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METHOD DEVELOPMENT FOR THE DETERMINATION OF ANTIBIOTICS AND ANTIBACTERIALS IN COW MILK USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Miss Urairat Koesukwiwat

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Chemistry
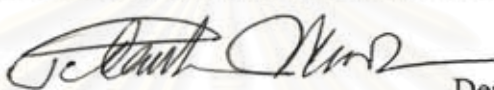
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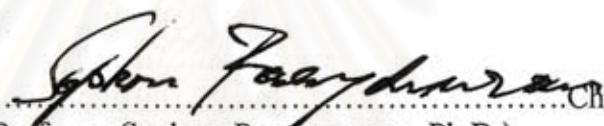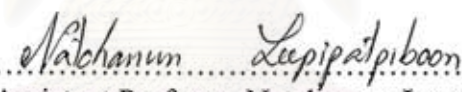Chulalongkorn University

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Thesis Title	Method development for the determination of antibiotics and antibacterials in cow milk using liquid chromatography-mass spectrometry
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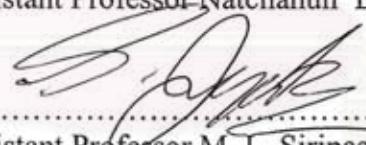(Assistant Professor M. L. Siripastr Jayanta)

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งานวิจัยนี้เป็นการพัฒนาวิธีวิเคราะห์สารตกค้างกลุ่มซัลโฟนามีด, เททราซัยคลิน และพีรี-
เมธามีน รวม 10 ชนิด ในน้ำนมวัวโดยใช้เทคนิคลิควิดโครมาโทกราฟี-แมสสเปกโทรเมทรี แบบ
electrospray ionization และตรวจวัดในรูปของประจุบวก วิธีการเตรียมสารตัวอย่างที่พัฒนาขึ้น
ประกอบด้วยการตกตะกอนโปรตีนในน้ำนมด้วยกรดและใช้สารละลายบัฟเฟอร์ McIlvain ในการ
ป้องกันการเกิดสารประกอบเชิงซ้อนระหว่างสารที่วิเคราะห์กับโลหะ จากนั้นจึงทำการแยกสารโดย
ใช้วัฏภาคของแข็งดูดซับแบบ co-polymer Oasis® HLB มีการศึกษาหาสภาวะที่เหมาะสมที่สุด
สำหรับสภาพไวและความสามารถในการแยกสารผสมทั้ง 10 ชนิด รวมทั้งศึกษาถึงปัจจัยที่มีผลต่อ
ประสิทธิภาพของวิธีการสกัดสาร พบว่าวิธีการเตรียมสารตัวอย่างที่พัฒนาขึ้นมามีประสิทธิภาพดี
ให้ค่าร้อยละการคืนกลับของสารทั้ง 10 ชนิด มากกว่า 70 เปอร์เซ็นต์ ขีดจำกัดต่ำสุดของวิธีการ
ตรวจวิเคราะห์มีค่าอยู่ในช่วง 0.48 -2.64 นาโนกรัมต่อกรัม ซึ่งมีค่าต่ำกว่าปริมาณสารตกค้างสูงสุดที่
อนุญาตให้มีอยู่ได้ในน้ำนมโคที่กำหนดโดย EU และ U.S. FDA ขีดจำกัดต่ำสุดของการวิเคราะห์หา
ปริมาณมีค่าอยู่ในช่วง 0.97 ถึง 8.64 นาโนกรัมต่อกรัม กราฟเทียบมาตรฐานสำหรับการวิเคราะห์หา
ปริมาณมีความเป็นเส้นตรงอยู่ในช่วงความเข้มข้นตั้งแต่ระดับ MQL ถึง 300 นาโนกรัมต่อกรัม
ความเที่ยงของวิธีวิเคราะห์ทั้งในวันเดียวกันและต่างวันกันที่ระดับความเข้มข้น MQL และ 5 เท่า
ของ MQL มีค่าสัมประสิทธิ์ความแปรผันน้อยกว่าร้อยละ 8.42 และ 9.97 ตามลำดับ ความแม่นของ
วิธีวิเคราะห์มีประสิทธิภาพดีให้ผลการวิเคราะห์อยู่ในช่วงที่ยอมรับได้สำหรับการตรวจวัดสาร
ปริมาณน้อยซึ่งกำหนดโดย AOAC การประเมินความแกร่งของวิธีการสกัดสารพบว่าค่าความเป็น
กรด-เบสของสารละลายเป็นปัจจัยสำคัญที่จำเป็นต้องมีการควบคุมสำหรับการสกัดสารทั้ง 10 ชนิด

ภาควิชา................เคมี................ ลายมือชื่อนิสิต...
สาขาวิชา................เคมี................ ลายมือชื่ออาจารย์ที่ปรึกษา...
ปีการศึกษา............2548................ ลายมือชื่ออาจารย์ที่ปรึกษาร่วม...................................

In this study, a challenging method for simultaneous extraction and determination of six sulfonamides, three tetracyclines, and pyrimethamine residue in cow milk was developed. The sample preparation method include the acidic deprotenization of milk and dechelation by metals with McIlvain buffer, followed by enrichment and clean-up using a co-polymeric solid phase extraction cartridge (SPE), Oasis® HLB. Reversed-phase LC-ESI-MS with positive ion mode was used for separation and detection of ten target analytes. Various factors affecting the sensitivity, separation, and extraction efficiency were optimized. Satisfactory recoveries for all compounds were obtained greater than 70%. Estimated method detection limits ranged from 0.48 to 2.64 ng/g which is far below the MRLs regulated by both EU and U.S. FDA. Method quantitation limits ranged from 0.97 to 8.64 ng/g. Linearity (R^2> 0.9990) for quantitative analysis was obtained over dynamic range from MQL values up to 300 ng/g. Intra-assay and intermediate precision were acceptable with RSD at MQL and 5-fold MQL levels less than 8.42 and 9.97 %. The method demonstrated satisfactory accuracy within an acceptable range of AOAC for trace analysis. The method was found to be robust and showed the pH of sample solution is a critical point for simultaneous extraction all compounds.

Department.................Chemistry.................Student's signature...Urairat Koesukwiwat

Field of study.............Chemistry................. Advisor's signature...Natchanun Leepipatpiboon

Academic year...............2005......................Co-advisor's signature...........................

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS AND SYMBOLS

SAs	Sulfonamides
SDZ	Sulfadiazine
STZ	Sulfathiazole
SMZ	Sulfamethazine
SMP	Sulfamethoxypyridazine
SMX	Sulfamethoxazole
SDM	Sulfadimethoxine
TCs	Tetracyclines
CTC	Chlortetracycline
OTC	Oxytetracycline
TC	Tetracycline
PYR	Pyrimethamine
HPLC	High performance liquid chromatography
UV	Ultraviolet
DAD	Diode array detector
FLD	Fluorescence detector
MS	Mass spectrometry
MS/MS, MS^n	Tandem mass spectrometry
LC-MS	Liquid chromatography-mass spectrometry
LC-ESI-MS	Liquid chromatography-electrospray-mass spectrometry
ESI	Electrospray ionization
APCI	Atmospheric pressure chemical ionization
PB	Particle beam
FAB	Fast atom bombardment
HPIC	High performance ion chromatography
CID	Collision induced dissociation
GC-MS	Gas chromatography-mass spectrometry
CE	Capillary electrophoresis
LOD	Limit of detection
MDL	Method detection limit

MQL	Method quantitation limit
RSD	Relative standard deviation
LLE	Liquid-liquid extraction
SPE	Solid-phase extraction
MSPD	Matrix solid phase dispersion
SLM	Supported liquid membrane
SPME	Solid phase microextraction
ppm	part pet million
ppb	part per billion
mL	milliter (s)
i.d.	internal diameter
g	gram (s)
cm	centimeter
mm	millimeter
μm	micrometer
nm	nanometer
M.W.	molecular weight
t_R	retention time
R^2	correlation coefficient
m/z	mass per charge ratio

CHAPTER I

INTRODUCTION

1.1   Problem Definition

For the time being, more and more people are interested in gaining good health and becoming more selective in choosing their foods. Basic foods such as cow's milk attract a lot of attention because it is a good source of essential nutrients, for instance, vitamin D, calcium, iodine and potassium, etc. Besides, most milk consumers are infants, children, pregnant women, seniors, and patients. Therefore, the quality of milk is a significant factor and has been the center of public concern. Good milk should contain no contamination or any residue of chemicals and drugs that may cause adverse effects to consumer's health.

