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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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PREPARATION OF MIXED-MODE MONOLITHIC SILICA CAPILLARY COLUMN WITH OCTADECYL AND AMINOPROPYL GROUPS FOR LIQUID CHROMATOGRAPHY

Miss Napug Samrejtuksing

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By	Miss Napug Samrejtuksing
Field of Study	Chemistry
Thesis Advisor	Puttaruksa Varanusupakul, Ph.D.
Thesis Co-adviser	Associate Professor Thumnoon Nhujak, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…… Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

…… Chairman
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

…….Thesis Advisor
(Puttaruksa Varanusupakul, Ph.D.)

……Thesis Co-advisor
(Associate Professor Thumnoon Nhujak , Ph.D.)

…… Examiner
(Monpichar Srisa-art, Ph.D.)

…… External Examiner
(Patcharin Chaisuwan, Ph.D.)

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มอนอลิทิกซิลิกาเป็นวัสดุพอลิเมอร์ ที่ใช้เป็นเฟสคงที่ในการแยกทางโครมาโทกราฟีอย่างแพร่หลาย เนื่องจากมอนอลิตเป็นวัสดุที่เตรียมง่าย สามารถปรับปรุงพื้นผิวด้วยหมู่ฟังก์ชันที่หลากหลาย มีความสามารถในการซึมผ่านดี และให้ประสิทธิภาพการแยกที่ดีกว่าคอลัมน์ที่บรรจุด้วยอนุภาคของแข็ง ในงานวิจัยนี้ เตรียมมอนอลิทิกซิลิกาคะพิลลารีคอลัมน์โดยผ่านกระบวนการโซล-เจล และทำการปรับปรุงพื้นผิวด้วย octadecyltrimethoxysilane และ aminopropyltri-methoxysilane (คอลัมน์ C_{18}-AP) ทดสอบและเปรียบเทียบพฤติกรรมทางโครมาโทกราฟีของคอลัมน์ C_{18}-AP และคะพิลลารีคอลัมน์ที่ปรับปรุงพื้นผิวด้วย octadecyltrimethoxysilane (คอลัมน์ C_{18}) โดยวิธีของ Tanaka พบว่า ทั้งสองคอลัมน์มีความสามารถในการแยกสารไม่มีขั้วใกล้เคียงกัน แต่คอลัมน์ C_{18}-AP มีความสามารถในการคัดสรรต่อขนาดและรูปร่างมากกว่าคอลัมน์ C_{18} สำหรับความสามารถในการแลกเปลี่ยนประจุ และการเกิดอันตรกิริยากับหมู่ไซลานอล คอลัมน์ C_{18}-AP แสดงแรงผลักทางไฟฟ้าสถิต เนื่องจากมีหมู่แอมิโนบนพื้นผิวเฟสคงที่ ดังนั้นจึงอาจกล่าวได้ว่าคอลัมน์ C_{18}-AP มีกลไกการแยกทั้งแบบริเวอร์สเฟส และการแลกเปลี่ยนประจุลบ เมื่อทดสอบประสิทธิภาพในการแยกสารของคอลัมน์ C_{18}-AP เปรียบเทียบกับคอลัมน์ C_{18} พบว่า ทั้งสองคอลัมน์แยกสารในกลุ่มเอคิลเบนซีนและกรดเบนโซอิกได้เหมือนกันด้วยกลไกรีเวอร์เฟส นอกจากนี้คอลัมน์ C_{18}-AP สามารถแยกแอนไอออนอนินทรีย์และกลุ่มเบนโซเอทแอนไอออนได้ดี ในขณะที่ไม่สามารถแยกได้ด้วยคอลัมน์ C_{18} เนื่องจากคอลัมน์ C_{18}-AP มีกลไกการแยกแบบการแลกเปลี่ยนประจุ อีกทั้งพีกในการแยกสารกลุ่มเบส เช่น อนุพันธ์อะนิลีนด้วยคอลัมน์ C_{18}-AP มีความสมมาตรมากขึ้น และเมื่อทำการแยกสารโพลิแอโรเมติกไฮโดรคาร์บอนด้วยคอลัมน์ C_{18}-AP ให้ค่าการแยกสารและประสิทธิภาพการแยกที่ดีกว่าคอลัมน์ C_{18} เนื่องจากคอลัมน์ C_{18}-AP มีความสามารถในการคัดสรรต่อขนาดและรูปร่างที่ดีกว่า

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สาขาวิชา เคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
ปีการศึกษา 2554	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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NAPUG SAMREJTUKSING: PREPARATION OF MIXED-MODE MONOLITHIC SILICA CAPILLARY COLUMN WITH OCTADECYL AND AMINOPROPYL GROUPS FOR LIQUID CHROMATOGRAPHY. ADVISOR: PUTTARUKSA VARANUSUPAKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. THUMNOON NHUJAK, Ph.D., 64 pp.

The silica monolith has been a substantial material used as stationary phase in chromatographic separation. Easy of preparation, versatile surface modification, better permeability and higher efficiency were the advantages of monolithic silica column comparing to silica packed column. In this work, The bared monolithic silica capillary column was first prepared via the sol-gel technique and followed by the post- modification with octadecylmethoxysilane and aminopropyltrimetho xysilane (C_{18}-AP column). The chromatographic behaviors of the C_{18}-AP column and monolithic silica capillary column modified with octadecyltrimethoxysilane (C_{18} column) were examined and compared according to Tanaka's test. The hydrophobicity of the C_{18}-AP column and the C_{18} column was similar while the shape selectivity of C_{18}-AP column was better than C_{18} column. For ion exchange capacity and silanophilic interactions, C_{18}-AP column showed repulsive electrostatic interactions due to the functionalized of aminopropyl groups on the stationary phase. Therefore, C_{18}-AP column could exhibit a reversed phase and a weak anion exchange separation behavior. The separation performances of C_{18}-AP column were then examined and compared to those of C_{18} column. The separation of alkylbenzenes and benzoic acid derivatives on both columns were similar which separated based on reversed phase mechanism. Inorganic anions and benzoate anions which were not separated on C_{18} column were successfully separated on C_{18}-AP column because of a weak anion exchange characteristic. Moreover, the improvement of peak tailing problem for separation of basic compounds such as aniline derivatives on C_{18}-AP column was observed. Lastly, the resolution of polyaromatic hydrocarbons (PAHs) separation on C_{18}-AP column was better than C_{18} column due to the better shape selectivity of C_{18}-AP column.

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LI ST OF ABBREVIATIONS AND SYMBOLS

APTMS	aminopropyltrimethoxysilane
C_{18}	octadecyl
C_{18}-AP	octadecyl and aminopropyl
E	separation impedance
k'	retention factor
I.D.	inner diameter
K	Permeability
L	total capillary length
LC	liquid chromatography
H	plate height
M	molar
mg L^{-1}	micro meter
mL	milligram per Liter
MTMS	methyltrimethoxysilane
N	number of theoretical plate
O.D.	outer diameter
ODS	octadecyltrimethoxysilane
PAHs	polycyclic aromatic hydrocarbons
PEG	poly(ethylene glycol)
RP	reversed phase
R_s	resolution
TEA	triethylamine
TMOS	tetramethoxysilane
t_0	retention time of marker
t_R	retention time of solute
WAX	weak anion exchange
α	selectivity

CHAPTER I

INTRODUCTION

1.1 Statement of purpose

In chromatographic separation, the efficiency of a column can be evaluated from plate height (*H*) which related to other chromatographic parameters and expressed as Eq. (1.1).

$$H = A + \frac{B}{u} + Cu \tag{1.1}$$

The Eq. (1.1) is called van Deemter equation where *u* is the linear velocity of the mobile phase; A is eddy diffusion coefficient which describes a multiple-path effect; B is the longitudinal diffusion coefficient and C is the mass-transfer coefficient. From this relationship, the efficiency of a column can be enhanced by decreasing A, B and C terms.

In liquid chromatography (LC), which is a powerful technique for quanlitative and quantitative analysis. A packed column with silica microparticles (2-5 μm) as stationary phase is widely used. The efficiency of the column is now improved by using small packing particles and small column diameters owing to a reduction of mobile phase consumption and sample volume. This provides better separation efficiency due to decreasing of eddy diffusion coefficient (A-term) and resistance to mass transfer (C- term) coefficients. As particle diameter is decreased, analyte molecules can diffuse to reach the particle surface with short distance. In addition, the C term is proportional to the square of the particle diameter. Using small packing particles will reduce the C-term and thus reduce the overall plate height. The columns packed with 1.5 μm particles have been reported [1-2] and commercially available. Though, it has not widely used in conventional HPLC [3] because they require a high pressure pump (> 10,000 psi) which is emploied in ultra-high pressure liquid

chromatography (UHPLC). This is a main drawback of using smaller particle sizes in which high efficiency is traded with high pressure drop [4].

Monolithic stationary phases which are an integrated continuous porous media without particular voids [5-8], as shown in Figure 1.1 have been recently attracted in liquid chromatography. Monolithic silica columns have been an alternative candidate to overcome the problem of high pressure associated with small particles in packed columns [9-10]. This type of stationary phase provides small-sized skeletons and wide through-pores as shown in Figure 1.2. Other advantages of monolith include an easy of preparation, versatile surface modification, higher phase ratios, and higher permeability compared with porous silica microparticles at similar separation efficiency [11-14].

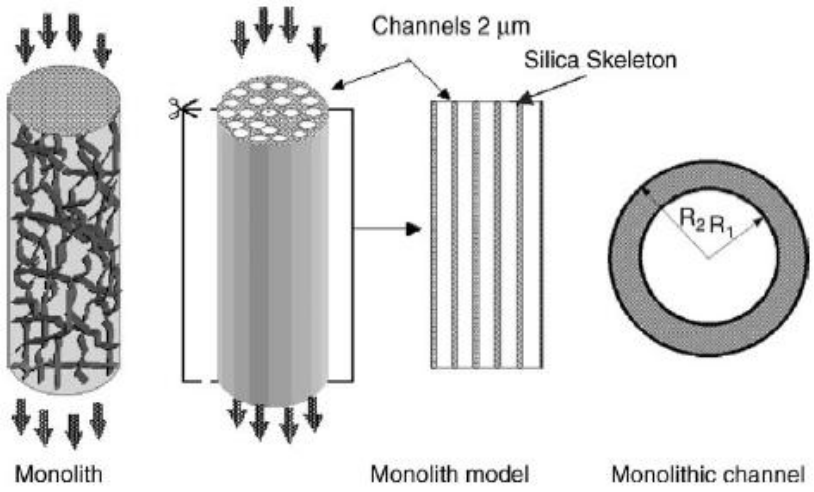
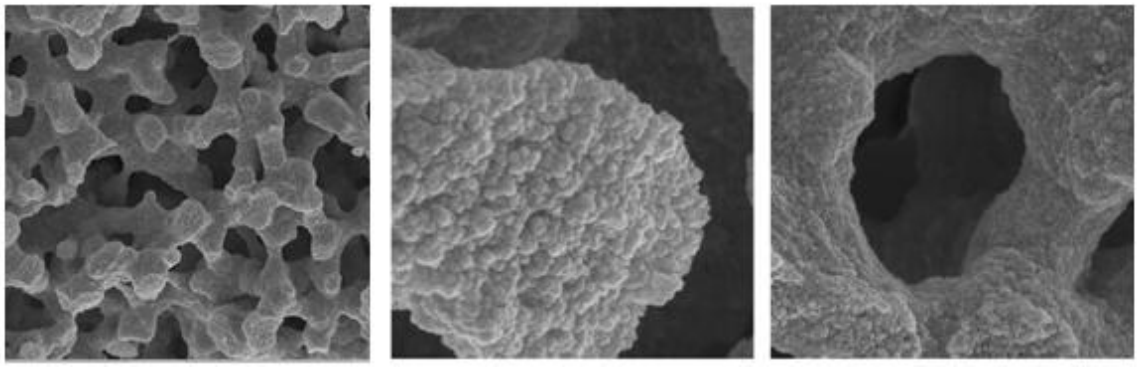


Figure 1.1 The sketch of the real and idealized monolith structure. [15]

Generally, monolithic materials can be devided into two groups, organic polymer- and silica-based. The organic polymer-based monoliths have excellent pH stability and were successfully applied for biological samples [16-19]. Unfortunately, the polymer-based monoliths swell or shrink when exposed to various organic solvents which typically used as mobile phase in LC. In comparison, silica-based monoliths provide higher mechanical strength and better organic solvent tolerance [20]. Moreover, it can be functionalized with various surface chemistries [21].

Therefore, they have been many reports on monolithic silica columns [4, 22]. They were mostly modified for a reversed phase (RP) mechanism [23-25]. Some of them reported for ion chromatography [26] and few researches presented a mixed-mode behavior [27-31].



(a) (b) (c)

Figure 1.2 Scanning electron microscopy (SEM) images of a typical porous structure of monolithic silica column (a), the mesoporous structure of the silica skeleton (b), and the macropores or throughpores of monolithic silica (c) [32].

Separation of basic and nitrogen containing compounds in reversed phase LC is usually problematic due to tailing peaks. The cause of this problem is an ion exchange interaction between a positively charged solute and a negatively charged silanol on the surface of silica stationary phase in a neutral pH mobile phase. There are several improvements. One action is the separation at low pH condition by using mobile phase at pH lower than 3, where the silanol group is protonated (pKa of silanol ~3.5). However, it is not an acceptable way due to the fluctuation of resolution and selectivity. A second way is to increase the buffer strength of a mobile phase. Although it decreases the interaction between basic compounds and the silanols, the precipitation of buffer salt in the instrument can occur, especially at the pump seals. Therefore the buffer concentration should be considered. Another improvement is to add a competing amine, such as triethylamine (TEA) in the mobile phase to reduce the availability of silanol group on the stationary phase and the interaction of the analyte with the silanols [33].

