

**Development of silica-based materials for chromatographic
separations 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クロマトグラフィー
一分離のためのシリカ系材料の開発

MAINA ESTHER WAMAITHA

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**A dissertation submitted to Gifu University in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in Material
Engineering**

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JAPAN

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Preface

The modern society today is specifically interested in the art technologies in search of solutions to the wide range of issues including industrial waste water management, food quality control, screening of criminal investigations and environmental pollutants. Chromatography has been one of the most extensively used analytical techniques for the analysis of these problems and has been seen to offer satisfactory results.

Since its discovery in 1903 by a Russian scientist Mikhail Semonovich Tswett, as he effectively separated green, orange and yellow plant pigments by use of filter paper extraction through calcium carbonate packed open glass column, fellow scientists have greatly developed it into liquid chromatography (LC), high performance liquid chromatography (HPLC) and gas chromatography (GC). This was due to the need for better samples separation with reduced time and solvent consumption.

HPLC became the preferred method of choice because it can withstand higher pressure, has higher sample detection capabilities, not limited to volatile or non-volatile samples and also has the possibility of achieving higher resolution during sample analysis. In the late 1980s, in order to further improve the reproducibility of the HPLC system, the packing materials were downsized ($< 10 \mu\text{m}$) with the packing being done at high pressure using slurry techniques.

These packing columns were further improved to monolithic columns that are sponge-like structures, which supposedly offered shorter diffusion distances and several solute dispersion paths. The backpressures experienced in the packing columns were overcome on monolithic columns usage. However, silica packed columns still dominates in the market due to their good chromatographic performance, ease of modification, mechanical strength and availability.

Capillary columns were used for the present study in order to achieve satisfactory results with reduced analysis time, sample concentration and eluent consumption. The synthesized silica based stationary phases were packed into capillary columns and were used to investigate the retention mechanisms of the prepared samples under different eluent conditions.

Chapter 1

Introduction

1.1 Chromatography

Chromatography has been observed to function as a bridge between science and discovery for centuries. It was originally used in the study of colored plant materials, thus its name; “chromatographic analysis” and has since been developed for compounds’ separation [1]. Chromatography was clearly implemented by Mikhail Semenovitch Tswett, a Russian chemist-botanist, who for the very first time described the separation of plants’ pigments. When he trapped calcium carbonate into a narrow glass tube and passed petroleum ether solution through the adsorbent, the pigments resolved in accordance to adsorption sequence. Later, when all the pigment solution had flowed through the glass tube, followed by a stream of pure solvent, the separation was observed to be complete [2].

Chromatography, by definition is a method of identification and separation of closely related complex compounds which are in a mixture. The basic principle involves passage of a solution containing both the analyte and the mobile phase (eluent) on the stationary phase (SP) such as immobilized silica on glass plates, paper or gels. The compounds with stronger affinity for the SP are adsorbed while compounds with lesser affinity flow out of the column resulting to separation. Over the past one hundred years, chromatographic techniques has been developed for better samples separations.

Cost effective analytical techniques is one of the key, and with thus, they are in high demand in many laboratories belonging in different research areas. In the field of any chromatographic separation, the prepared column is the heart of the entire chromatographic set-up, as it enables a better sample selectivity and resolution during samples separation.

Moreover, separation of these compounds with matrices of both low and high molecular weight and also compounds with different polarities can be very difficult. To solve this analytical problem, methods with reduced sample analysis, robustness, high sensitivity and resolution have been invented. For instance, high performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), normal phase liquid chromatography (NPLC), hydrophilic interaction liquid chromatography (HILIC), monolithic and packed columns have been reviewed as potential candidates [3].

As the name indicates, HPLC is a separation technique that uses pressurized pumps to pass the eluent together with the sample mixture through the stationary phase material packed in a column. In the column, the sample component differently interacts with the absorbent material, which results in their differing elution strength and consequently their separation. Their differences in intermolecular interactions and affinity between each of these analytes with the stationary phase relative to the mobile phase determines the analyte's retention time.

Isocratic elution and gradient elution modes exist as the two main HPLC elution modes during the sample analysis. In case of isocratic elution, constant mobile phase strength is used throughout the analysis whereas for the gradient elution, the percentage of the organic solvent being used as the mobile phase is gradually increased. When analyzing complex samples containing polar analytes, gradient elution is the preferred mode of elution [4]. Thus, separation of sample mixtures containing different compounds follows a differential migration flow through a packed column.

Chromatography can be categorized based on the type *i.e.* the physical conditions of the stationary phases and the type of interaction involved. The common and popular ones are based on solid stationary phases, namely, paper chromatography, thin-layer chromatography (TLC),

ion-exchange chromatography (IEC), liquid chromatography (LC), high-performance liquid chromatography (HPLC), hydrophilic interaction liquid chromatography (HILIC), normal-phase liquid chromatography (NPLC) and reversed-phase liquid chromatography (RPLC) as shown in **Table 1-1** below.

Table 1-11.1. Classification of chromatography

Type of chromatography	Abbreviation	Stationary phase	Mobile phase	Type of interaction
Paper chromatography	PC	Solid	Liquid	Partition
Thin-layer chromatography	TLC	Solid	Liquid	Adsorption
Ion-exchange chromatography	IEC	Solid	Liquid	Adsorption
Capillary liquid chromatography	CLC	Solid	Liquid	Adsorption
High-performance liquid chromatography	HPLC	Solid	Liquid	Adsorption
Ultra-high performance liquid chromatography	UPLC	Solid	Liquid	Adsorption
Hydrophilic interaction liquid chromatography	HILIC	Solid	Liquid	Adsorption
Normal-phase liquid chromatography	NPLC	Solid	Liquid	Adsorption
Reversed-phase liquid chromatography	RPLC	Solid	Liquid	Adsorption

1.1.1 Paper chromatography (PC)

Paper chromatography (PC) is a simple separation technique used to separate and identify compounds in a mixture. A paper is used as the stationary phase while a liquid solvent is used as the mobile phase. During sample separation, a sample mixture is spotted on one edge of this paper, which is then carefully immersed into the mobile phase tank. The mobile phase moves up

the paper through capillary action along with the component mixtures with varying distances depending on their affinity to both the stationary phase and the mobile phase [5].

1.1.2 Thin-layer chromatography (TLC)

TLC is a laboratory technique commonly used to assess organic compounds purity and also monitor the reaction progress. It has similar characteristics to paper chromatography but has relatively faster chromatography runs and better separations. Its stationary phase involves a polar adsorbent material like silica gel thinly coated onto a sheet of plastic or glass to form a TLC plate. A developing chamber which is half filled with the mobile phase is used as mobile phase's reservoir, and this plate is then carefully placed horizontally. In accordance to the targeted compounds to be separated, the mobile phase is chosen. During separations, spotting of a small amount of sample mixture is done near the bottom of this TLC plate, and due to the different affinity with the mobile phase and the stationary phase, separation is achieved. Through capillary action, the mobile phase rises slowly up the plate carrying along the sample in accordance to their affinity with the stationary phase [6].

1.1.3 Ion-exchange chromatography (IEC)

Ion-exchange chromatography is a separation technique in which ionizable samples are separated due to its stationary phase being specifically designed with charged or chargeable moieties. This stationary phase consists of exchangeable counterions such as single-charged monatomic ions (Na^+ , K^+ , Cl^-), double-charged monoatomic ions (Ca^{2+} , Mg^{2+}), polyatomic inorganic ions (SO_4^{2-} , PO_4^{3-}) and acids (COO^-). Quaternary alkyl ammonium salts together with sulphonic groups are used to prepare strong ion-exchangers, while the other charged groups are used as weak ion-exchangers [7-8].

Depending upon the interaction affinities of the analyte molecules with the charged stationary phases, these analytes are reversibly adsorbed where separation occurs with the mobile

phase passage through the stationary phase. Therefore, it can effectively be applied for the purification of peptides, amino acids, protein, and nucleic acids molecules. Due to its ease of modification and a large capacity to hold sample, it is thus becoming a versatile tool which is being widely used in LC.

1.1.4 Capillary liquid chromatography (CLC)

Analysis of a chemical sample mixture has complexity resulting from the mixture interaction amongst the number of components present, which greatly reduce the chances of successfully measuring analytes of interest. The most commonly employed analytical technique is HPLC. In HPLC, separation of these compounds is accomplished using a column prior moving to the detector.

Stainless steel packed column with an internal diameter range of 0.5 to 1.0 mm was developed by Horvath and his co-workers in the late 1960s [9, 10]. In 1970s, a micro column liquid chromatography (μ LC) was started at Nagoya University, Nagoya, Japan by Dr. Ishii's group.

From then on, μ LC systems with injection valves having low dead volume, low flow rates pumping system and detection volumes have been commercialized by number of manufactures [11]. Based on separation methods, the history of capillary and their related techniques was developed in accordance to the **Table 1-2** below. A capillary LC consists of a whole set-up that works hand in hand to bring about satisfactory results.

Table 1-2. Development of capillary-based separation techniques

Capillary-based separation technique	Year	References
μ LC	1974	[12]
Open-tubular capillary LC	1978	[13]
Packed micro capillary LC	1978	[14]
Fused-silica capillary	1979	[15]
Capillary zone electrophoresis	1981	[16]
Electrokinetic chromatography	1985	[17]
Capillary electrochromatography	1987	[18, 19]
Monolithic polymer capillary columns	1989	[20,21]
Capillary array electrophoresis	1992	[22]
Monolithic silica capillary columns	1998	[23]

1.1.4.1 Capillary liquid chromatography set-up

A capillary liquid chromatography system consists of a pump, which is connected to a sample-injection port, a column which is used as a separating unit, a detector, and a separation output which is shown in terms of chromatograms on the data-processor, and thus, each of these units are essential in performing the analysis (**Fig 1-1**). The pump is used to pump the eluent at a constant pressure and flow-rate, while using isocratic mode of separation, as the analyses are being performed. The sample is injected using a syringe through a manual injector into the flow path for analysis. The knob is initially set at the "load" position during sample injection, and then turned to the "inject" position as the sample is being injected into the sample loop. The eluent travelling from the pump through the loop, delivers the sample to the column in order to be separated. The separated samples are detected using a UV detector and the data converted into

electrical signal which is then showcased on the screen. A novel column is the heart of the entire system [24]. Capillary columns are classified into packed and monolithic columns.

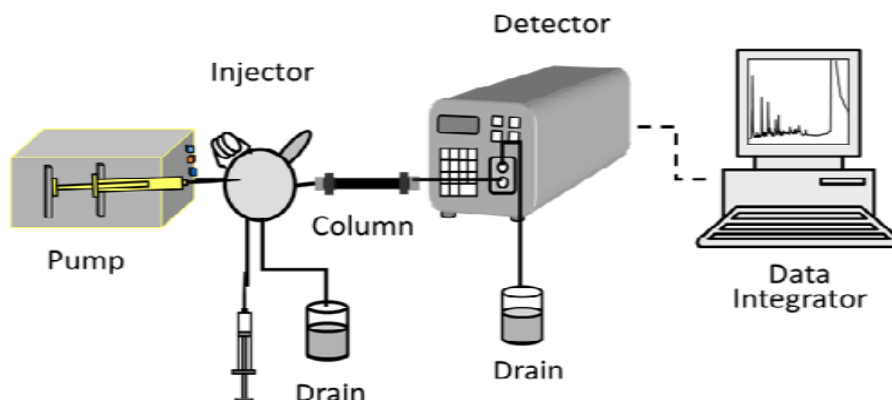


Fig. 1-1. The capillary liquid chromatography set-up [25]

1.1.4.1.1 Packed capillary columns

Packed columns are made up of fine particles fully packed in a column which is used as the column bed for the chromatographic separations. These columns comprise of a capillary column, packing materials and end plugs. A low mobile phase flow rate is used during separation due to the increased pressure inside the columns. These LC packing materials are categorized into two types; polymer and silica-based materials.

Polymer-based materials are more advantageous in comparison to silica-based materials, in that, they are chemically stable, thus can be operated under wide range of pH. However, silica-based packing materials still dominates in the market due to its availability, excellent physical properties, ease on modification, high separation efficiency and rigidity than the polymeric materials. The preparation of these materials is usually done through slight modification of the already existing conventional LC materials.

When packing these materials into the column, gases or liquids are used as medium of transferring them from the external reservoir to column tubing, in order to form a homogeneous and stable bed. A frit comprised of a glass wool is mounted at one of the capillary tube's end, and the packing particles are then pushed into the tube through the other end [26].

1.1.4.1.2 Monolithic capillary columns

Monolithic columns are prepared through polymerization reactions of both the organic and inorganic materials. This results to formation of a continuous skeleton as the stationary phase and has superior porosity. The porosity of a monolithic column results to high permeability of the mobile phase during chromatographic separations and hence rapid analysis and enhanced selectivity. Monolithic columns are classified into three groups, *i.e.* organic monolithic columns, inorganic monolithic columns and hybrid monolithic columns.

1.1.4.1.2.1 Organic monolithic columns

Organic monolithic columns are normally prepared through one pot reaction in which all the reagents; *i.e.* monomer, crosslinking agent, porogen and initiator are put in a capillary column or glass tube for polymerization reaction to occur. Initiation of polymerization reaction can be triggered by use of heat or UV irradiation. Optimization of polymer composition or reaction conditions can result to enhanced monolith porosity. The prepared stationary phase can thus be used for chromatographic separations [27-29].

1.1.4.1.2.2 Inorganic monolithic columns

These monolithic columns are prepared through polymerization reactions like one-pot reaction or sol gel process. One-pot polymerization reaction involves addition of all the reactant materials in one pot/vial while sol gel polymerization involves two main processes; hydrolysis and condensation reactions. For instance, tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS) being amongst the main reagents used, are first transformed into Si-O-Si from Si(OR)₄,

becoming silicon polymers and oligomers. A continuous skeleton made of silicone backbone having large pores is formed. The prepared monolith is highly advantageous as it highly permeable, has good mechanical stability and good thermal stability [30].

Other than silica, the oxides of alumina, zirconia, titania, hafnia, carbon, gold, silver, ceria, germinia, iron and hydroxyapatite inorganic monoliths have also been successfully prepared and used in chromatographic separations. The use of these inorganic monoliths together with their modified composites is still in its early stages and with thus, this field is inevitable, and a great deal of development is being made [31].

Both the organic and inorganic monolithic columns have some disadvantages like poor mechanical stability, sample retention reproducibility and repeatability, and also short column lifetime. This is so because certain reaction steps like cladding of the rods and drying is very difficult to achieve in an academic laboratory. In that case, combining the merits of both the organic and inorganic monolithic columns was realized as a way to enhance column's efficiency.

1.1.4.1.2.3 Hybrid monolithic columns

Hybrid monolithic columns are commonly synthesized through a sol gel process, and due to the incorporation of an organic moiety onto an inorganic surface, they have an enhanced separation capabilities, therefore creating a great deal of attention. Their advantages include; enhanced solvent resistance and good mechanical stability. For instance, Yan and his co-workers were able to effectively prepare a hybrid monolithic column consisting of both n-octyltriethoxysilane (C8-TEOS) and tetraethoxysilane (TEOS) in order to form octyl-hybrid monolithic column. The prepared monolith had about 180,000 plates, column efficiency during the separation of phenols and aromatic hydrocarbons (PAHs) [32].

1.1.5 Hydrophilic interaction liquid chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) has been evolving for the past few years as an efficient chromatographic technique used for separating of both polar and neutral analytes which are retained poorly in a reversed phase stationary phase. Polar stationary phases i.e. un-bonded silica is used as the adsorbent material while applying eluents similarly used during reversed phase separations. During separation, the hydrophobic eluent with water having relatively higher eluting strength is used to elute the analytes [33]. The complexity of HILIC stationary phase provides multiple interaction modes between stationary phase, eluent, analytes and stagnant water layers trapped within the stationary phase as observed by Gregor and his coworkers [34].

According to Hanai [35], the mechanism of HILIC is a combination of hydrophilic, hydrophobic, shape selectivity, hydrogen bonding and dipole-dipole interactions. However, some of these interactions are dependent on each other resulting to having some overlapping properties. For instance, electrostatic force and hydrogen bonding contributes to hydrophilic interactions while electrostatic force contributes to ion exchange interactions. Localized electrons contribute to both the π - π and dipole-dipole interactions. Thus, HILIC can be used as both normal and reversed-phase liquid chromatography.

1.1.6 Normal-phase liquid chromatography (NPLC)

In normal-phase liquid chromatography (NPLC), separation of compounds is done through adsorption on a polar stationary phase *i.e.* silica gel while using organic solvents as eluents. The selection of eluent determines the retention of analytes. NPLC have similar mechanism of interactions to HILIC. HILIC explains the retention mechanism of samples while NPLC explains the balance in polarity of the stationary phase and the eluent. Thus both terms can be used to describe the same liquid chromatography [35].

1.1.7 Reversed-phase liquid chromatography (RPLC)

RPLC is a powerful tool for chromatographic separations of moderately polar compounds, non-polar compounds, and amino acids. It is characterized by a non-polar stationary phase and a more polar mobile phase. Chronically in accordance to the chromatographic modes of separation, it was the second mode of separation after normal phase. Normal phase uses a polar stationary phase and a non-polar mobile phase hence 'straight', while the opposite was reversed-phase chromatography. These phases commonly consist of silica as the base material, which is chemically bonded to a hydrophobic surface amongst many other phases [36].

1.1.8 Size-exclusion chromatography (SEC)

In this type of a system, the column is filled with controlled pores size and the analyte is separated according to its molecular size. The smaller molecules are retained inside the pores of the packing materials while the larger molecules are washed out causing separation.

1.2 Objectives of the present research

Capillary liquid chromatography is a simple and powerful analytical technique used for compound's separation and identification which has greatly contributed to science and discovery. The present study presents capillary liquid chromatography columns that were used at low solvent consumption with high column efficiency and good sensitivity.

Chapter 2 describes the use of chromium alkoxide precursors as a modifier onto silica surface. Chromium is a very stable transitional element which exists as a hexavalent ion in solution forming complexes with other compounds. With thus, a need rose for incorporation of ligands, in order to effectively capture and close the chromium sites which could be interacting with the analytes that were being separated. Synthesis of metal organic framework silica (MOF-Si) was successful, however, the immobilization reaction was found to be a physical process.

Chapter 3 describes the preparation of a novel silica based stationary phase through click chemistry and the stationary phase formed was used for inorganic anions' separation.

Chapter 4 describes the retention of anions on a chemically bonded 4-amino butyric acid on the surface of silica gel. A simple 2-step reaction was followed in order to effectively synthesize the material. Selection of a suitable eluent resulted to the retention and separation of anions and thus, it worked as the anion-exchange stationary phase.

Chapter 5 describes the retention of anions on a silica gel immobilized with 7-amino heptanoic acid. Selection of a suitable mobile phase resulted to the separation of targeted anions, and the prepared column also worked as the anion-exchange stationary phase.

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Chapter 2

Development of silica based chromium metal-organic framework materials for chromatographic separations

2.1 Introduction

Heavy metal is a term used to describe metals with approximately 4.0 or higher specific gravity and is commonly distributed in rocks and soil. For industrial production of machineries, thermometers, batteries and blood pressure gauges, these metals are extracted from the earth's surface. Chromium is amongst the heavy metals which are used in various fields like in cement steel industries. However, these metals ions are heavily discharged into river streams posing a serious environmental and health concerns [1].

On the other hand, silica is widely used for liquid chromatographic separation due to its availability, excellent physical properties and ease on modification. However, silica based stationary phases have limited usable pH which results to residual silanols groups that causes peak tailing during chromatographic separations. Measures in order to extend its stability have been taken. For instance, Silva and his colleagues [2] were able to successfully incorporate titanium protective layer onto silica surface, which was able to improve the stability of silica surface on high pH eluent's applications. According to Hoth [3], oxides of zirconium, aluminum, titanium, iron and copper have been extensively used for chromatographic separations as they offer more pronounced chemical and thermal stability than silica and organic polymers. However, oxides of chromium have not been greatly researched on, which could also offer selective adsorbents for chromatographic separations [4, 5].

The surface of silica gel contains silanol groups which react with metal oxides to produce oxide surfaces which are associated with high thermal and chemical stability. Moreover,

modification of silica with organic functional groups could enhance the sorption capabilities of silica gel for chromium ions as they act as linkers.

Sorption process could either be physical sorption or chemical sorption. Physical sorption is where the organic linker is physically adsorbed whereas in chemical sorption processes, the organic linkers are chemically adsorbed onto the silica surface [6, 7]. According to Chmielarz's group [8], acetylacetonone ligands can be used to deposit the metal's active site on mesoporous silica. This is so because they are highly reactive towards the silanols through two different mechanism (i) hydrogen bonding mechanism: π -electrons of the acetylacetonone ligand with the proton on the surface of the silanol groups by hydrogen-bonding and (ii) ligand exchange mechanism: where the acetylacetonone ligand interacts with surface silanol groups resulting to the formation of covalent bond between the metal and surface oxygen which results in loss of a ligand. These methods have been shown to guarantee high and uniform dispersion of the active components in comparison to other methods like impregnation.

Coordination of these hybrids, *i.e.* organic linkers and transitional metals, is used in the formation of metal organic frameworks (MOF). These structures are having applications in different fields like separation, magnetism, gas storage and in catalysis. The coordination sites or bridging anions is often O⁻ and N⁻ resulting to formation of very stable, large surface area and open channels without the collapse of the framework. Most of these MOF organic linkers are rigid aromatic carboxylates, COO⁻ or N⁻ containing aromatic ligands [9]. Thus exploration of a ligand family with sufficient electrons for ligation purposes produces stable complexes [10].

The novelty of this research is the use of chromium alkoxide precursors onto silica surface in order to increase the silica column's lifetime for liquid chromatographic separations. Chromium is a stable transitional element which exists as a hexavalent ion in solution forming

complexes with other compounds [11]. Therefore, it is crucial for incorporation of ligands, in order to effectively capture and close the chromium sites which could be interacting with the analytes that were being separated. MOF-Si synthesis for chromatographic separation was successful, however, it was found to be a physical process.

2.2 Experimental

2.2.1 Chemical reagents

Silica gel (5 μ m), L-column silica (5 μ m), chromium (III) chloride hexahydrate ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$), toluene, acrylic acid, urea, distilled water, ethanol (EtOH), hydrochloric acid (HCl, 99%), toluene, methanol, isopropanol, polyethylene glycol (PEG, 10,000) were received from Wako Pure Chemicals Limited, Japan. Polypropylene glycol (PPG) was obtained from Sigma-Aldrich while Chromium (III) benzoylacetate ($\text{C}_{30}\text{H}_{27}\text{CrO}_6$) was obtained from Gelest (Morrisville, Japan). These chemicals were used without further purification.

