Modification of eluent-induced stationary phases and fabrication of dendrimer-modified stationary phases in capillary liquid chromatography

(キャピラリー液体クロマトグラフィーにおける溶離液誘導型固定相の改質とデンドリマー修飾固定相の作製)

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Contents

Preface

In today's society, analytical chemistry is making a contribution to a wide range of issues such as quality control of chemical, medical, food, semiconductor, metal and ceramics products in industrial companies, screening and detection of environmental pollutant, scientific criminal investigation. And it is also playing an important role in state-of-the-art technologies such as electronics, biotechnology, *etc*.

Chromatography has extensively developed as one of major methods in analytical chemistry. It has been more than 100 years since chromatography was discovered by the Russian scientist Mikhail Semonovich Tswett. The trigger of the start of chromatography is his observations with filter paper extraction, and he separated yellow, orange, and green plant pigments in leaves through an open glass column packed with calcium carbonate by draining petroleum ether. This seemingly easy technique has been expanded to gas chromatography (GC), liquid chromatography (LC) and thin-layer chromatography (TLC). The chromatographic method invented by Tswett requires a lot of time for analysis, since samples pass down through a column by gravity. The need for high-speed and better resolution analyses of samples led to the development of high performance liquid chromatography (HPLC) since the end of 1960s. Thereafter, HPLC system including stainless-steel columns, pumps, detectors, suppression technology and integrators has been updated to withstand higher pressure, enhanced sample detection capabilities and achieve higher resolution, and then HPLC has been commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and quantification far beyond previous techniques, and computers and automation provided convenience. Improvements in reproducibility were made as techniques such as micro-columns, affinity columns, and fast HPLC emerged. While the HPLC system has been improved, packing materials has been downsized and microparticulate packing materials (< 10 μm d_p) that are widely used today were developed and packed using high-pressure slurry techniques in the early 1970s. They provided even greater speed, efficiency, and sample capacity advantages. In the late

1980s, monolithic columns that have “sponge-like” like structure were developed, and they offer shorter diffusion distances and a number of flow paths for solute dispersion. The continuous porous rod structure is extremely permeable and provides large surface area for separation compared to packing materials. While high backpressure is arisen in association with reduction of particle size of packing materials, backpressure problems are easily overcome when using monolithic columns.

We used capillary columns which have smaller diameter compared to conventional columns. Capillary columns save consumption of eluent, samples and packing materials. They also simplify access to direct coupling of LC and mass spectrometry (MS) that make quantitative analysis of mixtures possible even when their separations are inadequate in LC. In this study, retention behavior of analyte and enhancement of detection will be discussed to improve chromatographic parameters through development of stationary phases and investigation of mobile phase effect.

Chapter 1

Introduction

1.1 Chromatography

The starting point of chromatography is on 21 March 1903 as Russian botanist Mikhail Semenovich Tswett (1872-1919) presented a lecture “On a new category of adsorption phenomena and their application to biochemical analysis” at the Biological Section of the Warsaw Society of Natural Sciences [1]. He reported separating different colored constituents of leaves by passing an extract of the leaves through a column of calcium carbonate, alumina, and sucrose in 1906. He coined the word **chromatography** from the Greek words “chroma” and “graphein” meaning “color” and “to write” respectively [2, 3].

Chromatography is a versatile technique for separating mixtures. Mix samples containing various components are injected into a mobile and a stationary phase which are held in equilibrium. Each component is distributed isostatically in the two phases continuously, and then be separated by the difference of the affinity of each component. In a place where the components are separated, it is called a stationary phase and it retains samples. The other is a mobile phase, which is equilibrated with the stationary phase, carries the samples. The samples distribute continuously in equilibrium between the mobile and the stationary phases [3, 4].

There are various chromatography methods which are based on the several combination states of a mobile and a stationary phase. Those are called gas chromatography (GC), liquid chromatography (LC) and supercritical-fluid chromatography (SFC), in which each mobile phase is a gas, liquid, and supercritical fluid, respectively [5].

LC is applicable for various samples, *i.e.* high-boiling, thermally unstable, high polarity and macromolecular substance; whereas GC is suitable only for samples with boiling point below approximately 300 degrees Celsius. However, in LC, the molecule diffusion speed is slow, owing to

the use of liquid as the mobile phase. Therefore, the drawback of LC is that the analysis time is longer compared to that of GC [6]. A solution to this problem is that the eluent should be potently passed through the column by an additional pressure. Currently, packing materials endurable to high pressure have been developed, and therefore, high speed separation of samples could be accomplished [4].

1.2 Ion chromatography

Ion chromatography (IC) is a physical separation method utilizing the affinity between ionic samples and an ion-exchange resin under the influence of hydrated ionic radius size and van der Waals interaction, *etc.*. The affinity between ionic samples and the ion-exchange resin is normally affected by multiple effects. In conventional IC, the separation mechanism is mainly caused by electrostatic interaction, thus ions containing high charge will have greater affinity (*i.e.* be retained longer) than that containing lower charges.

The ions in the sample and the mobile phase are continuously exchanged on the stationary phase in conformity with the relative affinity for the ion-exchange resin, sample ions are then separated [7].

IC has grown to become the method of choice for the determination of multiple inorganic cations and anions in solution since its introduction by Small *et al.* in 1975 [8], and is now the most widely applied analytical method for the determination of the ion composition of aqueous samples [9].

1.3 Capillary liquid chromatography

LC can be classified based on the column diameter. Columns with the diameter smaller than 0.075 mm are used in nano-LC and its flow rate is less than 1 μL/min, whereas the column diameter in μLC is 0.2-0.8 mm and its flow rate is 1-20 μL/min. Capillary LC is defined as the one involving nano-LC and μLC. Classification of LC, based on its general purpose, is shown in Table 1-1.

The future of capillary LC are attributed to the use of smaller-diameter columns and lower eluent flow rate. The lower flow rate saves solvents, reagents and packing materials, compared with

conventional LC using 4-6 mm ID columns [10].

Table 1-1 Classification of LC

Purpose	Classification	ID (mm)
Analytical	Nano-LC	~0.075
	μLC	0.2~0.8
	Semi-μLC	1.0~2.1
	Conventional LC	4.0~6.0
Preparative	Preparative LC	10~

Table 1-2 shows development history of capillary-based separation methods and their related techniques.

Table 1-2 Development history of capillary-based separation methods and their related technique

Year	Developed capillary separation methods and their related technique	References
1974	μLC	11
1978	Open-tubular capillary LC	12
1978	Packed microcapillary LC	13
1979	Fused-silica capillary	14
1981	Capillary zone electrophoresis	15
1985	Electrokinetic chromatography	16
1987	Capillary electrochromatography	17, 18
1989	Monolithic polymer capillary columns	19, 20
1992	Capillary array electrophoresis	21
1998	Monolithic silica capillary columns	22

1.4 Packing materials

Packing materials for LC are mainly divided into two types, *i.e.* silica- and polymer-based materials, and have respective advantages and disadvantages.

Silica packing materials generally have higher rigidity, theoretical plate number and retention ability than the polymeric materials; however, silica gels are chemically unstable and dissolve under basic conditions. Conversely, polymeric materials are chemically stable and can be operated in a wide range of pH. Nevertheless, silica materials generally have stronger hydrophilic property and longer life time than those of polymeric materials [23].

1.5 Monolithic materials

Monolithic materials are continuous porous rod structures characterized by mesopores and macropores. These pores provide monoliths with high permeability, a large number of channels, and a high surface area available for reactivity [24].

Since their inception in the late 1980s, a vast variety of useful porous monolithic materials have been described and their applications have been demonstrated [25, 26]. These monoliths were primarily used in chromatographic applications and emerged to address some of the well-known drawbacks of particulate sorbents such as the micrometer-sized bead use in packed high-performance liquid chromatography (HPLC) columns. Despite the immense popularity of bead-based separation media, slow diffusional mass transfer of macromolecular solutes into stagnant pool of the mobile phase present in their pores and the large void volume between packed particles remain significant issues in applications such as the rapid and efficient separation of macromolecules [27]. Monoliths are easy and fast to prepare, do not require frits, have low back pressure and can operate at high flow rates due to their permeability [28].

1.6 Terms of column efficiency

The separation efficiency of a column can be presented in terms of the number of theoretical plate. A theoretical plate is defined on the basis of distillation theory, by which each theoretical plate in chromatography can represent the image of a single equilibrium step. The number of theoretical plate can be calculated from the following equation.

$$N = 16\left(\frac{t_R}{w_b}\right)^2 \quad (1)$$

Where N is the number of plates of a column, t_R is the retention time, and w_b is the peak width measured at the base in the same units as t_R [3].

Height equivalent of one theoretical plate (HETP) which is the relationship between peak width and the distance can be calculated by dividing the length of the column, L, by N as shown in the following equation.

$$H = \frac{L}{N} \quad (2)$$

1.7 Van Deemter equation

Van Deemter equation indicates HETP calculated from these three independent factors below.

$$H = Ad_p + \frac{B}{u} + C{d_p}^2 u \quad (3)$$

The A term is eddy-diffusion parameter which is related to the difference between the uniformity and non-uniformity of the flow to and around a particle. It is proportional to the particle diameter of the packing material. The B term is diffusion coefficient which is diffusion of the analyte in the longitudinal direction, and inversely proportional to the flow-rate of the mobile phase. The C term is resistance to mass transfer coefficient of the analyte between mobile and stationary phase and related to both the particle diameter of the packing material and the flow-rate. When these three factors are totalized, HETP increases with increasing flow-rate of mobile phase as shown in Fig. 1-1.

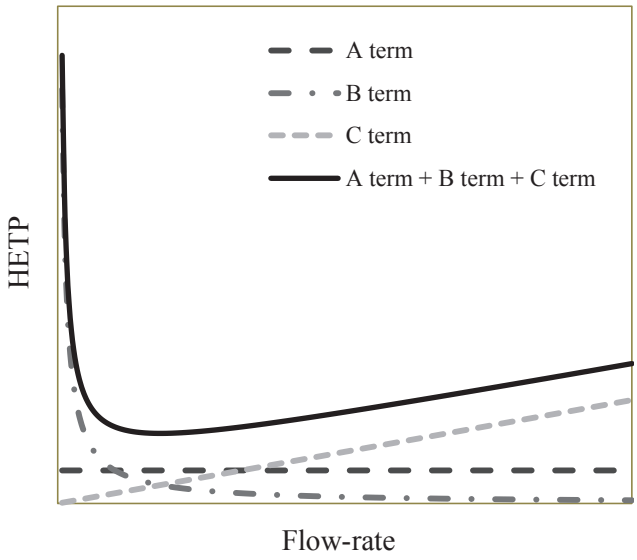

Fig. 1-1 Van Deemter plot

HETP increase with increasing flow-rate as shown in the figure, which means an increase of a flow-rate has a bad effect on the chromatographic resolution, and the lowest point in the curve shows the highest efficiency. If particle size is reduced by half, H is reduced by a factor of 2. Hence, it is possible to reduce band spreading within a column by utilizing smaller particles. As can be seen in van Deemter equation, if particle size is reduced, H is reduced by A and C terms.

For example, Fig. 1-2 shows a van Deemter plot of a 5 μm particle, a 3 μm particle and a 2 μm particle. As observed on the plot, a large 5 μm particle has a narrow optimal operating range. If the flow-rate of the mobile phase is too slow or too fast, an increase in H is observed, thus reducing chromatographic resolution and sensitivity. However, the 2 μm particle exhibits a much lower H value at a higher flow-rate. This means that a higher efficiency can be achieved in a faster time than with a column packed with larger 10 μm particles.

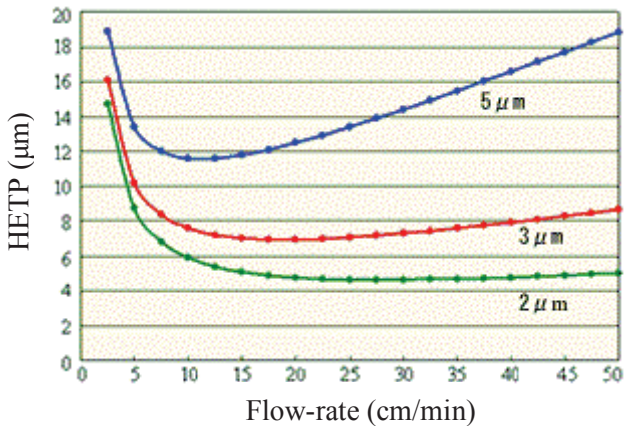

Fig. 1-2 Van Deemter plot comparing different sizes of particle [29]

1.8 Dynamic coating

Packing materials could be modified *via* two approaches, *i.e.* the chemical modification by covalent bonding and the dynamic coating by van der Waals attraction. The chemical modification is irreversible, which rarely get back to the original condition once modification is done. In contrast, the dynamic coating desorbs easily from the stationary phase by heating or reducing the pressure, due to the weaker interaction than that of chemical modification. However, the advantages of dynamic coating are shorter reaction interval and easy modification.

1.9 Conductivity detector

The conductivity detector detects ionic compounds by measuring electric conductivities based on the ionic property of the samples. If there are ions from the sample contained in the mobile phase flowing from the separation column outlet to the detector, then the electric conductivity in that area will vary according to the variation in ion concentration. It detects all ions existing in aqueous solutions.

Each ion has a characteristic constant called the equivalent conductivity, where the larger this value, the larger the detected peak. It is expressed in terms of Scm^2/mol units and indicates how easily

current flows per mole.

1.10 Objectives of the present research

IC has improved and discussed deeply for about 40 years. It is a very simple technique in principle, yet it has made a huge contribution to science as a powerful analytical method and it is a necessary method especially for ion analysis. Although studies about chromatography is getting saturated after rapid advancement of IC for 40 years, there are still challenges remaining such as downsizing of the system, improvement of column efficiency and enhancement of detection, since IC is being heavily used in several fields such as water quality analysis and management, and demand of IC is still growing in market. In the present research, capillary chromatography columns were used for low solvent consumption, high column efficiency and good sensitivity.

Chapter 2 describes the separation of inorganic anions on a hydrophobic column using crown ethers as eluent additive. Crown ethers which are adsorbed onto a reversed-phase column, form positively charged anion-exchange sites when they combine with eluent cations. Polymeric packing material was used to withstand under a wide range of pH. The column efficiency has been improved by using a conductivity detector with eluent containing potassium hydroxide.

Chapter 3 describes the preparation of a novel stationary phase possessing hyper hyperbranched polymer. The stationary phases were modified by simple reactions of ammonia and 1,4-butanedioldiglycidyl ether. Inorganic anions were separated on the stationary phase which consists of a tremendous amount of amine groups. The retention times of inorganic anions could be controlled by adjusting the length of the hyperbranched polymer.

Chapter 4 elucidated the fabrication of nucleotide-bonded zwitterionic stationary phases via a two-step covalent modification using 3-glycidyloxypropyltrimethoxysilane as the spacer. Two typical nucleotides *i.e.* cytidine5'-monophosphate and adenosine5'-monophosphate were reacted to the glycidyl-bonded silica gels through epoxy-ring opening reaction under various conditions. The resulted columns were evaluated for the separation of inorganic ions (3 anions and 5 cations) and polar

compounds (4 organic acids and 3 nucleobases) using a UV detector under ion-exchange and hydrophilic interaction chromatographic modes, respectively.

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Chapter 2

Separation of Inorganic Anions on Hydrophobic Stationary Phases Dynamically Modified with 18-Crown 6-Ethers

2.1 Introduction

Crown ethers are macrocyclic compounds that contains hydrogen, carbon and oxygen atoms repeat in a regular order, *i.e.* $(-CH_2-CH_2-O-)_n$. Cavities of crown ethers are materially filled with electrons by unshared pair of electrons. Therefore, crown ethers trap metal cations easily by electrostatic attractive force. Crown ether is called it because of the appearance of its molecular model and its ability to crown cations. The trivial names of crown ether consist of, in order: (1) the number and kind of hydrocarbon rings, (2) the total number of atoms in the polyether ring, (3) the class name, crown, and (4) the number of oxygen atoms in the polyether ring [1].

18-Crown 6-ethers (18C6E) compose of 18 atoms including 12 carbons and 6 oxygens working as a ligand for some metal cations and it has a particular affinity to potassium cations. The binding constant increases in the order $Li^+ < Na^+$, $Cs^+ < Rb^+ < K^+$ [2, 3].

In IC, crown ethers are added for obtaining better separation selectivity of cations. The simultaneous separation of common alkali, alkaline-earth and ammonium cations (Li^+, Na^+, NH_4^+, K^+, Mg^{2+} and Ca^{2+}) are difficult on traditional cation-exchange columns containing generally sulfonate or carboxylate groups. A quantitative determination of low concentration of ammonium on traditional cation-exchange is challenging, since sodium and ammonium often elute in similar retention behavior [4-7]. Crown ethers form complexes with several mono- and divalent cations and 18C6E specifically forms stable complexes with ammonium and potassium ions. Crown ethers have improved separation efficiency and selectivity of cations on cation separation columns in several studies [4-6, 8-13]. In some cases, it has also been reported that crown ethers could separate anions. Crown ethers which are adsorbed onto a reversed-phase column, form positively charged anion-exchange sites when they

combine with eluent cations [14]. It has been reported that crown ethers on a hydrophobic stationary phase could separate anions based on cations, which are trapped on the crown ethers, working as anion-exchange sites [15-17]. Silica gels and polymer resins coated with crown ether derivatives could separate cations and anions [17-19]. It has also been reported that the columns bonded with crown ethers could separate cations by a complex forming reaction [20-23] and anions on positively charged anion-exchange sites of crown ether complexes [22-24].

