong basic mobile phase interferences, the effect of hydroxide ions added probability.

The obtained results showed that the optimum concentration of buffer is 40 mM. The increase or decrease of buffer concentration to 40 mM decreased the capacity factors (K) of thiocyanate and nitrate, but did not influence the resolution

of other anions. To explain the chromatographic behavior of thiocyanate and nitrate, we must take into account that acetate anions selectively interact with the cationic ion-pair agents and thus lead to rapid elution of the thiocyanate and nitrate. Obviously, the acetate buffer concentration does not have a serious influence on the retention of hydrophilic ion-pairs that have very low elution times.A good resolution and optimal analysis time were obtained with 40 mM of buffer concentration at pH 3.2 (Figure 4).

Effect of organic modifier solvent and column temperature

The effect of organic modifier solvent on resolution was studied using increases in the percentage of methanol (10 to 40%, v/v) in the mobile phase containing 0.08 mM HTAB and 40 mM of acetate buffer at pH 5 (Figure 5). The retention of thiocyanate and nitrate are strongly influenced by the percentage of methanol in the mobile phase. The optimum methanol concentration was found to be 30%. The decrease or increase in methanol content to 30% caused a decrease in the resolution of anions. At this methanol percentage, hydrophobic interactions between ion-pairs and C18 column is optimum.

The effect of column temperature on the K values is presented in Figure 6. As shown in the figure, K values for
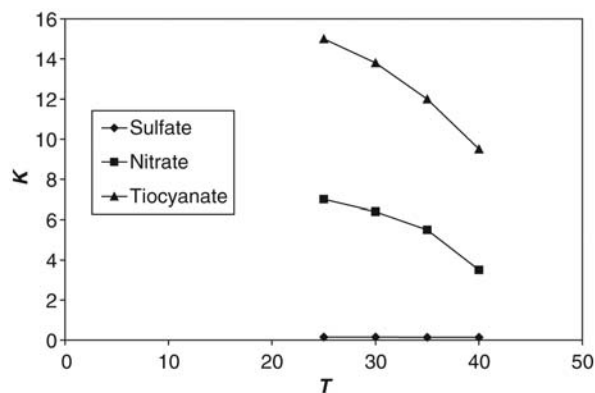
Figure 6. Dependence of capacity factor of anions on column temperature.

thiocyanate and nitrate decrease with an increase in column temperature, but the other anions are not influenced. Thus, 25°C was chosen as the optimum temperature.

Rate (kinetics) of equilibrium between ion-pair reagent and C18 column

In previous ion-pair HPLC methods, the equilibrium rate between ion-pair regent and C18 column was very slow and equilibrium was obtained after more than 2 hours (22).

In the present study, results show that a real equilibrium between HTAB and the C18 column was not obtained, even after 10 hours, and the retention times of nitrate and thiocyanate ions increase with time. Retention times of other anions that appeared in t_0 (i.e. sulfate) were constant the entire time (Figure 7). The speed of this instability will decrease after 70 min; the shape of the peaks and resolution between peaks is also suitable in this time. Hence, all quantitative and qualitative analyses were performed at 70 min after passing the mobile phase from the column.

Calibration graph and limit of detection

The least-squares regression (LSR) is a simple and reliable calibration model for quantitative analysis of different samples. The preliminary experiments showed that the peak height of the anion was directly proportional to its concentration in standard solutions. The regression equations were obtained from the LSR method and other statistical parameters such as limit of detection (LOD), relative standard deviation (RSD) and linear range (LR); the correlation coefficients of regression lines (r^2) for anions are presented in Table I. The LOD was obtained from $C_{LOD} = 3 \ S_b \ m^{-1}$, where S_b is the standard deviation of blank for ten replicate determination and m is the slope in the regression equations.

