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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF INORGANIC ANIONS ON A SILICA GEL COLUMN MODIFIED WITH A QUATERNARY AMMONIUM SALT

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SUMMARY

Silica gel columns dynamically modified with a quaternary ammonium salt have been investigated for the high-performance liquid chromatographic separation of inorganic anions. The parameters which affect the chromatographic retention behavior have been examined. The mobile phase is composed of an aqueous solution of salicylate, which competes with the analyte anions for active sites and maintains a background signal. The analyte anions are separated based on ion exchange and are detected by indirect photometry or indirect fluorometry. The latter allows detection of 6–15 pmoles (sub-ng level) of anions by means of a microbore column.

INTRODUCTION

Ion chromatography has advanced rapidly for the determination of ions since it was developed by Small *et al.*[1]. Meanwhile, several improvements have been accomplished. Single-column ion chromatography (without the use of a suppressor column) has been demonstrated[2], leading to a simplified system. Packing materials with low ion-exchange capacities are favored for both single-column and dual-column ion chromatography with conductometric detection, because they allow the use of lower eluting ion concentrations. The lower the concentration in the mobile phase, the lower the background noise is obtained in single-column ion chromatography. A low concentration is also favored in dual-column ion chromatography because then the suppressor column requires less frequent regeneration. There have been published several papers which deal with the synthesis of ion exchangers with low capacities[1–3].

Other approaches for the separation of ionic species include ion-pair chromatography[4–6], dynamic ion-exchange chromatography[7–10] and ion-interaction chromatography[11–13], where a hydrophobic counter ion is added to the mobile phase and reversed-phase alkyl-chain bonded silica, polystyrene, or polyacrylate packing materials are used. On the other hand, when the ion-interaction reagent is not added to the mobile phase after the equilibration, the retention mechanism involves ion exchange[14,15]. Ion-exchange columns obtained by dynamic modification[14] of an ODS

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column or static modification[15] of polystyrene resins, in which their ion-exchange capacities can be easily controlled by changing the coating conditions (concentration of the reagent, composition of the matrix solution, etc.) have been investigated.

Indirect photometric or fluorometric detection gives sensitive and universal detection of inorganic anions[16–20]. A visualization UV-absorbing[16–19] or fluorescing[20] reagent was added to the mobile phase. The visualization reagent maintains a background signal and competes (or interacts) with the analyte anions for the active sites. When the analyte anions elute, they are indirectly detected as negative peaks because the concentration of the visualization reagent in the analyte bands decreases. The visualization mechanism has been reported in the literature[12,13,16]. In the indirect detection method, the dynamic reserve (which is defined as the ratio between the background signal and its noise level), the concentration of the visualization reagent, and the displacement ratio (which is defined in this work as the number of visualization ions which are transferred by one analyte ion) all play important roles in the sensitivity that can be achieved. The concentration detectability (C_{lim}) at the detector is given by these parameters as follows.

$$C_{lim} = C_m/RD \tag{1}$$

where C_m is the concentration of the visualization reagent, R is the displacement ratio, and D is the dynamic reserve. The dynamic reserves for fluorescence and for absorption vary differently with the magnitude of the background signal. While the former maintains a $5 \cdot 10^3$ level until the concentration of the visualization reagent falls below 10^{-7} M[19], the latter is proportional to the concentration when the background absorption is below 1.0 a.u. The displacement ratio for monovalent ions is expected to be unity in ion-exchange chromatography (independent of the mobile phase concentration). Ion-exchange columns having very low capacities are required to demonstrate the separation of ions using a mobile phase with low concentrations of visualization reagent.

In contrast to previous work on ODS columns[14] or polystyrene resins[15], this paper describes the potential of silica gel columns, dynamically modified with a quaternary ammonium salt, for the high-performance liquid chromatographic separation of inorganic anions. The analyte anions were detected indirectly.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a reciprocating pump (MiniPump; LDC/Milton Roy, Riviera Beach, FL, U.S.A.) with a dampener or a syringe pump (Model 314; ISCO, Lincoln, NE, U.S.A.), a sample injector (Model 7520; Rheodyne, Cotati, CA, U.S.A.) with a 1-μl injection volume and a conventional silica gel column (HPLC-SI; Alltech, Deerfield, IL, U.S.A., 250 × 4.6 mm, 10 μm) or a microbore silica gel column (Microsphere Silica; Alltech, 250 × 1 mm, 10 μm). A fixed-wavelength (254 nm) UV detector (Model 260; Chromatronix, Berkeley, CA, U.S.A.) and a laser-based double-beam fluorometric detection system were used. The arrangement for the latter detection system was the same as the one reported previously[19,20]. The 325-nm UV beam of a HeCd laser (Model 4240NB; Liconix, Sunnyvale, CA, U.S.A.)