As describe above, the complicated method to reduce peak tailing on separation of basic compounds on reversed phase LC were reported. Hence, this work aims to prepare the silica-based monolith column that modified with octadecylmethoxysilane (ODS) and aminopropyltrimethoxysilane (APTMS) to provide a mixed-mode of reversed phase and a weak anion exchange to minimize peak tailing on separation of basic solutes.

1.2 Literature reviews

Silica-based monolithic stationary phases have been prepared via the sol-gel process and post modified with different functional groups to use as stationary phase in chromatographic separation. The preparation of silica-based monolith was previously used tetramethoxysilane (TMOS) as a starting material. However, it showed a shrinkage inside a fused silica capillary tube to attach silica skeleton to the inner walls of the capillary when prepared in large internal diameter column. Thus, methyltrimethoxysilane (MTMS) was added to resolve a shrinkage [34-36]. Kobayashi et al. [37] reported that a mixture of TMOS and MTMS in a 3:1 ratio is suitable to prepare the silica bared monolithic stationary phases.

Monolithic stationary phases are mostly developed for a reversed phase separation mechanism. Oscar et al. [22-23] prepared a monolithic silica capillary column with high efficiency by polymerization of octadecyl methacrylate using α, α′-azobis-isobutyronitrile (AIBN) as a free radical (abbreviated as ODM column). High efficiency and highly retentive monolithic silica capillary column was achieved when increasing monomer- and initiator concentration in polymerization reaction mixture. Then, a performance of separation on polar and non-polar compounds was compared between the ODM column and a monolithic silica capillary column modified with octadecylsilyl-(*N,N*-diethylamino)silane (abbreviated as the ODS column). Although the ODM column provided similar selectivity to the ODS column, it showed a higher differentiation for compounds with similar planar structures than the ODS column. Wasura et al. [24] compared the selectivity for halogenated compounds between an

ODS column and an ODM column. The ODM column provided greater selectivities for halogenated compounds and was capable for separation of isomeric dihalogenated compounds. This can conclude that the ODM column is an alternative for separation of halogenated compounds compared with the ODS column.

Moreover, a monolithic silica capillary column has been explored for ion analysis. Jaafar et al. [25] fabricated a monolithic silica capillary column modified with *N*-[3-(dimethylamino)propyl] acrylamidemethylchloride quaternary salt (DMAPAA-Q) by bonding 3-methacryloxypropyl moieties as an anchor to the silica surface. It exhibited strong anion exchange to separate an anion mixture containing iodate, bromate, nitrate, bromide, iodide, and thiocyanate completely. Good performance and large numbers of theoretical plates was observed.

Some mixed-mode monolithic silica capillary columns have been fabricated, but most of them were used in capillary electrochromatography (CEC). Ding et al [26] prepared an RP/WAX monolithic silica capillary column for CEC by using a one step sol gel procedure. The synthesis was optimized by changing the ratio of tetraethoxysilane (TEOS), aminopropyltriethoxysilane (APTES) and octyltriethoxysilane (C_8-TEOS) in the mixture. Although a single step synthesisprovided time saving, the ratios of the chemical modification reagents influence the continuous skeleton and large though-pores structure of the column. Ye et al. [27] prepared a monolithic silica capillary column and followed by a post modification with hexadecyltrimethoxysilane (HDTMS) and aminopropyltrimethoxy-silane (APTMS). The amino groups on the surface of the stationary phase were used to generate substantial anodic EOF as well as to provide electrostatic interaction sites for charged compounds at low pH. The separation of neutral solutes exhibited a RP chromatographic behavior. Anionic solutes were separated by a mixed-mode mechanism. Symmetrical peaks were obtained for basic solutes.

The mixed-mode monolithic stationary phases for solid phase extraction also presented. Zheng et al. [29] extracted sulfonamide on the silica monolith which provided both hydrophobic and strong cation exchange functional groups. Good extraction and compatibility for complex samples were reported.

There are few research articles that presented RP and anion exchange column for LC. Nogueira and co-worker [30] described the synthesis of monolithic RP/weak anion exchange (WAX) columns that funtionalized with *N*-(10-undecenoyl)-3-aminoquinuclidine onto thiol groups. The synthetic column exhibited the excellent separation of acidic hydrophilic peptides.

1.3 Scopes of this research

1.3.1 Preparation of a monolithic silica capillary column via the sol-gel process and functionalization of the monolithic silica capillary column with octadecyltrimethoxysilane (ODS) and aminopropyl-trimethoxysilane (APTMS) (C_{18}-AP column).

1.3.2 Study efficiency and chromatographic characterization of the C_{18}-AP column by Tanaka's procedure.

1.3.3 Separation of model neutral, acidic and basic compounds on the C_{18}-AP column by liquid chromatography.

1.4 The benefit of this research

Receiving of a monolithic silica capillary column modified with octadecyltrimethoxysilane and aminopropyltrimethoxysilane for reversed phase and weak anion exchange mechanisms on liquid chromatography.

CHAPTER II

THEORY

Introduction to liquid chromatography in this chapter is mostly quoted from references [38, 39] and information in monoliths is cited from Monolithic Silicas in Separation Science [40].

2.1 Liquid chromatography

Liquid chromatography is a chromatographic science for separation of mixtures. It is used in qualitative and quantitative analyses. In the separation process, the mixture sample is dissolved in fluid called the mobile phase which forced through a structure holding material called stationary phase. Each constituent in the mixtures has a different partition between mobile phase and stationary phase, causing them to be separated. Liquid chromatography can be historically divided into two different sub-classes, normal phase liquid chromatography (NPLC) and reversed phase liquid chromatography (RPLC). The difference of the two methods based on the polarity of the mobile phase and stationary phase. NPLC is the technique in which the stationary phase is more polarity than the mobile phase while the mobile phase in RPLC is more polarity than the stationary phase. Generally, a liquid chromatography system has five major components which are mobile phase reservoirs, pump, injection unit, column, and detector as shown in Figure 2.1

Figure 2.1 Schematic diagram of a typical HPLC instrument.

2.1.1 Mobile phase reservoirs

Mobile phase is a solvent which transfers sample mixtures through a stationary phase in a chromatographic column. The mobile phase normally can be a non-polar solvent (normal phase) or a polar solvent (reverse phase), depending on chromatographic method and type of detector. Moreover, the mobile phase using in liquid chromatography should be HPLC grade and filtered before entering into the system because it perhaps has impurities that can react with solutes. In addition, a separation operated by a constant mobile phase composition is called an isocratic elution. To enhance the separation efficiency, it frequently uses a gradient elution in which two or three solvents that differ significantly in polarity are varied by program which may be altered linearly or exponentially with time.

2.1.2 Pumping system

The function of the pump is to force mobile phase through the column at high pressure as well as to control flow rates. There are many types of pumps, providing constant pressure of constant flow rate. For a constant pressure pump, mobile phase flows at a constant pressure, but flow rate is varied relying on the flow resistance. A constant flow rate pump applies a steady flow rate to mobile phase, but the pressure will be fluctuated due to the flow resistance. Most commonly used pump types are pneumatic pumps and syringe pumps. Pneumatic pumps are inexpensive and pulse free, but they have limited capacity and pressure output as a result of flow rate on solvent viscosity and column back pressure. Meanwhile, syringe pumps produce a flow that tends to be independent of viscosity and back pressure. And the output is pulse free. Disadvantages include limited solvent capacity (~250 mL).

2.1.3 Injector (sample introduction)

An injection port commonly comprises an injection valve and a sample loop. A sample, typically dissolved in a mobile phase, is drawn into a syringe and injected into a sample loop via an injection valve. Then a valve rotor is rotated,

making the valve closes and the loop opens. This cause the sample injected into the stream of mobile phase.

2.1.4 HPLC column

Liquid chromatographic columns are usually made from stainless steel tubing, although glass or Tygon tubing is sometimes employed for lower pressure applications (< 600 psi). Most columns range in the length from 10 to 30 cm and have inside diameters from 2 to 5 mm. Column packings typically have particle sizes of 3 to 10 μm. Recently, microcolumns have become available with inside diameters of 1 to 4.6 mm and lengths of 3 to 7.5 cm. These columns, which are packed with 3- or 5-μm particles, have an advantage of speed and minimal solvent consumption. This latter property is of considerable importance because the high-purity solvents required for liquid chromatography are expensive and to dispose after use. Nonetheless, it has high back pressure.

2.1.5 Detector

A detector for HPLC is a device to visualize each component in the mixture that elutes off the chromatography column. A result shows as a peak signal on a chromatogram. There are many types of detectors, such as refractive index (RI), ultra-violet (UV), fluorescent (FL), radiochemical, electrochemical, near-infra red (Near-IR), mass spectroscopy (MS), nuclear magnetic resonance (NMR), and light scattering (LS). The most popular HPLC detector is a UV-visible detector. It is selective to detect a compound that can absorb ultraviolet light and shows the result as a plot of absorbance as a function of elution time.

2.2 Capillary liquid chromatography

Capillary LC, sometimes called micro LC, uses smaller column internal diameters than conventional LC. Thus, the amounts of injected solution must be controlled to avoid sample volume overload as well as a very low flow rate of a

mobile phase. The instrument that used for capillary column is similar to the conventional LC. But it requires a mobile phase flow rate through the column as small as 1 μL/min which exceed the lower limit of conventional LC pump (≈0.1 mL/min). Therefore, it is necessary to use a designed pump for capillary column that can operate flow rate as small as 1 μL/min. Microinjector is also crucial The injection volumes should be in the range of nanoliter to microliter. Split flow is an alternative method for using the injector and pump in conventional LC system. The splitter is usually placed before the capillary column to reduce the flow of mobile phase and injection volume. Obviously, tubing, fittings, injectors and detectors must be scaled accordingly to keep away from extra-column band broadening effects. To minimize extra-column volume, the detector cell volume should be at least 1/5 of the peak volume. In other word, if the detector cell has large path lengths, the detector cell broadens the peak until it will not differentiate good peak performance that previously provide from capillary column.

2.3 Liquid chromatography column

A column is the heart of HPLC. New stationary phase has been produced for the extensive range of sample and broad applications.

2.3.1 Packed column

Column packing is produced from bare particles or supports that sometimes modified with functional groups for application. Porous-silica particles in packed beds are the most commonly used. The physical configuration of particle packing influences the separation. The retention of analytes will increase with pore size decrease as well as column surface area increase. The size of the particles is especially determined to achieve good efficiency in packed column. Moreover, a particle size distribution is also very important. A narrow particle sized distribution is preferred to provide larger values of number of theoretical plates (*N*) and lower column pressure for comparable efficiency.

Several types of particles are currently available for HPLC. The most popular particle has diameters in the range of 1.5 to 5.0 μm. Up to now, much smaller spheres can be prepared by aggregation.

2.3.1.1 Pellicular particles

The stationary phase of pellicular particles which is coated with a very thin surface layer provide good efficiency (larger value of *N*) for macromolecules. It presents a suitable stationary phase mass transfer (smaller C-term). Moreover, these particles reduced retention and injection volume of sample due to their very low surface area.

2.3.1.2 Superficially porous particles

Superficially porous particles have a solid core with a porous outer shell. These particles typically have diameters of 2 to 5 μm, with porous shells of 0.25 to 0.5 μm in thickness. The surface area on porous shells is larger than that of pellicular particles. Besides, it provides longer retention and allows larger sample volume for injection. The thin, outer porous shell also gives faster separation, especially for macromolecules.

2.3.1.3 Perfusion particles

Perfusion particles contain very large pores (e.g. 400 to 800 nm), connected to a network of smaller pores (e.g. 30 to 100 nm). Because of their characteristics, solutes distribute into the perfusion particles by the combination of diffusion and flow of mobile phase through the particles. Thus, band broadening is reduced at high flow rates.

2.3.2 Monolithic column

Monoliths have been emerged since 1990. They represent an alternative material for column support. The monoliths refer to columns composed of an interconnected porous bed, as opposed to columns packed with distinct particles. Monoliths are synthesized as either silica- or polymer-based. The polymer-based monoliths have excellent pH stability. Although they show compatible with biological analysis [16-19], they suffer from shrinking or swelling under organic solvents typically used as mobile phase in LC. Accordingly, this work was interested in preparation of silica-based monoliths.

2.3.2.1 Silica-based monoliths

Silica monoliths are a single piece of porous material which commonly prepared as rods. The monolith includes two types of pores, macropores and mesopore. The macropores show diameters of ~2 μm. The Other displays diameters of ~10 nm. Generally, a conventional packed bed provide the interstitial volume about 40% of the column volume. While interstitial volume (defined as the volume of the macropores) of monoliths obtain as high as 80%. The diameters and total volume of the monolith macropores determine column permeability and the pressure required to achieve a certain flow rate. Because the macropores show quite large diameter. Thus the pressure for flow through a monolith is less than that for conventional columns of packed particles.

Monoliths can be used higher flow rates without loss in column efficiency comparison to particulate columns. There are some studies that describe that the separation of small molecule for commercial monolith columns give efficiencies equivalent to packed column with 4 to 5 μm particles [41]. Another study suggested that monolithic columns are only beneficial when high-resolution separations are needed [42]. The silica-based monoliths can be prepared reproducibly that is acceptable for routine analysis [41, 43].

Synthesis of silica-based monoliths

Silica-based monoliths can be synthesized through the sol-gel technique that is generally caused by two kinds of reaction, hydrolysis and polycondensation. The hydrolysis is initiated by mixing of water and alkoxide (M-OR) in the presence of alcohol as a cosolvent. The hydrolysis of M-OR produces M-OH. Then it subsequently condenses with another M-OH to produce polycondensed species including M-O-M linkage and water. A successive condensation leads to the growth of metalloxane oligomers that subsequently link together to form a gel network. In this case, Silicon alkoxide ($Si(OR)_4$) is used as an example and the process is expressed as follows;

Hydrolysis: $Si(OR)_4 + nH_2O \rightarrow Si(OH)_n(OR)_{4-n} + nROH$ (a)

Polycondensation: $\equiv Si\text{-}OH + \equiv Si\text{-}OH \rightarrow \equiv Si\text{-}O\text{-}Si\equiv + H2O$ (b)

$\equiv Si\text{-}OH + \equiv Si\text{-}OR \rightarrow \equiv Si\text{-}O\text{-}Si\equiv + ROH$ (c)

For overall kinetics of the reactions (a-c), acid catalysts are used to promote the reactions. Under acidic conditions, the mole ratio of water/alkoxide affects resultant gels. At high amount of water (water/alkoxide ≥ 4), gels with low cross-linking density are formed. They tend to be monolith when dried carefully. If the mole ratio of water/alkoxide ≤ 2, they are proper to form gel fibers and coating films.