The use of antibiotics in veterinary medicine began in the 1950's, with the use of tetracycline drugs as feed additives. Nowadays, many classes of antibiotics and antibacterials are widely used in dairy cattle management for preventing and treating several diseases, as well as, promoting growth in food producing animals. Among the diseases commonly found in dairy cattle, "mastitis" is the most important and is well recognized as being the major problem of serious wastage and undesirable milk quality. Mastitis is an inflammation of the cow's udder caused by a variety of micro-organisms, mostly bacteria, which gain access to the interior of the mammary glands through the teat canals. Several types of micro-organisms are known to cause mastitis such as *Steptococcus agalactiae* and *Staphylococcus aureus*. The micro-organisms live on the cows, specifically its udders, and the surrounding environment including the floor, feces, soil, feedstuffs, water, and milking equipment. In response to the bacterial invasion, leukocytes move from the blood stream into the milk in order to fight the infection. This constitutes the inflammatory response which is known as mastitis. In mild cases, frequent milking may help reduce the inflammation by removing infected organisms and their toxins, and help to rid the udder of cellular debris. However, in acute and chronic cases, antibiotic or antibacterial drugs are

necessary for treatment, and given by intramuscular injection or intramammary infusion. In some case, this can be administered orally by feed mixing. Drugs may also be added directly to foods, mainly to milk, to prolong its freshness during transportation.

The widespread use of veterinary drugs in dairy framing for lactating cows, pose a significant risk of leaving drug residue in milk. This is because most dairy farmers lack comprehension of physicochemical properties of drugs and diseases. Additionally, other factors that contribute to residue of drugs in milk include; extra label drugs used, routes of administration of drugs, treating for a longer or shorter time than recommended, inadequate withdrawal period, giving drugs to a non-recommended species or class of livestock, inadequate records of all treated cows, and contamination of drugs in feed and drinking water.

The presence of antibiotic or antibacterial drugs in cow's milk can provoke adverse health effects on consumers such as, allergic reactions in sensitive individuals; for instance, diarrhea, sore mouth, irritation, nausea, and carcinogenic characters. Antibiotic and antibacterial drugs can interfere with starter cultures for cheese and other dairy products. Moreover, prolong exposure of low level doses of antibiotic or antibacterial drugs could result in a resistant to harmful bacteria, which can transfer from foods to humans and initiate gene mutations. In addition to immediate adverse effects, long-term effects resulting from the exposure to low levels of antibiotic and antibacterial residues is escalating.

In Thailand, a common dairy cow's disease other than mastitis is metritis, respiratory infections, skin and soft tissue infections. Several groups of antibiotic and antibacterial drugs are administered in Thai dairy husbandry, the most common ones are sulfonamides (SAs) and tetracyclines (TCs). Sulfonamides are antibacterial and anti-infective drugs, often used in combination with other drugs such as "pyrimethamine" to increase their effectiveness for therapy. Pyrimethamine (PYR) is used as a potentiator. The combinations of sulfonamide and pyrimethamine may be used for therapy or prophylaxis of parasitic diseases, including coccidiosis and toxoplasmosis infection in cows. However, the choices of the combination depend on the type of illness.

Sulfonamide antibiotic drugs frequently used in Thai cow farms are sulfadiazine (SDZ), sulfathiazole (STZ), sulfamethazine (SMZ), sulfamethoxypyridazine (SMP), sulfamethoxazole (SMX), and sulfadimethoxine (SDM), as shown in Figure 1.1.

Sulfadiazine (SDZ)

Sulfathiazole (STZ)

Sulfamethazine (SMZ)

Sulfamethoxypyridazine (SMP)

Sulfamethoxazole (SMX)

Sulfadimethoxine (SDM)
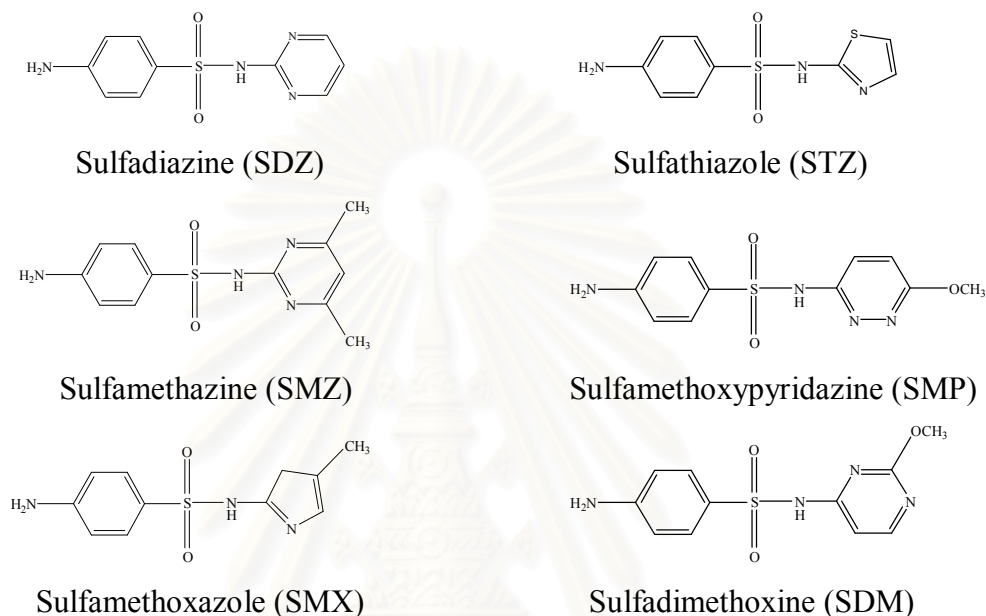
Figure 1.1 Chemical structures of sulfonamide antibacterials.

Tetracycline antibiotic drugs commonly used in Thai cow farms are oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) as shown in Figure 1.2.

Oxytetracycline (OTC)

Tetracycline (TC)

Chlortetracycline (CTC)
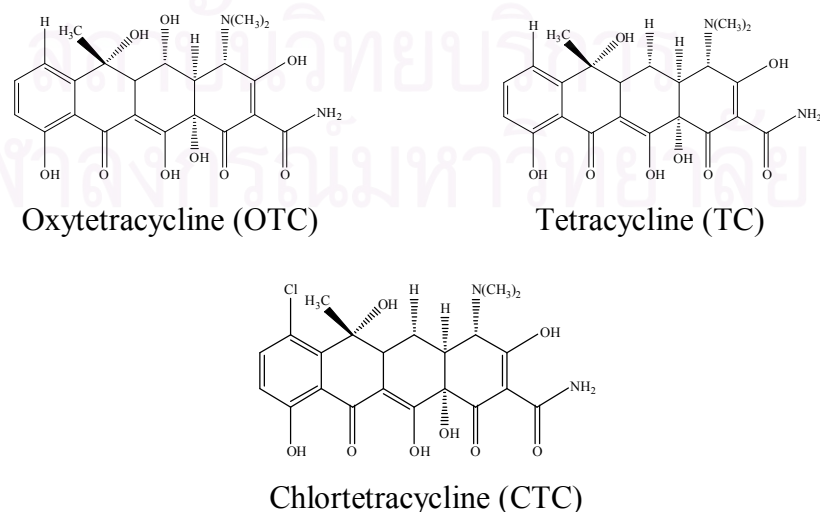
Figure 1.2 Chemical structures of tetracycline antibiotics.

The structure of pyrimethamine antibacterial drug regularly used in combination with sulfonamide drugs [1] is shown in Figure 1.3.
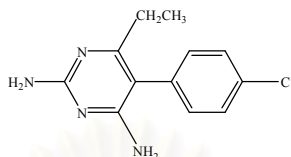
Figure 1.3 Chemical structure of pyrimethamine antibacterial.