was used as excitation source. The polarization of the beam was modulated at 100 kHz by an electrooptic modulator and the polarization-modulated beam was split into two beams by a calcite beam displacer. Each signal from the sample and the reference cell was collected by separate optical fibers to a common photomultiplier tube after passing several filters. The photomultiplier tube output was directed through an amplifier to a lock-in amplifier with a 1-s time constant. Two parallel quartz tubes were used as the flow cells and were fixed vertically. The same eluent was supplied to a reference flow cell by gravity with a flow-rate of *ca.* 0.3 ml/min. Both eluents flow upwards in the cell and laser beams hit the quartz tubes at right angles. Flow-rates for preparation of the column and the separation of the analytes were 1 ml/min for conventional HPLC and 50 μl/min for microbore HPLC unless otherwise noted.

Reagents

HPLC-grade methanol was supplied by Fisher Scientific (Fair Lawn, NJ, U.S.A.). Water employed in this work was pure water prepared with the Barnstead Nanopure II system (Barnstead, Division of Sybron, Boston, MA, U.S.A.). Cetyltrimethylammonium bromide (cetrimide), phenyltrimethylammonium chloride, tetramethylammonium bromide, and cetylpyridinium chloride monohydrate were supplied by Aldrich (Milwaukee, WI, U.S.A.). The other reagents used in this work were reagent grade and supplied by Fisher Scientific, unless otherwise noted. The mobile phase was degassed in an ultrasonic bath under vacuum. The reference solution for fluorometric detection was degassed the same way and then filtered with a Disposal Nylon 66 filter (Alltech, 0.45 μm). The sample was dissolved in pure water.

Preparation of the column

A quaternary ammonium salt was dissolved in methanol–10 m*M* potassium phosphate buffer (50:50) or 100% 10 m*M* potassium phosphate buffer. The prepared reagent solution was passed through the silica gel column for 4 h, followed by washing with water or methanol for 10 min. When a minipump was employed, the effluent was collected into the reagent reservoir for recycling. After the above dynamic modification, a $5 \cdot 10^{-4}$ *M* sodium salicylate aqueous solution (pH 6.9) or an aqueous solution of a mixture of sodium salicylate and salicylic acid (pH 4.7) was passed through the dynamically modified column, from which the breakthrough volume can be measured. In order to wash out the modified reagent from the column, methanol–0.05 *M* phosphoric acid (50:50) was passed through the column at least for 1 h, followed by washing with methanol for 10 min.

RESULTS AND DISCUSSION

Porous silica gel and zirconia gel dynamically modified with a quaternary ammonium salt were investigated for the HPLC separation of neutral species and ionic species[4,21–26]. The quaternary ammonium salt was added to the mobile phase. Retention of neutral species on the dynamically modified silica gel column is due to hydrophobic interactions between the analyte species and the hydrophobic layer on the silica surface. The retention mechanism for ionic species is more complex. Wall[27] has reported that silica gel and zirconia gel columns dynamically modified with a

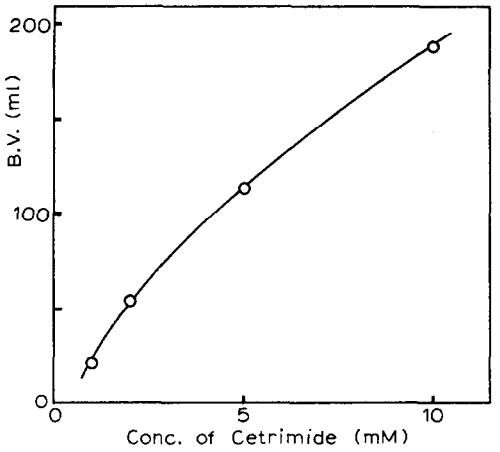

Fig. 1. Effect of the concentration of cetrimide on the breakthrough volume. Column: 250 × 4.6 mm. Eluent: $5 \cdot 10^{-4}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). pH of the reagent buffer: 6.35.

