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High-performance liquid chromatographic column-switching technique for the determination of intermediates of anaerobic degradation of toluene in ground water microcosm

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ABSTRACT

A reversed-phase liquid chromatographic column-switching system was used for the determination of phenol, benzoic acid and cresol (PBC) in the presence of toluene in ground water microcosm. A precolumn was connected in series with an analytical column via a column-switching valve. After the injection, as soon as PBC were eluted from the precolumn to the analytical column, the valve was switched so that the precolumn was between the analytical column and the UV detector. Toluene and other non-polar compounds were eluted from the precolumn in a very short time and detected along with the solvent front. Subsequently, PBC were separated on the analytical column and passed through the precolumn one more time before being detected by the UV detector. The total analysis time was 15 min. This technique facilitated the study of the basic mechanism and path way of anaerobic degradation of toluene in ground water aquifer.

INTRODUCTION

For high-performance liquid chromatographic (HPLC) analysis, isocratic elution is ineffective to separate compounds having widely disparate partition ratios (k). Gradient elution can provide a solution to this problem [1]. However, the column has to be brought back to its initial state to start reprogramming the solvent. The column-switching technique has been used to shorten the analysis time of complex mixtures without the use of gradient elution [2–4]. It was used with either one- or two-column configuration. Huber *et al.* [5] used column switching with a stop flow technique to determine toluene and the more-polar substituted phenols on a normal phase column. The separation time was reduced from 45 min to 8 min. Rocklin *et al.* [6] used a 4-way double stack slider-type valve with two cation columns to determine mono- and divalent cations in one run without stopping the flow of either column.

Alkylbenzenes are among the most tightly regulated classes of ground water contaminants. Fuel releases, either from leaking underground storage/transport systems or surface spills, are the most common route for alkylbenzenes to enter the subsurface environment. Alkylbenzenes constitute approximately 3 to 18% of petroleum-based fuels.

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The compounds of the so-called BTEX fraction (benzene, toluene, ethylbenzene and xylene) have relatively high solubility and mobility. The degradation of aromatic hydrocarbons has been extensively studied. For the last 10 years, laboratory and field evidences have shown that alkylbenzenes can be biodegraded under the anoxic conditions which are commonly found with fuel-contaminated aquifers [7–9]. As yet, little is known about the path ways used under anaerobic conditions or about the factors which limit or stimulate this activity. In our investigations on the fate of alkylbenzenes in the subsurface, we have used toluene as a model compound for biodegradation studies. It has been shown that phenol, benzoic acid and cresol (PBC) are all possible metabolic intermediates during anaerobic toluene degradation. A method for the determination of these intermediates, in the presence of toluene, is critical for researching the basic mechanisms and pathways involved.

The objective of this study was to develop a rapid HPLC method to determine PBC in ground water microcosms containing toluene. It is important that these compounds are quantified over the life of the experiment in order to identify changes in their concentrations. This will facilitate the study of the reduction process of toluene by anaerobic bacteria.

EXPERIMENTAL

Reagents and chemicals

Toluene, phenol, *m*-cresol and benzoic acid were purchased from Sigma (St. Louis, MO, USA). Analytical-reagent-grade phosphoric acid (H_3PO_4) (Fisher Scientific, Springfield, NJ, USA) was also used. Solvents used for chromatography were obtained as HPLC grade (Fisher Scientific, Fairlawn, NJ, USA). Water was purified in a Super-Q water purification system (Millipore, Bedford, MA, USA).

Reagent solutions

The mobile phase was prepared by mixing 250 ml of acetonitrile, 750 ml of water and 2 ml of H_3PO_4. It was sparged with helium at 10 p.s.i. for 15 min before use. A stock solution containing 500 ppm of phenol, benzoic acid and cresol was prepared in methanol–water (1:1, v/v). Working standard solutions were then prepared at the concentration range of 0.1–50 ppm by diluting the stock solution with water. Standard sulfate solution (1000 ppm) (Banco, Ft. Worth, TX, USA) was used to prepare the working standards range from 10 to 150 ppm.

