IN-PIPETTE TIP MONOLITHIC MICRO-SOLID PHASE EXTRACTION COUPLED WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR SIMULTANEOUS DETERMINATION OF RACTOPAMINE AND CLENBUTEROL IN ANIMAL URINE AND MEAT SAMPLES

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การสกัดแบบใช้สารน้อยด้วยของแข็งโมโนลิธในปิเปตทิปร่วมกับโครมาโทกราฟีของเหลวสมรรถนะสูงสำหรับการหาปริมาณแรคโตพามีนและเคลนบูเทอรอลในตัวอย่างปัสสาวะและเนื้อสัตว์

นางสาวเกศรา อาษานอก

มหาวิทยาลัยเทคโนโลยีสุรนารี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2561

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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เกศรา อายานอก : การสกัดแบบใช้สารน้อยด้วยของแข็งโมโนลิธในปิเปตทิปร่วมกับโครมาโทกราฟีของเหลวสมรรถนะสูงสำหรับการหาปริมาณแรคโตพามีนและเคลนบูเทอรอลในตัวอย่างปัสสาวะและเนื้อสัตว์ (IN-PIPETTE TIP MONOLITHIC MICRO SOLID PHASE EXTRACTION COUPLED WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR SIMULTANEOUS DETERMINATION OF RACTOPAMINE AND CLENBUTEROL IN ANIMAL URINE AND MEAT SAMPLES) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.พัชรินทร์ ชัยสุวรรณ, 82 หน้า

แรคโตพามีนและเคลนบูเทอรอลเป็นสารกลุ่มเบตาอะโกนิสท์ ซึ่งถูกจัดเป็นสารพิษที่มีการลักลอบใช้งานอย่างผิดกฎหมายโดยเกษตรกรผู้เลี้ยงสัตว์ในประเทศไทย เพื่อลดปริมาณไขมันและเพิ่มปริมาณเนื้อแดงในสัตว์ เนื่องจากความเป็นพิษของสารกลุ่มนี้ทำให้วิธีวิเคราะห์เพื่อควบคุมคุณภาพของอาหารและป้องกันการลักลอบใช้งานของแรคโตพามีนและเคลนบูเทอรอลในการปศุสัตว์มีความจำเป็นอย่างยิ่ง ในงานวิจัยนี้พัฒนาการสกัดแบบใช้สารน้อยด้วยของแข็งโมโนลิธในปิเปตทิปร่วมกับโครมาโทกราฟีของเหลวสมรรถนะสูงสำหรับการหาปริมาณแรคโตพามีนและเคลนบูเทอรอลในตัวอย่างปัสสาวะและเนื้อสัตว์ โดยทำการแยกบนซิลิกาคอลัมน์ที่ดัดแปลงให้มีหมู่ฟังก์ชันเป็น C_8 และเฟสเคลื่อนที่เป็น 25% (v/v) อะซิโตรไนไทรล์ในอะซิเตตบัฟเฟอร์ความเข้มข้น 20 มิลลิโมลาร์พีเอช 4.0 ที่มีไตรเอทิลอะมีนความเข้มข้น 2 มิลลิโมลาร์ ที่สภาวะการแยกดังกล่าวสารทั้งสองสามารถแยกจากกันได้ในเวลา 7 นาที ตัวดูดซับโมโนลิธสำหรับการสกัดสังเคราะห์จากกรดเมทาอคริลิก (Methacrylic acid) ซึ่งทำหน้าที่กำหนดคุณสมบัติทางเคมี และเอทิลีน ไกลคอล ไดเมทาคริเลท (Ethylene glycol dimethacrylate) ที่เป็นตัวเชื่อมโยงโครงสร้างโมโนลิธในออโตปิเปตทิป โดยการสังเคราะห์มีองค์ประกอบและสภาวะที่เหมาะสมคือ อัตราส่วนมอนอเมอร์ต่อตัวทำละลายเท่ากับ 18:82 และทำการพอลิเมอร์ไรด้วยความร้อนที่อุณหภูมิ 75 องศาเซลเซียสเป็นเวลา 1 ชั่วโมง 10 นาที โมโนลิธในปิเปตทิปนี้ได้ถูกเชื่อมต่อเข้ากับวาล์แบบสามทางและกระบอกฉีดยาเพื่อใช้สำหรับการสกัด จากการศึกษาสภาวะที่เหมาะสมพบว่า 10% (v/v) อะซิโตรไนไทรล์ในน้ำ (ปริมาตร 800 ไมโครลิตร) และ 30% (v/v) อะซิโตรไนไทรล์ในอะซิเตตบัฟเฟอร์ความเข้มข้น 200 มิลลิโมลาร์พีเอช 4.0 (ปริมาตร 150 ไมโครลิตร) เป็นสารละลายที่เหมาะสมสำหรับขั้นตอนการล้างและชะสารตามลำดับ ความเที่ยงของการเตรียมโมโนลิธในปิเปตทิปเปรียบเทียบระหว่างการเตรียมในชุดเดียวกันและระหว่างชุด จากประสิทธิภาพการสกัดพบว่าร้อยละส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์มีค่าน้อยกว่า 3.18 และความเที่ยงในการวิเคราะห์ที่คำนวณจากพื้นที่ใต้พีคของสารภายในวันเดียวกันและระหว่างวันมีค่าร้อยละส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์

น้อยกว่า 3.26 ความจุของวัสดุโมโนลิธสำหรับแรคโตพามีนและเคลนบูเทอรอลมีค่ามากกว่า 10 และ 20 ไมโครกรัมตามลำดับ การสกัดแบบใช้สารน้อยด้วยของแข็งโมโนลิธในปิเปตทิปสามารถใช้งานซ้ำได้อย่างน้อย 10 ครั้ง โดยยังคงมีประสิทธิภาพการสกัดที่ดีและสามารถเก็บได้ที่อุณหภูมิห้องนานกว่า 6 เดือน สำหรับการโหลดตัวอย่างปริมาตร 2.00 มิลลิลิตรและใช้สารละลายสำหรับชะปริมาตร 150 ไมโครลิตร สามารถเพิ่มความเข้มข้นของสารทั้งสองได้ถึง 12 และ 13 เท่า มีประสิทธิภาพการสกัดร้อยละ 92 และ 100 และมีขีดจำกัดการตรวจวัดเท่ากับ 1.3 และ 0.2 ไมโครกรัมต่อลิตรสำหรับแรคโตพามีนและเคลนบูเทอรอลตามลำดับ การสกัดแบบใช้สารน้อยด้วยของแข็งโมโนลิธในปิเปตทิปร่วมกับโครมาโทกราฟีของเหลวสมรรถนะสูงได้ประยุกต์ใช้สำหรับการหาปริมาณแรคโตพามีนและเคลนบูเทอรอลที่เติมลงในตัวอย่างปัสสาวะและเนื้อสัตว์ พบว่ามีค่าร้อยละการคืนกลับที่ดีในช่วง 84-114 สำหรับตัวอย่างปัสสาวะสัตว์และ 89-112 สำหรับตัวอย่างเนื้อสัตว์ วิธีการสกัดแบบใช้สารน้อยด้วยของแข็งร่วมกับโครมาโทกราฟีของเหลวสมรรถนะสูงนี้ใช้เวลาในการวิเคราะห์เพียง 12 นาทีต่อตัวอย่าง ตัวสกัดแบบใช้สารน้อยด้วยของแข็งโมโนลิธช่วยลดเวลา ปริมาตรตัวอย่างและสารละลายในกระบวนการสกัดอย่างมีนัยสำคัญ

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาเคมี ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2561 ลายมือชื่ออาจารย์ที่ปรึกษา _____

KESARA AR-SANORK : IN-PIPETTE TIP MONOLITHIC MICRO SOLID PHASE EXTRACTION COUPLED WITH HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR SIMULTANEOUS DETERMINATION OF RACTOPAMINE AND CLENBUTEROL IN ANIMAL URINE AND MEAT SAMPLES. THESIS ADVISOR : ASST. PROF. PATCHARIN CHAISUWAN, Ph.D. 82 PP.

MICRO SOLID PHASE EXTRACTION/MONOLITH/BETA-AGONISTS/ RACTOPAMINE/CLENBUTEROL

Ractopamine (RAC) and clenbuterol (CLEN) are two common β-agonists illegally used by Thai farmers to reduce fat and increase muscle mass in animal producing. As the residues of these compounds can cause the serious problems in human health, the analytical method for determination of these compounds is thus necessary for controlling food quality and preventing the use of the β-agonists in livestock. In this research, in-pipette tip monolithic micro-solid phase extraction (μ-SPE) off-line coupled with high performance liquid chromatography (HPLC) was developed for determination of RAC and CLEN in animal urine and meat samples. Rapid separation was carried out on silica-based C_8 column with optimum mobile phase of 25% (v/v) acetonitrile (ACN) in 20 mM acetate buffer pH 4.0 containing 2 mM triethylamine. RAC and CLEN could be separated within 7 min. Monolithic sorbent for the extraction, was *in-situ* synthesized from methacrylic acid as a functional monomer and ethylene glycol dimethacrylate as a crosslinker in auto-pipette tip. Monolithic composition of 18:82 (monomer:porogen) with thermal polymerization at 75°C for 1 hr and 10 min was the optimum condition for the synthesis of monolith. The monolith in-pipette tip was assembled with T-way valve

and syringes to perform the extraction. From the optimization, 10% (v/v) ACN in water (800 µL) and 30% (v/v) ACN in 200 mM acetate buffer pH 4.0 (150 µL) were the optimum solvents for washing and elution steps, respectively. The precision (%RSD) for the extraction efficiency from tip-to-tip and batch-to-batch preparations were less than 3.18% and inter-day and intra-day precisions of the analysis (calculated from peak area) were less than 3.26%. Capacities of the monolithic material were higher than 10 and 20 µg for RAC and CLEN, respectively. The in-pipette tip monolithic µ-SPE could be reused at least 10 times without loss of the extraction performance and stored at room temperature for at least 6 months. The sample loading of 2.00 mL with eluting volume of 150 µL provided pre-concentration factor of 12 and 13 with extraction efficiency of 92% and 100% and limits of detection of 1.3 and 0.2 µg L^{-1} for RAC and CLEN, respectively. The in-pipette tip monolithic µ-SPE was off-line coupled with HPLC for the determination of RAC and CLEN in spiked animal urine and meat samples. Good recovery was achieved in the range of 84-114% for urine samples and 89-112% for meat samples. Total analysis time for our µ-SPE-HPLC method was 12 minutes/sample. The µ-SPE in pipette tip significantly reduced the time, sample and solvent consumption in the process of extraction.

มหาวิทยาลัยเทคโนโลยีสุรนารี

School of Chemistry Student's Signature _____

Academic Year 2018 Advisor's Signature _____

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Kesara Ar-sanork

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LISTS OF ABBREVIATIONS

ACN	Acetonitrile
AIBN	Azobisisobutylronitrile
CE	Capillary electrophoresis
CLEN	Clenbuterol hydrochloride
DPX	Dispersion pipette extraction
E.E.	Extraction efficiency
EDMA	Ethylene glycol dimethacrylate
FT-IR	Fourier-transform infrared spectrometer
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MAA	Methacrylic acid
MCX	Mixed cation exchange
MeOH	Methanol
MIP	Molecularly imprinted polymer

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LISTS OF ABBREVIATIONS (Continued)

ND	Not detectable
NIP	Non-molecularly imprinted polymer
P.F.	Pre-concentration factor
RAC	Ractopamine hydrochloride
RSD	Relative standard deviation
SEM	Scanning electron microscope
SPD	Solid phase dispersion
SPE	Solid phase extraction
TEA	Triethylamine
μ-SPE	Micro-solid phase extraction

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CHAPTER I

INTRODUCTION

This chapter provides the introduction for β-agonists and principle for the material and methods employed in this thesis, including monolithic materials, solid phase extraction and high performance liquid chromatography.

1.1 β-Agonists

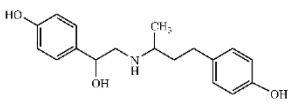
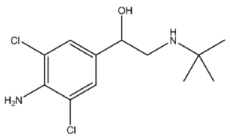
β-Agonists are synthetic phenylethanolamine compounds commonly used as treatments for respiratory disorders, asthma, tocolyics, bronchodilators and heart tonics (Shao et al., 2009; Guo et al., 2018). However, β-Agonists were found to be illegally used as animal feed additives by farmers to reduce fat and increase muscle mass in the animal producing (Xiu-Juan et al., 2013; Peng et al., 2018). The residual of these compounds in foods can cause negative effects on human health such as muscular tremors, cardiac palpitation, nervousness, headache, muscular pain, dizziness, nausea, vomiting, fever and chill. These compounds have been forbidden for the livestock in several regious such as European Union or EU (Council Directive 96/23/EC, 1996), China (The Announcement Ministry of Agriculture, No. 176, PR China, 2002), Japan and Thailand (Xiu-Juan et al., 2013).

However, the illegal use of these toxic compounds in Thailand was still recently reported (Figure 1.1).

Figure 1.1 The illegal uses of β-agonists recently reported by Thai newspapers (Found β-agonists in five thousand swine, 2018; Illegal uses of β-agonists in two Chachoengsao farms, 2018; β-agonists in USA pork, 2018; Toxicity of β-agonists, 2018).

Due to the illegal use of some β-agonists are still found, a reliable and accurate analytical method is necessary for controlling food quality and preventing the use of β-agonists in livestock. Ractopamine (RAC) and clenbuterol (CLEN) (Table 1.1) are two common β-agonists illegal used by Thai farmers.

Table 1.1 Chemical structures with IUPAC name, molecular weight and pKa for RAC and CLEN.

	Ractopamine	**Clenbuterol**
Chemical structure		
IUPAC Name	4,3,2-hydroxy-2,4-hydroxyphenyl ethyl-amino-butyl-phenol	4-amino-3,5-dichloro-a-tert-butylamino-methylbenzyl alcohol
Molecular weight	337.84	277.18
pKa	9.4	9.6

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1.2 Monolith

The term “monolith” is derived from the Greek “mono”, meaning “single” and “lithos”, meaning “rod”. In chromatography, monolith or monolithic material is a polymeric continuous single rod of porous material (Nema et al., 2014).

The single-piece material or monolith was firstly proposed in the late 1960s and early 1970s (Xie et al., 2002). The material is a sponge-like structure consisting of two different pores, mesopores and macropores (Figure 1.2). The mesopores are small pores located on the skeleton of the monolith. The macropores are large through pores allowing the solution to flow through. These two pores are the main factors govern permeability and surface area of the material while chemistry of the material depends on the functional groups on the surface of the monolith. The porous monolith permits to operate at relatively high flow rate due to the low back pressure (low flow resistance). The high porosity and high surface area of the material are good characteristics for both extraction and separation applications.

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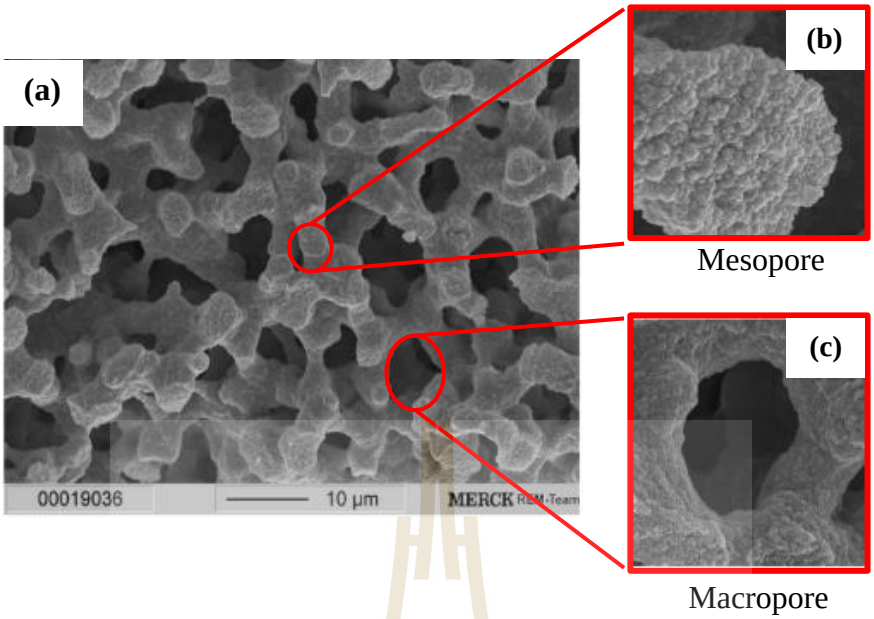
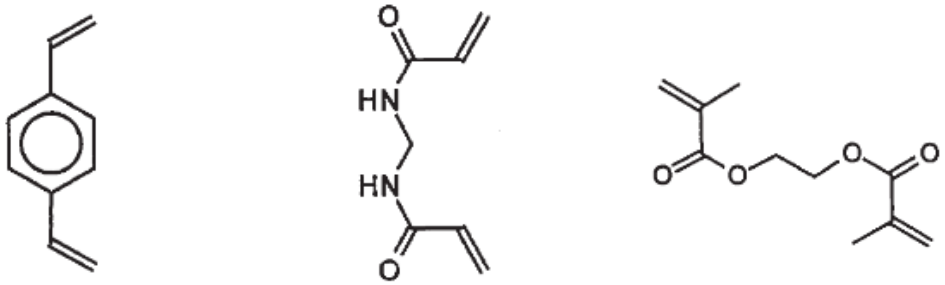


Figure 1.2 SEM images of (a) monolithic material (b) macropore and (c) mesopore (Cabrera, 2004).