There have been a number of reports on many chemical analysis techniques used for detecting antibiotic and antibacterial residue in foods. Because antibiotic and antibacterial residues in milk are present in very small amounts, the screening procedure should be rapid, reproducible, reliable, accurate, and able to detect with great sensitivity. Among these, the immunological or microbiological assays, based on the growth inhibitor of interested microorganisms, are commonly used to determine the presence of antibiotic and antibacterial residue in milk, because these methods are simple, fast, and inexpensive. However, these methods generally can not distinguish between members of a class of drugs present in milk, lack the sensitivity, and can provide only the semi-quantitative measurements of detected residues. Sometimes these methods give false-positive results by detecting drug residues at a level far below the officially mandated safe levels. Therefore, understanding the issues of drug residues is underpinned by the need for precise and sensitive techniques that are able to accurately qualify and quantify the residues at the low level these drugs commonly occur.

Consequently, many methods for measuring residuals of antibiotics and antibacterials in milk have been developed using high performance liquid chromatography (HPLC) with ultraviolet (UV), diode array (DAD) or fluorescence (FLD) detectors. However, in some cases, HPLC is not regarded as being sufficiently specific for use as a confirmatory technique in the existence of residual drugs. For a qualitative perspective, the isolation by HPLC is unable to provide an unequivocal identification of the mixture components because of incomplete separation that occurs in the chromatogram. As a result, precise and accurate quantitative determination of the analytes of interest is impossible. The use of mass-spectral data or a combination

of techniques based on different physico-chemical properties is desirable [2]. It is possible to use the mass spectra of many compounds that are sufficiently specific for identification purpose with a high degree of confidence for these overlapped peaks. Because compounds with similar or identical retention characteristics have quite different molecular weight and different mass spectra, they can be differentiated. Therefore, the combination of HPLC with mass spectrometry (MS) is very useful for simultaneous identification and quantitation of compounds that are not fully resolved chromatographically.

Recently, Public Health Agencies around the world have started to rely on detection by mass spectrometry (MS) for unambiguous confirmation of antibiotics and antibacterials in foodstuffs. The commission decision of the European Union (EU) states that "method based only on chromatographic analysis without the uses of molecular spectrometric detection are not suitable for use as confirmatory methods" [3, 4]. Residue data obtained, using mass spectrometry can represent the most definitive evidence, and where suitable equipment is available it is the confirmatory technique of choice. This technique can also be used for residue screening purposes. Mass spectrometric determination of residue is usually carried out in conjunction with a chromatographic separation technique to provide retention time, ion mass/charge ratio and ion abundance data simultaneously. Liquid chromatography-mass spectrometry (LC-MS) can provide good supporting evidence but the spectra generated are generally very simple, showing little characteristic fragmentation, and results produced from LC-MS are unlikely to be definitive. Therefore, additional confirmation of identity may be obtained by monitoring further reaction products of selected ion by tandem mass spectrometry (MS/MS or MS^n). In the present, LC-MS/MS is a more powerful technique, combining selectivity with specificity [2]. EI-MS or MS/MS performed with acquisition of spectra may provide good evidence of identity and quantity in many cases [5]. Therefore, research in new methodologies of MS, has significant advantages to analyze residual drugs with high sensitivity, selectivity and can perform both qualitative identification and quantitative determination simultaneously.

1.2   Regulation of Drug Residues in Milk

To safeguard human health, both European Union (EU) and U.S. Food and Drug Administration (U.S. FDA) have established in 1999, safe maximum residue limits (MRLs) for residues of veterinary drugs in animal tissues entering the human food chain [4, 6].   MRLs are the margins that ensure safety of human food from potentially harmful residues.   In 2001, the Thai's Ministry of Public Health established the MRLs levels of antibiotic and antibacterial drugs in cow's milk [7]. The enforced MRLs for some sulfonamides and tetracyclines in cow's milk by the EU, U. S. FDA and the Thai's Ministry of Public Health were collected in Table 1.1.

Table 1.1   Maximum residue limits (MRLs) of sulfonamides and tetracyclines in cow's milk stipulated by the EU, U.S. FDA and Thai's Ministry of Public Health.

Compounds	Maximum residue limit (MRL) (ppb)		
	EU	U.S. FDA	Thai's Ministry of Public Health
Sulfonamides			
Total sulfonamide residues	100	NC	NC
Sulfadiazine	NC	NC	NC
Sulfathiazole	NC	NC	NC
Sulfamethazine	NC	NC	NC
Sulfamethoxypyridazine	NC	NC	NC
Sulfamethoxazole	NC	NC	NC
Sulfadimethoxine	NC	10	NC
Tetracyclines			
Tetracycline	100	80	NC
Cholrtetracycline	100	30	NC
Oxytetracycline	100	30	100

* NC = not control.

However, the EU, U.S. FDA and the Thai's Ministry of Public Health currently do not regulate the residue safety level of pyrimethamine and other members of sulfonamides and tetracyclines in cow's milk.

1.3   Literature Reviews

Veterinary drugs show a range of variation in molecular structures, physicochemical properties and biological activities. Analytical methods available for antibiotic and antibacterial analysis reveal common analytical methodology.   The successes of these methods depend on sample preparation, which is difficult and controls the success of isolation of antibiotic and antibacterial drugs from the complex matrices. Commonly used techniques for the extraction and clean-up of cow's milk matrices involve liquid-liquid extraction (LLE), ultrafiltration, centrifugation, and solid-phase extraction (SPE) as regard to their potential for drug residue analysis.

The chromatographic methods commonly used for the determination of the residual drugs are HPLC coupled with UV, DAD, FLD, MS, and MS/MS.

1.3.1   Literature Reviews of The Determination of Sulfonamide
      Antibacterials

Smedley *et al*. [9-10] reported methods that were suitable for the determination of SAs at 10 μg/kg level in milk. The liquid-liquid extraction with chloroform-hexane was performed with 71 to 91% recoveries.

Tsai and Kondo [11] proposed the method to detect SA drugs by LC-FLD. Acetonitrile was used for milk protein precipitation followed by centrifugation. In a similar fashion, Fuh *et al*. [12] described a LC-ESI-MS method for the determination of SAs in meat. Acetonitrile and hexane were used as extractants. LODs were below 10 μg/kg and recoveries were better than 80%.

Rhijn *et al*. [13] demonstrated the applicability of ultrafiltration after protein precipitation with acetonitrile as the only sample preparation for LC-MS/MS analysis of SAs at trace level in milk. Analyte recoveries were better than 70% with

high sample throughput. This simple extraction procedure used a short analysis time which lowered the analysis cost. This method can also be applied to other banned veterinary drugs, dapsone and bensimidazoles with slight modifications, and produce good results.

Verzegnassi *et al*. [14] employed LC-ESI-tandem MS for the determination and confirmation of ten SAs in honey. They used trichloroacetic acid and dichloromethane in the liquid-liquid extraction step with recoveries ranging from 44-73%.

Postcolumn derivertization method was also used by Viñas *et al*. [15] to determine SA residues in food, including milk. *O*-phthaldehyde (OPA) combined with β-mercaptoethanol were used for derivertization. SAs were isolated from milk by extraction with trichloracetic acid, separated by a reverse phase column, and reacted with derivertizing agents. They described that the derivatization reaction made the detection limits of SAs down to 0.04-0.33 μg/g. In the same way, Stoev and Michailova [16] used fluorescamine as derivertizing reagent to produce fluorophores of SAs, after these drugs were extracted from matrix by SPE. This method could detect the residues at the lowest concentration with detection limits at 0.05 μg/kg.

The sample preparation based on protein precipitation and centrifugation is a very simple procedure, but separation from interferences was less than satisfactory. Also there was limited sensitivity and lack of specificity, regardless of the simplicity limit of these methods to screening purposes. Thus, SPE has been recognized as the technique for the extraction and clean-up of milk samples.