2.2.2 Activation of silica gel

Silica gel (5 μ m) was first activated using HCl following a previous method [12] with slight modification. In brief, 2 g of silica gel dissolved in 20 mL of 20% hydrochloric acid was added to a round bottomed flask and the mixture was refluxed at 70 $^{\circ}$ C for 4 hours. The sample was then filtered out, washed with water and acetone three times and later oven dried at 80 $^{\circ}$ C for 12 h. The sample was then stored in the desiccator ready for the next step.

2.2.3 Preparation of chromium solution

The chromium solution was first synthesized following a method by Kido's group [13] with slight modification. In brief, $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ (1.19 g), urea (2.68 g) and acrylic acid (0.8 g) were dissolved in a mixture of distilled water (1.82 mL) and EtOH (1.50 mL). Polypropylene glycol (1.88 mL) was then added to the solution and the solution was again ultra-sonicated for 20 min.

The solution was stirred under ultra-sonicator into a homogeneous green solution for 20 min and this mixture was denoted as solution A.

2.2.4 Preparation of Si-Cr Composites

The activated silica (0.4 g) was put into a round bottomed flask and solution A (0.2 g) suspended in 20 mL toluene was added drop wise. The mixture was refluxed for 24 h at 80°C. The sample was then washed with toluene, iso-propanol, methanol, water and finally with acetone three times subsequently. The sample was later oven dried at 80 °C for 12 h. The sample was denoted as Si-Cr.

2.2.5 Synthesis of Si-Cr benzoylacetate composites

PEG (10,000) (0.7 g) was dissolved in 5 mL ethanol and the solution ultra-sonicated at 45 °C for 30 min into a homogeneous solution. To the activated L-column silica (1 g), the PEG solution was added, and the mixture was again ultra-sonicated at 45 °C for 12 h. The sample (Si-PEG) was filtered and washed with acetone three times and later was oven dried at 45 °C for 12 h. Chromium (III) benzoylacetate (0.5 grams) dissolved in 40% acetone was added to the Si-PEG (1 g) and the mixture was ultra-sonicated at 45 °C for 3 days. The sample was washed three times with ethanol in order to remove any un-reacted materials. The sample was denoted as Si-Cr-BA as shown in **Fig. 2-1** below.

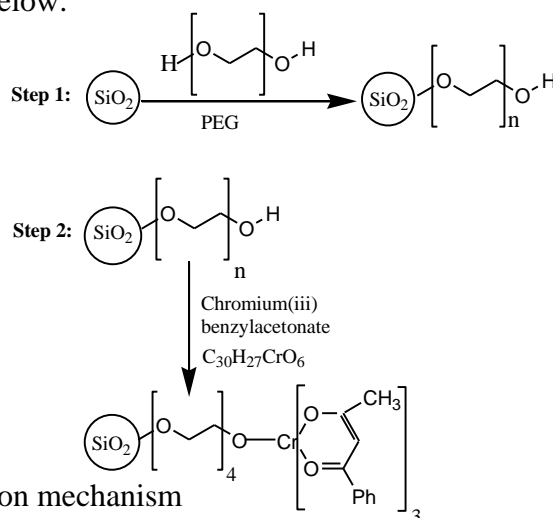


Fig. 2-1. The proposed reaction mechanism

2.2.6 Column packing and testing

Both the commercial silica (Si) and Si-Cr samples were packed into 10 cm capillary column (100 cm x 0.32 mm i.d.) and different analytes were separated, recorded and compared. The columns were downward packed individually using suspension of silicas in methanol using a syringe and the same solvent for propulsion purposes.

2.2.7 Sample characterization

2.2.7.1 Elemental and scanning electron microscope (SEM) analyses

The elemental analyzer, CHN 2400 Perkin-Elmer, was used to identify the elements present in both the Si-Cr and Si-Cr-BA samples. The samples (0.2 mg) were first heated in the ceramic crucibles in the temperature range of 30-1000 °C in the flowing air atmosphere with the heating rate of 10°C min⁻¹ [14]. The results were recorded and analyzed. The samples were again characterized using SEM.

2.2.7.2 FT-IR characterization

The IR spectra of all the samples were recorded at the wavelength range of 400-4000 cm⁻¹ using FT-IR Perkin Elmer Spectrum 400 spectrometer. The sample (0.1 mg) was placed on Gladi ATR™ PIKE technologies, Japan and was scanned 8 times. The spectra were recorded and analyzed.

2.2.7.3 BET adsorption isotherms

The Si-Cr powder was first oven dried at 150 °C for 180 min before measurements. It was then put inside a glass tube to be used as the adsorbate for nitrogen gas at 77 K, in order to form a monolayer sample. A certain volume (V) of nitrogen was adsorbed on the pore walls of Si-Cr and the corresponding partial pressure (p/p_o) was recorded. Measurement of specific surface area was done using the lower portion of adsorption-desorption isotherm which was used for pore analyses.

Physical gas adsorption is a technique of choice for examining the silica pore characteristic which determines the amount of gas adsorbed on silica gel which is a direct indication of the porous properties [15]. Thus the isotherm obtained from these adsorption measurements provided the information about surface area, pore volume and pore size distribution.

2.3 Results and discussions

2.3.1 Sample characterization

2.3.1.1 Si and Si-Cr elemental analysis

The elemental analysis for Si-Cr sample confirmed presence of carbon (C), hydrogen (H) and nitrogen (N) with an increment of 10.72 %, 1.43 % and 3.59 % from the initial percentages of 0.61, 1.63 and 0.21 respectively (**Table 2-1**). Chromium is a very stable transitional element, existing as a hexavalent ion in solution and thus forms complexes with organic moieties containing nucleophiles. Presence of acrylic acid, urea and polypropylene glycol during the synthesis of chromium solution provided the organic moieties like carboxyl and amide which formed coordinative bonds with chromium through hydrogen bonding. This assisted in regulating the formation of a highly cross linked network of chromium (III) hexahydrate which further formed coordinative bonds with commercial silica resulting to a formation of homogeneous Si-Cr particles [13]. With thus, the percentage increment in the analyzed elements was observed in the Si-Cr sample.

Table 2-1. Elemental analysis for both the commercial silica (Si) and synthesized silica (Si-Cr)

Sample	% C	% H	% N
Si	0.61	1.63	0.21
Si-Cr	11.33	3.06	3.80

2.3.1.2 Si and Si-Cr-BA elemental analysis

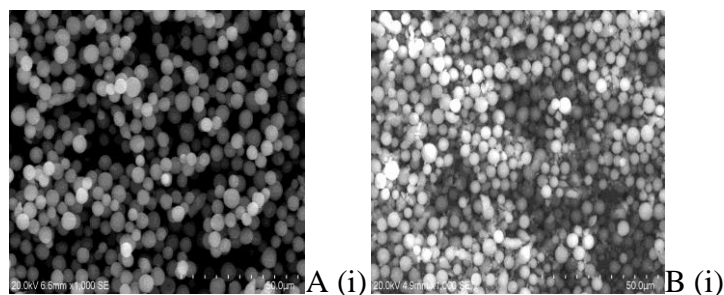
The Si-Cr-BA sample elemental analysis confirmed presence of C and H with an increment of 38.52 % and 2.61 % from the initial percentages of 0.35 and 1 % respectively (**Table 2-2**). Presence of PEG during the synthesis of Si-Cr-BA provided the carboxyl organic moieties linking the commercial silica surface with the chromium benzoylacetate complex ($C_{30}H_{27}CrO_6$) through hydrogen bonding. Thus, about thirty C (C30) atoms resulted from chromium complex and the rest eight (C8) resulted from the PEG ligand.

Table 2-2.Elemental analysis for both commercial silica (Si) and synthesized silica (Si-Cr-BA)

Sample	% C	% H	% N
Si	0.35	1	0
Si-Cr-BA	38.87	3.61	0

2.3.1.3 Scanning electron microscope for both Si and Si-Cr samples

When comparing the SEM images as shown in **Fig 2-2** of Si, *i.e.* A (i) and A (ii), and those of Si-Cr, *i.e.* B (i) and B (ii), the Si-Cr showed to have a smaller particles distribution. This may have been due to the replacement of an OH groups by O-Cr groups in silica pore gel. Thus, the incorporation of Cr (III) into tetrahedral geometry of Si (IV) was successful thereby increasing the lattice parameter [5].



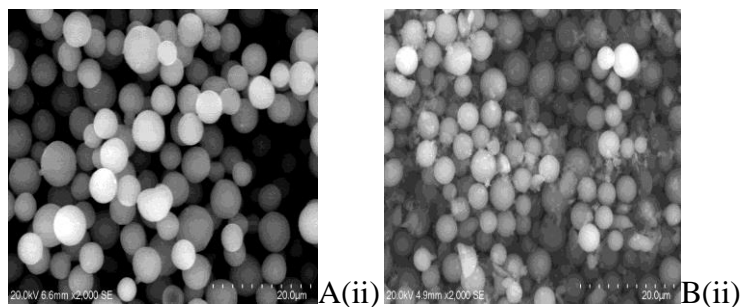


Fig. 2-2. SEM images of commercial silica (Si), A (i) magnification 1000x and A(ii) magnification 2000x, and synthesized silica (Si-Cr), B(i) magnification 1000X and B(ii) magnification 2000x, respectively

2.3.1.4 The FT-IR spectra for both Si and Si-Cr samples

The FT-IR spectra of both commercial silica (Si) {**Fig. 2-3 (a)**} and chromium modified silica gel (Si-Cr) {**Fig. 2-3 (b)**} were analyzed and recorded which confirmed successful incorporation of Cr (III) into the Si surface. The synthesized sample was green in color which was due to presence of trivalent chromium ions in the octahedral geometry. The IR absorption peaks at 468 cm^{-1} and 1088 cm^{-1} on commercial silica were due to asymmetric and symmetric stretching vibrations of the Si-O-Si bond. On Si-Cr sample analysis, these absorption peaks shifted to lower wavelength of 466.1 cm^{-1} and 1102.0 cm^{-1} respectively, which was a clear indication of Cr incorporation into silica tetrahedral framework. The absorption bands at 966 cm^{-1} caused by Si-O vibrations in Si-OH, was observed to shift to lower wavelength of 930 cm^{-1} on Si-Cr due to presence of chromium ions (**Table 2-3**). Similar results were observed by Bai's group [5].

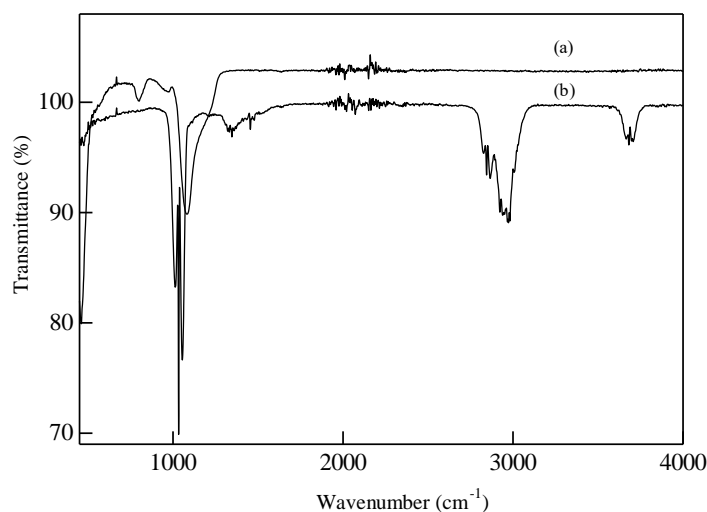


Fig. 2-3. FT-IR spectra for commercial silica (Si) (a) and synthesized silica (Si-Cr) (b)

Table 2-3. Assignment of the FT-IR absorption bands

Wavenumber (cm ⁻¹)	Assignment
468 cm ⁻¹	Si-O-Si Asymmetrical stretching
966 cm ⁻¹	Si-O-H stretching vibrations
1088 cm ⁻¹	Si-O-Si symmetrical stretching
1380.2 cm ⁻¹	Symmetrical vibration of methyl group
2925 cm ⁻¹	C-H stretching of the alkyl groups
3507.5 cm ⁻¹	O-H stretching vibration

2.3.1.5 The FT-IR spectra for both Si and Si-Cr-BA samples

The FT-IR spectra were recorded for both commercial silica, as shown in **Fig. 2-4(a)**, and Si-Cr-BA, as shown in **Fig. 2-4(b)**, and the absorption bands were assigned in accordance to **Table 2-4** below. The Si-Cr-BA sample contained both the PEG and benzoylacetate ligands as confirmed by the elemental analysis (**Table 2-2**). The carboxyl groups are the main groups in both the PEG and benzoylacetate structures, which may have undergone transformation during chromium metal complexes formation. These carboxyl groups contain C=O, C-O and O-H

vibration modes. The C=O stretch appeared at 1700.4 cm^{-1} , and due to the small differences in the lengths of C=O bonds in the carboxylic there may have been some sub-bands at 1710 cm^{-1} and 1698.5 cm^{-1} . On deprotonation of the carboxyl groups during the complex formation, this caused band in the 1700 cm^{-1} region disappear and new, very strong bands of asymmetric and symmetric stretching vibration of -COO^- appear. The broad spectrum at 3507.5 cm^{-1} was attributed to O-H stretching vibrations consequence of benzoylacetone and PEG ligand. The results were in consistence with previous research work [14, 16-17].

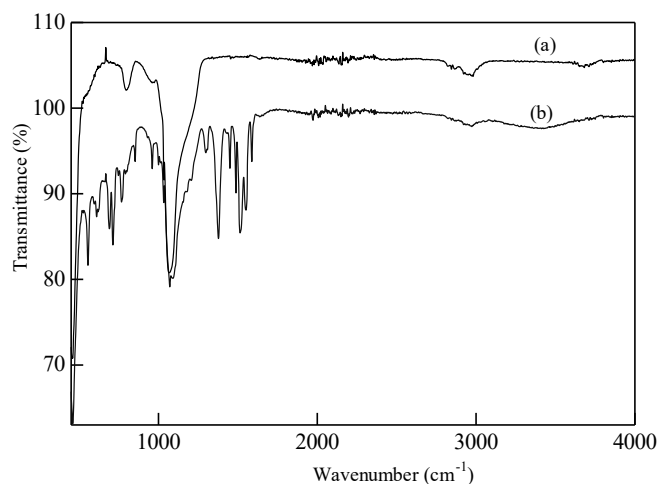


Fig. 2-4. FT-IR spectra for both the commercial silica (a) and synthesized silica (Si-Cr-BA)(Si) (b)

Table 2-4. Assignment of the FT-IR absorption bands

Wavenumber (cm^{-1})	Assignment
470 cm^{-1}	Si-O bending vibrations
583 cm^{-1}	Cr-O stretching vibrations
746 cm^{-1}	Mono substituted benzenes vibrations
800 cm^{-1}	Si-O-Si symmetrical stretching vibrations
1380.2 cm^{-1}	Symmetrical vibration of CH_3 in ligand

1525.6 cm ⁻¹	C=C assymetrical stretching of the ligand
1710 cm ⁻¹	Deprotonation of carboxyl group during complex formation
3507.5 cm ⁻¹	O-H stretching vibration

2.3.1.6 BET adsorption isotherms for both Si and Si-Cr samples

From the nitrogen adsorption isotherm, it was observed that the material had both macropores and mesopores structures. The nitrogen adsorption on Si-Cr sample was best described to be type IV in the BET classification, **Fig. 2-5 (A)**, as indicated by the square of the product moment correlation coefficient (R^2) value of 0.998 **Fig. 2-5 (B)**. This indicated that the mesopores structures had increased in N₂ uptake which confirmed excellent adsorption behavior resulting from modification of Si with the oxides of Cr [7]. This also showed that capillary condensation occurred during adsorption through cylindrical meniscus while desorption occurred through hemispherical meniscus, thus separating the vapor and the capillary condensed phases [15]. However, the presence of the oxides of chromium on silica backbone reduced the access of nitrogen into the skeletal base which thus resulted in gradual decrease in the specific surface area. The initial silica specific surface area ranges from 235-700 m²/g which gradually reduced to 200.89 m²/g specific surface area upon modification [4, 18]. This further supported the elemental, SEM and FT-IR sample analysis.

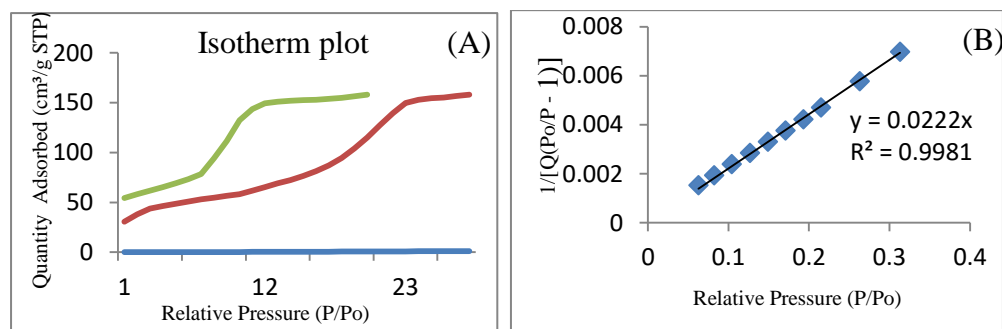


Fig. 2-5. Nitrogen adsorption isotherm at 77K with correlation coefficient of 0.998 (A) indicative of type IV in BET classification (B)

2.3.2 Chromatographic evaluation

2.3.2.1 Separation of mixed aromatic compounds

The present study was able to address a major problem of synthesizing a silica-chromium method that was able to produce an adsorbent which was stable under conditions of high flow and pressure, during the chromatographic evaluation. The Si-Cr column was found capable of separating mix aromatic compounds with retention time of 15 minutes (**Fig. 2-6**). The retention order was from phenol; the hydroxyl group on the phenol compound being the point of reference, thus from less non-polar to highly non-polar compound (diethyl phthalate). However, there was an observed peak broadening on peaks 1 and 2 (**Fig. 2-6**) which were due to the interaction of the given compounds (slightly polar) with the Cr open sites. Such a drawback could be fixed by replacement of the given ligands with rigid aromatic multi dentate ligands containing both O⁻ and N⁻ electron rich surfaces, so as to form stable complexes with chromium, effectively closing all the open Cr sites. These results were in line with the past research work [19].

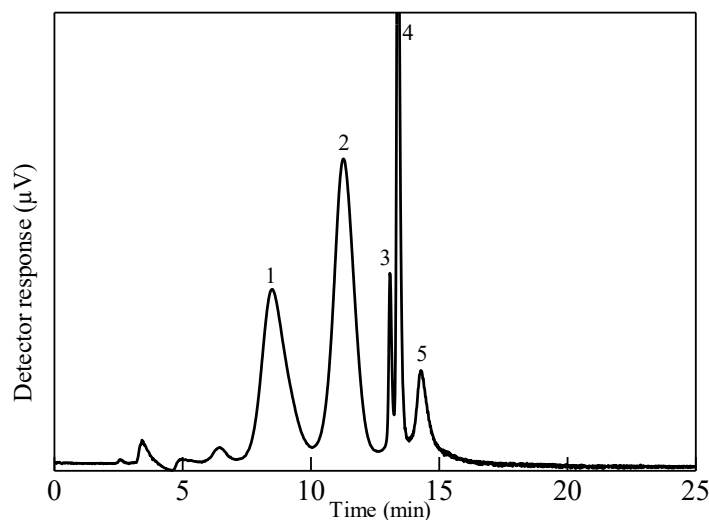


Fig. 2-6. Separation of mixed aromatic compounds; (1) phenol, (2) nitrobenzene, (3) acetophenone, (4) dimethyl phthalate and (5) diethyl phthalate on the synthesized silica (Si-Cr) column. Hexane: chloroform (90:10, v/v) was used

as the mobile phase, with a flow-rate of $2.0\mu\text{Lmin}^{-1}$, an injection volume of $0.2\mu\text{L}$ and at UV detection of 280 nm

2.3.2.2 Separation of alkyl benzenes

In order to effectively separate the alkyl benzenes compounds, the use of ligands ensured a homogeneous, cross linked chromium complex was formed. According to previous research works [4, 11], chromium surface is a non-polar surface, moreover, the incorporated ligands also increased the hydrophobicity of the commercial silica column **Fig. 2-7 (A)** to the synthesized silica surface (Si-Cr)**Fig. 2-7 (B)** which effectively separated non-polar compounds with about half an hour reduced retention time. The retention was in the order of less non-polar compound (benzene) to more non-polar compound (dodecyl benzene).

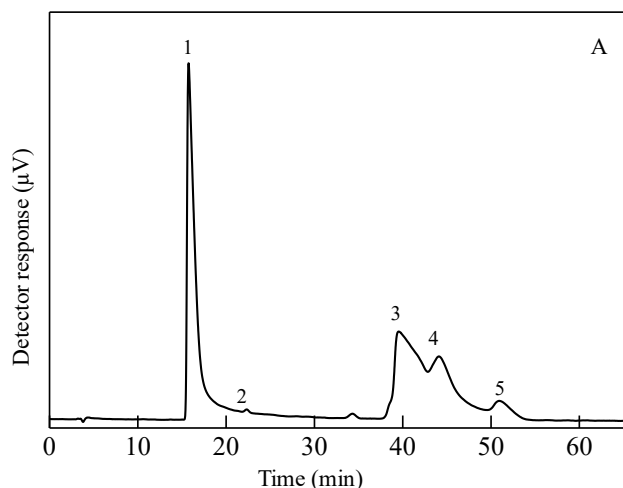


Fig. 2-7 (A).Separation of alkyl benzene; (1) benzene (2) toluene, (3) hexyl benzene, (4) octyl benzene (5) dodecyl benzene on commercial silica packed in a capillary column (A) of $100 \times 0.32\text{mm}$ i.d while using hexane-chloroform (94:6, v/v) as the mobile phase, a flow-rate of $2.0\mu\text{Lmin}^{-1}$, an injection volume of $0.2\mu\text{L}$ and UV detection at 254nm

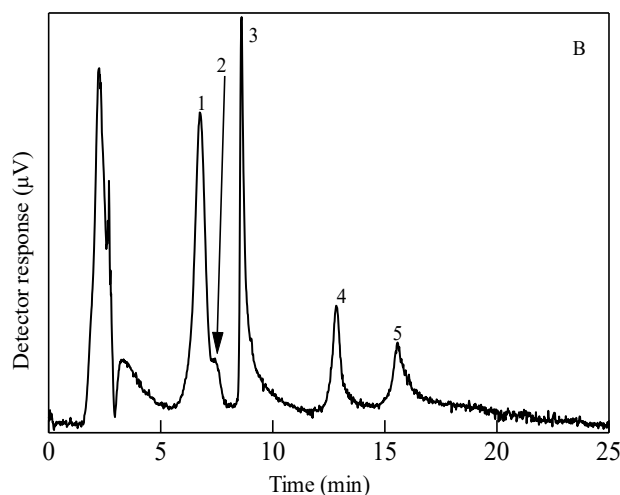


Fig. 2-7 (B). Separation of alkyl benzene; (1) benzene (2) toluene, (3) hexyl benzene, (4) octyl benzene (5) dodecyl benzene on synthesized silica (B) (Si-Cr). Operating conditions as shown in **Fig 2-7 (A)**

2.3.2.3 Separation of parabens

Parabens were separated on commercial silica (Si) and compared with both Si-Cr and Si-Cr-BA columns, as shown in **Fig. 2.8 (a)**, **Fig. 2-8 (b)** and **Fig. 2-9**, respectively. In comparison, the synthesized silicas (Si-Cr and Si-Cr-BA) were able to separate the parabens better than the commercial silica (Si). Moreover, separation on Si-Cr-BA column had reduced peak broadening and tailing in comparison with Si-Cr column. This was as a result of the increased hydrophobicity on column Si-Cr-BA (**Table 2-2**) resulting from the presence of benzoylacetate and PEG ligands in comparison to Si-Cr column (**Table 2-1**). The three benzoylacetate ligands present in the chromium complex formation, were also effective in closing the Cr open sites, thereby reducing the analytes-column interaction, thus the reduced tailing. The analytes were again separated in the order of polarity; with the less non-polar compounds eluting first while the more non-polar compound eluted last.