The monolith can be prepared from a mixture of functional monomer, crosslinking monomer, porogen and initiator *via* either thermal or photo polymerization. The role of each components is explained below.

1. Monomer or functional monomer is one of the important parameters for the preparation of monolith since the chemical properties of the monolith depend on the functional group of the monomers. Types of the monolith are therefore categorized into two types based on the monomers employed.

2. Crosslinking monomer (crosslinker) is monomer in monolithic synthesis for crosslinking of polymer chains. The mesopores and mechanical strength of the materials are related to the type and amount of the crosslinker. The popular crosslinking monomers, divinylbenzene, ethylene glycol dimethacrylate and N,N´-methylenebis (acrylamide) employed for the monolithic preparation are shown in Figure 1.3.



Divinylbenzene | Ethylene glycol dimethacrylate | N,N'-methylenebis (acrylamide)

Figure 1.3 Examples of crosslinking monomers for the preparation of monolith.

3. Porogen or porogenic solvent is a solvent employed to obtain the homogeneous polymerization solution. Type and ratio of porogen are usually optimized to adjust the pore size and porosity of the monolith. The solubility of the monomers is the main criteria for selecting the porogen.

4. Initiator is the compound used for starting or initiating the polymerization process. Type of initiator depends on the polymerization process or method. Azobisisobutyronitrile or AIBN is a popularly used initiator for thermal polymerization while N,N-didecyl-N-methylpoly (oxyethyl) ammonium propionate or DMPAP is typically used for photo polymerization.

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1.2.1 Type of monolith

Monolith can be categorized into two types which are organic and inorganic monoliths. The types of monolith depend on the chemical properties or type of the monomers employed.

1.2.1.1 Organic polymer-based monoliths or polymer monoliths

Organic polymer-based monoliths or polymer monoliths have been prepared by *in situ* polymerization using organic monomers. The polymerization solutions mainly contain monomers, porogens, and an initiator. The polymerization reaction is initiated by decomposition of an initiator to form radicals causing nucleation of polymer and polymer growth (Wu and Thompson, 2006). SEM images of an organic polymer-based monolith illustrated in Figure 1.4.

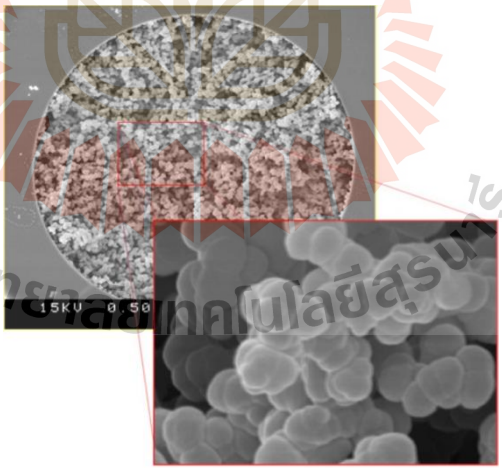
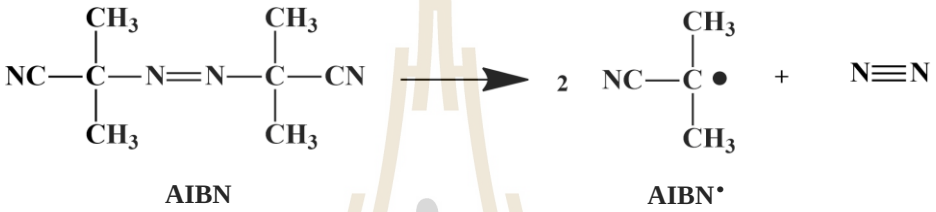


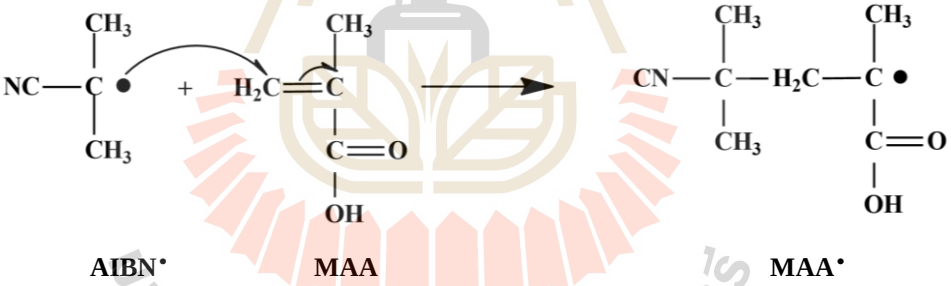
Figure 1.4 SEM images of organic-based monolith prepared in capillary column (Svec, 2011).

Methacrylate based monolith is the most common organic polymer-based monolith used in chromatography. The polymerization reactions for a methacrylate based monolith synthesized from methacrylic acid (MAA) monomer and ethylene glycol dimethacrylate (EDMA) crosslinker with azobisisobutyronitrile (AIBN) initiator are shown in Figure 1.5.

(1) Formation of initiation radical (AIBN•)



(2) Formation of MAA radical (MAA·)



(3) Growing of MAA radical chain (MAA chain·)

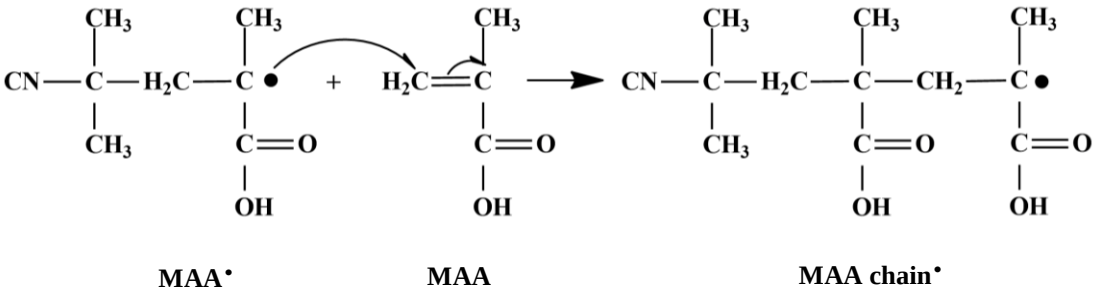


Figure 1.5 Radical polymerization reaction of MAA-EDMA monolith using AIBN initiator (Ramelow and Pingili, 2010).

(4) Growing of MAA-EDMA chain (MAA-EDMA)

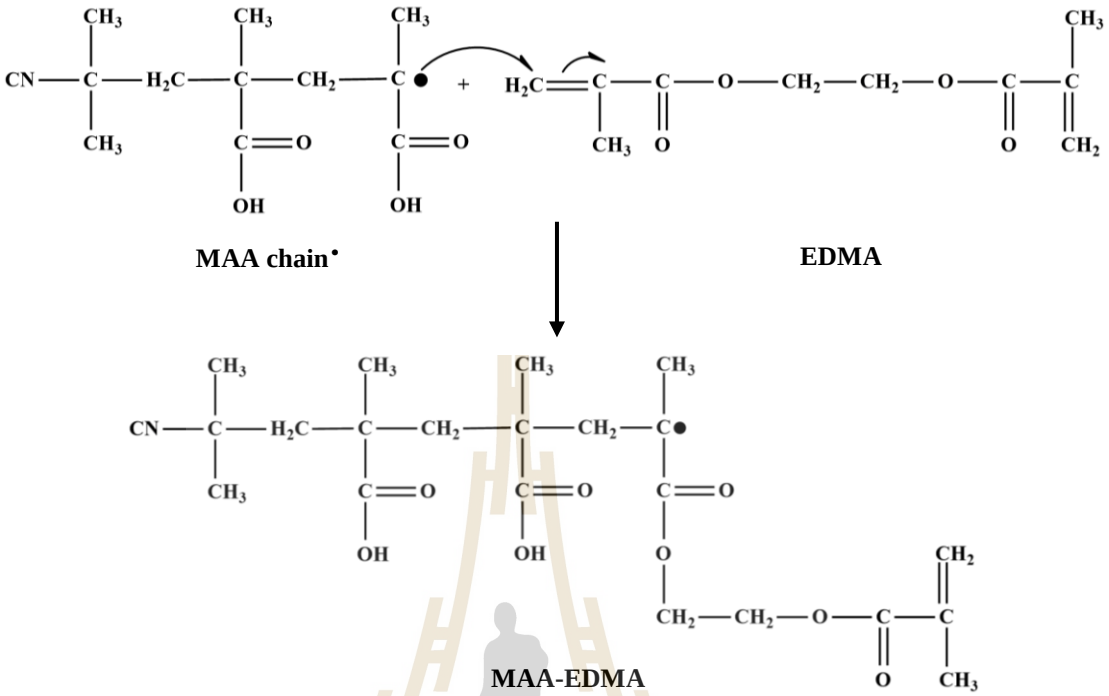


Figure 1.5 Radical polymerization reaction of MAA-EDMA monolith using AIBN initiator (Ramelow and Pingili, 2010) (continued).

Figure 1.6 shows examples of organic monomers reported for the organic polymer-based monolith including (1) hydrophilic monomers such as methacrylic acid, acrylamide, 2-acrylamido-2-methyl-1-propanesulfonic acid, glycidyl methacrylate, chloromethylstryrene, 2-vinyl-4,4-dimethylazlzctone, 4-acetoxystrylene, (2) hydrophobic monomers such as styrene, butyl methacrylate and (3) zwitterionic monomer (Xie et al., 2002)

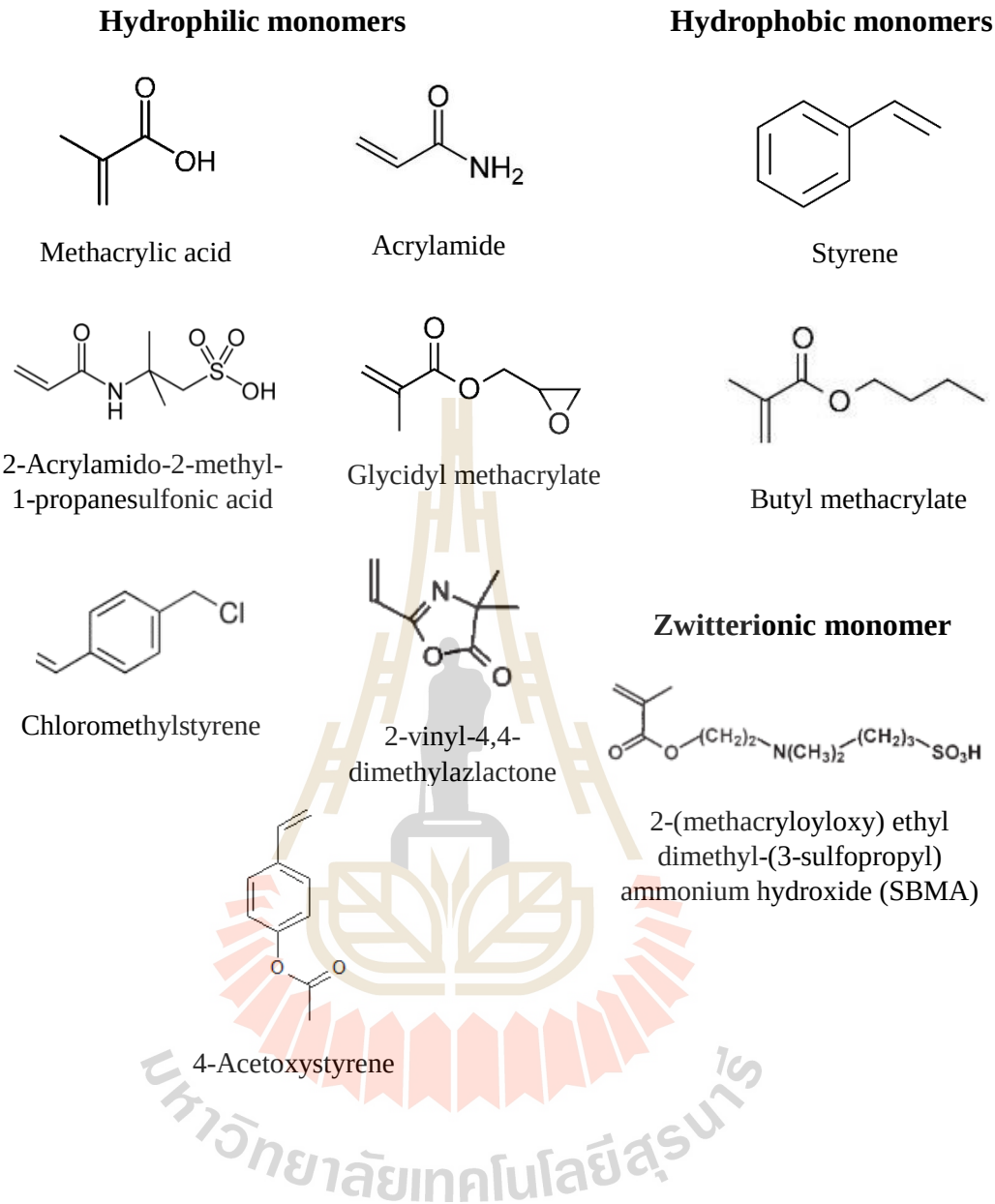


Figure 1.6 Examples of organic monomers for the preparation of organic polymer-based monoliths or polymer monoliths.

The advantages of this type of monolith is it can tolerate extreme changes in pH, from 1-14 (Nema et al., 2014) and it is simply synthesized in one step. However, swelling problem by organic solvents could be found for this type of monolith.

1.2.1.2 Inorganic polymer-based monoliths

Inorganic polymer-based monoliths are typically synthesized from alkoxysilane by a sol-gel method (Nema et al., 2014). The sol-gel method consists of two main steps, hydrolysis and condensation reactions (Figure 1.7). The alkoxysilane reacts with water to obtain free reactive silanol group. The polymerization is then occurred by condensation of the alkoxysilane.

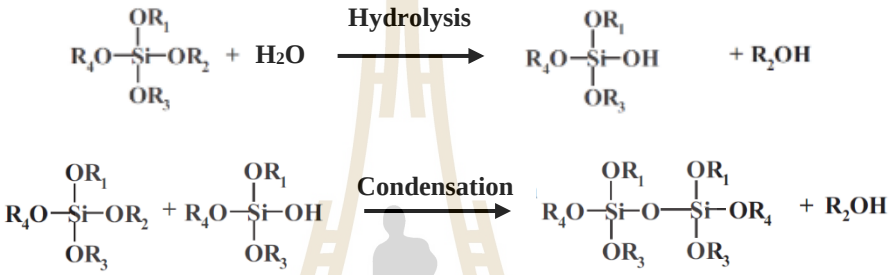


Figure 1.7 Hydrolysis and condensation reactions for synthesis of inorganic-based monolith (Nema et al., 2014).

Since most of the inorganic polymer-based monoliths are made from alkoxysilane, they are often called silica-based monoliths. Example of SEM images for this monolith is shown in Figure 1.8.

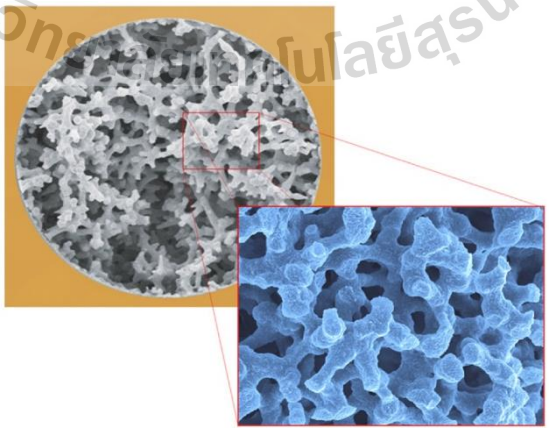


Figure 1.8 SEM images of inorganic-based monolith prepared in capillary column (Svec, 2011).

Inorganic-based monolith provides several advantages including high chromatographic separation efficiency, less swelling and good stability at extremely high temperatures (higher than 750°C) which is suitable to use as a stationary phase in gas chromatography. However, the limitation of this material is the narrow working pH range as the material is not stable at pH below 2 and above 8.

The monoliths have been widely used for chromatography such as stationary phase for liquid chromatography (Hussain et al., 2016; Hua-Liu et al., 2017; Gupta et al., 2016; Eeltink et al., 2017), ion-exchange chromatography (Allmaier et al., 2018) capillary electrochromatography (Xu et al., 2019) size exclusion chromatography (Huber et al., 2011).

In addition, the monolithic materials have been also developed for extraction by solid phase extraction (SPE) (Alwael et al., 2011; Xie et al., 2011; Du et al., 2014; Arabi et al., 2017; Fresco-Cala et al., 2018).

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1.3 Solid phase extraction (SPE)

In some chemical analysis, more than 70% of the analysis time is spent for sample preparation step and only 10-20% for the actual measurement for sample analysis. Therefore, sample preparation is an important key effecting on both the accuracy and analysis time.

Traditionally, liquid-liquid extraction (LLE) is used for sample extraction in various types of analytes and samples to reduce the sample matrices and increase the analytes concentration. The LLE method is widely used due to the simple apparatus and procedures. However, low extraction efficiency was usually found. In addition, the method is considered as not environmentally friendly as large amount of organic solvent.

Solid phase extraction or SPE have been employed as an alternative extraction method to LLE method due to the high extraction efficiency with low solvent consumption. Comparison of SPE and LLE methods are shown in Table 1.2.

Table 1.2 Comparison of SPE and LLE methods for sample preparation.