The final morphology control is associated with time of structure freezing as shown in Figure 2.2. If cooling rate is higher, the shorter time is allowed the coarsening phase-separating domains. On the other hand, the finer domains can be obtained by delaying the onset of phase separation.

Figure 2.2 Time evolution of a spinodally decomposing isotropic symmetrical system [44].

Both phase separation tendency and polycondensation rate are determined by the starting composition. Moreover all pore characteristics, including pore volume can be controlled by precisely adjusting the starting composition under a fixed temperature throughout the reaction.

The diameter of macropores and the thickness of silica skeletons are related to the characteristic sized of phase-separated domains that are developed in the course of sol-gel reaction. The term “domain size” usually denotes the sum of gel-phase size and its neighboring fluid-phase averaged over the sample. Even if a phase-separated wet gel is dried by removing the fluid-phase and the solvent, often accompanied by shrinkage, one can define the domain size as the sum of the diameter of the shrunk pore and its neighboring shrunk skeleton. Most silica sol-gel systems that phase separate in the course of a sol-gel transition contain three major components, silica, solvent and a phase separation inducer, and they separate into two phases. The distribution of the three major components to the two conjugate phases, one is rich in gelling silica and the other is rich in nongelling component, here

after denoted as gel-phase and fluid phase, determines the volume fractions of macropores and silica skeletons.

Silanol groups that are abundantly present on the surface of siloxane oligomers often strongly interact with the ligands of polymers or surfactants added as a phase-separation inducer. Hydrogen-accepting ligands, such as ether or carbonyl oxygens attractively interact with silanols to form hydrogen bonds. Many kinds of water-soluble polymers used to adsorb on the surface of silica oligomers, such as polyethylene. There are other kinds of water soluble polymers, such as poly(sodium styrenesulfonate), poly(acrylic acid), poly(ethyleneimine) and poly(allylamine) that do not specifically interact with silanols, but still can be used as a phase-separation inducer. However, it must do under acidic conditions.

On phase separation, the conjugate phases become one composed mainly of silica and an adsorbed additive, and the other containing a majority of solvent mixture. The volume fraction of the gel-phase is determined by the volume fractions occupied by silica and additive, and that of the fluid-phase by those of majority of solvents. In this case, the volume fraction of macropores forms after the removal of the fluid-phase can be controlled mainly by the volume fraction of solvent mixture. Since the domain size is controlled by the additive/Si ratio in principle, the pore size (domain size) and pore volume can be independently controlled (Figure 2.3).

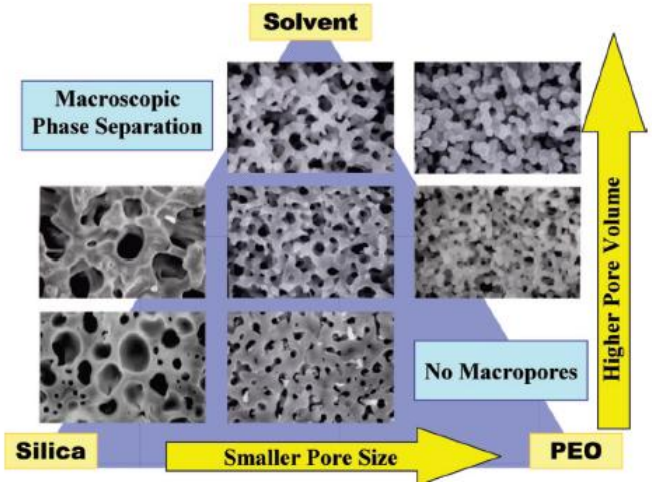


Figure 2.3 Schematic relations between starting composition and resultant gel morphologies in a pseudoternary [silica/poly(ethyleneoxide)/solvent] system [44].

In this case, controls over pore sizes by the concentration of additives necessarily influence the volume fraction of the fluid-phase, which become macropores afterwards.

The monoliticity, however, is hard to preserve even if the wet gel has well-connected micrometer-range open pores. Since the microscopic network of silica is determined by the acid-catalyzed polymerization, a substantial capillary stress corresponding to the pore size in the range of nanometers is exerted by the evaporation of solvents. To avoid or minimize undesired shrinkage and cracking during drying, an aging process under weakly basic condition is known to be effective in the case of pure silica gels.

Generally, monoliths are composed of a network of pores defined as macropore (>50 nm), which provide approximately 80% of the total porosity and mesopores (2-50 nm), which illustrate 10-15% of total porosity [4].

2.3.2.2 Organic polymer-based monoliths

Organic polymer-based monoliths are used in a wide range of applications. These columns can be made from polymerization reaction. The precursors for prepared organic polymer based monolith are initiator, cross-linker and monomer. They are directly formed into column tubes, including (0.5-mm diameter and smaller). The nature of the polymerizable material and the pore structure, it shows a wide pore which is suitable for separation biological molecules such as peptides, proteins, oligonucleotides, etc.

2.4 Mode of liquid chromatography

2.4.1 Normal phase

Normal phase is a classical mode of chromatographic separation. The stationary phase is polar and mobile phase is relatively nonpolar. The least polar component is eluted first; increasing the polarity of the mobile phase decreases the elution time.

2.4.2 Reversed phase

The stationary phase is nonpolar, often a hydrocarbon, and the mobile phase is relatively polar (such as water, methanol. or acetonitrile). Most commonly, the silica column is coated by a long hydrocarbon chain such as a C8 chain (n-octyl) or a C18 chain (n-octyldecyl). With this attachment, the long chain hydrocarbon groups are aligned parallel to one another and perpendicular to the particle surface, giving a brush- or bristle-like structure. From this appearance, it shows physical adsorption. As a result, molecules of mobile phase then compete with analyte molecules for positioning on the organic surface. Moreover, the used mobile phase is often an aqueous solution containing various concentrations of solvents, such as methanol, acetonitrile, or tetrahydrofuran. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture while they are passing

through the column. Nonpolar compounds, which are more hydrophobic, will tend to interact with hydrocarbon groups on the stationary phase. They will spend more time on the column.

2.4.3 Ion exchange chromatography (IEC)

These applications are useful for analytical separations. The stationary phase includes ion-exchange resins used to separate charged species. Separation is based on electrical charge. This technique characterized by the nature and strength of acidic or basic functions on their surfaces and the types of ions that attract and retain. Cation exchange is used to retain and separate positively charged ions on a negative surface. Conversely, anion exchange is used to retain and separate negatively charged ions on a positive surface.

2.4.4 Mixed-mode

Mixed-mode chromatography is a type of chromatographic method in which multiple interaction modes take place between the stationary phase and solutes in the column [45, 46]. It is distinctly different from single mode chromatography because the second interaction cannot be too weak, and the two interactions should both contribute to the retention of the solutes. From the secondary interaction, such as peak tailing, many research tried to eliminate and minimize to procedure. Mixed-mode chromatography became a new technique for improving separation power by using suitable approaches such as the connection of two columns with different modes, the mixing of two types of stationary phases in a column, the packing of one mixed-mode stationary phase into a column [45, 47, 48], and the chemical synthesis of new stationary phases with different functional groups.

2.5 Theory in liquid chromatography

2.5.1 Chromatographic retention

Dead or void time (t_M or t_0) is defined as the amount of time that mobile phase migrates on the column. It is an important parameter for identifying analyte peaks. All components spend time (t_M) in the mobile phase. To measure t_M, an unretained species can be added if one is not already present in the sample or the mobile phase. The unretained molecule generally used to measure t_M are thiourea ($SC(NH_2)_2$), uracil ($C_4H_4N_2O_2$), etc because these compounds are not retained on the stationary phase. They can transfer through the column at the same rate as the mobile phase.

Retention time (t_R) is the time taken after sample injection for analyte peaks to reach the detector. Each analyte in a sample has a different retention time.

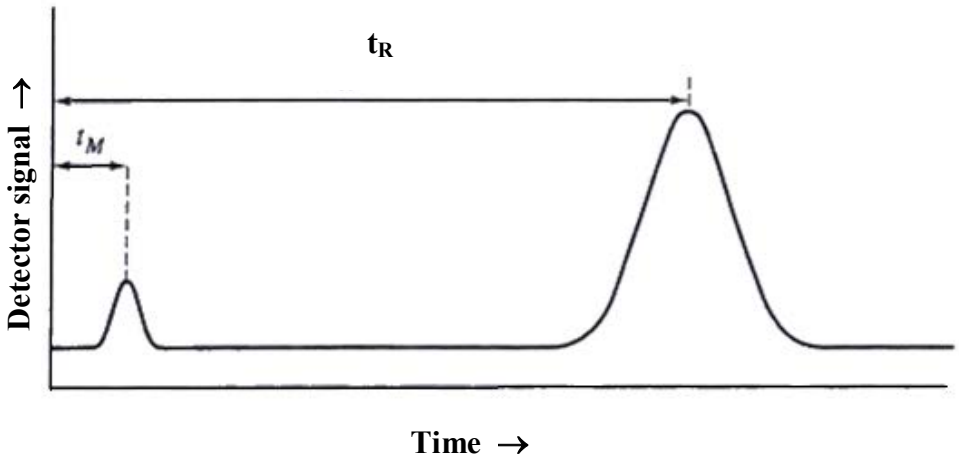


Figure 2.4 Shown the void time (t_M) and retention time (t_R).

The retention measurements are taken from the chromatogram in order to quantify the analytes. Generally, the best way to identify it by the capacity or retention factor (k') which is given by the equation 2.1. It uses an experimental information that is widely used to compare the migration rates of solutes on columns. The retention factor (k') can evaluate from the ratio of the time that analyte spends in

the stationary phase relative to time it spend in the mobile phase. Nonetheless, this can be varied with column length and mobile phase flow rate.

$$k' = \frac{t_R - t_0}{t_0} \tag{2.1}$$

Where t_R is the retention time of the solute and t_0 is retention time of the unretained solute travelling in the same speed of the mobile phase.

t_R and t_M are readily obtained from a chromatogram Figure 2.4. When the retention factor of a solute is much less than unity, it means that the solute emerges from the column at a time near the void time. When the retention factor is larger than perhaps 20-30, elution times become inordinately long. Ideally, separations are performed under conditions in which the retention factors for the solutes in a mixture lie in the range between 1 and 5.

2.5.2 Band broadening and column efficiency

2.5.2.1 Plate theory

The efficiency of chromatographic column is measured by two related terms: (1) plate height H and (2) number of theoretical plates N. the two are related by the equation 2.2.

$$N = \frac{L}{H} \tag{2.2}$$

where L is the length of the column packing (usually in centimeters).

Normally, the efficiency of chromatographic column increases as the number of theoretical plate become greater and as the plate height becomes smaller. Column type, mobile phase and stationary phase affected to the efficiency of column.

2.5.2.2 Rate theory

The van deemter equation can explain complex physical interactions and effects that lead to zone broadening and lower column efficiencies. The equation is defined as

$$H = A + \frac{B}{u} + Cu \qquad (2.3)$$

where H is the plate height in centimeters and u is the linear velocity of the mobile phase in centimeter per second. The term A is eddy diffusion which describes the multiple-path effect. While B term is the longitudinal diffusion coefficient and C term is mass-transfer coefficient.

2.5.2.2.1 Eddy diffusion

The eddy diffusion occurs as a result of multiple flow paths through a packed column bed. Each analyte molecule travels through a column with different flow paths, which causes dispersion (different path lengths). The band broadening is independent of flow rate, but depends on the arrangement and sizes of particles within the column; band broadening due to eddy diffusion increases for poorly packed columns. The eddy diffusion is minimal, when small spherical particles materials are packed uniformly in a column.

2.5.2.2.2 Longitudinal diffusion

The longitudinal diffusion in column chromatography is a band broadening process in which solutes diffuse from the concentrated center of a zone to the more dilute regions ahead of and behind the zone center that is toward and opposed to the direction of flow of the mobile phase. Peak broadening always contributes at very low flow rates below the optimum plate height. Moreover, the longitudinal diffusion can cause band width increasing with time, and this occurs

whether or not the mobile phase is flowing. The time spent by the band during its passage through the column varies inversely with the flow rate, so the contribution to band width from longitudinal diffusion decreases for faster flow.

2.5.2.2.3 Mass transfer coefficients

This term relates to the mass transfer of sample component between the mobile phase and the stationary phase, which can be divided into two terms stationary phase mass transfer (C_s) and mobile phase mass transfer (C_m).

2.5.2.2.3.1 The stationary phase mass transfer term (C_s)

It influences when the stationary phase is immobilizes liquid. With smaller C_s, the support particles must have thin film as well as the diffusion coefficient of solute in the film must have large value. Some sample molecules will penetrate further into a particle pore (by diffusion) and spend longer time before leaving the particle. During this time other molecules will have moved a shorter distance into the particle and spent less time before leaving the particle. Molecules that spend less time in the particle will move further along the column, with a consequent increase in band width. This contribution to band broadening increases as the flow rate increases.

2.5.2.2.3.2 The mobile phase mass transfer term (C_m)

This term describes the contribution to peak broadening in the mobile phase. Because the linear velocity of the mobile phase is lower closer to the column walls (or the stationary phase particles) than in the center (or further away from the particles), the analyte molecules experience different velocities. This phenomenon shows peak broadening.