Analytical application

The applicability of the proposed method for simultaneous determination of anions in different environmental matrices was checked by the standard addition method. The results are presented in Table II for five different water samples. The recovery of the anions from water samples was satisfactory, which
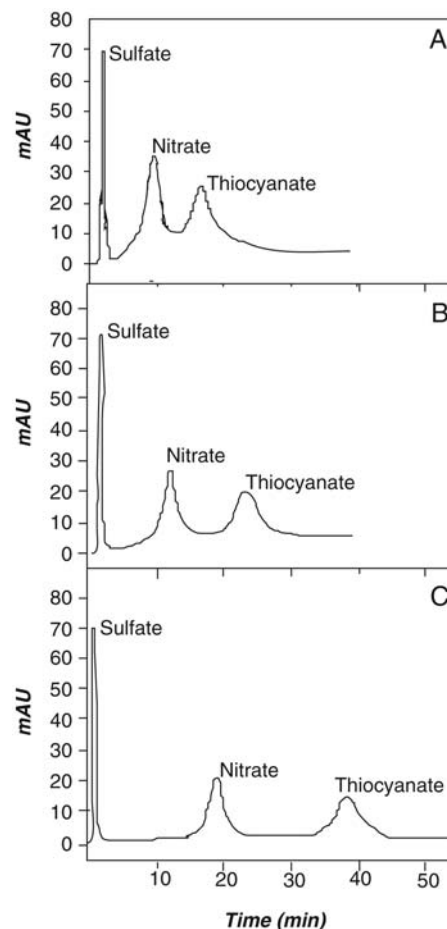

Figure 7. Chromatograms of sulfate and nitrate and thiocyanate: at 40 min HPLC (A); at 70 min after HPLC (B); at 250 min after HPLC (C).

Table I

Regression Equations and Statistical Parameters for the Determination of Anions

Analyte	Regression equation	LR (mM)	RSD%	LOD (mM)	r^2
SO_4^{2-}	$S = 11.33C + 4.333$	2.5–40	0.5	0.12	≥ 0.99
NO_3^-	$S = 25.88C - 3.526$	1.0–10	3.6	0.054	≥ 0.99
SCN^-	$S = 4.5028C + 2.3575$	1.0–10	4.7	0.31	≥ 0.99

Table II

Recovery of Sulfate, Nitrate and Thiocyanate in Different Water Samples

Sample	Sample	Added (mM)	Recovery	RSD%
Damavand mineral) water (Damavand Mountain, Iran)	Sulfate	7	91%	0.79
	Nitrate	1	118%	5.89
	Thiocyanate	2	89.7%	5.5
Chaloos River water (Iran)	Sulfate	7	95%	0.6
	Nitrate	1	109%	6.7
	Thiocyanate	2	92.6%	5.8
Tap water (Ghazvin City, Iran)	Sulfate	7	94%	0.54
	Nitrate	1	101.7%	4.9
	Thiocyanate	2	96.3%	5
Barajin Aqueduct (Ghazvin City, Iran)	Sulfate	7	90%	0.68
	Nitrate	1	113%	7.8
	Thiocyanate	2	87.5%	7.3
Tap water (Tehran City, Iran)	Sulfate	7	93%	0.47
	Nitrate	1	117.9%	4.01
	Thiocyanate	2	98.1%	6

indicated the capability of the proposed method for the simultaneous determination of anions. The recovery values calculated for the spiked samples were 90, 101 and 87% for sulfate, nitrate and thiocyanate, respectively, which confirms the accuracy of the procedure and its independence from matrix effects.

Conclusions

The described photometric ion-pair HPLC method has been proved to be a simple, sensitive, reproducible and accurate technique for the separation and simultaneous determination of nitrate and thiocyanate. The resolution (R^s) between nitrate and thiocyanate was 1.96 at 70 min. The R^s was obtained from $R^s = 2(t_{r2} - t_{r1})/w_1 + w_2$, where t_r and w are retention time and peak width. Analysis time for the separation and determination of analytes was approximately 30 min. This method was successfully applied to quantitative measurement of the anions in different water samples.

Acknowledgments

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