Parameter	**LLE method**	**SPE method**
Sample/solvent consumptions	Large	Medium-less
Extraction time	Long	Medium-short
Extraction efficiency	Medium	High
Apparatus use	Simple	Complex
Easily automated	No	Yes
Cost	Low-medium	High

1.3.1 Solid phase extraction mechanism

The extraction by SPE is based on the partitioning of the analytes between the liquid sample and the surface of the solid sorbent. When sample is loaded or flowed through the solid sorbent, analyte(s) can adsorb on the sorbent by several interactions such as hydrophobic, hydrophilic, columbic interaction and hydrogen bond. The interaction of the analyte(s) with the sorbent can be expressed in the term of distribution coefficient (K_D) defined below.

$$K_D = \frac{C_{sorbent}}{C_{sample}}$$

Where $C_{sorbent}$ is concentration of the analyte on the solid sorbent and C_{sample} is concentration of the analyte in sample solution.

1.3.2 Solid phase extraction procedure

To carry out the SPE, the procedure is based on four basic steps including (1) conditioning (2) loading (3) washing and (4) eluting. Schematic for basic SPE procedures are shown in Figure 1.9.

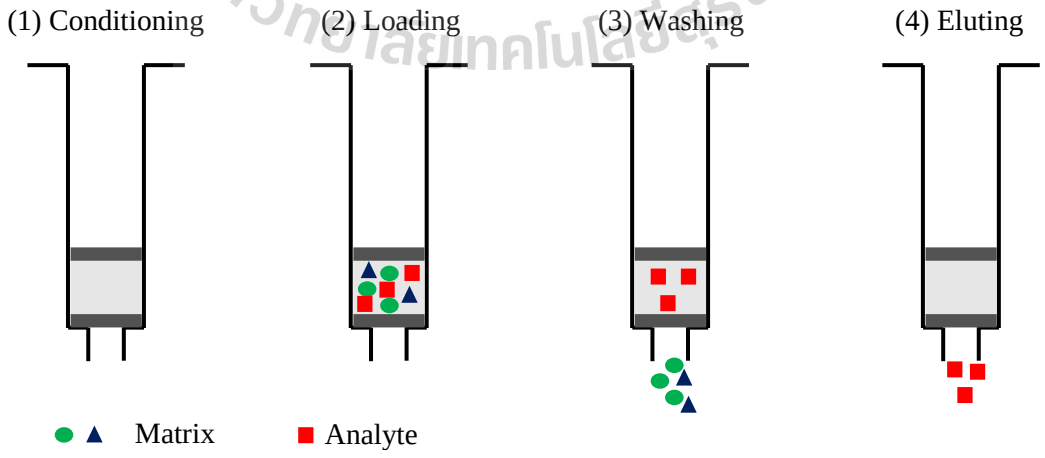

Figure 1.9 Solid phase extraction procedures.

(1) Conditioning

Before the extraction, the solid sorbent is conditioned with a suitable solvent(s) to activate the functional group at the surface of the solid sorbent before performing the extraction (Figure 1.10).

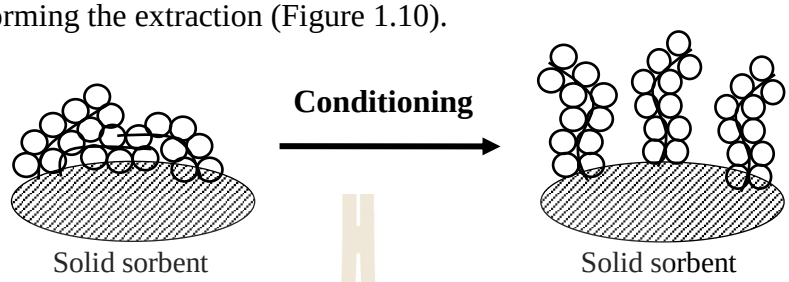

Figure 1.10 Activation of functional groups on the surface of the solid sorbent (modified from James, 1999).

(2) Loading

After conditioning step in which the sorbent is ready to interact with the analyte(s), sample solution is loaded or flowed through the solid sorbent. The analyte(s) are adsorbed on the active sites of the solid sorbent *via* various possible interactions such as hydrophobic, hydrophilic, coulombic interaction and hydrogen bond, depending on the functional groups of the compounds and the active sites of the sorbent. During this step, some of the sample matrices possibly also retain on the solid sorbent.

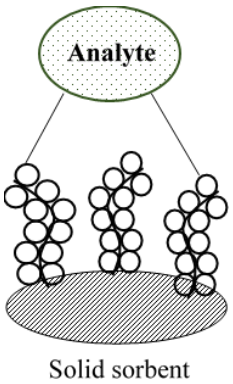

Figure 1.11 The adsorption of analyte on solid sorbent in the loading step.

(3) Washing

Since the sample matrices may also interact and retain on the surface of the sorbent, a washing solvent is required to remove the unwanted compounds before sample elution. An optimum washing solvent should be able to remove the matrices retained on the sorbent as much as possible without loss of the trapped analyte(s).

(4) Eluting

The analyte(s) is eventually desorbed from the sorbent by an eluting solvent. In order to increase concentration of the analyte(s), small volume of eluting solvent is employed. Reconstitution or re-dissolving step is usually performed to enhance the pre-concentration factor or when the eluting solvent is not compatible with the analytical method.

Typical SPE methods require much less solvent than used in LLE (5-20 mL/sample) (Shao et al., 2009; Xiu-Juan et al., 2013). In order to further reduce the chemicals and solvent consumption, the SPE was miniaturized in various devices and designs, such as capillary, the channels of micro-fluidic device, polypropylene tubing or in pipette tips (Alwael et al., 2011) SPE in pipette tips (Figure 1.12(b)) provides several advantages, including ease of use, low sample and solvent consumption and suitability for field or on-site analysis.

(a) (b)

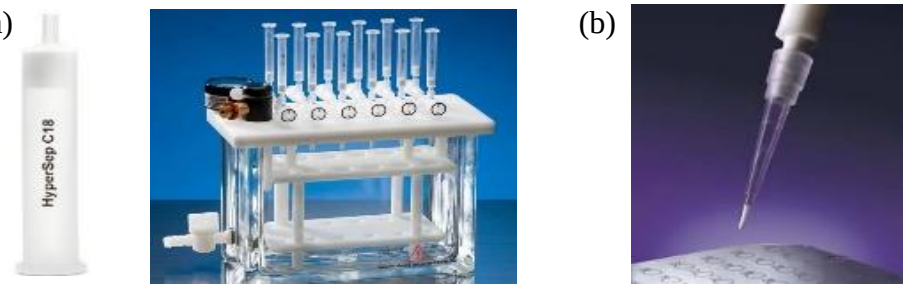

Figure 1.12 (a) SPE cartridges with a chamber and a vacuum pump (b) a commercial micro-pipette tip SPE.

Monolith is an interesting material for pipette tip-based micro-solid phase extraction (in-pipette tip μ-SPE) since it can be *in situ* prepared in the pipette tip with tailor-made chemical and physical properties. The material can be held in the tip without need of a frit. The comparisons of organic monolithic and classical particle based sorbents for SPE technique are summarized in Table 1.3.

Table 1.3 Comparison of organic monolithic and classical particle based sorbents for SPE.

Parameter	**Organic monolithic sorbent**	**Classical particle based sorbent**
Synthesis or packed procedure	Simple	Complicated, time consuming
Functionality	Various	Limited
Porosity	High	Low
Mass transfer	Fast	Medium
Surface area	Medium-high	High
Capacity	Medium-high	High
pH range	Wide	Narrow-wide*
Back pressure	Low	High
Cost	Low	Medium-high

* Depends on type of the sorbent.

1.4 High performance liquid chromatography (HPLC)

HPLC is a powerful modern separation technique based on two different phases, stationary phase and liquid mobile phase. The first chromatographic method was established in 1906s by M. S. Tswett for separating of pigments color in plant. In HPLC, the analytes are separated in a column consisting of extremely small particles of stationary phase to interact with the analytes. A solvent is applied by mean of high pressure pump to dissolve the analytes and elute them from the column. The analytes with different chemistry interact with the stationary phase differently and therefore migrate from the column with different speed. The separation is thus achieved.

1.4.1 Principle of the separation

The mechanism for liquid chromatography (LC) or HPLC is similar with that explained for the SPE (described in Section 1.3.1). The difference between SPE and LC is the separation in LC is performed under continuous flow of the mobile phase. When sample is introduced to the column, the analytes can distribute between two different phases, stationary phase (solid support) and mobile phase (liquid solvent). The affinity of the analyte with the stationary phase is expressed in terms of the distribution coefficient (K_D) which is the ratio of the analyte concentration in the stationary phase ($C_{\text{stationary phase}}$) to concentration of the analyte in the mobile phase ($C_{\text{mobile phase}}$).

$$K_D = \frac{C_{stationary\ phase}}{C_{mobile\ phase}}$$

Figure 1.13 shows a graphical picture for separation of a sample containing components A and B. Due to the higher interaction of the component A with the stationary phase, the component A moves slower and retains in the column longer than component B. As the separation time passes by, higher resolution is achieved (Figure 1.13(c)).

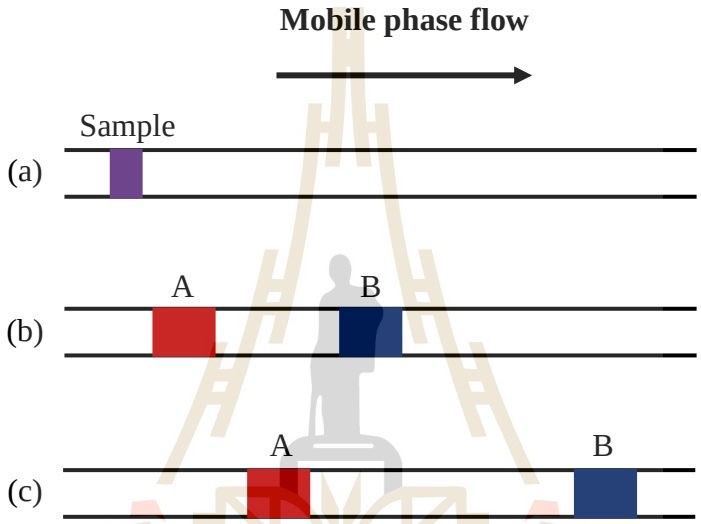

Figure 1.13 Schematic of the chromatographic separation of two analytes on a column.

1.4.2 Mode of LC separation

Mode of the separation depends on the separation mechanism or functional groups of the stationary phase and the analyte. Table 1.4 Shows five common LC separation modes.

Table 1.4 Shows five common LC separation modes.

Separation mode	Stationary phase	Type of analyte
Reversed phase	Hydrophobic materials such as alkyl chain bonded particle	Hydrophobic molecule
Normal phase	Hydrophilic materials such as silica or alumina	Hydrophilic molecule
Ion exchange	Ion-exchange resin	Ionic analyte
Ion paring	Hydrophobic materials such as alkyl chain bonded particle	Ionic analyte
Size exclusion	Porous beads	Large or macromolecule

1.4.3 Instrumentation of HPLC

A HPLC system typically comprises of solvent reservoir (mobile phase), high pressure pump, an injector, column, detector and data acquisition, as shown in Figure 1.14.

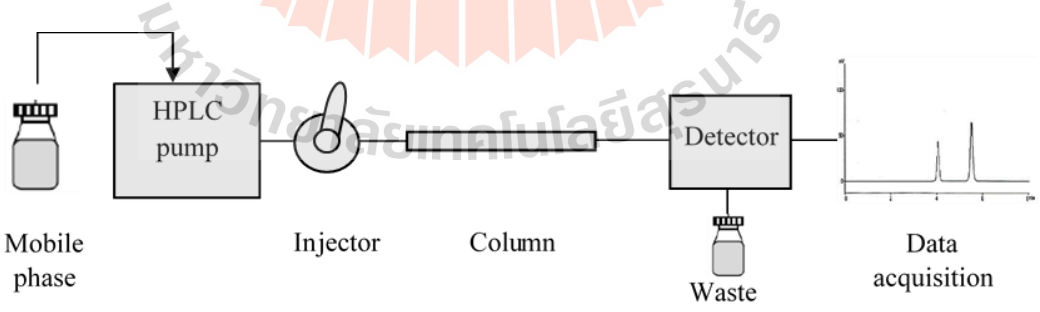

Figure 1.14 Basic components of a high performance liquid chromatography system.

1.5 Research objectives

1. To develop a portable micro-extraction method based on monolithic solid phase extraction for simple and low cost extraction of ractopamine and clenbuterol.

2. To develop the accurate and reliable analytical method based on high performance liquid chromatography for determination of ractopamine and clenbuterol in animal urine and meat samples.

CHAPTER II

LITERATURE REVIEWS

Due to the illegal use of RAC and CLEN in livestock, many analytical methods have been developed to examine the amounts of RAC and CLEN in animal feed, animal urine and tissue. This chapter summarizes the extraction methods reported and analytical methods developed for RAC and CLEN in various types of samples.

2.1 Extraction methods for RAC and CLEN

Sample extraction is generally performed to decrease the sample matrix interference (sample clean up) and/or increase the analyte concentration (sample pre-concentration) before the analysis. The extraction methods reported for RAC and CLEN are given in this section.

2.1.1 Solid phase extraction (SPE)

SPE is widely used for both sample clean up and pre-concentration. The method provides high extraction efficiency with simple procedures. Several types of sorbent and designs for the extraction of RAC and CLEN were published.

2.1.1.1 Commercial SPE cartridge

SPE cartridges are the most common SPE commercially available. Various types of commercial SPE cartridges were employed for the extraction of β-agonists as shown in Table 2.1. For this method, the extraction time varied from 20-50 minutes with solvent consumption between 5-20 mL.

Table 2.1 Extraction methods using commercial SPE cartridges for sample pre-treatment of β-agonists samples.

Sorbent	Number of β-agonists	Sample	Extraction time (min)	Reference
HLB and MCX cartridge	16	Liver, kidney, meat	>40	(Shao et al., 2009)
C_{18} SPE cartridge	5	Pig feed, urine, liver	>20	(Wang et al., 2010)
MCX cartridge	12	Animal feed	>50	(Wang et al., 2013)
MCX and C_{18} SPE cartridge	22	Muscle, liver	>30	(Guo et al., 2018)

MCX=mixed cation exchange, HLB=hydrophilic lipophilic balance

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2.1.1.2 Molecularly imprinted polymer (MIP)

Several types of MIP materials were also synthesized for selective extraction by SPE. The MIPs for β-agonists were reported as followed.

Xu et al. (2010) proposed MIP coated stir bar for simple and fast extraction. The MIP was synthesized using RAC as the template for extraction of RAC and its analogues (CLEN, isoxsuprine and fenoterol). The developed MIP-coated stir bar demonstrated higher selectivity and capacity (2.4 to 3.3 times) than the non-molecularly imprinted polymer (NIP)-coated stir bar. The developed MIP coated stir bar could be reused at least 40 times with no loss of extraction performance (Xu et al., 2010).

Yan et al. (2013) developed ionic liquid modified dummy molecularly imprinted microspheres for the extraction of CLEN and clorprenaline in urine samples. The 1-allyl-3-ethlyimidazolium bromide ionic liquid was employed as a co-functional monomer with phenylephrine as a dummy template for specific extraction of the two analytes. The ionic liquid did not provide only good adsorption capacity and selectivity but also preventing the template leakage during the extraction (Yan et al., 2013)

Wang et al. (2015) proposed a novel molecularly imprinted polymer for selective extraction of five β-agonists consisting of CLEN, RAC, brombuterol, bromchlorbuterol and clorprenaline in pork tissue samples. Phenylethanolamine A as the dummy template and *p*-vinylbenzoic acid as the functional monomer were polymerized *via* thermal polymerization. The results showed that the MIP was more selective to the five β-agonists than NIP. The extraction capacities obtained from the MIP were 2.7 to 3.4 times higher than those obtained from the NIP (Wang et al., 2015).

And Qiu et al. (2016) fabricated ractopamine MIP nanotube membrane on anodic alumina oxide nanopore surface (AAO@MIP). The MIP nanotube membranes

was synthesized by using RAC as the template and MAA as the functional monomer by atom transfer radical polymerization. The MIP nanotube membranes provided specific extraction of RAC and its analogues including CLEN, epinephrine, terbutaline and dopamine. The AAO@MIP significantly improved the adsorption capacity (Qiu et al, 2016).

Although, the MIP sorbent provided higher extraction selectivity compared with the NIP sorbent, the synthesis requires multi-preparation step with relatively long preparation time. In addition, the use of template can increase the cost of analysis.

2.1.2 Dispersive solid phase extraction

Mastrianni et al. (2018) proposed the determination of ten β-agonists including RAC and CLEN in pork meat samples. The extraction method was performed by using a commercial dispersive pipette extraction (from DPX Technologies) before analysis by LC-MS/MS. Loose resin was employed as a sorbent. The extraction time was 20 min/sample (Mastrianni et al. 2018).

2.1.3 Liquid liquid extraction (LLE)

The classical extraction LLE was employed as a sample pre-treatment method for analysis of eight β-agonists in pork samples by an electrochemical sensor (Lin et al., 2013) and for rapid analysis of RAC, CLEN and salbutamol in urine samples using surface-enhance Raman spectroscopy (Zhai et al., 2012). To extract the analytes from sample matrices, 40-100 mL ethyl acetate was used as the extractant. The average extraction time was 50 min/sample. The method was simple but consumed large amount of solvent.

2.2 Analytical methods for determination of RAC and CLEN

Various analytical methods have been reported for determination of β-agonists such as HPLC, capillary electrophoresis, electrochemical sensors, Raman spectroscopy and colorimetric methods.