Inorganic anions have been separated on triacontyl-functionalized silicas modified with crown ethers in a previous study [15]. The aim of this study was to apply the previous study to electrical conductivity detection which is the typical detection method for ion chromatography. The retention behavior of inorganic anions investigated on a stationary phase dynamically modified with 18C6E in IC. Silica gels cannot be used under basic conditions and they will be dissolved especially in eluents with pH more than 10. In order to overcome this drawback, polymeric columns, which are relatively stable over a wide range of pH, were applied to the separation of anion.

Additionally, the second approach is to use a polymer monolithic column, which has larger porosity as well as surface area than the particulate column, for improving the separation of target anions.

2.2 Experimental

2.2.1 Chemicals

L-column2 ODS (mean particle diameter, 5 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan) and PLRP-S (mean particle diameter, 8 μm; Agilent Technologies, Santa Clara, CA, USA) were used as the stationary phase. Oasis MCX (The mean particle diameter, 30 μm; Waters, Massachusetts, USA) was used as the suppressor. 18C6E, dibenzo-16-crown-6, 2,2'-azobis(isobutyronitrile), sodium chloride, potassium chloride, sodium iodate, hydrochloric acid and potassium hydroxide were purchased from Wako Pure Chemical Industries (Osaka, Japan). 1,4-butanediol, 1-propanol, toluene, ammonium chloride, sodium nitrate, sodium nitrite, sodium iodide,

sodium thiocyanate and ethylbenzene were purchased from Nacalai Tesque (Kyoto, Japan). Lithium chloride, cesium chloride, magnesium chloride and calcium chloride were purchased from Yoneyama Yakuhin Kogyo (Osaka, Japan). HPLC grade acetonitrile was purchased from Kanto Chemical (Tokyo, Japan). All the other reagents and solvents were purchased from Tokyo Chemical Industry (Tokyo, Japan) unless otherwise specified. Microcolumns used in this study were prepared in our laboratory and packed into a fused-silica capillary tube with polyimide coating (100 mm × 0.32 mm I.D.; GL Sciences, Tokyo, Japan) by dispersing packing materials in methanol. Distilled deionized water used for preparing all reagents was purified with a Simplicity UV water distillation system (Merck Millipore, Darmstadt, Germany).

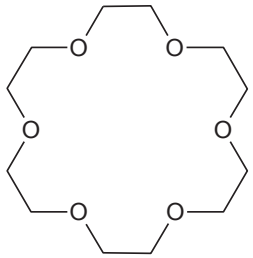

Fig. 2-1 Chemical structure of 18-crown-6-ether

2.2.2 Apparatus

The HPLC system used in this study comprised a microfeeder (L.TEX Corporation, Tokyo, Japan) equipped with a gas-tight syringe (0.5 mL; Ito, Fuji, Japan) as a pump, an injector M-435 Micro Injection Valve of 0.2μL injection volume (Upchurch Scientific, Oak Harbor, WA, USA) , a UV detector UV-970 (JASCO, Tokyo, Japan), a conductivity detector Tracedec Contactless Conductivity detector (Innovative Sensor Technologies, GmbH, Germany) ,a data processor CDS-Lite (LA soft, Chiba, Japan) and a microcolumn prepared from a fused-silica capillary tube (100 mm × 0.32 mm i.d.; GL Sciences, Tokyo, Japan) and The inlet pressure was monitored by an L.TEX-8150 Pressure Sensor (L.TEX).

2.2.3 Preparation of C18 polymer monolithic column

The fused-silica capillaries were pretreated by rinsing with acetone and water, and then flushed using a syringe pump at a flow rate of 0.25 μL /min with 0.2 mol/L of sodium hydroxide and 0.2 ml/L HCl for 30 min. Finally, 20%(w/w) ethanol solution of 3-(trimethoxysilyl)propyl methacrylate with an apparent pH adjusted to 5 using acetic acid was pumped through the capillary at a flow rate of 0.25 μL/min for 1 h. The capillary was then dried in a stream of nitrogen, and left at room temperature for overnight before use.

C18 polymeric monoliths were prepared in the pretreated capillaries using in-situ polymerization of a mixture that comprised the different ratios of stearyl methacrylate and ethylene dimethacrylate, 0.56 mL 1,4-butanediol, and 0.14 mL 1-propanol. For an initiator, 3 μg 2,2'-azobis(isobutyronitrile) was used. This polymerization mixture was sonicated for 10 min, then purged with nitrogen for 3 min in order to remove oxygen and finally introduced into the capillaries. Both ends of the capillary were sealed with PTFE stoppers and the capillary was placed in a water bath. Polymerizations ware carried out at temperatures of 60°C for 24 h. After the polymerization reaction was completed, both ends of the capillary were cut to adjust their lengths to 10 cm and the unreacted components were removed by flushing with acetonitrile using a syringe pump [25].

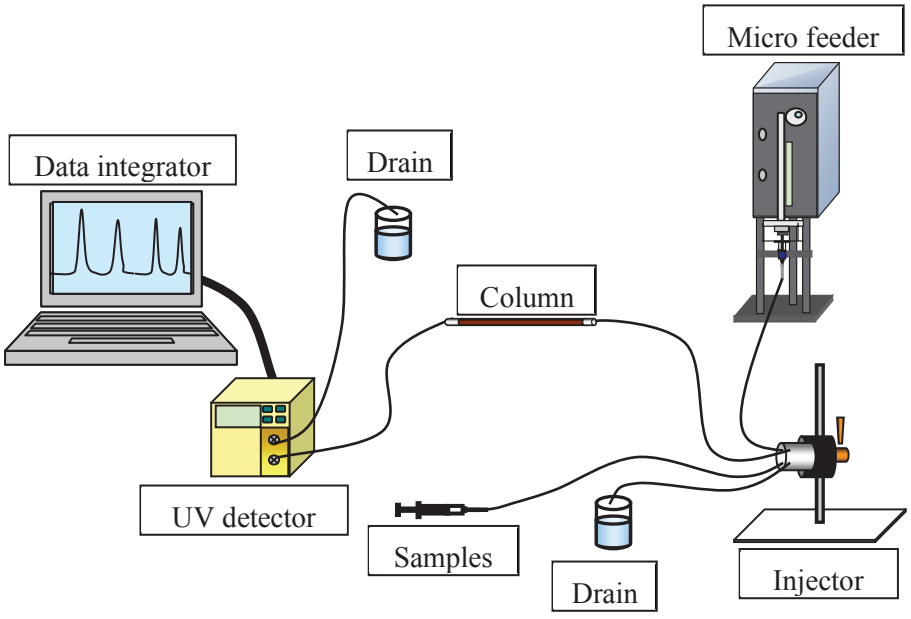

Fig. 2-2 Illustration of the capillary LC system

2.2.4 Preparation of styrene-divinylbenzene copolymer monolithic column

The fused-silica capillaries were pretreated by the same procedures as described in session 2.2.2. Styrene-divinylbenzene copolymeric monoliths were prepared in pretreated capillaries using in situ polymerization of a mixture that comprised 24% styrene, 16% divinylbenzene, 42% dodecanol as macroporogen, and 18% toluene as microporogen. For an initiator, 1% 2,2'-azobis(isobutyronitrile) was used. This polymerization mixture was sonicated for 10 min, and then purged with nitrogen for 3 min in order to remove oxygen and finally introduced into the capillaries. Both ends of the capillary were sealed with PTFE stoppers and the capillary was placed in a water bath. Polymerizations were carried out at temperatures of 60°C for variable polymerization times. After the polymerization reaction has completed, both ends of the capillary were cut to adjust their lengths to 10 cm and the unreacted components were removed by flushing with acetonitrile using a syringe pump [26-28].

2.3 Results and discussion

2.3.1 Measurement on the silica packing column

Five common inorganic anions were separated on the ODS column bonded with octadecyl chain, as shown in Fig. 2-3. The eluent contained 18C6E and potassium chloride. The 18C6E adsorbed on the stationary phase by hydrophobic interaction and form inclusion complexes with potassium cations by ion-dipole interaction, and the trapped cations retained the target anions.

Elution order of the anions is mainly affected by their ionic radius sizes. The smaller the ion radius is, the larger the charge density will be, and the larger the force to attract a solvent, thus causing the solvent to intercept anions from adsorbing onto the surface of the ion-exchange resin. The thiocyanate ion was retained for a longer time than anything else owing to the extra hydrophobic interaction that occurred on the surface of the ODS stationary phase.

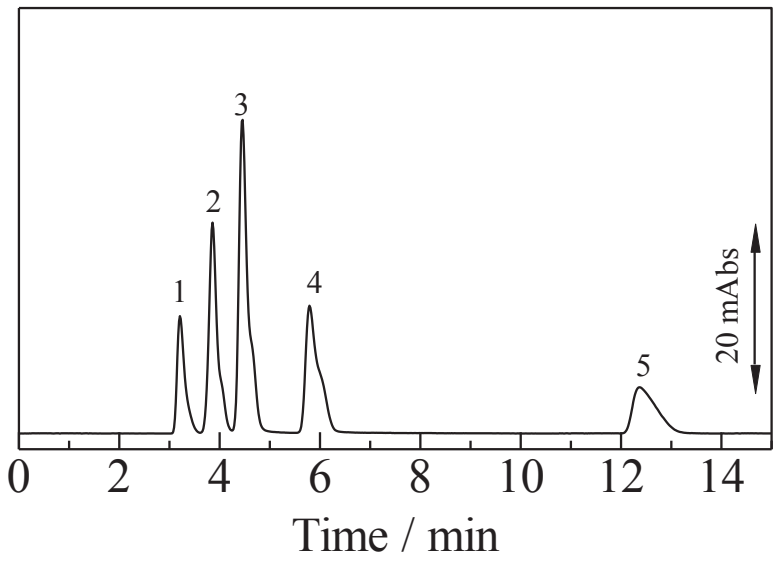

Fig. 2-3 Separation of five common inorganic anions on the ODS column. Column: L-column2 ODS (100 × 0.32 mm I.D.); eluent: 1 mM 18C6E + 10 mM KCl; analytes: 0.4 mM each of 1 = IO_3^-, 2 = NO_2^-, 3 = NO_3^-, 4 = I^-, 5 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

2.3.2 Measurement on styrene-divinylbenzene copolymer packing column (polystyrene packing column)

The use of polystyrene packing column, *i.e.* a kind of polymeric packing material, which can be operated in a wide range of pH, was also investigated. The separation of five common inorganic anions was achieved on the polystyrene packing column using 5 mM 18C6E, 10 mM potassium chloride and the addition of 4% acetonitrile, as shown in Fig. 2-4. Apparently, the peaks of nitrite and nitrate slightly overlapped each other owing to the broad peaks obtained. This could be due to the fact that the particle size of the polystyrene gel used was 8 μm, while those of the ODS was 5 μm; by using smaller polystyrene particles could improve the peak shape.

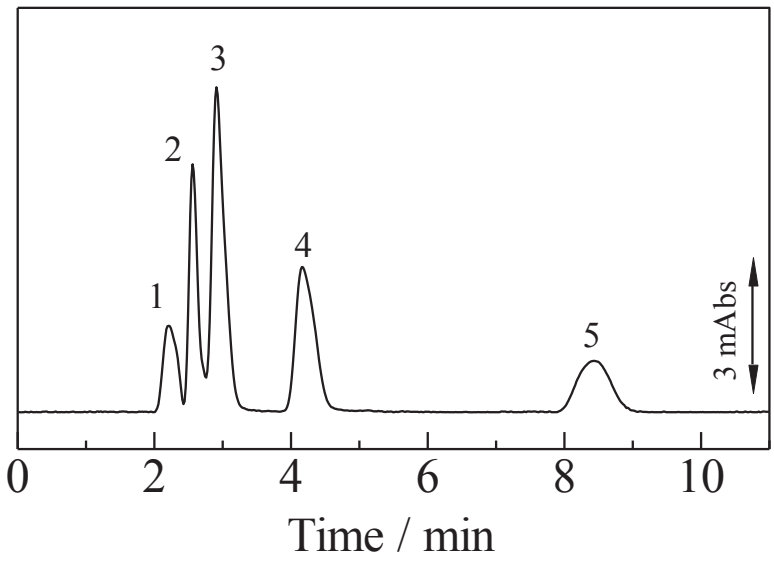

Fig. 2-4 Separation of five common inorganic anions on the polystyrene column. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + 10 mM KCl + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_2^-, 3 = NO_3^-, 4 = I^-, 5 = SCN^- ; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

Eluents that contained either 18C6E or potassium chloride were also used to investigate whether crown ethers could really trap potassium ions and works as the ion-exchange sites, and the results are shown in Fig. 2-5. The use of both eluents could not achieve any retention of anions. Both 18C6E and potassium chloride are needed to be contained in the eluent in order to retain anions, and hence crown ethers trap potassium ions and work as the ion-exchange sites.

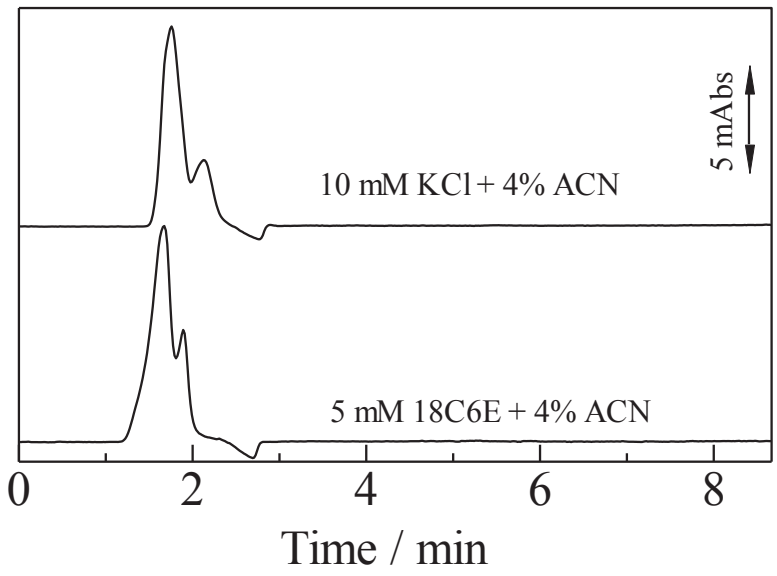

Fig. 2-5 Effect of 18C6E or KCl on the polystyrene column. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E or 10 mM KCl + 4% ACN; analytes: 0.1 mM each of IO_3^-, NO_3^-, I^-, SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

2.3.3 Effect of acetonitrile

The effect of the addition of acetonitrile in the eluent was also evaluated using the polystyrene column, and it was found that the retention of the anions increased with decreasing acetonitrile concentration, as shown in Fig. 2-6.

In addition, retention times of thiocyanate ion (SCN^-) and the passage of time were plotted on a vertical axis and a horizontal axis respectively in Fig. 2-7. Acetonitrile was added to the eluent for permeation into pores of the packing materials. Fig.2-7 shows that the retention time of the thiocyanate ion slightly decreased with the passage of time, indicating that the aqueous mobile phase was pulled out of the pore of the hydrophobic packing materials and thus resulting in shorter retention time [29]. The retention time was, however, stabilized when the addition of acetonitrile was more than 4%.