However, in Si-Cr-BA column, there was no peak when ACN was used as a mobile phase as Cr sites have more affinity for N, *i.e.* the cyano group present in ACN than O, and methanol was thus used as both a mobile phase and a modifier, inhibiting the analytes-column interaction up to optimum 55% [19].

In comparison to the two synthesized column, Si-Cr was more stable during the separation analysis in comparison to Si-Cr-BA. This may have been due to presence of both O⁻ and N⁻ ligands during the Cr complex formation (**Table 2-1** and **2-2**) [9-10]. However, separation with reduced peak tailing was achieved on Si-Cr-BA column due to the better ligand coverage on all the possible open Cr sites.

However, the synthesis process on Si-Cr-BA column was found to be physical process (**Fig. 2-10**) and with thus, no further analysis was carried out. Other than the type of the ligand used for stable Cr complex formation as seen in **Tables 2-1** and **2-2**, the modification process may also have been a physical process due to the synthesis method. The chemical modification process may have been attained if it was carried out in a Teflon lining under elevated pressure and temperature (hydrothermal treatment/ microwave treatment) [7]. A bit of difference with original commercial silica (Si) and ligands desorbed from the commercial silica (Si-Cr-BA), FT-IR spectra as shown in **Fig. 2-10(e)** and **2-10(d)** respectively, was attributed to a partial loss in the crystalline structure of the framework after the separation applications.

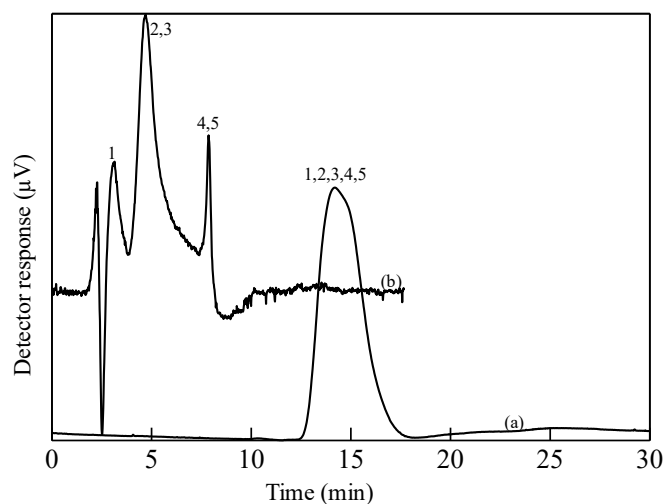


Fig. 2.8. Separation of parabens; (1) 4-hydroxy benzoic acid, (2) methyl paraben, (3) ethyl paraben, (4) propyl paraben and (5) butyl paraben (a) commercial silica (Si) and (b) synthesized silica (Si-Cr) packed in a 100×0.32 mm i.d. capillary column. Hexane: chloroform (98:2, v/v) was used as the mobile phase, with a flow-rate of $2.0 \mu\text{Lmin}^{-1}$, injection volume of $0.2 \mu\text{L}$ and UV detection at 280 nm

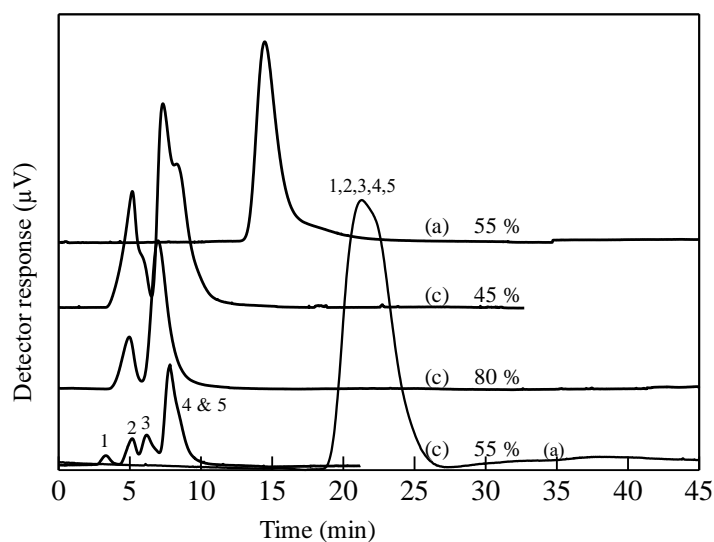


Fig. 2.9. Separation of parabens; (1) 4-hydroxy benzoic acid, (2) methyl paraben, (3) ethyl paraben, (4) propyl paraben and (5) butyl paraben (a) commercial bare silica (Si) and (c) synthesized silica Si-Cr-BA. Mobile phase used was 45%, 55% and

80% MeOH with a flow-rate of $2.0 \mu\text{Lmin}^{-1}$ injection volume of $0.2 \mu\text{L}$ and UV detection at 280nm

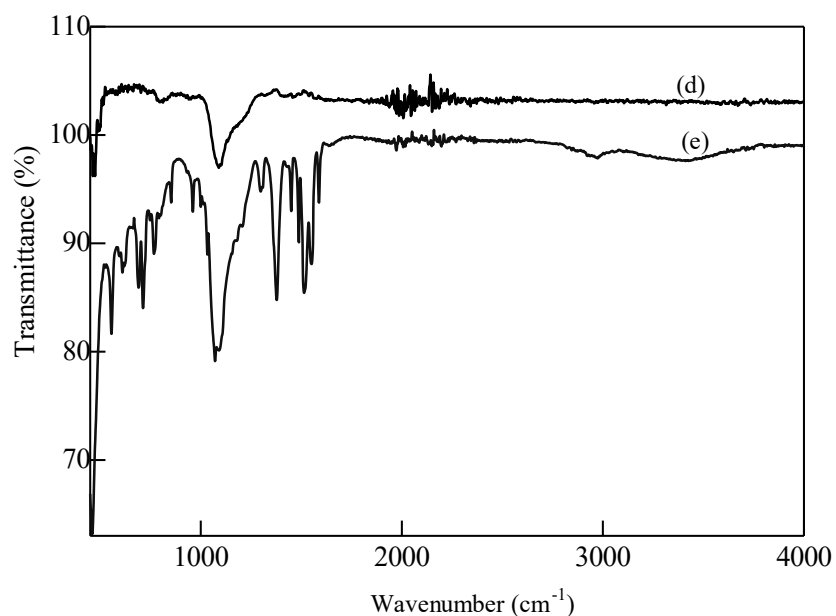


Fig. 2-10. FT-IR spectra for both the synthesized silica (Si-Cr-BA) (e) and used column (Si-Cr-BA) (d)

2.4 Conclusions

Modification process was successful; however, physical-sorption process on Si-Cr-BA column may have been involved. Chemical-sorption is the best modification process for preparation of stationary phases to be used in chromatographic separation fields. No peak was observed when using ACN as a mobile phase in parabens separation on this column. This was due to the interaction of cyano group in ACN with the open Cr-sites. The optimal MeOH used as the mobile phase was 55%. MeOH also acted as stationary phase modifier inhibiting interaction between the paraben analytes and Cr open sites. Use of the appropriate multi dentate ligand together with ligand-Cr ratio is important in MOF-Cr synthesis for separation applications. Silica

can be used as a carrier in drug delivery applications/ sorption (adsorbent) of heavy metals like chromium from waste water.

2.5 References

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Chapter 3

Development of a novel stationary phase for ion exchange liquid chromatography *via* click reaction

3.1 Introduction

Liquid chromatography (LC) is a well-known analytical technique used for separation of different compounds. It can be categorized into different modes of separation, *i.e.* reversed-phase LC (RPLC), normal-phase LC (NPLC), ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), hydrophilic interaction chromatography (HILIC) and size-exclusion chromatography (SEC) amongst others [1]. As a branch of LC, IEC has been well-established over the past years since its first initiation by Small [2] for the ionic samples separation. From then on, researchers have made a considerable effort in preparation of these ion exchange stationary phases [3]. These phases have charged functionalities, where ionic analytes are separated through electrostatic forces. For instance, on this chromatographic media, inorganic anions and other charged molecules are mainly separated.

Covalently bonded silica based stationary phases still dominates as the best, most popular separation medium due to its good chromatographic performance, mechanical strength and easy during preparation process [4]. The most commonly used stationary phases covalently bonded onto silica surface is C18-alkyl chains, which have been widely utilized for reversed-phase separation mode. Other reactive groups like $-\text{NH}_2$, $-\text{COOH}$, have also been immobilized onto silica surface, but sometimes with low selectivity and efficiency. This could be due to some side reactions between the silica supports and the other functional groups resulting in inefficient immobilization process, which generally affects the separation efficiency [5].

Cetyltrimethylammonium modified silica was evaluated for the inorganic anions detection in seawater samples by our research group [6-7]. The quaternary ammonium anion was

introduced onto silica surface forming two layers, where the first layer was introduced through electrostatic interaction and the second layer worked as anion-exchange site [6]. Later, inorganic anions were effectively separated using pyridine functionalized silica-based stationary phase; the inorganic anions were retained on a protonated pyridine under acidic eluent conditions in ion-exchange mode [7].

On further development to these findings, a novel, facile and a powerful strategy for covalently immobilizing these functionalities onto silica support through click chemistry was reported [8-9]. Since then, it has attracted a great attention in the modern chemistry. These reactions have excellent advantages like high selectivity, no side reactions, functional groups tolerance and the reactions are done under mild conditions. According to Chu's group research work, the mechanism of CuAAC is highly favored by different factors. For instant, the solvents used during the reaction promotes ligand exchange and consequently promoting the formation of polynuclear copper acetylides, the complexes' reactivity and the ligands present during this reaction [1]. It is thus useful class of selective chemical reactions which affords high yields with tolerance to variety of solvents and functional groups. Many researchers have been able to effectively immobilize highly functionalized molecules onto silica surface with terminal alkyne and azido modified silica surface [10]. Liu and his co-workers immobilized propargylamine onto azide-silica through click chemistry. The stationary phase had good selectivity in inorganic anions separation under ion exchange mechanism [11].

Depending on the functionalities on click modified silica stationary phases, several separation modes, e.g., RPLC, chiral separation, HILIC and IEC have been identified. Guo and his group functionalized silica through click chemistry and their usage in HPLC chromatographic separations of compounds. They were able to effectively bond di- and oligosaccharides on silica

gel for HILIC and it was assessed to be an excellent chromatographic phase for the polar compounds separation. This further demonstrated the power of click chemistry in the preparation of complex bonded stationary phases [5].

A novel click-arginine-stationary phase was also synthesized by covalently linking arginine onto silica gel through click reaction. This stationary phase exhibited a good HILIC property, excellent selectivity and high capacity towards glycopeptides analytes and thus become a promising glycoproteomics analytical material [11].

Additionally, Huang and colleagues covalently linked a glycosyl amino acid (glycosyl phenyl glycine) onto silica *via* click chemistry. Again, the material exhibited to be a typical HILIC characteristic which was found capable of separating high polar compounds like nucleosides and bases in a simple eluent composition of acetonitrile and water. It also showed better separation ability with longer retention during the separation of polar organic acids [12].

Liu and coworkers were able to effectively synthesize azide-silica and later attached propargylamine through click chemistry. This stationary phase had good selectivity in inorganic anions separation under ion exchange mechanism. In combination with the free amino group attached to the prepared material, the triazole ring played a role in the separation [11].

Futhermore, Guo and coworkers developed a lysine attached onto silica stationary phase through click reaction for ion exchange mechanism through click chemistry for the inorganic ions analysis. The synthesized material demonstrated good performance and effective separation ability for the common inorganic anions while using aqueous sodium sulfate as eluent. In accordance to the above highlighted research observations, many stationary phases i.e NPLC, RPLC and HILIC modes have been prepared through click chemistry for the polar compounds' separation and complex samples except for IC stationary phases [5].

With thus, a novel silica based stationary phase was developed through click chemistry for inorganic anions' separation in this study.

3.2 Experimental

3.2.1 Apparatus

A capillary LC system comprised of a micro-feeder (L.TEX-8301 Corporation, Tokyo, Japan) equipped with a gas-tight syringe (0.5mL; Ito, Fuji, Japan) was applied as a pump, 0.2 μ L as the injection volume, was injected through a M435 micro-injection valve (Upchurch Scientific, Oak Harbor, WA, USA), and a UV-970 UV detector (JASCO, Tokyo, Japan) with a capillary flow cell (75 μ m; JASCO) attached, was used to carry out the chromatographic measurements. The pump flow-rate was kept at 1.0 μ L min⁻¹, and the detector was operated at 210 nm. CDS-Lite ver 5.0 data processor (LAssoft, Nagareyama, Chiba, Japan) was used to acquire all the data.

Fourier transform infrared spectroscopy (FT-IR) was used during the characterization of the silicas. The FT-IR spectra of all the samples were recorded at the wavelength range 400-4000 cm⁻¹ using FT-IR Perkin Elmer Spectrum 400 spectrometer. The sample (0.1 mg) was placed on Gladi ATRTM PIKE technologies, Japan and was scanned 8 times. The spectra were recorded and analyzed.

3.2.2 Reagents and chemicals

Spherical silica 5 μ m particle diameter (Chemical Evaluation and Research Institute, Tokyo, Japan) was used. 3-Isocyanatopropyl-triethoxysilane, propargyl bromide, tetrahydrofuran (THF), sodium azide (NaN₃) and sodium chloride (NaCl) were purchased from FUJIFILM Wako Pure Chemical Co-operation (Osaka, Japan), tetrabutylammonium iodide and L-ascorbic acid sodium salt (Nacalai Tesque INC., Japan), while dimethyl sulfoxide (DMSO) and 1-chloro octadecane were obtained from Kanto Chemicals Co., INC, Japan. A simple UV water purification system

was used to produce ultrapure water in the laboratory, which was used in the preparation of all solutions used in this study.

3.2.3 Preparation of alkynyl-modified silica

A previous method by Guo [5], for the synthesis of alkynyl-modified silica gel was followed with slight modification. To the anhydrous THF (20 mL) was added a solution of 3-isocyanatopropyltriethoxysilane (1.73 g) and propargyl bromide (0.462 g). The mixture was heated at 85° C with constant stirring for 12 h, which was then cooled to room temperature. Silica gel (2 g) was added to the resultant mixture and this, was stirred for 32 h at 110°C and later filtered. The obtained product was rinsed with 15 mL dichloromethane, 15 mL methanol and 7.5 mL acetone, the solid was collected through filtration using 0.45µm filter paper.

3.2.4. Preparation of 1-azido octadecane

1-azidooctadecane was prepared by a method previously described with slight modification[8]. Sodium azide (0.715 g) and tetrabutylammonium iodide (2.0 mg) were dissolved in DMSO (22 mL). 1-chloro octadecane (3.33 g) was added to the solution and the mixture was heated at 83° C for 16 h. Later, water was added to the solution mixture in order to quench it, which was then extracted with hexane using a separating funnel, to obtain a whitish oil layer.

3.2.5. Synthesis of the stationary phase

The synthetic procedure used in the preparation of this stationary phase is as described by Guo [5] with slight modification. To a solution of 1-azidooctadecane (0.44 g) in 15 mL MeOH/ H₂O (60:30), L-ascorbic acid sodium salt (50 mg) dissolved in 2 mL H₂O, CuSO₄ (25 mg) dissolved in 2 mL H₂O and alkyne-silica (0.49 g) was added into the mixture while slowly stirring. The resultant solution was stirred at room temperature for 5 days. The obtained solid product after filtration was then washed with water (100 mL), methanol (100 mL), 0.1 mol L⁻¹EDTA solution

(400 mL), water (100 mL) and acetone (200 mL) in that succession. The obtained solid product was dried at 60 °C for 24 h in the oven as shown in **Fig. 3-1** below.

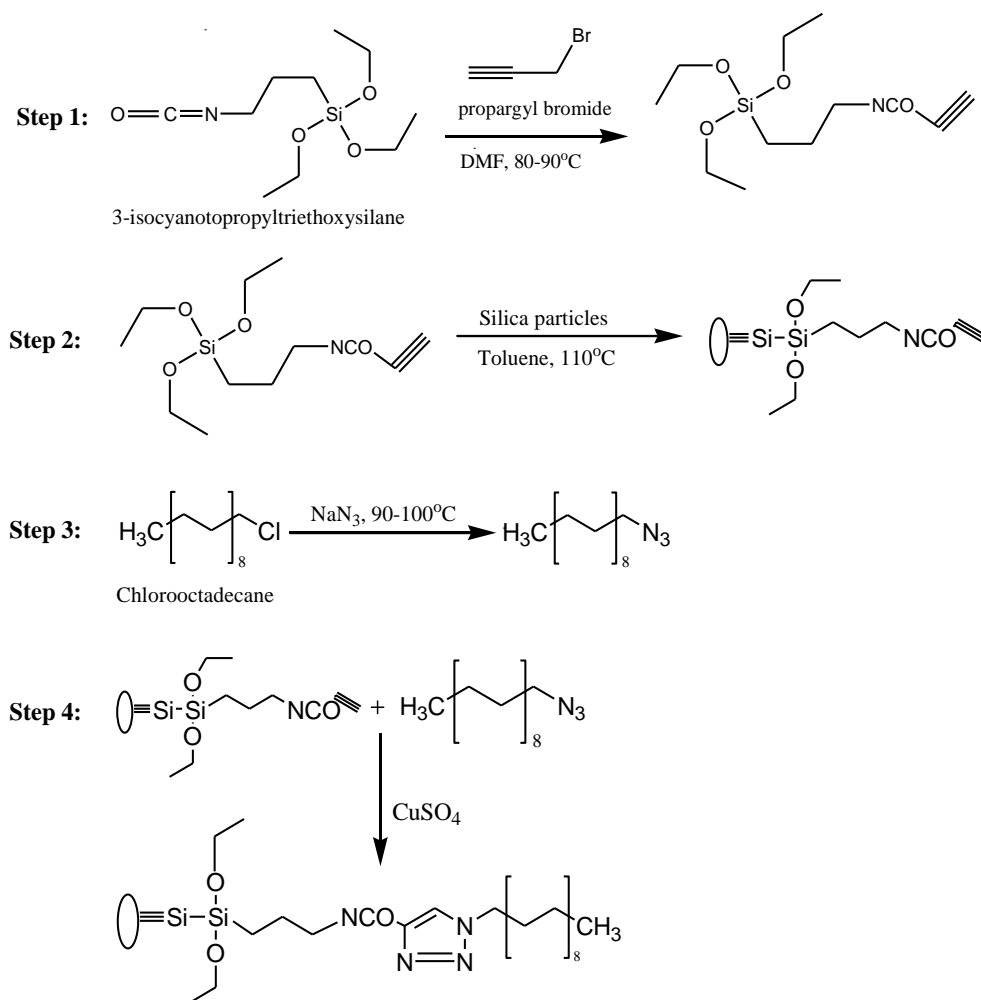


Fig. 3-1. Scheme for the expected reactions

3.3. Results and discussions

3.3.1 FT-IR characterization

Bare silica, intermediate silica and clicked silica were characterized by FT-IR as shown in **Fig 3-2 (a, b, and c)** respectively. On bare silica modification with 3-isocyanatopropyl-triethoxysilane and propargyl bromide, the IR spectra displayed an increase of absorptions intensities at due to C–H stretching indicating a largely ordered structure of the hydrocarbon chains, as comprehensively discussed in **Table 3-1** below. These spectral changes were clear proofs of a

successful reaction and an immobilization procedure. This was further confirmed using elemental analysis as shown in **Table 3-2** below

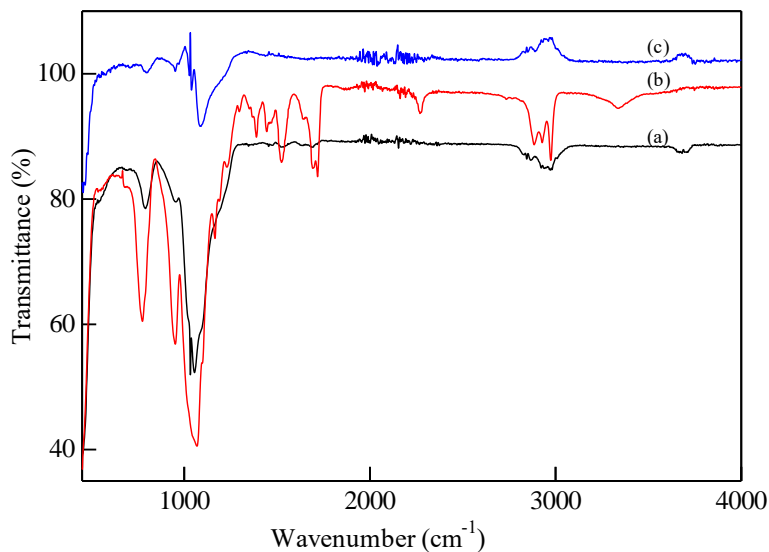


Fig. 3-2. FT-IR characterization of bare silica (a), intermediate silica (b) and clicked modified silica (c)

Table 3-1. Assignment of the FT-IR absorption bands for bare silica, intermediate silica and clicked modified silica

Wavenumber(cm^{-1})	Peak assignment
477.1 cm^{-1}	Si-O bending vibrations
800.3 cm^{-1}	Si-O-Si symmetrical stretching
1380.2 cm^{-1}	Symmetrical vibration of CH_3
1585-1600 cm^{-1}	C-C stretching of aromatic ring
1710.5 cm^{-1}	Asymmetric stretching of C=O
2925 cm^{-1}	C-H stretch of alkyl groups
3507.5 cm^{-1}	O-H stretching vibration

Table 3-2. Elemental analysis of both bare and modified silica samples

Sample	% C	% H	% N
Silica	0.35	1	0
Modified Silica	9.62	2.16	2.05
Increment	9.27%	1.16%	2.05%

3.3.2 Inorganic anion's retention behavior

The prepared click alkyl-azide silica bonded stationary phase was achieved through the reaction of alkyne silica and azido-octadecane in presence of Cu catalyst to produce a triazole ring. This ring could have served as an ion exchange site as it is considered to be a basic aromatic heterocycle. After the protonation of this ring, the eluent cations may have been trapped resulting in formation of ion exchange site. The ions were separated in the order of iodate, bromate, bromide, nitrate, iodide, and thiocyanate which is a similar observation made to common IC columns (**Fig 3-3**).