2.2.1 High performance liquid chromatography-ultraviolet detection (HPLC-UV)

HPLC-UV method is one of the most common analytical techniques employed for analysis of organic compounds including RAC and CLEN. Most of the methods were carried out by reversed-phase HPLC using commercial packed-based C_{18} column and aqueous-organic solvent as the mobile phase. Although the method could separate the analytes from the sample matrices, sample pre-treatment for clean up and pre-concentration is still needed. The method is therefore coupled with various types of sample preparation techniques especially SPE. The LOD of the HPLC-UV coupled off-line with SPE was in the range of ppt-ppb with total analysis time between 30-40 min/sample. The main advantages of this technique are good selectivity, simple operation and relatively low operation cost. The HPLC-UV methods are shown in Table 2.2.

Table 2.2 The HPLC-UV methods for determination of β-agonists.

Column	Mobile phase	LOD (ppb)	Sample pre-treatment	Extraction /analysis time (min)	Reference
Agilent C_{18}	64:36 (v/v) ACN, 0.1% formic acid and 50 mM ammonium acetate	<0.05	SPE	30/18	(Yan et al., 2016)
R Stech C_{18}	25:75 (v:v) Water:MeOH containing 0.25% trifluoroacetic acid pH 2.8	0.07 µg/kg	SPE	>30/6	(Liu et al., 2011)
Beta basic C_{18}	MeOH:50 mM ammonium phosphate buffer pH 2 (gradient elution)	0.5	SPE	30/18	(Blomgren et al., 2002)
Shimadzu C_{18}	40:60 (v:v) MeOH:0.2% formic acid pH 4	3.0	SPE	>20/10	(Wei et al., 2014)

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2.2.2 High performance liquid chromatography-tandem mass spectrometry detection (HPLC-MS/MS)

A highly selective MS/MS detection coupled with HPLC was also developed for the analysis of β-agonists in complex samples. The HPLC-MS/MS was carried out on reverse phase C_{18} column with the mobile phase consisted of organic solvent (acetonitrile or methanol) and 0.1% formic acid using gradient elution. Wang et al. (2015) proposed the method to separate five β-agonists (CLEN, RAC, brombuterol, bromchlorbuterol and clorphenaline) (Wang et al., 2015). Mastrianni et al. (2018) also developed the HPLC-MS/MS for the analysis of ten β-agonists in pork tissue (Mastrianni et al., 2018). Although the method showed excellent selectivity and sensitivity with fast separation, this technique required not only special technical skills for the operation but also high price instruments.

2.2.3 Capillary electrophoresis (CE)

Wang et al. (2010) successfully developed a CE-UV method for determination of five β-agonists, CLEN, RAC, metoprolol, isoxsuprine and salbutamol in pig feed, urine and liver. Under the optimum condition, the target analytes could be separated in a bare fused silica using H_3PO_3-NaB_4O_7 buffer pH 9.0 as background electrolyte in 15 min. The method was simple, fast and cheap. Another advantage is the method consumed very low amount of sample and solvents in micro-liter and milliliter, respectively. However, the method has drawbacks in terms of low sample loading capacity and low sensitivity. The special operational skills are also needed (Wang et al., 2010).

2.2.4 Raman spectroscopy

Zhai et al. (2012) developed a method based on Raman spectroscopy for the analysis of three β-agonists, RAC, CLEN and salbutamol in swine urine samples. Liquid liquid extraction method was used to extract the target analytes before Raman analyzes. The LOD in the range of 2-100 ppb was observed with the analysis time of 30 min (Zhai et al., 2012).

2.2.5 Colorimetric method

He et al. (2011) reported colorimetric detection of thirteen β-agonists by using gold nanoparticles. The colorimetric method is based on the color changing of the gold nanoparticles. When gold nanoparticles are induced by the β-agonists, aggregation of gold nanoparticles caused color change from red to blue. However, the method could not be directly applied to real samples due to the low sensitivity and selectivity of the method (He et al., 2011).

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CHAPTER III

MATERIALS AND METHODS

This chapter provides the information for materials and methods employed in this research. The details for the preparation of chemical and sample solutions are also given. The experiments for the extraction, HPLC method and the analysis in real samples as well as the criteria for evaluation of method performances are fully explained.

3.1 Reagents and chemicals

Reagents and chemicals used are as followed:

Table 3.1 List of reagents and chemicals.

Reagent and chemical	Grade	Supplier
Acetonitrile (ACN)	Analytical	Sigma-Aldrich (USA)
Azobisisobutylronitrile (AIBN)	Analytical	Sigma-Aldrich (USA)
Clenbuterol hydrochloride (CLEN)	Analytical	Sigma-Aldrich (USA)
Decanol	Analytical	Sigma-Aldrich (USA)
Di-sodium hydrogen phosphate (Na_2HPO_4)	Analytical	Carlo Erba (Italy)
Ethylene glycol dimethacrylate (EDMA)	Analytical	Sigma-Aldrich (USA)

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Table 3.1 List of reagents and chemicals (Continued).

Reagent and chemical	Grade	Supplier
Glacial acetic acid	Analytical	Sigma-Aldrich (USA)
Potassium dihydrogen phosphate (KH_2PO_4)	Analytical	Carlo Erba (Italy)
Ractopamine hydrochloride (RAC)	Analytical	Sigma-Aldrich (USA)
Sodium hydroxide (NaOH)	Analytical	Sigma-Aldrich (USA)
Toluene	Analytical	Sigma-Aldrich (USA)
Triethylamine (TEA)	Analytical	Sigma-Aldrich (USA)
Zinc sulphate ($ZnSO_4$)	Analytical	Sigma-Aldrich (USA)
Methacrylic acid (MAA)	Analytical	Sigma-Aldrich (USA)
Methanol (MeOH)	Analytical	Sigma-Aldrich (USA)
Phosphoric acid (H_3PO_4)	Analytical	Carlo Erba (Italy)
Potassium chloride (KCl)	Analytical	Sigma-Aldrich (USA)

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3.2 Instrumentations

The details for the apparatus and equipments used are listed in the table below.

Table 3.2 List of instruments and apparatus.

Instrument	Model	Company
Analytical Balance	AB 104-S	Metter Toledo (Thailand)
Centrifuge	CP80WX	Hitachi (Japan)
Fourier-transform infrared spectrometer (FT-IR)	TENSOR 27-Hyperion	Burker (Germany)
High performance liquid chromatography (HPLC)	HP 1100	Agilent (USA)
Hot air oven	ULE600	Memmert (Germany)
SB C_8 column	4.6 mm×75 mm, 3.5 µm	Agilent (USA)
pH meter	827 pH Lab	Metrohm (Switzerland)
Polypropylene pipette tip	1,000 µL	Axygen (USA)
Scanning electron microscope (SEM)	S-3400N	Hitachi (Japan)
T-way valve	-	Upchurch (USA)
Tygon tube	-	Upchurch (USA)

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3.3 Chemical preparation

3.3.1 Standard solutions of RAC and CLEN

Stock standard RAC and CLEN solutions at the concentration of 500 mg L^{-1} were prepared by dissolving 0.0050 g of the pure standards and making up to 10.00 mL with DI water in volumetric flasks. To construct the calibration curves, series of working standard solutions were daily prepared by diluting the stock solutions with DI water to obtain the desired concentration in the range of 25-5,000 µg L^{-1} for RAC and 5-10,000 µg L^{-1} for CLEN. The solutions were directly loaded through the in-pipette tip monolithic µ-SPE for extraction before the analysis by HPLC method. For HPLC measurement with no extraction, the solutions were filtered through 0.22 µm nylon syringe filter before injecting into the HPLC system.

3.3.2 20 mM Acetate buffer pH 4.0 and 5.0

116 µL Glacial acetic acid was diluted with ~80 mL of DI water. The pH was adjusted to pH 4.0 or 5.0 by adding small amount of 0.1 M NaOH. The solution was then made up to 100.00 mL in a volumetric flask with DI water.

3.3.3 20 mM Phosphate buffer pH 2.0, 6.0, 7.0 and 9.0

Phosphate buffers pH 2.0, 6.0, 7.0 and 9.0 were prepared by dissolving 123 µL H_3PO_4, 0.2772 g KH_2PO_4 and 0.2839 g Na_2HPO_4 in ~80 mL DI water, respectively. Small volume of 0.1 M NaOH was added into the solutions to adjust the pH before making up to 100.00 mL in volumetric flasks.

3.3.4 Mobile phases for HPLC analysis

Mobile phases of 25% (v/v) of acetonitrile in aqueous buffer at different pH were investigated to find an optimum mobile phase composition for the separation of RAC and CLEN. The mobile phases were prepared by pipetting 25.00 mL of acetonitrile into volumetric flasks and made up to 100.00 mL with the buffer solutions (20 mM acetate buffer pH 4.0, 5.0 or 20 mM phosphate buffer pH 6.0). To improve the peak shape, 280 µL of TEA additive was added into 25% (v/v) acetonitrile in 20 mM acetate buffer pH 4.0. The mobile phases were filtered through 0.2 µm cellulose membrane before use.

3.3.5 50 mM Zinc sulphate

Zinc sulphate solution was prepared by dissolving 0.8050 g of $ZnSO_4$ in DI water to the final volume of 100.00 mL. The solution was used to precipitate protein in meat samples before the extraction.

3.3.6 Polymerization solution for synthesis of monolithic sorbent

The polymerization composition was modified from our previous work (Chaloemsuwiwattanakan et al., 2016). The polymerization solution contained MAA and EDMA monomers, porogenic solvents of toluene and 1-decanol and AIBN initiator. The ratio of monomers to porogenic solvents was varied from 15:85 to 23:77 as shown in Table 3.3.

Table 3.3 Weight of monomers, porogens and initiator for preparation of the monolith in pipette tip.

Monomer:Porogen (g:g)	Monomer (g)		Porogen (g)		Initiator (g)
	MAA	EDMA	Toluene	1-Decanol	
15:85	0.0150	0.1350	0.1020	0.7480	0.0015
18:82	0.0180	0.1680	0.0984	0.7216	0.0018
21:79	0.0210	0.1890	0.0948	0.6952	0.0021
23:77	0.0230	0.2070	0.0924	0.6776	0.0023

3.4 HPLC-UV experiment

Reversed-phase chromatographic separation of RAC and CLEN was performed on Zorbax SB-C_8 column (4.6 mm×75 mm, 3.5 µm, Agilent, USA) connected with 20 µL sample loop. The column was equilibrated with the mobile phase for ~30 min or until stable baseline was observed. The separation was carried out at the flow rate of 0.7 mL min^{-1} and detection wavelength at 210 nm.

3.5 Preparation of monolith in pipette tip

Monolithic sorbent was *in situ* synthesized inside auto-pipette tip made from polypropylene *via* thermal polymerization method. The tips were washed with 300 µL MeOH and dried before use. The cleaned pipette tip was sealed with Parafilm® and Scotch® tape at the narrow end before filling in with 60 µL of the homogenous polymerization solution prepared in Section 3.3.6. The top of tip was then sealed with Parafilm® and Scotch® tape before keeping in an oven at certain temperatures for thermal polymerization (see Figure 3.1). Polymerization temperature and time were varied to find the optimum synthesized condition. The observed monolith was washed

with 300 µL MeOH to remove the unpolymerized reagents. The in-pipette tip monolith was stored at room temperature until use.

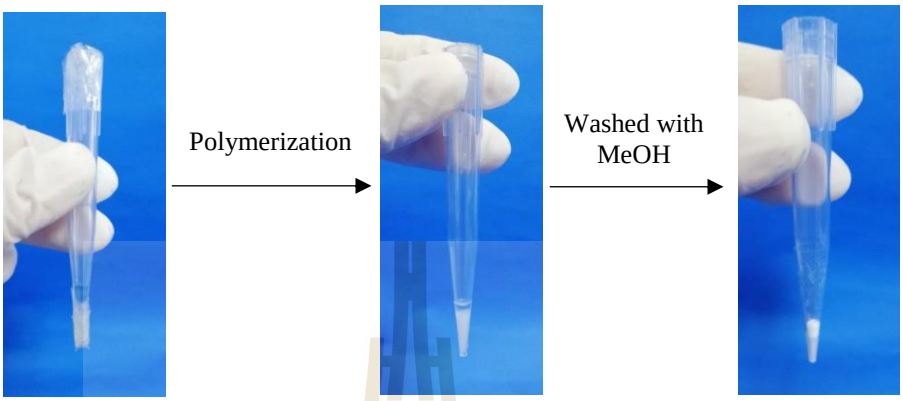


Figure 3.1 Synthesis of in-pipette tip monolith.

3.6 Monolith characterization

The synthesized monolith in pipette tips were characterized by FT-IR and SEM techniques. To investigate the functional group of the monolithic sorbent, the monolith was crushed into powder before characterization by FT-IR. For SEM characterization, the monolith in-pipette tip was cut around 4 mm from narrow end and coated with gold.

3.7 In-pipette tip monolithic µ-SPE devices

The in-pipette tip monolith can be used with both auto-micropipette device (Figure 3.2(a)) and T-way valve (Figure 3.2(b)). In this work, the T-way device was selected as it was easier to operate, more convenient to change the loading solvents and the extraction time was shorter. To assemble the in-pipette tip monolith with T-way valve, the in-pipette tip monolith was cut from narrow end around 1.5 cm and connected with Tygon tube. To carry out the extraction, the solutions were accurately pipetted into

the syringe before manual loading. Inlet 1 was used for loading of samples and washing solvent, while eluting solvent was loaded at inlet 2.

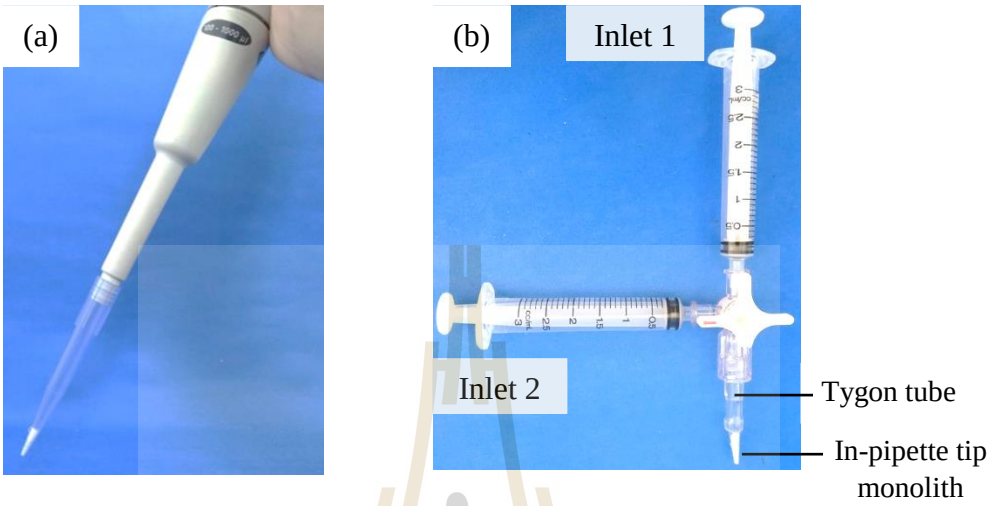
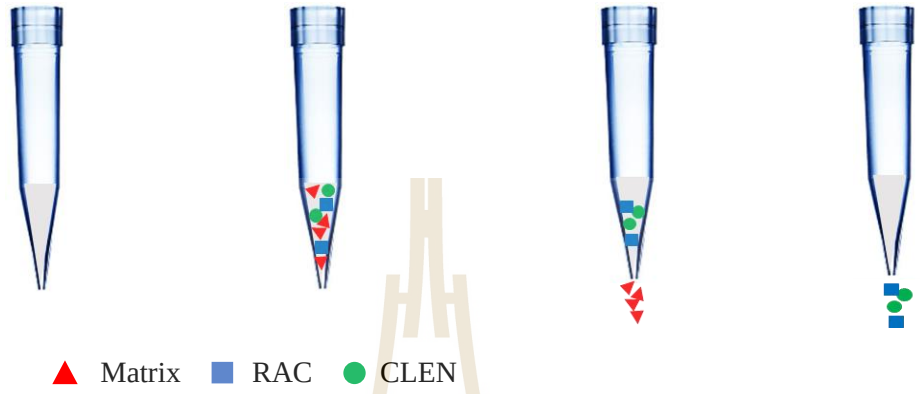


Figure 3.2 In-pipette tip monolithic µ-SPE used with (a) auto-micropipette device (b) T-way valve device.

3.8 Optimization of in-pipette tip monolithic µ-SPE condition

300 µL MeOH was loaded through the sorbent to activate the nonpolar functional groups on the surface of the monolithic sorbent followed by 50 µL of DI water. The analyte solutions were then loaded into the tips before washing and eluting solvents. Important parameters for washing and eluting solvent were investigated such as type and volume of solvents. The eluates were directly subjected to the HPLC-UV system described in Section 3.4.

Conditioning	Loading	Washing	Eluting
MeOH 300 µL H_2O 50 µL	Sample solution	Washing solvent	Eluting solvent



3.9 Sample pre-treatment

Meat samples (pork and beef) were purchased from three local markets in Nakhon Ratchasima, Thailand. For meat samples, protein matrix was removed according to our method reported previously (Chaloemsuwiwattanakan, et al., 2016). The samples pooled were manually cut into small pieces. Accurate weight about 0.2 g of homogeneous sample was taken into centrifuge tube and mixed with 10.00 mL of 50 mM $ZnSO_4$. The sample solution was vortexed and centrifuged at 3500 rpm for 15 min to precipitate protein. The supernatant was collected and filtered through 0.22 µm nylon syringe filters.

Animal urine samples (swine and cattle) were obtained from three local farms in Nakhon Ratchasima, Thailand. 1.00 mL of pooled urine samples were pipetted into the volumetric flasks and made up to 10.00 mL with DI water. The samples were filtered through 0.22 µm nylon syringe filters. The pre-treated samples were extracted by the method explained in section 3.8.