Suhren and Heeschen [17] demonstrated the residues of some SAs and sulphone dapsone in milk using HPLC-UV. SPE column contained cation exchange cartridges that were applied for extraction and preconcentration of analytes, with results showing better than 70% recoveries. Moreover, this method was used to study the contamination of other drugs, tetracycline and penicillin drugs. With this method, interference disturbed the analyte peaks. Therefore, Balizs *et al*. [18] selected silica cartridge for the clean-up after extraction with chloroform-acetone.

Ito *et al*. [19] compared the efficiency of two types of anion-exchange SPE cartridges for the extraction of SAs in animal entrails. They reported better than 98%

recovery of SAs from primary/secondary amine cartridge of strong retention power to retain SAs. Detection limit of this method was 0.03 μg/g.

In 2002, Korean researchers [20] measured the residual SAs in meat by HPLC-APCI-MS. LiChrolut-EN® SPE was used in sample preparation procedure.

LC-ESI-MS with single quadrupole, was applied to identify trace of 14 SAs in milk and eggs by Cavaliere and co-workers [21]. Nonporous graphitized carbon black sorbent SPE was used for extraction and purification of SAs after protein precipitation with acetonitrile.

Combs [22] used HPLC-APCI-MS for the analysis of SAs in biological tissues after extraction with supercritical fluid extraction (SFE). Detection limit of this method was about 25 μg/kg for SDM.

Additionally, matrix solid phase dispersion (MSPD) is a technique of interest, for extracting contaminants and particularly drugs from biological matrices. The method was first developed by Baker in 1989 [6]. MSPD offers distinct advantages over classical sample pretreatment procedures, such as reduced solvent consumption and eliminates the possibility of emulsion formation. Moreover, MSPD constitutes a significant advance in simplicity and efficiency, and makes it possible to screen more samples.

A multi-residue method for the isolation and determination of veterinary drug residues in meat is described by Boulaire *et al*. [23]. MSPD, C_{18} sorbent, was chosen as SAs extractant by directly blending with matrix samples followed by eluting with suitable organic solvent.

Kishida and co-workers [24] studied the ability of different MSPD sorbents and concluded that normal-phase and neutral aluminum oxide sorbents were suitable for the extraction and the purification of SAs. Other researchers, selected crystobalite sand as extracting sorbents for twelve SAs residues in cattle tissues [25] and milk [26], followed by the analysis by LC-ESI-MS.

Nowadays, Oasis® HLB is a popular hydrophilic-lipophilic water-wettable reversed phase sorbent SPE. It is made from a balanced ratio of two monomers, the hydrophilic N-vinylpyrrolidone and the lipophilic divinylbenzene, which allows simultaneous extraction of a wide range of analytes including acid, basic and neutral compounds in only one procedure. This reduces analysis time. Moreover, it provides

superior reversed phase SPE with resistance to extreme pH, has high retention capacity, reproducibility, and recoveries even when the sorbent runs dry during the extraction procedure. It is the more rugged and robust sample preparation method available today.

Göbel *et al.* [27] used Oasis® HLB SPE combined with LC-MS/MS for simultaneous determination of SAs in wastewater. Thus the residue levels of these drugs as low as 10 ng/L can be determined. In contrast to the above study [28], samples were extracted through an anion-exchange cartridge, to reduce interference from organic matter, in tandem with Oasis® HLB cartridges.

Recently, supported liquid membrane (SLM) has been recognized for the extraction of pharmaceuticals in biological matrices. Msagati *et al.* [29] used SLM as a sample clean-up technique for SAs. They reported that SAs were enriched with SLM and selective solvents, resulting in low interfering compounds and excellent extraction efficiency. However, factors affecting the extraction efficiency, such as donor pH, acceptor pH, enrichment time and membrane solvent were manipulated to obtain the optimum chromatographic procedure.

Numerous methods employed for the analysis of SA residues in food and biological matrices were based on the HPLC technique. Gas chromatography-mass spectrometry (GC-MS) can be applied to determine of these compounds as well. Reeves [30] used GC-MS to confirm the presence of SAs in milk. C_8 SPE sorbents were selected for the clean-up procedure and derivatization was done with N-methyl-N-trifluoroacetyl. Moreover, capillary electrophoresis (CE) technique was also applied to analysis of the SAs in meat as reported by Fuh *et al.* [31].

1.3.2 Literature Reviews of The Determination of Tetracycline Antibiotics

Fletouris and co-workers [32] reported a HPLC-UV method was able to quantify the residue of TCs in milk at low-concentration level. They described that the sample should be acidified to pH lower than 3 for complete dissociation of TCs from milk proteins. TCs were extracted with acid and acetonitrile followed by re-extracted as ion-pairs with TBA reagent.

Eksborg *et al*. [33-34] described the addition of ion-pair reagent in the mobile phase as a powerful technique in regulating the retention and separation of TCs. Use of mobile phase containing oxalic acid is reported by Oka and Uno [35] to reduce the peak tailing of TCs on the reversed phase column.

Several methods were employed with direct injection of sample supernatant of protein precipitation without complex sample preparation and clean-up steps. Thomas [36] diluted samples with EDTA-phosphate buffers and centrifuged for re-suspending milk solids, followed by direct injection of the filtrate into HPLC. Moats [37] extracted and deprotenized milk samples with hydrochloric acid and acetonitrile. Trichloroacetic acid was used to dilute milk samples by Furusawa [38]. Furusawa *et al*. [39] used an ultrasonic homogenizer in the extraction procedure without any organic solvent.

Because, it is possible to detect TCs by fluorescence detection, a few methods employing this technique were developed. Blanchflower *et al*. [40] presented a technique for the HPLC-FLD analysis of TCs. This approach was based on the conversion of TCs to its more highly fluorescent iso-derivatives. However, the conversion process required a lengthy 2.5 hour procedure after samples extraction.

Sokol *et al*. [41], Walsh *et al*. [42] and Nagy *et al*. [43] used C_{18} SPE for the clean-up of TCs after extraction with EDTA-McIlvain buffer. Recoveries exceeded 85% with LOD of about 50 ng/g. They explained that TC antibiotics have tendency to combine with protein, and form chelated complexes with metal ions. Therefore EDTA-McIlvain buffer, phosphate buffer or hydrochloric acid must be employed to facilitate the extraction.

The method based on HPLC coupled with a MS detector for a more reliable identification and determinations of TCs in biological matrices appeared in 1991. A particle beam (PB) device with negative ion chemical ionization [44-46], fast atom bombardment (FAB) or thermospray [47] were employed as LC interfaces. However, interfaces commonly selected for TCs analysis are APCI and ESI.

Blanchflower *et al*. [48] detected TCs in muscle using APCI-MS. Samples were extracted into glycine-HCl and clean-up using C_8 SPE. Recoveries ranged from 59 to 72 % and 0.01 µg/g LOD were reported. However, the addition of oxalic acid

in the mobile phase was necessary to improve the resolution and peak shapes of TCs and EDTA was used for improving the recoveries.

Oka *et al*. [49-50] used EDTA-McIlvaine buffer to extract TCs, clean-up use C_{18} SPE, followed by analysis through LC-ESI-MS/MS. This method could detect TCs residual at 0.1 μg/g with recoveries ranging from 55 to 79 %.

Nakasawa *et al*. [51] applied LC-APCI-MS/MS for the analysis of TCs in milk. TCs were extracted with Na_2EDTA-McIlvaine buffer and separated from co-extracting using C_{18} SPE. The recoveries ranged from 60 to 88 % with LOD at 1.0 μg/g. Kennedy *et al*. [52-53] used the same method to study the metabolism of CTC in eggs.

Brono *et al*. [54] developed LC-ESI-MS for analyzing TCs in milk at levels well below the tolerance levels. Milk samples were diluted with EDTA solution, followed by clean-up on carbon black SPE cartridge. Average recoveries of TCs ranged between 72 to 96 %.

Furusawa [55] explained a simple extraction method for the isolation of TCs in milk. Milk samples were directly applied to a C_8 SPE column and water was used as eluent with the average recoveries better than 80 %.