mixture of a non-ionic and an anionic surfactant can separate tyrosinyl peptides in the cation-exchange mode. The initial surface monolayer is formed rapidly by interaction of polar groups of the non-ionic surfactants with gel hydroxyls. Then, a partial bilayer is formed more slowly by hydrophobic interaction of the long alkyl chains of the bound non-ionic surfactant and those of the anionic surfactant dissolved in the mobile phase. On the other hand, it has been suggested that the quaternary ammonium reagent forms a monolayer on the silica gel surface[22,24]. If the quaternary ammonium reagents electrostatically interact one-to-one with silanol groups and form a monolayer on the silica gel surface, the modified surface is expected not to have a net charge. In this work, we found that silica gel columns dynamically modified with quaternary ammonium salts with a long alkyl chain retain anions even if the eluent does not include the quaternary ammonium reagent after the dynamic modification. This observation implies that electrostatic interaction between the analyte anions and the modified surface occurs. Either a double layer of the quaternary ammonium reagent is formed on the silica surface, as suggested by Wall[27], or there exists another structure of the dynamically generated phase.

We have examined the preparation conditions using cetrimide as the quaternary ammonium reagent. Fig. 1 shows the effect of the concentration of cetrimide on the breakthrough volume for salicylate after modification. A volume of 200 ml of each solution was prepared with methanol–10 m*M* phosphate buffer (pH 6.35) (50:50) and the effluent was recycled during the modification. The breakthrough volume increased with increasing concentration of cetrimide. In other words, the amount of cetrimide adsorbed on the silica gel increased with increasing concentration of cetrimide. The amounts of salicylate adsorbed on the modified surface can be calculated from the breakthrough volume, *e.g.*, 94 μmoles of salicylate anions were adsorbed on the column modified with 10 m*M* cetrimide. Strictly speaking, the recycling method does not give precise adsorption data because the concentration of the reagent gradually decreases during the modification. In fact, when the column is modified with 2 m*M* cetrimide in methanol–10 m*M* phosphate buffer (pH 6.35)

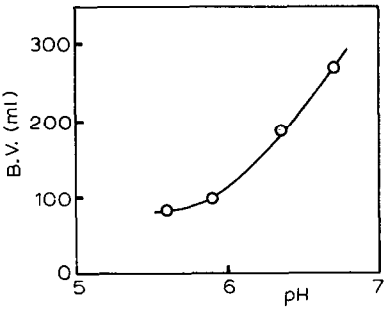

Fig. 2. Effect of pH of the reagent solution on the breakthrough volume. Operating conditions are the same as in Fig. 1 except the concentration of the reagent is 10 m*M*.

(50:50), the breakthrough volumes are 54 ml for the recycling method and 85 ml for the non-recycling method, respectively. Salicylate and the analyte anions were not retained on the bare silica column or on the modified column after washing with methanol–0.05 *M* phosphoric acid (50:50).

The pH of the modifying solution affects the amount of cetrimide adsorbed on the silica surface because the dissociation of silanol groups is affected by the pH of the eluent. Fig. 2 shows the effect of the pH of the reagent solution on the breakthrough volume for salicylate. The breakthrough volume increases with increasing pH of the buffer solution. The apparent pH of the methanol–buffer solution is not considered here. Fig. 3 shows relationships between the retention time of the analyte anions and the breakthrough volume. The larger the breakthrough volume, the longer is the retention time. This is consistent with increasing capacity of the column.

Table I shows breakthrough volumes obtained for columns modified with different reagents. Each reagent, prepared with methanol–10 m*M* phosphate buffer (pH

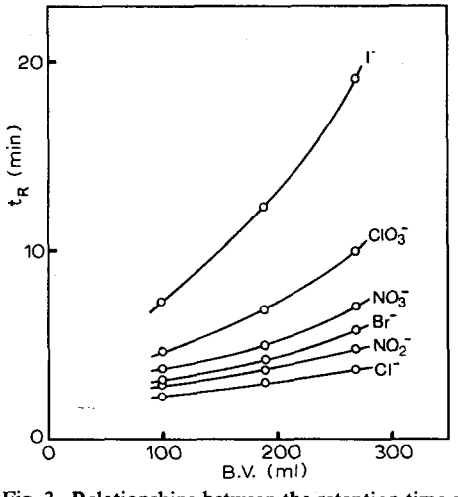

Fig. 3. Relationships between the retention time and the breakthrough volume. Column: 250 × 4.6 mm. Mobile phase: $5 \cdot 10^{-4}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 1.0 ml/min.