2.5.3 Separation

2.5.3.1 Selectivity

The selectivity (α) is defined as the column capacity to separate between two peaks of analytes. It can characterize from the ratio of retention factor of an examined band (solute B) and an adjacent band (solute A) in which solute B is a strongly retained species and solute A is a rapidly eluted species. According to this definition, α is always greater than unity. If α = 1, it means the analytes have the same retention time and co-elute. The selectivity (α) can be calculated by equation 2.4.

$$\alpha = \frac{k'_B}{k'_A} \tag{2.4}$$

Although the selectivity (α) describes the ability of column to separate between two peaks of analyte. It evaluates from the band center and does not account with peak width.

2.5.3.2 Shape selectivity

Shape selectivity has been described as the two separate manifestations of steric exclusion: steric interaction [49]. The stationary phase can indicate this chromatographic property based on their molecular structures rather than other physical or chemical differences of the solutes [50].

Slot model is one of the retention models for explaining the shape selectivity behavior. Planar molecules are preferentially retained over their nonplanar molecules because they interact more strongly with the bonded phase than the nonplanar molecules. The bonded phase consists of a number of narrow “slot” into which the solute molecules can penetrate, as can be seen in Figure 2.5. Planar molecules would be able to fit more easily into these narrow slots and interact strongly with the stationary phase. Whereas the nonplanar molecules would not

penetrate as far into the slots, and so interact less strongly with the stationary phase [51].

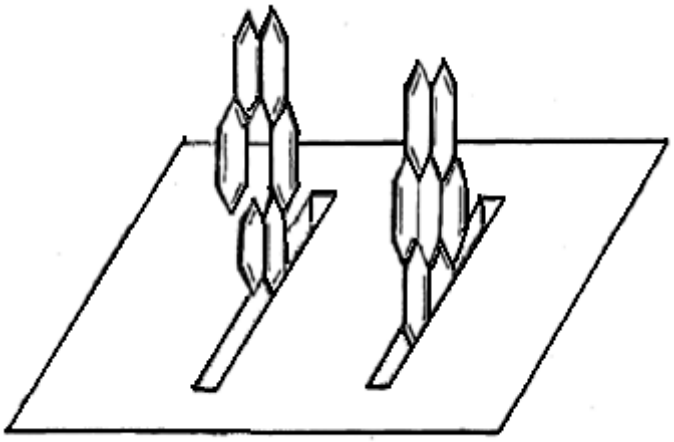


Figure 2.5 Slot model [51].

2.5.3.3 Resolution

The resolution (R_s) of a column is a quantitative measurement of the ability of the column to separate two bands of analytes which are related to their widths. The resolution (R_s) of a column is defined as equation 2.5.

$$R_s = 1.18\left(\frac{t_{R1} - t_{R2}}{w_{h1} - w_{h2}}\right) \tag{2.5}$$

where w_{h1} and w_{h2} are the bandwidth at half-height (min) for adjacent bands 1 and 2. t_{R1} and t_{R2} are the retention times for adjacent bands 1 and 2 (min). Baseline resolution is the resolution value which is equal or above 1.5 for two peaks with similar size.

CHAPTER III

Experimental

3.1 Instrument and apparatus

3.1.1 Liquid chromatography (LC) system consists of

- Jasco PU-980 Intelligent HPLC pump, Japan
- Rheodyne 7125 injector, Germany
- Jasco UV-2075 plus intelligent UV-Visible Detector, Japan
- Power Chrom 280 Data acquisition

In this work, the conventional LC system was modified for the use of capillary column by put in a T-splitter between an injector and a capillary column as shown in Figure 3.1. This will reduce the volumetric flow of mobile phase to fit a capillary column.

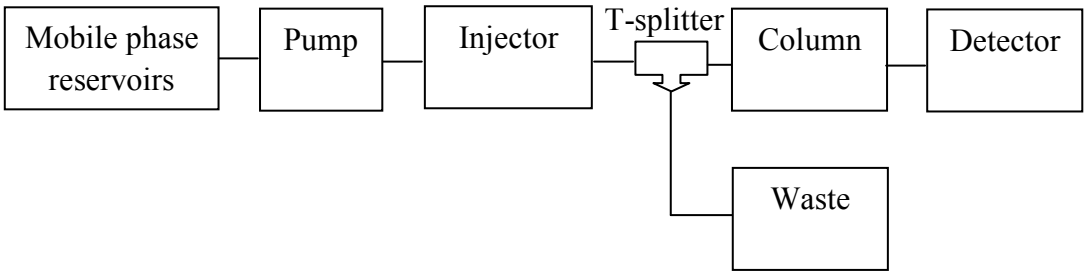


Figure 3.1 The schematic diagram of a capillary liquid chromatography.

3.1.2 Milli-Q, Ultrapure water systems, with Millipak® 40 Filter unit 0.22 µm, Model Millipore ZMQS5VOOY, Millipore, Billerica, MA, U.S.A.

3.1.3 pH meter, Model 744, Metrohm, Herisau, Switzerland.

3.1.4 Micropipettes 10-100, 100-1000 µm and tips, Eppendorf, Hamburg, Germany.

3.1.5 Volumetric flask 10.00, 25.00, 250.00 and 1000.00 mL

3.1.6 Beakers 10, 100, 250, 500, 1000 mL.

3.2 Chemicals

3.2.1 Preparation of a mixed-mode monolithic silica capillary column

Tetramethoxysilane (TMOS), methyltrimethoxysilane (MTMS), poly(ethylene glycol) (PEG, MW 10,000) (Merck, Germany) and urea (Sigma-Aldrich, Germany) were used to prepared the monolithic silica capillary column. The column was then modified with octadecylmethoxysilane (ODS) and aminopropyltrimethoxysilane (APTMS) (Acros Organics, Belgium). All reagents were in analytical grade.

3.2.2 Chromatographic characterization by Tanaka's test

Reagents in Tanaka's test are listed in Table 3.1, which were purchased from Fluka, Sigma-Aldrich and Merck. All probes were prepared in methanol at a concentration of 50 mg L^{-1}.

3.2.3 Separation performance of modified monolithic silica capillary columns

A mixture of ethylbenzene, propylbenzene, butylbenzene and pentylbenzene (Fluka, Germany) at a concentration of 50 mg L^{-1} in methanol and a mixture of 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and 3,4,5-trihydroxybenzoic acid (Sigma-Aldrich, Germany) at a concentration of 50 mg L^{-1} in methanol were used for a separation in the reversed phase mechanism. A mixture of 2-fluorobenzoic acid, 3-chlorobenzoic acid, and 3,4-dichlorobenzoic acid (Sigma-Aldrich, Germany) at a concentration of 50 mg L^{-1} in methanol and a mixture of iodide, iodate and bromide (Sigma-Aldrich, Germany) at a concentration of 50 mg L^{-1} in water were used for a separation in the anion exchange mechanism. The separation of basic compounds was tested by a mixture of aniline, *N*-methylaniline and *N, N*-dimethylaniline at a concentration of 50 mg L^{-1} in methanol. Lastly, the shape

selectivity of the column was tested by a mixture of triphenylene, benzo[a]anthracene and benzo[k]fluoranthene at a concentration of 50 mg L^{-1} in methanol.

3.3 Preparation of a mixed-mode monolithic silica capillary column

3.3.1 Preparation of a bared silica monolithic capillary column

A bared silica monolithic capillary column was prepared according to Kobayashi′s procedure [37], as can be seen in Figure 3.2. Briefly, a capillary column (300 cm × 198.5 μm I.D. × 354.4 μm O.D.) was pretreated with 1 M NaOH at 40 °C for 3 hours and washed with water. Then, the column was flushed with 1 M HCl at 40 °C for 2 hours and washed with water and acetone. A mixture of TMOS and MTMS (3:1), PEG, urea and acetic acid was introduced into the pretreated capillary column, and aged at 40 °C overnight. Then, the capillary column was treated with urea and heated from 40 °C to 120 °C at a rate of 1.33 °C/min and kept at 120 °C for 3 hours. Finally, the synthesized silica monolithic capillary column was washed with methanol to flush out other reaction residuals for 7 days, and heated at 330 °C for 24 hours.

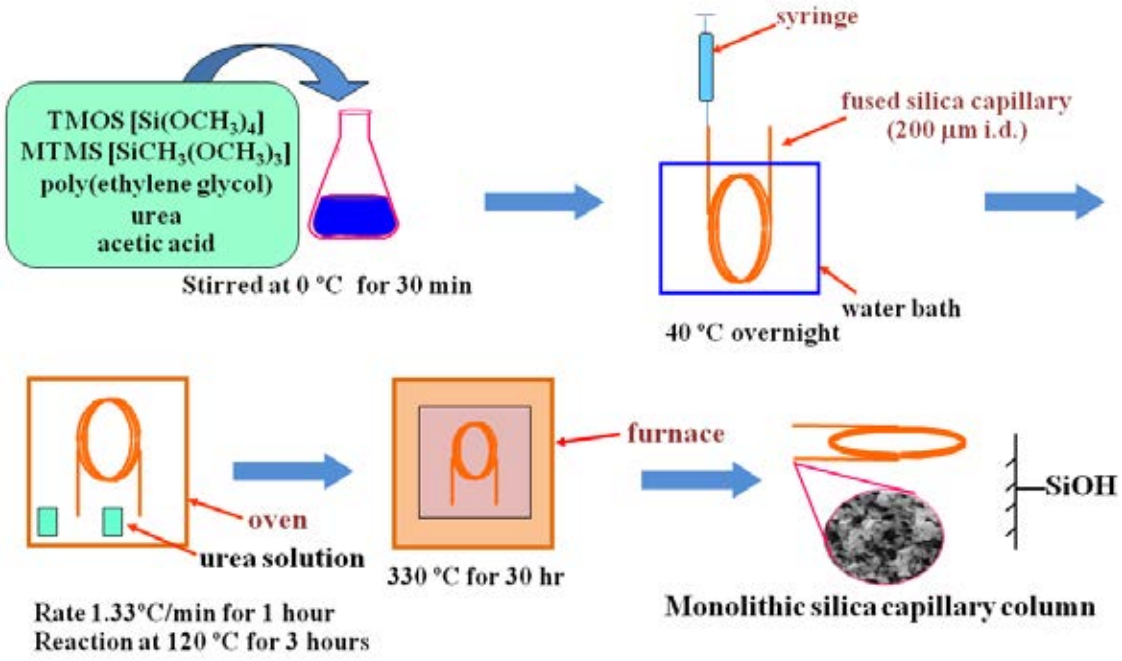


Figure 3.2 The diagram for preparation of monolithic silica capillary column

3.3.2 Modification of bared silica monolithic capillary column

A bared silica monolithic capillary column was functionalized with ODS and APTMS (C_{18}-AP column), which was followed the method previously reported by Ye et al. [28]. 10% (v/v) of a mixture of ODS and APTMS (9:1) in toluene was flowed through the monolithic silica capillary column by nitrogen gas for 1 hour, and then left the reaction to occur at 110 °C for 1 hour. This step was repeated three times and the final reaction was done overnight. Finally, the monolithic silica capillary column was flushed with methanol to remove unreacted reactants.

The monolithic silica capillary column functionalized with ODS (C_{18} column) was done with the same procedure as the C_{18}-AP column. 10% (v/v) of a ODS in toluene was applied instead of 10% (v/v) of a mixture of ODS and APTMS.

3.4 Chromatographic characterization by Tanaka's test

The chromatographic characterizations of modified monolithic silica capillary column (40 cm × 198.5 μm I.D. × 354.4 μm O.D.) were followed by Tanaka′s procedure [52], which commonly used to determine hydrophobicity, shape selectivity and polarity of reverse phase columns. Chromatographic characterization probes and a mobile phase system used for each test were summarized in Table 3.1.

Mobile phase of 30% CH_3OH was adjusted to pH 2.7 and 7.6 using 0.02 M phosphate buffer.

Table 3.1: Characterization scheme by Tanaka [52]

Properties of Stationary Phase	Parameter	Probes	Mobile Phase
Hydrophobicity	α_{CH2}	Pentyl benzene Butyl benzene	80% CH_3OH
Shape Selectivity	$\alpha_{T/O}$	Triphenylene o-terphenyl	80% CH_3OH
Ion Exchange Capacity at pH 2.7	$\alpha_{A/P}$	Benzylamine Phenol	30% CH_3OH, pH 2.7
Ion Exchange Capacity at pH 7.6	$\alpha_{A/P}$	Benzylamine Phenol	30% CH_3OH, pH 7.6
Silanophilic Interaction	$\alpha_{C/P}$	Caffeine Phenol	30% CH_3OH

3.5 Separation performance of the C_{18}-AP monolithic silica capillary columns by HPLC

The separation performances of the C_{18}-AP column were examined and compared to the C_{18} column. All separations were performed at the mobile phase linear flow velocity of 1 mm s^{-1}.

3.5.1 Reversed phase mechanism

3.5.1.1 Separation of alkylbenzene

Separation of alkylbenzene, containing 50 mg L^{-1} of ethylbenzene, propylbenzene, butylbenzene, and pentylbenzene was performed using a mobile phase of 80% methanol.

3.5.1.2 Separation of benzoic acid derivatives

Separation of benzoic acid derivatives consist of 4-hydroxybenzoic acid, 3,4- dihydroxybenzoic acid, and 3,4,5- trihydroxybenzoic acid. They were prepared in methanol at a concentration of 50 mg L^{-1}. Separation was performed using a mobile phase of MeOH: 0.02 M phosphate, pH 2.5 (25: 75).

0.02 M phosphate buffer pH 2.5 was prepared by dissolving 0.31 g. sodium dihydrogen phosphate (NaH_2PO_4) in 100 mL of water and adjusted pH of the solution using 0.02 M phosphoric acid (H_3PO_4).

3.5.2 Anion exchange mechanism

3.5.2.1 Separation of inorganic anions

Anions including iodide, iodate and bromide, were separated using a mobile phase of MeOH: 0.05 M phosphate, pH 3.0 (10: 90).