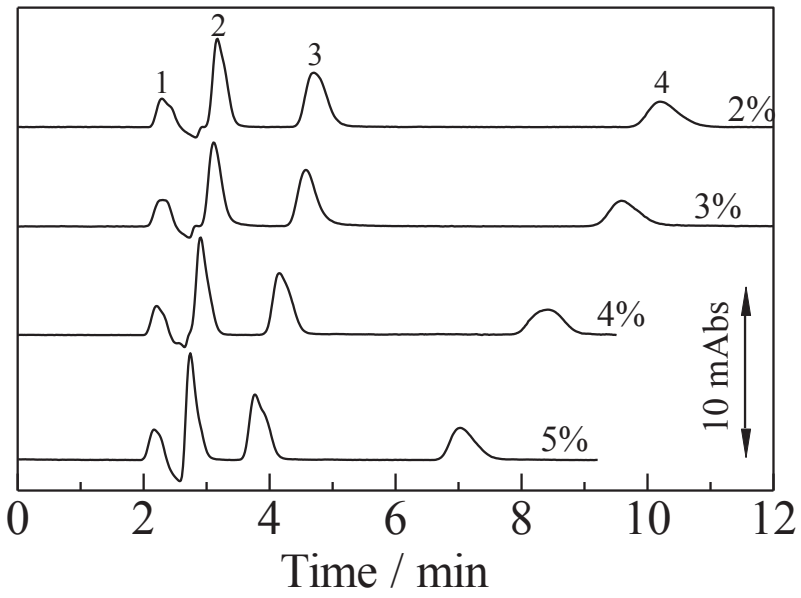

Fig. 2-6 Effect of acetonitrile on the retention of common anions. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + 10 mM KCl + different concentrations of ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

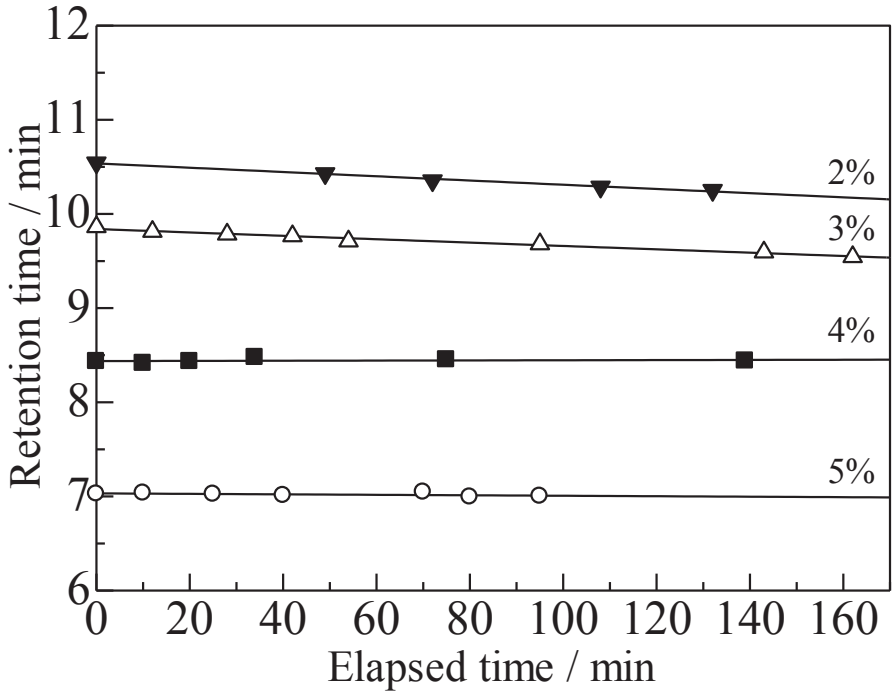

Fig. 2-7 Retention behavior of thiocyanate ion on the polystyrene column over time. Operating conditions were the same as Fig. 2-6.

2.3.4 Effect of 18-crown 6-ether concentration

The effect of 18C6E concentration on the retention of anions was examined by using different concentrations of 18C6E together with 10 mM potassium chloride and 4% acetonitrile as the eluent on the polystyrene column, and the results are shown in Fig. 2-8. The retention time of the anions increased with increasing 18C6E concentration. The increase in the retention time is due to the increase in the amount of adsorbed 18C6E on the polystyrene column. When 9 mM 18C6E was used as the eluent, the retention time did not increase obviously. It is inferred that excess 18C6E passed through the stationary phase because the surface of the polystyrene gel had been saturated with 18C6E.

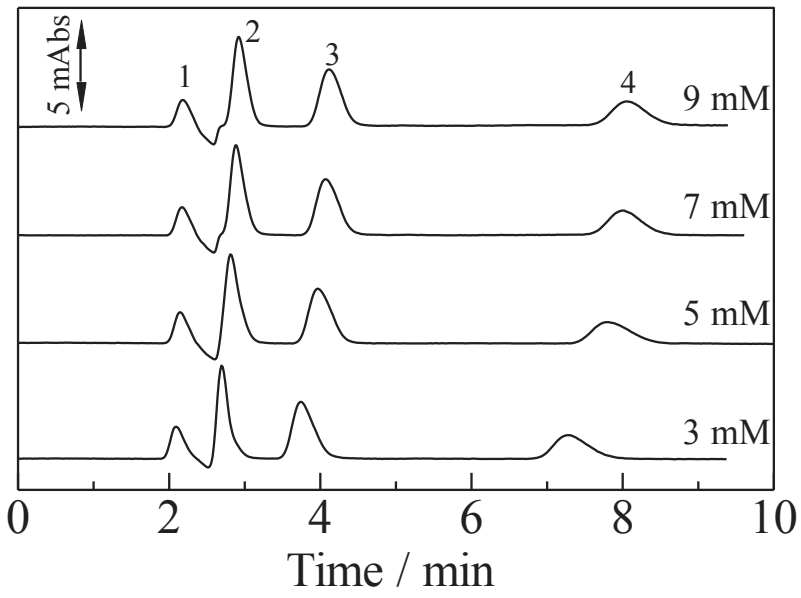

Fig. 2-8 Effect of 18C6E concentration on the retention of common anions. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: different concentrations of 18C6E (as indicated) + 10 mM KCl + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

2.3.5 Effect of eluent cation

Fig. 2-9 shows that the separation of the anions by using multiple different cations. The retention time increased in the order calcium chloride < magnesium chloride < lithium chloride < sodium chloride < cesium chloride < ammonium chloride < rubidium chloride < potassium chloride.

The size of the cavity of 18C6E is 2.6-3.2 Å and form inclusion complexes adequately with cations with sizes in between 0.80–0.97 fold of the size of the cavity. Table 2-1 [30] indicates that potassium cation is better fitted with the size of the cavity, thereby when the potassium chloride was used as the eluent, it gave the strongest retention. Additionally, Table 2-2 [15] indicates the association constants for 1:1 complexation between 18C6E and metal ion in the order: sodium chloride < cesium chloride < ammonium chloride < rubidium chloride < potassium chloride.

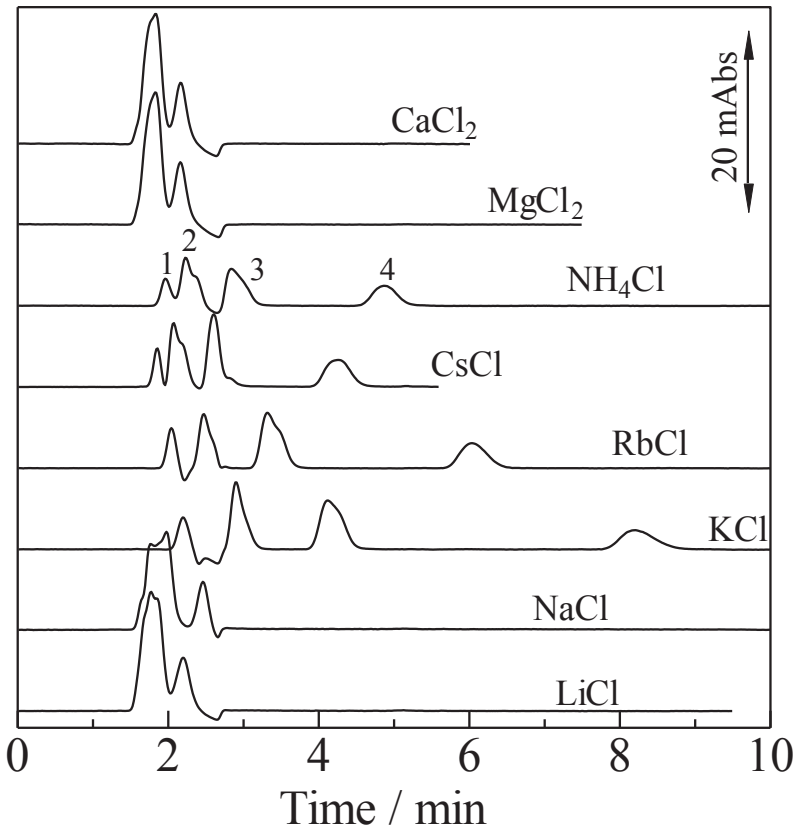

Fig. 2-9 Effect of eluent cation on the retention of common anions. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + 10 mM salt (as indicated) + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 µL; flow-rate: 4.2 µL/min; detector: UV; wavelength of UV detection: 210 nm.

Table 2-1 Size of cations [30]

Monovalent cations		Divalent cations	
Cation	Diameter (Å)	Cation	Diameter (Å)
Li^+	1.20	Ca^{2+}	1.98
Na^+	1.90	Mg^{2+}	1.30
K^+	2.66		
Rb^+	2.96		
Cs^+	3.34		
NH_4^+	2.84		

Table 2-2 Association constants for 1:1 complexation between 18C6 and metal ion [15]

Monovalent cations			Divalent cations		
Cation	Log K	Temperature	Cation	Log K	Temperature
Li^+	~0	27 ± 1°C	Ca^{2+}	<0.5	25°C
Na^+	0.80 ± 0.10	25°C	Mg^{2+}	-	
K^+	2.03 ± 0.10	25°C			
Rb^+	1.56 ± 0.02	25°C			
Cs^+	0.99 ± 0.07	25°C			
NH_4^+	1.23 ± 0.06	25°C			

2.3.6 Effect of salt concentration

The effect of salt concentration on the retention of anions on the polystyrene column was examined by using 5 mM 18C6, different concentrations of potassium chloride and 4% acetonitrile as the eluent, and the results are shown in Fig. 2-10. The retention time of the anions increased with increasing potassium chloride concentration. The increase in the retention time is due to the increase in the amount of trapped potassium ion on 18C6, hence the ion exchange capacity increases. However, when salt concentration was 80 mM, the retention time decreased owing to the increase of chloride concentration in the eluent, the elution strength then increased. Thus the reciprocal effect arises as the concentration of potassium chloride increases, and then its balance controls the retention ability.

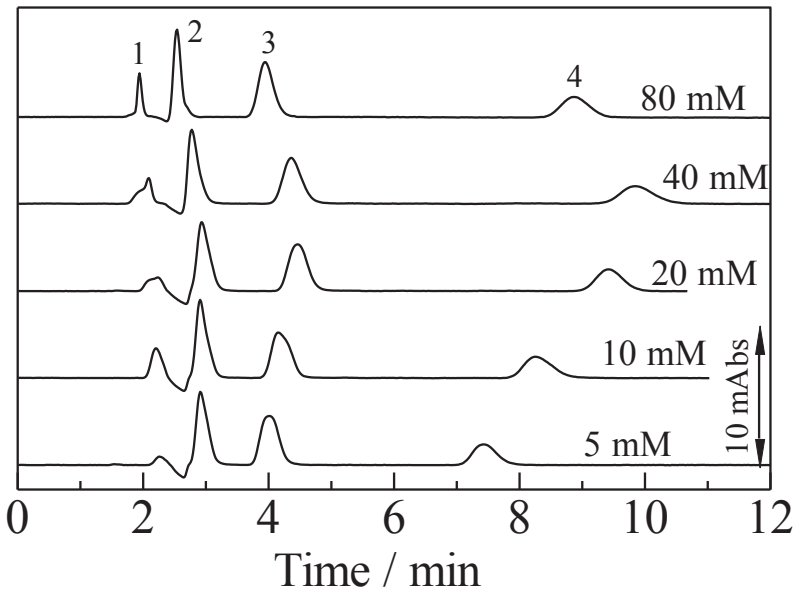

Fig. 2-10 Effect of salt concentration on the retention of common anions. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + different concentrations of KCl (as indicated) + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

2.3.7 Use of basic eluent

The effect of basic eluent was investigated by using 5 mM 18C6E, 10 mM potassium hydroxide, 4% acetonitrile as the eluent. No significant difference was observed in the retention of the anions as compared to the eluent contained potassium chloride when the eluent contained potassium hydroxide was used, as shown in Fig. 2-11.

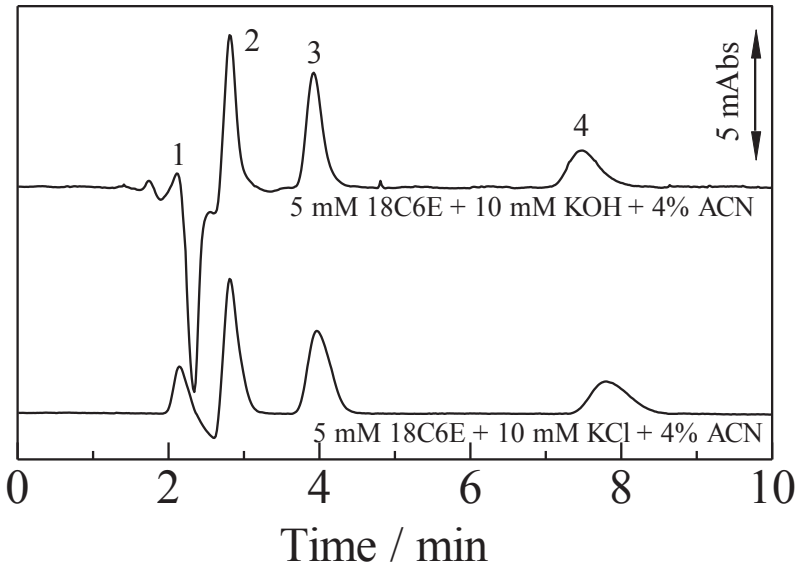

Fig. 2-11 Effect of basic eluent. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + 10 mM KCl or KOH + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

2.3.8 Use of conductivity detector

When the eluent contained potassium hydroxide was used, no significant difference was observed in the retention of the anions as compared to the eluent contained potassium chloride. Therefore, the detection of anions was also demonstrated with the eluent containing potassium hydroxide by using a contactless conductivity detector, which is expected to have higher sensitivity compared to UV detection. The conductivity of the eluent was suppressed by using a suppressor column (100 mm × 0.32 mm I.D.) packed with Oasis MCX. Comparing Figs. 2-11 and 2-12, it was found that the

interfering water dip (which appeared between the IO_3^- and NO_3^- peaks in Fig. 6) was not observed, and the iodate peak intensity was greatly enhanced in Fig. 2-12.

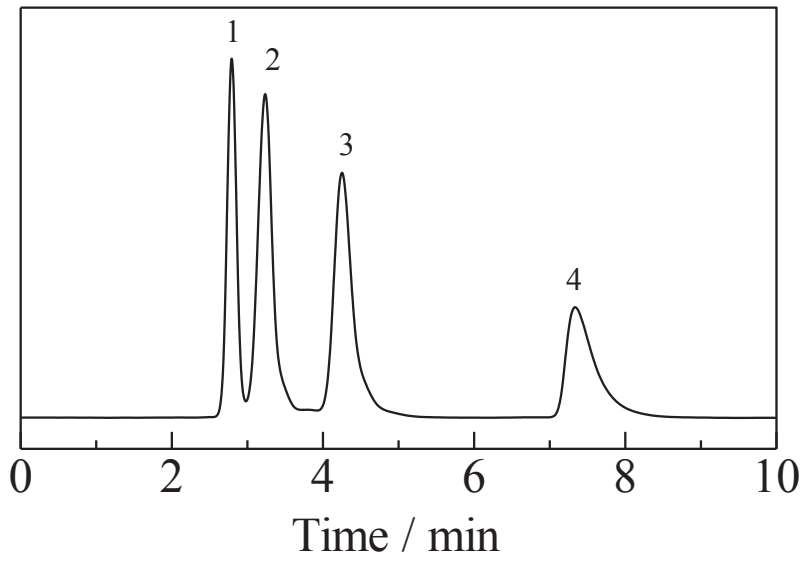

Fig. 2-12 Separation of four common inorganic anions on the polystyrene column by using a contactless conductivity detector. Column: PLRP-S (100 × 0.32 mm I.D.); suppressor: Oasis MCX (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + 10 mM KOH + 4% ACN; analytes: 0.1 mM each of IO_3^-, NO_3^-, I^-, SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: contactless conductivity detector.

2.3.9 Measurement using the polymer monolithic columns

The separation of anions was investigated on the different ratios of C18 contained in the polymeric monolith columns using 5 mM 18C6E, 10 mM potassium chloride and 4% acetonitrile as the eluent. However, the anions were not retained on the C18 polymeric monolith column. Fig. 2-13 illustrates that the separation of hydrophobic compounds (naphthalene and pyrene were used in this study) on the C18 polymeric monolith using 70% acetonitrile as the eluent, and the results showed that the more the stearyl methacrylate (indicated as SMA in the chromatograms) was contained, the larger the retention was.

Although it was speculated that the 18C6E could not plentifully adsorb on the C18 polymeric monolith due to the weak hydrophobic interaction, which causing it could not retain anions,

nevertheless, the polystyrene monolithic column, which has similar hydrophobic property to that of the ODS packing column, as can be seen from Fig. 2-14, could not retain anions as well. These results, however, could be due to the fact that one of the retention mechanisms of the hydrophobic compounds on the polystyrene columns involves π-π interactions of the benzenoid aromatic compounds.