TABLE I

BREAKTHROUGH VOLUMES FOR $5 \cdot 10^{-4}$ *M* SALICYLATE

Reagent	*Concentration (mM)*	*Breakthrough volume (ml)*
Cetyltrimethylammonium bromide	2	85
Cetylpyridinium chloride	2	74*
Phenyltrimethylammonium chloride	10	0
Tetramethylammonium bromide	10	0

* After washing with methanol–water (50:50) for 50 min and with methanol for 1 h.

6.35) (50:50), was passed through the conventional silica gel column by the non-recycling method. The breakthrough volumes were measured using $5 \cdot 10^{-4}$*M* sodium salicylate. The columns modified with cetrimide and cetylpyridinium chloride retain salicylate and analyte anions. The selectivity of the columns modified with these two reagents is very similar. On the other hand, the columns modified with phenyltrimethylammonium chloride and tetramethylammonium bromide do not retain salicylate or analyte anions. For tetramethylammonium bromide, a phosphate buffer reagent solution (without methanol) was also examined, but the breakthrough volume for salicylate was again zero. Nevertheless, all four reagents adsorb on the silica surface during modification, which can be deduced from the observation that the modified surfaces are more hydrophobic than the bare silica surface. For the columns modified with phenyltrimethylammonium chloride and cetylpyridinium chloride, adsorption of these reagents on the silica surface can be confirmed by monitoring the effluent with a UV detector when washing with 0.05 *M* phosphoric acid–methanol (50:50) solution.

On the other hand, when pure methanol or acetonitrile was passed through an ODS column which was previously coated with cetylpyridinium chloride, it took a large volume to wash out the reagent. This means that the interaction between cetylpyridinium chloride and the ODS surface is very strong. This was also observed with the silica gel column dynamically modified with the cetylpyridinium reagent. From these observations, it may be concluded that cetrimide and cetylpyridinium chloride form a double layer on the silica surface, while phenyltrimethylammonium chloride and tetramethylammonium bromide form only a monolayer on the silica surface. The first layer of the reagent is formed by electrostatic interaction between nitrogen with a positive charge and a silanol group, while the secondary layer is formed by the hydrophobic interaction between the cetyl groups of the first layer and those of the reagent in the treating solution. It can be assumed that methyl or phenyl groups are not hydrophobic enough to form a secondary layer. The electrostatic interaction between the silica surface and the first layer is much stronger than the hydrophobic interaction between the first and second layer. Cetylpyridinium chloride is convenient for estimating the amount of the reagent adsorbed on the column. When 2 m*M* cetylpyridinium chloride in methanol–phosphate buffer (pH 6.35) (50:50) was passed through a conventional column for 4 h at 1.0 ml/min, followed by washing with methanol–water (50:50) for 50 min and with methanol for 1 h, 228

μmoles of the reagent were adsorbed on the surface. From the breakthrough data, 37 μmoles of salicylate were adsorbed on the modified surface. Assuming that each ammonium ion on the secondary layer interacts with one salicylate anion and hydrophobic adsorption of sodium salicylae on the modified surface is negligible, it can be estimated that 191 μmoles of the reagent form the first layer and 37 μmoles of the reagent (locally adsorbed on the first layer) form the secondary layer. Although we have no information about the surface properties of the silica gel used in this work, the surface coverage in the above case is roughly estimated to be 4%[23]. The retention mechanism of anions on the dynamically modified silica surface involves anion exchange, which is the same as in previous work, in which reversed-phase materials coated with cationic surfactants were used as the stationary phase[14,15].