3.10 Analytical method performances

3.10.1 Repeatability of the in-pipette tip monolithic µ-SPE

To measure the reliability of the preparation method for the monolith in pipette tip. Triplicate extraction of a standard mixture containing 150 µg L^{-1} RAC and 100 µg L^{-1} CLEN were performed at the optimum condition by using the in-pipette tip monoliths synthesized from the same batch (tip-to-tip) and between batches (batch-to-batch). The repeatability in terms of %RSD was calculated from peak areas obtained from HPLC analysis of the two compounds.

3.10.2 Reusability of the in-pipette tip monolithic µ-SPE

To evaluate the robustness or reusability of the in-pipette tip monolithic µ-SPE, extraction of a standard solution containing 150 µg L^{-1} of RAC and 100 µg L^{-1} of CLEN at the optimum condition, was repeatedly carried out for 10 times (n=3 tips). The results were reported in terms of %RSD for peak areas of the two analytes.

3.10.3 Calibration curve (linearity) and breakthrough study

Breakthrough or capacity and linearity of the fabricated in-pipette tip monolithic µ-SPE monolith were investigated by increasing the concentration of the mixture standard solutions. The concentration ranges for RAC and CLEN were 25-5,000 µg L^{-1} and 5-10,000 µg L^{-1}, respectively. The mixed standard solutions were extracted at the optimum extraction conditions and analyzed by the developed HPLC method (Section 3.4). The peak areas for the two analytes were measured and plotted against the concentrations. The breakthrough or capacity is the maximum amount of

the analytes that can be adsorbed on the sorbent. The regression coefficient (r^2) was used to evaluate the linearity of the calibration curves.

3.10.4 Limit of detection (LOD) and limit of quantification (LOQ)

A low concentration of a mixed standard containing 12.5 µg L^{-1} for RAC and 2.5 µg L^{-1} for CLEN was extracted using the in-pipette tip monolithic µ-SPE and analyzed by the HPLC method to find the LOD and LOQ of the method based on the definitions of signal-to-noise (S/N) ratios of 3 and 10, respectively. The peak area was used as the signal while noise was measure from baseline drift in the HPLC chromatograms.

3.10.5 Precision

The precision for analysis of RAC and CLEN by our developed in-pipette tip monolithic µ-SPE-HPLC method within the day and between days were investigated. Triplicate analysis of spiked samples containing RAC and CLEN at 1000 µg L^{-1} was performed within a day and in three days. The peak areas were used to calculate %RSD for intra-day precision and inter-day precision.

3.10.6 Stability

In order to study the stability of the in-pipette tip monolithic µ-SPEs, the tips were dried and stored at room temperature for several months. To test the tip performance after long-term storage, the tips were used for extraction using the optimum conditions. The extraction results were compared with those obtained from newly prepared in-pipette tip monolithic µ-SPE.

3.10.7 Accuracy

Accuracy of the developed method was evaluated in terms of % recovery. The samples were spiked with standard RAC and CLEN before extraction and HPLC analysis. The analysis results from non-spiked and spiked samples were used for calculating the %recovery by the following equation.

$$\%\text{Recovery} = \frac{C_{\text{spiked sample}} - C_{\text{non-spiked sample}}}{C_{\text{standard added}}} \times 100$$

Where $C_{\text{spiked sample}}$ is the concentration of analyte found in spiked sample;

$C_{\text{non-spiked sample}}$ is the concentration of analyte found in non-spiked sample;

$C_{\text{standard added}}$ is the concentration of analyte added.

To examine the accuracy of the analysis in meat and urine samples. The samples were spiked by standard solutions of 10 mg L^{-1} RAC and 10 mg L^{-1} CLEN. Two levels of spiked concentration investigated were 50 µg L^{-1} and 1,000 µg L^{-1}. For meat samples, 0.2000 g of samples were weighted into 50 mL centrifuge tubes and added with 50 µL of the standard solutions. Another set of the samples were spiked with 1,000 µL of the standard solutions. 10.00 mL of 50 mM $ZnSO_4$ was added into the samples for protein precipitation. The samples were then vortexed and centrifuged at 3500 rpm for 15 min. The supernatants were collected and filtered through 0.22 µm nylon syringe filters.

For urine samples, 1.00 mL of pooled urine samples (swine and cattle) were pipetted into 10 mL volumetric flasks and spiked with 50 µL of the RAC and CLEN standard solutions. Another set of the samples were added with 1,000 µL of the

standard solutions. The samples were made up to final volume with DI water. The samples were filtered through 0.22 µm nylon syringe filters.

To perform the analysis, the pre-treated samples were extracted by the method explained in section 3.8. and analyzed by HPLC method summarized in 3.4.

3.10.8 Pre-concentration factor (P.F.) and extraction efficiency (E.E.)

P.F. and E.E. were defined as followed:

Pre-concentration factor (P.F.)

$$\text{P.F.} = \frac{\text{The analyte concentration after extraction } (C_2)}{\text{The analyte concentration in the sample before extraction } (C_1)}$$

$$\text{P.F} = \frac{C_2}{C_1}$$

Extraction efficiency (E.E.)

$$\text{E.F.} = \frac{\text{Mole of the analyte in the sample loaded}}{\text{Mole of the analyte in the extractant}}$$

$$E.E = \frac{C_2 V_2}{C_1 V_1} \times 100$$

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where V_1 is sample loading volume (mL); V_2 is eluting solvent volume (mL); C_1 is analyte concentration before extraction (µg L^{-1}) and C_2 is analyte concentration after extraction (µg L^{-1}).

CHAPTER IV

RESULTS AND DISCUSSIONS

In this chapter, the results for method development for analysis of two important β-agonists, RAC and CLEN are shown. The method was based on micro-extraction using lab-made in-pipette tip monolithic micro-solid phase extraction off-line coupled with high performance liquid chromatography. The results for the monolith in tip fabrication, characterization and analytical method development and performances are shown and discussed. The potential of our method for the analysis RAC and CLEN in real samples are also demonstrated.

4.1 Development of HPLC method

The HPLC-UV method was developed for the separation of RAC and CLEN after extraction by the fabricated in-pipette tip monolithic μ-SPE. Reversed-phase chromatographic separation was carried out on a silica-based C_8 column. The mobile phase of 25% (v/v) ACN in aqueous buffer was studied. The effect of mobile phase pH at 4.0, 5.0 and 6.0 and addition of TEA additive, were investigated. The results in Figure 4.1 shows that at the lower pH of 4.0 and 5.0 (Figure 4.1(a) and (b)), RAC and CLEN came out from the column faster. This could be explained that the secondary amine of RAC and CLEN could be fully protonated resulting in less interaction with

the nonpolar column. TEA was also added to the mobile phase to improve the peak shape by reducing peak tailing for CLEN. The mobile phase of 25% (v/v) ACN in 20 mM acetate buffer pH 4.0 containing 2 mM TEA was thus selected for further investigation.

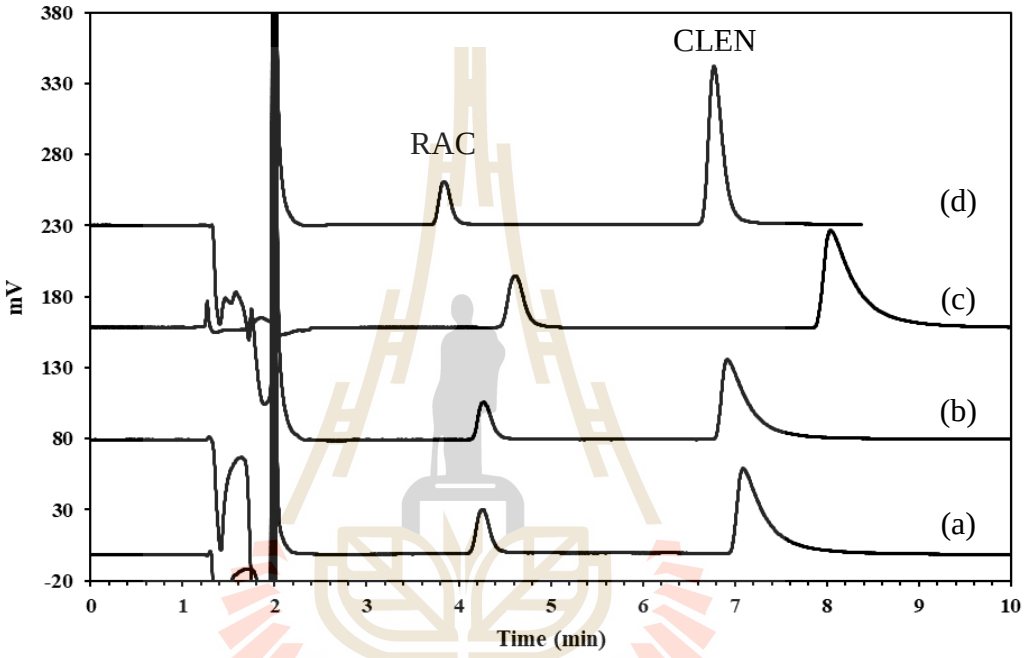

Figure 4.1 Chromatograms of standard mixture containing 5 mg L^{-1} RAC and 5 mg L^{-1} CLEN. Separation condition; column C_8, mobile phase of 25% (v/v) ACN in 20 mM acetate buffer pH (a) 4.0 (b) 5.0 (c) 6.0 and (d) 4.0 containing 2 mM TEA, injection volume of 20 µL, flow rate of 0.7 mL min^{-1} and detection wavelength of 210 nm.

4.2 Synthesis and characterization of in-pipette tip monolith

Sample extraction is usually required for analysis of high matrix containing sample such as biological, environmental and food samples. The solid phase extraction (SPE) was selected in this work as high extraction performance can be achieved with simple apparatus and procedures. In this thesis, the SPE was synthesized from a porous monolithic material and miniaturized in auto-micro pipette tip. The synthesis of MAA and EDMA co-polymer was carried out in auto-pipette tip *via* thermal polymerization as explained in Section 3.5. The chemistry of the monolithic material depends on type of monomers while morphology such as skeleton and porosity, depends on type and amount of the porogenic solvent (porogen). Co-polymer of polar MAA and non-polar EDMA was selected as it is stable at the extraction condition, simply prepared and polarity-tunable. Two important parameters, polymerization temperature and amount of porogen were investigated. The temperature was varied from 60°C to 75°C. As expected, the time to complete the polymerization decreased with increasing of polymerization temperature due to the increasing of polymerization rate. Polymerization time were reduced from 4 hr to 1 hr and 10 min by increasing the temperature from 60°C to 75°C (data are shown in APPENDIX A). However, at temperature higher than 75°C, melting of the Parafilm® and Scotch® tape was observed. Therefore, the temperature of 75°C was an optimum temperature for synthesis of the monolithic sorbent in pipette tip.

For extraction, the sorbent should provide high surface area with good permeability (flow through ability of the solution) so that the high extraction efficiency with high speed can be achieved. In order to adjust the porosity of the monolithic sorbent, the ratios of monomers to porogens in the polymerization solution were varied from 15:85 to 23:77. It was found that the composition did not only affect the

permeability (Table 4.1) but also attachment of the material on the surface of the pipette tips. The high amount of porogen at 85% caused gel-like monolith structure and dropping of the monolith from the end of the tip as also reported previously (Zhang and Chen, 2013). The dropping of monolith could be eliminated by increasing monomer ratio from 18% to 23%. Good morphology and well attachment of the monolith in the tip was observed with success rate for the preparation of 95%. Another important key for the preparation is the cleanliness of the inner wall of the pipette tips before processing the polymerization. Without proper tip cleaning, the preparation success rate could be reduced. The SEM images in Figure 4.2 show the monolith formed inside the tip with good wall attachment. By compromising the surface area and extraction time, the composition of monomers to porogens of 18:82 was selected for further investigation.

Table 4.1 The permeability and preparation success rate with various ratio of monomers to porogens.

Ratio of monomer:porogen (g:g)	Permeability*	Preparation success rate **
15:85	High	30%
18:82	Medium	95%
21:79	Less	95%
23:77	Less	95%

* Tested by flowing a solution of MeOH through the synthesized materials.

** Percent of monolith formed with good morphology and well retaining in the tips (n = 20).

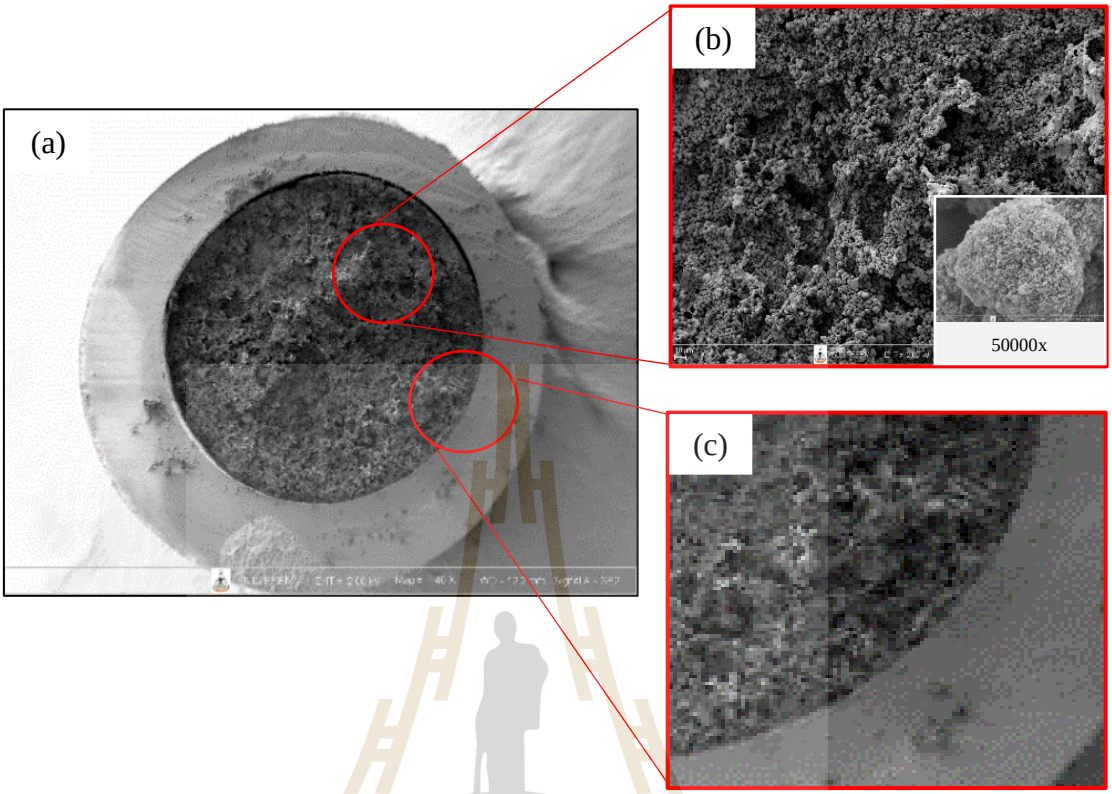

Figure 4.2 SEM images of the representative in-pipette tip monolith (a) cross section of monolith prepared from 18:82 monomer:porogen, polymerization at 75°C for 1 hr and 10 min, (b) image magnification of 300x and 50000x and (c) attachment of the monolith with the inner surface of the pipette tip.

FT-IR was used to characterize the functional groups of the MAA-EDMA monolithic material synthesized in pipette tip. The FT-IR spectrum in Figure 4.3 shows the peaks at around 1146 cm^{-1} from C-O stretching, 1723 cm^{-1} from C=O stretching, 2800-2900 cm^{-1} from C-H stretching and 3567 cm^{-1} from O-H stretching. The results were similar with these reported by Jin et al. (Jin et al., 2013).

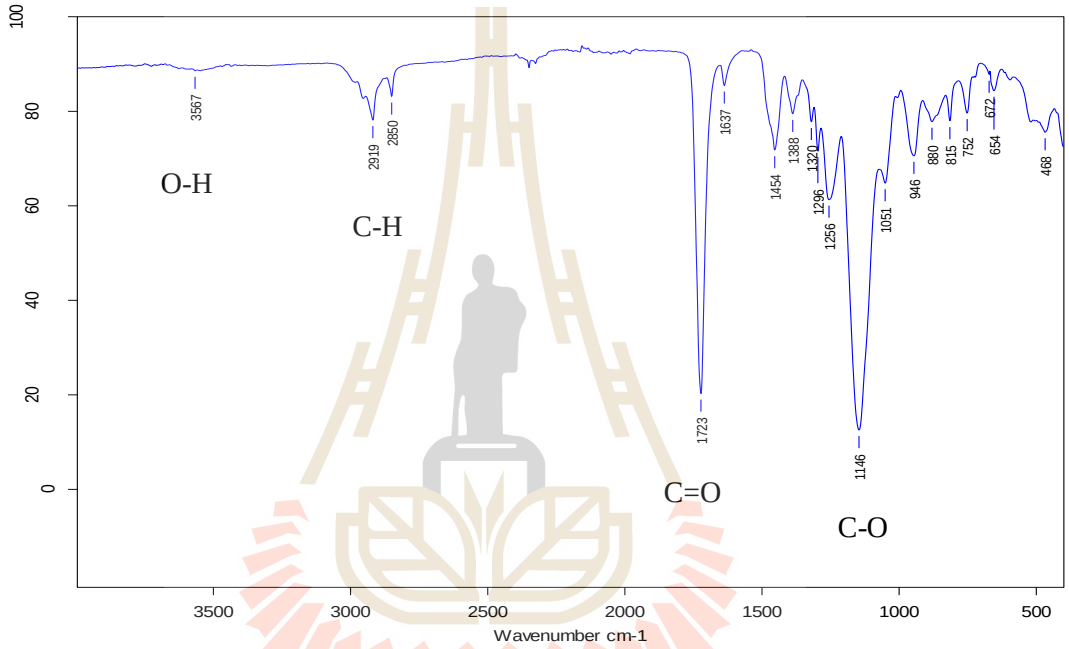

Figure 4.3 FT-IR spectrum of MAA-EDMA monolith synthesized in pipette tip.