0.05 M phosphate buffer pH 3.0 was prepared by dissolving 0.78 g. sodium dihydrogen phosphate (NaH_2PO_4) in a 100 mL of water and was adjusted pH of the solution using 0.05 M phosphoric acid (H_3PO_4).

3.5.2.2 Separation of benzoate anions

Benzoic acid derivatives including 2-fluorobenzoic acid, 3-chlorobenzoic acid, and 3,4-dichlorobenzoic acid, were separated using a mobile phase of MeOH: 0.02 M phosphate, pH 6.7 (60: 40).

0.02 M phosphate buffer pH 6.7 was prepared by dissolving 0.28 g disodium hydrogen phosphate (Na_2HPO_4) in a 100 mL of water and was adjusted to the required pH using 0.02 M sodium dihydrogen phosphate (NaH_2PO_4).

3.5.3 Separation of basic compounds

Separation of aniline derivatives containing aniline, *N*-methylaniline, and *N, N*-dimethylaniline was performed using a mobile of MeOH: 0.01 M phosphate, pH 7.5 (55: 45).

0.01 M phosphate buffer pH 7.5 was prepared by dissolving 0.14 g. disodium hydrogen phosphate (NaH_2PO_4) in a 100 mL of water and was adjusted pH of solution using 0.01 M phosphoric acid sodium dihydrogen phosphate (NaH_2PO_4).

3.5.4 Shape selectivity

Separation of polycyclic aromatic hydrocarbons (PAHs) was performed to evaluate the shape selectivity of the columns. A mixture of PAHs containing 50 mg L^{-1} of triphenylene, benzo[a]anthracene, and benzo[k]fluoranthene was separated using a mobile phase of 60% and 70% MeOH

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preparation of the mixed-mode monolithic silica capillary column

In this work, a monolithic silica capillary column was first prepared from a mixture of tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMS) according to Hara et al [21] to overcome the problem of shrinkage of silica skeleton when prepared in a large internal diameter capillary column (> 50 μm) [53]. Figure 4.1 shows SEM images of the cross-section of the prepared a monolithic silica capillary column. The phase homogeneity which has co-continuous morphology of silica skeletons and through-pore was obtained. Moreover, the good attachment of silica monoliths to the inner wall of the capillary column was observed.

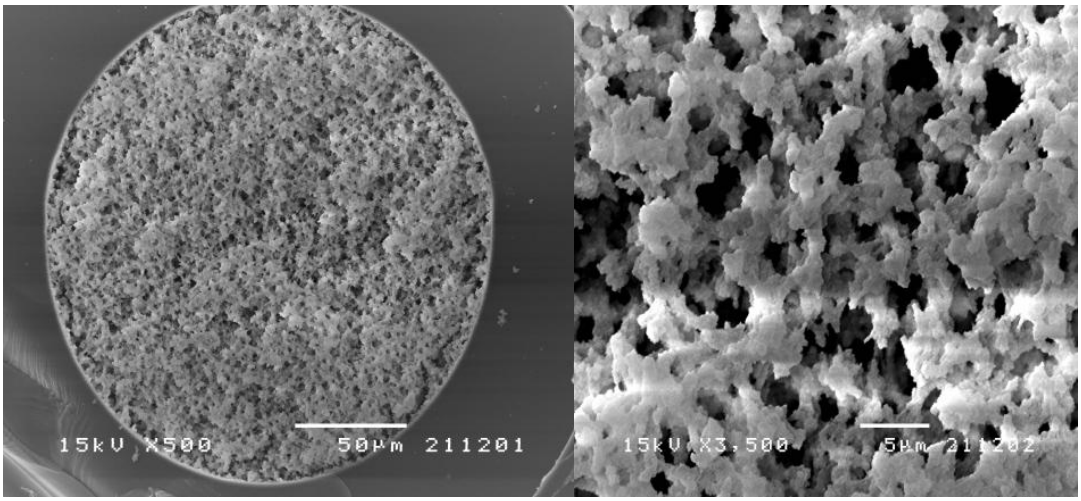


Figure 4.1 Scanning electron micrograph (SEM) images of the cross-sectional view of a bared silica monolithic capillary column.

Even though the morphology of the prepared monolithic silica capillary column was satisfied, it showed a poor efficiency (acceptable plate height, H, ≤ 10 μm.). This may be caused from the poor temperature control during the stage of mesoporous formation [40] when raising the temperature slowly from 40 to 120 °C for 1 hour in urea. So, Wasura, my colleague and a Ph.D. candidate at Department of Chemistry, Faculty of Science, Chulalongkorn University, gave an acceptable efficiency monolithic silica capillary columns which was synthesized and evaluated the column efficiency at Tanaka's laboratory as a gift to modify and study in this work. However, the plate height of the monolithic silica capillary column obtained from our LC system was 5-10 μm higher than that evaluated at Tanaka's laboratory. This may cause by the difference of the detection system. On-column detector was set at Tanaka's laboratory while our LC system used a u-cell shape in the detector which caused an extra column band broadening. Therefore, the value of plate height after surface modification evaluated by our LC system was normalized by the factor of this extra column band broadening.

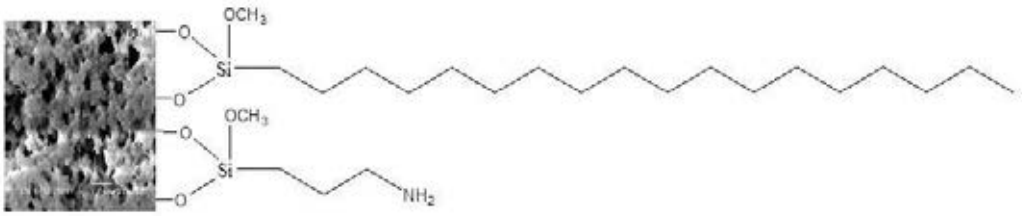
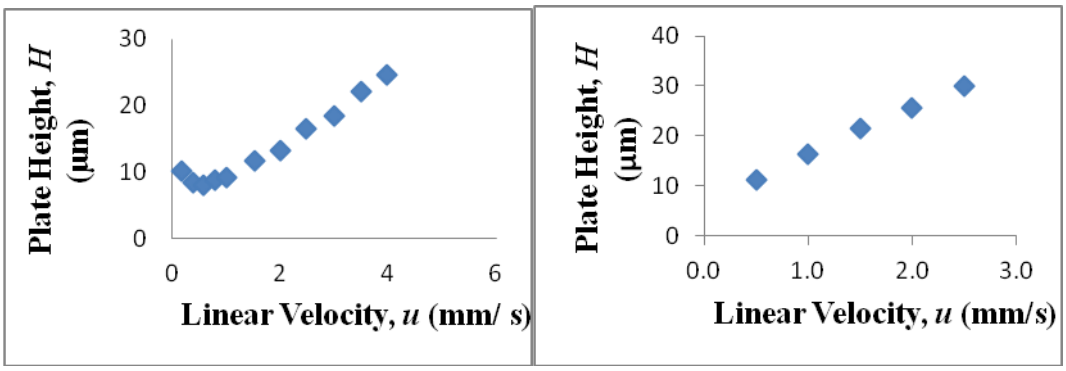


Figure 4.2 The proposed structure of stationary phase in the C_{18} –AP column.

The stationary phase containing octadecyl and aminopropyl groups in the monolithic silica capillary column (C_{18}–AP column) was functionalized by the mixture of octadecylmethoxysilane (ODS) and aminopropyltrimethoxysilane (APTMS) at a ratio of 9:1 because this ratio gave better both hydrophobic and anion-exchange according to the report by Ye et al [28]. The proposed structure of mixed-mode monolithic silica stationary phase embedded with the octadecyl group for a reversed phase mechanism (RP) and the aminopropyl group for a weak anion exchange mechanism (WAX) is shown in Figure 4.2, which was adapted from Ye et al [28].

The plate heights as a function of linear velocity of mobile phase (van Deemter plot) were measured and plotted. Figure 4.3 (a) shows the van Deemter plot for stationary phase C_{18} column which obtained from Wasura's results and performed at Tanaka's laboratory. This curve illustrated a V-shape corresponding to the chromatographic theory. The optimum efficiency (minimum *H*) was achieved at a linear velocity of 0.5 mm/s. Figure 4.3 (b) is the van Deemter plot for the C_{18}–AP column which is the mixed-mode RP/WAX column. This curve did not present a V-shape yet because the lowest linear velocity performed for this column was 0.5 mm/s. Though, similar curve to that of the C_{18} column would be observed if linear velocities lower than 0.5 mm/s were carried out.



(a) (b)

Figure 4.3 Van Deemter plots obtained from the C_{18} column (a) which is obtained from Wasura's result [24] and C_{18}–AP column (b) with uracil as a solute. Mobile phase: 80% methanol.

The pressure drop and permeability of the C_{18}–AP column were further investigated. The relationship between a linear velocity and a column pressure drop for C_{18}–AP column in 80% methanol at 25 °C is shown in Figure 4.4. The increase in linear velocity of mobile phase produced a higher pressure drop of the column.

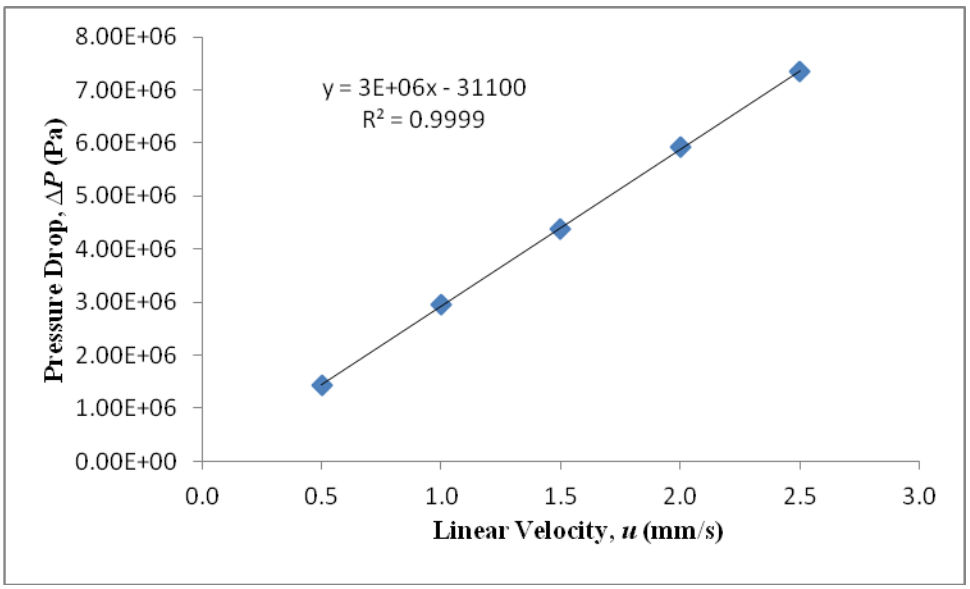


Figure 4.4 A plot of the column back pressure against the linear velocity of mobile phase. Mobile phase: 80% methanol.

The permeability (K) was calculated using Eq. (4.1), where u, η, L, $\mathcal{E}$, and ΔP stand for the linear velocity of the mobile phase, the viscosity of the mobile phase, the column length, the porosity of the column and the column backpressure, respectively [54]. This parameter describes the degree of obstruction that the column bed opposes to the stream of mobile phase.

$$K = \frac{u\eta L\varepsilon}{\Delta P} \tag{4.1}$$

Normally, the porosity ($\mathcal{E}$) presumes to be 0.90 [55] for a monolithic silica capillary column and 0.65 for a particle-packed column [55, 56]. The viscosity of the mobile phase in this study, which was 80% methanol, was 1.12 x 10^{-3} Pa.s [40]. Thus, the C_{18}–AP column modified with ODS and APTMS possessed K value of 13.0 x 10^{-14} m^2. From the correlation between average pore size, skeleton size, and permeability of monolithic silica capillary columns in Table 4.1, the average macropore size and skeleton size in the C_{18} –AP column can be approximated to be ~ 2.0 µm and ~1.0 µm, respectively.

Table 4.1 Correlation between average pore size, skeleton size, and permeability of monolithic silica capillary columns. [57-58]

Permeability (10^{-14} m^2)	Macropore size (μm)	skeleton size (μm)
130	8.0	2.0
56	4.5	2.0
25	2.8	1.4
19	2.0	1.5
15	2.2	1.1
8	2.0	1.0

The permeability of monolithic silica capillary column is normally reported greater than 8×10^{-14} m^2 [59], while a particle packed column (silica based, 5 μm particle) has a permeability of 2 x 10^{-14} m^2 [55]. Therefore, the C_{18}-AP column (K = 13.0 x 10^{-14} m^2) provided a good permeability and better than that of the particle packed column ~6 times. This would benefit the fast separation at high linear velocities or highly efficient separation using long column.

Separation impedance (E) which describes the total performance of column based on the required time and column backpressure to produce one theoretical plate is calculated by Eq. (4.2) where t_0, ΔP, η, N and H stand for the elution time of an unretained solute, the column backpressure, the viscosity of the mobile phase, the theoretical plate number, and plate height, respectively.

$$E = \frac{t_0 \Delta P}{N^2 \eta} = \left(\frac{\Delta P}{N}\right) \times \left(\frac{t_0}{N}\right) \times \left(\frac{1}{\eta}\right) = \frac{H^2}{K} \qquad (4.2)$$

From Eq. (4.2), the C_{18}-AP column at a linear velocity of 1 mm/s gave E of 2,097 while the particle packed column provided E ~6,000 [59]. This can conclude that the C_{18}-AP column provided higher separation efficiency (lower separation impedance) and permeability at lower column backpressure compared to a column packed with 5 μm particles.