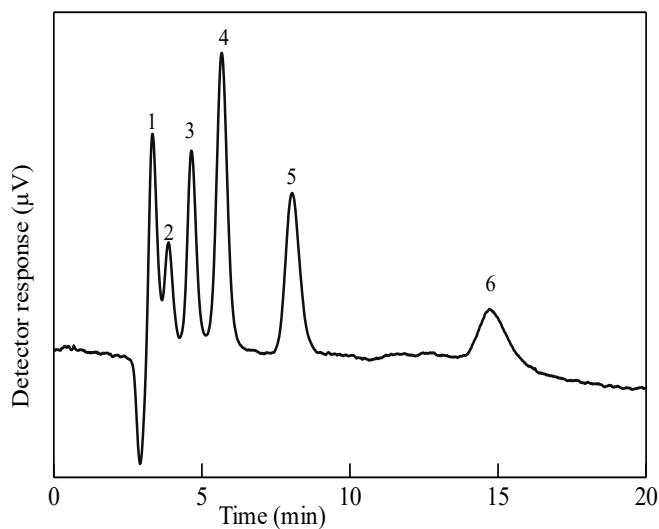


Fig 3-3. Separation of inorganic anions on a click alkyl-azide silica bonded stationary phase column 100 x 0.32 mm I.D, while using 100 mM NaCl as eluent, a flow-rate of $1.5 \mu\text{L min}^{-1}$ and UV wavelength detection at 210 nm. Analytes: 1= IO_3^- , 2= BrO_3^- , 3= Br^- , 5= I^- , 6= SCN^- , 1 mM each while 4 = NO_3^- (0.5 mM)

3.3.3 The effect of eluent concentration

The inorganic anion's retention behavior was investigated under different eluent concentration as shown in **Fig 3-4**. The analyte anions retention in the column decreased with increase in sodium chloride concentration. The typical chromatogram is as shown in **Fig. 3-4** of the tested sample anions was well separated in less than 20 min using 70mM NaCl as eluent. The observed decreased retention time on increased eluent concentration was due to the increase in the amount of trapped sodium ions on click alkyl-azide silica bonded stationary phase column and hence, the ion-exchange capacity increased. This consequently increased the eluent's elution strength [13].

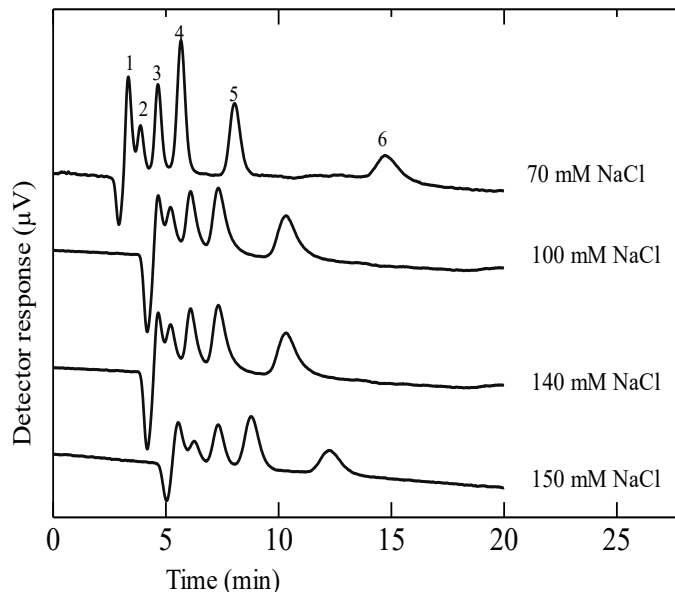


Fig 3-4. Separation of inorganic anions using different eluent concentration on click alkyl-azide silica bonded stationary phase column, column 100 x 0.32 mm I.D, and operating conditions as shown in **Fig. 3-3**.

3.3.4 Repeatability tests

The repeatability of the prepared stationary phases was assessed as shown in **Fig 3-5** below while using 100 mM NaCl. The repeatability of retention time during the separation of the inorganic anions was assessed after column preparation and after one week later. The

observation made was that the repeatability of the retention time was not very good. That is, the retention time of the assessed inorganic anions was observed to decrease with increasing analysis time, meaning that the long-term stability of the prepared stationary phase remained to be a big challenge [19]. This may have been as a result of the exposure of the click modified material to light, thus resulted to degradation of the ion exchange active sites.

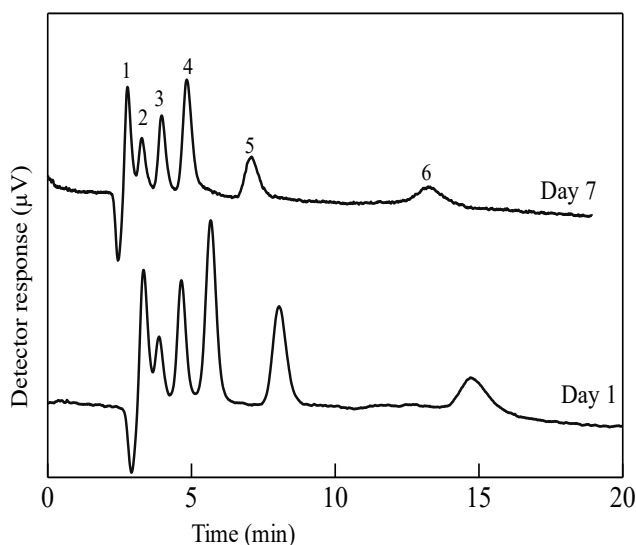


Fig 3-5. Repeatability assessment during the separation of inorganic anions on a click alkyl-azide silica bonded stationary phase column, and other operating conditions as shown in **Fig. 3-3**.

3.3.5 Column application

3.3.5.1 Gifu University laboratory tap water

Tap water was collected from Gifu university laboratory, which was then filtered through a 0.45- μm membrane filter prior to injection. The prepared column was used to assess this sample while using 70mM NaCl as eluent. Tap water was confirmed to contain both had nitrate and iodide ions as shown in **Fig. 3-6** below.

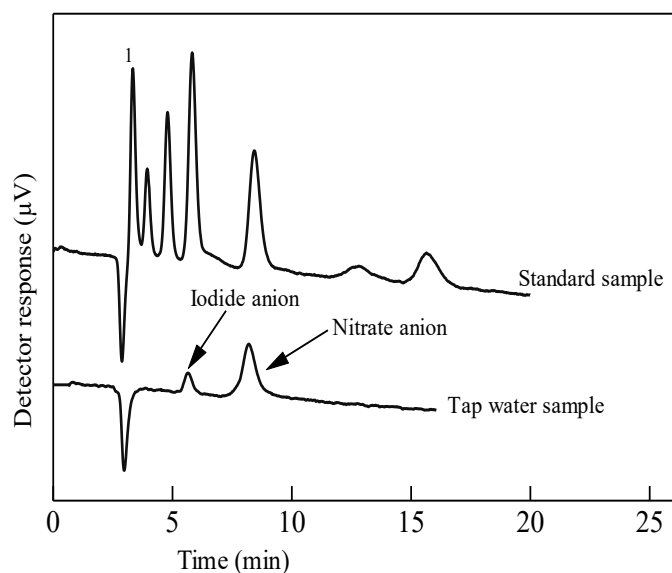


Fig 3-6.Real sample column application for tap water samples analysis using the click alkyl-azide silica bonded stationary phase column, and other operating conditions as in **Fig. 3-3**.

3.3.5.2 Saliva sample analysis

A saliva sample was collected from a healthy non-smoker, which was then filtered through a 0.45 µm membrane filter prior to injection. The inorganic anions present were examined using the prepared column where 70mM NaCl as was used as the eluent. As shown in **Fig 3-7**, this sample contained nitrate, iodide and thiocyanate anions. The saliva sample peak's retention time was observed to be slightly different when comparing it with the standard sample, due to matrix interaction; however, confirmation was done through spiking the saliva sample, and consequently peak height increment.

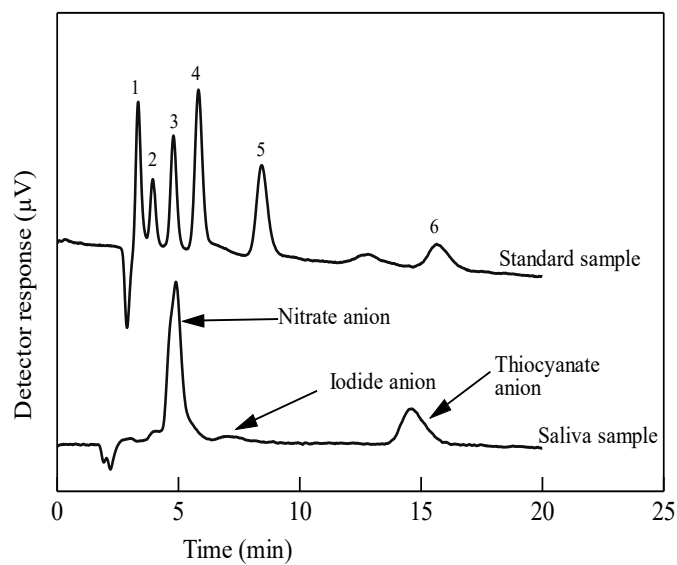


Fig 3-7.Real sample column application for saliva sample analysis using and other operating conditions as in **Fig. 3-3**.

3.4 Conclusions

This study demonstrated ions are separated using stationary phases with ionizable functional groups. The triazole ring that was formed during click reaction could be responsible for the separation of inorganic anions thus acting as ion exchange site. Separation of the inorganic anions could be achieved using 70mM NaCl as eluent. The inorganic anions retention as observed to be affected by the concentration of eluent.

3.5 References

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Chapter 4

A novel covalently bonded 4-amino butyric acid silica stationary phase for capillary ion chromatography

4.1 Introduction

Monitoring the inorganic anions' concentrations, *i.e.* bromide (Br^-), nitrate (NO_3^-), iodide (I^-) and nitrite (NO_2^-) anions in rivers, lakes or seawaters is essential for marine pollution research and environmental conservation. Concentrations of both the NO_2^- and NO_3^- anions in aquatic environmental water is responsible for algal blooms and eutrophication pollution, as nitrate is the main source of nitrogen for the phytoplankton growth in seawater. Eutrophication blocks sunlight passage to the aquatic life and its overgrowth may deplete off oxygen present in the water, creating a dead zone for living organisms [1].

On the other hand, inorganic anions, *i.e.* iodide and bromide anions are naturally occurring in aquatic environment. Chlorination of this water for drinking purposes is essential as it kills microbes and bacteria responsible for the waterborne diseases like typhoid, cholera and dysentery outbreak. I^- and Br^- anions present in water are rapidly oxidized during chlorination to hypiodous acid (HOI) and hypobromous acid (HOBr) respectively. The natural occurring organic matter reacts with these oxidants (HOBr and HOI) to form carcinogenic compounds [2].

Determination of these inorganic anions is done by ion chromatography (IC) with an ultraviolet (UV) detector. This IC analytical technique was developed in 1975 by Small and his co-workers [3].

The most commonly applied IC stationary phases are silica and polymer based. Silica based stationary phases have rich silanol groups on its surfaces, which allows easy introduction of many functionalities onto the gel. Moreover, the rich silanol groups on silica surface may

affect biological sample's separation, due to their high rigidity, fast mass transfer and resistance to organic solvents [4].

Basically, most IC stationary phases have chargeable functional groups, where the difference in repulsive forces or electrostatic attraction between the ionic analytes and the prepared stationary phase results to their separation, through ion exchange mechanism [5]. With thus, many researchers have been interested in developing various types of silica stationary phases for IC.

For instance, our group dynamically modified silica gel with a cetyltrimethylammonium which effectively separated inorganic anions. This was done by the passage of cetyltrimethylammonium ion's solution through the column [6]. Later in the research, we fabricated monolithic silica modified with cetyltrimethylammonium ion for the separation of five inorganic anions in seawater matrix while using a capillary column. In order to stabilize the anions' retention time, cetyltrimethylammonium chloride was added as a modifier for the mobile phase. In their research findings, nitrate anion had a retention factor (K) of 3.2 and a plate height (H) of 87 μm while using 500 mmol/L sodium chloride with 0.1 mmol/L of cetyltrimethylammonium chloride (CTAC) as the mobile phase [7]. They made further advancement by modifying the monolithic silica with dilauryldimethylammonium bromide (DDAB). This was observed to have higher stability in anions' retention in comparison to CTAC modified monolithic silica, which thus allowed for bromide ion's determination in seawater without tedious pretreatments [8].

Additionally, Sun and his co-workers [9] effectively modified silica with benzimidazole, and the stationary phase was evaluated as reversed phase and anion exchange mixed mode. Moreover, imidazole [10], poly (1-allylimidazole) [11], phenyl amine [12], 8-quinolinol [13],

pyridine [14], dipyridine [15], phenylpropanolamine [16] modified silicas have been investigated as potential mixed mode stationary phase liquid chromatography. The prepared columns had multi-interactions including π - π stacking, electrostatic forces, hydrogen bonding, hydrophobic and anion-exchange interactions. Thus different analytes including phthalates, phenols, organic and inorganic anions, poly aromatic hydrocarbons and anilines were effectively separated.

Nesterenko and his group effectively prepared zwitterionic stationary phases with different amino acids like valine, glutamine, asparagine, lysine and tryosine by first linking 3-glycidyloxypropyltrimethoxysilane (GPTMS) on silica surface, which was then followed by an epoxy ring attack using the targeted amino acid [17-20].

The purpose of this study was to investigate the retention of anions on a chemically bonded 4-amino butyric acid (ABA) onto the surface of silica gel. A simple 2-step reaction was followed in order to effectively synthesize the material. Selection of a suitable eluent resulted to the retention and separation of anions and thus, it worked as the anion-exchange stationary phase.

4.2 Experimental

4.2.1 Apparatus

A capillary LC system was used to carry out the chromatographic measurements. It comprised of a L.TEX-8301 micro-feeder fitted with a 0.5mL gas-tight syringe as a pump, an injector with an injection sample volume of 0.2 μ L-a M435 microinjection valve (Upchurch Scientific, Oak Harbor, WA, USA), and a JASCO, Tokyo, Japan, UV detector (UV-970) fitted with a flow cell capillary (75 μ m; JASCO) attached. The pump flow rate was kept at 1.0 μ L min⁻¹, and the detector was operated at 210 nm. CDS-Lite ver 5.0 data processor (LAssoft, Nagareyama, Chiba, Japan) was used to acquire all the data. A centrifuge (IEC610-2-020, Kubota, Tokyo, Japan) was used for centrifuging the mixture solution during the cleaning off the un-reacted residue on the prepared stationary phase.

4.2.2 Reagents

All the chemicals used were of high quality. Sodium chloride, hydrochloric acid (HCl), 4-aminobutyric acid (ABA) and 3-isocyanatopropyl-triethoxysilane (ICPT) were acquired from Wako Pure Chemical Manufacturers (Osaka, Japan) while Kanto Chemical (Tokyo) provided methanol and acetonitrile. A simple UV water purifier system produced the ultrapure water which was used to prepare all solutions used in this study. Silica gel (3 μm) was used as the stationary phase's base materials (Chemical Evaluation and Research Institute, Tokyo, Japan). The silica gel was first dried at 120°C for 5 h before use while toluene was dried using molecular sieves 3A (1/16" pellets) for *ca.* 5 h, and a reflux equipment was used as the reaction vessel.

4.2.3 Preparation of the stationary phase

The ABA stationary phase (**Fig 4-1**) was prepared following a method by previous researchers [21-22] with slight modification. Dried porous silica gel (0.2 g) was placed in the reaction reflux equipment, and 1.2 mL dry toluene together with 0.1 mL of ICPT was added to the vessel. The reaction was carried out at 110°C with continuous stirring for 18 h. The ICPT-bonded silica gel was washed with methanol and was then dried at 70°C for 6 h. The reacted silica gel was reacted with ABA at 90°C for 24 h, and was finally washed twice with ethanol and water as shown in **Fig 4-1**. The synthesized material was then packed into the capillary column, while using ethanol as propulsion solvent. HCl (30 mM) was then passed through the packed column for 30 minutes.

The ABA stationary phase, **Fig. 4-1(a)**, was also prepared following a method by previous report [23] with slight modification. Silica gel was first dried at 120° C for 5 h before use while toluene was dried using molecular sieves 3A (1/16" pellets) for *ca.* 5 h, and a reflux equipment was used as the reaction vessel. Briefly, dried porous silica gel (0.1 g), 4-aminobutyric acid (0.1 g), 0.1 mL of 3-isocyanopropyltriethoxysilane, ethanol (0.3 ml) and water (0.5 mL) were all mixed together in reflux equipment. The reaction was refluxed with continuous stirring for 24 h

at 90° C. The reaction was later cooled to room temperature, filtered and the obtained material was washed three times with toluene and ethanol consequently. The obtained synthesized material was packed into the capillary column and ethanol was used as the propulsion solvent. Hydrochloric acid (30 mM) was passed through the packed column for 30 minutes. The prepared column also gave satisfactory results. Moreover, another possible reaction mechanism route was also proposed to guarantee similar results as shown in **Fig 4-1(b)**.

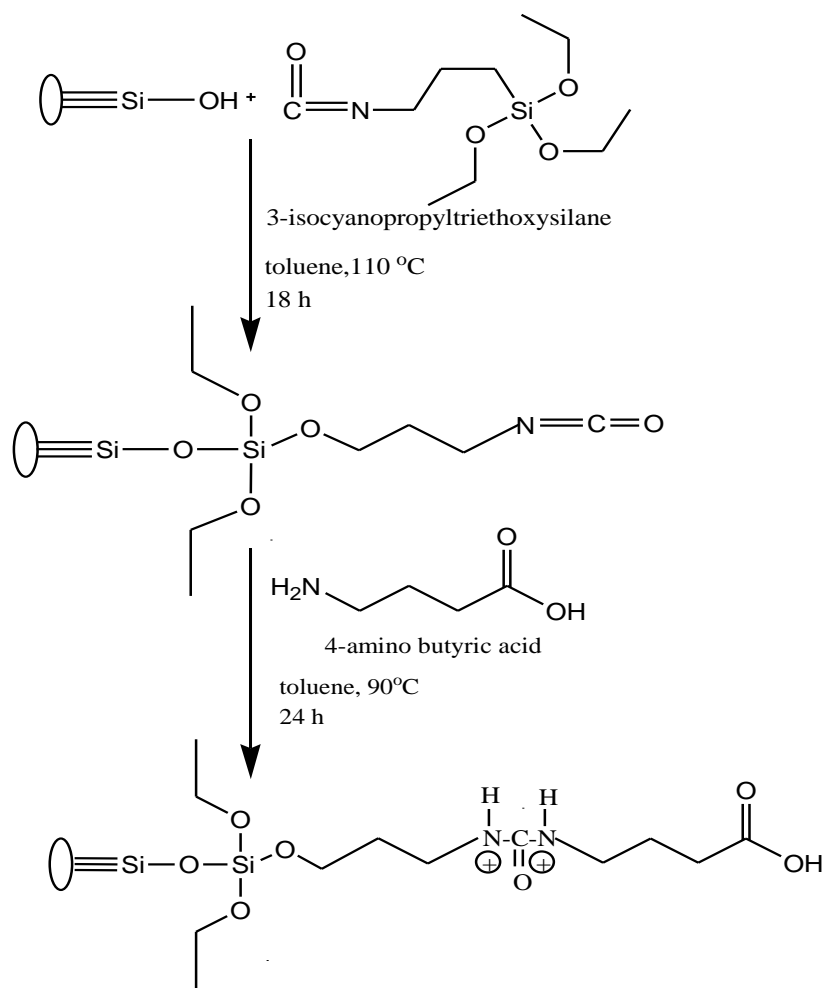


Fig. 4-1. The proposed reaction mechanism for 4-aminobutanoic acid-modified column

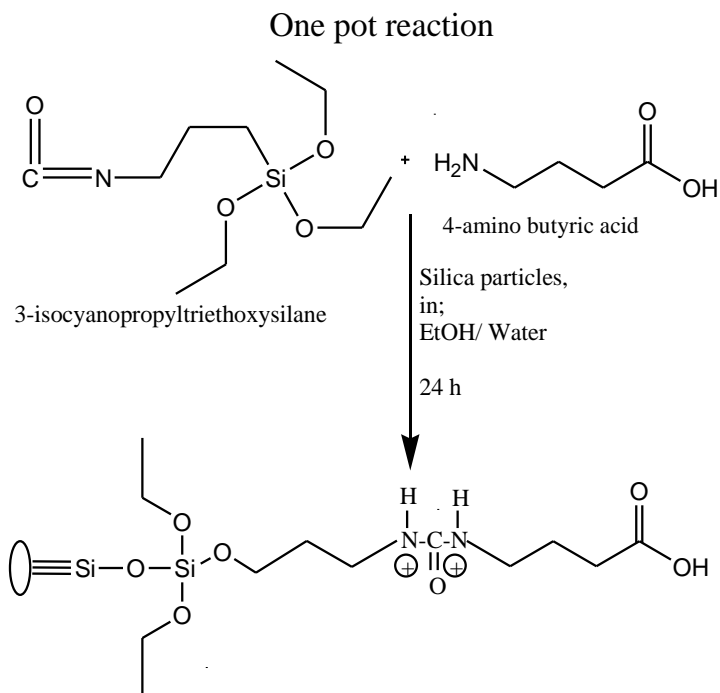


Fig. 4-1 (a). Possible reaction mechanism for 4-aminobutanoic acid-modified column

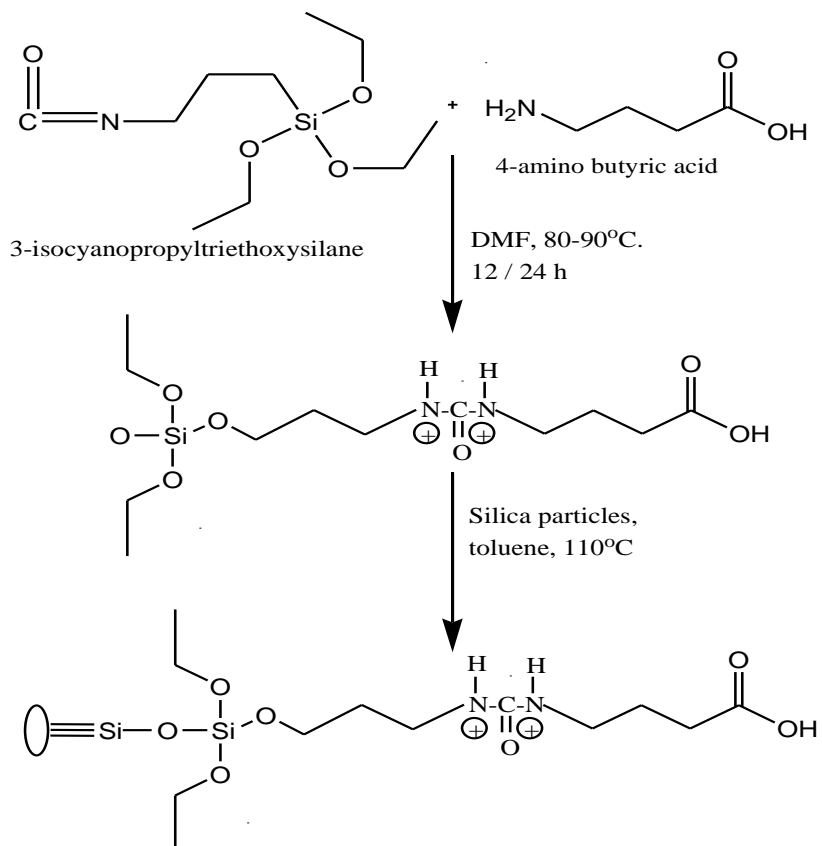


Fig. 4-1 (b). Possible reaction mechanism for 4-aminobutanoic acid-modified column

4.3 Results and discussions

4.3.1 Inorganic anion's retention behavior

The prepared ABA-silica bonded stationary phase was achieved through the reaction of ICPT with 4-amino butyric acid which guaranteed the covalent incorporation of 4-amino butyric acid onto the silica gel to yield a secondary amine. The surface was able to effectively retain and separate the inorganic anions within reasonable time. It was suggested that the expected mode of inorganic anions separation was due to the primary amine group's protonation, on the ABA modified silica surface, which may have acted as cationic site interacting with the inorganic anions through electrostatic interaction, for the effective separation. On this synthesized stationary phase, the inorganic anions were separated through the ion-exchange mode with an elution order of iodate, bromate, bromide, nitrate, iodide, and thiocyanate. This elution order was observed to be similar to that of common IC while using 80 mM sodium chloride in 0.5 mM HCl as the eluent while subjecting a flow rate of 1 $\mu\text{L}/\text{min}$, as shown in **Fig. 4-2**.