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The in-pipette tip monolithic µ-SPE obtained from the optimum condition, are shown in Figure 4.4.

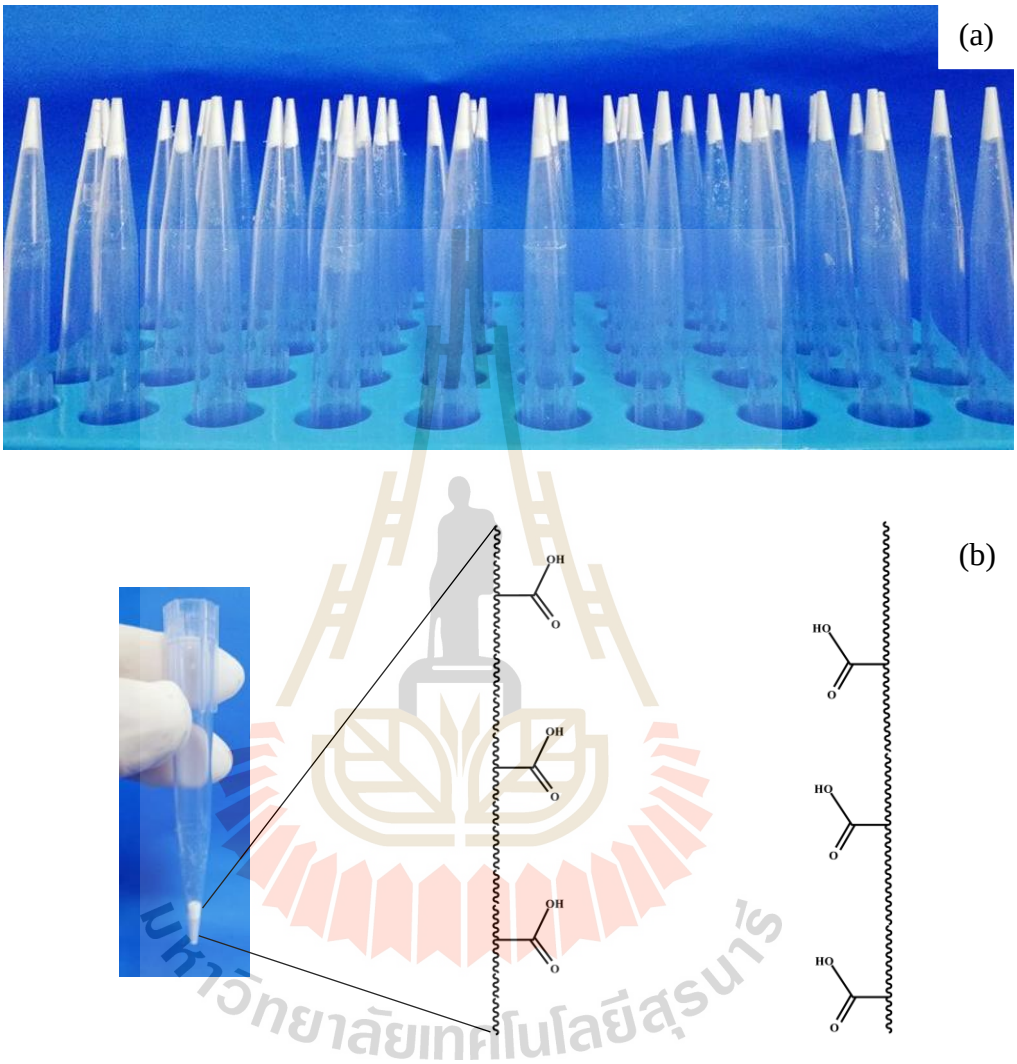
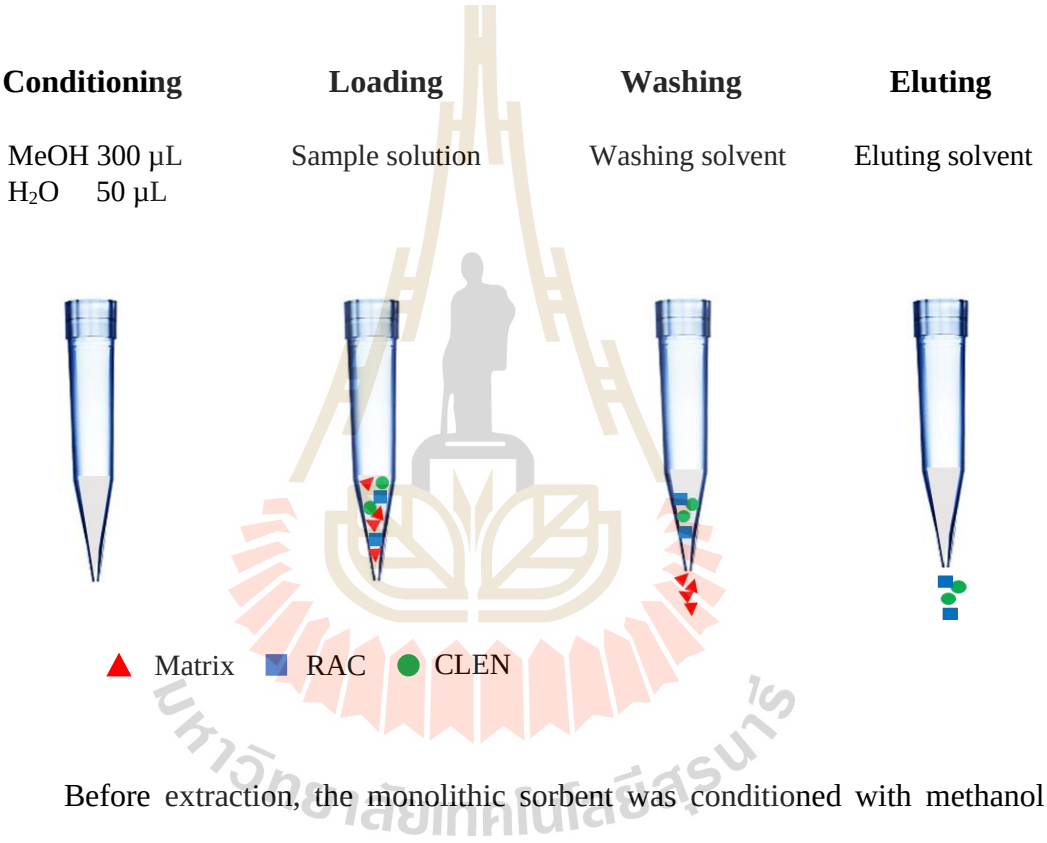


Figure 4.4 (a) Images of in-pipette tip monolithic µ-SPE synthesized from 18:82 monomers:porogens at condition as explained in Section 3.5. (b) functional groups on the surface of the MAA-EDMA monolithic sorbent in pipette tip (~~~~ is the hydrophobic skeleton of the monolith).

4.3 Optimization for in-pipette tip monolithic µ-SPE

To perform the extraction, the pipette tip was connected with T-way valve and syringes as described in Section 3.7 (Figure 3.2(b)). Extraction procedure for in-pipette tip monolithic µ-SPE consisted of four important steps, conditioning, loading, washing, and eluting.



Before extraction, the monolithic sorbent was conditioned with methanol to activate functional groups on the surface of the monolithic sorbent then washed with water to remove the conditioning MeOH solvent before sample loading. The washing step is usually required to remove the unwanted compounds that could also adsorb on the sorbent before eluting of the trapped analytes from the sorbent. To obtain the good extraction performance, washing and eluting solvents were investigated.

4.3.1 Washing solvent

During sample loading step, some matrices in the sample can also adsorb on the surface of the sorbent. The matrices weakly trapped can be removed from the sorbent by a suitable solvent called washing solvent. The washing solvent can help to reduce the matrices interfering and contamination on the HPLC system. Basically, the washing solvent should be able to remove the matrices which possibly retain on the monolithic sorbent without loss of the trapped analytes. As the main component of the monolithic sorbent is EDMA, the interaction is like in reversed-phase chromatography. Organic solvent of ACN was hence selected as the washing solvent additive for reducing the interaction between the compounds and the sorbent. Mixtures of ACN and water from 10% to 25% (v/v) ACN, 800 μL were examined and compared with the control washing solvent (0%ACN, water). After washing, the trapped standard RAC and CLEN were eluted with 150 μL of 30% (v/v) ACN in 200 mM acetate buffer pH 4.0. The eluates were subjected into the HPLC system. The HPLC results showed that only 10%ACN was adequate to elute the adsorbed matrices.

In addition, ACN concentration higher than 15% significantly could desorb the relatively polar RAC from the sorbent (Figure 4.5). Washing solvent of 10%ACN, 800 μL was thus selected as the optimum washing solvent.

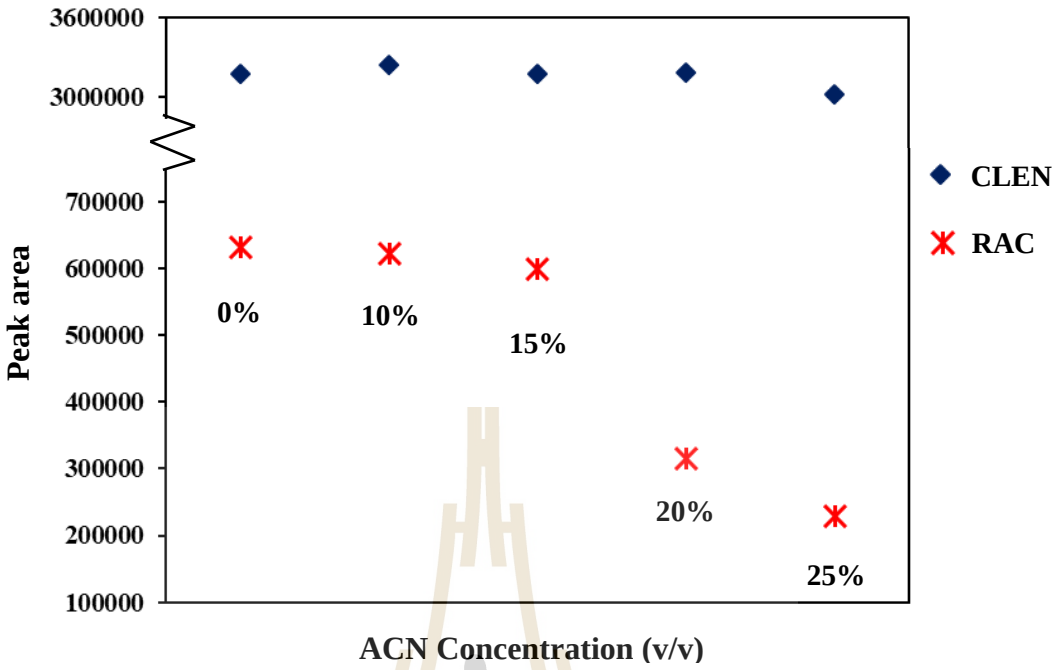


Figure 4.5 Effect of ACN concentration in washing solvent on signals obtained from spiked urine samples containing 1 mg L^{-1} RAC and 1 mg L^{-1} CLEN. Extraction conditions; 300 µL conditioning solvent of MeOH and 50 µL water, 800 µL washing solvent volume, 2.00 mL sample loading, 150 µL eluting solvent (30% (v/v) ACN in 200 mM acetate buffer pH 4.0). Separation condition; C_8 column, mobile phase of 25% (v/v) ACN in 20 mM acetate buffer pH 4.0 containing 2 mM triethylamine, injection of 20 µL, flow rate of 0.7 mL min^{-1} and detection wavelength of 210 nm.

4.3.2 Eluting solvent

In this work, the eluate was directly subjected into the HPLC system. The eluting solvent is therefore important for both extraction efficiency and pre-concentration factor. To obtain the high P.F., the eluting solvent should completely desorb the trapped analytes with minimum volume.

Since the samples were loaded through the monolith at the pH around 6 or 7 (aqueous environment without pH adjustment), RAC and CLEN could interact on the sorbent surface *via* both hydrophobic interaction with the main hydrophobic skeleton of the monolithic sorbent and hydrophilic interactions due to H-bonding/coulombic interaction with the carboxylate groups on the surface of the monolith as shown in Figure 4.6.

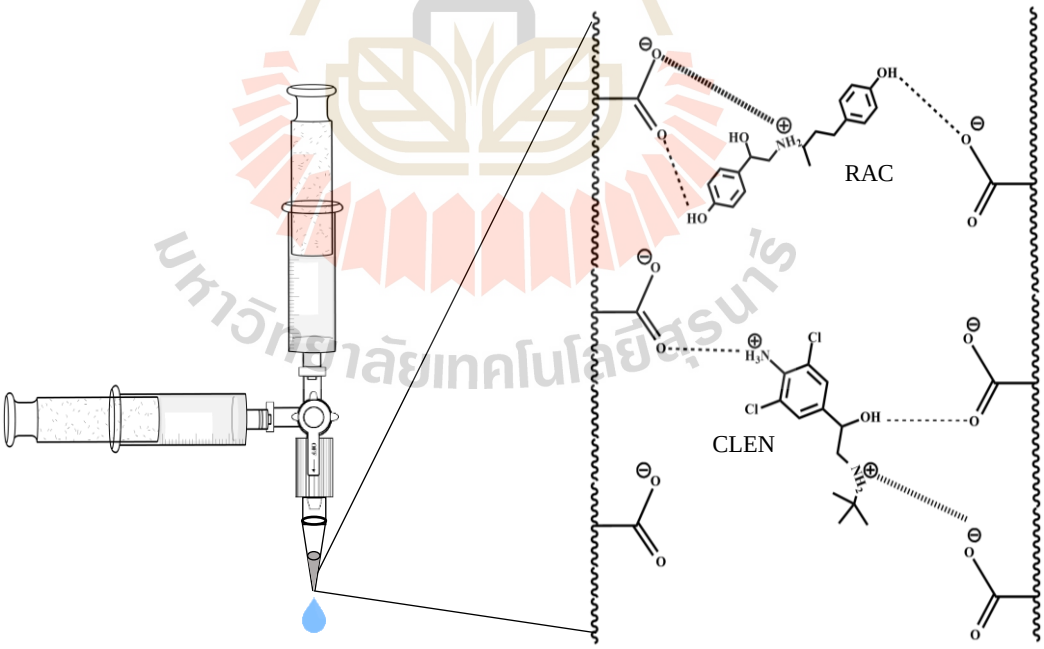


Figure 4.6 Predicted interaction between the analytes and MAA-EDMA monolithic sorbent in pipette tip.

To find an optimum eluting solvent, several parameters including effect of buffer pH, organic solvent concentration, type of buffer, buffer concentration and salt additive, and volume of the eluting solvent were investigated.

The first examined parameter was the effect of buffer pH 30% (v/v) ACN in 20 mM buffer pH 2.0, 4.0, 7.0 and 9.0 were investigated. The recovery from HPLC analysis of the two analytes in Figure 4.7 shows that the efficient elution could be achieved from acidic solutions since both the carboxylate groups and the secondary amine groups were fully protonated. The interactions between the analytes and the sorbent were thus reduced. The adsorbed RAC and CLEN were therefore released from the material. From this study, the eluting pH of 4.0 was selected as the pH of 2.0 can reduce the life time of the silica-based HPLC column.

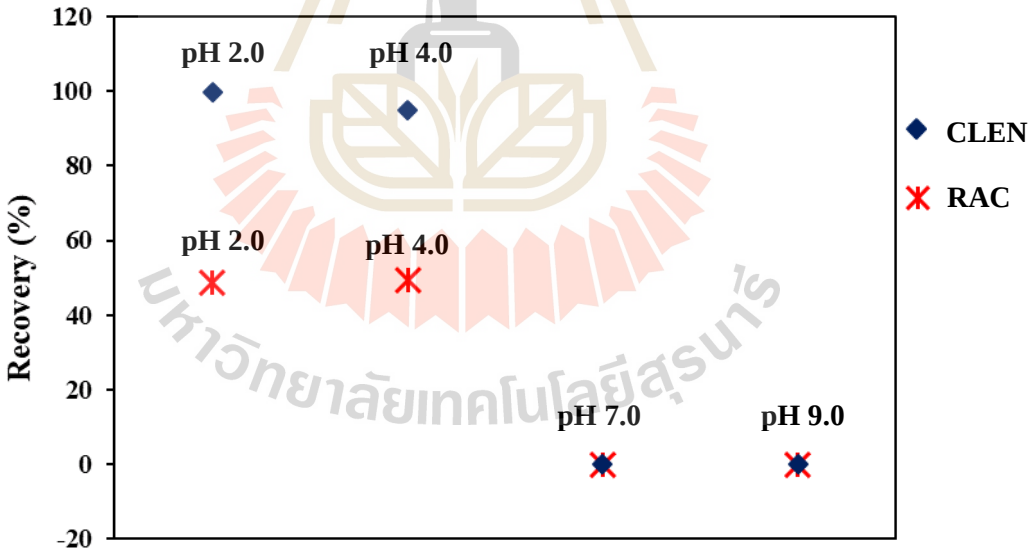


Figure 4.7 Effect of pH in the eluting solvent on extraction of 1 mg L^{-1} RAC and 1 mg L^{-1} CLEN in spiked urine samples. Other conditions are in Figure 4.5. Condition; 800 µL washing solvent of 10%(v/v) ACN in water.