4.2 Chromatographic characterizations

Recently, a lot of researches have been presented novel synthesized monolithic column characterized by different techniques, such as diffused IR spectra, scanning electron microscope (SEM), elemental analysis, etc. However, the chromatographic test using chemical probes provides better information and demonstrates the actual chromatographic behavior of the columns. Therefore, this work aimed to characterize the chromatographic behaviors of a C_{18}-AP column containing octadecyl and aminopropyl groups as a stationary phase and compared to a C_{18} column containing an octadecyl group as a stationary phase by Tanaka's test. Retention factors of compounds and values of parameters in Tanaka's test for both C_{18} and C_{18}-AP columns are summarized in Table 4.2.

Table 4.2 Chromatographic characterizations of C_{18} and C_{18}-AP columns by Tanaka's test.

Properties of Stationary Phase	Probes	C_{18} column		C_{18}-AP column	
		k'	α	k'	α
Hydrophobicity	amylbenzene	0.458	1.35±0.01	0.552	1.40±0.02
	butylbenzene	0.339		0.394	
Shape Selectivity	triphenylene	0.633	1.41±0.04	0.812	2.06±0.02
	o-terphenyl	0.450		0.393	
Ion Exchange Capacity at pH 2.7	benzylamine	0.045	0.16±0.01	(-0.101)	(-0.18)±0.01
	Phenol	0.286		0.558	
Ion Exchange Capacity at pH 7.6	benzylamine	0.550	2.07±0.01	0.280	0.37±0.08
	Phenol	0.266		0.768	
Silanophilic Interaction	Caffeine	0.292	1.11±0.01	0.365	0.48±0.02
	Phenol	0.263		0.767	

4.2.1 Hydrophobicity

The hydrophobicity of column can be evaluated from the ratio of retention factors of amylbenzene and butylbenzene, which is the selectivity of methylene increment (α_{CH2}). The retention factors of amylbenzene and butylbenzene on the C_{18} column (0.458 and 0.339) were lower than those of C_{18}-AP column (0.552

and 0.394). This may result from the C_{18}-AP column having an aminopropyl group which also provides hydrophobicity as an octadecyl group. So, there is higher carbon content and density of ligands to retain non-polar compound compared to the C_{18} column. However, α_{CH2} of both columns were similar (1.35±0.01 for C_{18} column and 1.40±0.02 for C_{18}-AP column). This means both columns have similar hydrophobicity, which would result in similar separation performance based on the reversed phase mechanism.

4.2.2 Shape selectivity

The shape selectivity is defined as the capability to differentiate between planar and nonplanar analytes of similar molecular weights and sizes. It can be calculated from the ratio of the retention factors of triphenylene (planar) and *o*-terphenyl (nonplanar), ($\alpha_{TR/O}$). As shown in Table 4.2, the retention of triphenylene on C_{18}-AP column ($k \leftarrow 0.812$) was higher than that on C_{18} column ($k \leftarrow 0.633$) while the retention of *o*-terphenyl on C_{18}-AP column ($k \leftarrow 0.393$) was lower than that on C_{18} column ($k \leftarrow 0.450$). As a result, the shape selectivity of C_{18}-AP column ($\alpha_{TR/O}$ = 2.06±0.02) was better than that of C_{18} column ($\alpha_{TR/O}$ = 1.41±0.04). It implied that C_{18}-AP column, containing octadecyl and aminopropyl groups, has more efficiency to differentiate between planar and nonplanar compounds than the C_{18} column, containing only an octadecyl group. The higher shape selectivity may arise from the strong interaction of planar compounds with the stationary phase explained by the slot model of shape selectivity. The solute molecules can penetrate through the slot on the C_{18}-AP column better than on the C_{18} column because it has aminopropyl group which showed electrostatic interaction with solute molecules.

4.2.3 Ion exchange capacity and silanophilic interactions

In this part, ion exchange capacity and silanophilic interactions of the column were evaluated from the ratio of the retention factors of benzylamine and phenol ($\alpha_{A/P}$) using the mobile phase of 30% methanol at pH 2.7 and pH 7.6. In

Tanaka's test, the retention behaviors at pH lower than 3 were observed to evaluate silanol activity. At this pH, the majority of the residual silanols was protonated and can react with positively charge analytes. The retention behaviors at pH higher than 7 were observed to evaluate ion exchange capacity in which most of residual silanols were deprotonated and ionized.

At a pH 2.7 condition, benzylamine eluted before the void volume marker on the C_{18}-AP column and resulted in the k' of -0.101 while phenol can retain on the column ($k' = 0.558$). In comparison to the C_{18} column, lower silanol activity was achieved for the C_{18}-AP column. This can be affected from the protonation of aminopropyl group which functionalized on the C_{18}-AP column. The ionic repulsion by an electrostatic interaction between amionpropyl group on the stationary phase and benzylamine was occurred and resulted in shielding of the silanols. At a pH 7.6 condition, benzylamine retained better than phenol on the C_{18} column, whereas it occurred in a reversed elution order on the C_{18}-AP column. This can explain that the silanol activity of free silanol group on the C_{18} column interacted with the protonated benzylamine while the C_{18}-AP column has a positive charged aminopropyl group which provided repulsive electrostatic interactions with protonated benzylamine.

The silanophilic interaction was evaluated from the ratio of retention factors of caffeine and phenol ($\alpha_{C/P}$) using the mobile phase of 30% methanol. The C_{18} columns ($\alpha_{C/P} = 1.11 \pm 0.01$) gave more $\alpha_{C/P}$ than the C_{18}-AP column ($\alpha_{C/P} = 0.48 \pm 0.02$). But again, a reversed elution order of caffeine and phenol on C_{18} and C_{18}-AP columns were observed, similar to the separation of benzylamine and phenol at pH 7.6 condition. This can be explained that the C_{18} column has silanophilic interaction. But the C_{18}-AP column presented the complicated interaction caused by a repulsive electrostatic interaction of the aminopropyl group functionalized on the stationary phase.

Even though the tests are useful to probe polarity and silanophilic interactions in conventional RP column as in C_{18} column, it may less inform for

mixed-mode RP/WAX column as in C_{18}-AP column because WAX function may exert shielding effect which may prohibit basic compounds to penetrate to the residual silanols. However, it demonstrated the influence of WAX site for retention of basic compounds in C_{18}-AP column.

4.3 Separation performance of the C_{18} and C_{18}-AP monolithic silica capillary columns by HPLC

4.3.1 Reversed phase mechanism

A reversed phase mechanism of the mixed-mode monolithic silica capillary column (C_{18}-AP column) was investigated by separation of neutral and hydrophobic solutes (alkylbenzenes) and acidic compounds at a low pH condition (benzoic acid derivatives) and compared to the RP stationary phase (C_{18} column).

4.3.1.1 Separation of alkylbenzenes

Figure 4.5 shows chromatograms obtained from separation of alkylbenzenes with the C_{18} and C_{18}-AP columns. The elution order of these compounds on both columns was similar, which is thiourea < ethylbenzene < propylbenzene < butylbenzene < pentylbenzene, according to their hydrophobicity. Moreover, both columns exhibited the similarity in selectivity and separation performance as shown in Table 4.3. The reversed phase mechanism in the C_{18}-AP column was achieved and similar to the C_{18} column.

Table 4.3 Retention time and selectivity of alkylbenzene on the C_{18} and the C_{18}-AP columns.

Peaks	Solutes	C_{18} column		C_{18}-AP column	
		t_R	k′	t_R	k′
1	Thiourea	6.62		6.52	
2	Ethylbenzene	7.90	0.1934	7.98	0.2239
3	Propylbenzene	8.30	0.2538	8.52	0.3067
4	Butylbenzene	8.87	0.3399	9.29	0.4248
5	Pentylbenzene	9.65	0.4577	10.38	0.5920

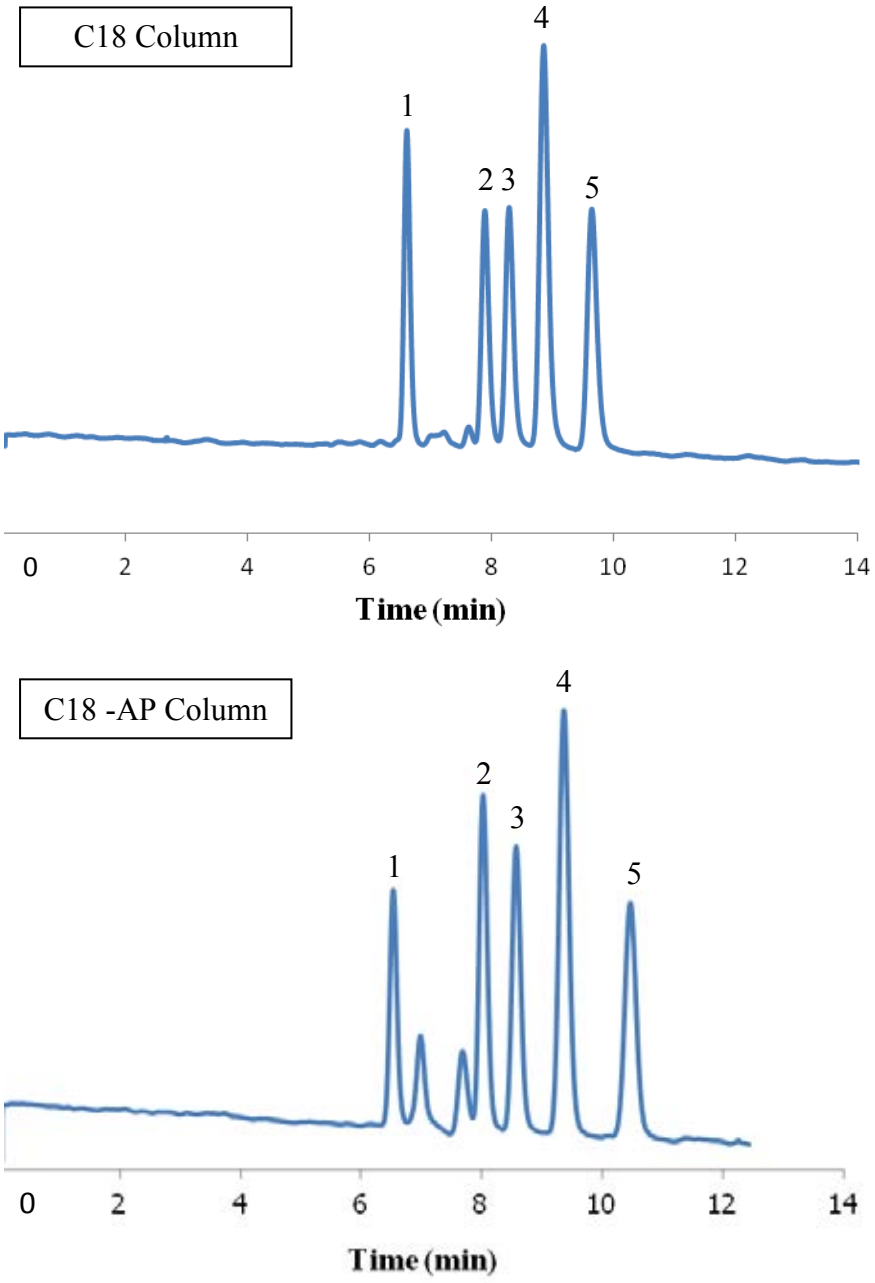


Figure 4.5 The separation of alkylbenzene derivatives mixture composed of (1) thiourea; (2) ethylbenzene; (3) propylbenzene; (4) butylbenzene and (5) pentylbenzene using C_{18}- and C_{18}–AP columns. Conditions: mobile phase, 80% MeOH; linear velocity: u=1.0 mm/s, detection wavelength = 210 nm.

4.3.1.2 Separation of benzoic acid derivatives

The analysis of acidic compounds on the C_{18}-AP column carried out by using mobile phase at low pH. The acidic mobile phase is used to suppress the ionization of acidic compounds which would lead to ion exchange interaction between a negatively charged solute and positively charged WAX moieties. Therefore, the benzoic acid derivatives were separated using methanol: 0.02 M phosphate buffer pH 2.5 (25:75). In this condition, the analytes were neutral according to their degrees of ionization (α), as shown in Table 4.4. Therefore, the separation of benzoic acid derivatives on the C_{18}-AP columns should be mainly based on the typical reversed phase mechanism. Chromatograms of the separation using the C_{18} and C_{18}-AP columns are shown in Figure 4.6. The order of elution was thiourea < 3,4,5 trihydroxybenzoic acid < 3,4 dihydroxybenzoic acid < 4-hydroxybenzoic acid which related to log P_{ow} which is the distribution coefficient of a neutral compound between octanol (nonpolar phase) and water (polar phase) [31]. Retention time (t_R) and retention factor (k′) on both columns were comparable as can be seen in Table 4.5.

Table 4.4 pK_a, Log octanol/water coefficients (Log P_{ow}), and degree of ionization (α) of benzoic acid derivatives.

Solute	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}	log P_{ow}	α [a] (%)
4- hydroxybenzoic acid	4.58	9.23	-	-	1.37	0.008
3,4-dihydroxybenzoic acid	4.50	8.70	12.80	-	1.15	0.010
3,4,5-trihydroxy benzoic acid	4.48	4.47	4.47	4.41	0.70	0.012

Note[a]: the degree of ionization (α) was calculated on the examined condition (mobile phase, MeOH: 0.02 M phosphate, pH 2.5 (25: 75))

Table 4.5 Retention time and selectivity of alkylbenzene on the C_{18} and the C_{18}-AP columns.