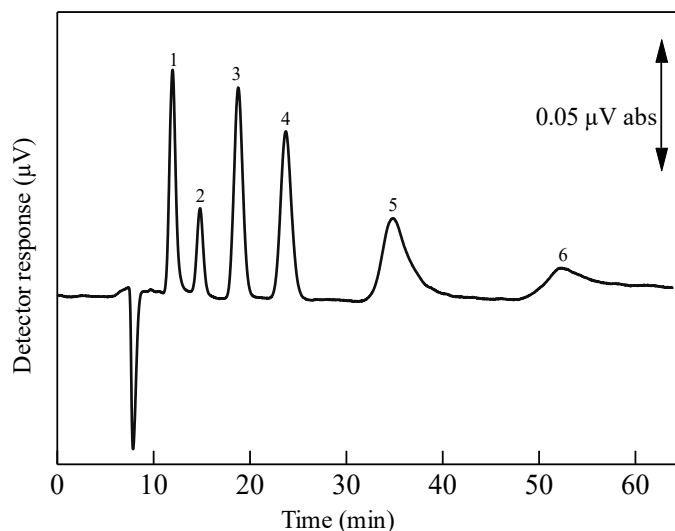


Fig. 4-2. Separation of inorganic anions on a ABA modified silica column, (column 100 x 0.32 mm I.D), while using sample mixture of (1) IO_3^- , (2) BrO_3^- , (3) Br^- , (4) NO_3^- , (5) I^- , and (6) SCN^- at a concentration of 1mM each and 0.5mM NO_3^- , an

eluent's concentration of 80 mM NaCl in 0.5 mM HCl as the eluent, and at a flow rate of 1.0 $\mu\text{L}/\text{min}$, while using UV wavelength of 210 nm

4.3.2. The effect of eluent concentration

Salt concentration effect on the analyte anions' retention was investigated using sodium chloride as the eluent as shown in **Fig.4-3**. As expected, the analyte anions' retention on the column decreased with increased eluent's concentration. This is so because, with the increased eluent's concentration, the eluting strength also increased [22-23].

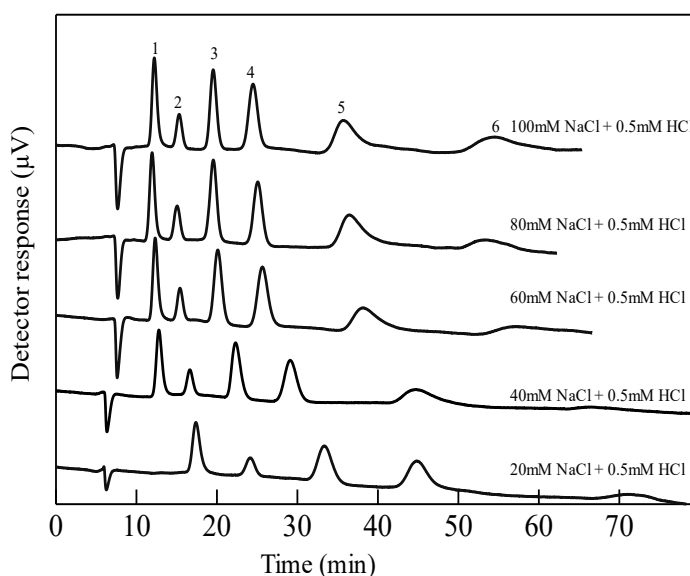


Fig.4-3. Separation of inorganic anions using different eluent concentration on an ABA-bonded silica, and other operating conditions as shown in **Fig. 4-2**.

4.3.3 Repeatability studies

The repeatability of the prepared stationary phases was assessed as shown below using 40 mM NaCl + 0.5 mM HCl, 60 mM NaCl + 0.5 mM HCl and 80 mM NaCl + 0.5 mM HCl (**Fig. 4-4. to 4-6.**) with six, four and three continuing chromatographic runs respectively. The relative standard deviation (RSD) values of the analytes retention time were 1.42, 1.66, 1.43, 1.10, 0.94, and 1.72% respectively while using 40 mM NaCl + 0.5 mM HCl (**Table 4-1**). When 60 mM

NaCl + 0.5 mM HCl and 80 mM NaCl + 0.5 mM HCl were used as the mobile phases, RSD values were between 0.57-1.75% and 0.12-2.76%, as can be seen in **Table 4-1.** and **4-2.**, respectively. Good repeatability with RSD value less than 2% in almost all repeatability assessments was observed with some peak signal being relatively sharp.

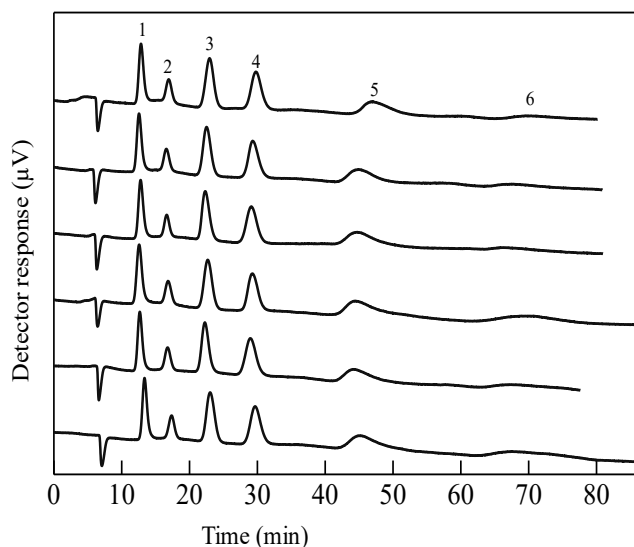


Fig.4-4. Repeatability assessment during the separation of inorganic anions on an ABA-bonded silica, while using 40 mM NaCl + 0.5 mM HCl as eluent, and operating conditions as in **Fig. 4-2.**

Table 4-1. RSD values of the separation of inorganic anions on an ABA-bonded silica while using 40 mM NaCl + 0.5 mM HCl as eluent, operating conditions as in **Fig. 4-4.**

Anion	Average	S.D	RSD (%)
IO ₃ ⁻	12.6858	0.1797	1.42
BrO ₃ ⁻	16.8515	0.2798	1.66
Br ⁻	22.6305	0.3238	1.43
NO ₃ ⁻	29.3485	0.3233	1.10
I ⁻	44.6153	0.4184	0.94
SCN ⁻	68.6892	1.1814	1.72

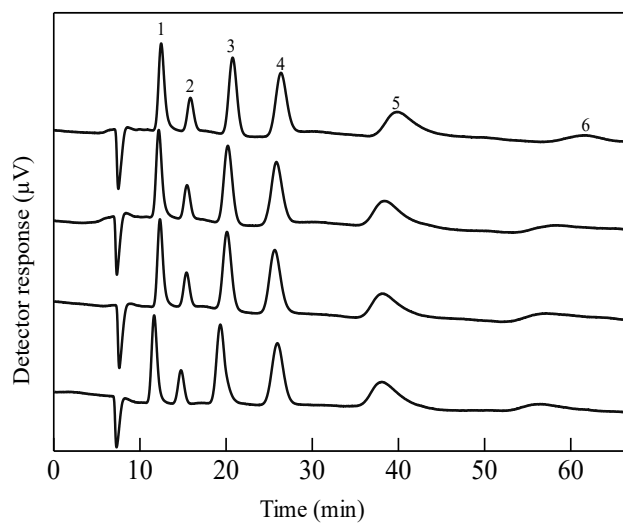


Fig.4-5. Repeatability assessment during the separation of inorganic anions on an ABA-bonded silica, while using 60 mM NaCl + 0.5 mM HCl as eluent, and other operating conditions as shown in **Fig. 4-2**.

Table 4-2. RSD values of the separation of inorganic anions on an ABA-bonded silica while using 40 mM NaCl + 0.5 mM HCl as eluent, operating conditions as in **Fig. 4-5**.

Anion	Average	S.D	RSD (%)
IO_3^-	12.3280	0.1407	1.14
BrO_3^-	15.5800	0.2468	1.58
Br^-	20.3577	0.3567	1.75
NO_3^-	25.8153	0.1540	0.57
I^-	38.1637	0.1120	0.29
SCN^-	57.2213	0.6834	1.19

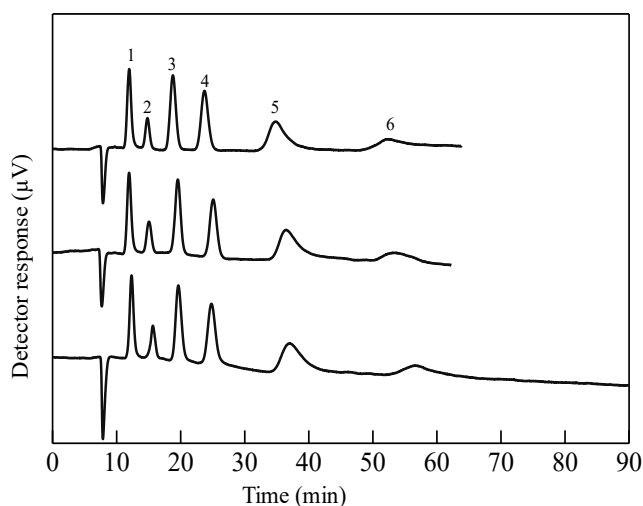


Fig.4-6. Repeatability assessment during the separation of inorganic anions on an ABA-bonded silica, column 100 x 0.32 mm I.D, while using 80 mM NaCl + 0.5 mM HCl as eluent, operating conditions as in **Fig. 4-2**.

Table 4-3. RSD values of the separation of inorganic anions on an ABA-bonded silica while using 80 mM NaCl + 0.5 mM HCl as eluent, operating conditions as in **Fig. 4-6**.

Anion	Average	S.D	RSD (%)
IO_3^-	11.9540	0.0141	0.12
BrO_3^-	15.3555	0.4250	2.76
Br^-	19.5915	0.0583	0.29
NO_3^-	24.936	0.1980	0.79
I^-	36.706	0.4186	1.14
SCN^-	52.906	0.8457	1.59

4.3.4 Effect of eluent cation

The effect of different chloride solutions *i.e.* NaCl (a), KCl (b), LiCl (c), RbCl (d), CsCl (e) at 20 mM + 0.5 mM HCl each and 10 mM CaCl_2 + 0.5 mM HCl (f) were used as eluents in order to investigate their effect on anions' retention as shown in **Fig. 4-7**. The retention time was observed not to be affected by these eluent cations.

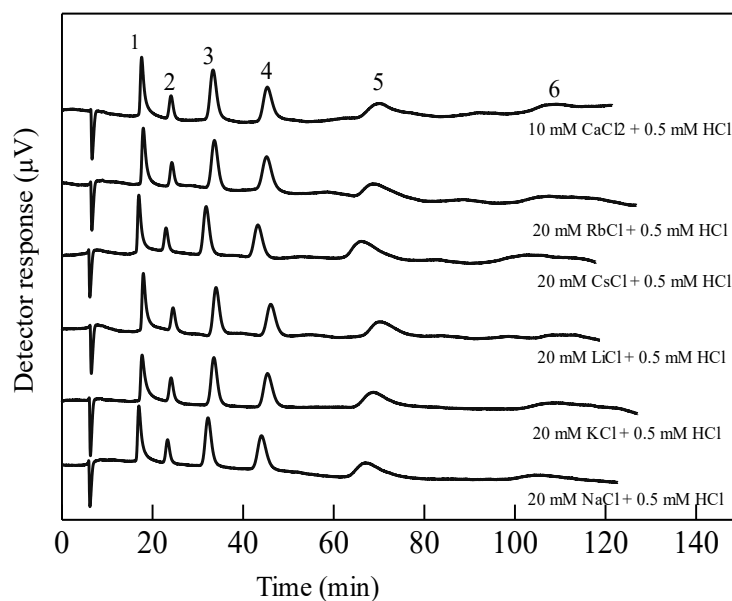


Fig.4-7.Evaluation of inorganic anions at different eluent cations on an ABA-bonded silica, other operating conditions as shown in **Fig. 4-2**.

4.3.5 Effect of pH

In order to investigate the effect of pH on inorganic anions' retention as shown in **Fig 4-8**, the prepared column was again evaluated. The procedure was carried out while using 40 mM NaCl solutions as the mobile phase whose pH value had been adjusted to; 2.49, 3.59, 3.98, 5.55 and 6.96. Below, **Fig. 4-8** demonstrates the separation of iodate, bromate, bromide, nitrate and iodide on the prepared column at pH value close to neutral (pH 6.96) and at acidic eluent conditions (pH 2.49, 3.59, 3.98 and 5.55). The anions were more retained in the column under acidic condition, while they were less retained on the stationary phase under neutral condition. In addition, the elution order was iodate, bromate, bromide, nitrate, iodide and thiocyanate ions. This observation shows that the 4-amino butyric acid functionality and the terminal weak acid group produced a variable capacity anion exchanger. 4-Amino butyric acid is a weakly basic amino acid with the pK_a values at 25°C of 4.01. This means that the ABA stationary phase

employed in this work was protonated at a lower pH, while it had no charge at a neutral or higher pH. Thus, the prepared stationary phase worked as an anion-exchange stationary phase at a lower pH [9, 17].

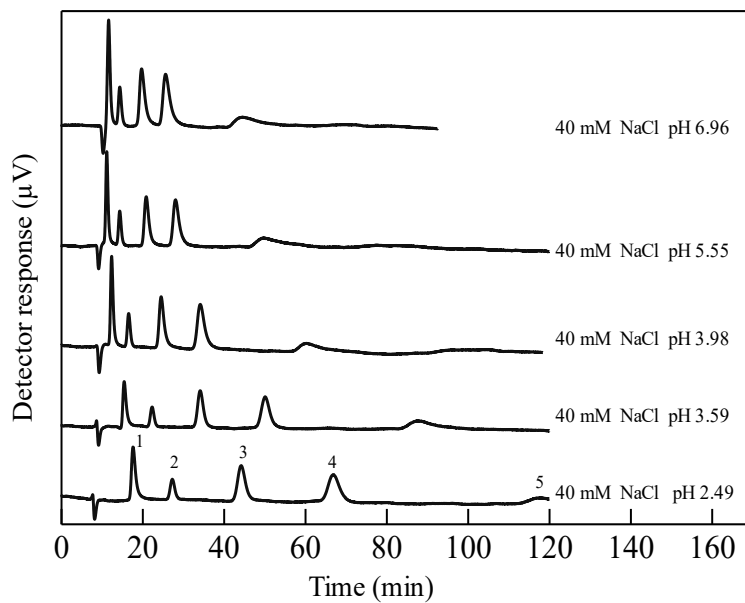


Fig.4-8.Evaluation of inorganic anions at various eluent pH on an ABA-bonded silica while using 40 mM NaCl as eluent, other operating conditions as in **Fig. 4-2**.

4.3.6 Column application

Water samples; Gifu university laboratory tap water (**Fig 4-9**), Chubu Centrair Japan Airport water (**Fig 4-10**) and Shinmaiko seawater (**Fig 4-11**) were first collected. These samples were filtered through a 0.45 µm membrane filter prior to injection. The ABA column was used for the assessment these water samples while using 120mM NaCl + 0.5mM HCl as eluent. Tap water had nitrate ions as confirmed through increase in peak height after spiking the sample with 0.5 mM nitrate ion (0.25 ml). On the other hand, Central Japan Airport waters and Shinmaiko seawaters were confirmed to contain both the bromide and nitrate ions.

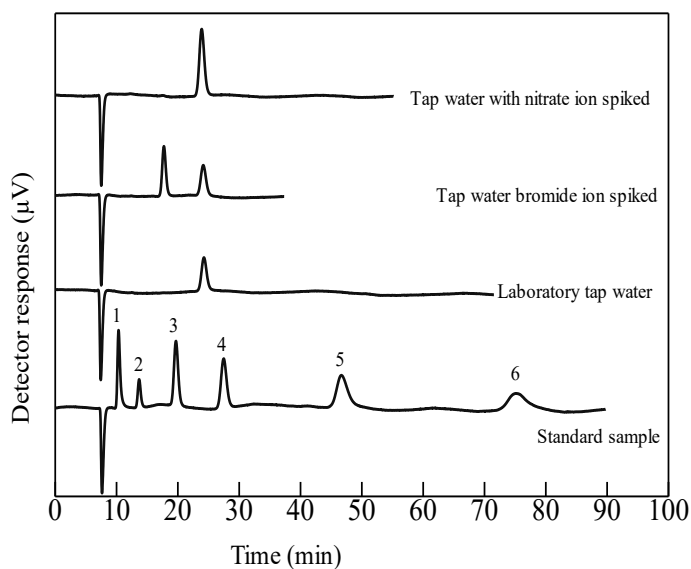


Fig.4-9.Evaluation of inorganic anions in laboratory tap water on an ABA-bonded silica while using 120 mM NaCl + 0.5 mM HCl as eluent, other operating conditions as shown in **Fig. 4-2**.

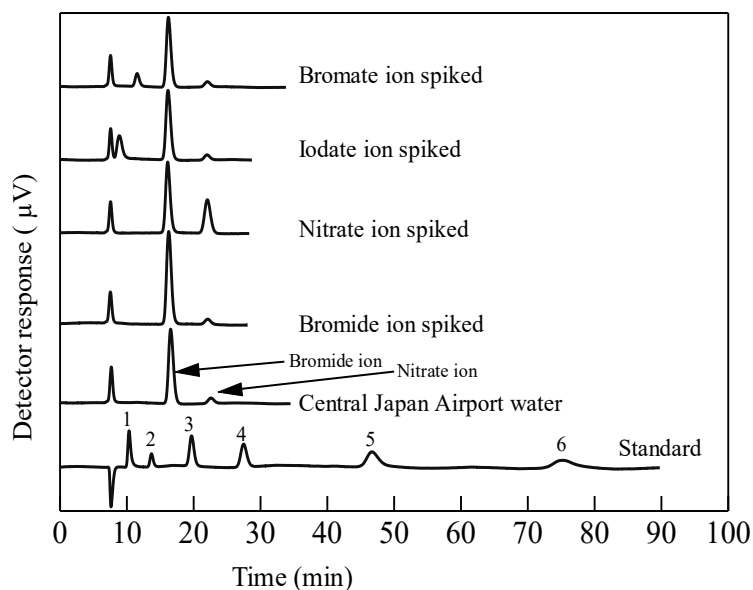


Fig .4-10.Evaluation of inorganic anions in Central Japan Airport water on an ABA-bonded silica while using 120 mM NaCl + 0.5 mM HCl as eluent, other operating conditions as shown in **Fig. 4-2**.

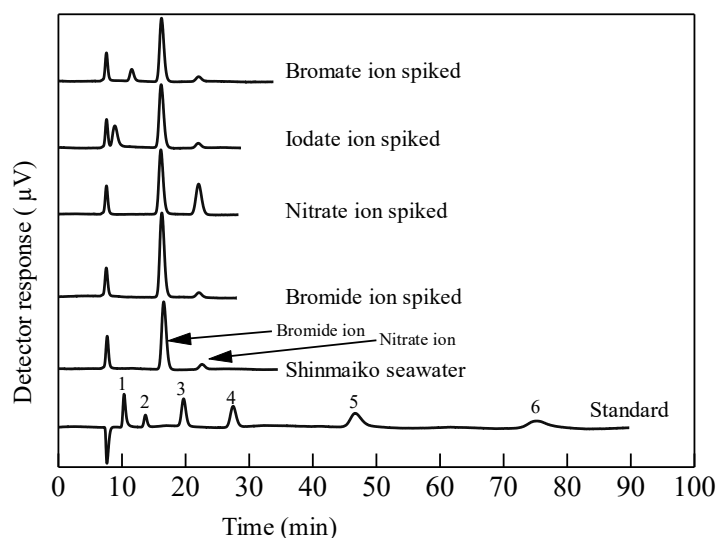


Fig.4-11.Evaluation of inorganic anions in Shinmaiko sea water on an ABA-bonded silica while using 120 mM NaCl + 0.5 mM HCl as eluent, other operating conditions as shown in **Fig. 4-2**.