The amount of ACN in 20 mM acetate buffer pH 4.0 was also varied from 30 to 50% (v/v). It was found that the concentration of ACN in the studied range provided similar elution performance (Figure 4.8). However, 30% (v/v) ACN in 20 mM acetate buffer pH 4.0 was selected due to the better peak shape (sharper peak).

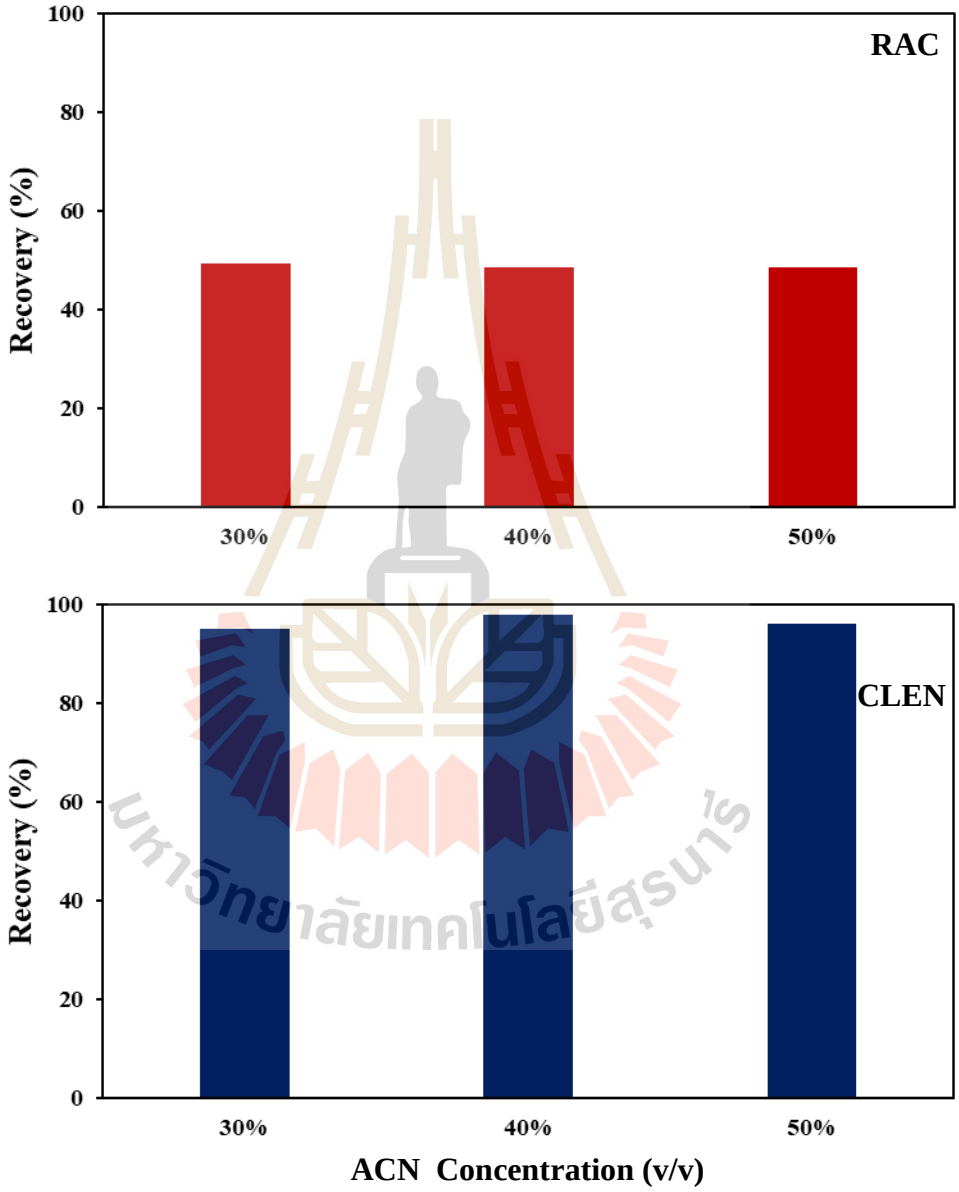


Figure 4.8 Effect of ACN concentration in the eluting solvent of ACN in 20 mM acetate buffer pH 4.0 on extraction of 1 mg L^{-1} RAC and 1 mg L^{-1} CLEN in spiked urine samples. The conditions are as described in Figure 4.7.

Although the high extraction efficiency was achieved for CLEN, the elution of RAC was not satisfactory (Figure 4.8). To improve the elution efficiency for RAC, type and concentration of buffer and salt additive were investigated. The results in Figure 4.9 demonstrated that acetate buffer provided much higher extraction recovery compared with that obtained from phosphate buffer (Figure 4.9(a) and (b)). In addition, significant improvement was observed from increasing the acetate buffer concentration from 20 mM to 200 mM (Figure 4.9(b) and (d)). The addition of KCl only slightly improved the RAC extraction (Figure 4.9(b) and (c)). The eluting volume of 150 µL was found to be minimum volume required for complete elution of RAC and CLEN.

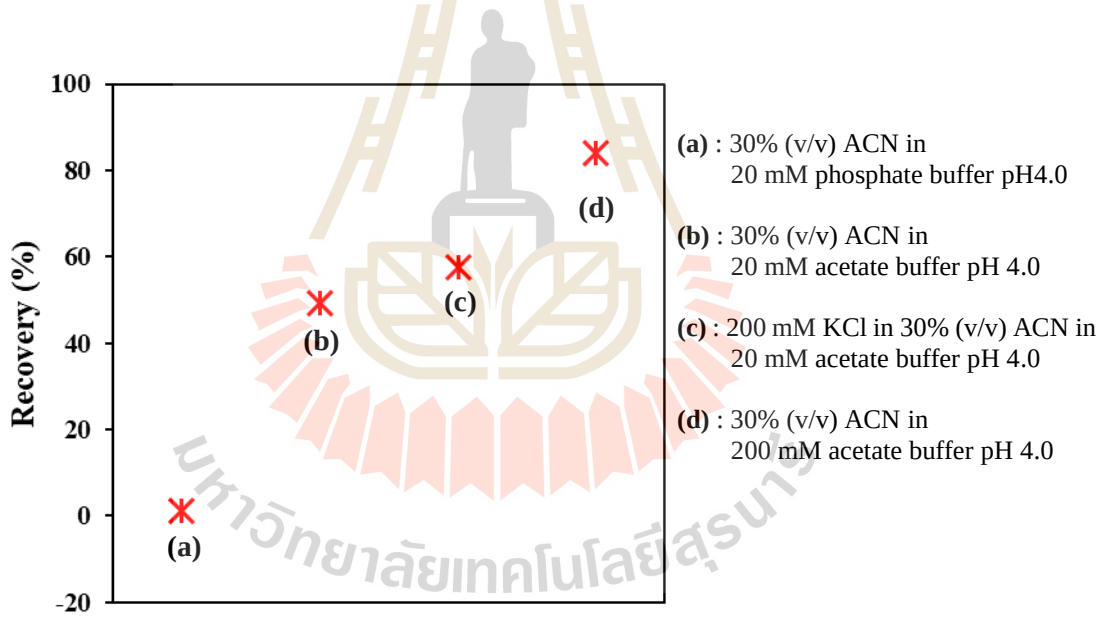


Figure 4.9 Effect of type of buffer, buffer concentration and salt additive in the eluting solvent on extraction of 1 mg L^{-1} RAC in spiked urine samples. The conditions as described in Figure 4.7.

The optimum condition for the extraction of RAC and CLEN using the in-pipette tip monolithic µ-SPE is summarized below.

Table 4.2 Optimum condition for RAC and CLEN pipette tip monolithic µ-SPE.

Step	Solvent	Volume
Conditioning	MeOH	300 µL
	H_2O	50 µL
Loading	Sample or solution	2000 µL
Washing	10% (v/v) ACN in water	800 µL
Eluting	30% (v/v) ACN in 200 mM acetate buffer pH 4.0	150 µL

4.4 Analytical method performances

4.4.1 Repeatability of the in-pipette tip monolithic µ-SPE

To evaluate the precision of the in-pipette tip monolith preparation, the extraction using tip-to-tip and batch-to-batch in-pipette tip monolith were investigated. The extracted amount of RAC and CLEN were monitored by HPLC method. The repeatability and precision in terms of %RSD are shown in Table 4.3. The low %RSD of peak areas less than 3.18% indicated that the preparation method is repeatable and reliable.

4.4.2 Reusability of the in-pipette tip monolithic µ-SPE

The reusability of the in-pipette tip monolithic µ-SPE was examined by regenerating the used monolith in tip before re-extracting a standard mixture of RAC and CLEN. It was found that the tips could be reused at least 10 times without loss of

extraction performance. The %RSD for signal in terms of peak areas for RAC and CLEN for 10 times extractions (n=3 tips) were 2.36 and 4.66, respectively (data are shown in APPENDIX B).

4.4.3 Breakthrough and calibration curve (linearity) study

Breakthrough or capacity is the highest amount of the analyte that can be adsorbed on the material. This property is related with the chemistry or functional groups and surface area or active sites of the material. In order to find the capacity of our monolith in tip, the loaded amount of RAC and CLEN on the tips were varied. At loading volume of 2.00 mL, the extracted analytes linearly increased with the analyte concentration as shown in Figure 4.10. The results showed wide dynamic linearity range for both RAC and CLEN (25-5,000 µg L^{-1} and 5-10,000 µg L^{-1} for RAC and CLEN, respectively). From the calibration curves, it could be concluded that the monolith in pipette tip could adsorb RAC and CLEN more than 10 and 20 µg, respectively. Good linearity with correlation coefficients $\geq$ 0.9999 was observed.

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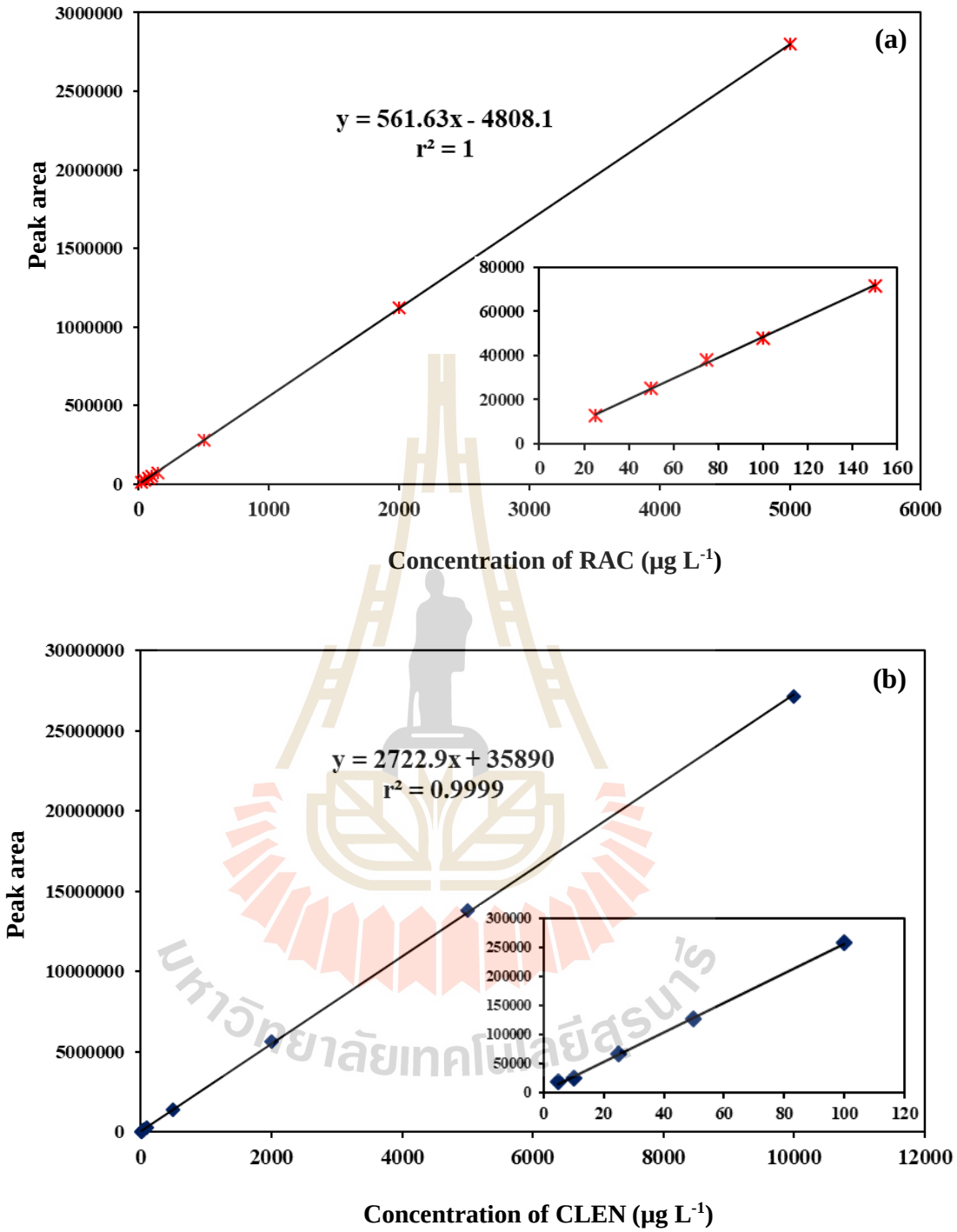

Figure 4.10 Calibration curves of standard (a) RAC and (b) CLEN. The conditions are as described in Figure 4.7.

4.4.4 LOD and LOQ

Sensitivity of the method in terms of LOD and LOQ was evaluated. The LODs obtained from our method were 1.3 and 0.2 µg L^{-1} for RAC and CLEN, respectively. LOQs of 4.3 µg L^{-1} for RAC and 0.7 µg L^{-1} for CLEN were achieved. The LODs and LOQs obtained were relatively low compared with the other reported work as shown in Table 4.4 and adequate for real applications as the found concentration of RAC and CLEN were reported between 2 µg L^{-1} and 100 ug L^{-1} for urine and meat samples, respectively.

4.4.5 Precision

Precision of the method for RAC and CLEN analysis using the in-pipette tip monolithic µ-SPE off-line coupled with HPLC, was examined. Inter-day and intra-day precisions were studied as described in Section 3.10.5. The precision was evaluated in terms of %RSD for peak areas observed from HPLC analysis. The precision for both inter-day and intra-day were less than 3.26. The full results for these experiments are shown in Table 4.3.

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4.4.6 Stability

In order to find lifetime of the in-pipette tip monolith, stability of the tips was performed (see details in Section 3.10.6). It was found that the tips could keep dry and stored at room temperature for at least 6 months without loss of performance and physical change.

Table 4.3 Summary for analytical method performances.

	RAC	CLEN
In-pipette tip monolithic µ-SPE		
Repeatability		
• Tip-to-tip (n=3)*	3.18	2.66
• Batch-to-batch (n=3)*	2.83	1.27
Reusability	2.36	4.66
Breakthrough	>10 µg	>20 µg
Stability	At least 6 months	
Extraction time	5 min	
In-tip monolithic µ-SPE – HPLC		
Calibration curve (µg L^{-1})	25-5000	5-10000
Slope	561±0.95	2722±9.62
Intercept	-4808±1807	35889±36464
r^2	1.0000	0.9999
LOD (µg L^{-1})	1.3	0.2
LOQ (µg L^{-1})	4.3	0.7
Precision		
Intra-day (n=3)*	1.70	3.26
Inter-day (n=3)*	3.26	2.73
Analysis time	12 min	

*%RSD calculated from peak area

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Table 4.4 Comparison of the method from this work with other works for the analysis of RAC and CLEN.

Extraction method	Detection method	Sample	LOD (ng g^{-1})		Sample/solvent consumption**	Reconstitute	Analysis time (min)	Reference
			RAC	CLEN				
MIPs membrane	HPLC-DAD	Pork meat	0.07*	0.15*	5 g/>10 mL	X	>40	(Qui et al., 2016)
MIPs SBSE	HPLC-DAD	Pork, liver, feed	0.1*	0.21*	5 g/>170 µL	X	60	(Qiu et al., 2010)
DPX	LC-MS/MS	Pork meat	0.2	0.4	0.25 g/>850 µL	✓	26	(Mastrianni et al., 2018)
In-tip µ-SPE	HPLC-UV	Meat, urine	1.3*	0.2*	0.2 g,1 mL/950 µL	X	12	This work
NS-SFODM	CZE	Swine feed	1.1	1.5	2 g/>50 µL	✓	>27	(Wang et al., 2015)
MSPD	HPLC-DAD	Porcine liver	6.1	8.4	0.2 g/ >13 mL	✓	>30	(Zhu et al., 2014)
C_{18}-SPE	CE-ED	Pig feed, urine, liver	90*	100*	2-10 g/ >5 mL	✓	30	(Wang et al., 2010)

* unit of µg L^{-1}, ** total volume of solvent used

MIPs: Molecularly Imprinted Polymers, SBSE: Stir Bar Sorptive Extraction, DPX: Dispersive Pipette Extraction, SFODM: nitrogen-blowing salt-induced solidified floating organic droplet microextraction, MSPD: Micro-solid phase dispersion.