Sczesny *et al*. [56] simultaneously determined TC residues in eggs using a microbiological assay coupled with HPLC. TCs were easily extracted with acetonitrile and centrifuged to precipitate milk protein. This method can be employed to confirm residual TCs at 20 ng/g with recoveries of 71 to 109 %.

Recently, Viñas *et al*. [57] described a method for residue analysis of TCs in honey by varying types of SPE sorbents. The best result was obtained using phenyl sorbent for sample clean-up. Moreover, extraction with mild acidic solvent containing EDTA may be used to release protein bound TCs. Detection limits of this method ranged between 15 to 30 ng/g.

Alternatively, several recent studies emphasized the use of copolymeric solid phase extraction cartridges. For instance, Oasis® HLB allowed simplified sample clean-up to isolated TCs from various biological matrices. HPLC-DAD method was optimized for the determination of TCs in milk and muscle by Cinquina and co-workers [58]. They used trichloroacetic acid for milk protenization, extracted

with McIlvaine buffer and followed by isolation on Oasis® HLB. Recoveries were higher than 81 % with the detection capability ranging between 117 to 131 ng/g.

Anderson *et al.* [59] used Oasis® HLB SPE for the isolation and clean-up of TCs in milk after extraction with succinic acid. LC-UV coupled with MS/MS was employed to determine and confirm residual TCs. Recoveries were greater than   75 % with the confirmation level at 50 to 300 ng/g. In addition, Oasis® HLB was successfully applied to determine residual TCs in other matrices, such as water [60, 61].

Obviously, many chromatographic techniques were described in the literature for the analysis of TCs in food and foodstuffs. Most published methods used reversed phase HPLC coupled with many detectors, such as UV, DAD, FLD, MS and MS/MS. When using HPLC determination, the sample preparation procedures were complicated and lengthy. Therefore, a better technique is need for TC residues analysis.

Radioimmunoassay, Charm II, have been used for the detection of residual TCs in milk [62-63]. It was observed that the residual levels detected by this method tended to be higher than those obtained by LC methods.

Capillary electrophoresis (CE) is being extensively used for the analysis of antibiotic residues in foods as well. Chen and Gu [64] used CE for simultaneous determination of TCs in bovine milk.

In 1999, Lock *et al.* [65] used SPME with a Carbowax/templated resin for the extraction of TCs in milk, and then analyzed by LC-ESI-MS. Cooper and co-workers [66] applied the on-line metal affinity chromatography HPLC to analyze TCs. Moreover, Ding *et.al.* [67] explained how the specificity of high performance ion chromatography (HPIC) can be used to analyze TCs residues in milk and milk powder.

1.3.3 Literature Reviews of The Determination of Pyrimethamine Antibacterial

Fuh and co-workers [68] explained a newly LC-ESI-MS method for the determination of PYR. Acetonitrile was used to extract this compound from samples.

Additionally, acetic acid and sodium acetate were added into the mobile phase to achieve adequate LC separation and to enhance the collision induced dissociation (CID) fragments of the analytical molecules.

Coleman *et al*. [69] studied the excretion of pyrimethamine in biological fluid. After isolating the analyte from the matrix, it was then separated using ion-pair liquid chromatography. This method is well suited for the analysis of other antimalarial drugs, i.e. chloroquine, sulfadiazine and sulfadoxine.

1.3.4 Literature Reviews of The Simultaneous Determination of Sulfonamide and Pyrimethamine

Astier and co-workers [70] studied the pharmacokinetic of antimalarial drugs in human plasma, by observing the plasma drug's concentration using UV-HPLC. Combinations of sulfadoxine and pyrimethamine were used for the treatment of malaria in children. After extraction and clean-up with C_8 SPE, the compounds were separated on a C_{18} column showing good efficient extraction ranging between 82 to 93 %. This method was feasible and was recommended for drug monitoring and pharmacological studies, to confirm or define appropriate dosages.

Nevado *et al*. [71] quantified SAs and PYR by HPLC. The results of this method were compared with those obtained by capillary electrophoresis (CE). Similar results when applied in veterinary commercial formulations were reported.

1.3.5 Literature Reviews of The Simultaneous Determination of Sulfonamide and Tetracycline

Lindsey and co-workers [72] presented a method for trace analysis of SAs and TCs in groundwater and surface water, by SPE and LC/MS with positive-ESI. These compounds can be prevented from chelating with metals in the matrix by adding a metal chelating agent, Na_2EDTA, to the sample, followed by extraction with Oasis® HLB cartridges. They reported quantitative recoveries of SAs and TCs ranging from 84 to 130% and 89 to 101%, respectively.

1.4 Purpose of The Study

In Thailand, cow's milk including fresh milk, processed milk and other milk products are very popular among Thai consumers in all age groups. Because its high nutrition, inexpensiveness and availability. It is very necessary to control the level of contaminants, such as veterinary drug residues in cow's milk, because drug residues entering the food chain can provoke potential adverse side effects to human health. Therefore, a reliable sample preparation technique, and a selective and sensitive analytical method capable of detecting residues of antibiotic and antibacterial drugs in cow's milk and their products at trace level are very important.

From the presented literature reviews, various methods have been developed to determine the residues of sulfonamides, tetracyclines and pyrimethamine in cow's milk, edible tissues and biological samples. Most reported analysis methods can detect the residue of drugs in each group, as specified for the members of sulfonamide group, but the procedure is not suitable for tertracyclines and pyrimethamine. Because of the difference in structural and chemical properties of sulfonamides, tetracyclines, and pyrimethamine, simultaneous determination of these drugs is likely to be difficult. Up to now, there is no analytical method developed for simultaneous determination of sulfonamides, tetracyclines and pyrimethamine drugs residue in cow's milk.

In the present, the most reliable and acceptable chromatographic technique used for confirmation of antibiotics and antibacterials residue in food and foodstuffs, is the LC-MS/MS. Because it can perform both identification and quantization simultaneously, with high selectivity and specificity at trace level analysis. However, the limitation of an instrument and the facility of our laboratory can provide only the LC-MS with single quadrupole instrument. Thus, LC-MS provides good evidential data that can be used for confirmation analysis of residual sulfonamides, tetracyclines, and pyrimethamine in milk at trace levels with high reliable.

This research aimed to develop a new sample preparation technique for simultaneous extraction of multi-residue sulfonamides, tetracyclines, and pyrimethamine in cow's milk using liquid chromatography-mass spectrometry

(LC-MS) with single quadrupole as a chromatographic analysis. Antibiotics and antibacterials used in this study comprise of three classes drugs;

       1. Sulfonamide drugs: sulfadiazine, sulfathiazole, sulfamethazine, sulfamethoxypyridazine, sulfamethoxazole, and sulfadimethoxine.

       2. Tetracycline drugs: chlortetracycline, oxytetracycline, and tetracycline.

       3. Pyrimethamine.

A new sample preparation technique was developed using solid phase extraction (SPE) technique, which allowed simultaneous quantitative extraction of these drugs in cow's milk down to low-concentration levels, and provided a significant improvement in extraction speed and time. All procedures were validated for the determination of these drugs at the levels regulated by the European Union (the MRL level $\leq$ 100 ppb).

CHAPTER II

THEORY

2.1 Liquid Chromatography [73-74]

Liquid chromatography is one type of chromatography used for separation in which the components to be separated are distributed between two phases. One of which is the stationary phase, while the other is the mobile phase that moves over it in a definite direction. High performance liquid chromatography (HPLC) is the term used to describe liquid chromatography, where the liquid mobile phase is mechanically pumped through a column that contains fine particles of solid-core stationary phase. In general, a HPLC system consists of four component parts. However, the use of sensitive mass detector limit the specification of each component as will be explained.

2.1.1 Pump

The popular pump used today is the reciprocating pump that consists of a cylindrical chamber which is filled and then emptied by back and forth motions. The pump must provide stable flow rates and must be pulse free and able to deliver constant flow of mobile phase.

2.1.2 Sample Introduction (Injector)

The most widely used method of sample introduction in HPLC is based on sampling loops. From a mass spectrometry perspective, the injector is of little concern other than the fact that any bubbles introduced into the injector may interrupt the liquid flow, resulting in an unstable response from the mass spectrometer.