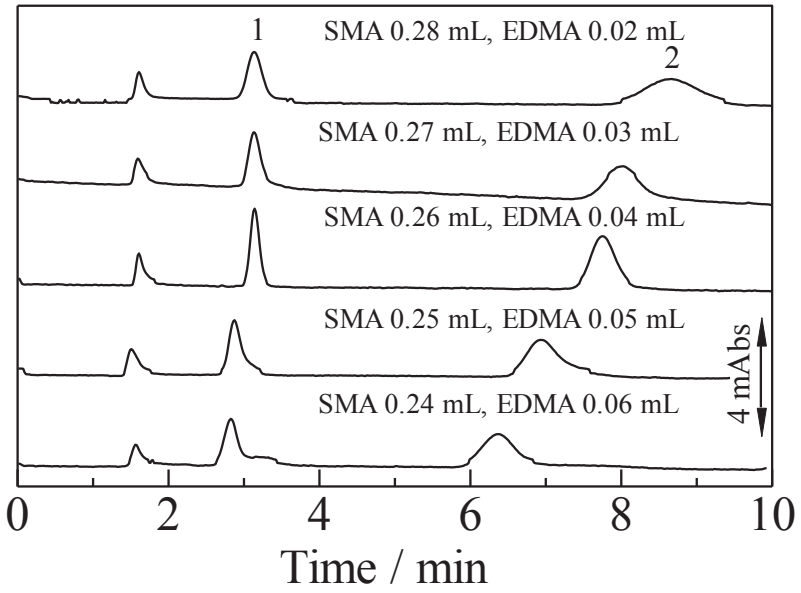

Fig. 2-13 Separation of hydrophobic compounds on the C18 polymer monolithic column. Column: C18 polymer monolith (100 × 0.32 mm I.D.); eluent: 70% ACN; analytes: 1 = 0.001% naphthalene, 2 = 0.0005% pyrene; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 254 nm.

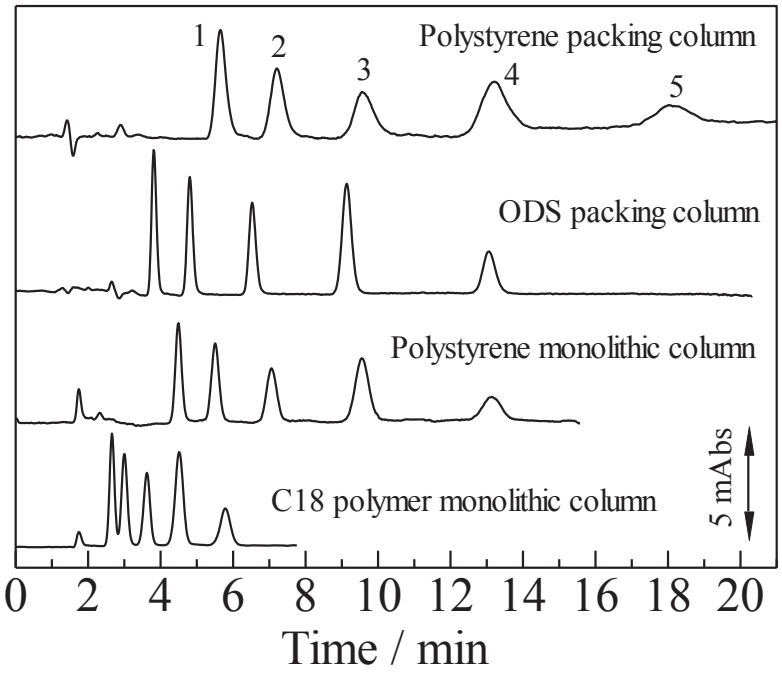

Fig. 2-14 Separation of hydrophobic compounds on several columns. Column: as indicated (100 × 0.32 mm I.D.); eluent: 70% ACN; analytes: 0.005% each of 1 = toluene, 2 = ethylbenzene, 3 = n-propylbenzene, 4 = n-butylbenzene, 5 = amylbenzene; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

For gaining a higher hydrophobicity on the polystyrene monolithic column, the column is modified with C18 functional groups by the expected reaction shown in Fig. 2-15. 1-Chlorooctadecane was reacted to the polystyrene monolithic column by Friedel-Crafts alkylation, and the retention behavior of hydrophobic compounds on the prepared column was determined as shown in Fig. 2-16. After the Friedel-Crafts reaction, although the hydrophobicity on the column increased, that is estimated from the increase of retention times of the hydrophobic compounds, anion samples could not be separated on the column when 18C6E and potassium chloride were used as eluent.

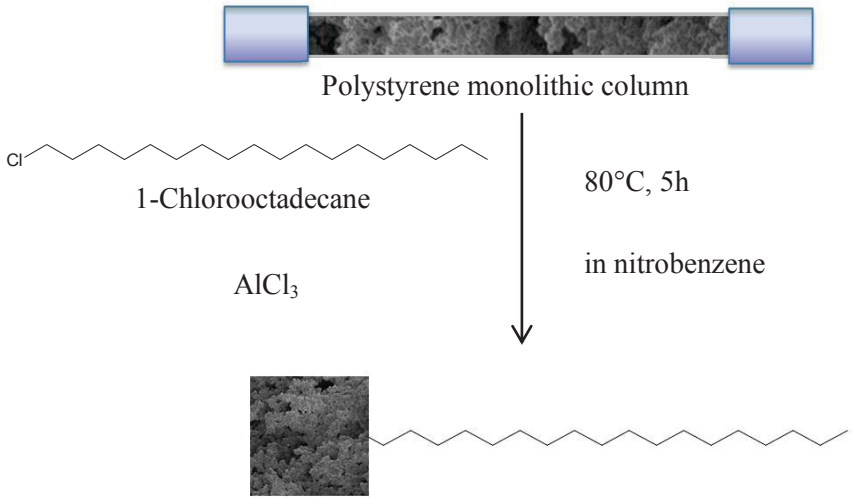

Fig. 2-15 Expected reaction of alkylated polystyrene monolithic column

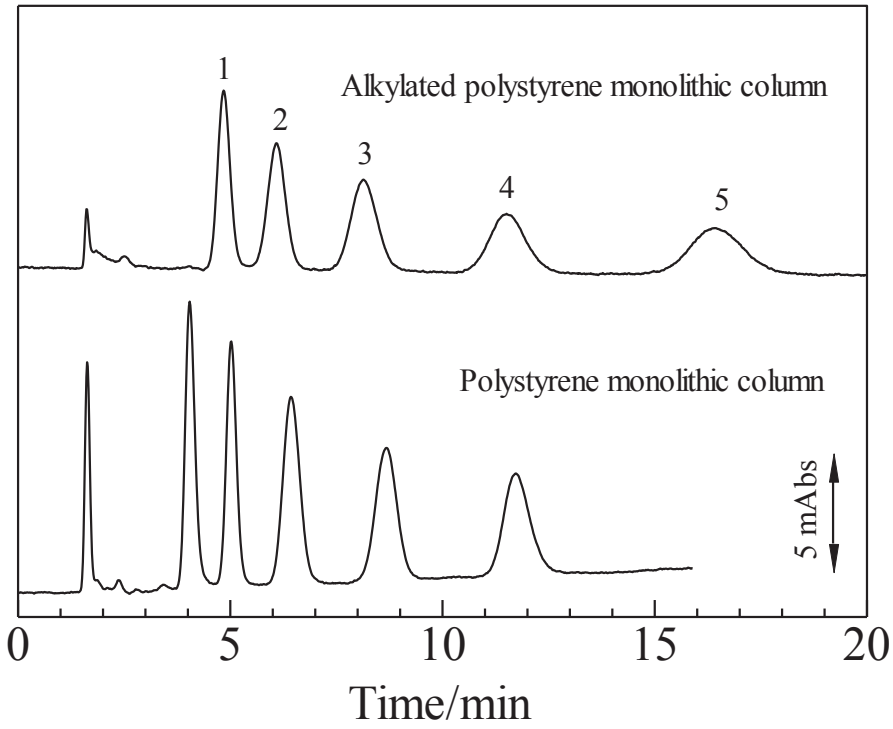

Fig. 2-16 Comparison of retention times of hydrophobic compounds between the alkylated polystyrene monolithic column and the polystyrene monolithic column. Column: as indicated (100 × 0.32 mm I.D.); eluent: 70% ACN; analytes: 0.005% each of 1 = toluene, 2 = ethylbenzene, 3 = n-propylbenzene, 4 = n-butylbenzene, 5 = amylbenzene; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

2.3.10 Separation of anion samples on bis(2-ethylhexyl) maleate (BEHM) modified stationary phases

We attempted to separate anion samples using a different monolithic colum and other 18C6E derivatives *i.e.* benzo-18-crown 6-ether (B18C6E) and divenzo-18-crown 6-ether (D18C6E) in mobile phases. When the monolithic column modified with BEHM as a monomer, anion samples were slightly separated with the eluent containing B18C6E and potassium chloride (Fig. 2-16). D18C6E can not be used as eluent since D18C6E hardly dissolve in solvent with low concentrate of ACN.

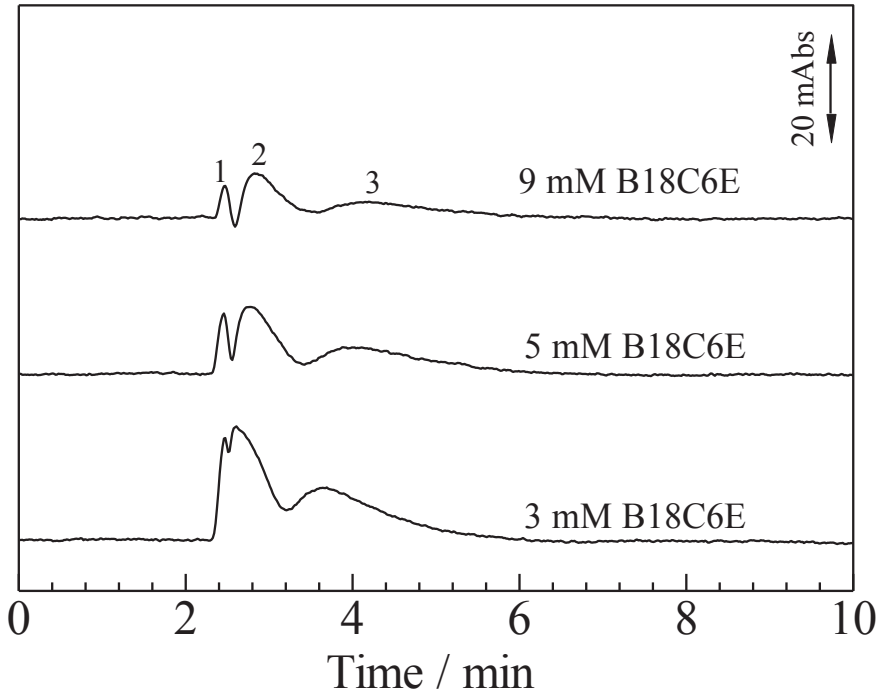

Fig. 2-17 Separation of anion samples on BEHM column. Column: as indicated (100 × 0.32 mm I.D.); eluent: different concentrations of B18C6E (as indicated) + 40 mM KCl; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

2.4 Conclusions

The polystyrene packing column could be used to separate common anions by using 18C6E, potassium chloride and acetonitrile as the eluent; the same separation could also be achieved on the ODS packing column. It was found that 18C6E adsorbed on the stationary phase, and then, the potassium cations contained in the eluent were trapped on the 18C6E and worked as the anion-exchange sites. The retention of anions under both neutral and basic conditions could be carried out using the polystyrene packing column; however, the results showed that the pH of the eluent did not have significant effect on the retention of anions. As for the effect of organic solvent additive (in this study, acetonitrile was used), although the retention of the anions increased with decreasing acetonitrile concentration, it decreased with the passage of time, indicating that the aqueous mobile phase was pulled out of the pore of the hydrophobic packing materials and thus resulting in shorter retention time. In addition, the sensitivity of the anions was enhanced when a contactless conductivity detector was used in replacement to the conventional UV detector.

Polymer monolithic columns were also fabricated in order to improve the separation efficiency on the anions, which was assumed could be achieved due to the higher porosity and larger surface area of the monolithic columns comparing to those of the packed columns. The results showed that the polymer monolithic columns could not retain anions even under the same eluent condition with the polystyrene packing column, though the columns have hydrophobic property.

Further improvement can be expected by using polymer monolithic columns; however, producing monolithic columns with good repeatability still remain as a challenging task.

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Chapter 3

Investigation of Chromatographic Performance of Hyperbranched Amine-modified Stationary Phases in Capillary Liquid Chromatography

3.1 Introduction

In multibranched polymers there are mainly three types of polymers starburst polymers, hyper branched polymers and dendrimers which characters show in Table 1.

Table 3-1 Classification of multibranched polymers and their characters

	Branching degree	productivity	functionality
Starburst polymer	Low	High	Low
Hyperbranched polymer	Middle	High	Middle
Dendrimer	High	Low	High

Tomalia et al. have first synthesized the new class of topological macromolecules dendrimers [1]. In contrast to the divergent method, in which dendrimer chains are synthesized from the core, proposed by Tomalia et al., Frechet et al. has suggested a new dendrimer synthesis method where the hyperbranched molecules are first synthesized and then attached to the core, which they coined it the convergent method [2].

Dendrimers are defined as highly branched macromolecules possessing a large number of end groups and a unique three-dimensional shape. The total number of functional groups on a dendrimer surface increases exponentially as a function of the core multiplicity and the generation [3]. Dendrimers are synthesized with a regular structure, whereas hyperbranched polymers allow imperfectly branched or irregular structure, which are easily prepared and can be a good alternative to

dendrimers [4, 5]. Applications of dendrimers as stationary phases for HPLC have been studied in some papers [4-10].

In this study, hyperbranched amine-modified capillary columns containing multiple terminal amine functional groups were prepared by repeating simple reactions of amine and diglycidyl. The retention behavior of some common inorganic anion samples (IO_3^-, NO_3^-, I^-) on the prepared column was investigated by ion chromatography. The eluent contained sodium chloride in neutral eluent conditions, and sodium chloride and hydrochloric acid in acidic conditions were used as the mobile phases. In addition, an elemental analysis was conducted to confirm the progress of the reactions. The anion exchange capacities were determined by breakthrough curves.

3.2 Experimental

3.2.1 Chemicals

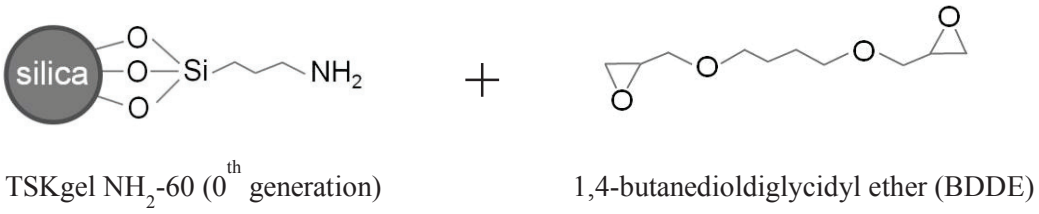
TSKgel NH_2-60 were obtained from Tosoh Corporation (Mean particle diameter, 5 μm; Yamaguchi, Japan). Sodium chloride, sodium iodate, hydrochloric acid and dimethylamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ethylamine, sodium nitrate, sodium iodide, ammonia solution (28%) and ethylbenzene were purchased from Nacalai Tesque (Kyoto, Japan). 1,4-butanedioldiglycidyl ether (BDDE) was obtained from Sigma–Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile was purchased from Kanto Chemical (Tokyo, Japan). All the other reagents and solvents were purchased from Tokyo Chemical Industry (Tokyo, Japan) unless otherwise specified. Microcolumns used in this study were prepared in our laboratory and the developed stationary phases were packed into a fused-silica capillary tube with polyimide coating (100 mm × 0.32 mm i.d.; GL Sciences, Tokyo, Japan) by using the slurry-packing technique with methanol used as the packing solvent. Distilled deionized water used for preparing all reagents was purified with a Simplicity UV water distillation system (Merck Millipore, Darmstadt, Germany).

3.2.2 Apparatus

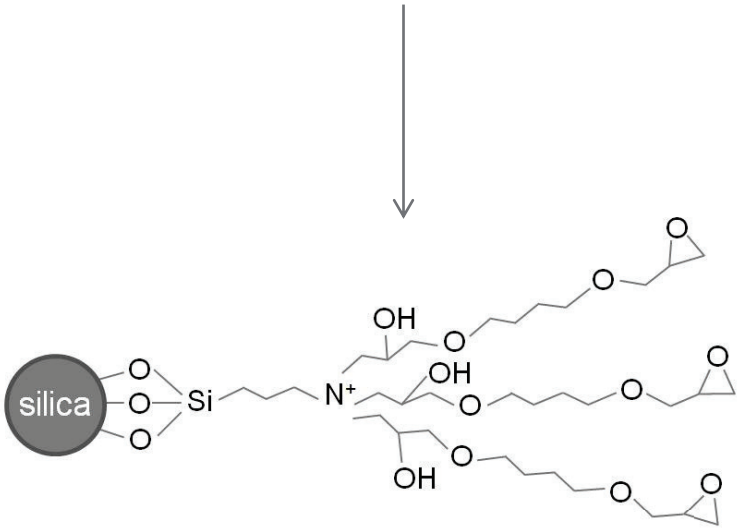
The HPLC system used in this study comprised a microfeeder (L.TEX Corporation, Tokyo, Japan) equipped with a gas-tight syringe (0.5 mL; Ito, Fuji, Japan) as a pump, an injector M-435 Micro Injection Valve with 0.2 μL injection volume (Upchurch Scientific, Oak Harbor, WA, USA) , a UV detector UV-2075 (JASCO, Tokyo, Japan), a data processor CDS-Lite (LA soft, Chiba, Japan) and a microcolumn prepared from a fused-silica capillary tube (100 mm × 0.32 mm I.D.; GL Sciences, Tokyo, Japan) and the inlet pressure was monitored by an L.TEX-8150 Pressure Sensor (L.TEX).