Peaks	Solutes	C_{18} column		C_{18}-AP column	
		t_R	k′	t_R	k′
1	Thiourea	6.57		6.42	
2	gallic acid	6.92	0.0533	6.86	0.0685
3	3,4-dihydroxybenzoic acid	7.45	0.1339	7.47	0.1636
4	p-hydroxybenzoic acid	8.39	0.2770	8.52	0.3271

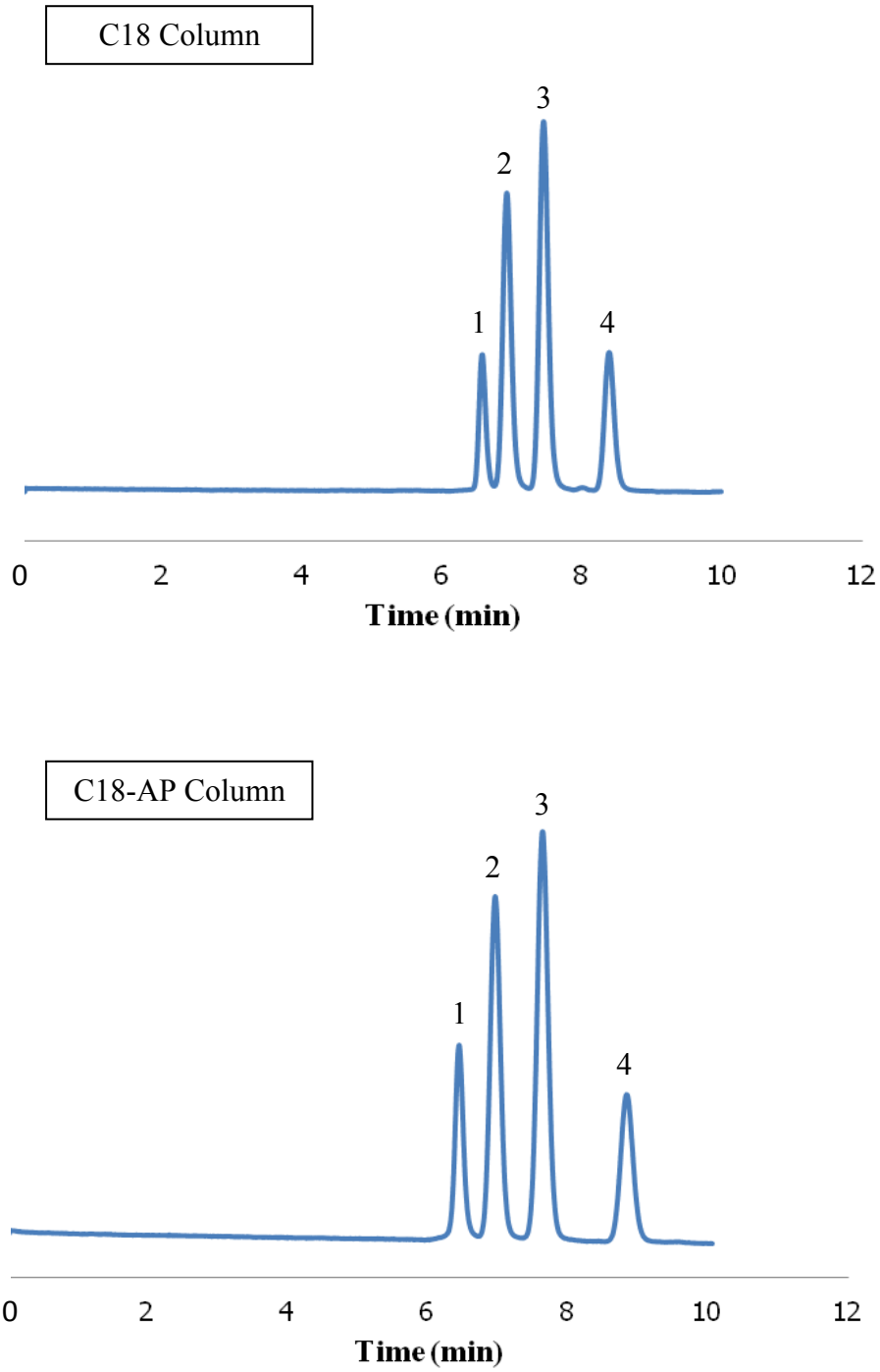


Figure 4.6 The separation of benzoic acid derivatives mixture composed of (1) thiourea; (2) 3,4,5-trihydroxybenzoic acid; (3) 3,4-dihydroxybenzoic acid; (4) 4-hydroxybenzoic acid using C_{18}- and C_{18}–AP columns. Conditions: mobile phase, MeOH: 0.02 M phosphate, pH 2.5 (25: 75); linear velocity: u=1.0 mm/s, detection wavelength = 210 nm.

4.3.2 Anion exchange behavior

4.3.2.1 Separation of inorganic anions

Generally, inorganic anions cannot separate on conventional C_{18} column unless using ion pair information or separated on an anion exchange column [60-61]. From the proposed structure of stationary phase in C_{18}-AP column, it was also possible for separation of inorganic anions because of the aminopropyl group (WAX). Figure 4.7 shows the chromatogram for the separation of inorganic anions mixture consists of iodate, bromide and iodide on the C_{18}-AP column. These solutes eluted according to their ionic strength as the elution order was iodate < bromide < iodide.

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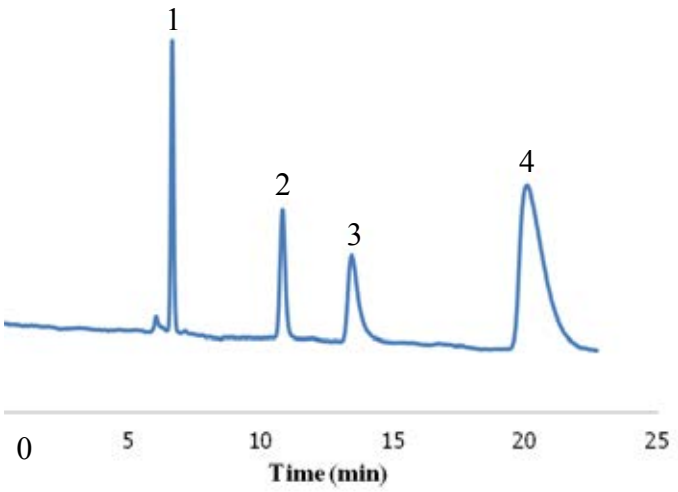

Figure 4.7 The separation of an inorganic anion mixture composed of (1) thiourea; (2) iodate; (3) bromide; (4) iodide on the C_{18}–AP column. Conditions: mobile phase, MeOH: 0.05 M phosphate, pH 3.0 (10: 90); linear velocity: u=1.0 mm/s, detection wavelength = 210 nm.

4.3.2.2 Separation of benzoate anions

The separations of benzoate anions on the C_{18}- and C_{18}-AP columns were performed using a mobile phase of MeOH: 0.02 M phosphate, pH 6.7 (60: 40). At this condition, the analytes were completely deprotonated according to

their degree of ionization (α) as shown in Table 4.6. The benzoate anions were hard to separate on the C_{18} column while the separation on C_{18}-AP column can be achieved as shown in Figure 4.8. Symmetrical peak shape and good efficiency were observed on the C_{18}-AP column, which may be attributed to WAX mechanism via aminopropyl group (WAX). The contributions of weak electrostatic interaction such as dipole-dipole interaction with benzoate anions and hydrophobic interaction from octadecyl group result in different retentions of the benzoate anions. The elution order of analytes was 3-fluorobenzoic acid < 3-chlorobenzoic acid < 3,4-dichlorobenzoic acid because 3,4-dichlorobenzoic acid, which is disubstituted benzoic acid, was more polarity than monosubstituted benzoic acids (3-fluorobenzoic acid and 3-chlorobenzoic acid) and the affinity of 3- fluorobenzoic acid was more than that of 3-chlorobenzoic acid owing to its density of charge.

Table 4.6 pK_a, and degree of ionization (α) of benzoate anion derivatives

Solutes	pK_a	α^b (%)
3-fluorobenzoic acid	3.93	99
3-chlorobenzoic acid	3.82	100
3,4-dichlorobenzoic acid	3.70	99

Note[b]: the degree of ionization (α) was calculated on the examined condition (mobile phase, MeOH: 0.02 M phosphate, pH 6.7 (60: 40))

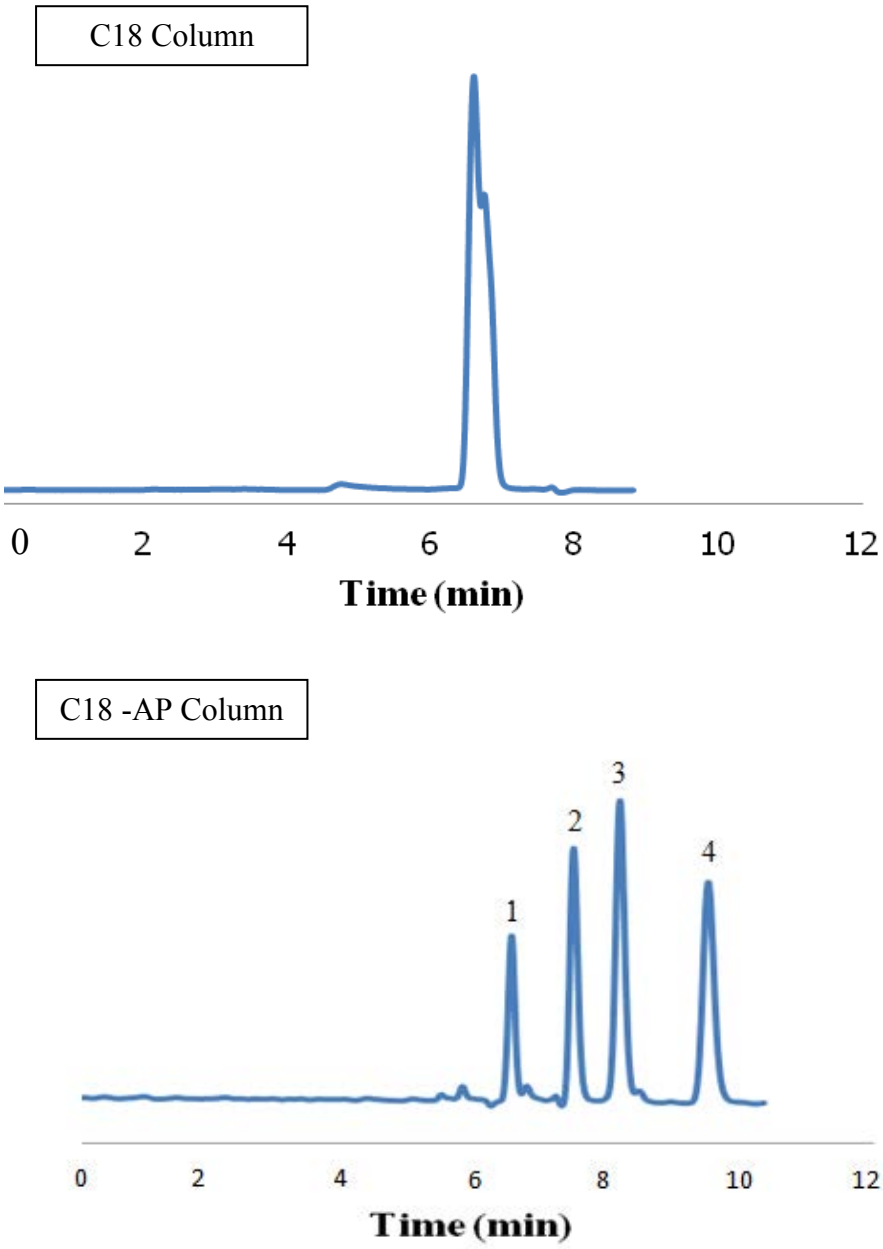

Figure 4.8 The separation of a benzoate anion mixture composed of (1) thiourea; (2) 3-fluorobenzoic acid; (3) 3-chlorobenzoic acid; (4) 3,4-dichlorobenzoic acid on the C_{18}- and C_{18} –AP columns. Conditions: mobile phase, MeOH: 0.02 M phosphate, pH 6.7 (60: 40); linear velocity: u=1.0 mm/s, detection wavelength = 210 nm.

4.3.3 Separation of basic compounds

Commonly, conventional RP silica-based columns are rarely used to separate basic and nitrogen-containing compounds at neutral pH mobile phase due to peak tailing. The mixed-mode RP/WAX monolithic column (C_{18}-AP column) is an alternative approach to overcome this problem. In this study, three aniline derivatives which were aniline, *N*-methylaniline, *N, N*-dimethylaniline were selected to investigate their peak shape as shown in Figure 4.9. It was observed that peak shape of all solutes were symmetrical on the C_{18}-AP column, but peak tailing on the C_{18} column as asymmetry factors in Table 4.7. For C_{18}-AP column were closer to one than those for C_{18} column. Aminopropyl groups on the surface of C_{18}-AP column minimized the adsorption of basic solutes to the residual silanols but it was not strongly enough to shielding the interaction of *N,N*-dimethylaniline to the silanols.