2.1.3 Mobile Phase

Mobile phase plays an important part in the separation mechanism. In HPLC, the separation mechanism is the relative interaction of an analyte with both the mobile phase and stationary phases that determines analyte retention characteristics. Since, buffers are widely used in LC-MS for controlling the degree of ionization and charge of the analyte, the selection of mobile phase composition and quality of the mobile phase is an important.

2.1.4 Stationary Phase (Column)

The majority of HPLC analyses employ reversed-phase systems where the columns contain chemically modified silica stationary phases. Generally, the column has an internal diameter of 4.6 mm and operates at high flow rate which is too high to be directly introduced to the MS. Because of high selectivity and sensitivity of the LC-MS, good results can only be obtained at low flow rate. One way to reduce flow is to reduce the internal diameter of the LC column, for example, microbore column has 2.1 mm internal diameter and compatible with low flow rate. Further miniaturization of the column dimension is widely accepted when limited sample quantities are available.

2.1.5 Detector

The choice of detector is often crucial to the success of a particular HPLC method. Many are available for routine use, including ultraviolet (UV), fluorescence, electrochemical conductivity, refractive index, and mass spectrometer. Detectors can be classified according to the property that forms the basis of the detection.

2.2 Mass Spectrometry (MS) [75-79]

Generally, chromatography can be regarded as the separation of the components of a mixture to allow for identification and quantitation of all of them. Identification is initially carried out on the retention characteristic, thus this is not sufficient to allow unequivocal identification. Mass spectrometer detects the m/z ratio of each analyte and allows for the differentiation of compounds with similar retention

characteristics. It provides the absolute identification based not only on the molecular weight of the analyte but also structural information for the molecule under investigation. Moreover, the MS sensitivity is useful for identification purposes and provides quantitative information, usually to low levels, with high accuracy and precision.

The combination of the separation power of HPLC with the detection power of MS is called "Hyphenated Technique". LC-MS has recently become popularized from the demands of sensitive and selective analyte detection method for complex biologicals, environmental, and food matrices.

2.2.1 Ionization Methods

2.2.1.1 Electron Ionization (EI)

In EI, the analyte of interest in the gas phase is bombarded with high energy electrons, usually at 70 eV. The electrons ejected from a heated filament are accelerated through an electric field to form an electron beam. The analyte interacts with an electron beam and absorbs some of this energy to form the molecular particle [M]. The analyte is further ionized by removal of a single electron, yielding a radical cation $[M^{+\bullet}]$, called molecular ion, where the m/z corresponds to the molecular weight of the analyte.

$$M + e^- \text{ (70 eV)} \longrightarrow M^{+\bullet} (\sim 5 \text{ eV}) + 2e^- (\sim 65 \text{ eV})$$

However, excess energy (5 eV) can bring the molecule to fragments or possible spatial arrangements of atoms within the molecule. The information of EI spectrum can be extrapolated to an unequivocal molecular structure.

2.2.1.2 Chemical Ionization (CI)

CI is a technique that has been developed to reduce the fragmentation associated with ionization. It is term a "soft ionization technique".

In CI, the vapor phases of analyte molecules are introduced into the mass spectrometer source containing a reagent gas. The mixture is then bombarded with electrons, initiating the ionization processes. Ion-molecule interactions then take

place between the reagent gas ions and the neutral analyte molecules in the high-pressure region of the mass spectrometer source. For example, the process of chemical ionization mass spectrometry using methane as the reagent gas described as follows:

$$CH_4 + e^- \longrightarrow CH_4^{+\bullet} + 2e^-$$
$$CH_4^{+\bullet} \longrightarrow CH_3^+ + H^\bullet$$
$$CH_4^{+\bullet} + CH_4 \longrightarrow CH_5^+ + CH_3^\bullet$$
$$CH_3^+ + CH_4 \longrightarrow C_2H_5^+ + H_2$$
$$CH_5^+ + M \longrightarrow MH^+ + CH_4$$
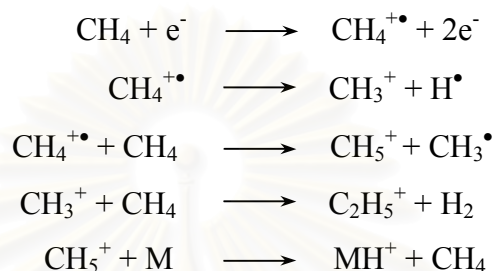
CI leads to the formation of adducts of reagent ions with analyte molecules in relatively low energy processes that lead to little fragmentation. The m/z of the ion observed in the molecular ion form does not give the molecular weight directly but arises from the combination of the analyte with adduct.

2.2.1.3   Fast-Atom Bombardment (FAB)

FAB is one of the soft-ionization techniques which utilize a high-energy beam of Xe atom or Cs^+ ions to sputter the analyte and matrix from the probe surface that placed in the source of the mass spectrometer. When the beam of fast-moving ions is directed to the solution on the probe, the matrix will absorb the incident energy and transfer energy to the analyte molecules to facilitate ionization.

FAB matrix (*m*-nitrobenzylalcohol or glycerol) is a nonvolatile liquid material that the analyte is dissolved. It serves to replenish the probe surface with new sample and minimize sample damage from the high-energy particle beam.

2.2.1.4   Matrix-Assisted Laser Desorption Ionization (MALDI)

MALDI is an ionization method that permits the analysis of polar, thermally labile, and high molecular weight compounds with high sensitivity.

Ion formation in MALDI is achieved by directing a pulsed laser beam onto a sample suspended in a matrix that dried on the laser target. The matrix absorbs the

laser light energy causing the matrix material to vaporize. Then, the vaporized matrix reacts with analyte molecules and transfers excess energy to facilitate the ionization.

MALDI matrix is a nonvolatile solid material that absorbs the laser radiation. Common matrix used in MALDI are nicotinic or sinapinic acids.

2.2.2 Interfaces of LC-MS [74-75, 77]

There are many incompatibilities between liquid chromatography and mass spectromety as follows:

1. The HPLC mobile phase is pumped at high flow rate and operates at atmospheric pressure, while the MS operates under conditions of high vacuum (around 10^{-6} torr). Therefore, it is not possible to introduce the eluate from HPLC column directly into the source of MS.

2. Incompatible of solvent composition such as non-volatile mobile phase additives, buffer, and ion-pair reagent.

3. Most of analytes that separate by HPLC are relatively non-volatile and/or thermally labile compounds. Therefore, the ionization of these analytes by vigorous methods is not possible.

In order to solve the flow rate incompatibility, the mass spectrometer was developed by using LC-MS interfaces. The interface is designed to remove mobile phase while still passing the maximum amount of analytes into the mass spectrometer. Thus, the characteristics of an ideal LC-MS interface are the following:

1. The interface should cause no reduction in chromatographic performance.

2. No uncontrolled chemical modification of the analyte should occur during its passing through the interface into the MS.

3. Ionization efficiency is extremely important for trace level analysis or for when polar and/or labile analytes are involved.

4. Minimize chemical background.

5. Reliable, easy to use and low maintenance.

6. Simple and inexpensive.

7. Interface operation should be compatible with all chromatographic conditions.

8. Operation of the interface should not compromise the vacuum requirements of the MS.

9. Mass spectra produced should provide unambiguous moleccular weight information.

10. The MS should provide reproducible and interpretable information.

11. The interface should provide quantitative information with reproducibility, low limits of detection and have a linear response.

2.2.2.1 Moving-Belt Interface

The operation of this type of interface may be divided into four stages as follows:

1. The application of the LC eluate consisting of mobile phase and dissolved analytes, to a continuously moving-belt.

2. Removal of the mobile phase by passage of the belt under an infrared heater or fan and through a reduced pressure in a number of differentially pumped regions. Thus, analytes remain on the continuously cycling belt and are transported into the MS source.

3. Flash desorption/vaporization of the analytes at the tip of the moving-belt provides the anlytes in a gaseous state into the ion source of the MS.