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4.5 Pre-concentration factor (P.F.) and extraction efficiency (E.E)

To find the pre-concentration factor (P.F.) and extraction efficiency (E.E.) of the extraction method, spiked pooled swine and cattle urines were extracted at the optimum condition with loading volumes from 2.00-20.00 mL. The pre-concentration factor linearly increased with increasing of sample loading volume from 2.00-10.00 mL while the extraction efficiency slightly decreased (Figure 4.11). Co-elution of RAC and sample matrix in the HPLC chromatograms was found when the loading volume was more than 10.00 mL. The high extraction efficiency indicates good interaction between the analyte and the sorbent with high mass transfer rate. The P.F. is readily increased by increasing sample loading volume with small increasing of extraction time due to the high permeability of the sorbent. Therefore, the optimum loading volume depends on the sample volume availability, sample matrix and pre-concentration needed. Another advantage of the extraction method is the wide capability of the technique as the device could also employ sample volumes in the range of micro-liters, which may not be able to handle by the typical SPE cartridges or classical liquid-liquid extraction procedure.

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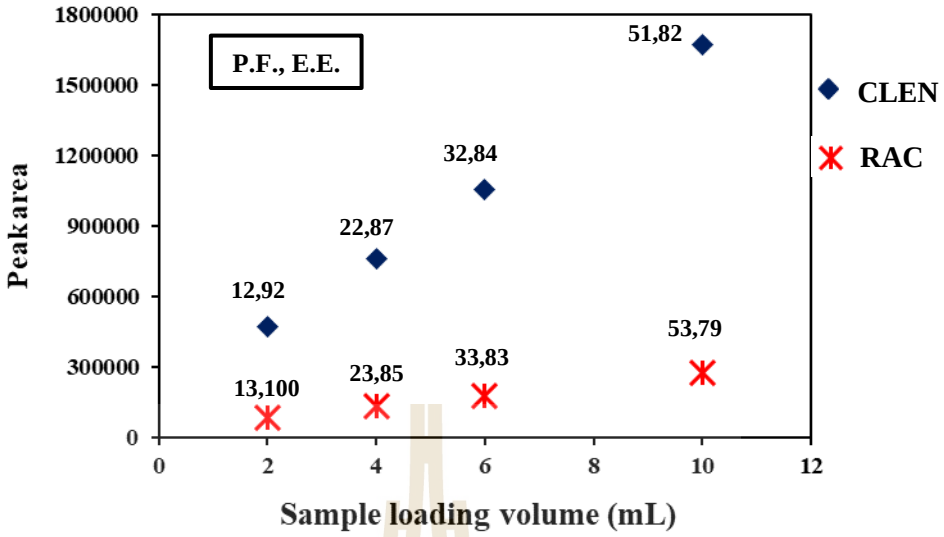

Figure 4.11 Effect of sample loading volume on pre-concentration factor (P.F.) and extraction efficiency (E.E.) (pooled spiked swine urine samples containing 100 µg L^{-1} RAC and 100 µg L^{-1} CLEN). The conditions are as described in Figure 4.7.

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4.6 Application of in-pipette tip monolithic µ-SPE

The method was applied for determination of RAC and CLEN in four types of samples which were swine urine, cattle urine, pork and beef samples. The samples were spiked with standard RAC and CLEN at two concentration levels of 50 and 1,000 µg L^{-1}. After sample pre-treatment as explained in Section 3.9, the spiked and non-spiked samples were extracted by the developed µ-SPE method. At the optimum condition with 2 mL sample loading, the extraction time was 5 min with solvent consumption of 425 µL. For HPLC analysis, the two analytes were separated in 7 min. It was found that no RAC and CLEN were detected in non-spiked samples. It could be concluded that the samples contained no RAC and CLEN or the RAC and CLEN in the samples were lower than 1.3 and 0.2 µg L^{-1}, respectively. The accuracy of the method was evaluated in terms of percent recovery. The recoveries achieved were between 84-114% for urine samples and 83-112% for meat samples. The analysis results are shown in Table 4.5. The chromatograms for spiked samples are in Figure 4.12(a), it is clearly seen that the extraction did not only significantly enhance the analyte signals but also efficiently cleaned up the sample as only RAC and CLEN were found in the samples after the extraction. This implied that at the optimum condition, the extraction method was highly selective to the two analytes. Without the extraction, the analysis could not be performed due to the interfering by matrix signals and low analyte signals (Figure 4.12(b)).

Table 4.5 Determination of RAC and CLEN in spiked meat and urine samples using the developed in-pipette tip monolithic µ-SPE coupled with HPLC-UV method.

Sample	Concentration of RAC (µg L^{-1})		Recovery (%)	Concentration of CLEN (µg L^{-1})		Recovery (%)
	Added	Found		Added	Found	
Swine urine	0	ND	-	0	ND	-
	1000	887.8	89	1000	904.8	90
	50	49.5	99	50	56.8	114
Cattle urine	0	ND	-	0	ND	-
	1000	898.3	90	1000	938.5	94
	50	51.7	103	50	42.2	84
Pork	0	ND	-	0	ND	-
	1000	1082.5	108	1000	1091.0	109
	50	50.6	101	50	44.3	89
Beef	0	ND	-	0	ND	-
	1000	1120.2	112	1000	1114.0	111
	50	41.4	83	50	56.0	112

ND = Not detectable

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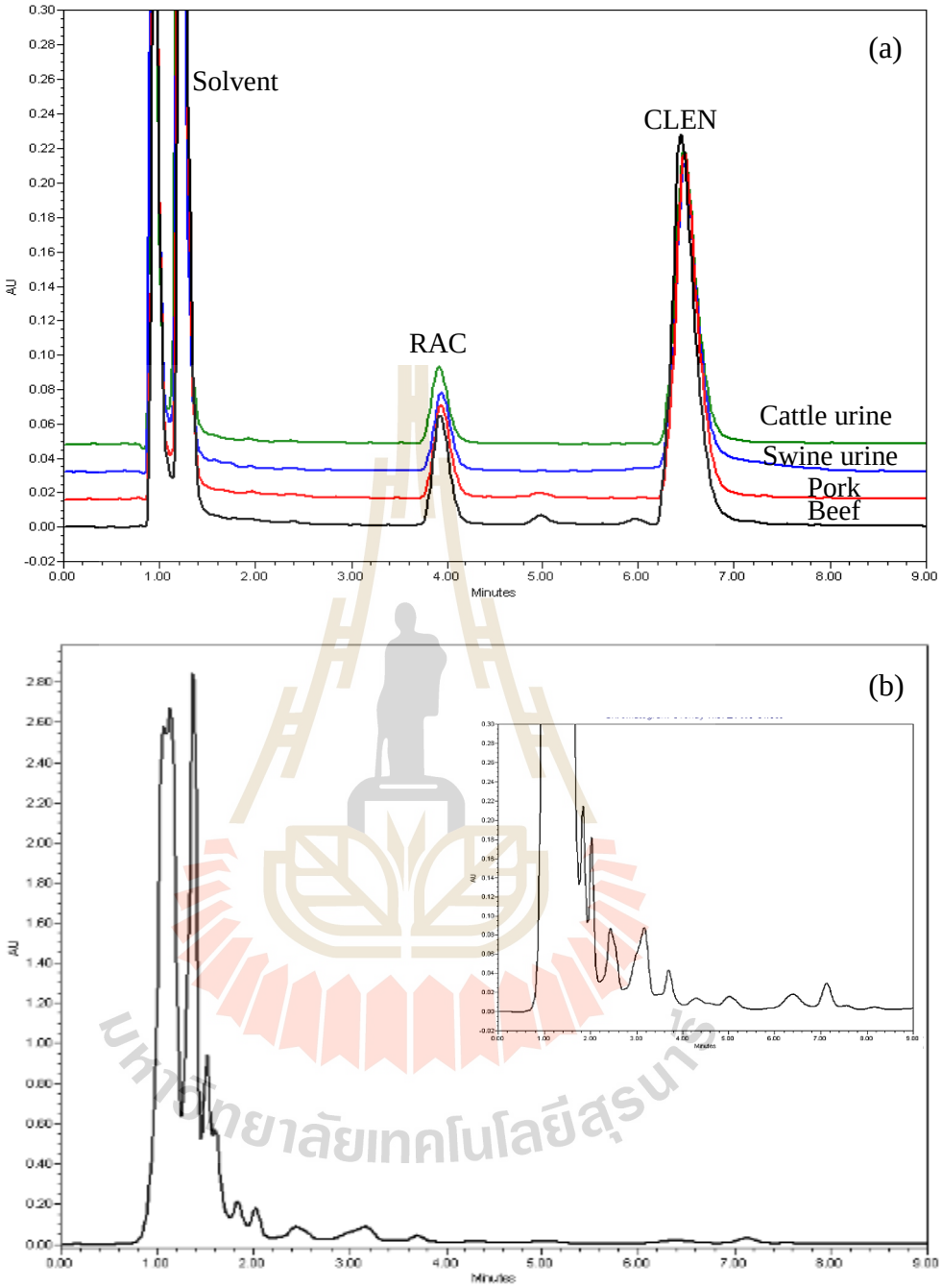

Figure 4.12 Representative chromatograms of spiked samples containing 1 mg L^{-1} RAC and 1 mg L^{-1} CLEN (a) with in-pipette tip monolithic µ-SPE before HPLC analysis (b) urine sample without in-pipette tip monolithic µ-SPE. Other condition as described in Figure 4.7.

CHAPTER V

CONCLUSIONS

This thesis aims to develop a portable, low cost and green micro-extraction method and an accurate, reliable and fast analytical technique for the determination of two important RAC and CLEN in animal urine and meat samples. The green micro-extraction device was fabricated by *in situ* synthesis of porous MAA-EDMA monolithic material in auto-pipette tip at the monomer to porogen ratio of 18:82 and polymerization temperature of 75°C for 1 hr and 10 min. The extracted analytes were analyzed by a high separation efficiency HPLC method. SEM and FT-IR data confirmed the formation and attachment of the synthesized monolith in the tip. The preparation success rate of 95% was achieved. By assembling with a T-way valve, micro-solid phase extraction was performed without need of flow controlling system. The optimum conditions for the extraction for RAC and CLEN were 300 µL of conditioning solvent (MeOH), 800 µL of washing solvent (10% (v/v) ACN in water) and 150 µL of eluting solvent (30%(v/v) ACN in 200 mM acetate buffer pH 4.0). At 2 mL sample loading, the pre-concentration of 12 and 13 were achieved with %extraction efficiency of 92% and 100% for RAC and CLEN, respectively. However, the pre-concentration was readily increasing by increasing the sample loading volume. The extraction was performed in only 5 min with organic solvent consumption of about 425 µL. In order to quantitate the two analytes, an HPLC method was developed. RAC and CLEN could be separated on a silica-based C_8 column with optimum mobile phase of 25% (v/v)

ACN in 20 mM acetate buffer pH 4.0 containing 2 mM triethylamine. The separation was achieved in 7 min.

Off-line coupling of the developed micro-extraction and HPLC method was applied for the analysis in spiked swine urine, cattle urine, pork and beef. The urine samples were diluted and directly loaded into the monolithic in-pipette tip device while meat samples required protein precipitation by 50 mM $ZnSO_4$ before the extraction. Good accuracy in terms of %recovery higher than 84% were observed. The method provided low LODs and LOQs (< 1.3 ppb and 4.3 ppb, respectively) with good inter-day and intra-day precisions (%RSD < 3.26). The in-pipette tip monolith could be reused at least 10 times without the loss of extraction performance.

The in-pipette tip monolith significantly improved the sensitivity and selectivity of the RAC and CLEN analysis by HPLC. This extraction method is environmentally friendly as it requires organic solvent in micro-liters volume only. In addition, the device is simple to prepare, cheap, portable and can be performed without need of special apparatus. The device could be an interesting alternative choice for other applications especially field analysis, screening test and clinical point of care as on-site sampling can be simply preformed.

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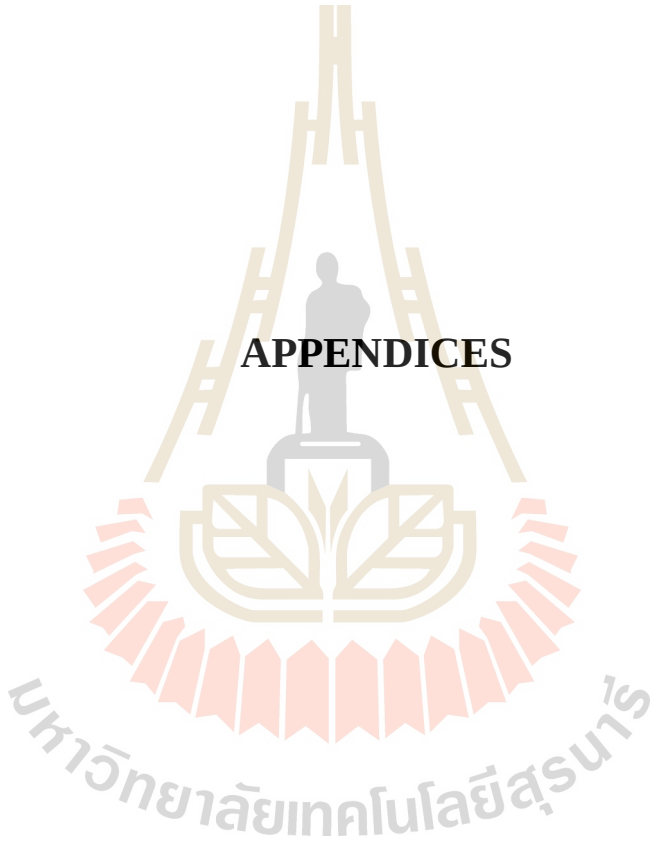
APPENDICES



APPENDIX A

EFFECT OF POLYMERIZATION TEMPERATURE AND TIME FOR SYNTHESIS OF MONOLITH IN MICRO PIPETTE TIP

Table 1A Polymerization time and temperature for synthesis of monolith in pipette tip.

Temperature (°C)	Time
60	4 hr
70	2 hr
75	1 hr 10 min

*Monolith composition= monomers:porogens 18:82

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APPENDIX B

REUSABILITY OF IN-PIPETTE TIP MONOLITHIC MICRO-SOLID PHASE EXTRACTION

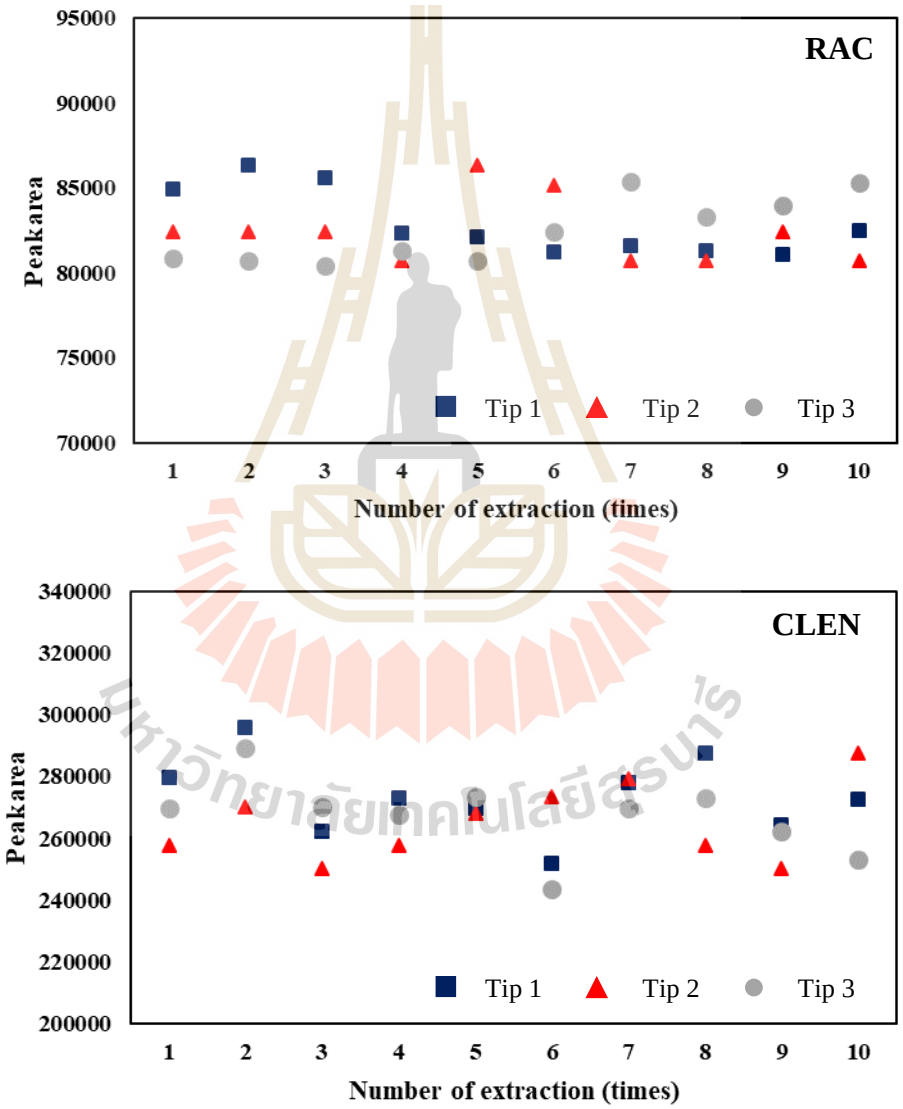

Figure 1B The distribution of signal in terms of peak area for 10 times extractions (n=3 tip).

Table 1B Summary for mean, S.D. and %RSD of peak area for RAC and CLEN.

	RAC			**CLEN**		
	Tip 1	Tip 2	Tip 3	Tip 1	Tip 2	Tip 3
Mean±S.D	82905± 1965	82386± 1952	82443± 1937	273525± 12682	265216± 12568	267188± 12291
%RSD	2.37	2.37	2.35	4.64	4.74	4.60
%RSD (Mean)		2.36			4.66	

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