3.2.3 Preparation of hyperbranched amine-modified columns

TSKgel NH_2-60, which is a silica gel containing primary amine groups, were dried in an oven at 120°C. BDDE was then reacted to the packing materials in water solvent at 80°C for 3 hours (reaction A), and subsequently ammonia was reacted to it in water solvent at 80°C for 3 hours (reaction B). Reaction A and B were repeated several times as shown in Fig. 3-1. Here we call these stationary phases 0^{th}, 0.5^{th}, 1^{st} generation in order.

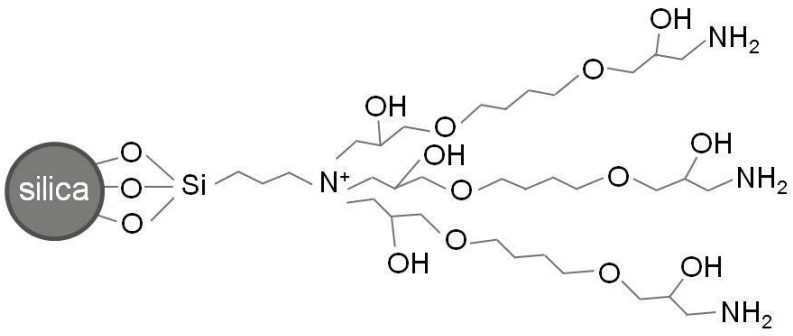


TSKgel NH_2-60 (0^{th} generation) 1,4-butanedioldiglycidyl ether (BDDE)



BDDE modified NH_2-60 (0.5^{th})

Ammonia



Ammonia-BDDE modified NH_2-60 (1^{st})

Fig. 3-1 Reaction for hyperbranched amine-modified columns

3.3 Results and discussion

3.3.1 Separation of inorganic anions under neutral condition

The retention times of anion samples such as iodate, nitrate and iodide were determined with the eluent of 100 mM sodium chloride in the different numbers of generation as shown in Fig. 3-2. Black lines show chromatograms of 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 6^{th} and 9^{th} generations. The 0^{th} generation of dendrimer column did not separate anions due to no positive charge. Over 0.5^{th} generation of columns, anions were separated due to the positive charges of the quaternary ammonium groups and retention times increased with an increase of generation.

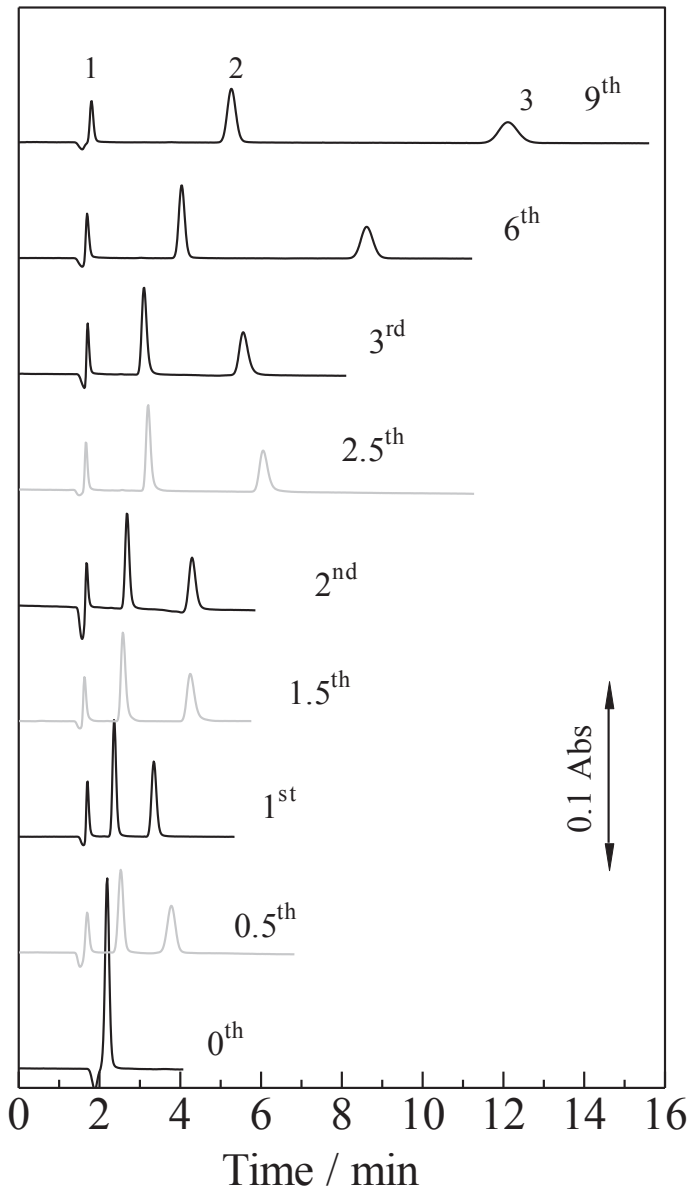


Fig. 3-2 Separation of inorganic anions on hyperbranched amine-modified columns. Column: as indicated (100 × 0.32 mm I.D.); eluent: 100 mM NaCl; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-; injection volume: 0.2 μL; flow-rate: 4.0 μL/min; detector: UV; wavelength of UV detection: 210 nm.

Fig. 3-3 shows the retention times of iodide, nitrate and iodate against the generations of hyperbranched amine-modified columns. Gray points are retention times of .5th generations. The increase of retention time was observed after BDDEs were reacted, *i.e.* from 0.5th to 1.5th and then to 2.5th generations, due to the formation of quaternary ammonium groups on the stationary phases. On

the other hand, after ammonia was reacted, the retention time was decreased as can be observed from 0.5th to 1st, from 1.5th to 2nd, and from 2.5th to 3rd generations, respectively. Considering the fact that exterior amine groups should not provide a bad effect to the retention of anions, it is therefore expected that the silica gels might have been dissolved under the basic condition during the reactions with ammonia.

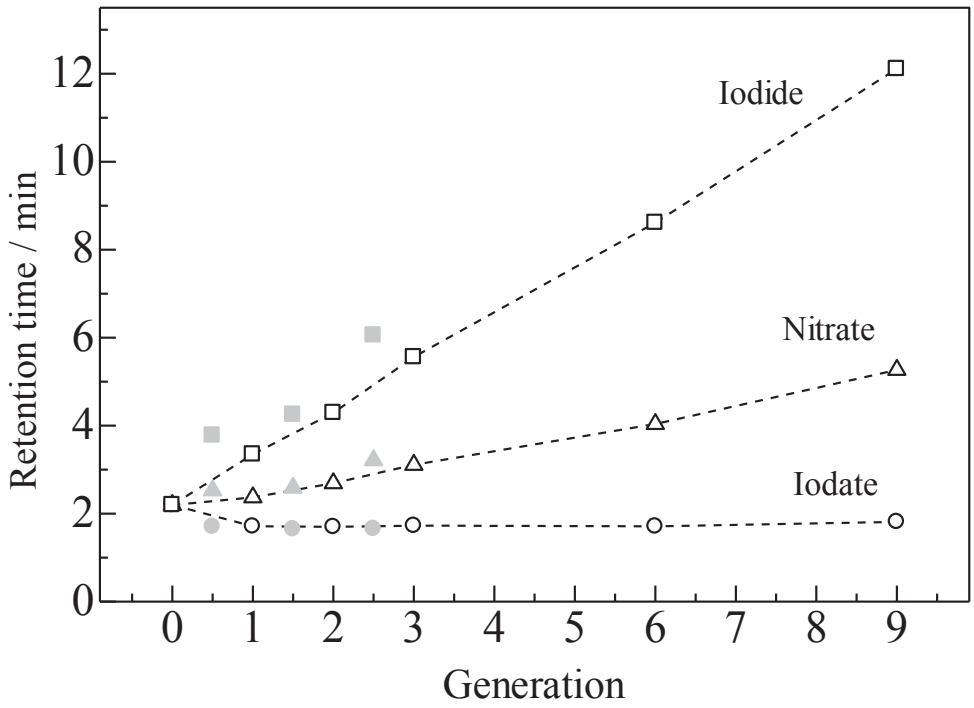


Fig. 3-3 Retention times of inorganic anions against generations of hyperbranched amine-modified columns

3.3.2 Separation of inorganic anions under acidic condition

The retention times of inorganic anions were also determined under acidic eluent condition as shown in Figs. 3-4, while the retention times of inorganic anions against each generation of the resulted stationary phases are shown in Fig. 3-5. 1 mM hydrochloric acid was added for the acidic conditions and it was found that the retention times of the analytes were longer than those under neutral eluent condition. Under the acidic eluent condition, exterior primary amine groups on ends of hyperbranched amine chains possess positive charges by protonation. Therefore the 0th generation of column which only consists of primary amine groups could even separate those anions. It was also

found that the analytes anions eluted in the reverse order of common ion exchange order under acidic condition in the 0^{th} generation. In the 0.5^{th} generation, the elution order was reversed to general order of ion exchange. Hence this difference indicates that the reaction from 0.5^{th} generation to 1^{st} generation has been prompted. In addition, the retention times of iodate decreased while those of iodide increased. It could be due to the increased hydrophobicity in the longer hyperbranched amine chains. Therefore, hydrophilic anion *i.e.* iodate was little retained on the stationary phase while hydrophobic anion *i.e.* iodide was retained more strongly.

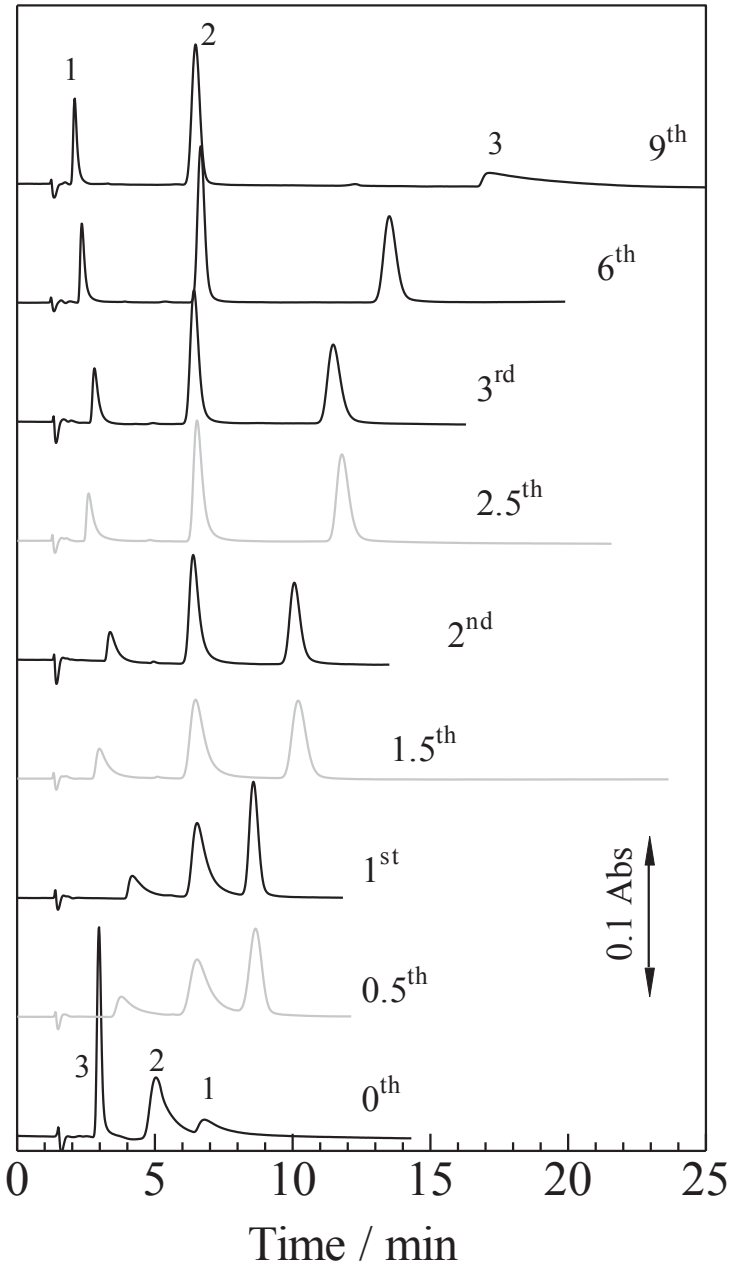


Fig. 3-4 Separation of inorganic anions on hyperbranched amine-modified columns under acidic

condition. Column: as indicated (100 × 0.32 mm I.D.); eluent: 500 mM NaCl + 1 mM HCl; analytes: 0.5 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-; injection volume: 0.2 μL; flow-rate: 4.0 μL/min; detector: UV; wavelength of UV detection: 210 nm.

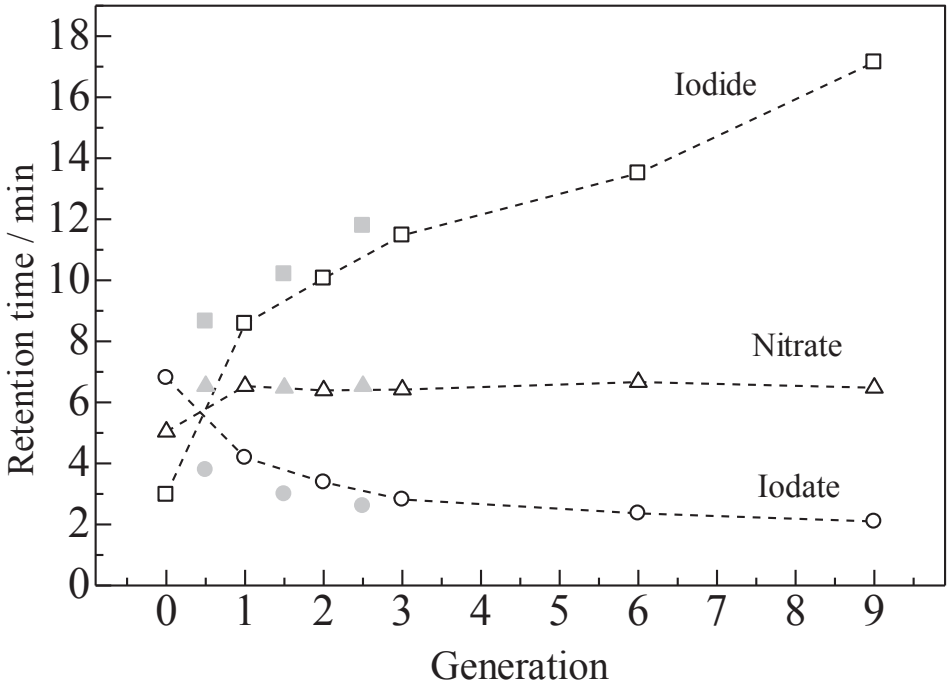


Fig. 3-5 Retention times of inorganic anions against generations of hyperbranched amine-modified columns under acidic eluent conditions

3.3.3 Characterization of hyperbranched amine-modified columns

The growth of amine group chains was evaluated by elemental analysis. Table 3-2 shows content of hydrogen, carbon and nitrogen in the stationary phases, and relative ratios of mol number calculated when contents of nitrogen is 1, were described in parentheses. The ratios of carbon contents increased in 0.5^{th}, 1.5^{th}, 2.5^{th} and 3.5^{th} generations. Therefore it is presumed that BDDEs were reacted significantly, whereas it seems ammonia was hardly reacted.

Table 3-2 Content of hydrogen, carbon and nitrogen in hyperbranched amine-modified columns

Generation	**H** [g (mol)]	**C** [g (mol)]	**N** [g (mol)]
0^{th}	1.61 (14.53)	4.17 (2.37)	1.54 (1)
0.5^{th}	2.48 (29.46)	10.60 (7.93)	1.17 (1)
1^{st}	2.43 (28.38)	10.29 (7.57)	1.19 (1)
1.5^{th}	2.64 (33.26)	12.06 (9.60)	1.10 (1)
2^{nd}	2.71 (30.87)	11.95 (8.58)	1.22 (1)
2.5^{th}	3.09 (34.64)	14.73 (10.40)	1.24 (1)
3^{rd}	3.00 (33.09)	13.46 (9.35)	1.26 (1)
3.5^{th}	3.12 (33.88)	14.89 (10.19)	1.28 (1)

Fig. 3-6 shows the relationship of the ion exchange capacity and the generation of hyperbranched stationary phases. The ion exchange capacities increased with increasing reaction cycle of hyperbranched stationary phases, which indicates amount of amines in the stationary phases increased. In addition, the amounts of ion exchangers after protonation were approximately 4-5 folds larger comparing to those under the neutral condition, indicating the existence of tertiary or lower grades of amines beside quaternary ammonium groups.

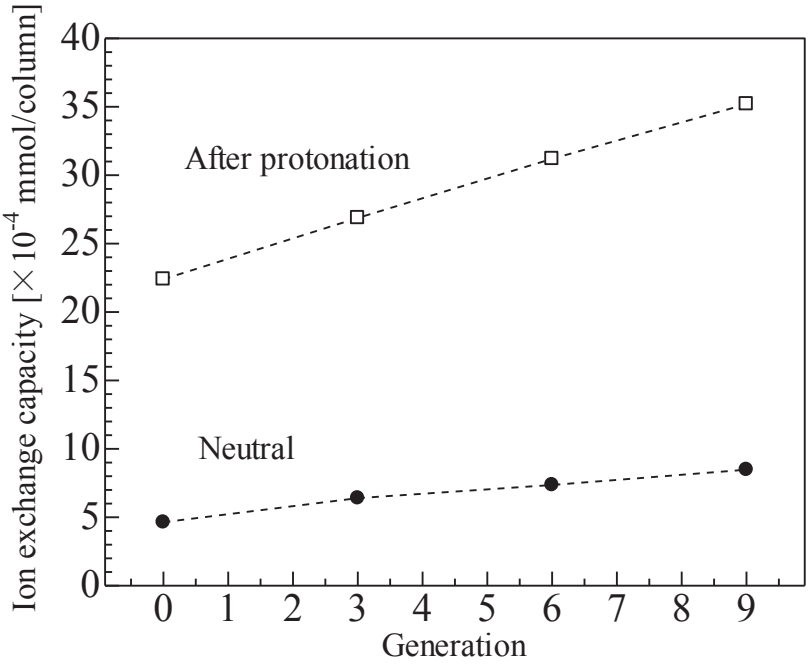


Fig. 3-6 Ion exchange capacity of hyperbranched amine-modified columns

3.3.4 Comparison with other hyperbranched columns modified with different grades of amines

The retention behaviors of inorganic anions on the column reacted with different grades of amine were also investigated and the results are shown in Fig. 3-7 (neutral condition) and Fig. 3-8 (acidic condition). The ammonia-reacted column has the highest retention property for anions. It is presumed that more BDDE could react to terminal primary amine groups. Lower grade of amine have higher retention behavior since more BDDEs can react at the terminal of amine.

From Fig. 3-8, it was also found that the retention of anions decreased severely at the 6th generation after modification with tertiary amines. This could be due to the lower p*K*b of dimethylamine (p*K*b = 3.27) comparing to those of ethylamine (p*K*b = 3.37) and ammonia (p*K*b = 4.76), which lead to the higher possibility of the deterioration of the silica gels.

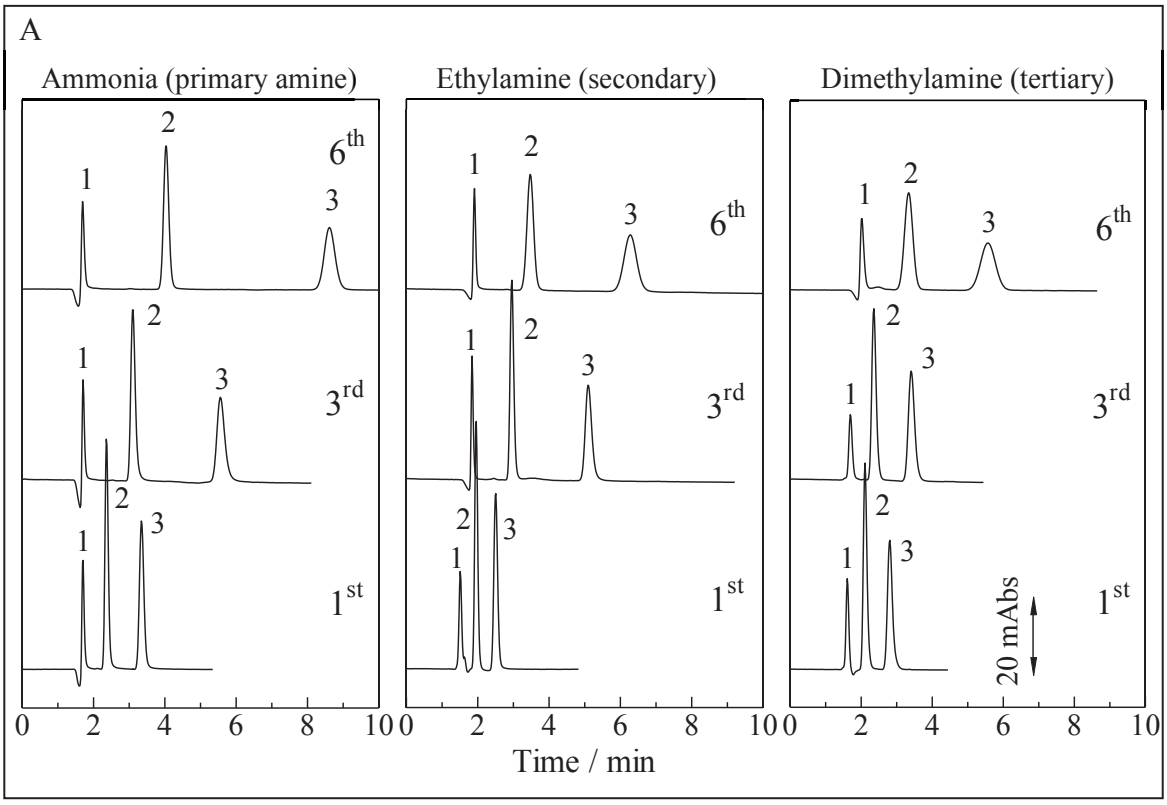


Fig. 3-7 Separation of inorganic anions on hyperbranched columns modified with different grades of amines under neutral condition. Column: as indicated (100 × 0.32 mm I.D.); eluent: 100 mM NaCl; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-; injection volume: 0.2 μL; flow-rate: 4.0 μL/min; detector: UV; wavelength of UV detection: 210 nm.

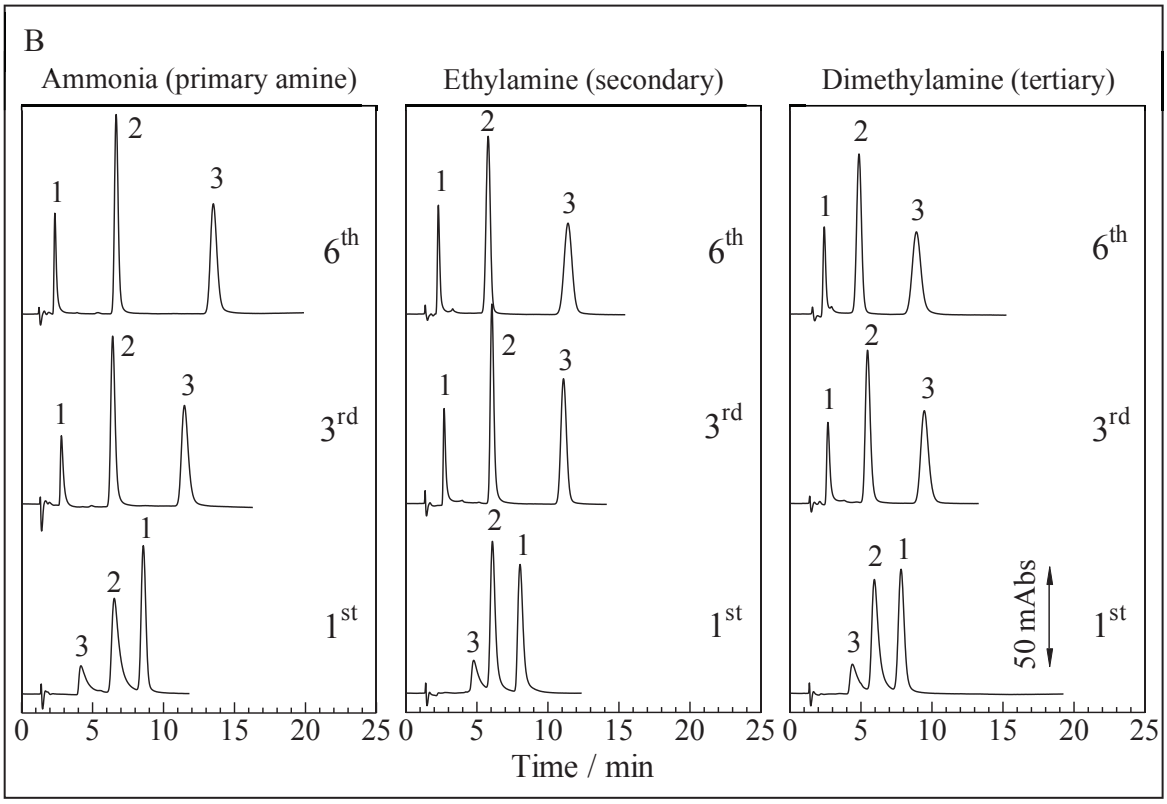


Fig. 3-8 Separation of inorganic anions on hyperbranched columns modified with different grades of amines under acidic condition. Column: as indicated (100 × 0.32 mm I.D.); eluent: 500 mM NaCl + 1 mM HCl; analytes: 0.5 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-; injection volume: 0.2 μL; flow-rate: 4.0 μL/min; detector: UV; wavelength of UV detection: 210 nm.

3.3.5 Separation of hydrophobic compounds

The retention behavior of hydrophobic compounds such as toluene, ethylbenzene and n-propylbenzene are shown in Fig. 3-9. The retention times increased with increasing generation of hyperbranched stationary phases. It indicates the hyperbranched chanins are hydrophobic and the chains grew by the reactions. The increase of hydrophobicity might affect the increase of retention times of the hydrophobic inorganic anion *i.e.* iodide in Figs. 3-3 and 3-5.

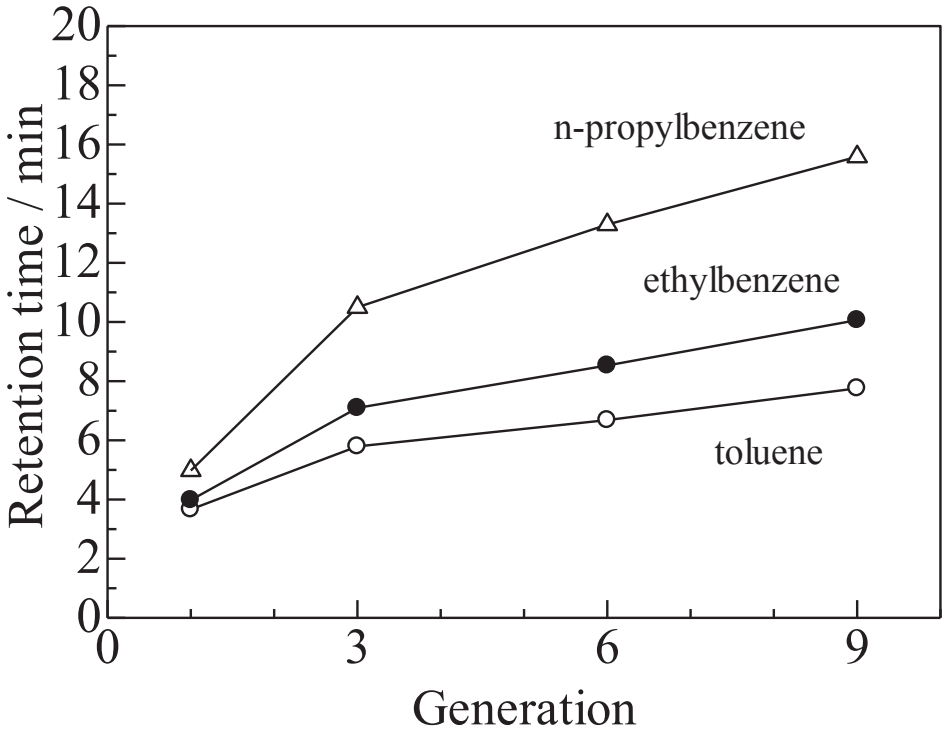


Fig. 3-9 Separation of hydrophobic compounds. Column: as indicated (100 × 0.32 mm I.D.); eluent: 70% ACN; analytes: 0.005% each of samples; injection volume: 0.2 μL; flow-rate: 4.0 μL/min; detector: UV; wavelength of UV detection: 210 nm.

3.4 Conclusions

The retention behavior of inorganic anions on hyperbranched amine-modified capillary columns was investigated. Comparing to neutral eluent conditions, the retention time of inorganic anions increased significantly under acidic eluent conditions; some amine functional groups remained as primary, secondary or tertiary amines, and were protonated under acidic eluent condition, thus causing the increased retention time. It was found that the retention time of inorganic anions increased with an increase of reaction cycles. In addition, the growth of amine group chains was confirmed by elemental analysis, *i.e.* by calculating the increase of nitrogen content. The anion exchange capacities were determined by breakthrough curves and the values showed an increase with the growth of dendritic chains. Therefore, the anion exchange capacity could be controlled by varying the number of reaction cycles.

References

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Chapter 4

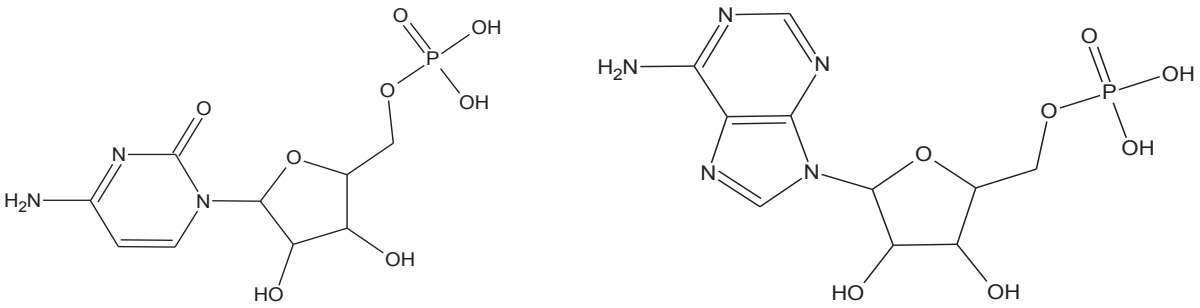
Development of Nucleotide-bonded Stationary Phases for Capillary Liquid Chromatography

4.1 Introduction

Miniaturization of chromatographic systems has attracted much attention since the 1970s [1] and the development of capillary-based separation systems has grown tremendously especially after the invention of fused-silica capillaries [2]. With the increasing concern of environmentally friendly analysis, capillary systems do not only reduce the amount of solvents, reagents and packing materials used, but we can also expect ultra-high sensitive and selective analysis of the most complex samples when it is directly connected to a mass spectrometer (MS) or a tandem MS/MS detection unit. Capillary columns also make the development of novel stationary phases so much easier and more importantly, affordable [3-7].

Since ion chromatography was introduced in 1975, even though there are many types of stationary phases have been developed, ion-exchange chromatography still remains as the most common and widely used separation methods especially for the separation of inorganic anions and cations, and recently it has also been proven to be extremely useful for the separation of amino acids, as can be seen from the number of papers published in recent years [8]. Among these stationary phases, zwitterionic stationary phases, which contain both positive and negative charges, attract much attention due to their ability for higher efficient separations (caused by the shorter diffusion paths provided by the existence of oppositely charged layers on the surface) and changes of a wide range of selectivity (by using fixed concentrations of ligands and varying pH and ionic strength of mobile phases) [9]. However, performing simultaneous separation of both anions and cations with high efficiency still remains as a challenging task.

In this study, nucleotide-bonded zwitterionic stationary phases were prepared via a two-step covalent modification. Nucleotides are generally recognized as the monomers or subunits of deoxyribonucleic acid (DNA) as well as ribonucleic acid (RNA), and they contain at least a nucleoside (which is a nitrogenous base) and a phosphate group. Therefore, under certain eluent conditions, nucleotides could be charged positively and/or negatively; and this characteristic favors its application in both ion-exchange and hydrophilic interaction chromatographic separations. Cytidine5'-monophosphate and adenosine5'-monophosphate were chosen in this study and their chemical structures are shown in Fig. 4-1.



Cytidine5'-monophosphate Adenosine5'-monophosphate

Fig. 4-1 Chemical structures of the nucleotides used in this study.

4.2 Experimental

4.2.1 Chemicals

Reagents employed were of guaranteed reagent grade and were obtained from Nacalai Tesque (Kyoto, Japan), unless otherwise noted. Sulfuric acid, *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), sodium iodate, potassium sulfate, phenol, salicylic acid, adenine and thymine were purchased from Wako Pure Chemical Industries (Osaka, Japan), while copper(II) sulfate pentahydrate was supplied by Yoneyama Yakuhin Kogyo (Osaka). Cytidine5'-monophosphate and 3-glycidyloxypropyltrimethoxysilane (GPTMS) were obtained from

Tokyo Chemical Industry (Tokyo, Japan), whereas methanol, acetonitrile (ACN) and adenosine5'-monophosphate were obtained from Kanto Chemical (Tokyo). Ultrapure water was produced in the laboratory by using a Simplicity UV water purification system (Millipore, Molsheim, France), and all solutions used in this study were prepared using this ultrapure water.

The stationary phase materials employed were silica gels with average particle diameter and mean pore diameter of 5 μm and 12 nm, respectively (Chemical Evaluation and Research Institute, Tokyo, Japan). The silica gels were dried at 120°C for 5 h before use. Toluene was dried in laboratory using molecular sieves 3A (1/16" pellets) for overnight (approx. 12 h), and stainless-steel tubing with 100 x 4.6 mm I.D. was used as the reaction vessel.

4.2.2 Apparatus

The capillary LC system used in this study was constructed by an L.TEX-8301 Micro Feeder (L.TEX corporation, Tokyo, Japan) equipped with an MS-GAN050 gas-tight syringe (0.5 mL, Ito, Fuji, Japan) as a micro pump, an M-435 micro valve injector (Upchurch Scientific, Oak Harbor, WA, USA) with an injection volume of 0.15 μL, a 100 mm x 0.32 mm I.D. microcolumn and a UV-2070 detector (JASCO, Tokyo, Japan). A capillary flow cell (75 μm; JASCO) was attached to the UV detector. All data was acquired using CDS-Lite ver5.0 data processor (LAsoft, Nagareyama, Chiba, Japan). Elemental analysis of the stationary phases was carried out by using a JM10 Micro Corder (J-Science Lab, Kyoto, Japan). A model 5220 centrifuge (Kubota, Tokyo, Japan) was used for washing the reaction products.

4.2.3 Preparation of stationary phases

The nucleotide-bonded stationary phases were prepared similar to that of our previous work with some modifications on each reaction step and the second reaction solvent was optimized in this study [10-11]. The dried porous silica gel was firstly reacted to GPTMS using dried toluene as the solvent and the reaction was carried out at 110°C for 18 h in the reaction vessel. The vessel was sometimes shaken manually during the reaction. After that, the GPTMS-bonded silica gel was washed with

methanol and then dried at 75°C for 6 h. Cytidine5'-monophosphate and adenosine5'-monophosphate were reacted to the silica gel through epoxy-ring opening reaction under different solvents. Fig. 4-2 shows the series of the expected reactions when cytidine5'-monophosphate.

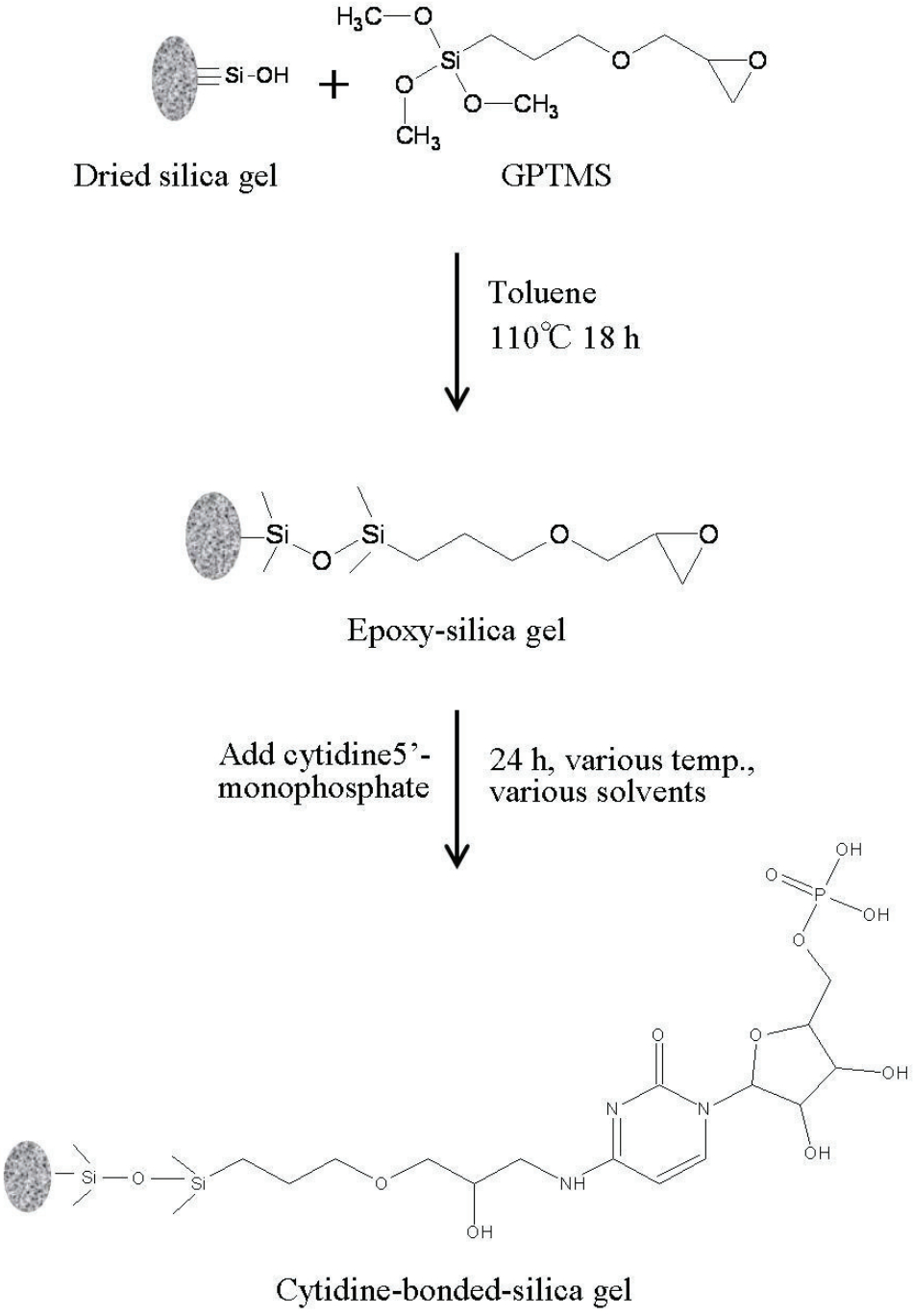


Fig. 4-2 Scheme for the expected reactions.

4.3 Results and discussion

4.3.1 Elemental analysis

In order to optimize the bonded amount of nucleotides on the silica gel stationary phase, various reaction solvents such as toluene, DMF, DMSO, THF and ACN were used at various temperatures with the reaction time kept constant at 24 h. As can be seen from both tables 4-1 and 4-2, DMF as the reaction solvent with reaction temperature at 120 degree Celsius.

Table 4-1 Elemental analysis of cytidine-bonded-silica gels under various conditions.

Reaction solvent	N / wt %	Nucleotide content / mmol g-1
① Toluene, 110 ℃	0.14	0.03
② DMF, 90 ℃	0.86	0.20
③ DMF, 120 ℃	0.98	**0.23**
④ DMSO, 120 ℃	0.14	0.03
⑤ THF, 65 ℃	0.56	0.12
⑥ ACN, 80 ℃	0.91	0.22

Table 4-2 Elemental analysis of adenosine -bonded-silica gels under various conditions.

Reaction solvent	N / wt %	Nucleotide content / mmol g-1
① DMF, 90 ℃	0.47	0.07
② DMF, 120 ℃	0.66	**0.10**
③ DMSO, 120 ℃	0.35	0.05
④ ACN, 80 ℃	0.30	0.04

4.3.2 Separation of anions

Figures 4-3 and 4-4 showed the separation of anions on cytidine- and adenosine-bonded stationary phases, respectively. As can be seen from the chromatograms, cytidine-bonded stationary phase did not have any retention on the analytes anions while adenosine-bonded stationary phase showed slight retentions towards those analytes.

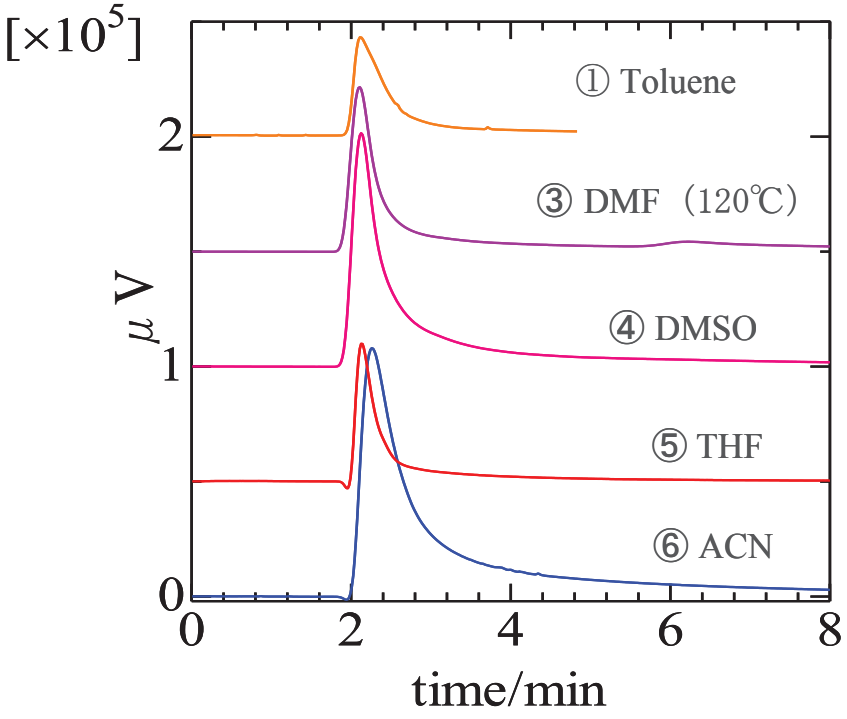


Fig. 4-3 Separation of anions on cytidine-bonded stationary phase

Column: Cytidine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 10 mM NaCl + 1 mM HCl + 3% ACN; Sample: I^-, NO_3^-, IO_3^-, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

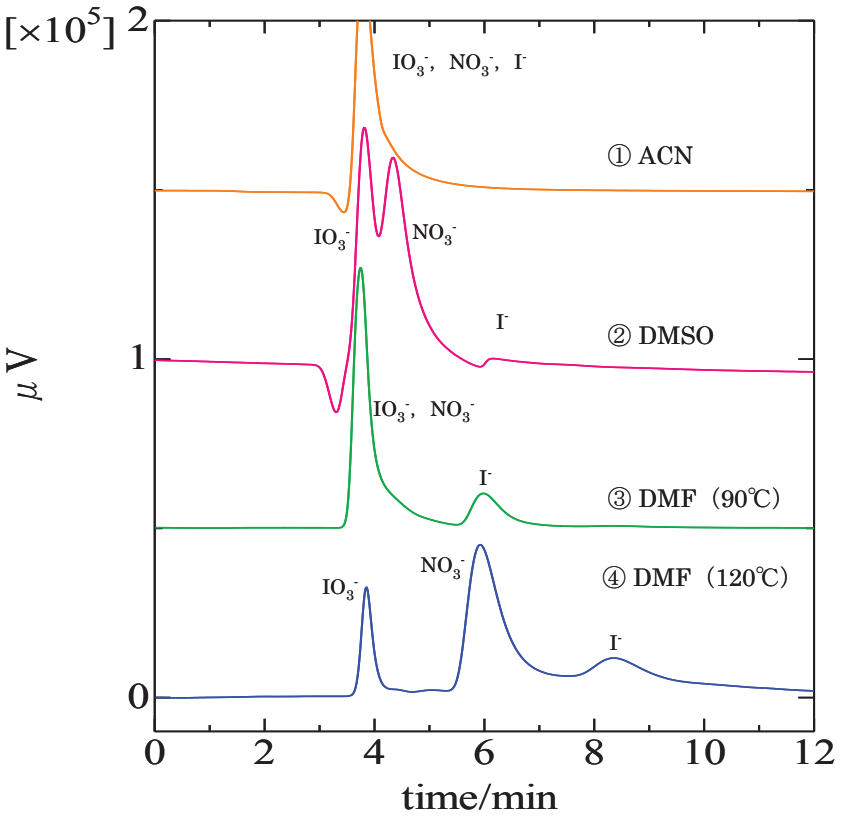


Fig. 4-4 Separation of anions on adenosine-bonded stationary phase

Colum: Adenosine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 10 mM NaCl + 1 mM HCl + 3% ACN; Sample: I^-, NO_3^-, IO_3^-, 0.5 mM each; Flow-rate: 2.0 μL/min; Wavelength of UV detection: 210 nm.

4.3.3 Separation of cations

Figures 4-5 and 4-6 showed the separation of cations on cytidine- and adenosine-bonded stationary phases, respectively. As can be seen from the chromatograms, all monovalent and divalent cations were eluted as individual groups; and the resulted stationary phases did not have enough separation selectivity towards each individual monovalent or divalent cations.

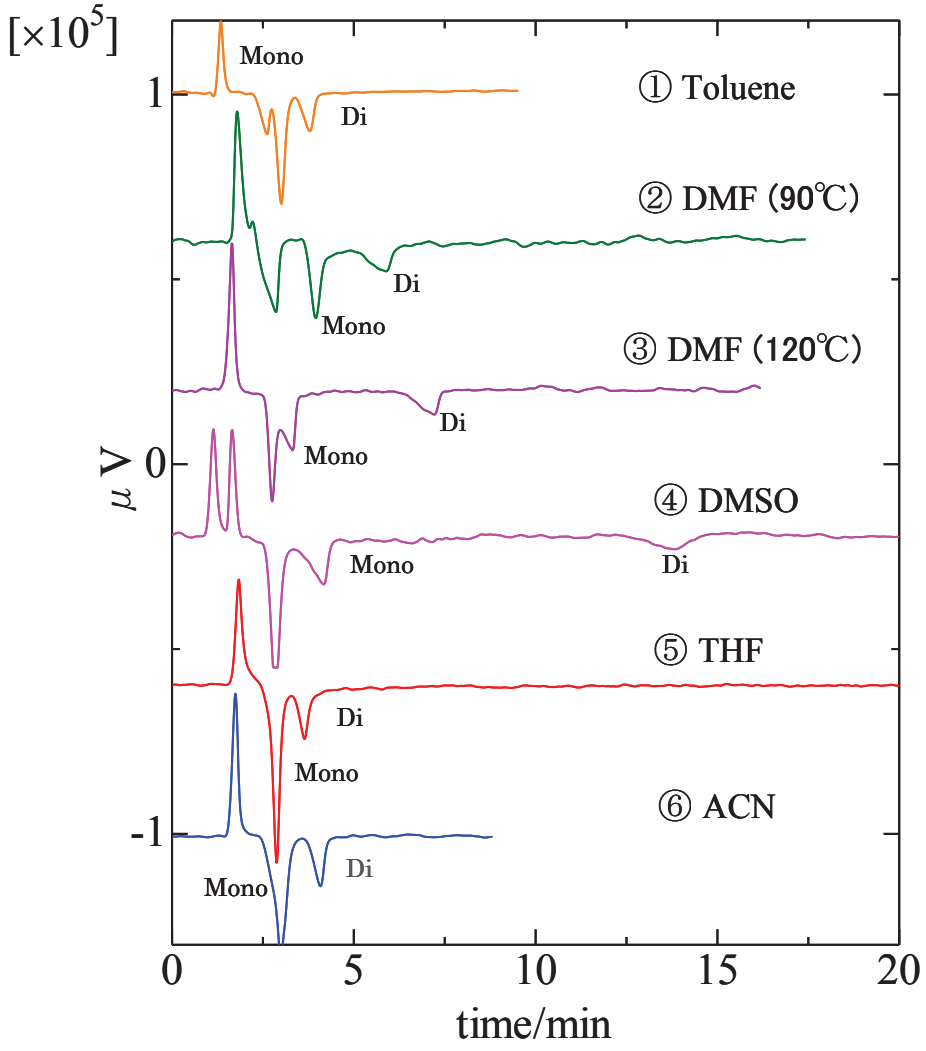


Fig. 4-5 Separation of cations on cytidine-bonded stationary phase

Column: Cytidine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 1 mM $CuSO_4$; Sample: Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

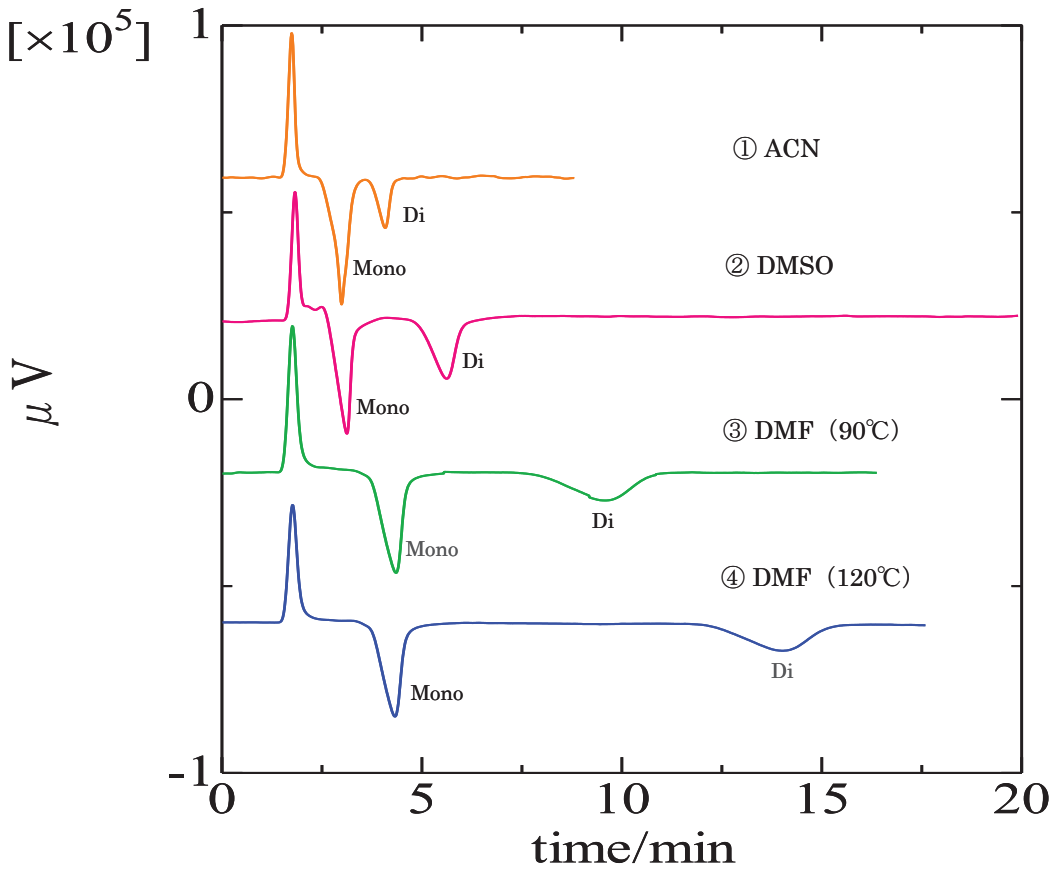


Fig. 4-6 Separation of cations on adenosine-bonded stationary phase

Column: Adenosine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 1 mM $CuSO_4$; Sample: Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

Under neutral eluent condition, however, the separation selectivity of the adenosine-bonded stationary phase was improved, and the separation profile is shown in Fig. 7.

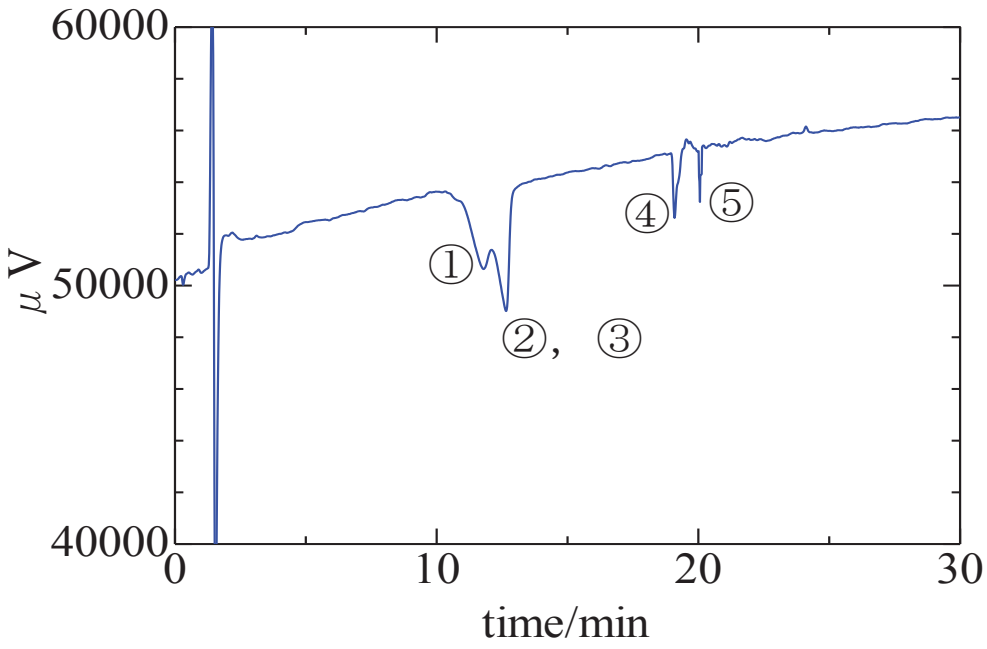


Fig. 4-7 Separation of cations on adenosine-bonded stationary phase under neutral eluent condition Column: Adenosine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 1 mM BETMAC; Sample: ①Na^+, ②K^+, ③NH_4^+, ④Mg^{2+}, ⑤Ca^{2+}, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

4.3.4 Separation of organic acids

Figures 4-8 and 4-9 showed the separation of organic acids on cytidine- and adenosine-bonded stationary phases, respectively. As can be seen from the chromatograms, both stationary phases showed fairly good retention and separation efficiency towards the analytes. The retention of organic acids increased with increasing ACN concentration, showing both stationary phases could be operated under typical HILIC conditions.

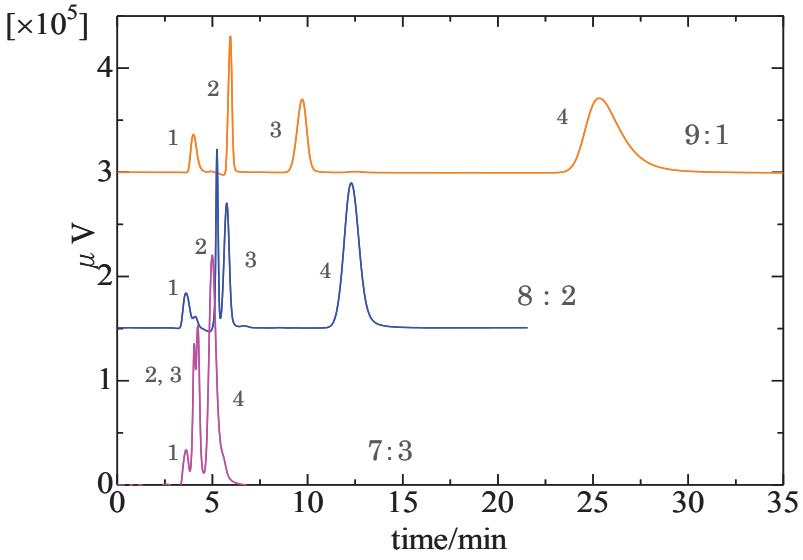


Fig. 4-8 Separation of organic acids on cytidine-bonded stationary phase

Column: Cytidine-bonded silica, 100 x 0.32 mm I.D.; Eluent: ACN:10 mM ammonium acetate, ratio as indicated; Sample: 1=phenol, 2=salicylic acid, 3=benzoic acid, 4=phthalic acid, 1 mM each; Flow-rate: 2.0 μL/min; Wavelength of UV detection: 254 nm.

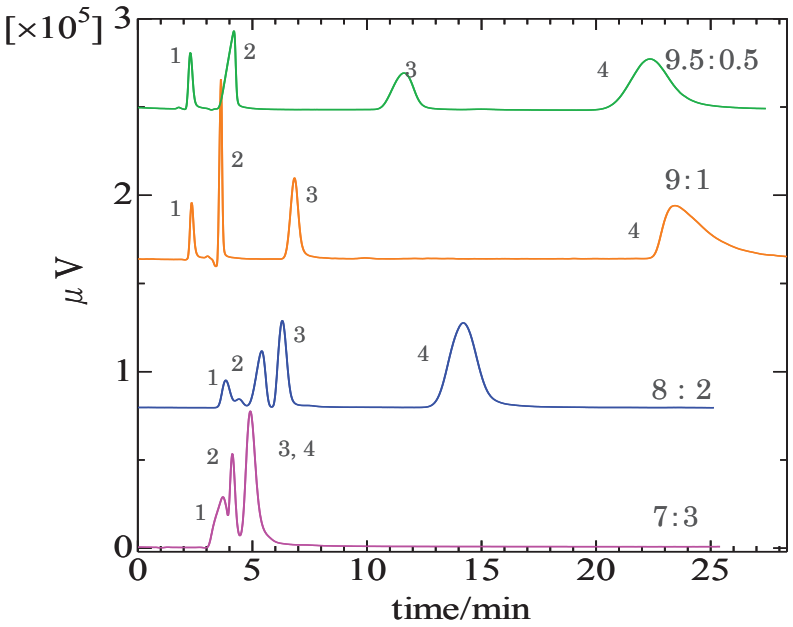


Fig. 4-9 Separation of organic acids on adenosine-bonded stationary phase

Column: Adenosine-bonded silica, 100 x 0.32 mm I.D.; Eluent: ACN:10 mM ammonium acetate, ratio as indicated; Sample: 1=phenol, 2=salicylic acid, 3=benzoic acid, 4=phthalic acid, 1 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 254 nm.

4.4 Conclusions

Cytidine- and adenosine-boned bonded stationary phases were prepared *via* epoxy-ring opening reaction under various conditions. The resulting nucleotide-bonded silica gels were then packed into 0.32 mm fused-silica tubes (10 cm in length) and the columns were evaluated for the separation of inorganic ions (3 anions and 5 cations) and polar compounds (4 organic acids and 3 nucleobases) using a UV detector under ion-exchange and HILIC modes, respectively. The results showed that both cytidine- and adenosine-bonded columns could separate mono- and divalent cations, respectively, using indirect UV detection with copper(II) sulfate used as the eluent. Adenosine-bonded column was found to have longer retention for organic acids under HILIC mode, and it was assumed that the distance between the phosphate and secondary amine groups in the structure plays an important role in the separation of these polar compounds.

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Chapter 5

Conclusions and future perspectives

The present study has been focused on capillary liquid chromatography, especially ion chromatography and developing separation columns for the separation of inorganic anion samples and hydrophobic compounds. Retention behavior of inorganic anions has been investigated on hydrophobic columns dynamically modified with 18-crown-6 ether (18C6E) and hyperbranched amine-modified stationary phases.

1. The polystyrene packing column could be used to separate common anions by using 18C6, potassium chloride and acetonitrile as the eluent; the same separation could also be achieved on the ODS packing column. It was found that 18C6E adsorbed on the stationary phase, and then, the potassium cations contained in the eluent were trapped on the 18C6E and worked as the anion-exchange sites. The retention of anions under both neutral and basic conditions could be carried out using the polystyrene packing column; however, the results showed that the pH of the eluent did not have significant effect on the retention of anions. As for the effect of organic solvent additive (in this study, acetonitrile was used), although the retention of the anions increased with decreasing acetonitrile concentration, it decreased with the passage of time, indicating that the aqueous mobile phase was pulled out of the pore of the hydrophobic packing materials and thus resulting in shorter retention time. In addition, the sensitivity of the anions was enhanced when a contactless conductivity detector was used in replacement to the conventional UV detector.

   Polymer monolithic columns were also fabricated in order to improve the separation efficiency on the anions, which was assumed could be achieved due to the higher porosity and larger surface area of the monolithic columns comparing to those of the packed columns. The results showed that the polymer monolithic columns could not retain anions even under the same eluent condition with the polystyrene packing column, though the columns have hydrophobic property.

Further improvement can be expected by using polymer monolithic columns; however, producing monolithic columns with good repeatability still remain as a challenging task.

2. The retention behavior of inorganic anions on hyperbranched amine-modified capillary columns was investigated. Comparing to neutral eluent conditions, the retention time of inorganic anions increased significantly under acidic eluent conditions; some amine functional groups remained as primary, secondary or tertiary amines, and were protonated under acidic eluent condition, thus causing the increased retention time. It was found that the retention time of inorganic anions increased with an increase of reaction cycles. In addition, the growth of amine group chains was confirmed by elemental analysis, i.e. by calculating the increase of nitrogen content. The anion exchange capacities were determined by breakthrough curves and the values showed an increase with the growth of dendritic chains. Therefore, the anion exchange capacity could be controlled by varying the number of reaction cycles.

3. Cytidine- and adenosine-boned bonded stationary phases were prepared *via* epoxy-ring opening reaction under various conditions. The resulting nucleotide-bonded silica gels were then packed into 0.32 mm fused-silica tubes (10 cm in length) and the columns were evaluated for the separation of inorganic ions (3 anions and 5 cations) and polar compounds (4 organic acids and 3 nucleobases) using a UV detector under ion-exchange and HILIC modes, respectively. The results showed that both cytidine- and adenosine-bonded columns could separate mono- and divalent cations, respectively, using indirect UV detection with copper(II) sulfate used as the eluent. Adenosine-bonded column was found to have longer retention for organic acids under HILIC mode, and it was assumed that the distance between the phosphate and secondary amine groups in the structure plays an important role in the separation of these polar compounds.

For the future developments of the current studies, hydrophobic columns dynamically modified with crown ethers should be applied to polymeric monolithic column, which leads to low backpressure and rapid analysis, and the hyperbranched polymer can be applied to the separation of complicated mixtures of polar and non-polar compounds since a large number of functional groups are present within the polymer.

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Fig. 2-7 Retention behavior of thiocyanate ion on the polystyrene column over time.Operating conditions were the same as Fig. 2-6.

Fig. 2-8 Effect of 18C6E concentration on the retention of common anions. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: different concentrations of 18C6E (as indicated) + 10 mM KCl + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

Fig. 2-9 Effect of eluent cation on the retention of common anions. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + 10 mM salt (as indicated) + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

Fig. 2-10 Effect of salt concentration on the retention of common anions. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + different concentrations of KCl (as indicated) + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

Fig. 2-11 Effect of basic eluent. Column: PLRP-S (100 × 0.32 mm I.D.); eluent: 5 mM 18C6E + 10 mM KCl or KOH + 4% ACN; analytes: 0.1 mM each of 1 = IO_3^-, 2 = NO_3^-, 3 = I^-, 4 = SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 210 nm.

Fig. 2-12 Separation of four common inorganic anions on the polystyrene column by using a contactless conductivity detector. Column: PLRP-S (100 × 0.32 mm I.D.); suppressor: Oasis MCX; eluent: 5 mM 18C6E + 10 mM KOH + 4% ACN; analytes: 0.1 mM each of IO_3^-, NO_3^-, I^-, SCN^-; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: contactless conductivity detector.

Fig. 2-13 Separation of hydrophobic compounds on the C18 polymer monolithic column. Column: C18 polymer monolith (100 × 0.32 mm I.D.); eluent: 70% ACN; analytes: 1 = 0.001% naphthalene, 2 = 0.0005% pyrene; injection volume: 0.2 μL; flow-rate: 4.2 μL/min; detector: UV; wavelength of UV detection: 254 nm.

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detection: 210 nm.

Fig. 4-5 Separation of cations on cytidine-bonded stationary phase

Column: Cytidine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 1 mM $CuSO_4$; Sample: Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

Fig. 4-6 Separation of cations on adenosine-bonded stationary phase

Column: Adenosine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 1 mM $CuSO_4$; Sample: Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

Fig. 4-7 Separation of cations on adenosine-bonded stationary phase under neutral eluent condition

Column: Adenosine-bonded silica, 100 x 0.32 mm I.D.; Eluent: 1 mM BETMAC; Sample: ①Na^+, ②K^+, ③NH_4^+, ④Mg^{2+}, ⑤Ca^{2+}, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

Fig. 4-8 Separation of organic acids on cytidine-bonded stationary phase

Column: Cytidine-bonded silica, 100 x 0.32 mm I.D.; Eluent: ACN:10 mM ammonium acetate, ratio as indicated; Sample: 1=phenol, 2=salicylic acid, 3=benzoic acid, 4=phthalic acid, 1 mM each; Flow-rate: 2.0 μL/min; Wavelength of UV detection: 254 nm.

Na^+, K^+, NH_4^+, Mg^{2+}, Ca^{2+}, 0.5 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 210 nm.

Fig. 4-9 Separation of organic acids on adenosine-bonded stationary phase

Column: Adenosine-bonded silica, 100 x 0.32 mm I.D.; Eluent: ACN:10 mM ammonium acetate, ratio as indicated; Sample: 1=phenol, 2=salicylic acid, 3=benzoic acid, 4=phthalic acid, 1 mM each; Flow-rate: 4.0 μL/min; Wavelength of UV detection: 254 nm.

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List of publications

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List of presentations

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1. Itsuya Kawase, Lee Wah Lim, Toyohide Takeuchi: 2nd International Young Scientists Conference on Analytical Sciences "Pure and Applied Sciences for the Future", (Andalas University, Padang, Indonesia), September 17-18, 2013

2. Itsuya Kawase, Lee Wah Lim, Toyohide Takeuchi: 44th Annual Meeting of Union of Chemistry-Related Societies in Chubu Area, (Shizuoka University, Japan), November 2-3, 2013

3. Itsuya Kawase, Lee Wah Lim, Toyohide Takeuchi: The 8th Asia Pacific Symposium on Ion Analysis, (International Conference Hall Makuhari Messe, Japan), August 31 to September 3, 2015

4. Itsuya Kawase, Lee Wah Lim, Toyohide Takeuchi: The 6th Annual Basic Science International Conference, (Atria Hotel and Conference, Malang, Indonesia), March 2 - 3, 2016

5. Itsuya Kawase, Lee Wah Lim, Toyohide Takeuchi: The 33rd Ion Chromatography Symposium, (Kumamoto City international exchange hall, Japan), December 1-2, 2016

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