4.4 Conclusions

This study demonstrated that ions are usually separated using stationary phases with charged or chargeable functional groups in LC. ABA bonded stationary phase provided the anion-exchange site which allowed us to separate of inorganic anions and as it was expected, ion exchange mechanism was involved in the retention of these analyte anions. The retention time of the analyte anions was carefully controlled by a good selection of the eluent. The eluent concentration greatly affected the analyte anions' retention on the synthesized stationary phase.

4.5 References

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Chapter 5

A novel covalently bonded 7-amino heptanoic acid silica stationary phase for capillary ion chromatography

5.1 Introduction

Capillary columns are at the core of chromatographic separation techniques. According to a review by Lim [1], these columns were first invented for gas chromatography (GC), but due to the majority of the target compounds being neither volatile nor stable to be separated on a GC system, a vision was therefore realized to use glass beads packed columns for liquid chromatography (LC). LC was invented in 1967, and it is generally attributed to Horvath and co-workers [2]. Since then, an era of LC systems was then developed. The majority of these columns are still packed columns, with monolithic columns being the best alternatives [3].

Packed capillary columns are made by slurry packing of the silica materials under pressure with particles $< 15\mu\text{m}$ in diameter. On the other hand, open-tubular capillary columns are fused-silica capillaries with an inner diameter in the range of $< 100\ \mu\text{m}$ while monolithic column consists of tailored polymer matrix through photo or thermally initiated polymerization of monomers with the desired functional groups or through sol-gel processes [4].

Packed capillary columns for ion exchange chromatography (IEC) were invented by Small and his coworkers [5], are probably the most frequently used chromatographic media for the identification and separation of ionic samples, mainly, inorganic anions and cations, peptides and carbohydrates. Since then, this technique has been applied to solve a wide range of analytical problems, such determination of the composition of aqueous samples and as quality control. Ion exchangers with silica as the base material still dominates as the best stationary phases due to

its good mechanical strength, easy modification processes and excellent chromatography performance [6].

These IEC stationary phases are being utilized owing to their charged moieties. Cyanuric chloride quaternary ammonium salt was linked covalently onto silica surface, forming a strong anion exchanger for the separation of single charged inorganic anions while using tartaric acid as eluent by Lin and his group [7].

Additionally, a methyl-capped poly (ethylene oxide) bonded silica gel was developed by our group and the stationary phase was evaluated for the inorganic anions' effective separation, with the retention behavior examined to be ion exchange mechanism [8]. Later, we observed that a pyridine stationary phase could also be used for ion-exchange separation of inorganic anions under acidic condition. The protonated pyridine group acted as an effective ion exchange site when operated in acidic condition [9].

Basically, ionically charged functional groups; negative or positive charged sites, are attached to the stationary phase and the retention mechanism is ion exchange [10-12]. The eluent cation must be sufficiently attracted to the ion-exchange stationary phase, and at a very low eluent concentration, in order to effectively separate the sample anions [7]. That is, due to the differences in the electrostatic interactions, the ionic analytes are separated prior to moving into the detector [13].

In accordance to previous research findings [14], hydrophilic interaction liquid chromatography (HILIC) columns with zwitterionic functional groups could also simultaneously separate anions and cations in active pharmaceutical components while using acetonitrile-ammonium formate buffer as the mobile phase. On the other hand, Arai and coworkers [15]

demonstrated that diol-columns could be used to achieve excellent separation of anions using acidic eluent with presence of H^+ being very critical.

Zwitterionic stationary phases are mainly prepared by using amino acids as they have both an acid (carboxylic acid, *i.e.* negative charge) and basic (amine; *i.e.* positive charge) functional groups within the same molecule [16]. The opposite charge on groups within a molecule is based upon the location and the pK_a at neutral pH, and thus the prepared stationary phase could have cation, anion or zwitterions exchange properties, depending on the structure of the amino acids [6]. In aqueous solutions, amino acids acts as weak acids and bases, *i.e.* the carboxylic acid functionality is the dominant form at low pH and they exist in equilibrium between the neutral molecules and the zwitterionic form at intermediate pH [17].

This led Nesterenko and coworkers to investigate some amino acid functionalized silica based ion exchangers by immobilizing amino acids with different structures *i.e.* asparagine, valine, arginine, lysine, glutamine and tryrosine, on the surface of a monolithic silica column, on which, according to their research findings, these phases they exhibited zwitterionic properties. They prepared the phase by first covalently linking GPTMS onto silica surface, which was immediately followed by the attack of the epoxy ring by the amino acid of interest [18-20].

The purpose of this study was to investigate the retention of anions on a silica gel immobilized with 7-amino heptanoic acid. Two simple step reactions were followed. Selection of a suitable mobile phase resulted to the separation of targeted anions, and the prepared column worked as the anion-exchange stationary phase.

5.2 Experimental

5.2.1 Reagents

High quality reagents were used in this study. Hydrochloric acid (HCl), 7-amino heptanoic acid (AHA) and sodium chloride were purchased from Wako Pure Chemical Manufacturers (Osaka,

Japan) and used as obtained. All solutions used were prepared using ultra-pure water using a simple UV water purifier system. Silica gel (3 μm) obtained from Chemical Evaluation and Research Institute, Tokyo, Japan, was first dried at 120°C for 5 h before use and used as the base material.

5.2.2 Apparatus

A capillary LC system comprised of a L.TEX-8301 micro-feeder and fitted with a 0.5mL gas-tight syringe was used as a pump. An M435 microinjection valve (Upchurch Scientific, Oak Harbor, WA, USA) as an injector with an injection sample volume of 0.2 μL -a M435 microinjection valve, and a JASCO, Tokyo, Japan, UV detector (UV-970) fitted with a flow cell capillary (75 μm ; JASCO) were also attached. The pump flow-rate was kept at 2.5 $\mu\text{L min}^{-1}$, and the detector was operated at 210 nm. All the data was acquired using a CDS-Lite ver 5.0 data processor (LAssoft, Nagareyama, Chiba, Japan). The modified silica was washed and separated using a centrifuge (IEC610-2-020, Kubota, Tokyo, Japan).

5.2.3 Preparation of stationary phases

The AHA stationary phase was prepared following a method by previous researchers [21-23] with slight modification. Dried porous silica gel (0.2g) was placed in the reaction reflux equipment, and 1.2 mL dry toluene together with 0.1 mL of 3-chloropropyltrimethoxysilane were added to the vessel. The reaction was refluxed with continuous stirring for 24 h. The reaction was later cooled to room temperature, filtered and the obtained material was washed two times with toluene and ethanol consequently. The obtained chloropropyl silica (ChprSil) was dried using an oven at 65°C for 8h. The chloro group of the ChprSil was then reacted with AHA at 90°C for 24 h, and was finally washed twice with ethanol and water as shown as shown in **Fig 5-1**. The obtained synthesized material was packed into the capillary column and ethanol was used

as the propulsion solvent. Hydrochloric acid (30 mM) was passed through the packed column for 30 minutes.

The AHA stationary phase, **Fig. 5-1(a)**, was also prepared following a method by a previous report [24] with slight modification. Silica gel was first dried at 120° C for 5 h before use while toluene was dried using molecular sieves 3A (1/16" pellets) for *ca.* 5 h, and a reflux equipment was used as the reaction vessel. Briefly, dried porous silica gel (0.1 g), 7-aminoheptanoic acid (0.1 g), 0.1 mL of 3-chloropropyltrimethoxysilane, ethanol (0.3ml) and water (0.5 ml) were all mixed together in reflux equipment. The reaction was refluxed with continuous stirring for 24 h at 90° C. The reaction was later cooled to room temperature, filtered and the obtained material was washed three times with toluene and ethanol consequently. The obtained synthesized material was packed into the capillary column and ethanol was used as the propulsion solvent. Hydrochloric acid (30 mM) was passed through the packed column for 30 minutes. The prepared column also gave satisfactory results. Moreover, another possible reaction mechanism route was also proposed to guarantee similar results as shown in **Fig 5-1(b)**.

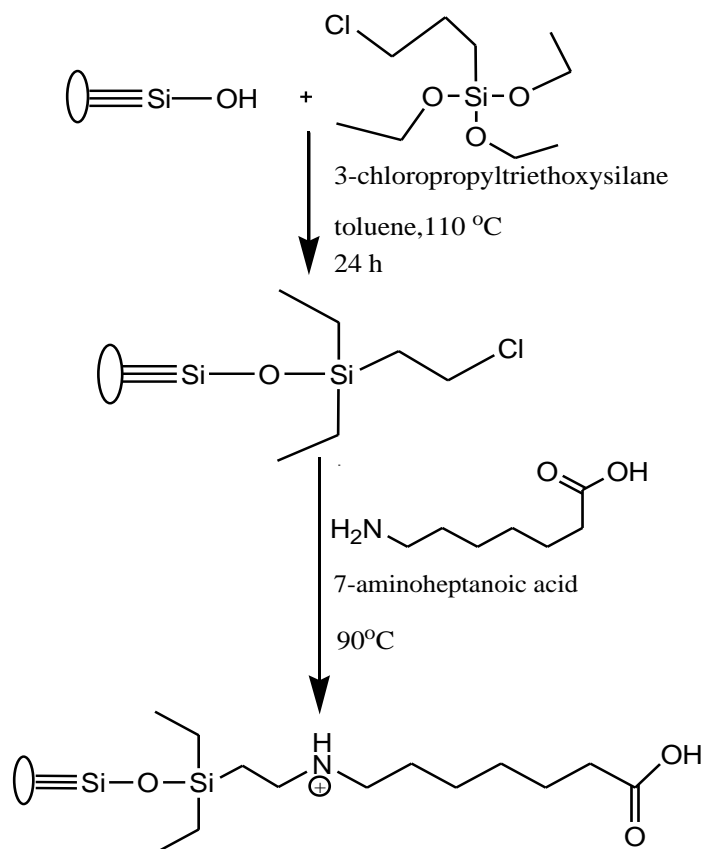


Fig. 5-1. Reaction for 7-aminoheptanoic acid-modified column

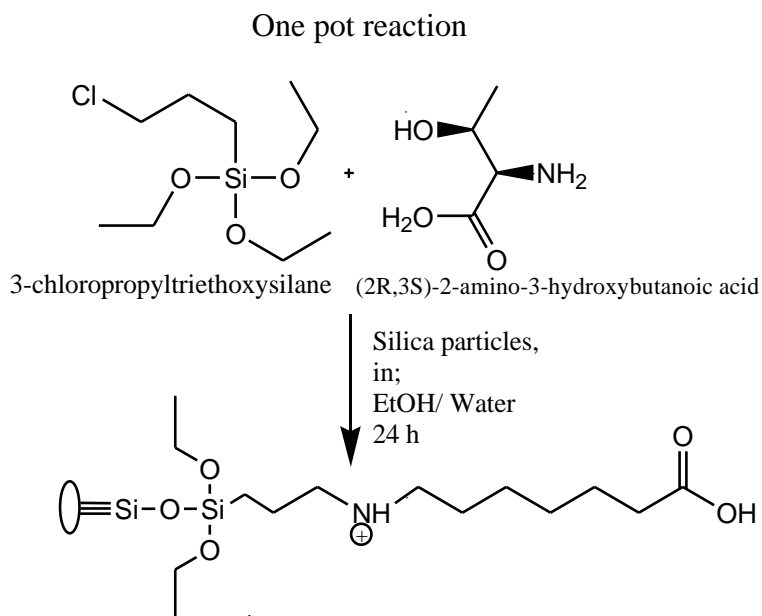


Fig. 5-1 (a). Possible reaction mechanism for 7-aminoheptanoic acid-modified column

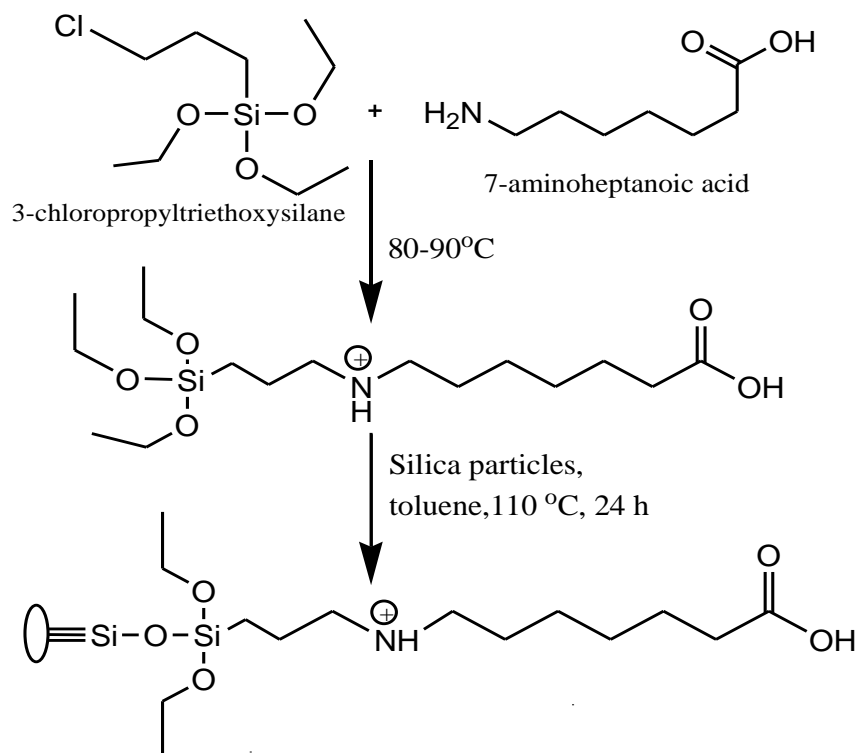


Fig. 5-1 (b). Possible reaction mechanism for 7-aminoheptanoic acid-modified column

5.3 Results and discussions

5.3.1 Retention behavior

The prepared stationary phase was able to effectively retain and separate the inorganic anions. It was suggested that the expected mode of inorganic anions separation was due to the secondary amine group's protonation, on the AHA modified silica surface, which may have acted as cationic site interacting with the inorganic anions through electrostatic interaction, for the effective separation. The elution order was in the order of iodate, bromate, nitrite, bromide, nitrate and iodide and the retention mechanism was deduced to be ion-exchange mechanism, while using 60 mM sodium chloride. This elution order was observed to be similar to that of common IC as shown in **Fig. 5-2**.

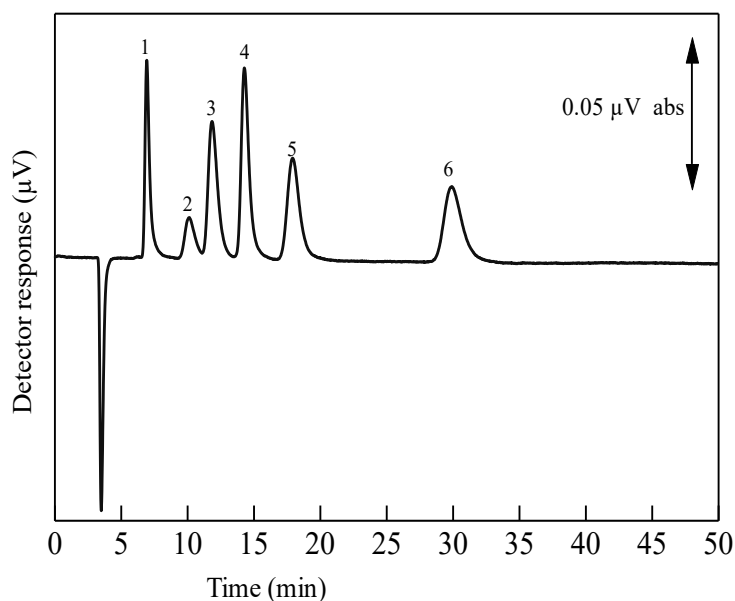


Fig. 5-2. Separation of inorganic anions on a AHA modified silica column, (column 100 x 0.32 mm I.D), while using a sample mixture of (1) IO_3^- , (2) BrO_3^- , (3) NO_2^- (4) Br^- , (5) NO_3^- , (6) I^- , at a concentration of 1mM each and 0.5mM NO_3^- , and at an eluent's concentration of 60 mM NaCl as the eluent, and a flow-rate of 2.5 $\mu\text{L}/\text{min}$, while using a UVdetection wavelength of 210 nm

5.3.2 The effect of eluent concentration

The six anions used in this study were observed to be affected by the salt concentration as shown in **Fig 5-3**. As the NaCl concentration was increased from 20mM NaCl to 120mM NaCl, the anions' retention was observed to be decreased. Thus, it was understood that with an increase in elution strength, the anions' retention was decreased [21-23].

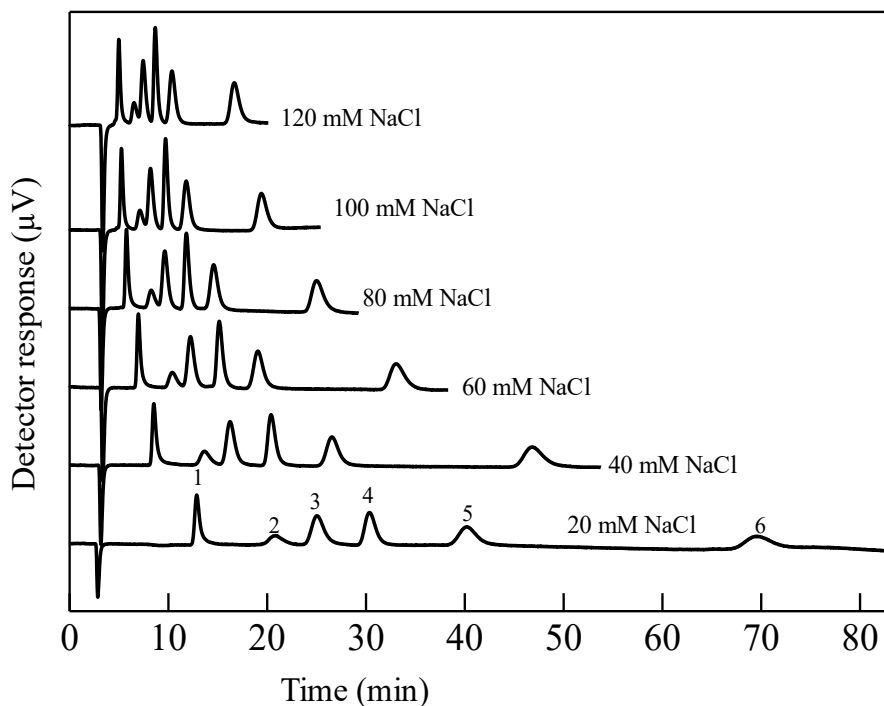


Fig. 5-3. The effect of various eluent's concentrations on analytes' retention with sample mixture as shown in **Fig 5-2**, with a flow-rate of $2.5\mu\text{L}/\text{min}$ and UV detection wavelength of 210nm , on the prepared column

5.3.3 Repeatability tests

The prepared column's repeatability was assessed as shown in **Fig 5-4.**, **Fig 5-5.**, **Fig 5-6.**, **Fig 5-7.** and **Fig 5-8** using 40mM NaCl , 60mM NaCl , 80mM NaCl , 100mM NaCl and 120mM NaCl , respectively. With repeated chromatographic runs ($n = 4-6$) relatively sharp peaks with excellent repeatability having relative standard deviation (RSD) value of $<2\%$ for anions' retention time as shown in **Tables 5-1**, **5-2**, **5-3**, **5-4** and **5-5** was obtained.

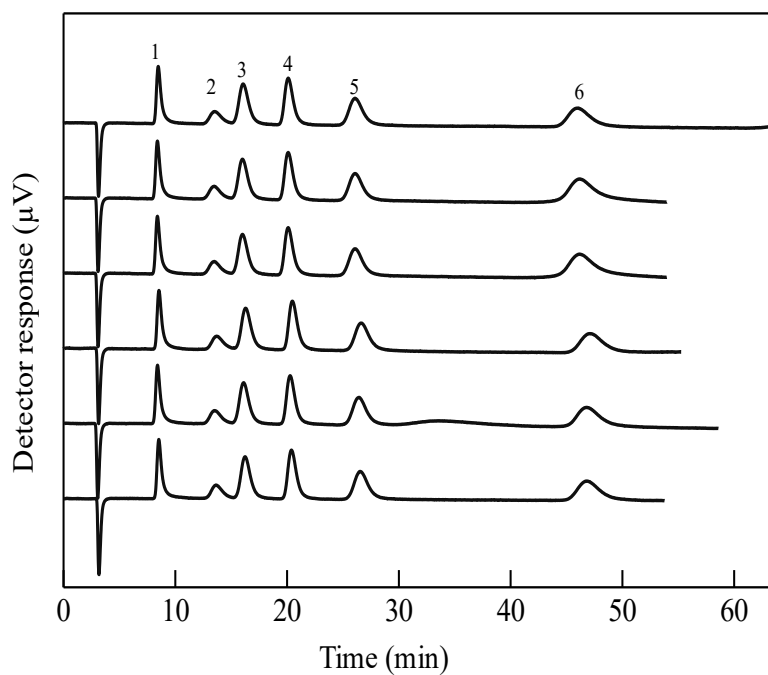


Fig. 5-4. Repeatability tests for the separation of inorganic anions with sample mixture as shown in **Fig. 2.1** Eluent's concentration used was 40mM NaCl with a flow-rate of 2.5 μ L/min and at a UV detection wavelength of 210 nm

Table 5.1. RSD values of the retention time during the separation of inorganic anions on an AHA-bonded silica while using 40 mM NaCl as eluent, and other operating conditions as in **Fig. 5-4**.

Anion	Average	S.D	RSD (%)
IO ₃ ⁻	8.514	0.0971	1.14
BrO ₃ ⁻	13.69	0.242	1.76
Br ⁻	16.23	0.252	1.54
NO ₃ ⁻	20.40	0.327	1.60
I ⁻	26.57	0.521	1.96
SCN ⁻	46.95	0.739	1.57

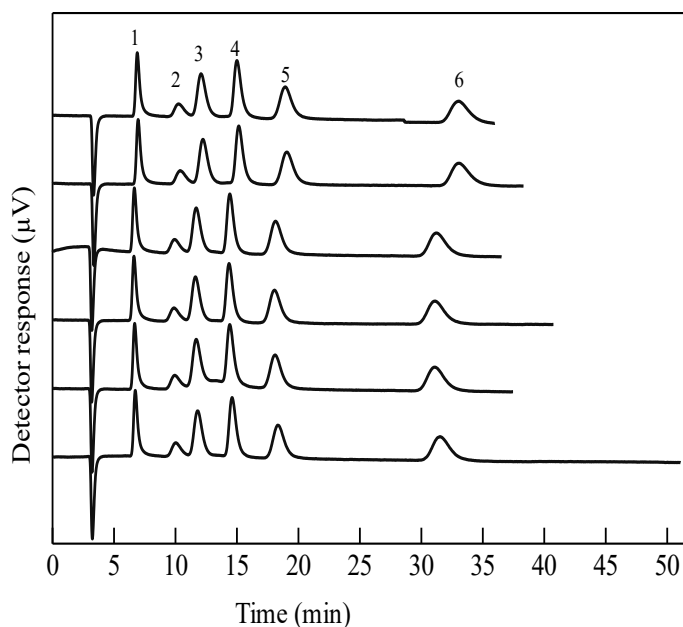


Fig. 5-5. Repeatability tests for the separation of inorganic anions using eluent's concentration of 60mM NaCl with sample mixture and operating conditions as shown in **Fig. 5-1**.