Other parameters which can affect the chromatographic properties of the prepared columns involve the modification time, the concentration of the phosphate buffer solution, the composition of the treating solution (organic solvent *versus* aqueous solution), properties of the packing material (*e.g.*, surface area), etc. In fact, when the column was modified with 10 m*M* cetrimide dissolved in methanol–water (pH 5.8) (50:50) for *ca.* 17 h at 0.5 ml/min, the breakthrough volume was 3.4 times smaller than that obtained with 10 m*M* cetrimide dissolved in methanol–10 m*M* phosphate buffer (pH 5.9) (50:50) for 4 h at 1.0 ml/min. This difference in the breakthrough volume is mainly due to the effect of potassium phosphate. However, effects of the other parameters mentioned above have not been examined in detail in this work.

Fig. 4 demonstrates the indirect photometric detection of 10 nmoles of each inorganic anion using the silica gel dynamically modified with 10 m*M* cetrimide. It should be noted that the mobile phase does not contain cetrimide but salicylate. The analyte anions which are transparent at 254 nm give negative peaks, while iodide ion gives a positive peak due to its absorption. Iodate ion is not detected because its absorption just cancels the change of the background signal. Nearly the same chromatograms were obtained with the column modified with cetylpyridinium chloride. The retention order of the inorganic monovalent anions obtained with the modified

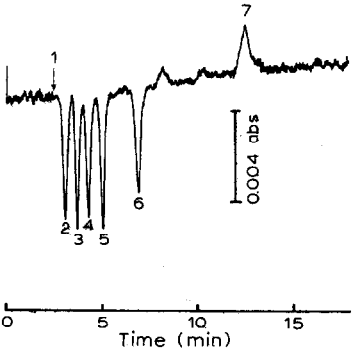

Fig. 4. Indirect photometric detection of inorganic monovalent anions. Column: 250 × 4.6 mm. Mobile phases: $5 \cdot 10^{-4}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 1.0 ml/min. Sample (10 nmoles each): 1 = iodate, 2 = chloride, 3 = nitrite, 4 = bromide, 5 = nitrate, 6 = chlorate, 7 = iodide. Detector: UV.

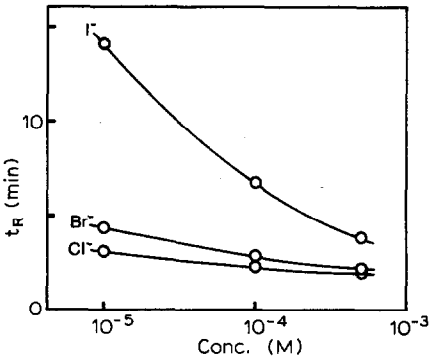

Fig. 5. Retention time *versus* the logarithm of the concentration of salicylate in the eluent. Column: 250 × 4.6 mm, dynamically modified with 2 m*M* cetrimide in methanol–10 m*M* phosphate buffer (pH 6.35) (50:50). Mobile phase: salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 1.0 ml/min.

silica columns is very similar to that observed with the common ion-exchange column[16]. Fluoride anion is not detected in this system, which may be due to its irreversible adsorption on the modified surface. The modified silica columns are very stable because cetrimide (or cetylpyridinium chloride) is not soluble in the mobile phase.

Fig. 5 shows the retention times of the analyte anions as a function of the logarithm of the concentration of salicylate in the eluent. The retention times of the analyte anions increase with decreasing concentration of salicylate. In ion-exchange chromatography, a linear relationship between the logarithm of the adjusted retention time and the logarithm of the eluent concentration was observed[4]. However, the ordinate in Fig. 5 is not plotted as the logarithmic scale of the adjusted retention time because it is difficult to estimate the void volume, and because the pH of the eluent changes depending upon its concentration, *viz.*, 4.7 for $5 \cdot 10^{-4}$ *M*, 5.4 for $1 \cdot 10^{-4}$ *M* and 5.7 for $1 \cdot 10^{-5}$ *M*.

In double-beam laser-based indirect fluorometry, the dynamic reserve maintains a $5 \cdot 10^3$ level until the concentration of the visualization reagent falls below 10^{-7} *M*[21]. Thus, indirect fluorescence provides lower detectabilities at low visualization reagent concentrations than indirect photometry. Also, the ion-exchange column should have quite low ion-exchange capacities so that the analyte ions can be eluted in reasonable time. The ion-exchange capacity of dynamically modified columns can be controlled by the preparation conditions, and is thus well-suited for this detection method.