4. Cleaning of the belt with a heater or wash bath to removal any involatile.

5. Materials or excess sample prior to the application of further LC eluate and a repeat of steps 1-4.
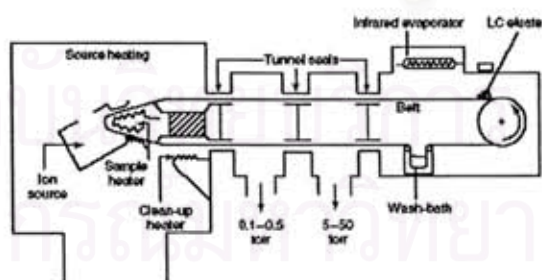
Figure 2.1 Moving-belt interface.

2.2.2.2 Direct-Liquid-Introduction (DLI) Interface

The DLI interface was made available commercially 1980. The DLI interface is shown in Figure 2.2.
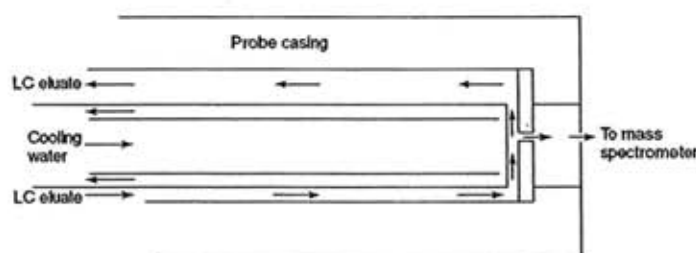
Figure 2.2 Direct-liquid introduction LC-MS interface.

A pinhole at the end of a probe joins with a desolvation chamber and connects to the ion source of MS. The eluate from a HPLC column is circulated through the probe and when it reaches the pinhole, the vacuum in MS draws an eluate into the desolvation chamber and subsequently to the source of MS, where analytes are ionized by CI-type processes, known as "solvent-mediated chemical ionization".

2.2.2.3 Continuous-Flow Fast Atom Bombardment (CF-FAB) Interface

The CF-FAB interface or dynamic FAB was developed to study nonvolatile and thermally labile compounds. This interface is a modification of the FAB ionization technique that allows continuous on-line refreshing of the HPLC eluate on the FAB target. The construction of CF-FAB interface as shown in Figure 2.3.
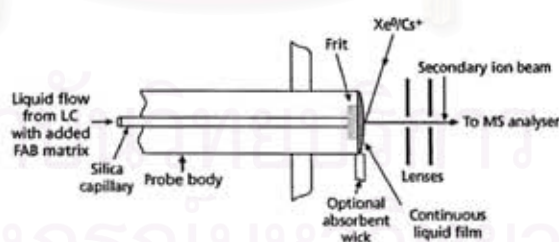
Figure 2.3 Continuous-flow fast atom bombardment interface.

When linking HPLC to a FAB system, FAB matrix will be mixed with the LC effluent and transported to the probe tip. Then, fast-atoms bombard the sample and ions are sputtered out of the solution into the gas phase and drawn to the high vacuum of the MS. Excess mobile phase has to be removed from the probe tip by an

absorbent pad situated adjacent to the area subjected to atoms or ions bombardment, thus the stability of the ion current can be a problem.

2.2.2.4   Particle-Beam Interface

The construction of particle-beam interface as shown in Figure 2.4.
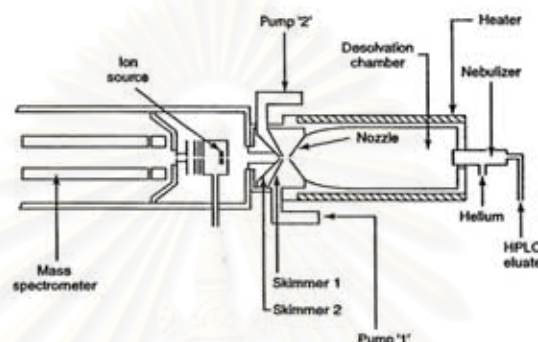
Figure 2.4 Particle-beam interface.

In general, the procedure used in this interface consists of four stages, as follows:

1. Nebulization of the HPLC eluate.
2. Desolvation of the droplet so formed.
3. Removal of the solvent vapour and nebulizing gas.
4. Ionization of the analyte.

The LC eluent is forced through a small nebulizer to form a stream of uniform droplets produced under atmospheric pressure. Then, the droplets are carried by high velocity gas stream into a desolvation chamber that provides sufficient heat to allow for complete evaporation into sub-micron diameter particles or solid particles. The analytes are transferred into the source of the mass spectrometer where they are vaporized and ionized using EI or CI. This interface is capable of producing EI spectra belonging solely to the analyte. The molecular weight limit of the particle-beam interface is around 1000 Da.

2.2.2.5 Thermospray Interface

The schematic of thermospray interface is illustrated in Figure 2.5 and its operation can be described as follows:

1. The formation of droplets from the HPLC eluate.
2. Charging of these droplets.
3. Desolvation of the droplets.
4. Formation of ions of the analyte.
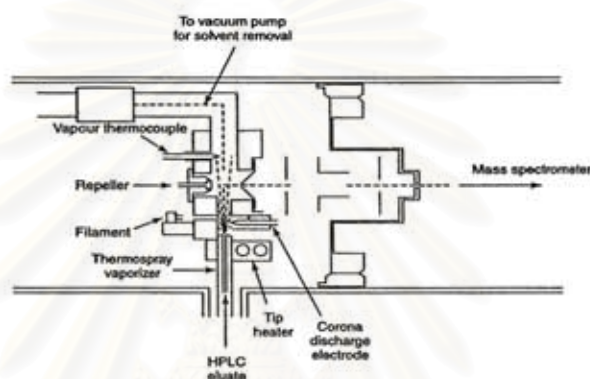
Figure 2.5 Thermospray interface.

The HPLC eluate flows through a heated capillary, which creates a spray of superheated mist. The temperature of this capillary is controlled to completely vaporize the liquid. The vapor so produced acts as a nebulizing gas which aids the breaking-up of the liquid stream into droplets. When the droplets travels through a heated region, the desolvation takes place and analyte ions are formed by means of ion-molecule reactions or ion evaporation. Subsequently, these ions are directed through the entrance of MS.

The control of the vaporization process is very important because over heating will result in the decomposition of analyte and if insufficient heat is applied, vaporization does not occur. There are two classes of ionization processes:

1. *Real thermospray*, charging of the droplets is due to a buffer in the mobile phase. Both positively and negatively charged droplets are formed due to the fluctuation in anion and cation density. Ions formed are transferred to the gas phase by evaporation of the solvent or by ion evaporation.

2. *When no buffer is used,* filament or discharge electrode is used to generate plasma of ions from the mobile phase. Protons are transferred at the interface of the liquid droplet and in the gas phase.

2.2.2.6 Electrospray Ionization (ESI) Interface

Electrospray ionization (ESI) is the most successful atmospheric pressure ionization method used in mass spectrometry today. The basic diagram of electrospray is shown in Figure 2.6.
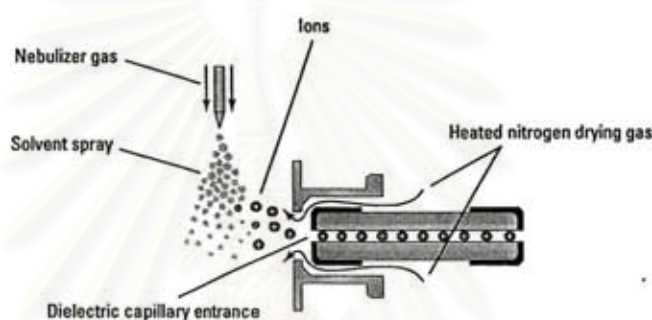
Figure 2.6 Electrospray ionization (ESI) interface.