Sample preparation

Aquifer solids were collected using the aseptic, anaerobic coring procedure as described by Leach *et al.* [10]. Sealed, collected cores were placed in an anaerobic chamber where 50 g of mixed, saturated core material were added aseptically to 160 ml of sterilized serum bottles, filled with 90 ml of a distilled water–sterile spring water (50:50, v/v) mixture (Byrd's Mill Spring-Municipal water supply for the city of Ada, OK, USA, pH 7.4, (total alkalinity 320 $CaCO_3$ equiv.) and amended with ammonium phosphate (10 m*M* ammonium and 5 m*M* phosphate final concentration) and sodium sulfate (1 m*M* final concentration). Reducing conditions were maintained by the addition of sodium sulfide (*ca.* 1 m*M* final concentration). Resazurin (0.0001%, w/v) was added as a redox indicator. Microcosms were inoculated with 10 ml of a toluene-degrading culture enriched from contaminated aquifer material. Microcosms were then spiked with toluene, phenol, cresol and benzoic acid or a mixture of these compounds (20 ppm total concentration), or were unamended. The bottles were sealed with PTFE-faced butyl rubber stoppers and aluminum crimp seals. Microcosms were incubated in an anaerobic chamber at 20°C and protected from light. Controls were constructed as above with the exception that the serum bottles were autoclaved for one hour on two successive days after core addition and before being filled and spiked. The microcosms were repetitively sampled by introduction of a syringe needle down the side of a partially opened stopper and removing an aqueous subsample. Sulfate concentrations in the microcosms were also determined in accordance with the US Environmental Protection Agency (EPA) method 375.2 [11].

Liquid chromatograph

The chromatograph consisted of a Model M590 pump (Waters Assoc., Milford, MA, USA), a Water autosampler Model 710B, a six-port automated column-switching valve (Waters), a Waters UV detector Model 484 at 200 nm. A NewGuard RP-18 precolumn with 7-μm particles (1.5 cm × 3.2 mm

I.D., Applied Biosystems, Santa Clara, CA, USA) for column 1 and an Econosphere C_{18} column with 3-μm particles (10 × 0.46 cm I.D., Alltech Assoc., Deerfield, IL, USA) for column 2 were used. The switching valve was controlled by time-programmable external events of the M590 pump and the autosampler. During the method development, three elution methods were performed, as follows.

Isocratic method. The mobile phase was acetonitrile–water (25:75, v/v) with 0.2% H_3PO_4 at a flow-rate of 1 ml/min. The switching valve was at the position 1 (Fig. 1) enabling column 1 to be between the injector and column 2. An aqueous solution containing 1 ppm of PBC and 25 ppm of toluene was injected (50 μl). The total analysis time was 40 min.

Gradient method. The conditions were similar to the isocratic method, except that gradient elution was used. A Model 600E pump (Waters) was used to generate a gradient program between solvent A (same as used in the isocratic method) and solvent B (acetonitrile) at a flow-rate of 1 ml/min. The gradient profile was shown in Table I. The total analysis time was 25 min.

Column-switching method. The column configuration during the analysis was also shown in Fig. 1. Other conditions were the same as in the isocratic method. The total run time was 15 min.

RESULTS AND DISCUSSION

PBC were quantified with the isocratic method on the reversed-phase column within 15 min using a weak mobile phase (25% acetonitrile). However, it took at least 35 min to elute toluene with the same conditions (Fig. 2). The gradient elution method, on the other hand, reduced the run time to 25 min. First, the isocratic elution was used to elute PBC and then the amount of acetonitrile in the mobile phase was increased to 100% to elute toluene. It