Mass detectability is improved by using micro HPLC columns and a laser detection system. On the other hand, concentration sensitivity cannot be improved by reducing column dimensions. A laser is naturally suitable for small-volume detection. Fig. 6 demonstrates indirect fluorometric detection of inorganic anions with a microbore silica gel column, dynamically modified with cetrimide, in which 200 pmoles of each analyte is injected. The limit of detection is 6–15 pmoles (sub ng level) at S/N = 2. Many unknown peaks also appear in the chromatogram, which interfere with the separation of the analytes. The sample flow cell is connected to the outlet union of the column via stainless-steel tubing (35 mm × 0.33 mm I.D.), 1/16 in.

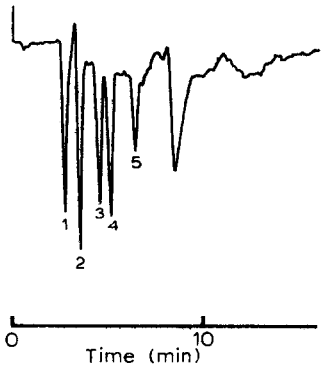

Fig. 6. Indirect fluorometric detection of inorganic anions. Column: 250 × 1 mm, dynamically modified with 1 m*M* cetrimide in methanol–10 m*M* phosphate buffer (pH 6.35) (50:50) for 4 h at 50 μl/min. Mobile phase: $5 \cdot 10^{-5}$ *M* salicylate (sodium salicylate–salicylic acid, 90:10). Flow-rate: 50 μl/min. Sample: (200 pmoles each): 1 = iodate, 2 = chloride, 3 = nitrite, 4 = bromide, 5 = nitrate.

zero-dead-volume union and PTFE tubing (100 mm × 0.19 mm I.D.). Total dead volume is 5.8 μl. The observed peak volume of chloride ion is 20 μl. Band broadening due to the above connecting parts can be estimated to be *ca.* 8% by using the Taylor equation[28]. On the other hand, band broadening due to injection parts is much smaller than the above value.

The displacement ratio between the monovalent anion and the salicylate anion is expected to be unity in ion-exchange chromatography. The displacement ratio can be calculated from the peak height and the peak volume. The change of concentration of the visualization reagent is calculated from the peak height and the background signal, while the concentration of the analyte is calculated from the peak volume using the following equation.

$$C_{max} = \sqrt{\frac{8}{\pi}} \frac{C_0 V_i}{V_w} \tag{2}$$

where C_{max} is the concentration of the analyte at top of the peak, C_0 is the concentration of the analyte in the sample solution, V_i is the injection volume and V_w is the peak volume. The observed displacement ratios are less than unity and decrease with decreasing concentration of salicylate, *e.g.*, 0.29 for $5 \cdot 10^{-4}$ *M*, 0.20 for $1 \cdot 10^{-4}$ *M* and 0.15 for $5 \cdot 10^{-5}$ *M*. This result may be due to the fact that salicylate molecules adsorbed on the hydrophobic groups can compensate for the induced signal derived from ion exchange between salicylate and analyte anions. The unknown peaks that appeared in the chromatogram may be due to complex interactions of salicylate (hydrophobic and electrostatic interactions). We did not use eluents with concentrations of salicylate less than $1 \cdot 10^{-5}$ *M* for fluorometric detection because the displacement ratio became very small (less than 0.1) and unknown peaks severely interfered with the analyte peaks.

CONCLUSIONS

Silica gel columns dynamically modified with quaternary ammonium salts with a long alkyl chain are suitable as stationary phases for the separation of inorganic anions. The retention times can be controlled by changing the preparation conditions. The formation of a double layer of the quaternary ammonium reagent with a long alkyl chain on the silica surface is suggested to explain the chromatographic retention behavior. Laser-based double-beam indirect fluorometry allows detection of sub-ng levels of inorganic anions by means of a microbore column. The advantage of dynamically modified silica gel columns lies in their low price, easy control of the column performance, and easy regeneration (compared to ODS or polystyrene columns). In addition, when a void appears on the top of the column due to hydrolysis of silica gel, fresh silica gel packing material can be manually replaced onto the column with a spatula. Finally, dynamical modification makes it straightforward to scale down to micro LC and open-tubular LC, with improved mass detectability.

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