Table 5-2. RSD values of the retention time during separation of inorganic anions on an AHA-bonded silica while using 60 mM NaCl as eluent, other operating conditions as in **Fig. 5-5**.

Anion	Average	S.D	RSD (%)
IO_3^-	6.745	0.0750	1.11
BrO_3^-	10.06	0.192	1.91
Br^-	11.84	0.234	1.97
NO_3^-	14.63	0.228	1.56
I^-	26.57	0.397	2.16
SCN^-	46.95	0.621	1.96

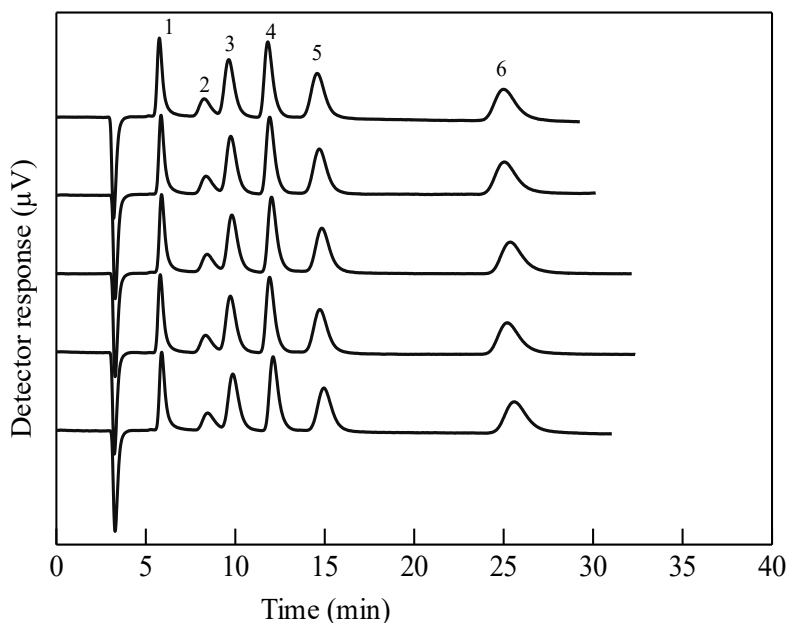


Fig. 5-6. Repeatability tests for the separation of inorganic anions using eluent's concentration of 80mM NaCl with sample mixture and operating conditions as shown in **Fig. 5-2**.

Table 5-3. RSD values of the retention time during the separation of inorganic anions on an AHA-bonded silica while using 80 mM NaCl as eluent, other operating conditions as in **Fig. 5-6**.

Anion	Average	S.D	RSD (%)
IO_3^-	5.831	0.0487	0.83
BrO_3^-	8.363	0.0662	0.79
Br^-	9.724	0.0638	0.66
NO_3^-	13.06	1.560	1.95
I^-	14.74	0.109	0.74
SCN^-	25.22	0.207	0.82

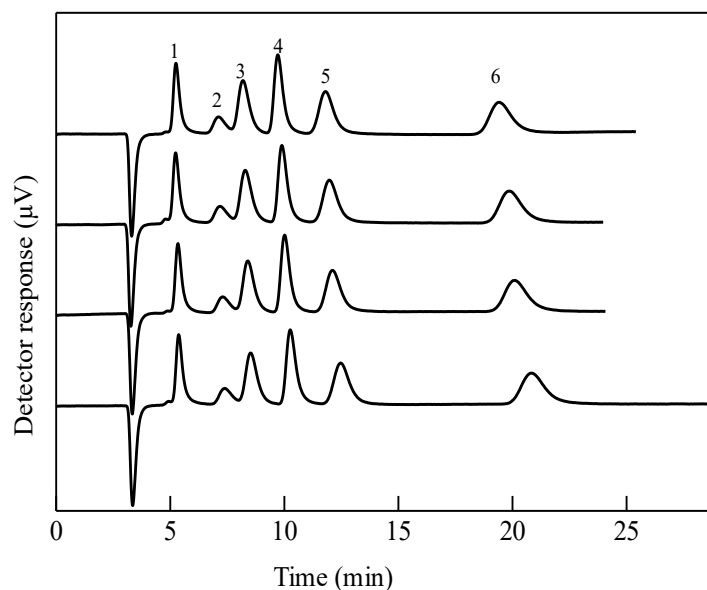


Fig. 5-7. Repeatability tests for the separation of inorganic anions using eluent's concentration of 100mM NaCl with sample mixture and operating conditions as shown in **Fig. 5-2**.

Table 5-4. RSD values of the retention time during the separation of inorganic anions on an AHA-bonded silica while using 100 mM NaCl as eluent, other operating conditions as shown in **Fig. 5-7**.

Anion	Average	S.D	RSD (%)
IO_3^-	5.302	0.0658	1.24
BrO_3^-	7.248	0.116	1.60
Br^-	8.349	0.145	1.73
NO_3^-	10.23	0.371	3.54
I^-	12.09	0.283	2.34
SCN^-	20.63	0.476	2.30

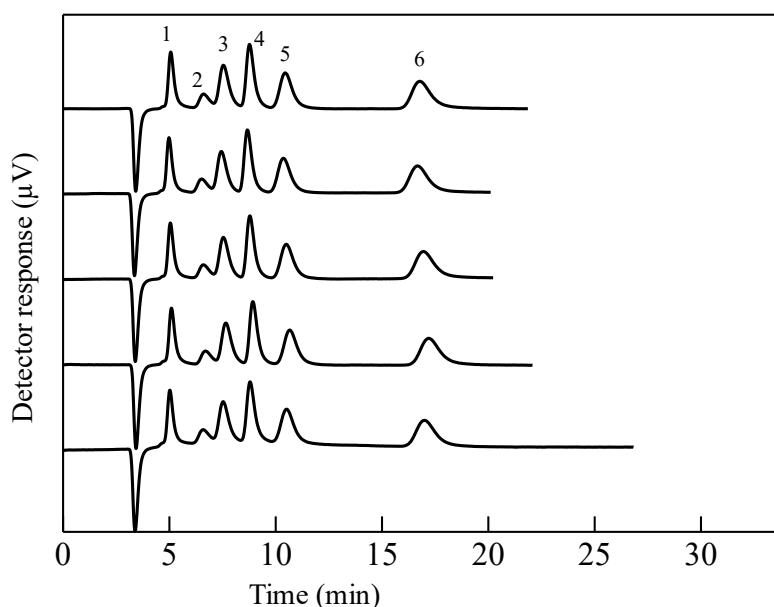


Fig. 5-8. Repeatability tests for the separation of inorganic anions using eluent concentration of 120mM NaCl with sample mixture and other operating conditions as shown in **Fig. 5-2**.

Table 5-5. RSD values of the retention time during the separation of inorganic anions on an AHA-bonded silica while using 120 mM NaCl as eluent, other operating conditions as in **Fig. 5-8**.

Anion	Average	S.D	RSD (%)
IO_3^-	5.061	0.0310	0.61
BrO_3^-	6.604	0.0635	0.96
Br^-	7.543	0.0760	1.01
NO_3^-	8.789	0.0910	1.04
I^-	10.50	0.1086	1.03
SCN^-	16.92	0.2041	1.21

5.3.4 Effect of the flow rate

The prepared column had good mechanical strength and relatively low mobile phase resistance which made it possible for us to operate it at high flow rate, resulting to high speed anion's separations. As shown below in **Fig. 5-9**, chromatographic separation of seven anions was achieved at different flow rates, *i.e.* 2.5 $\mu\text{L}/\text{min}$ (A), 4.5 $\mu\text{L}/\text{min}$ (B), 6.0 $\mu\text{L}/\text{min}$ (C) and 6.5 $\mu\text{L}/\text{min}$ (D) while using 120mM NaCl as eluent. As it was expected, the elution time decreased with increasing flow rates. It can also be observed that the height equivalent to a theoretical plate (HETP) increased with increasing flow-rate within the tested flow-rates.

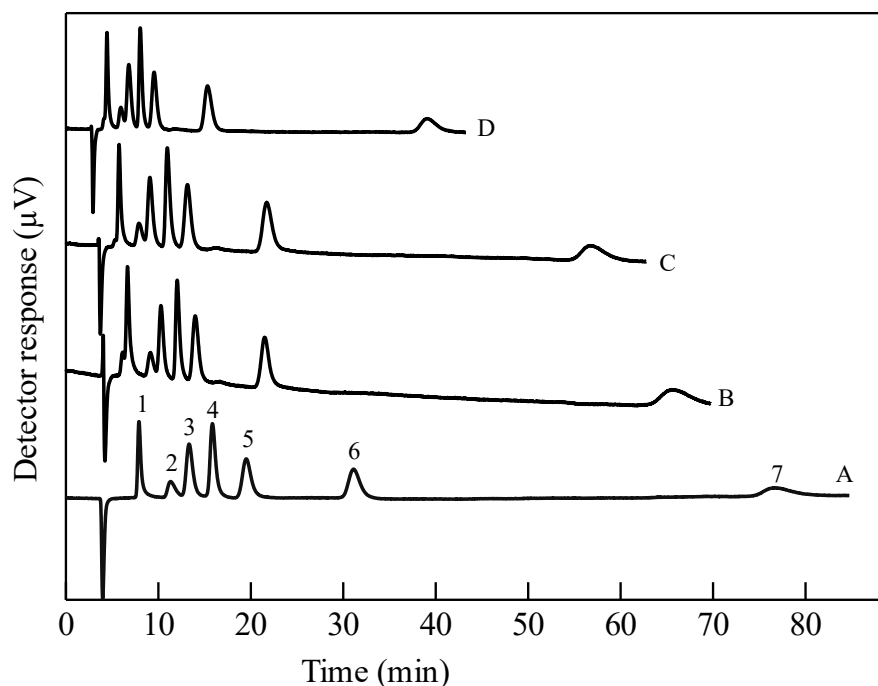


Fig. 5-9. Separation of inorganic anions at various flow-rates; 2.5 $\mu\text{L}/\text{min}$ (A), 4.5 $\mu\text{L}/\text{min}$ (B), 6.0 $\mu\text{L}/\text{min}$ (C) and 6.5 $\mu\text{L}/\text{min}$ (D) on the prepared column. The mobile phase used was 120 mM NaCl and at UV detection wavelength of 210 nm. Other operating conditions as shown in **Fig 5-2**

5.3.5 Effect of pH

The effect of pH was evaluated in the retention of the inorganic anions as shown in **Fig. 5-10** below. This was done using 120mM NaCl as the eluent at pH values of (a) 3.56, (b) 4.63, (c) 5.48, (d) 6.45 and (e) 7.42. The anions were observed to be more retained in the column under acidic condition, while they were less retained under neutral condition. The elution order was iodate, bromate, nitrite, bromide, nitrate, iodide and thiocyanate ions. This meant that the prepared stationary phase was protonated at a lower pH, while it has no charge at a neutral or higher pH. Thus, the prepared stationary phase worked as an anion-exchange stationary phase at a lower pH [18, 21].

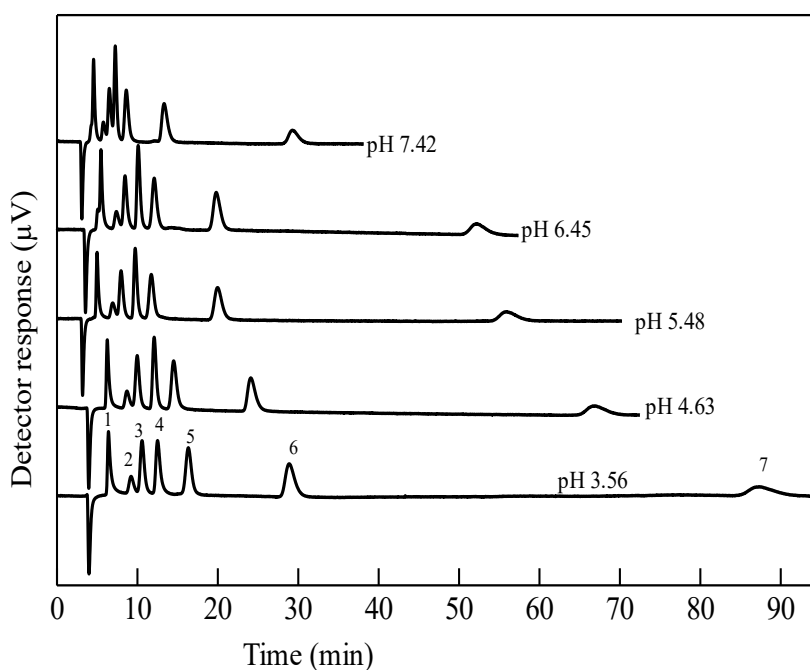


Fig.5-10.Retention of inorganic anions at various eluent pH while using 120 mM NaCl as eluent at different concentration of (a) 3.56, (b) 4.63, (c) 5.48, (d) 6.45 and (e) 7.42, a flow rate of 4.5 μ L/Min and UV detection wavelength of 210 nm. Other operating conditions as shown in **Fig 5-2**.

5.3.6 Real sample application

5.3.6.1 Saliva sample analysis

Saliva sample was collected from a healthy non-smoker, which was then filtered through a 0.45 μm membrane filter prior to injection. The inorganic anions present were examined using the prepared column while using 120mM NaCl as eluent. As shown in **Fig 5-11**, this sample contained nitrite, bromide, nitrate and thiocyanate anions. The saliva sample peak's retention time was observed to be similar when comparing it with the standard sample.

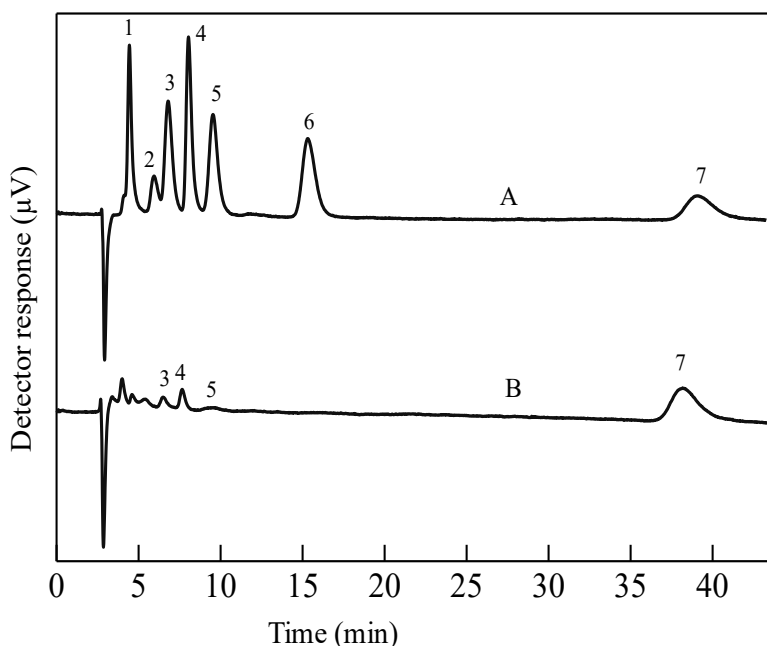


Fig. 5-11. Separation of inorganic anions contained in saliva sample on the prepared column. The mobile phase used was 120 mM NaCl, with flow rate of 6.5 $\mu\text{L}/\text{min}$ and other operating conditions as shown in **Fig. 5-9**, for both the standard sample (A) and saliva sample (B)

5.3.6.2 Tap water sample assessment

Water sample; Gifu university laboratory tap water, was first collected and filtered through a 0.45 μm membrane filter prior to injection. The AHA column was used for the assessment this

water sample while using 120mM NaCl as eluent. Tap water was confirmed to have nitrate ions as shown in **Fig. 5-12**.

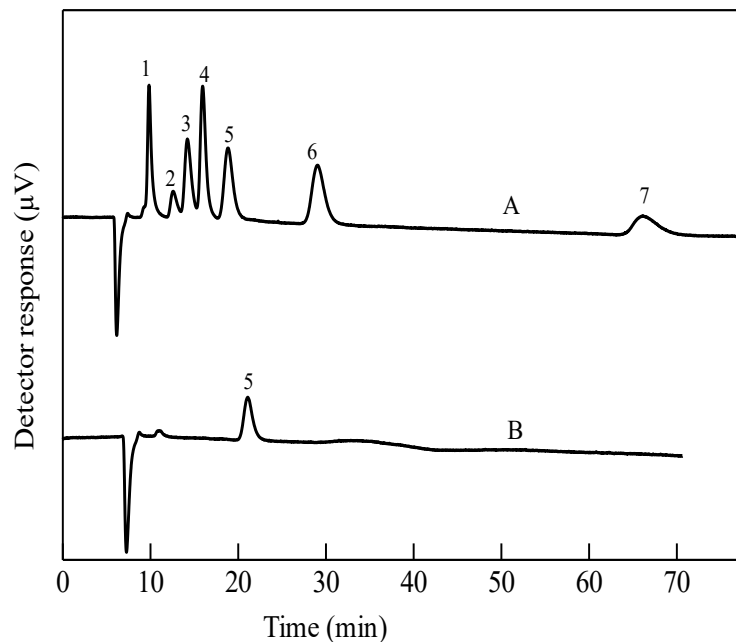


Fig. 5-12. Separation of inorganic anions contained in laboratory tap water on the prepared column, while using 120 mM NaCl as eluent, a flow rate of 4.5 $\mu\text{L}/\text{min}$ and other operating conditions as shown in **Fig. 5-9**, for both the standard sample (A) and laboratory tap water (B)

5.3.6.3 Sea water sample assessment

Water sample; Shinmaiko seawater was first collected and then filtered through a 0.45 μm membrane filter prior to injection. The AHA column was used for the assessment of this water sample while using 120mM NaCl as eluent. Shinmaiko sea waters were confirmed to contain both the bromide and nitrate ions as shown in **Fig 5-13**.

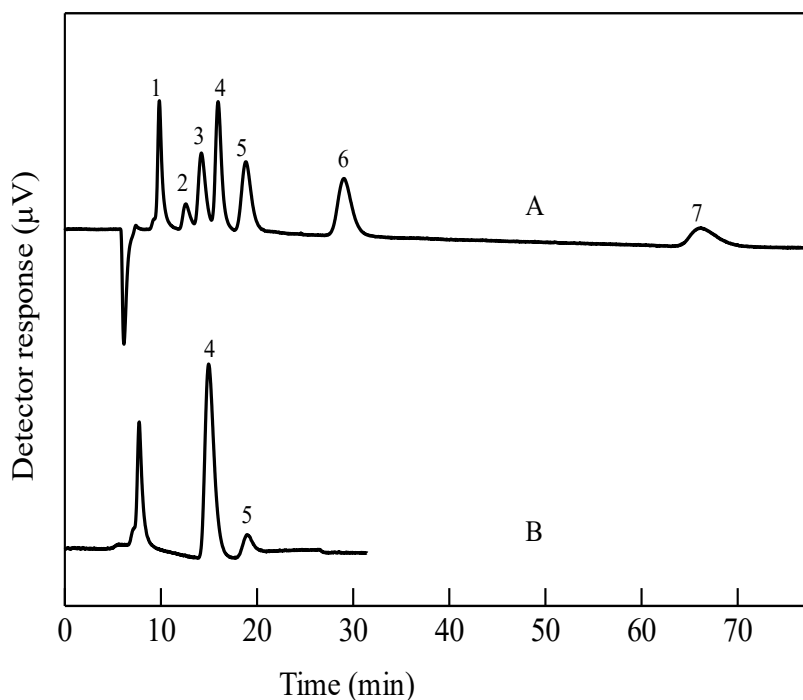


Fig. 5-13. Separation of inorganic anions contained in laboratory tap water on the prepared column, while using 120 mM NaCl as the mobile phase, a flow rate of 4.5 $\mu\text{L}/\text{min}$ and other operating conditions as shown in **Fig. 5-9**, for both the standard sample (A) and Shinmaiko seawater (B)

5.4 Conclusions

Stationary phases with charged or chargeable functional groups are frequently used as stationary phase in IEC. AHA-bonded stationary phase provided the anion-exchange site which allowed us to separate of inorganic anions and as it was expected that ion-exchange mechanism was involved in the retention of these analyte anions. The retention time of the analyte anions was carefully controlled by a good selection of the eluent. The eluent's concentration greatly affected the analyte anions' retention on the synthesized material.

Comparison between ABA-modified silica column in chapter 4 and AHA-modified silica column in this chapter was done, and the analytes retention results obtained for AHA silica

gel was observed to be better in terms of column's chemical stability and reproducibility. This was due to the fact that AHA modified silica formed stable, homogenous, smooth surface unlike ABA modified silica. This research work results is in line with the computation modeling of molecular interaction by silico software by past research work [25-26]

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Chapter 6

Threonine-bonded silica stationary phase for capillary ion chromatography

6.1 Introduction

Silica-based stationary phases still dominates as the best, most popular separation medium due to their good chromatographic performance, mechanical strength and easy during preparation process. These phases have rich silanol groups on their surfaces, which allows easy introduction of many functionalities onto the silica gel. Moreover, the rich silanol groups on silica surface may affect biological sample's separation, due to their high rigidity, fast mass transfer and resistance to organic solvents [1].

Most commonly used stationary phases covalently bonded onto silica surface are C18-alkyl chain, which have previously been utilized for reversed-phase separation mode [2].

Nevertheless, determination of inorganic anions is also crucial as earlier highlighted in the previous chapters. Basically, stationary phase's surface have chargeable functional groups, where the difference in electrostatic attraction between the ionic analytes and the prepared stationary phase results to their separation, through ion exchange mechanism [3]. Thus, many researchers have been interested in developing various types of silica stationary phases for ion chromatography (IC).

Sun and coworkers [4] effectively modified silica with benzimidazole, and the stationary phase was evaluated for reversed-phase and anion-exchange mixed mode separation. Moreover, imidazole [5], poly(1-allylimidazole) [6], phenyl amine [7], 8-quinolinol [8], pyridine [9], dipyridine [10], phenylpropanolamine [11] modified silicas have been investigated as potential mixed mode stationary phases for liquid chromatography (LC). The prepared columns had multi-

interactions including π - π stacking, electrostatic forces, hydrogen bonding, hydrophobic and anion-exchange interactions. Thus different analytes including phthalates, phenols, organic and inorganic anions, poly aromatic hydrocarbons and anilines were effectively separated.