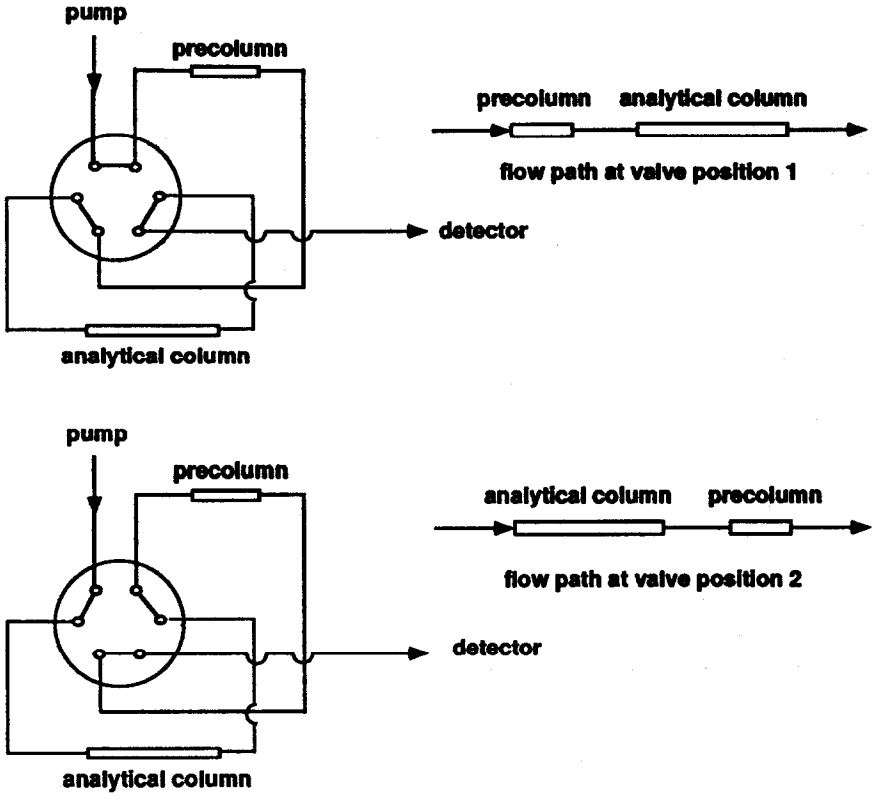

Fig. 1. Flow scheme of the six-port column-switching valves and mobile phase flow path.

TABLE I

GRADIENT PROFILE FOR THE GRADIENT METHOD

Time (min)	Flow-rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
Start	1	100	0
7.0	1	100	0
7.5	1	0	100
13.0	1	0	100
13.2	2	0	100
17.0	2	0	100
17.5	2	100	0
22.0	2	100	0
22.5	1	100	0
25.0	1	100	0

took 10 min at a flow-rate of 2 ml/min to bring back to its initial condition after toluene was eluted at 14 min (Fig. 2). By using the column-switching method, the total run time is 15 min for the PBC like the isocratic method while toluene was eluted close to the solvent front.

The strategy of the proposed column-switching technique was to elute polar compounds (PBC) from column 1 to column 2. Immediately after cresol was in column 2, the valve was switched so that the column 1 was between column 2 and the detector. Since column 1 was short, non-polar compounds, including toluene were eluted from the column quite rapidly and detected along with the solvent front. Subsequently, PBC were separated on the higher efficiency column (column 2) and column 1 one more time before they were quantified by the UV detector.

The mobile phase used in the column-switching method was the same as used in the isocratic method. It exhibited a good separation among PBC. The selection of column 1 was crucial to ensure that it separated PBC from toluene and allowed enough time for the valve switching. The NewGuard columns consisting of different packing materials (RP-2, RP-4, RP-8, phenyl, RP-18 and polymeric) were evaluated. An aqueous solution containing toluene and PBC was injected onto the NewGuard column and eluted with the isocratic mobile phase. The RP-18 exhibited the best retention of toluene (at 1.4 min) from cresol (0.8 min) which had the longest retention time among PBC (Fig. 3). Therefore, the RP-18 column was chosen for the experiment.

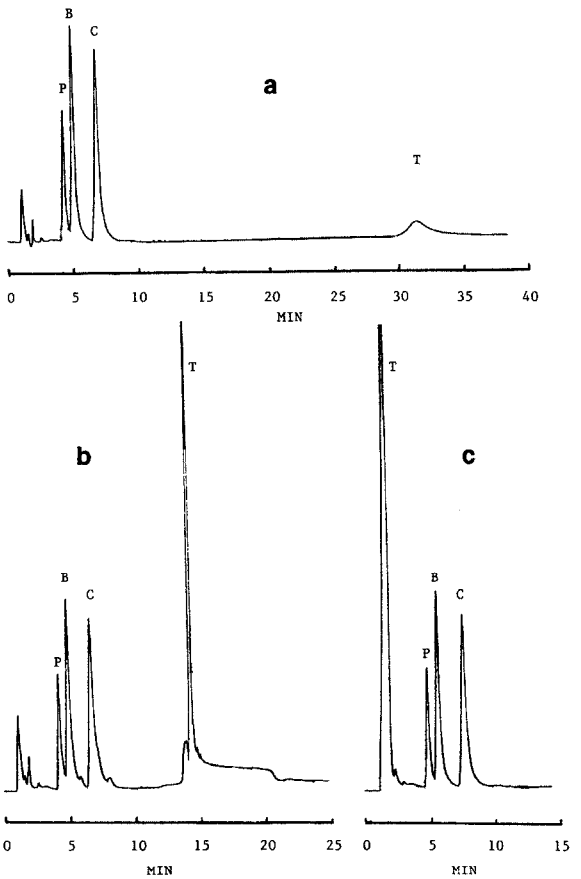

Fig. 2. Chromatograms of aqueous samples containing 1 ppm of PBC and 25 ppm of toluene using (a) isocratic method, (b) gradient method and (c) column-switching method. Peaks: P = phenol; B = benzoic acid; C = cresol; T = toluene.

Next, the proper switching time was examined by injecting 50 μl of PBC in aqueous solution (1 ppm) and varying the switching time after the injection from 0.6 to 1.4 min. The peak areas of PBC were recorded and plotted *versus* the switching times (Fig. 4). At 1.1 min after the injection, PBC were eluted from column 1 while toluene was still retained. Thus, the switching time at 1.1 was chosen. To observe the effect of toluene concentration on the column-switching method, the aqueous solution containing 1 ppm of PBC was added with toluene at concentrations of 0.5, 12.5, 25, 50 and 100 ppm. These samples were analyzed and the chromato-

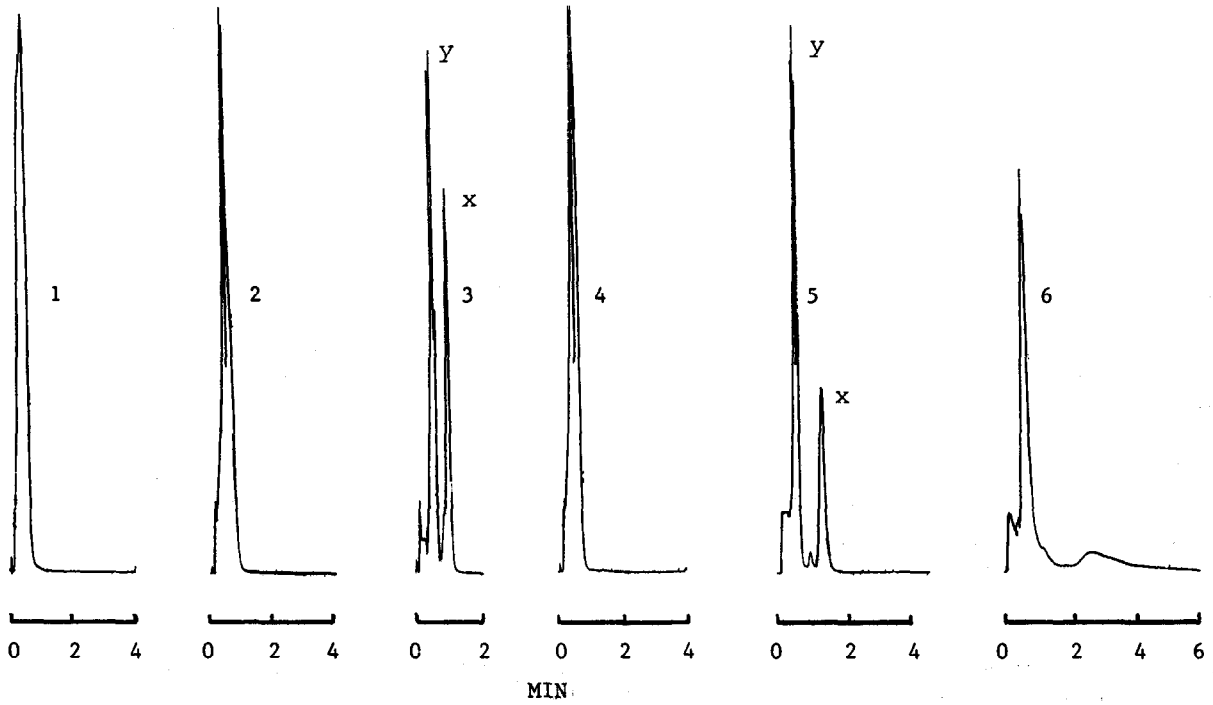

Fig. 3. Chromatograms of aqueous samples containing 1 ppm of PBC and 25 ppm of toluene using isocratic method on the NewGuard columns: (1) RP-2, (2) RP-4, (3) RP-8, (4) phenyl, (5) RP-18 and (6) polymeric. x = Toluene, y = PBC. Injection volume = 50 μl.