Table 4.7 Peak asymmetry of aniline derivertives on C_{18}- and C_{18}-AP column

Peaks	Solutes	C_{18} column	C_{18}-AP column
		Asymmetry	Asymmetry
2	Aniline	1.31	1.18
3	N-methylaniline	1.48	1.23
4	N, N-dimethylaniline	1.75	1.40

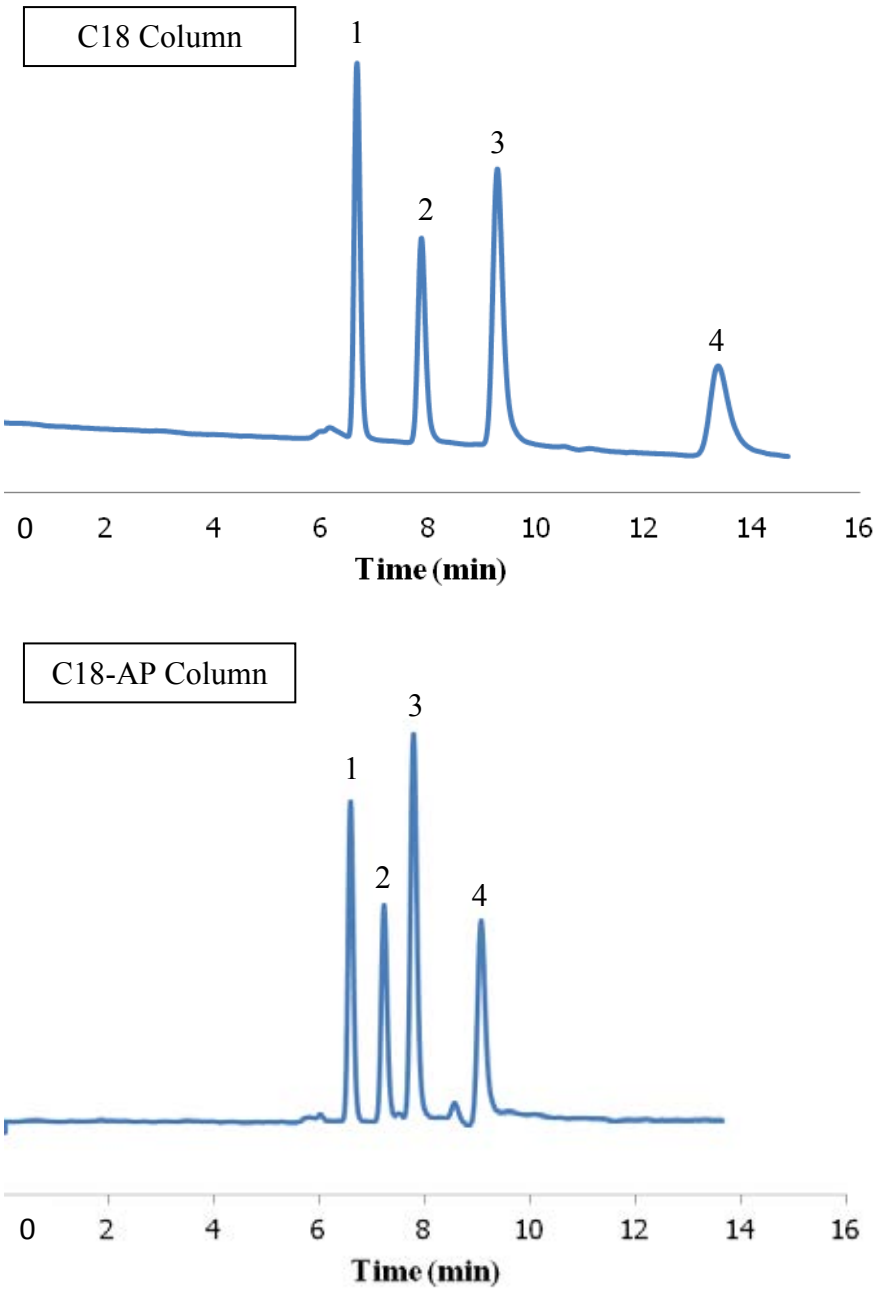

Figure 4.9 The separation of an aniline derivatives mixture composed of (1) thiourea; (2) aniline; (3) *N*-methylaniline; (4) *N, N*-dimethylaniline using C_{18}- and C_{18}–AP column. Conditions: mobile phase, MeOH: 0.01 M phosphate, pH 7.5 (55: 45); linear velocity: u=1.0 mm/s, detection wavelength = 210 nm.

4.3.4 Separation of polycyclic aromatic hydrocarbons (PAHs)

Separation of PAHs was performed to evaluate the shape selectivity performance of the C_{18}-AP column. A mixture of triphenylene, benz[a]anthracene, and benzo[k]fluoranthene was separated by C_{18} and C_{18}-AP columns using 70% methanol as a mobile phase. As shown in Figure 4.10, the separation resolution of triphenylene and benz[a]anthracene which are isomers on the C_{18}-AP column was better than that on the C_{18} column. It can explain that the C_{18}-AP column not only provide hydrophobic interaction from octadecyl group but also give weak electrostatic interaction from aminopropyl group. The positively charged aminopropyl group can interact with electrons that resonance on cyclic aromatic on PAHs. However, the separation on C_{18} column also can be achieved when increasing the content of water in mobile phase as shown in Figure 4.11, analysis time and band broadening of the peaks were increased.

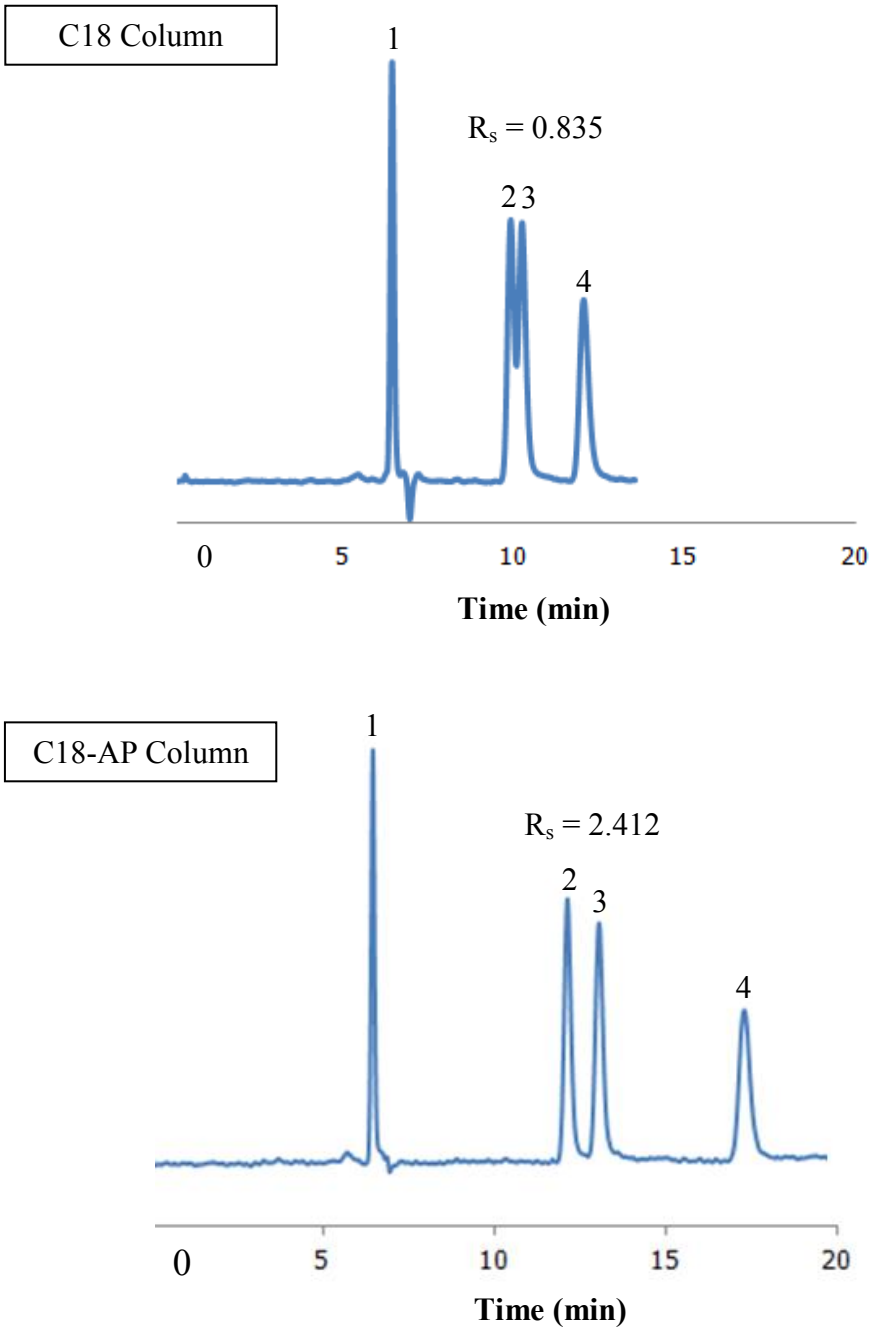

Figure 4.10 The separation of a PAHs mixture composed of (1) thiourea; (2) triphenylene; (3) benz[a]anthracene; (4) benzo[k]fluoranthene on C_{18} column and C_{18}–AP column. Conditions: mobile phase: 70% MeOH; linear velocity: *u*=1.0 mm/s, detection wavelength = 210 nm.

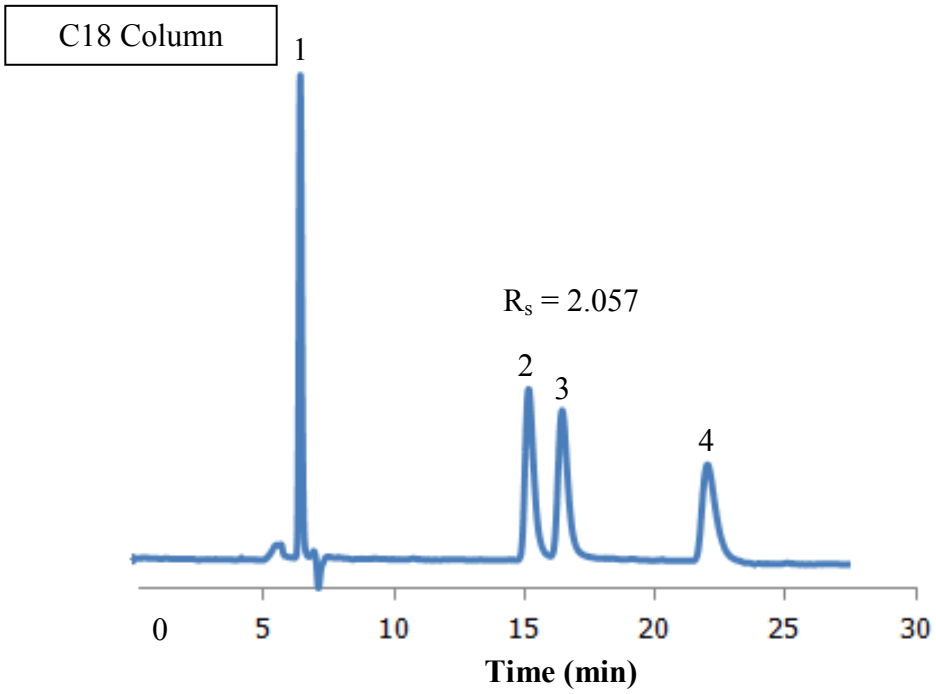

Figure 4.11 The separation of a PAHs mixture composed of (1) thiourea; (2) triphenylene; (3) benz[a]anthracene; (4) benzo[k]fluoranthene on C_{18} column. Conditions: mobile phase: 60% MeOH; linear velocity: u=1.0 mm/s, detection wavelength = 210 nm.

CHAPTER V

CONCLUSION

5.1 Conclusion

The mixed mode RP/WAX monolithic silica capillary column for liquid chromatography was first prepared via the sol-gel process to achieve bare silica monolith and then post modified with octadecyltrimethoxysilane and aminopropyltrimethoxysilane (abbreviated as C_{18}-AP column). The synthetic monolithic silica capillary column possessed a permeability, $K = 12.9 \times 10^{-14}$ m^2. Comparison to a particle packed column with 5 μm, the permeability of C_{18}-AP column showed ~6.5 times higher. From the permeability value, it can approximate macropore size and skeleton size of the C_{18} –AP column, to be 2- and 1 μm, respectively. The total performance can be described by separation impedance value (E) in which the C_{18}-AP column provided E = 2,097. This value can sum up that the C_{18}-AP column gave higher separation efficiency and permeability than the 5 μm, packed column at lower column backpressure. The chromatographic characterization on the C_{18}-AP column and monolithic silica capillary column modified with octadecyltrimethoxysilane (abbreviated as C_{18} column) were determined and compared according to Tanaka′s procedure. Although, the hydrophobicity of both columns was similar, the C_{18}-AP column has higher shape selectivity than C_{18} column. For ion exchange capacity and silanophilic interactions, the C_{18} column exhibited the silanol effect owning to large $\alpha_{C/P}$ as well as large $\alpha_{A/P}$ values above pH 7 while the C_{18}-AP column showed repulsive electrostatic interactions due to the functionalized of aminopropyl groups on the stationary phase. This can indicate that octadecyl- and aminopropyl groups were functionalized on silica monolithic surface successfully. Thus, the C_{18}-AP column showed both reversed phase (RP) chromatographic behavior and a weak anion exchange (WAX) characteristic. The separation performances of C_{18}-AP column were then examined and compared to those of C_{18} column. The separation of alkylbenzenes and benzoic acid derivatives at

low pH on both columns was based on reversed phase mechanism and both provide good performance. The separation of inorganic anions and benzoate anions which were hard to separate on the C_{18} column was successfully achieved on the C_{18}-AP column because of a weak anion exchange characteristic. Moreover, the improvement of peak tailing problem for separation of basic compounds on the C_{18}-AP column was observed. It can be explained that the aminopropyl group was shielded the adsorption of the positively charged basic compounds to the silanol group on the stationary phase. Furthermore, good resolution and efficiency on the separation of PAHs was observed on the C_{18}-AP column.

5.2 Suggestion of future work

Although mixed-mode monolithic silica capillary column was successful in improvement of peak tailing in separation of aniline derivatives, it cannot reduce peak tailing of *N,N*-dimethylaniline. This may be due to the fact that an aminopropyl group is a weak anion exchange group, it is not strong enough to minimize adsorption of basic compound. To enhance the separation performance of basic compounds in future work, it may require a strong anion exchange functional group.

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VITA

Miss Napug Samrejtuksing was born on Wednesday 29th October 1986, in Bankok, Thailand. She graduated with a Bachelor's degree of Science in Chemistry, from Thammasat University in 2009. Then she studied for a Master's degree of Science in Analytical Chemistry, the Department of Chemistry, the Faculty of Science, Chulalongkorn University. In 2012, she graduated with a Master's degree of Science.

Poster presentation and proceeding

"Preparation of the mixed-mode monolithic silica capillary column with octadecyl and aminopropyl groups for liquid chromatography" Napug Samrejtuksing, Thumnoon Nhujak and Puttaruksa Varanusupakul. Poster presentation and proceeding, Pure and Applied Chemistry International Conference (PACCON 2012), The Empress Hotels, Chiangmai, Thailand, 11-13 January, 2012.