1. *The mechanism of electrospray ionization*

ESI usually takes place at atmospheric pressure and the electrospray process occurs by the same four steps as in thermospray. The ESI source comprises of two electrodes, the electrospray capillary and the counter electrode. Electrospray spectra are produced by passing a HPLC eluate through a high voltage metal capillary (typically 3-4 kV). The applied potential difference between both electrodes causes the liquid stream to break into fine threads that disintegrate to small droplets, so called electrospray. Under the influence of the applied electric field, ions of the same polarity migrate toward the liquid at the capillary tip. The liquid surface is drawn out of the capillary forming a "Taylor cone" as illustrated in Figure 2.7.
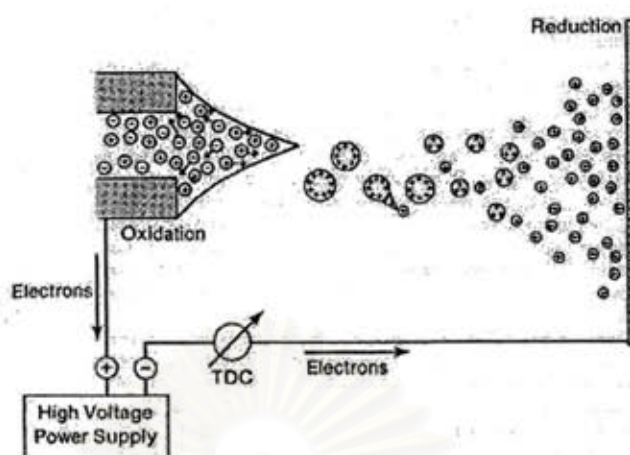
Figure 2.7 Schematic of Taylor cone in ESI.
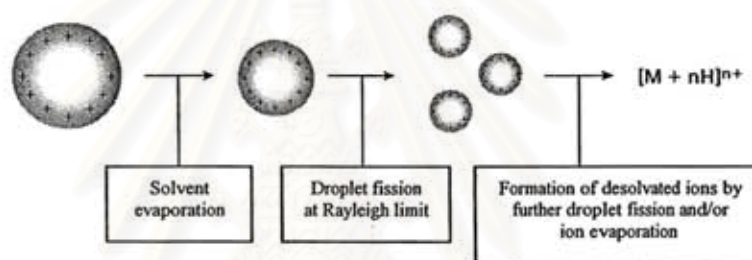
Figure 2.8 Schematic of coulombic explosion.

When an excess of ions of one polarity at the surface of the liquid reaches the point that repulsive forces between charges are sufficient to overcome the surface tension of the liquid, one ion polarity is emitted from the capillary, so called "coulombic explosion" as shown in Figure 2.8. These droplets shrink by solvent evaporation followed by a number of coulombic explosions, leading to very small charged droplets. A series of such explosions then take place until a point is reached at which an appropriated amount of analyte ions dissolved in these droplets are produced. The ions are transferred through a set of focusing devices into the mass analyzer. The actual mechanism of ion production from the very small charged droplets can be explained in two models:

(1) Ion-evaporation model, the droplets become smaller until a point is reached at which the surface charge is sufficiently high for direct ion evaporation into the gas phase.

(2) Charge residue model, repeated coulombic explosions take place until droplets of a single ion are formed. Evaporation of solvent continues until ions are formed in the vapour phase.

An important factor that will affect the production of ions by the elctrospray process and the mass spectra is the HPLC characteristics. Because the ions are being generated directly from the mobile phase by electrospray, therefore, the identity and the concentration of any buffer and the flow rate of mobile phase are important considerations.

Desolvation of the droplets formed and ionization of analytes is favoured by the initial production of small droplets. Therefore, a mobile phase with high surface tension and high viscosity should be avoided. Moreover, the small droplets are observed when a high buffer concentration is used.

The flow rate of HPLC affects the size of the droplets formed that resulting in the number of charges on each droplet. In general, the smaller diameter of the spraying capillary, the narrower droplet size distribution is obtained leading to higher efficiency in transferring of sample to the mass spectrometer.

Electrospray is most efficient when operating at flow rates between 5-10 μL/min. Microbore columns are now available.

The use of electrospray in conjunction with pneumatically assisted nebulization at high liquid flow rates, also known as "Ion spray[®]". Ion spray using a probe that provides a flow of nitrogen gas concentrically to the mobile phase stream, which aids the formation of droplets sprayed from the bulk liquid and allows a flow rate around 200 μL/min to be used.

The alternative is to employ a heated source inlet as shown in Figure 2.9. A heated capillary is located directly in line with the electrospray probe so that droplets produced by the electrospray process enter through this capillary to the lens system and mass spectrometer.
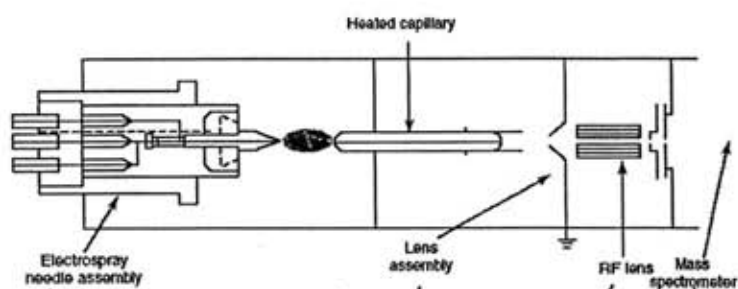
Figure 2.9 Schematics of a heated capillary elctrospray interface.

2. *Sample types*

The ability to produce ions using electrospray ionization is dependant on the solution chemistry of the analyte. The electrospray system can be considered as an electrochemical cell, in positive ion mode, an oxidation reaction occurs at the capillary tip and a reduction reaction occurs at the counter electrode (the production of negative ions). For this reason, the compounds that can be studied are:

(1) Ionic compounds that are intrinsically charged in solution.

(2) Neutral or polar compounds that may be protonated (for positive ion mode) or deprotonated (for negative ion mode) under the solution condition employed.

(3) Non-polar compounds that undergo oxidation or reduction at the electrospray capillary tip.

Moreover, the properties of the solvent, such as its viscosity, conductivity, surface tension and polarity all affect the electrospray process. However, a crucial importance parameter that has a direct effect on the ionization is the pH of the solution. The production of positive ion is favored at acidic pH.

The electrospray process is susceptible to competition effects. All polar or ionic species in solution are not only derived from the analytes, but it derived from buffers or additives as well. The best analytical sensitivity will be obtained from a solution with a single analyte, thus competition is not possible, at low flow rate and with the narrowest diameter electrospray capillary. If excess electrolyte materials are present, competition of each species become present and the efficiency of analyte ionization depend upon the concentration of each species present and the relative efficiency of the conversion of each to the gas phase.

Electrospray does not provide only ions from molecular species, but it also consists of a number of fragment ions depending on molecular weight of the analyte. Unlike most ionization techniques, if the analyte contains more than one site of protonation or deprotonation, multiple charged ions are usually observed. Thus, the most striking application is that of high molecular weight, thermalabile, polar biomolecules such as peptides, proteins, oligonucleotides, etc. The production of multiple charged ions using electrospray ionization effectively extends the mass range of the mass spectrometer by a factor directly related to the number of charges attached to the analyte molecule.

3. *Structural information from electrospray ionization*

The great strength of MS is its ability to generate structural information from the analyte. The electrospray is the "softest" ionization process, the transfer of ions to the gas phase is a low energy process that does not disrupt their structures. However, there are two ways in which fragmentation can be generated.

The first is convinently done by applying tandem mass spectrometry (MS-MS). The strength of MS-MS is its ability to select a single ion and induce fragmentation and mass analyze the fragment ions. The second, known as "cone voltage" or "in-source" fragmentation. Ions are fragmented within the source of the MS by the application of a voltage between the nozzle and the skimmer.

Electrospray allows the study of the three-dimensional structure of compounds, particularly proteins, biomolecules and can be applied to a wide range of compounds. However, the effect of HPLC conditions, such as pH, may have on the appearance of an electrospray spectrum and the conformational deductions that may be made from them.

Advantages:

- Ionization occurs directly from solution.
- Mobile phase flow rate from nL/min to in excess of 1 mL/min can be used, thus allowing conventional and microbore columns to be employed.
- Electrospray ionization produces multiply charged ions of the intact solute molecule. This allows the study of molecules with molecular weights well outside its normal range.

- For high molecular weight materials, an electrospray spectrum provides a number of independent molecular weight determinations from a single spectrum and thus increased precision.
- