

**Development of polar stationary phases and investigation  
of retention behavior 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  
極性固定相の開発と保持挙動の解明

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## Preface

Chromatography was discovered by a Russian scientist named Mikhail Semenovitch Tswett in 1903; he separated chlorophylls with calcium carbonate and that was the first separation based on the adsorption mechanism. Since then, various kinds of separation techniques have appeared and been developed. Chromatography has diverged based on its separation mechanism. Now it becomes an indispensable method in analytical field. Chromatography is a separation method by utilizing the interaction between mobile phase and stationary phase. These phases are generally classified based-on their physical states, *i.e.* solid, liquid and/or gas. Liquid chromatography (LC) has general-purpose properties because specimen materials must be able to be dissolved in a carrier and liquid mobile phases can be considered better compatible than anything else.

Since the development of capillary LC, performances of the same kind of analysis could be achieved by using less consumption of solvent and analyte. In contrast, it is recognized that the performance (separation efficiency) of LC decreases by down-sizing the system due to the fact that the system can be easily affected by the surrounding circumstances such as a change in temperature or a little quiver. Nevertheless, capillary LC has many advantages over the conventional LC, *i.e.* low consumption of mobile phase, mobile additives as well as stationary phase, decreased amount of waste (especially organic solvents), *etc.* Its low cost and environmentally friendly features favor the development of novel stationary phase, for instance, when research activities carried out in the laboratory.

The establishment of capillary-based techniques allows scientists to study more easily on the development and improvement of chromatographic methods. Investigation of synthesis of stationary phases has been conducted in order to optimize all kinds of complicated separations. Monolithic columns provide a breakthrough that can avoid troubles caused by particle packed columns. Monolithic columns have through-pores as well as meso-pores on the skeleton and this double-pore characteristic greatly reduces the back pressures under chromatographic measurements. Monolithic columns are generally divided into two types, *i.e.* polymer-based and silica-based. Polymer-based

monolithic columns could be synthesized by easy polymerization and used under a wide range of pH. The preparation of polymer-based monolithic column involves monomers, cross-linkers and porogens. Various types of chemicals were employed to form expected structure and attach functional groups. Silica-based columns were prepared by sol-gel technology. Silanol groups of the surface of silica-based monolith could be modified. And silica-based monolithic columns could show the solvent resistance and mechanical strength.

In ion chromatography, charged functional groups on the surface of stationary phases are the most important points of ion-exchange mechanism. Generally, ionic analyte are retained by electrostatic interaction. There are several ways to introduce electrostatic forces on stationary phases. It is presumed that zwitterionic functional groups could attract both anion and cation samples, whereas repulse them. In this study, zwitterionic reagents were successfully bonded to (what kind of) stationary phases, and zwitterionic surfactant and ion-pair reagents were also dynamically coated on hydrophobic stationary phases. Although the retention mechanism of ionic samples on zwitterionic stationary phases is complex, they have the ability to separate both anion and cation samples within the same column.

Another main application of zwitterionic stationary phases is for hydrophilic interaction chromatograph (HILIC). HILIC is a very useful method especially when it comes to real samples analysis. Samples that are not retained on reversed-phase LC can be separated by the HILIC mode. Stationary phases that have polar structure can be applied for HILIC mode. These polar structure produce hydration layer and hydrophilic samples are attracted by partition. Major functional groups are bare silica, diol, imidazole, pyridine and those with zwitterionic groups such as sulfobetaine.

In this study, various kind of stationary phases for HILC were employed to show the resolution of retention mechanism of inorganic anions under HILIC. It unveiled that electrostatic interaction and hydrophilic partition competed for anion separation under the HILIC condition. Development of zwitterionic monolithic columns and investigation of anion and cation separation were conducted. Reaction condition, ratio of monomer, cross-linker and porogen were also optimized.



# Chapter 1

## Introduction

### 1.1 Chromatography

Chromatography is a method that separates mixed components using two inter-connected phases, which are called stationary phase and mobile phase. Chromatography conducts separation by taking advantage of physical or chemical interaction between stationary phase and mobile phase, and it is normally categorized according to the condition of mobile phase. If the mobile phase is a liquid, it is called liquid chromatography (LC), and for the case when a gas is used, it is called gas chromatography (GC), *etc.* [1].

In GC, due to the gas phase, diffusion speed of molecules is fast, which leads to the fact that equilibrium can be achieved very fast and short-time analysis is also obtainable. On the other hand, diffusion speed of respective components in liquid is slower than that of GC, so LC requires longer time to achieve equilibrium and longer analysis is needed, and so this is one of the flaws of LC. However, GC has a different problem, that non-volatile materials or high molecular weights substance cannot be analyzed by GC. Most of these materials are evaluated by LC. Other types of chromatography are shown in Table. 1-1 [2].

To shorten the analysis time, the mobile phase was flowed by adding a pressure. At the same period of time, other equipments and materials for chromatography systems were also developed. Chromatography was originally invented by Tswett, *i.e.* a Russian botanist, in 1903. He successfully separated the color constituents from a plant pigment, and he then named the method “chromatography” with its original meaning “to write with color”. In present, there are many modifications and improvements to the chromatography system, and high performance liquid chromatography, *i.e.* more widely known as HPLC, is the improved chromatograph and has been used in various fields such as life sciences, environment, medicals, pharmaceuticals, foods, and *etc.* [3,4].

Table 1-1 Classification of chromatography [2]

Mobile phase	Stationary phase	Name of chromatography
Gas	Gas	Gas chromatography
	Liquid	
Liquid	Solid	Liquid chromatography
	Liquid	
Supercritical fluid		Supercritical fluid chromatography
Shape of separation bed		
Tubular		Column chromatography
Planar		Thin layer chromatography
		Paper chromatography
Type of interaction		
Adsorption		Adsorption chromatography
Partition		Partition chromatography
Ion-exchange		Ion-exchange chromatography
Size-exclusion		Size-exclusion chromatography
Permeation		Gel permeation chromatography
Filtration		Gel filtration chromatography

## 1.2 Ion exchange chromatography

Materials that have ionic functional group(s) are called ion-exchange resins, and they are utilized in ion-exchange chromatography (IEC) or more specifically they are used as the stationary phase. The main retention mechanism is ion exchange by electrostatic interaction between stationary phase and sample ions. Ionic materials are attracted by either negative or positive charges inner stationary phase [5-8].

Typical ion exchangers are sulfonic acid or carboxylic acid groups for cation separation, and quaternary ammonium is often used for anion separation. And three other types of ammonium groups (*i.e.* primary, secondary and tertiary) also can work as anion exchangers after they are protonated. Examples of ion-exchangers are shown in Table 1-2.

The performance of this mode depends on the exchange capacity, structure of functional group(s), base material, particle size, and *etc.* In addition, composition of mobile phase also affects the retention of samples. By adjusting these parameters, separation of analyte ions can be achieved.

Table 1-2 Types and functional groups of common ion-exchangers

Type	Functional groups	Structure
Strongly basic anion exchanger	Quaternary ammonium	$-\text{CH}_2\text{N}^+(\text{CH}_3)_3$
Weakly basic anion exchanger	Amine	$-\text{CH}_2\text{NH}_2$
Strongly acidic cation exchanger	Sulfonic acid	$-\text{SO}_3^-$
Weakly acidic exchanger	Carboxylic acid	$-\text{COO}^-$

### 1.3 Zwitterionic stationary phase

Zwitterionic stationary phase has both negative and positive charges. Many reports regarding zwitterionic stationary phase have been published [9-14]. This type of stationary phases have been invented and developed to aim simultaneous separation of cations and anions. The existence of both charges also causes the stationary phase to be highly polarized due to the attraction and repulsion within the stationary phase, and thus it exhibits alternative ion selectivity to those obtained by the conventional anion and cation exchangers, and therefore it has the potential of optimization in IC separation.

Zwitterionic stationary phase can be obtained by covalently attaching zwitterionic molecules to a stationary phase or by dynamically coating zwitterionic surfactants on a hydrophobic stationary phase [15-18]. In covalently-bonded zwitterionic stationary phases, the important factors are distance

between the positive and negative charges as well as the position which charge is located at the external site. In case of dynamic modification (coating), it is considered that the retention behavior depends on the chaotropic properties of the analytes ion [19].

#### **1.4 Monolithic column**

Monolithic stationary phases could be synthesized by chemical reaction in tubes. The first introduction in capillary columns was in late 1980s [20-22]. In contrast to packed columns, measurements with monolithic columns show low pressure due to the unique structure of the skeleton and through pores, and separation in higher flow rate could be achieved. Generally, base materials of monolithic stationary phases are categorized into two types, organic polymer-based and silica-based. Organic polymer-based monolithic columns are prepared by polymerization of mixture, which are monomer, cross-linker, porogens and initiator. The advantages of polymeric columns are the resistance properties under a wide range of pH and the easy preparation. Lots of approaches for preparation of matrix have been conducted. In silica-based monolithic columns, they are prepared through sol-gel technology. The advantages of silica monoliths are their strong mechanical stability and solvent resistance.

#### **1.5 Hydrophilic interaction chromatography (HILIC)**

Hydrophilic interaction chromatography (HILIC) has been attracting a lot of attention since it was coined in 1990 [24]. It is normally considered as one type of the normal-phase chromatography. In HILIC, large amount of organic solvent, usually acetonitrile (ACN) is contained in eluent, and stationary phases having high polarity are normally used. In most cases, functionalized groups such as amino, diol and amide are covalently bonded to the stationary phase [25]. It is defined that separation of analytes is dominated by hydrophilic interaction. A water layer forms on the surface of the stationary phase and polar compounds could be retained by partition. The unique nature of HILIC is that it can retain polar compounds which are too hydrophilic for the reversed-phase liquid

chromatography (RPLC). So, HILIC is a better approach which can cover the field that is beyond the control of RPLC, and it has been widely utilized for the separation of various kinds of samples. The popular applications of HILIC are the determination of nucleic acid-based derivatives, vitamins, sugars and amino acids [25, 26]. Obviously, it is expected that HILIC is the most useful method for biological samples [27].

## 1.6 Capillary LC

LC system can be categorized by their column diameter in Table. 1-3 [28]. Generally, diameter of column for capillary LC is less than 1.0 mm. On the other hand, over 4.0 mm diameter of columns is often used for conventional LC. The biggest advantage of capillary LC is its environmental friendly feature, which means low amount of mobile phases and waste. Extremely small amount of sample volume is also sufficient for the analysis. As the results, expensive reagents for mobile phase additive and scarce samples such as biological samples are also applicable. Nevertheless, it is well-known that the performance (separation efficiency) of LC decreases by down-sizing the system due to the fact that the system can be easily affected by the surrounding circumstances such as a change in temperature or a little quiver.

Table. 1-3 Classification of LC columns based on their I.D. [28]

Purpose	Classification	I.D. / 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 ~

## 1.7 Objectives of the research

A lot of commercial stationary phases for HILIC are available. These stationary phases have polar functional groups to form hydration layer. Most of the functional groups can be charged negatively or positively. Residual silanol groups on silica-based columns can be deprotonated and negatively charged while amine groups can be protonated and charged positively. That means retention mechanism of HILIC is not explained by simple interaction [29]. Chapter 2 shows the specific phenomena of inorganic separation in HILIC mode and provides resolution.

Many papers of zwitterionic monolithic columns have been published [30-33]; those papers discussed applications of these columns for mainly HILIC mode. Chapter 3 shows an easy one-pot synthesis of zwitterionic monolithic columns. The reaction condition was optimized and the prepared columns were evaluated by capillary ion chromatography. Results were reviewed by comparison with commercially available packing columns.

It is known that the amino groups have the ability to react with other reagents by the lone electron pair and is expected to form zwitterionic structure. And a number of articles, detailing stationary phases dynamically coated with zwitterionic molecules, have been published. However, there is still much room to study on the zwitterionic stationary phase. It is expected that zwitterionic stationary phase can separate anions and cations simultaneously and be applied to alternative chromatography. In chapter 4, several types of zwitterionic stationary phases were prepared and the characteristic(s) of each phase was investigated.

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## Chapter 2

# Retention behavior of inorganic anions in hydrophilic interaction chromatography

### 2.1 Introduction

Hydrophilic interaction chromatography (HILIC) has been attracting a lot of attention since it was coined in 1990 [1]. In HILIC, a large amount of organic solvent, usually acetonitrile (ACN) is contained in the eluent, and stationary phases having high polarity are normally used. In most cases, the stationary phase for HILIC are bare silica or functionalized groups such as amino, diol, silica and amide which are covalently bonded to the silica backbone [2]. A water layer forms on the surface of the stationary phase and polar compounds could be retained by partition. The unique nature of HILIC is that it can retain polar compounds which are too hydrophilic for the reversed-phase liquid chromatography (RPLC). So, HILIC is a better approach which can cover the field that is beyond the control of RPLC, and it has been widely utilized for the separation of various kinds of samples. The popular applications of HILIC are the determination of nucleic acid-based derivatives, vitamins, sugars and amino acids [3,4]. Obviously, it is expected that HILIC is a most useful method in biological samples [5].

Ion-exchange chromatography (IEC) is the most common method used to separate and determine ionic samples. Usually ionic functional groups which have negative or positive charge are chemically bonded to the base materials for IEC stationary phase. The main retention mechanism is ion exchange by electrostatic interaction between stationary phase and analyte ions. There are several factors which affect the retention of inorganic ions and their elution order. For examples, they are the strength of electrostatic interaction, the adsorption of analyte ions to the base material of the stationary phase, and the degree of hydration of the analyte ions as well as the surface of the ion exchange sites. Especially, the degree of hydration has a major influence to the elution order. The selectivity of anion exchange chromatography was studied by using methanol, acetonitrile, *N,N*-dimethylformamide and

their mixtures; drastic changes of retention time for inorganic anions were observed according to the composition of mobile phase [6].

Besides, some stationary phases, where there is no positive/negative charge, could achieve the separation of ions by dynamically coating ionic reagents such as ion-pairs reagents or surfactants [7-10]. It was also found that some inorganic anions could be separated on C30 stationary phase by hydrophobic interaction [11]. Separation of inorganic anions in the partition mode was observed by the C30 stationary phase coated by poly(ethylene glycol) (PEG) [12,13]. PEG covers the surface of the C30 and forms layer on it. This layer could be adjusted by the eluent concentration and optimization of the eluent was investigated. Chemically bonded poly(oxyethylene) stationary phase is also examined [14]. It was reported that inorganic anions could be separated by ion-exchange mode. This mechanism is considered that several poly(oxyethylene) chains catch the eluent cation such as sodium ions and potassium ions and they can attract analyte anions.

Recently, it has been found that HILIC stationary phase can also be applied for ion separation. HILIC stationary phase which is a diol-type has achieved ion separation under acidic condition [15]. Mixed mode of HILIC/anion-exchange was investigated on a latex coated monolith column, which implied that the mechanism depends on the partition for kosmotropic anions, while anion-exchange dominates for chaotropic anions [16]. Zwitterionic stationary phase is often used to evaluate the effect of both charges [4, 17-21]. Nucleoside, water soluble vitamins, benzoic acid derivatives and basic compounds were employed as analyte samples [4]. Three types of zwitterionic stationary phases, which have different negative and positive charges' ratio each, were prepared and various experiment results suggested that protonation of stationary phase and analyte sample is controllable by adjusting the pH. Hence, retention of samples is affected by the electrostatic attraction or repulsion.

Based on the above concepts, there is still plenty of room that has not been understood in HILIC separation. In this paper, we investigated the retention behavior of inorganic anions by using HILIC stationary phases such as TSKgel NH<sub>2</sub>-60, Polar-Imidazole, TSKgel Amide-80, Polar-Pyridine

and ZIC-HILIC. Base material of all stationary phases was silica gel.

## **2.2 Experimental**

### **2.2.1 Reagents and chemicals**

Reagents employed were of guaranteed reagent grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise noted. Sodium bromate, sodium bromide, sodium iodate, sodium iodide, sodium nitrate, sodium nitrite, sodium thiocyanate and ammonium acetate, were obtained from Nacalai Tesque (Kyoto, Japan). HPLC grade acetonitrile was obtained from Tokyo Chemical Industry (Tokyo, Japan). Ultrapure water was prepared 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.

TSKgel NH<sub>2</sub>-60 and TSKgel Amide-80 were obtained from Tosoh Corporation (Yamaguchi, Japan). Polar-Imidazole and Polar-Pyridine were obtained from Sepax Technologies (Newark, USA) while ZIC-HILIC was obtained from Merck Millipore (Darmstadt, Germany).

### **2.2.2 Apparatus**

In this work, all experiments were conducted by using a capillary LC system constructed by a microfeeder (L.TEX Corporation, Tokyo, Japan) equipped with a gas-tight syringe (0.5 mL; Ito, Fuji, Japan) as a pump, an M-435 micro injection valve (Upchurch Scientific, Oak Harbor, WA, USA) with an injection volume of 0.2  $\mu$ L, a microcolumn prepared from a fused-silica capillary tube (100 mm  $\times$  0.32 mm I.D.; GL Sciences, Tokyo, Japan), a UV detector (JASCO, Tokyo, Japan) with the wavelength 210 nm, and a data processor (CDS-Lite ver 5.0; LA soft, Chiba, Japan). The inlet pressure was monitored by an L.TEX-8150 pressure sensor (L.TEX). Separation columns were immersed into a water bath for temperature controlled at 20°C throughout the study.

### 2.2.3 Structures of stationary phases employed in this study

Schematic structures of stationary phases employed in this study are shown in Fig. 2-1.

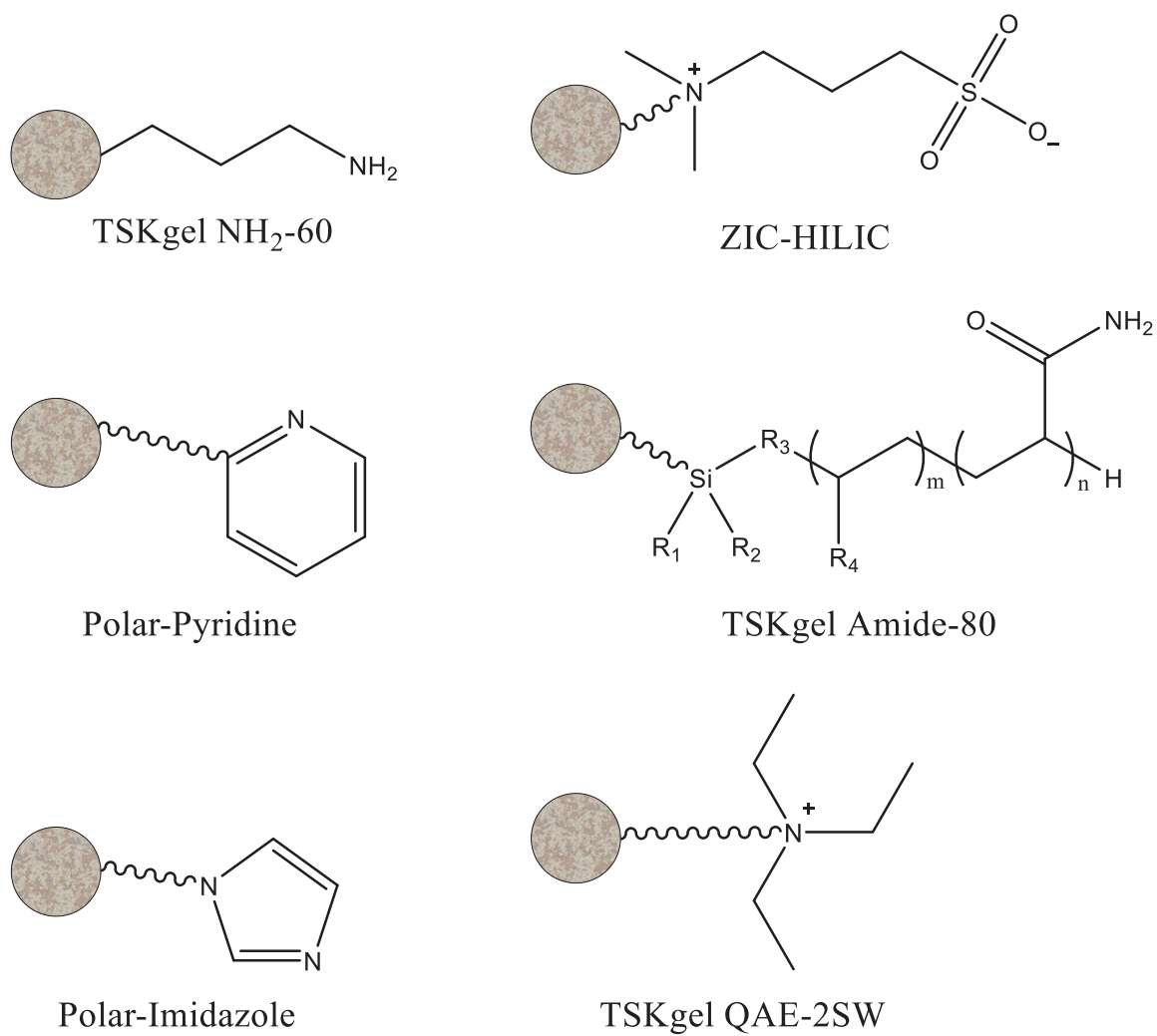


Fig. 2-1 Schematic diagram of employed stationary phases

## 2.3 Results and discussion

### 2.3.1 Retention behavior of inorganic anions under IEC and HILIC modes

Fig. 2-2 compared the difference of elution order of inorganic anions between IEC mode and HILIC mode. As for the IEC mode (Fig. 2-2 A; upper trace), IC-Anion-PW<sub>XL</sub> and 200 mM sodium chloride were employed as the stationary phase and eluent, respectively. As can be seen from Fig. 2-2 A, the elution order of IEC is  $\text{BrO}_3^- < \text{NO}_2^- < \text{Br}^- < \text{NO}_3^- < \text{I}^- < \text{SCN}^-$ . On the other hand, TSKgel NH<sub>2</sub>-60 and 30 mM ammonium acetate containing 70 % ACN were employed as the stationary phase and eluent, respectively, and the separation was carried out under HILIC mode (Fig. 2-2 B); opposite elution order was observed, *i.e.*  $\text{SCN}^- < \text{I}^- < \text{NO}_3^- < \text{Br}^- < \text{NO}_2^- < \text{BrO}_3^-$ . These results suggested that hydration degree of the analyte anions is the most important factor deciding the elution order. Anions which have smaller radii have stronger electrostatic force per unit area and therefore would be strongly hydrated. And furthermore, the radius of strongly hydrated anion became big and it has weak electrostatic interaction to the anion-exchange site. As a result, small anions retain weakly on the stationary phase and big anions have opposite nature. In HILIC mode, a lot of ACN is added in the eluent; and since ACN promotes the desolvation of analyte anions, small hydrated anions after desolvation can be strongly attracted to the stationary phase. And therefore the elution order became reversed under the HILIC mode.

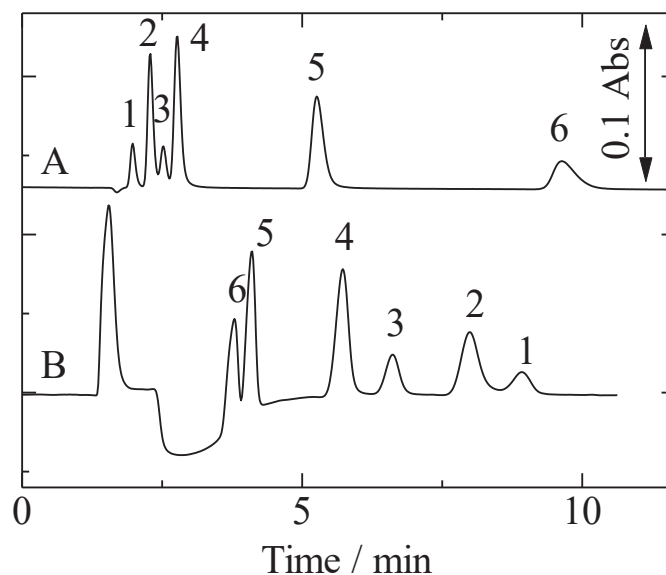


Fig. 2-2 Separation of inorganic anions under IEC (A) and HILIC (B) modes.

Columns: TSKgel IC-Anion-PW<sub>XL</sub> (A), TSKgel NH<sub>2</sub>-60 (B), 100 × 0.32 mm i.d. Eluents: 200 mM NaCl (A), 30 mM CH<sub>3</sub>COONH<sub>4</sub> with 70 % ACN additive (B). Flow-rate: 4.0 μL min<sup>-1</sup>. Analytes: 1 = BrO<sub>3</sub><sup>-</sup>, 2 = NO<sub>2</sub><sup>-</sup>, 3 = Br<sup>-</sup>, 4 = NO<sub>3</sub><sup>-</sup>, 5 = I<sup>-</sup>, 6 = SCN<sup>-</sup>, 0.5 mM each. Injection volume: 0.2 μL. Wavelength of UV detection: 210 nm.

### 2.3.2 Retention behavior of inorganic anions on various HILIC stationary phases

Figs. 2-3 and 2-4 show the retention behavior of inorganic anions. Sodium chloride and sodium perchlorate are used as salt in Fig. 2-3 and Fig. 2-4, respectively. The concentration of salt and ACN were kept constant 20 mM and 70 %. SCN<sup>-</sup>, I<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, Br<sup>-</sup>, NO<sub>2</sub><sup>-</sup> and BrO<sub>3</sub><sup>-</sup> are used as analyte anions. The results indicate that TSKgel NH<sub>2</sub>-60 and Polar-Imidazole could retain the analyte anions. TSKgel Amide-80 also could retain them weakly while Polar-Pyridine did not show any retention. ZIC-HILIC retained them but showed the asymmetric peaks in Fig. 2-4. The pK<sub>a</sub> values of conjugate acid for methylamine, imidazole and pyridine are 10.62, 7.00 and 5.29, respectively. That means TSKgel NH<sub>2</sub>-60 is protonated, Polar-Imidazole is partially protonated and Polar-Pyridine is little

protonated under the neutral condition. For this reason, it is expected that TSKgel NH<sub>2</sub>-60 and Polar-Imidazole could retain the analyte anions by electrostatic interaction. TSKgel Amide-80 is non-protonable and it does not work *via* electrostatic interaction but partition. ZIC-HILIC has both positive and negative charges in its structure. In this case, analyte anions can be attracted and repulsed by both charges and the elution order showed irregular and peaks became asymmetry. Therefore, TSKgel NH<sub>2</sub>-60 and Polar-Imidazole were selected to investigate the effect of other parameters in the following experiments.

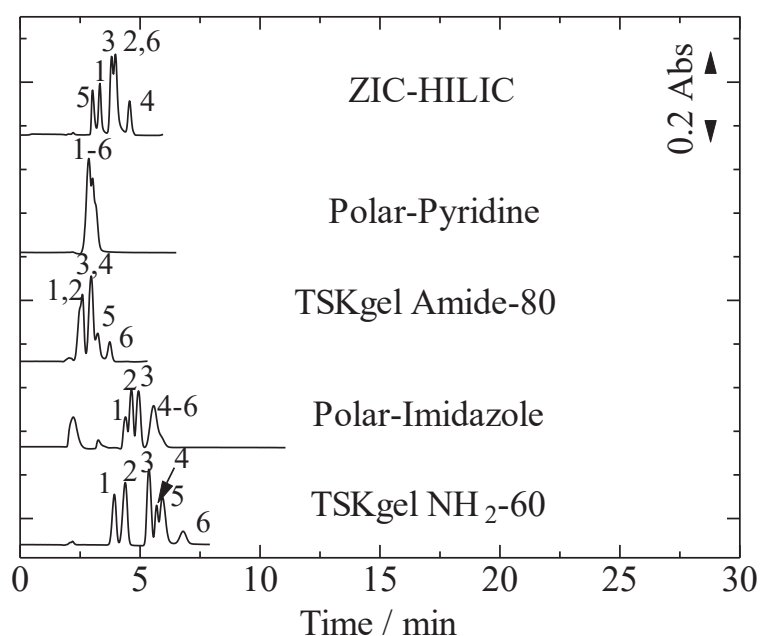


Fig. 2-3 Retention behavior of inorganic anions on various HILIC stationary phases using sodium chloride salt.

Columns: TSKgel NH<sub>2</sub>-60, Polar-imidazole, TSKgel Amide-80, Polar-Pyridine, ZIC-HILIC, 100 × 0.32 mm I.D. Eluent: 20 mM NaCl with 70 % ACN. Flow-rate: 3.0 μL min<sup>-1</sup>. Analytes: 1 = BrO<sub>3</sub><sup>-</sup>, 2 = NO<sub>2</sub><sup>-</sup>, 3 = Br<sup>-</sup>, 4 = NO<sub>3</sub><sup>-</sup>, 5 = I<sup>-</sup>, 6 = SCN<sup>-</sup>, 0.5 mM each. Injection volume: 0.2 μL. Wavelength of UV detection: 210 nm.

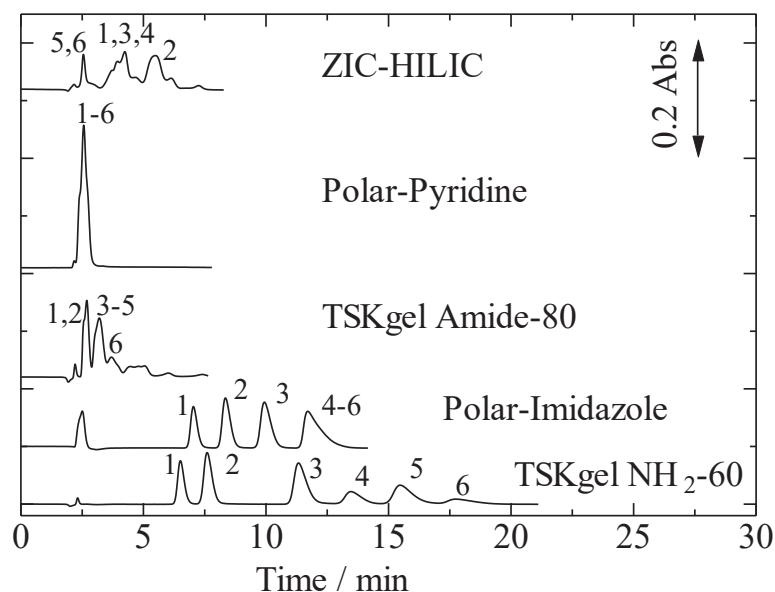


Fig. 2-4 Retention behavior of inorganic anions on various HILIC stationary phases using sodium perchlorate salt.

Columns: TSKgel NH<sub>2</sub>-60, Polar-imidazole, TSKgel Amide-80, Polar-Pyridine, ZIC-HILIC, 100 × 0.32 mm I.D. Eluent: 20 mM NaClO<sub>4</sub> with 70 % ACN. Flow-rate: 3.0 μL min<sup>-1</sup>. Analytes: 1 = BrO<sub>3</sub><sup>-</sup>, 2 = NO<sub>2</sub><sup>-</sup>, 3 = Br<sup>-</sup>, 4 = NO<sub>3</sub><sup>-</sup>, 5 = I<sup>-</sup>, 6 = SCN<sup>-</sup>, 0.5 mM each. Injection volume: 0.2 μL. Wavelength of UV detection: 210 nm.

### 2.3.3 Effect of salt species

Fig. 2-3 and Fig. 2-4 also show the effect of salt species for TSKgel NH<sub>2</sub>-60 and Polar-Imidazole. The investigated salt species are sodium chloride and sodium perchlorate. As can be seen, sodium chloride leads to shorter retention time while sodium perchlorate caused longer retention time, respectively. Usually it is considered that, when sodium perchlorate is used, retention time of analytes should be shorter than that observed when sodium chloride was used. This is because perchlorate can retain on the stationary phase stronger than chloride, and that means perchlorate prevents the retention of analyte anions and leads to shorter retention time eventually. But in this case, converse retention result was observed. This could be explained by the hydration for each chloride and perchlorate become weak due to high ACN concentration. The ACN can disturb the hydration of



elution anions and their hydrated ionic radii become smaller than those in water. Thus, chloride can retain stronger than perchlorate and shorter retention time of analyte anions was observed.

#### 2.3.4 Effect of salt concentration

Retention behavior was investigated with various concentrations of sodium perchlorate under HILIC condition. The concentration of ACN was maintained at 70% while sodium perchlorate was varied from 10 to 40 mM, the chromatograms are shown in Figs. 2-5 and 2-6. The retention time of all samples decreased with increasing sodium perchlorate concentration; as commonly observed under the conventional IEC mode. Figs. 2-7 and 2-8 show the logarithm of retention factor ( $k$ ) of analytes versus the logarithm of the eluent (*i.e.* sodium perchlorate) concentration. Three anions, thiocyanate, iodide and nitrate are plotted for Polar-Imidazole in Fig. 2-8 because other samples, bromide, nitrite and bromate were coeluted. It is widely known that the plots of  $\log k$  versus  $\log$  eluent concentration should be straight lines and the slopes should be  $-1$ , when monovalent anions are employed for both analytes and eluent. The slopes obtained in Fig. 2-7 were  $-0.44$ ,  $-0.43$ ,  $-0.43$ ,  $-0.43$ ,  $-0.44$ , and  $-0.45$  for thiocyanate, iodide, nitrate, bromide, nitrite, and bromate, respectively. The slopes obtained in Fig. 2-8 were  $-0.69$ ,  $-0.68$  and  $-0.73$  for thiocyanate, iodide and nitrate, respectively. Although the slopes do not attain the theoretical values, they can be considered straight lines (with linear correlation coefficients,  $R^2 > 0.99$ ). These results imply that ion-exchange mode works but it is not the only retention mechanism involved in the retention of these anions under the HILIC condition.

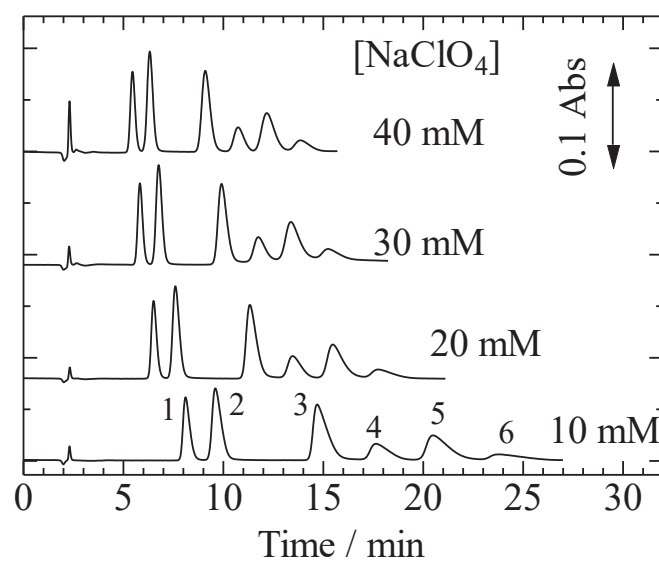


Fig. 2-5 Separation of inorganic anions on a TSKgel NH<sub>2</sub>-60 column with different eluent concentrations.

Column: TSKgel NH<sub>2</sub>-60, 100 × 0.32 mm I.D. Eluents: NaClO<sub>4</sub> with 70 % ACN, the concentration of NaClO<sub>4</sub> as indicated. Flow-rate: 3.0 μL min<sup>-1</sup>. Analytes: 1 = BrO<sub>3</sub><sup>-</sup>, 2 = NO<sub>2</sub><sup>-</sup>, 3 = Br<sup>-</sup>, 4 = NO<sub>3</sub><sup>-</sup>, 5 = I<sup>-</sup>, 6 = SCN<sup>-</sup>, 0.5 mM each. Injection volume: 0.2 μL. Wavelength of UV detection: 210 nm.

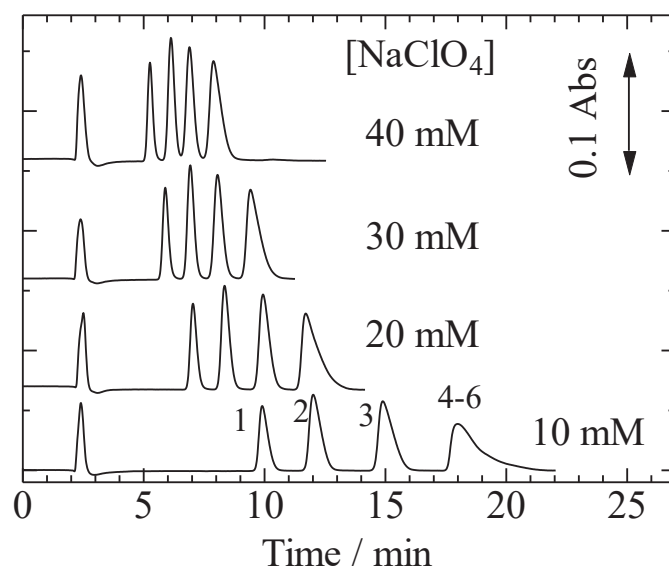


Fig. 2-6 Separation of inorganic anions on a Polar-imidazole column with different eluent concentrations.

Column: Polar-Imidazole,  $100 \times 0.32$  mm I.D. Eluents:  $\text{NaClO}_4$  with 70 % ACN additive, the concentration of  $\text{NaClO}_4$  as indicated. Flow-rate:  $3.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{BrO}_3^-$ , 2 =  $\text{NO}_2^-$ , 3 =  $\text{Br}^-$ , 4 =  $\text{NO}_3^-$ , 5 =  $\text{I}^-$ , 6 =  $\text{SCN}^-$ , 0.5 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

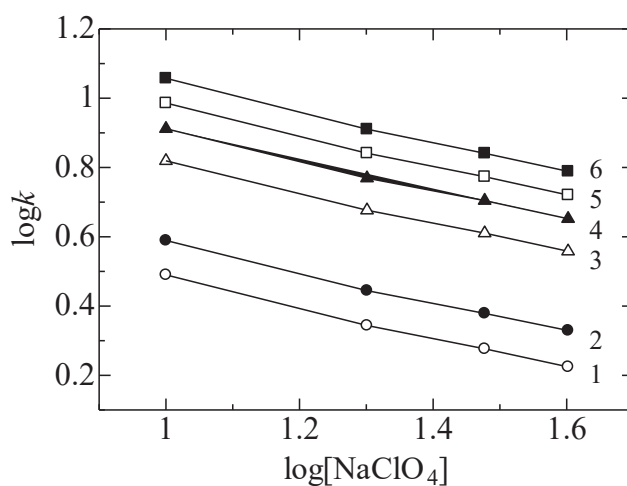


Fig. 2-7 Plotting of logarithm of the retention factor ( $k$ ) versus logarithm of the  $\text{NaClO}_4$  concentration given by a TSKgel  $\text{NH}_2$ -60 column.

Operating conditions as in Fig. 2-5.

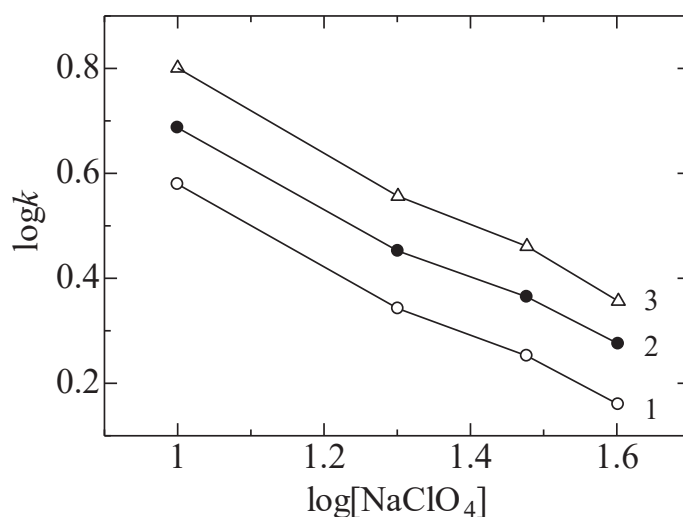


Fig. 2-8 Plotting of logarithm of the retention factor ( $k$ ) versus logarithm of the  $\text{NaClO}_4$  concentration given by a Polar-Imidazole column.

Operating conditions as in Fig. 2-6.

### 2.3.5 Effect of ACN concentration

Figs. 2-9 and 2-10 illustrate the effect of ACN concentration. The various concentration of ACN was investigated in the range of 40-70%. 20 mM sodium perchlorate was utilized in these experiments and the chromatograms are shown in Fig. 2-9. As can be seen in Fig. 2-9, the retention time for each anion increased with increasing ACN concentration. All anions could not be separated at 40% ACN whereas the separation of all samples was achieved when the ACN concentration was greater than 60%. In Fig. 2-10, similar phenomenon was observed. The fact that retention time became longer when the amount of ACN increased is a typical mechanism of HILIC.

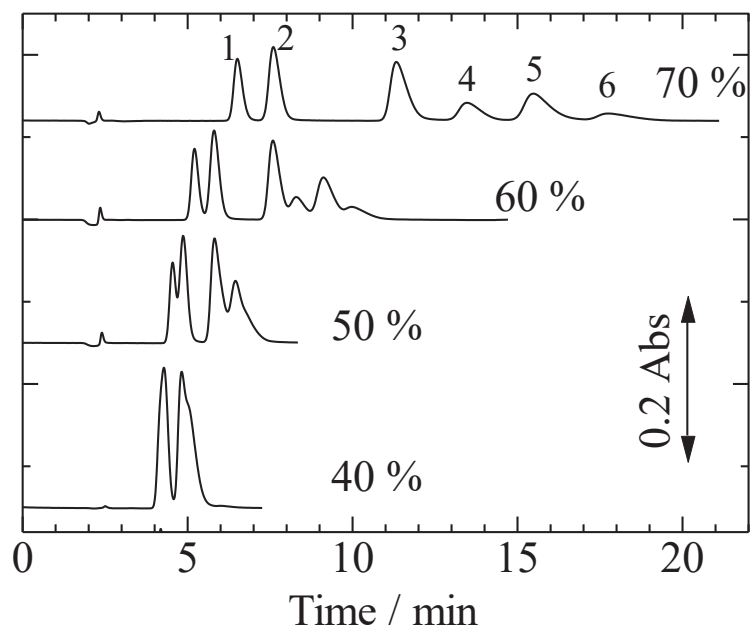


Fig. 2-9 Separation of inorganic anions on TSKgel NH<sub>2</sub>-60 column with different concentration of ACN.

Column: TSKgel NH<sub>2</sub>-60, 100 × 0.32 mm I.D. Eluents: 20 mM NaClO<sub>4</sub> + ACN with concentration as indicated. Flow-rate: 3.0 μL min<sup>-1</sup>. Analytes: 1 = BrO<sub>3</sub><sup>-</sup>, 2 = NO<sub>2</sub><sup>-</sup>, 3 = Br<sup>-</sup>, 4 = NO<sub>3</sub><sup>-</sup>, 5 = I<sup>-</sup>, 6 = SCN<sup>-</sup>, 0.5 mM each. Injection volume: 0.2 μL. Wavelength of UV detection: 210 nm.

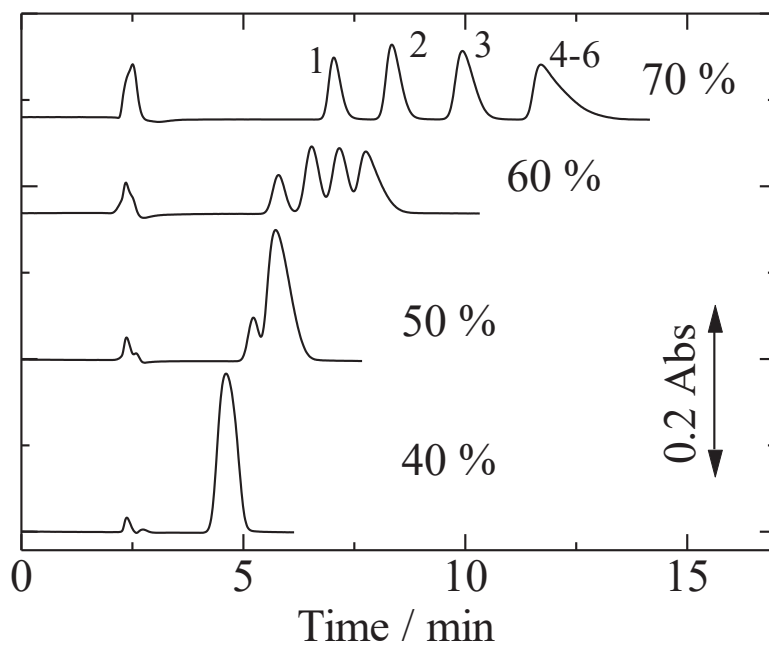


Fig. 2-10 Separation of inorganic anions on Polar-Imidazole column with different concentration of ACN.

Column: Polar-Imidazole,  $100 \times 0.32$  mm I.D. Eluents: 20 mM  $\text{NaClO}_4$  + ACN with concentration as indicated. Flow-rate:  $3.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{BrO}_3^-$ , 2 =  $\text{NO}_2^-$ , 3 =  $\text{Br}^-$ , 4 =  $\text{NO}_3^-$ , 5 =  $\text{I}^-$ , 6 =  $\text{SCN}^-$ , 0.5 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

### 2.3.6 Effect of ACN concentrations with another stationary phase (cross-checking experiment)

In order to compare the effect of stationary phases, investigation of various concentration of ACN was conducted with TSKgel QAE-2SW, which is a silica-based stationary phase with quaternary ammoniums as the ion exchange sites.  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{I}^-$  and  $\text{SCN}^-$  are chosen as analyte anions. The concentration of ACN was examined from 0% to 80%, and sodium chloride was kept at 50 mM, the separation results are summarized in Fig. 2-11. ACN 0% means the retention mechanism is solely depend on ion exchange mode with elution order:  $\text{IO}_3^- < \text{NO}_2^- < \text{NO}_3^- < \text{I}^- < \text{SCN}^-$ , which is the standard order for IEC [22]. As increasing of ACN, gradually the elution order became reverse, and the retention times of each sample are represented in Fig. 2-12. Elution order became completely reversed at high ACN concentration. In this study, the same phenomenon was observed when TSKgel NH<sub>2</sub>-60 was used as the stationary phase. A lot of ACN can promote the desolvation of analyte anions, and their hydrated ionic radii became smaller. As the result, small hydrated anion after desolvation has strong electrostatic force per unit area and can interact strongly to the stationary phase. After the reversed elution order was observed at the 70 % ACN, the retention time of  $\text{IO}_3^-$  became longer at the 80% ACN. These results imply that HILIC mode appeared and partition is one of the retention mechanisms.

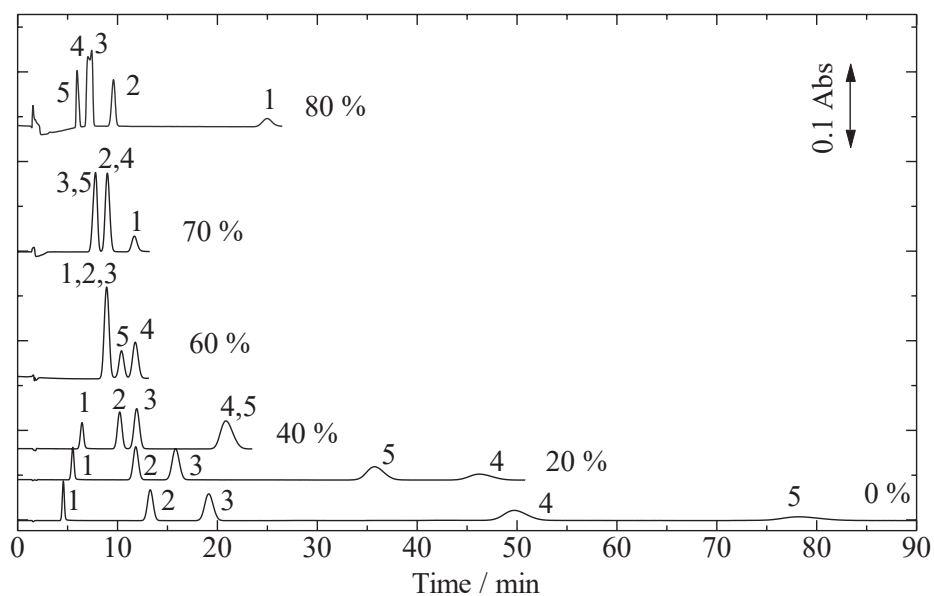


Fig. 2-11 Separation of inorganic anions on a TSKgel QAE-2SW column with different ACN concentrations.

Column: TSKgel QAE-2SW,  $100 \times 0.32$  mm I.D. Eluents: 50 mM NaCl with ACN additive, the concentration of ACN as indicated. Flow-rate:  $3.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{IO}_3^-$ , 2 =  $\text{NO}_2^-$ , 3 =  $\text{NO}_3^-$ , 4 =  $\text{I}^-$ , 5 =  $\text{SCN}^-$ , 0.5 mM each. Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.

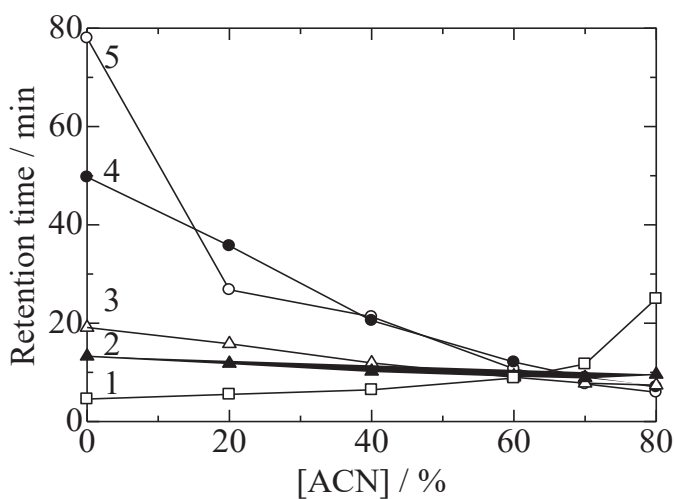


Fig. 2-12 Retention time as a function of the ACN concentration in the eluent.

Operating conditions as in Fig. 2-11



## 2.4 Conclusions

Stationary phases that are suitable for the separation of inorganic anions under HILIC condition are TSKgel NH<sub>2</sub>-60 and Polar-Imidazole. Elution order of inorganic anions was reversed in HILIC mode in comparison with IEC mode when TSKgel NH<sub>2</sub>-60 and Polar-Imidazole were used as the stationary phase. High concentration of ACN facilitates the desolvation of analyte anions as well as anions that are in the eluent. It is presumed that the retention of anions is affected by the electrostatic interaction but the retention mechanism is not purely based on ion-exchange mode. Partition is also competing for retention mechanism due to the fact that retention increases as increasing the concentration of ACN.

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## Chapter 3

# Preparation of zwitterionic monolithic columns in capillary ion chromatography

### 3.1 Introduction

Monolithic columns are generally categorized into two types, *i.e.* one is organic polymer base, and another is silica base. Advantages of monolithic columns are derived from their high porous structures. It is consisted by mesopores and through-pores, which enable higher liner flow late and low pressure. Another representative merit of monolithic columns is easy preparation. Fabrication of skeleton and modification of structure could be led by simple chemical reaction. So many kinds of monolithic stationary phases have been synthesized to meet various needs.

Several types of zwitterionic (ZIC) stationary phases were developed for ion chromatography. Zwitterionic structure has both anion and cation exchange sites. The most popular structure is the one that is covalently bonded zwitterionic molecules [1]. ZIC-HILIC, produced by Merck, has sulfobetaine functional groups on its structure [2]. Positively charged and negatively charged groups are covalently bonded to silica particles. 1,3-propane sultone was reacted to imidazolium groups as a quaternizing reaction to form zwitterionic structure [3-4]. Jiang and coworkers reported phosphorylcholine type zwitterionic stationary phase that was synthesized by graft polymerization [5]. In addition, a study that showed how to control the ratio of positively and negatively charged groups was also reported [6]. An effect of the length of alkyl chain between the positively and negatively charged groups was also studied [7].

Another approach is to dynamically coat the stationary phases with zwitterionic surfactants [8-11]. These stationary phases are usually used for reversed-phase chromatography and the surfactants are normally attracted *via* hydrophobic interaction. In most of cases, zwitterionic chemicals are added to the mobile phase to keep the concentration.

Monolithic columns were introduced in the late 1990s [12-14]; many scientists have made

attempts to synthesize zwitterionic monolith columns since then. Polymer-based monolithic columns are synthesized by *in-situ* polymerization. Monomers, cross-linkers, porogens and initiators are important materials for synthesizing monolithic columns. Zwitterionic monolithic stationary phase was prepared based on the thermal-initiated copolymerization of *N,N*-dimethyl-*N*-(3-methacryl-amidopropyl)-*N*-(3-(sulfopropyl) ammonium betaine and ethylene glycol dimethacrylate [15,16]. Another monomer *i.e.* 2-methacryloyloxyethyl phosphorylcholine was also used to synthesize phosphorylcholine-type zwitterionic monolithic column [17]. Silica monolith was also applied for attachment of lysine (2,6-diaminohexanoic acid) groups [18]. The resultant stationary phase has zwitterionic nature.

The main and ultimate aim to synthesize zwitterionic stationary phases was simultaneous separation of cation and anion samples. And it is well known that zwitterionic stationary phases can be applied for hydrophilic interaction chromatography (HILIC). HILIC is considered as one of the normal phase chromatography. High concentration of polar organic solvent, usually acetonitrile (ACN), and polar stationary phases are chosen [19]. By taking advantage of easy preparation of monolithic columns, optimization for HILIC separation was fulfilled. Buffer salt concentration in mobile phase was investigated [20]. In most cases, when organic polymer-based monolithic columns were synthesized, ethylene dimethacrylate (EDMA) was often used as the cross-linker. Three different types of cross-linkers were utilized to confirm the influence on the retention of analytes [21].

Although zwitterionic monolithic stationary phases have been applied for HILIC mode many times, there are few reports regarding separation of cations and anions. Zwitterionic structure caused attraction and repulsion to inorganic analyte samples. In this study, zwitterionic monolithic columns were synthesized and applied to capillary ion chromatography.

## 3.2 Experimental

### 3.2.1 Reagents and materials

Reagents employed were of guaranteed reagent grade and were obtained from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise noted. Sodium bromide, sodium iodate, sodium iodide, sodium nitrate, sodium nitrite, sodium thiocyanate, ammonium chloride and benzyltrimethylammonium chloride were obtained from Nacalai Tesque (Kyoto, Japan). 2-(Methacryloyloxy)ethyl 2-(trimethylammonio)ethyl phosphate and 3-(trimethoxysilyl)propyl methacrylate were obtained from Tokyo Chemical Industry (Tokyo, Japan). Lithium Chloride, magnesium chloride hexahydrate and potassium chloride were obtained from Yoneyama Yakuhin Kogyo (Osaka Japan) [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide was obtained from Sigma-Aldrich (St. Louis USA) Ultrapure water was prepared 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. ZIC-HILIC was obtained from Merck Millipore (Darmstadt, Germany). All packing materials are packed in fused-silica capillary tube (100 mm × 0.32 mm i.d).

### 3.2.2 Apparatus

In this work, all experiments were conducted by using a capillary LC system constructed by a syringe pump YSP-101 (YMC, Kyoto, Japan) equipped with a gas-tight syringe (0.5 mL; Ito, Fuji, Japan) as a pump, an C4-1004-.2 internal sample injector (VICI Valco Instruments, Houston, USA) with an injection volume of 0.2 µL, a microcolumn prepared from a fused-silica capillary tube (100 mm × 0.32 mm i.d.; GL Sciences, Tokyo, Japan), a UV detector (JASCO, Tokyo, Japan) with the detection wavelength was set at 210 nm, and a data processor (CDS-Lite ver 5.0; LA soft, Chiba, Japan). The inlet pressure was monitored by an L.TEX-8150 pressure sensor (L.TEX). Separation columns were operated under room temperature (controlled at 25°C) throughout the study.

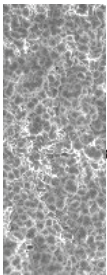
### 3.2.3 Preparation of monolithic column

0.2 M NaOH, 0.2 M HCL and 20 % (v/v) 3-(trimethoxysilyl)propyl methacrylate in ethanol were passed through fused-silica capillary tube in a sequential order for thirty minutes each to attach methacrylate groups on the inner wall of the capillary tube. Then the tube was washed by ethanol and dried by passing nitrogen. A mixture of monomer, cross-linker, porogen and initiator was then filled in the pre-treated tube and it was sealed both sides. The capillary was dipped in water bath at 60 °C for 18 hours. After reaction, methanol was flashed through the tube to wash out the residuals.

### 3.3 Results and discussion

#### 3.3.1 Preparation of zwitterionic sulfobetaine monolithic column

Monolithic columns were prepared by one-pot reaction, as seen in Fig. 3-1. Adjustable parameters were weights of each chemicals, zwitterionic monomer, EDMA and methanol. Table. 3-1 shows the all specific values employed in this study. The reaction temperature and duration was kept constant 60 °C and 18 h in all situations. Differences among columns A, B, C and D were concentration of methanol. The ratio between zwitterionic monomer to EDMA was one to one. Column A could not be passed mobile phase due to the fact that it had lowest concentration of methanol, which means there was not large enough space of through pore. Columns C and D could be inspected the utility and show 3 peaks (3 samples coeluted). Column D had highest concentration of methanol that caused low density of polymer skeleton. As the result, it did not show a clear chromatogram. Columns E, F, G and H are distinguished from columns A, B, C and D based on the different ratio between zwitterionic monomer and cross-linker. The ratio of zwitterionic monomer is three times of EDMA. Amounts of methanol were varied from 130 mg to 200 mg. Column E showed good separation of anions but the pressure was 3.3 MPa that was still high taking into account a property of monolithic column. Column F, G and H showed better results and Column F is the best of all others. The exact amounts of each reagent were 60.6 mg of zwitterionic monomer, 20.4 mg of EDMA and 140.4 mg of methanol, 27.3 wt %, 9.1 wt % and 63.3 wt % respectively. Column F was chosen for further investigation. Scanning electron microscopy (SEM) photos of cross-sectional surface of Column F was shown in Fig 3-2.



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Table 3-1 Compositions of various polymerization conditions.

Column	Monomer (mg)	Cross-linker (mg)	Porogen (mg)
A	40	40	160
B	40	40	200
C	40	40	220
D	40	40	250
E	60	20	130
F	60	20	140
G	60	20	150
H	60	20	200

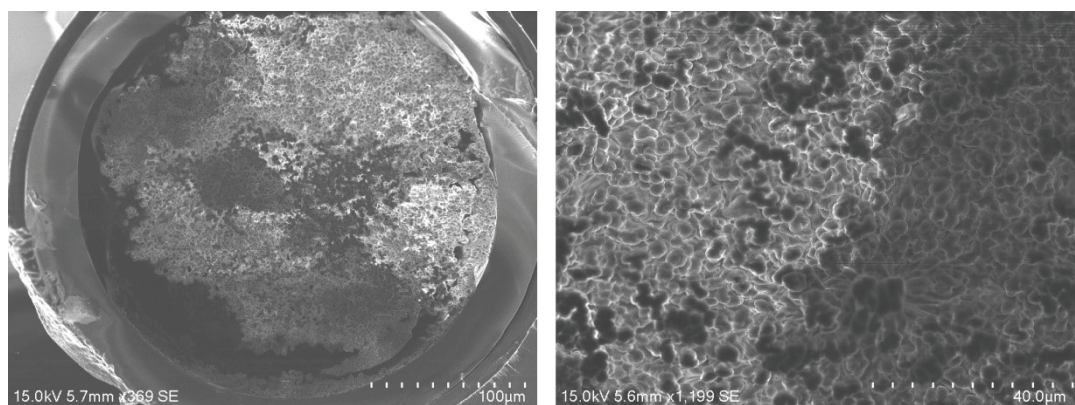


Fig. 3-2 SEM photos of zwitterionic sulfobetaine monolithic column (Column F).

### 3.3.2 Evaluation of zwitterionic monolithic column and comparison with ZIC-HILIC column

#### 3.3.2.1 Separation of inorganic anions

In order to measure utilities of zwitterionic monolith column, ZIC-HILIC was employed to compare the results.  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{I}^-$  and  $\text{SCN}^-$  were selected as analytes samples. Although zwitterionic structure can repulse samples which have negative charges, it can be expected that these samples could be retained. Fig. 3-3 showed the results of inorganic anions separation. All samples were separated on both columns, and elution order of samples was same one obtained by ion exchange chromatography. Retention times of samples on zwitterionic monolithic column are longer than those on ZIC-HILIC. The result may indicate that zwitterionic monolithic columns is more positively charged or less negatively charged than ZIC-HILIC.

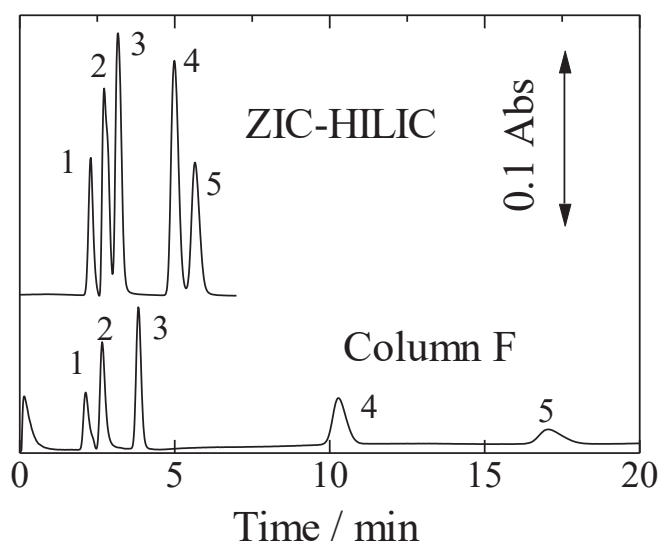


Fig. 3-3 Separation of inorganic anions.

Columns: ZIC-HILIC and zwitterionic monolithic column,  $100 \times 0.32$  mm i.d. Eluent: 100 mM NaCl.

Flow-rate:  $3.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{IO}_3^-$ , 2 =  $\text{NO}_2^-$ , 3 =  $\text{NO}_3^-$ , 4 =  $\text{I}^-$ , 5 =  $\text{SCN}^-$ , 0.5 mM each.

Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

### 3.3.2.2 Pressure of zwitterionic monolithic column vs ZIC-HILIC

Pressures were monitored and recorded all times. Flow rate was increased from 2.0  $\mu\text{L} / \text{min}$  to 5.0  $\mu\text{L} / \text{min}$ . As seen in Fig. 3-4, pressure was lower than 1.0 Mpa on zwitterionic monolithic column even when it was operated at 5.0  $\mu\text{L} / \text{min}$ . And increment rate is pretty slight, whereas pressures are higher on ZIC-HILIC column and increment rate is also steep. This is the major advantage of monolithic columns.

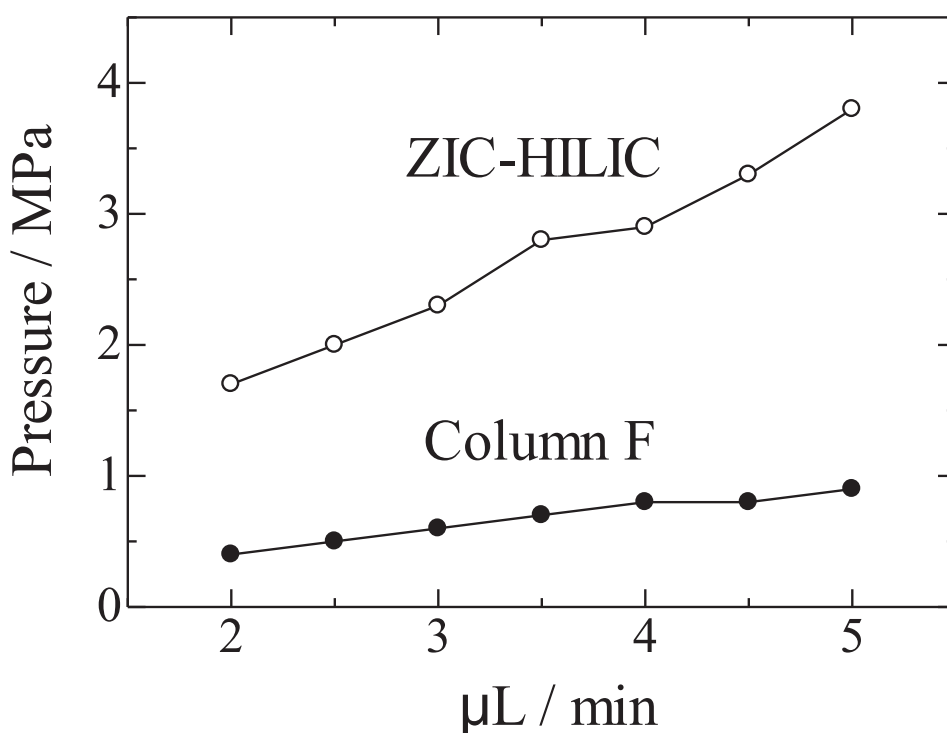


Fig. 3-4 Plotting of pressures versus flow rate on zwitterionic monolithic column and ZIC-HILIC.

Columns: ZIC-HILIC and zwitterionic monolithic column,  $100 \times 0.32$  mm i.d. Eluent: 100 mM NaCl.

Flow-rate: 2.0 – 5.0  $\mu\text{L} \text{min}^{-1}$ . Analytes: 1 =  $\text{IO}_3^-$ , 2 =  $\text{NO}_2^-$ , 3 =  $\text{NO}_3^-$ , 4 =  $\text{I}^-$ , 5 =  $\text{SCN}^-$ , 0.5 mM each.

Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.

### 3.3.2.3 van Deemter plot of zwitterionic monolithic column vs ZIC-HILIC

van Deemter plot is figured out in Fig. 3-5 to find optimum flow rate. Numbers of theoretical plates ( $N$ ) and height equivalent of one theoretical plate (HETP) were calculated below equations (1) (2), where  $tr$  is retention time,  $H$  is peak height,  $A$  is an area of peak and  $L$  is column length.  $\text{SCN}^-$  was selected as a test sample for calculation of HETP. Table.3-2 shows the  $N$  of  $\text{SCN}^-$  on each stationary phase. Then, calculated HETP were plotted against flow rate in Fig. 3-4. Obviously, HETP given by zwitterionic monolith column are lower than those on ZIC-HILIC. This result means that zwitterionic monolithic column is more suitable for anion separation.

$$N = 2\pi \left( \frac{tr \cdot H}{A} \right)^2 \quad (1)$$

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

Table 3-2 Numbers of theoretical plates of  $\text{SCN}^-$  with various flow rates.

Flow rate ( $\mu\text{L} / \text{min}$ )	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Monolithic column	3038	2535	2284	2084	1948	1891	1627
ZIC-HILIC	1375	1211	1052	1055	924	815	636

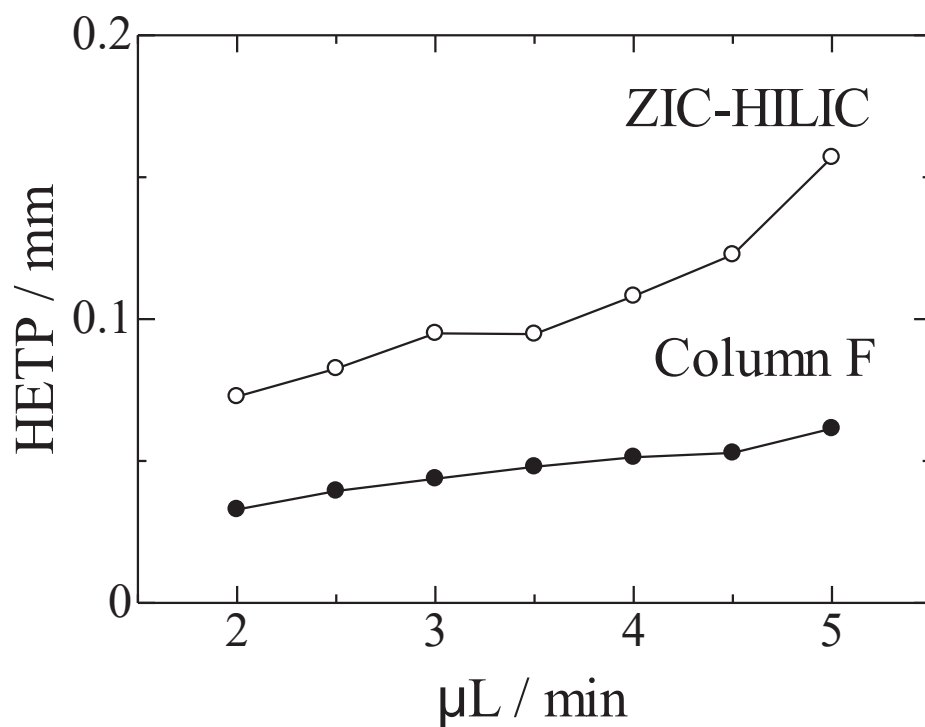


Fig. 3-5 van Deemter plot of zwitterionic monolithic column and ZIC-HILIC.

Columns: ZIC-HILIC and zwitterionic monolithic column,  $100 \times 0.32$  mm i.d. Eluent: 100 mM NaCl.

Flow-rate:  $3.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{IO}_3^-$ , 2 =  $\text{NO}_2^-$ , 3 =  $\text{NO}_3^-$ , 4 =  $\text{I}^-$ , 5 =  $\text{SCN}^-$ , 0.5 mM each.

Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.  $\text{SCN}^-$  was selected to calculate HETP.

### 3.3.3 Effect of eluent cation

Zwitterionic stationary phases have both positive and negative charges. So it can be presumed that cations present in the mobile phases could influence in the retention behavior of anionic samples. Fig. 3-6 shows the retention of analyst anions separated with various eluents that contained deferent cations. The concentrations of chloride were kept constant, *i.e.* 100 mM. Retention times of all samples became longer as the cations varied from  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$  to  $\text{Mg}^{2+}$ . This could be explained by the retention strength of cations. The order of retention strength is  $\text{Li}^+ < \text{Na}^+ < \text{K}^+ < \text{Mg}^{2+}$  on cation exchange site. Cation that has strong retention such as  $\text{Mg}^{2+}$  screens the negative charges on the external cation exchange groups, and analyte anions were more retained by the quaternary ammonium groups.

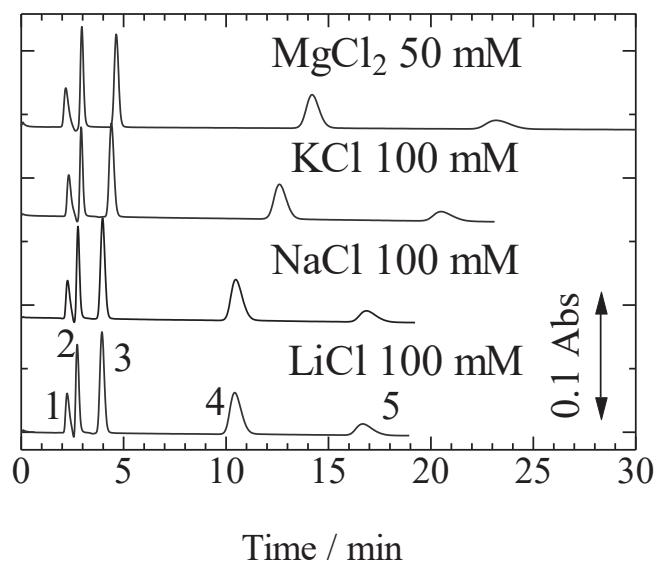


Fig. 3-6 Separation on inorganic anions under various mobile phases that contained different kinds of cation.

Column: Zwitterionic monolithic column (Column F), 100 × 0.32 mm i.d. Eluents: as indicated.

Flow-rate: 3.0  $\mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{IO}_3^-$ , 2 =  $\text{NO}_2^-$ , 3 =  $\text{NO}_3^-$ , 4 =  $\text{I}^-$ , 5 =  $\text{SCN}^-$ , 0.5 mM each.

Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.

### 3.3.4 Effect of acid in mobile phase

The retention behavior of inorganic anions was also investigated under acidic condition. In this study, analyte  $\text{NO}_2^-$  was replaced by  $\text{Br}^-$ .  $\text{NO}_2^-$  is not suitable for this measurement because  $\text{NO}_2^-$  cannot be deprotonated and does not behave as an anion analyte. Fig. 3-7 shows the comparison of anions separation between neutral and acidic conditions. Retention times given by acidic condition are longer than those by neutral condition. This is because protons can screen the negative charges on the external cation exchange groups, and anion exchange groups attract analyte samples more strongly.

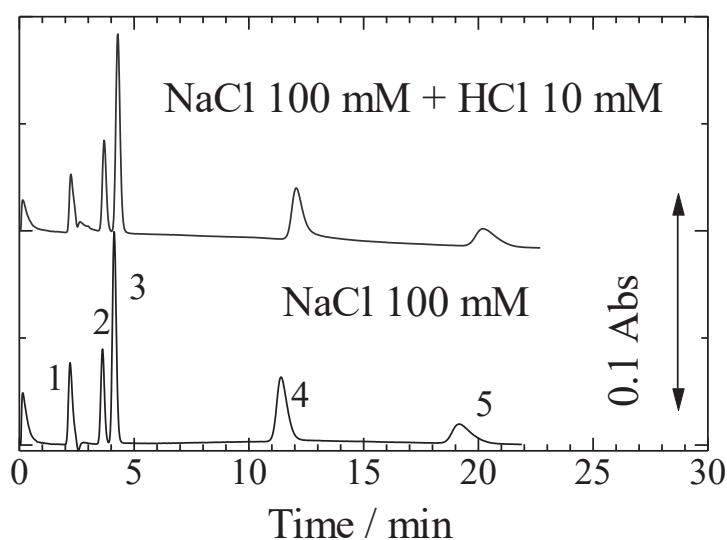


Fig. 3-7 Separation of inorganic anions under neutral and acidic conditions.

Column: Zwitterionic monolithic column (Column F),  $100 \times 0.32$  mm i.d. Eluents: as indicated.

Flow-rate:  $3.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{IO}_3^-$ , 2 =  $\text{Br}^-$ , 3 =  $\text{NO}_3^-$ , 4 =  $\text{I}^-$ , 5 =  $\text{SCN}^-$ , 0.5 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

### 3.3.5 Separation of cation samples

Zwitterionic monolithic column can be applied for cation separation. Fig. 3-8 showed the result of cation separation. Salt for eluent was 20 mM benzyltrimethyl ammonium chloride that is neutral reagent. Divalent cations  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  could be retained and separated. On the other hand, monovalent cations  $\text{Na}^+$ ,  $\text{NH}_4^+$  and  $\text{K}^+$  were coeluted. It is considered that cation exchange capacity is not enough to retain monovalent cations.

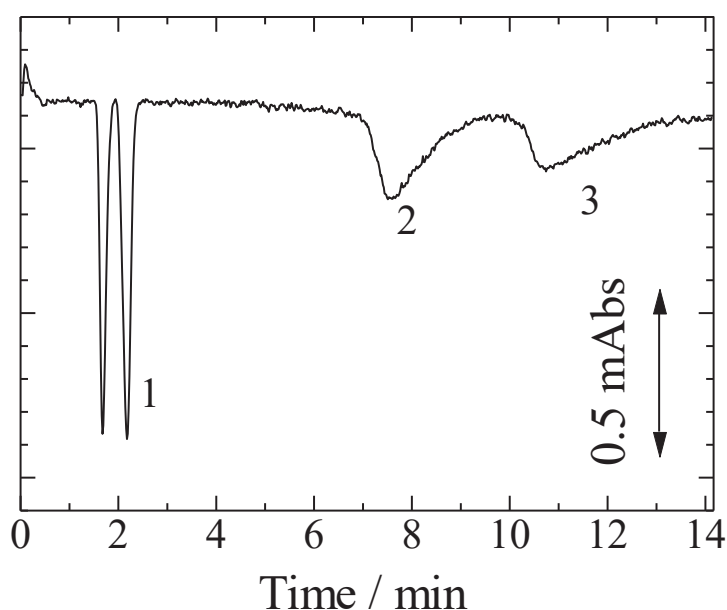


Fig. 3-8 Separation of cation samples on zwitterionic monolithic column.

Column: Zwitterionic monolithic column (Column F),  $100 \times 0.32$  mm i.d. Eluent: 20 mM benzyltrimethyl-ammonium chloride (background 0.4 Abs). Flow-rate:  $5.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{Na}^+$ ,  $\text{NH}_4^+$ ,  $\text{K}^+$ , 2 =  $\text{Mg}^{2+}$ , 3 =  $\text{Ca}^{2+}$ , 1.0 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.



### 3.3.6 Preparation of zwitterionic phosphocholine monolithic column

Phosphorylcholine is also well known for its zwitterionic structure. 2-(Methacryloyloxy)ethyl 2-(trimethylammonio)ethyl phosphate was employed as zwitterionic monomer. The way of preparation of phosphorylcholine zwitterionic monolithic columns was the same as in Session 3.3.1., Fig. 3-9 shows the schematic diagram of the expected reaction. Optimization for synthesis condition was conducted. The best ratio of monomer, cross-linker and porogen were 19.6, 60.8 and 211.5 mg, respectively, equivalent to 27.3, 9.1 and 63.3 wt % if converted to weight ratio. SEM photos of cross-sectional surface of this column is shown in Fig. 3-10.

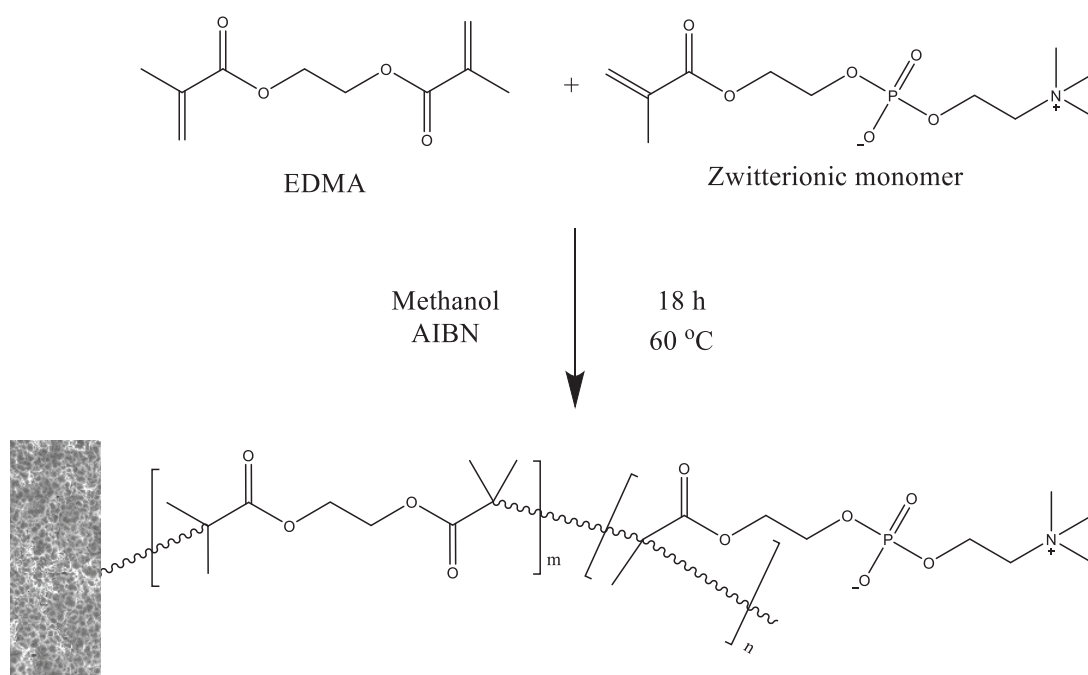


Fig. 3-9 Expected synthesis steps for preparation of zwitterionic phosphocholine monolithic column.

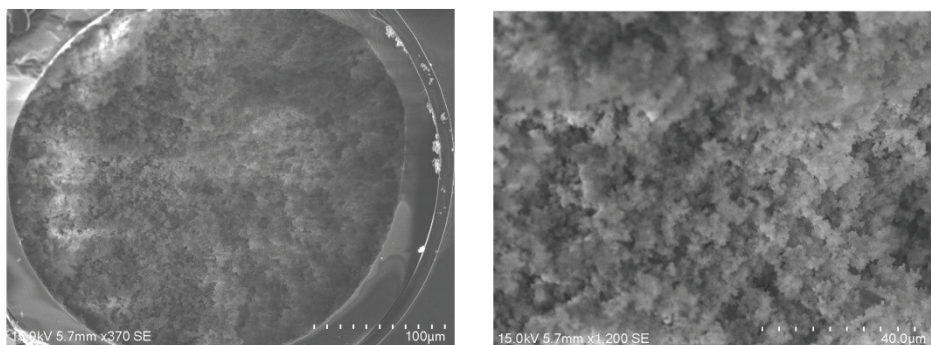


Fig. 3-10 SEM photos of zwitterionic phosphocholine monolithic column.

### 3.3.7 Retention behavior of anions

Inorganic separation was conducted by prepared monolithic column. Fig. 3-11 shows the result. As seen in it,  $\text{I}^-$  and  $\text{SCN}^-$  were retained but  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  could not be separated. The pressure was 0.3 MPa that is too low. It implies that through pores are too large to retain samples. And that caused samples could not be attracted to the surface of meso-pores.

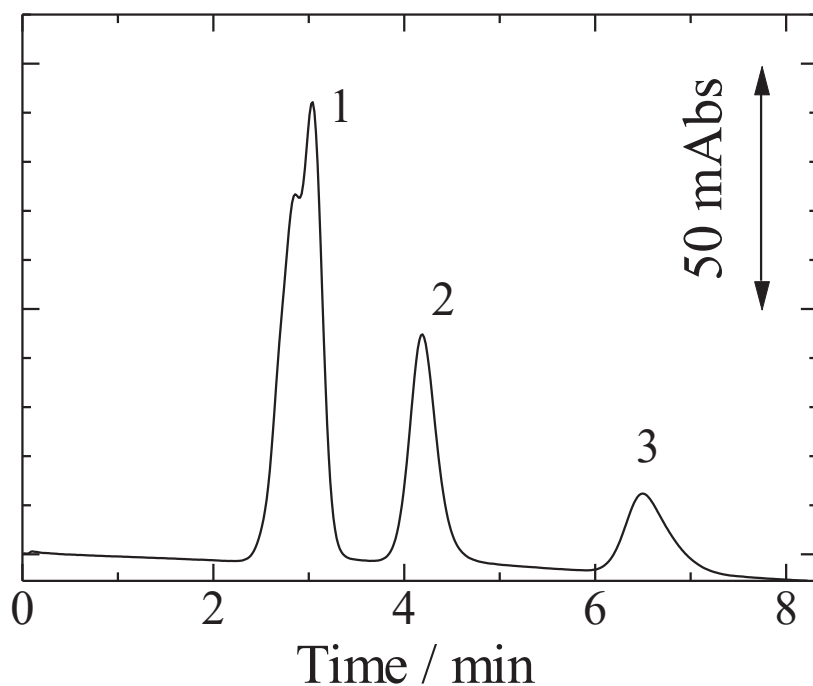


Fig. 3-11 Separation of inorganic anions on zwitterionic phosphorylcholine monolithic column

Column: zwitterionic phosphorylcholine monolithic column  $100 \times 0.32$  mm I.D. Eluent: 100 mM NaCl. Flow-rate:  $3.0 \mu\text{L min}^{-1}$ . Analytes: 1 =  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , 2 =  $\text{I}^-$ , 3 =  $\text{SCN}^-$ , 0.5 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

### 3.4 Conclusion

Synthesis condition was optimized for both zwitterionic monomers, *i.e.* *N,N*-dimethyl-*N*-(3-methacryl-amidopropyl)-*N*-(3-(sulfopropyl) ammonium betaine and 2-(methacryloyloxy)ethyl 2-(trimethylammonio)ethyl phosphate. Points that are important were to decrease porogen as low as possible and increase the ratio of zwitterionic monomer. Much amount of porogen will cause low density of polymer skeleton and these stationary phases cannot work in ion chromatography mode. Ratio of zwitterionic monomer determines the capacities of ion exchange. Analytes anions could be separated on the sulfobetaine-type monolithic column (Column F) and it could demonstrate low HETP and pressure compared to ZIC-HILIC. Predictively, composition of eluent has influence to retention behavior of inorganic anions. Monovalent cations could not be separated, that may imply that sulfobetaine functional groups are not suitable for attraction of cations. Although it can be expected that zwitterionic phosphorylcholine monolithic column could show equivalent efficacy as the sulfobetaine, the suitable condition was not found.

### 3.5 References

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## **Chapter 4**

### **Preparation of covalently bonded zwitterionic stationary phases**

#### **4.1 Introduction**

Ion chromatography (IC) is the method established by Small [1]. The aim of invention and application of IC is to inspect liquid samples. There are many situations when IC is necessary, for examples, inspection of environmental water and monitoring drainage water produced by factory. In order to inspect various samples, IC system has been improved. At infancy of IC, the system was equipped with a column and a suppressor. Anions separated through a column were enhanced intensity and electric conductivities of eluents were decreased. Fritz invented non-suppressor systems that were easy to use [2]. The defect of the system is increment of noise due to eluents that have a slightly higher background. Dynamic coating of ion-pair or surfactant on ODS is innovative approaches [3-6]. Negatively/positively or zwitterionic compounds were applied for this system. The next goal for IC was simultaneous separation of anions and cations. Tanaka and his co-workers achieved simultaneous separation by ion-exclusion and cation exchange mode [7]. Usually, separation of ionic samples is based on electrostatic interaction [8-12]. Preparation of zwitterionic stationary phases seems extensional approach to separate anions and cations simultaneously. Many papers regarding zwitterionic stationary phases have been reported [13-18]. Tertiary amine could be quaternary ammonium by chemical reaction. The resultant materials have zwitterionic structures [15, 17]. Zwitterionic stationary phases have positive and negative charge, and that means they can work attraction and repulsion against ionic samples. In this chapter, expedients to synthesize zwitterionic stationary phases were shown and the practicalities were investigated.

#### **4.2 Experiment**

##### **4.2.1 Reagents and material**

Reagents employed were of guaranteed reagent grade and were obtained from Wako Pure

Chemical Industries (Osaka, Japan), unless otherwise noted. Ammonium chloride, potassium chloride, sodium bromate, sodium bromide, sodium iodate, sodium iodide, sodium nitrate, sodium nitrite, sodium sulfite, and sodium thiocyanate were obtained from Nacalai Tesque (Kyoto, Japan). Lithium chloride, magnesium chloride hexahydrate, calcium chloride dehydrate, copper(II) sulfate and potassium chloride were obtained from Yoneyama Yakuhin Kogyo (Osaka Japan). 3-Glycidyloxypropyl-trimethoxysilane (GPTMS) was obtained by Tokyo Chemical Industry (Tokyo, Japan) Purified water was produced in the laboratory by using a Simplicity UV distillation system (Millipore, USA). All solutions used in this work were prepared using this purified water. Toluene, which was used as the reaction solvent, was dried before use. L-column silica gel (5  $\mu\text{m}$  particle diameter; Chemicals Evaluation and Research Institute, Saitama, Japan) and Develosil ODS-5 (5  $\mu\text{m}$  particle diameter; Nomura Chemical, Seto, Japan) were used for the preparation of stationary phases.

#### 4.2.2 Apparats

All experiments were conducted by using a capillary LC system constructed by an microfeeder (L.TEX Corporation, Tokyo, Japan) equipped with a gas-tight syringe (0.5 mL; Ito, Fuji, Japan) as a pump, an injector with an injection volume of 0.2  $\mu\text{L}$  (Rheodyne, Cotati, CA, USA), a microcolumn prepared from a fused-silica capillary tube (100 mm  $\times$  0.32 mm i.d.; GL sciences, Tokyo, Japan), a UV detector (JASCO, Tokyo, Japan), and a data processor (CDS-Lite ver 5.0; LA soft, Chiba, Japan). Outline drawing of this system is shown in Fig. 4-1.

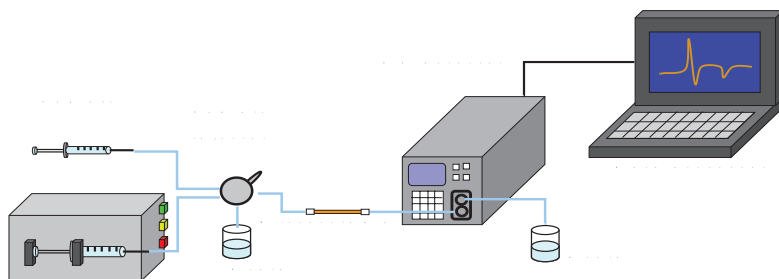


Fig. 4-1 Diagram of the capillary LC system

## 4.2.3 Preparation of stationary phases

### 4.2.3.1 Preparation of zwitterionic stationary phases

#### 4.2.3.1.1 Preparation of tertiary amino stationary phase

Covalently bonded zwitterionic stationary phase was prepared *via* a series of chemical reactions. Firstly, tertiary amino stationary phase was prepared. 3-(*N,N*-dimethylaminopropyl)-trimethoxysilane {3-(*N,N*-DMAP)-TMS } (0.1 mL) and dry silica gel (0.2 g) were mixed, and anhydrous toluene (3 mL) was added as the reaction solvent. The reaction was undergone for 24 h at 110°C, as shown in Fig. 4-2. The obtained 3-(*N,N*-DMAP)-TMS-bonded silica was washed by methanol [19].

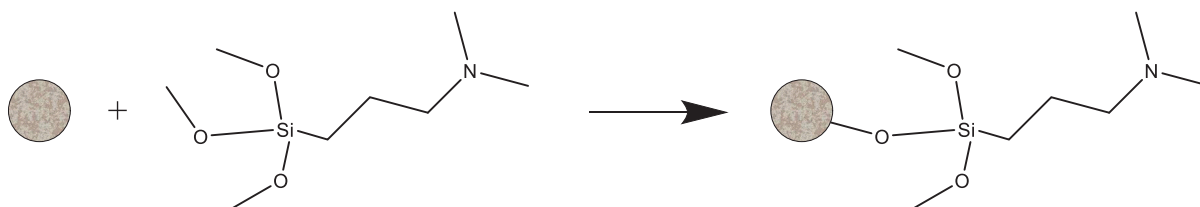


Fig. 4-2 Synthesis of tertiary amino stationary phase

#### 4.2.3.1.2 Attachment of sulfo group to tertiary amino stationary phase

Furthermore, 3-(*N,N*-DMAP)-TMS-bonded silica which was obtained (2.3.1.1) was used for a quaternizing reaction with 1,3-propane sultone (0.1 g) to form the zwitterionic stationary phase (Fig. 4-3). The reaction condition was similar as those described previously.

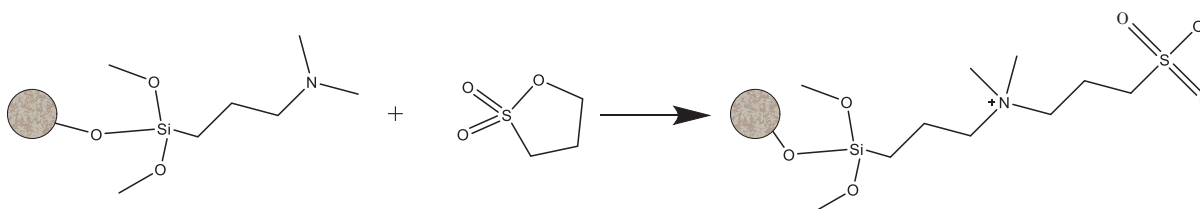


Fig. 4-3 Quaternizing reaction with 1,3-propane sultone

### 4.2.3.2. Preparation of alternative zwitterionic stationary phase

#### 4.2.3.2.1 Preparation of glycidyl bonded silica

In this study, tertiary amino stationary phase was also prepared by an alternative route. 3-glycidyloxypropyl-trimethoxysilane (GPTMS) (0.1 mL) was attached to dry silica gel (0.1 g) (Fig. 4-4). Anhydrous toluene was chosen as the reaction solvent and the mixture was heated to 110°C for 24 h. The obtained glycidyl bonded silica was washed and applied for two different reactions, *i.e.* one is to form tertiary amino stationary, and another one is to elaborate the sulfo group.

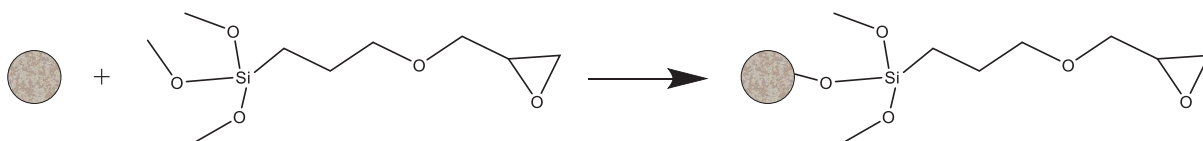


Fig. 4-4 Attachment of glycidyl group to silica gel

#### 4.2.3.2.2 preparation of tertiary amino stationary phase via glycidyl group

Diethylamine (0.1 mL) was reacted with glycidyl bonded silica (Fig.4-5). Reaction solvent was anhydrous toluene and temperature was 90°C for 24 h. The obtained diethylamine bonded silica was washed by methanol and dried out at 90°C for 2 h. Dried diethylamine bonded silica was added into 10 mM H<sub>2</sub>SO<sub>4</sub> (20% ACN) and stirred for 18 h at room temperature to open the epoxy groups which were not reacted.

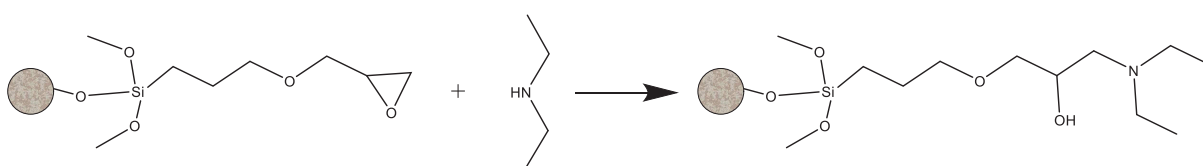


Fig. 4-5 Synthesis of tertiary amino stationary phase *via* glycidyl group



#### 4.2.3.2.3 Application of glycidyl group

Glycidyl group can be reacted with sodium sulfite and form sulfo group. Glycidyl bonded silica and  $\text{Na}_2\text{SO}_3$  (0.1 g) were mixed and stirred in water for 24 h at room temperature (Fig. 4-6).

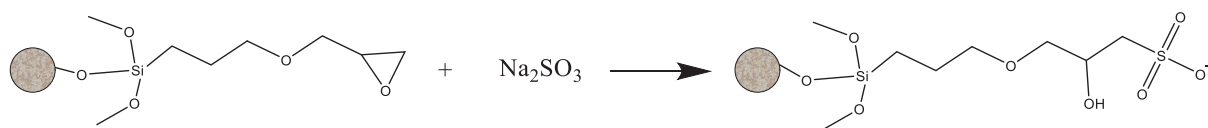


Fig. 4-6 Synthesis of sulfo group

#### 4.2.3.2.4 Attachment of sulfo group to tertiary amino stationary phase via glycidyl group

The way of quaternizing reaction with 1,3-propane sultone (0.1 g) was completely the same as the previous one (2.3.1.2). The reaction scheme is shown in Fig. 4-7

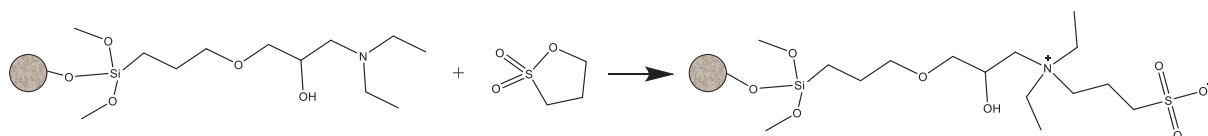


Fig. 4-7 Quaternizing reaction with 1,3-propanesultone via glycidyl group

#### 4.2.3.3 Hydrophobic phase dynamically coated with zwitterionic molecules

Sulfobetaine surfactants were coated by hydrophobic adsorption on the ODS column (Fig. 4-8). To stabilize and maintain a constant amount of the sulfobetaine surfactant on the surface of the stationary phase, all the eluents used always contained 5 mM of the surfactant.

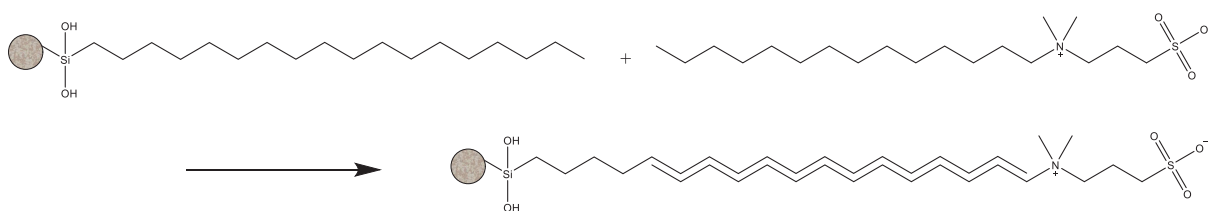


Fig. 4-8 Dynamically coating of zwitterionic surfactants on ODS column

### 4.3 Results and discussion

#### 4.3.1 Investigation of stationary phases with covalently bonded zwitterionic molecules

##### 4.3.1.1 Retention behavior of inorganic anions on dimethylamine-bonded silica

Fig. 4-9 shows the separation of five common inorganic anions. Mobile phase was 200 mM sodium chloride including 1 mM hydrochloric acid to protonate tertiary amino group. Five anions were separated and the result indicated that tertiary amino groups were successfully bonded to silica gel and protonated tertiary amino groups could work to retain analyte anions.

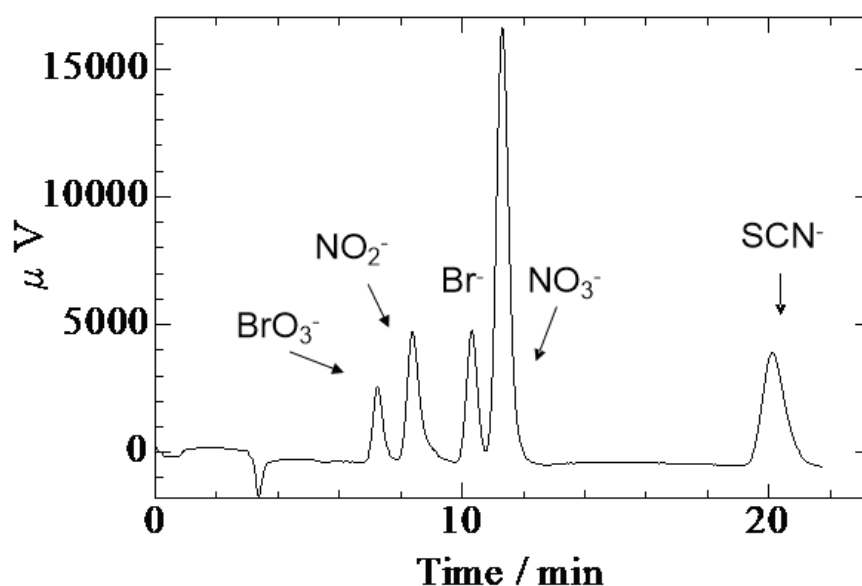


Fig. 4-9 Retention behavior of inorganic anions on the tertiary amino group.

Column: 3-(*N,N*-DMAP)-TMS bonded silica, 100  $\times$  0.320 mm i.d. Eluent: 200 mM sodium chloride in 1 mM of hydrochloric acid. Flow-rate: 3.0  $\mu\text{Lmin}^{-1}$ : Analytes:  $\text{BrO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{Br}^-$ ,  $\text{NO}_3^-$ ,  $\text{SCN}^-$ , 0.5 mM each. Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.

#### 4.3.1.2 Retention behavior of inorganic cations on sulfobetaine-dimethylamine-bonded silica

Retention behavior of inorganic cations on sulfobetaine-dimethylamine-bonded silica is shown in Fig. 4-10. 5 mM copper sulfate was used to conduct indirect detection. Monovalent sodium ion and bivalent magnesium ion were chosen as the samples. As seen in the figure, both sodium ion and magnesium ion could not be retained on the stationary phase. The result means that quaternizing reaction of dimethylamine with 1,3-propane sultone was not successfully carried out and the produced stationary phase did not possess negative charges.

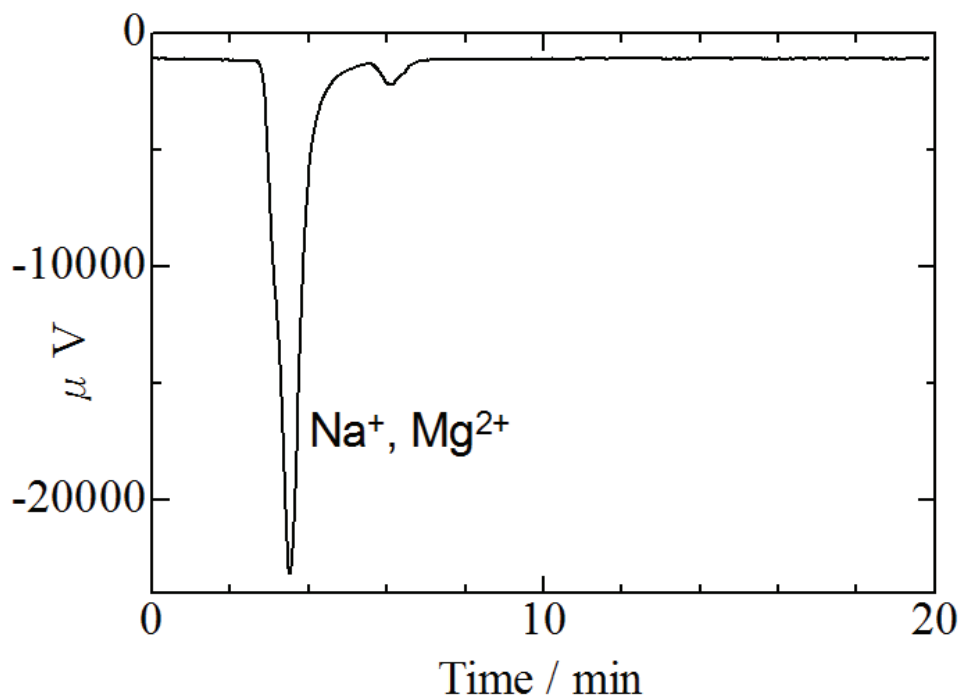


Fig. 4-10 Retention behavior of inorganic cations on sulfobetaine-dimethylamine-bonded silica. Column: Sulfobetaine-dimethylamine-bonded silica,  $100 \times 0.320$  mm i.d. Eluent: 5 mM copper sulfate. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ ; Analytes:  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , 1.0 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 230 nm.

### 4.3.2 Investigation of stationary phases with covalently bonded zwitterionic molecules by means of glycidyl groups

#### 4.3.2.1 Retention behavior of inorganic anions on diethylamine-bonded silica

Fig. 4-11 shows the separation of three inorganic anions. To protonate tertiary amino group, 100 mM sodium chloride in 1 mM of hydrochloric acid was used as mobile phase. As can be seen from the figure, all three anions could be separated. This result indicates that diethylamine was bonded to the glycidyl group and could work as anion exchangers after protonation.

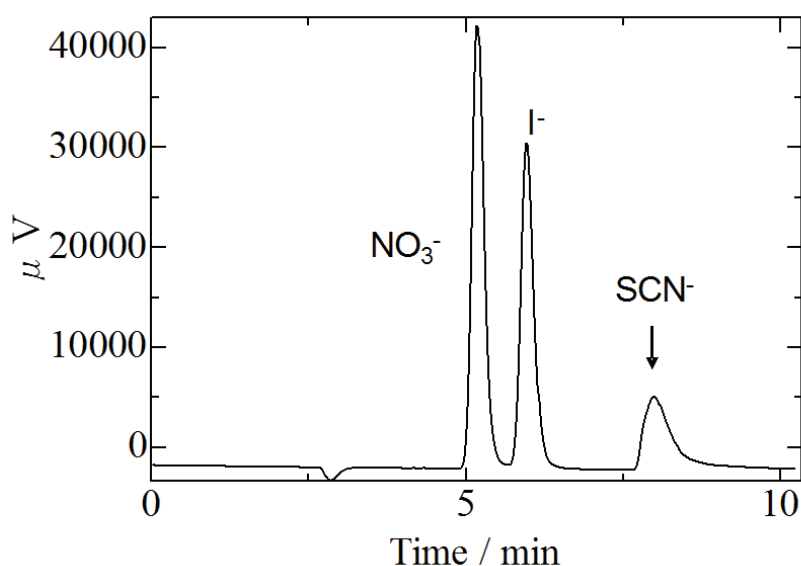


Fig. 4-11 Retention behavior of inorganic anions on diethylamine-bonded silica.

Column: Diethylamine-bonded silica,  $100 \times 0.320$  mm i.d. Eluent: 100 mM sodium chloride in 1 mM of hydrochloric acid. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ : Analytes:  $\text{NO}_3^-$ ,  $\text{I}^-$ ,  $\text{SCN}^-$ , 0.5 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

#### 4.3.2.2 Another usage of glycidyl group

It is known that glycidyl groups can be reacted with sodium sulfite and be added sulfo groups into structure. Sulfo group has negative charges and it is often used as cation exchangers. Fig. 4-12 shows the result of five inorganic cations separation. All five cation samples could be separated. This means that sodium sulfite could be reacted with glycidyl groups and sulfo groups are added.

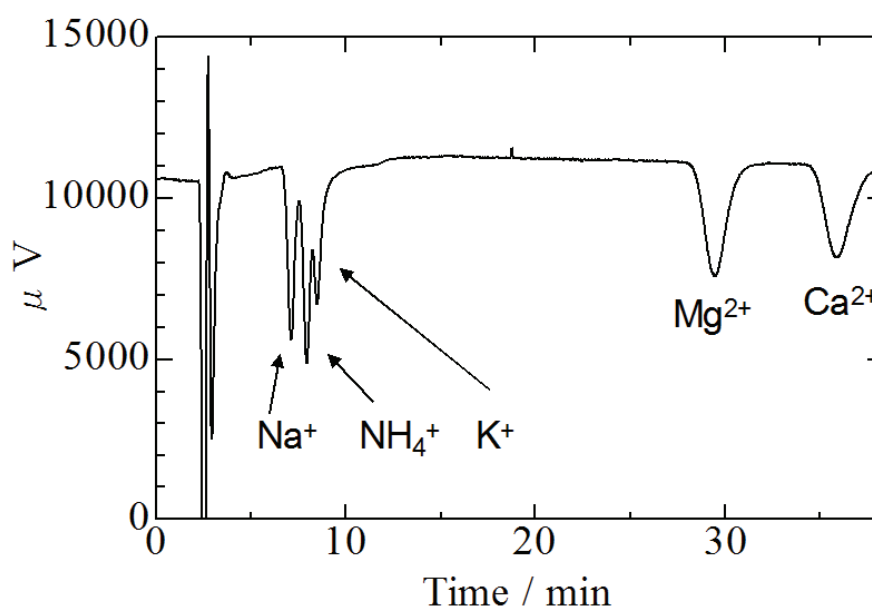


Fig. 4-12 Retention behavior of inorganic cations on sulfo group bonded silica.

Column: Sulfo group bonded silica, 100 × 0.320 mm i.d. Eluent: 5 mM copper sulfate. Flow-rate: 3.0  $\mu\text{Lmin}^{-1}$ : Analytes: Na<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, K<sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, 1.0 mM each. Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 230 nm.

#### 4.3.2.3 Retention behavior of inorganic cations on sulfobetaine-diethylamine-bonded silica

Fig. 4-13 shows the retention behavior of five inorganic cations. Bivalent magnesium ion and calcium ion were retained and could be separated. But monovalent sodium ion, potassium ion and ammonium ion could not be retained. This is presumed that cation exchange resin does not have enough selectivity to separate all five cations and the positive charges on the quaternary ammonium groups could repulse analyte cations.

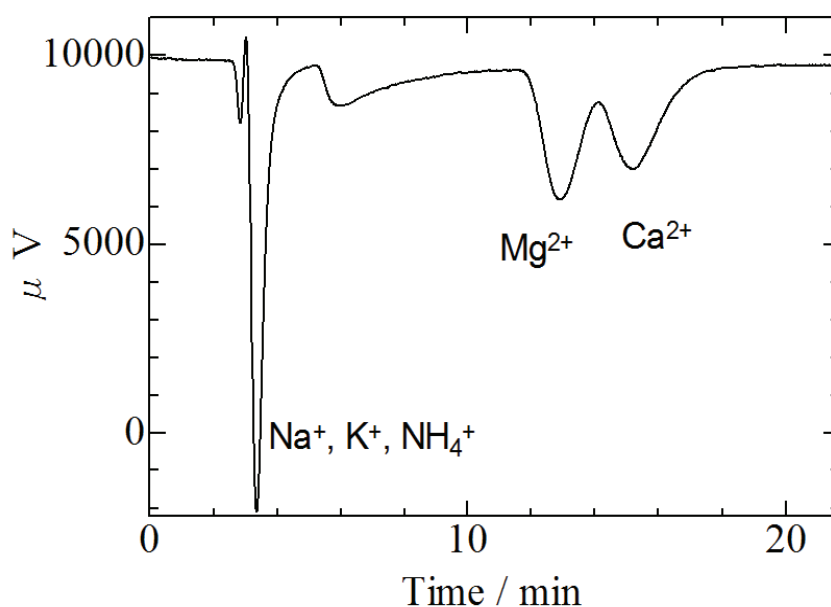


Fig. 4-13 Retention behavior of inorganic cations on sulfobetaine-diethylamine-bonded silica.

Column: sulfobetaine-diethylamine-bonded silica,  $100 \times 0.32$  mm i.d. Eluent: 5 mM copper sulfate.

Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ ; Analytes:  $\text{Na}^+$ ,  $\text{NH}_4^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ , 1.0 mM each. Injection volume:  $0.2 \mu\text{L}$ .

Wavelength of UV detection: 230 nm.

#### 4.3.2.4 Retention behavior of inorganic anions on sulfobetaine-diethylamine-bonded silica under neutral condition

It is considered that quaternary ammonium group can work as anion exchanger under neutral eluent condition. 100 mM sodium chloride was used as mobile phase. Fig. 4-14 shows the result of retention behavior of three inorganic anions. As shown in the figure, all of samples are weakly retained and could not be separated. Under neutral condition, negative charges on external sulfo groups could prevent analyte anions from approaching to quaternary ammonium group.

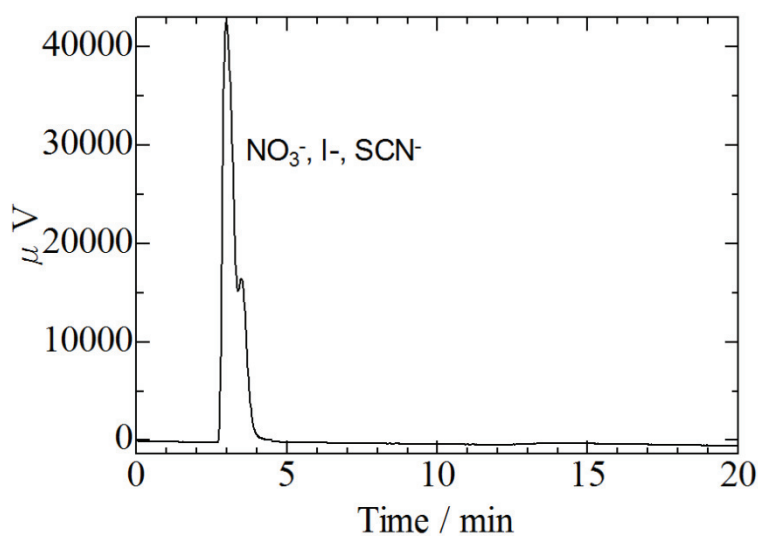


Fig. 4-14 Retention behavior of inorganic anions on sulfobetaine-diethylamine-bonded silica.

Column: Sulfobetaine-diethylamine-bonded silica,  $100 \times 0.32$  mm i.d. Eluent: 100 mM sodium chloride. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ . Analytes:  $\text{NO}_3^-$ ,  $\text{I}^-$ ,  $\text{SCN}^-$ , 0.5 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

#### 4.3.2.5 Retention behavior of inorganic anions on sulfobetaine-diethylamine-bonded silica under acidic condition

The prepared zwitterionic stationary phase is considered that it can be applied to inorganic anion separation under neutral eluent condition, but it could not show separation of inorganic anions. Utility of the stationary phase was investigated under acidic eluent condition. Fig. 4-15 shows the retention behavior of three inorganic anions. Analyte anions could be retained and separated. This result indicates that tertiary amino groups which were not reacted with 1,3-propane sultone remained in the column and they were protonated. Both of positive charges, protonated tertiary amino groups and quaternary amino groups attracted analyte anions.

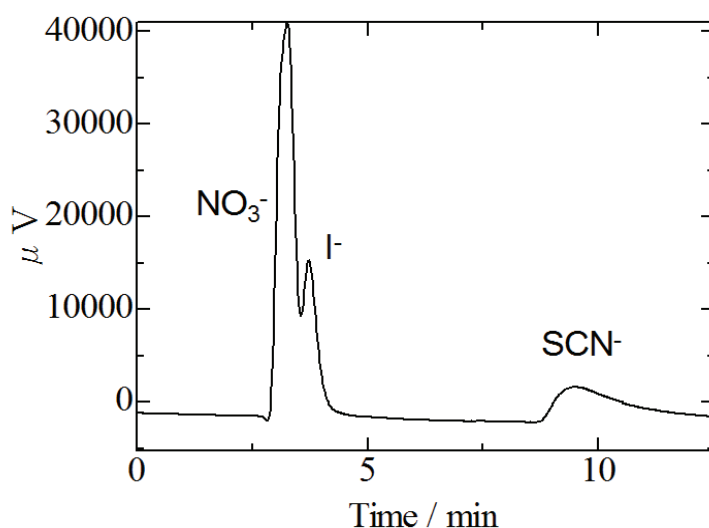


Fig. 4-15 Retention behavior of inorganic anions on sulfobetaine-diethylamine-bonded silica under acidic eluent condition.

Column: Sulfobetaine-diethylamine-bonded silica,  $100 \times 0.32$  mm i.d. Eluent: 100 mM sodium chloride in 1 mM of hydrochloric acid. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ : Analytes:  $\text{NO}_3^-$ ,  $\text{I}^-$ ,  $\text{SCN}^-$ , 0.5 mM each. Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.



### 4.3.3 Investigation of hydrophobic phase dynamically coated with zwitterionic molecules

#### 4.3.3.1 Retention behavior of inorganic anions under neutral eluent condition

Dynamically coated zwitterionic stationary phase was obtained by passing sulfobetaine surfactant through ODS column. 5 mM of the surfactant was added to the mobile phase at all time in order to stabilize and maintain a constant amount of the sulfobetaine surfactant on the surface of the stationary phase. Fig. 4-16 and Fig. 4-17 show the separation of four inorganic anions under neutral eluent condition using sodium chloride and sodium sulfate as the eluent, respectively.

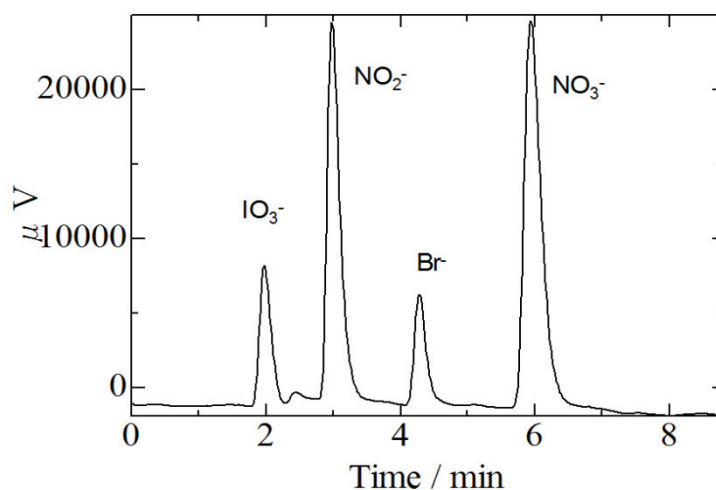


Fig. 4-16 Retention behavior of inorganic anions on sulfobetaine surfactant-coated ODS under neutral eluent condition.

Column: Sulfobetaine surfactant-coated ODS,  $100 \times 0.32$  mm i.d. Eluent: 30 mM sodium chloride containing 5 mM sulfobetaine surfactants. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ : Analytes:  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{Br}^-$ ,  $\text{NO}_3^-$ , 0.5 mM each. Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.

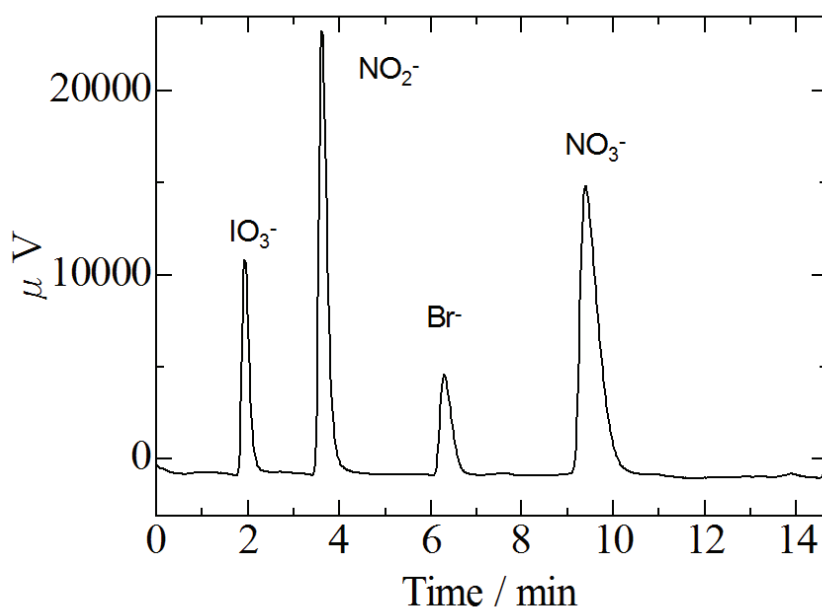


Fig. 4-17 Retention behavior of inorganic anions on sulfobetaine surfactant-coated ODS under neutral eluent condition.

Column: Sulfobetaine surfactant-coated ODS,  $100 \times 0.32$  mm i.d. Eluent: 30 mM sodium sulfate containing 5 mM sulfobetaine surfactants. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ . Analytes:  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{Br}^-$ ,  $\text{NO}_3^-$ , 0.5 mM each. Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.

In IEC, if sodium sulfate is used as the mobile phase, the retention time of analyte anions should decrease in comparison with sodium chloride, because doubly charged  $\text{SO}_4^{2-}$  retain strongly on stationary phase and they interrupt the retention of analyte anions. But these results show inverse phenomenon. This is considered that  $\text{SO}_4^{2-}$  which has large hydration energy cannot interact electronically with anion exchanger on the hydrophobic stationary phase. Due to this character, retention time of analyte anions increased in sodium sulfate mobile phase. And this implies the effect of hydrophobic interaction might have influence to the retention behavior. However, the elution order of the analyte anions was the same as that observed under common ion chromatography conditions.

#### 4.3.3.2 Effect of the eluent concentration under acidic condition

Fig. 4-18 shows the retention behavior of inorganic anions under various concentration of sodium chloride in 1 mM hydrochloric acid. Retention time of all samples, except iodate, decreased with increasing sodium chloride concentrations. Although this implicates that this retention mechanism can be explained by ion exchange mode, the relationship between the retention factor and concentration of elution, which is shown in Fig. 4-19, showed that for the retention of anions was not purely based on ion exchange mode.

Compared with neutral condition (Fig. 4-16), retention time of bromide, nitrate, and nitrite increased. This is because that acidic condition (*i.e.*  $H^+$ ) screens the negative charges on the external cation exchange groups, and analyte anions were retained by the quaternary ammonium groups.

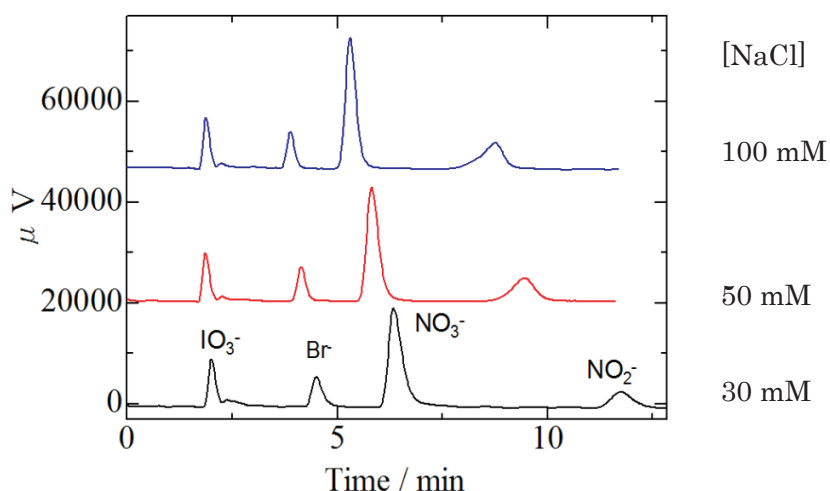


Fig. 4-18 Retention behavior of inorganic anions on sulfobetaine surfactant-coated ODS with different eluent concentrations.

Column: Sulfobetaine surfactant-coated ODS,  $100 \times 0.32$  mm i.d. Eluents: 30, 50, 100 mM NaCl (from below) in 1.0 mM hydrochloric acid containing 5 mM sulfobetaine surfactants. Flow-rate:  $3.0 \mu L \cdot min^{-1}$ ; Analytes:  $IO_3^-$ ,  $NO_2^-$ ,  $Br^-$ ,  $NO_3^-$ , 0.5 mM each. Injection volume: 0.2  $\mu L$ . Wavelength of UV detection: 210 nm.

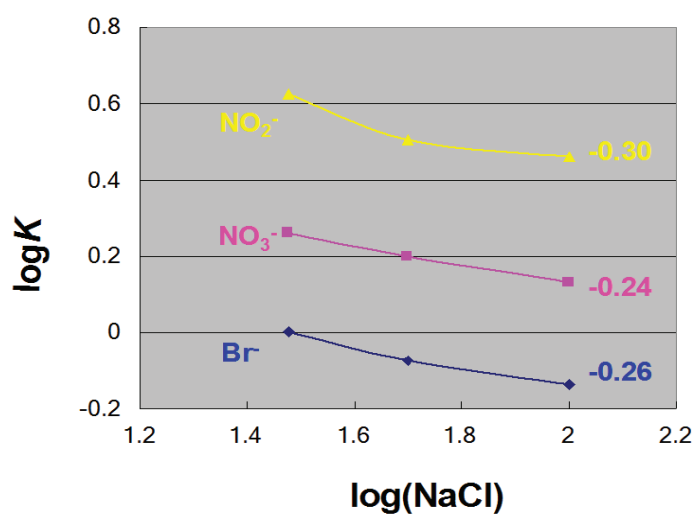


Fig. 4-19 Logarithm of the retention factor of analyte anions versus the logarithm of the sodium chloride concentration.

Column: Sulfobetaine surfactant-coated ODS,  $100 \times 0.32$  mm i.d. Eluents: 30, 50, 100 mM NaCl in 1.0 mM hydrochloric acid containing 5 mM sulfobetaine surfactants. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ . Analytes:  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{Br}^-$ ,  $\text{NO}_3^-$ , 0.5 mM each. Injection volume:  $0.2 \mu\text{L}$ . Wavelength of UV detection: 210 nm.

Fig. 4-20 shows the effect of various sodium sulfate concentrations in 0.5 mM sulfuric acid. As can be seen from the figure, iodate, bromide, and nitrate were not so affected while nitrite was deeply affected, and comparing to sodium chloride condition as shown in Fig. 4-18, it showed irregular elution order. This is considered that this strange retention behavior depends on hydration energy of nitrite. Under these acidic conditions, the pH of mobile phases was about 3.3, and  $pK_a$  of nitrous acid is 3.38. This means that undissociated nitrous acid and nitrite coexist and they are particularly affected by the influence of the hydration energy.

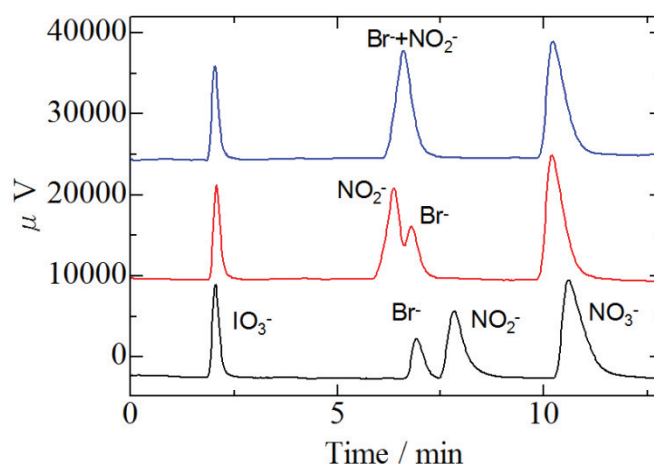


Fig. 4-20 Retention behavior of inorganic anions on sulfobetaine surfactant-coated ODS with different eluent concentrations.

Column: Sulfobetaine surfactant-coated ODS,  $100 \times 0.32$  mm i.d. Eluents: 30, 50, 100 mM  $\text{Na}_2\text{SO}_4$  (from below) in 0.5 mM sulfuric acid containing 5 mM sulfobetaine surfactants. Flow-rate:  $3.0 \mu\text{Lmin}^{-1}$ ; Analytes:  $\text{IO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{Br}^-$ ,  $\text{NO}_3^-$ , 0.5 mM each. Injection volume: 0.2  $\mu\text{L}$ . Wavelength of UV detection: 210 nm.

## 4.4 Conclusions

Inorganic anion separations by using tertiary amino groups which were produced during the process of zwitterionic stationary phase synthesis were confirmed, and the elution order of the analyte anions was the same as that observed under common ion chromatographic conditions. Attachment of sulfo groups to dimethylamine was not achieved. Pyridine groups and imidazole groups also could not react with 1,3-propane sultone. Attachment of sulfo groups to diethylamine was achieved because the electron density of diethylamine is greater than dimethylamine and high electron density on amino group enables quaternizing reaction with 1,3-propane sultone. The prepared zwitterionic stationary phase by using diethylamine showed retention and separation of bivalent cations, but monovalent cations were not retained because the column showed insufficient selectivity to separate all five cations, and the positive charges on the quaternary ammonium groups could repulse analyte cations. Separation of inorganic anions on dynamically coated zwitterionic stationary phase could be achieved, and retention time of bromide, nitrate, and nitrite increased under acidic condition compared with neutral condition. This fact infers that acidic condition (*i.e.*  $H^+$ ) screens the negative charges on the external cation exchange groups, and analyte anions were retained by the quaternary ammonium groups. Cations were not retained on dynamically coated zwitterionic stationary phase even under neutral conditions. This is due to that cations have large hydration energies in order to form the hydration spheres and they cannot retain on this stationary phase. Iodate with a large hydration energy could not be retained on hydrophobic zwitterionic stationary phase under all conditions, and polarizable anions such as  $I^-$  and  $SCN^-$  are strongly retained and the elutions were not confirmed due to their less hydration water character.

## 4.5 References

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## Chapter 5

### Conclusions

The stationary phases that are suitable for separation of inorganic anions under HILIC condition are TSKgel NH<sub>2</sub>-60 and Polar-Imidazole. Elution order of inorganic anions was reversed in HILIC mode in comparison with IEC mode when TSKgel NH<sub>2</sub>-60 and Polar-Imidazole were used as the stationary phase. High concentration of ACN facilitates the desolvation of analyte anions as well as anions that are in the eluent. It is presumed that the retention of anions is affected by the electrostatic interaction but the retention mechanism is not purely based on ion exchange mode. Partition is also competing for retention mechanism due to the fact that retention increases as increasing the concentration of ACN.

Synthesis condition was optimized for both zwitterionic monomers, *N,N*-dimethyl-*N*-(3-methacryl-amidopropyl)-*N*-(3-(sulfopropyl) ammonium betaine and 2-(methacryloyloxy)ethyl 2-(trimethylammonio)ethyl phosphate. Points that are important were to decrease porogen as low as possible and increase the ratio of zwitterionic monomer. Much amount of porogen will cause low density of polymer skeleton and these stationary phases cannot work as ion chromatography mode. Ratio of zwitterionic monomer determines the capacities of ion exchange. Analytes anions could be separated on the sulfobetaine type monolithic column (Conlumn F) and it could demonstrate low HETP and pressure compared to ZIC-HILIC. Predictively, composition of eluent has influence to retention behavior of inorganic anions. Monovalent cations could not be separated, that may imply that sulfobetaine functional groups are not suitable for attraction of cations. Although it can be expected that zwitterionic phosphorylcholine monolithic column can show equivalent efficacy of sulfobetaine type, it could not work for ion chromatography.

Inorganic anion separations by using tertiary amino groups which were produced during the process of zwitterionic stationary phase synthesis were confirmed, and the elution order of the analyte anions was the same as that observed under common ion chromatographic conditions. Attachment of



sulfo groups to dimethylamine was not achieved. Pyridine groups and imidazole groups also could not react with 1,3-propane sultone. Attachment of sulfo groups to diethylamine was achieved because the electron density of diethylamine is greater than dimethylamine and high electron density on amino group enables quaternizing reaction with 1,3-propane sultone. The prepared zwitterionic stationary phase by using diethylamine showed retention and separation of bivalent cations, but monovalent cations were not retained because the column showed insufficient selectivity to separate all five cations, and the positive charges on the quaternary ammonium groups could repulse analyte cations. Separation of inorganic anions on dynamically coated zwitterionic stationary phase could be achieved, and retention time of bromide, nitrate, and nitrite increased under acidic condition compared with neutral condition. This fact infers that acidic condition (*i.e.*  $H^+$ ) screens the negative charges on the external cation exchange groups, and analyte anions were retained by the quaternary ammonium groups. Cations were not retained on dynamically coated zwitterionic stationary phase even under neutral conditions. This is due to that cations have large hydration energies in order to form the hydration spheres and they cannot retain on this stationary phase. Iodate with a large hydration energy could not be retained on hydrophobic zwitterionic stationary phase under all conditions, and polarizable anions such as  $I^-$  and  $SCN^-$  are strongly retained and the elutions were not confirmed due to their less hydration water character.

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## List of publications

[1] Nobuyuki TAKAYAMA, Lee Wah LIM and Toyohide TAKEUCHI

“Retention Behavior of Inorganic Anions in Hydrophilic Interaction Chromatography”

*Analytical sciences*, (Published on May 10, 2017)

[2] Nobuyuki TAKAYAMA, Lee Wah LIM and Toyohide TAKEUCHI

“Optimization and investigation of zwitterionic monolithic stationary phases for ion chromatography”

*Analytical sciences*, (Published on May 10, 2017)

## List of presentations

### Oral Presentation

- [1] Nobuyuki Takayama, Lee Wah Lim, Toyohide Takeuchi: 44<sup>th</sup> Annual Meeting of Union of Chemistry-Related Societies in Chubu Area, (Shizuoka University, Japan), November 2-3, 2013
  
- [2] Nobuyuki Takayama, Lee Wah Lim, Toyohide Takeuchi: 30<sup>th</sup> Ion Chromatography Symposium, (Toyota Central R&D Laboratory, Japan), November 28-29, 2013
  
- [3] Nobuyuki Takayama, Lee Wah Lim, Toyohide Takeuchi: 34<sup>th</sup> Summer Seminar of Analytical Chemistry in Chubu Area, (Mihoen Hotel, Shizuoka, Japan), August 31-September 1, 2014
  
- [4] Nobuyuki Takayama, Lee Wah Lim, Toyohide Takeuchi: The 65<sup>th</sup> Annual Meeting of The Japan Society for Analytical Chemistry (Hokkaido university, Hokkaido, Japan), September 14-16
  
- [5] Nobuyuki Takayama, Lee Wah Lim, Toyohide Takeuchi: 33<sup>rd</sup> Ion Chromatography Symposium, (Kumamoto City International Center, Kumamoto, Japan), December 1-2, 2016

### Poster Presentation

- [1] Nobuyuki Takayama, Lee Wah Lim, Toyohide Takeuchi: 11<sup>th</sup> Takayama Forum, (Takayama City Library, Japan), November 11-12, 2011

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