Nesterenko's group also effectively prepared zwitterionic stationary phases with different amino acids like valine, glutamine, asparagine, lysine and tryosine by first linking 3-glycidyloxypropyltrimethoxysilane (GPTMS) on silica surface, which was then followed by an epoxy ring attack using the targeted amino acid [12-14].

The purpose of this study was to investigate the retention of anions on a threonine chemically bonded silica gel. A simple one-pot reaction was followed in order to effectively synthesize the material. Selection of a suitable eluent resulted to the retention and separation of anions and thus, it worked as the anion-exchange stationary phase.

6.2 Experimental

6.2.1 Apparatus

A capillary LC system comprised of a L.TEX-8301 micro-feeder and fitted with a 0.5mL gas-tight syringe was used as a pump. An M435 micro-injection valve (Upchurch Scientific, Oak Harbor, WA, USA) as an injector with an injection sample volume of 0.2 μ L, and a JASCO, Tokyo, Japan, UV detector (UV-970) fitted with a capillary flow-cell (75 μ m; JASCO) were also attached. The pump flow-rate was kept at 2.5 μ L min⁻¹, and the detector was operated at 210 nm. All the data was acquired using a CDS-Lite ver 5.0 data processor (LAssoft, Nagareyama, Chiba, Japan). The modified silica was washed and separated using a centrifuge (IEC610-2-020, Kubota, Tokyo, Japan).

6.2.2 Reagents

High quality reagents were used in this study. Hydrochloric acid (HCl), D-threonine and sodium chloride were purchased from Wako Pure Chemical Manufacturers (Osaka, Japan) and used as

obtained. All solutions used were prepared using ultra-pure water using a simple UV water purifier system. Silica gel (3 μm) obtained from Chemical Evaluation and Research Institute, Tokyo, Japan, was first dried at 120°C for 5 h before use.

6.2.3 Preparation of stationary phases

The threonine bonded stationary phase was prepared following a method by a previous researcher [15] with slight modification as shown in **Fig 6-1** below. Briefly, dried porous silica gel (0.2g), D-threonine (0.2 g), and toluene (1.5 mL) mixed with 0.2 mL of 3-chloropropyltrimethoxysilane were placed in the reflux equipment. The reaction was refluxed with continuous stirring for 24 h at 90°C. The reaction was later cooled to room temperature, filtered and the obtained material was washed two times with toluene and ethanol consequently. The obtained synthesized material was packed into the capillary column and ethanol was used as the propulsion solvent. Hydrochloric acid (30 mM) was passed through the packed column for 30 min.

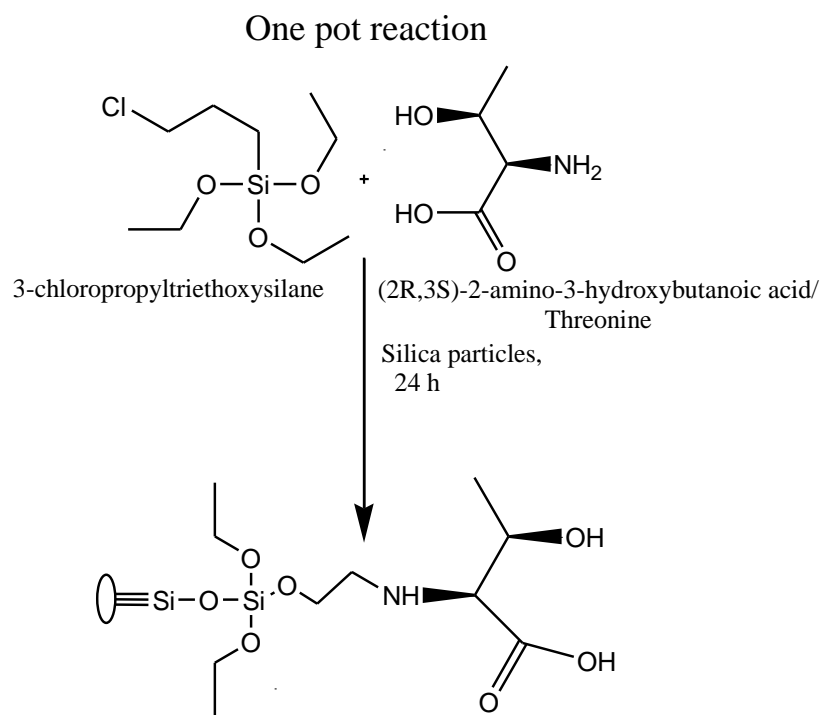


Fig. 6-1. One-pot reaction for threonine bonded silica modified column

6.3 Results and discussions

6.3.1 Inorganic anions retention behavior

The prepared threonine-bonded stationary phase was able to effectively retain and separate the inorganic anions. The expected mode of inorganic anions separation was due ion exchange attributed to the secondary amine group's protonation, on the surface of the modified silica. The elution order was in the order of iodate, bromate, nitrite, bromide, nitrate and iodide while using 10^{-6} mM sodium chloride. This elution order was observed to be similar to that of common IC as shown in **Fig. 6-2**.

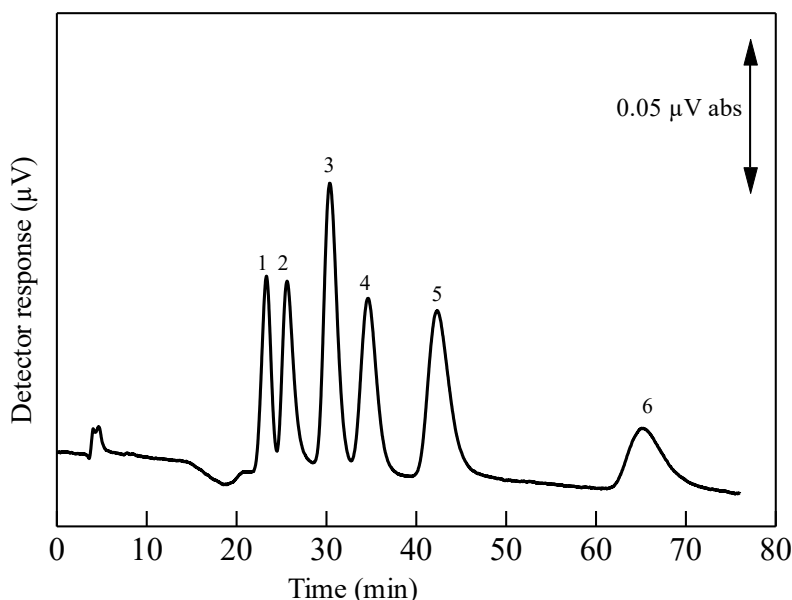


Fig. 6-2. Separation of inorganic anions on a threonine-modified silica column (100 x 0.32 mm I.D), while using a sample mixture of (1) IO_3^- , (2) BrO_3^- , (3) NO_2^- , (4) Br^- , (5) NO_3^- , (6) I^- , at a concentration of 1mM each and 0.5mM for NO_3^- , and at an eluent's concentration of 10^{-6} mM NaCl as the eluent, and a flow-rate of 1.0 $\mu\text{L}/\text{min}$, while using a UV detection wavelength of 210 nm

6.3.2 The effect of eluent concentration

The six anions used in this study were observed to be affected by the salt concentration as shown in **Fig 6-3**. As the NaCl concentration was reduced from 10 mM NaCl to 1 mM NaCl, the anions'

retention was observed to increase. Thus, it was understood that with an increase in elution strength, the anions' retention was decreased and vice versa [21-23].

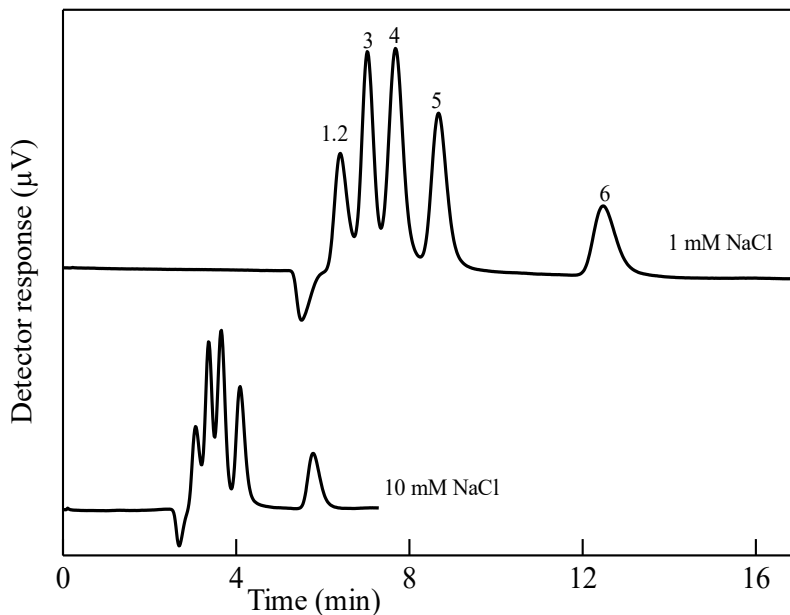


Fig. 6-3. The effect of various eluent's concentrations on analytes' retention with sample mixture as shown in **Fig 6-2**, with a flow rate of $1.0 \mu\text{L}/\text{min}$ and UV detection wavelength of 210nm , on the prepared column

6.3.3 Repeatability tests

The prepared column's repeatability was assessed as shown in **Fig 6-4**, **Fig 6-5** and **Fig 6-6** while using 1 mM NaCl , 10^{-4}mM NaCl and 10^{-6}mM NaCl respectively. With repeated chromatographic runs of $n = 3, 4$ or 5 , relatively sharp peaks with relatively good repeatability was achieved, however, there was an observed room for column's improvement.

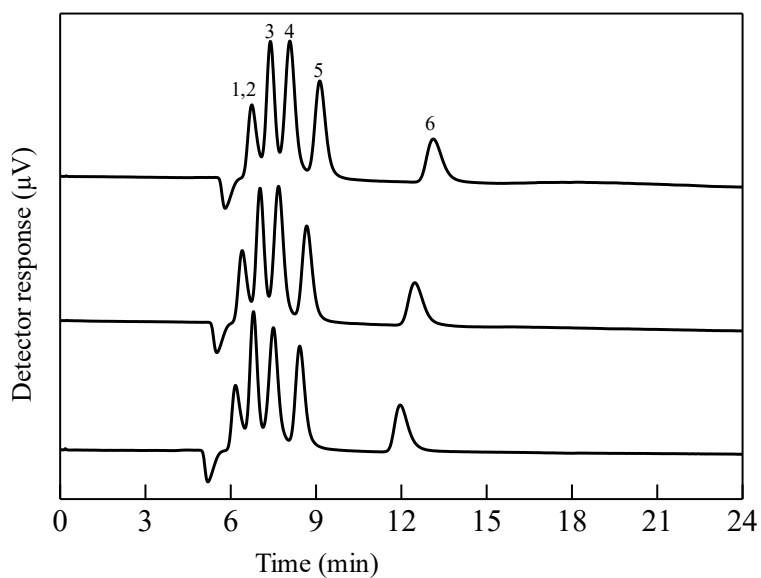


Fig. 6-4. Repeatability tests for the separation of inorganic anions using eluent's concentration of 1 mM NaCl with sample mixture and operating conditions as shown in **Fig. 6-2**

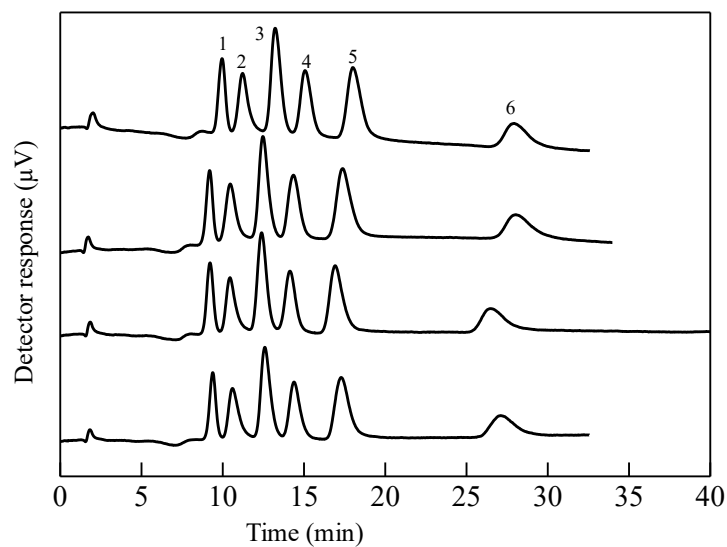


Fig. 6-5. Repeatability tests for the separation of inorganic anions using eluent's concentration of 10^{-4} mM NaCl with sample mixture and operating conditions as shown in **Fig. 6-2**.

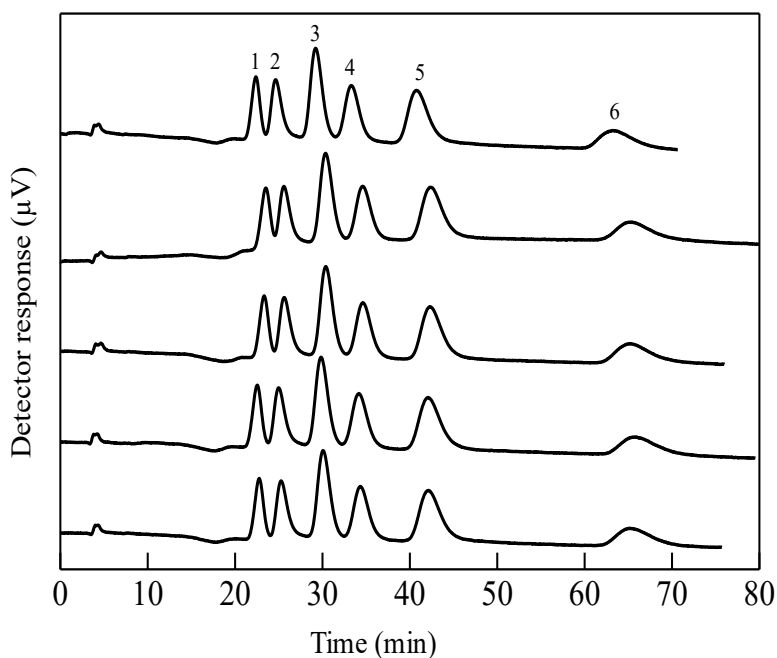


Fig. 6-6. Repeatability tests for the separation of inorganic anions using eluent's concentration of 10^{-6} mM NaCl with sample mixture and operating conditions as shown in **Fig. 6-2**.

6.4 Conclusions

Stationary phases with charged or chargeable functional groups are frequently used as stationary phase in IEC. Threonine-bonded stationary phase provided the anion-exchange site which allowed us to separate of inorganic anions and as it was expected that ion exchange mechanism was involved in the retention of these analyte anions. The retention time of the analyte anions was carefully controlled by a good selection of the eluent. The eluent's concentration greatly affected the analyte anions' retention on the synthesized material.

6.5 References

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Chapter 7

Conclusions and future perspectives

7.1 Conclusions

In chapters 2, 3, 4, 5 and 6, the study involved developing novel separation columns for the separation of various compounds on capillary liquid chromatography. Retention behavior of inorganic anions was investigated on these columns dynamically modified through; metal organic framework (MOF), click reactions, 4-amino butanoic acid, 7-amino heptanoic acid and threonine respectively as described below.

Chapter 2 presents the study on silica based chromium metal-organic framework materials that was able to address a major problem of synthesizing a silica-chromium adsorbent able to produce a homogeneous surface for the chromatographic evaluation. The Si-Cr column was found capable of separating mix aromatic compounds with retention time of 15 min. The retention order was from phenol, the hydroxyl group on the phenol compound being the point of reference, thus from less non-polar to highly non-polar compound (diethyl phthalate). However, there was an observed peak broadening on peaks which were due to the interaction of the given compounds (slightly polar) with the Cr open sites. Such a drawback could be fixed by replacement of the given ligands with rigid aromatic multi dentate ligands containing both O⁻ and N⁻ electron rich surfaces, so as to form stable complexes with chromium, effectively closing all the open Cr sites.

Chapter 3, on the other hand, presents click modified silica that was first confirmed using FT-IR spectroscopy. On bare silica modification with 3-isocyanatopropyl-triethoxysilane and propargyl bromide, the FT-IR spectra displayed an increase of absorptions intensities at due to C-H stretching indicating a largely ordered structure of the hydrocarbon chains. These spectral

changes were clear proofs of a successful reaction. FT-IR spectra of the silica alkyne groups disappeared as observed on the FT-IR spectra of the click-silica alkylazide, which indicated that the intermediate azide group was almost exhausted in the final immobilization procedure. The prepared click alkyl-azide silica bonded stationary phase was achieved through the reaction of alkyne silica and azido-octadecane in presence of Cu catalyst to produce a triazole ring. This ring could serve as ion exchange site as it is considered to be a basic aromatic heterocycle. The ions were separated in the order of iodate, bromate, bromide, nitrate, iodide, and thiocyanate which is a similar observation made to common IC columns. The inorganic anions' retention behavior was investigated under different eluent concentration. The analyte anions retention in the column decreased with increase in sodium chloride concentration.

Chapter 4 presents α -4-amino butyric acid silica packing column that could be used to separate common anions by using sodium chloride as the eluent. It was suggested that the expected mode of inorganic anions separation was due to the secondary amine group's protonation, on the modified silica surface, which may have acted as cationic site interacting with the inorganic anions through electrostatic interaction, for the effective separation. The elution order was in the order of iodate, bromate, nitrite, bromide, nitrate and iodide and the retention mechanism was deduced to be ion-exchange mechanism, while using sodium chloride as eluent. This elution order was observed to be similar to that of common IC. The anions used in this study were observed to be affected by the salt concentration. As the NaCl concentration was increased, the anions' retention was observed to be decreased. Thus, it was understood that with an increase in elution strength, the anions' retention was decreased. The prepared column's repeatability was assessed with repeated chromatographic runs of $n = 4, 5$ or 6 , where the results had relatively sharp peaks with excellent repeatability with RSD value of 2% for anions'

retention time was obtained. The pH of the eluent also affected the retention of the anion. The anions were more retained in the column under acidic condition, and less retained on the stationary phase under neutral condition. This meant that the prepared stationary was protonated at a lower pH, while had no charge at a neutral pH. Thus, the prepared stationary phase worked as an anion-exchange stationary phase at a lower pH.

Chapter 5 presents a 7-amino heptanoic acid silica packing column that could be used to separate common anions by using sodium chloride as the eluent. As expected, the mode of inorganic anions separation was due to the secondary amine group's protonation, on the modified silica surface, which may have acted as cationic site interacting with the inorganic anions through electrostatic interaction, for the effective separation. The elution order was in the order of iodate, bromate, nitrite, bromide, nitrate and iodide and the retention mechanism was deduced to be ion-exchange mechanism, while using sodium chloride as eluent. This elution order was observed to be similar to that of common IC. The anions used in this study were observed to be affected by the salt concentration. As the NaCl concentration was increased, the anions' retention was observed to be decreased. Thus, it was understood that with an increase in elution strength, the anions' retention was decreased. The prepared column's repeatability was assessed with repeated chromatographic runs of $n = 5$ or 6 , where the results had relatively sharp peaks with excellent repeatability with RSD value of 2% for anions' retention time was obtained. The pH of the eluent also affected the retention of the anion. The anions were more retained in the column under acidic condition, and less retained on the stationary phase under neutral condition. This meant that the prepared stationary was protonated at a lower pH, while had no charge at a neutral pH. Thus, the prepared stationary phase worked as an anion-exchange stationary phase at a lower pH.

The same behavior was observed with threonine modified silica in chapter 6.

Comparison between the analytes retention results obtained for both the 4-amino butyric acid modified and 7-amino heptanoic acid silica gel, both the chemical stability and the reproducibility of 7-amino heptanoic acid modified silica was better than 4-amino butyric acid. This can be explained by the fact that 7-amino heptanoic acid modified silica formed stable, homogenous, smooth surface unlike 4-amino butyric acid modified silica. This research work results are in line with the computation modeling of molecular interaction, i.e. molecular simulations using Silico analysis by past researcher [1-3]. In his research work, Silico software was used to demonstrate three dimensional chemical structure models between analytes and stationary phase's molecular interaction. This further assisted in understanding the quantitative interactions involved.

7.2 Future perspective

For the future developments of the current studies, silica-based chromium metal-organic framework materials 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 surface. Amino acid modified columns on the other hand, should be applied to ion exchange chromatography, which leads to low backpressure and rapid sample analysis [1-2].

Simulation softwares like Chrom X or ChromSword, amongst others can be used for the development of model based processes, which can work hand in hand with laboratory experimental work. This can be done through importing liquid chromatogram UV signals into this Silico software for calibration of models and simulation of any further laboratory experiment. Thus, they can assist in saving time and laboratory reagents and also in avoiding tedious unsuccessful experiments [3-4].

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- [3] T. Hanai, *Separations*,6, (2019), 2.
- [4] T. Hanai, *J. Sep. Sci.*,37, (2014), 3013–3014

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Chapter 2

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Fig. 2-1. FT-IR spectra for both the synthesized silica (Si-Cr-BA) (e) and used column (Si-Cr-BA) (d)

Chapter 3

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List of presentations

1. Esther Wamaitha Maina, Lee Wah Lim, Toyohide Takeuchi
“Development of a Novel Inorganic Material for Chromatographic Separation”
The 36thTakayama forum which was held in Takayama City library, Japan, on the 29th - 30thAugust, 2017 (Poster Presentation)
2. Esther Wamaitha Maina, Lee Wah Lim, Toyohide Takeuchi
“Development of Silica Based Chromium Metal-Organic Framework Materials for Chromatographic Separations”, the 14th Asian Conference on Analytical Sciences Symposium, which was held in Jakarta Conventional Center, Jakarta, Indonesia, on the 4th-6thApril, 2018(Oral Presentation)
3. Esther Wamaitha Maina, Lee Wah Lim, Toyohide Takeuchi
“Development of Silica-Based Materials for Chromatographic Separations”, an international conference held by the 6th Asian Network for Natural and Unnatural Materials (ANNUM VI), at Gifu Satellite Campus, Gifu, Japan, on the 27th -28th July, 2018(Poster Presentation) [Best presentation award]
4. Esther Wamaitha Maina, Lee Wah Lim, Toyohide Takeuchi
“Development of Chromium-Silica Hybrid Materials for Chromatographic Separations”, an international conference by The Society for Chromatographic Sciences, held in Toyohashi, Japan, on the 7th -9thNovember, 2018 (Poster Presentation)

5. Esther Wamaitha Maina, Lee Wah Lim, Toyohide Takeuchi

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