grams were examined (Fig. 5), The amount of toluene up to 100 times of the PBC concentration did not affect the separation of PBC.

It was highly recommended to pump at least 50 ml of acetonitrile through the columns after 25 injections to elute non-polar compounds from the reversed-phase column. Linearity data for concentrations of 0.1, 0.5, 1.5 and 10 ppm are shown in Table II. Concentrations as high as 50 ppm did not cause the carry-over in subsequent runs. The detection limit of the method was defined by the lowest amount that gave the signal three times higher than baseline noise. The recovery of the Bird's mill treated ground water spiked with 1 and 10 ppm of PBC is also shown in Table II. The accuracy is excellent with relative standard deviations range from 0.3 to 6.6%.

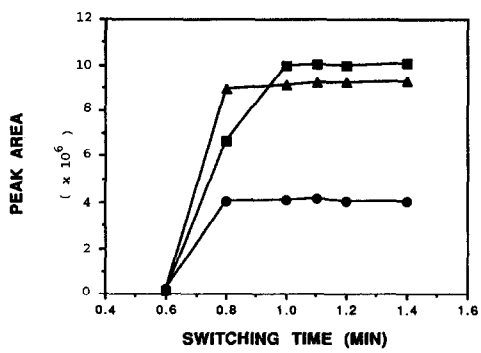

Fig. 4. Elution of PBC from the NewGuard column (RP-18) at different column-switching times. Samples: ● = phenol; ▲ = benzoic acid; ■ = cresol.

Suspension cultures of the sulfate-reducing, toluene-degrading consortium were able to degrade benzoic acid alone or preferentially in the presence of cresol and phenol (Fig. 6). The onset and cessation of benzoic acid removal correlated well to the reduction of sulfate (Fig. 7). The ratio of benzoic acid removed to sulfate reduced is close to the calculated values for the oxidation of benzoic acid to carbon dioxide coupled to sulfate reduction. Benzoic acid has been shown to be an intermediate of toluene, phenol, and cresol oxidation under anaerobic conditions by several groups and might be ex-

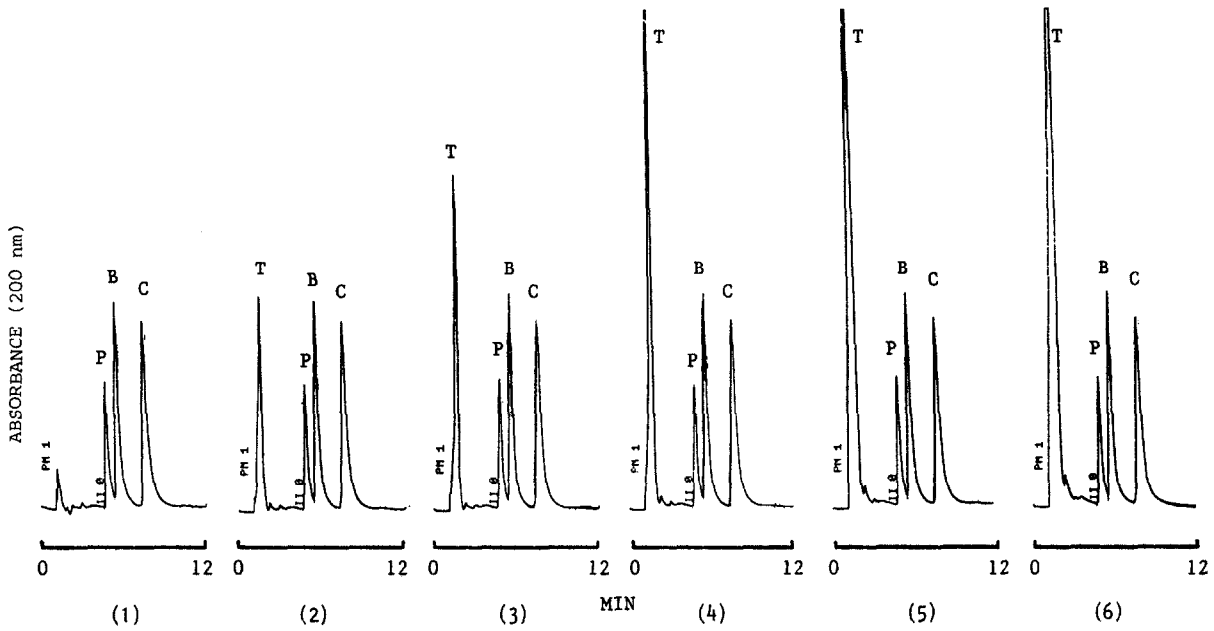

Fig. 5. Chromatograms of aqueous samples containing 1 ppm of PBC and toluene at the concentration of (1) 0 ppm, (2) 5 ppm, (3) 12.5 ppm, (4) 25 ppm, (5) 50 ppm and (6) 100 ppm. Injection volume = 50 μl.

pected to be the most easily degradable of the tested substrates [7,8,12,13]. Research continues to degradability of cresol and phenol in this system.

CONCLUSIONS

The use of the column-switching method permitted the determination of PBC in the presence of non-polar compounds particularly toluene under the isocratic condition in a very short time. It eliminated the need for re-equilibration of the analytical column and produced a constant spectral background. Complete analysis required less than 15 min. The method was simple, reproducible especially through automation with commercially available equipment. This technique could be also applied for

TABLE II

LINEARITY AND ACCURACY OF THE COLUMN-SWITCHING METHOD

Compound	Detection limit [a] (ppm)	Concentration range (ppm)	Linearity			Accuracy	
			Slope	Intercept	Correlation of coefficient	Mean[b]	Relative standard deviation (%)
Phenol	0.05	0.1–10	$1.69 \cdot 10^{-6}$	−0.13	0.998	97.41 (a)	0.64
						100.07 (b)	3.37
Benzoic acid	0.05	0.1–10	$1.51 \cdot 10^{-6}$	−4.49	0.999	100.98 (a)	6.59
						99.18 (b)	5.47
Cresol	0.05	0.1–10	$1.10 \cdot 10^{-6}$	−9.00	0.999	100.18 (a)	1.48
						99.22 (b)	0.34

[a] Signal-to-noise ratio = 3.

[b] (a) Spike level is 1 ppm with 6 replicates; (b) spike level is 10 ppm with 6 replicates.

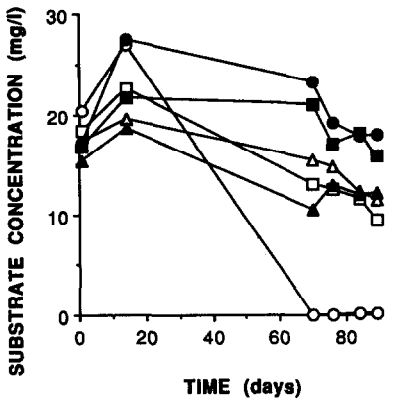

Fig. 6. Relationship between the incubation period and the concentration of PBC in the ground water microcosms. Samples: △ = phenol; □ = cresol; ○ = benzoic acid; ▲ = phenol control; ■ = cresol control; ● = benzoic acid control.

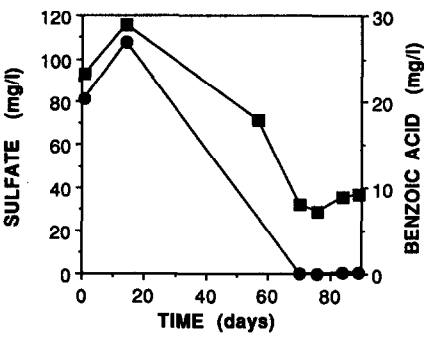

Fig. 7. Relationship between the incubation period and the concentration of sulfate (■) and benzoic acid (●) in the ground water microcosms.

the determination of other polar compounds in the present of non-polar interferences in an aqueous sample.

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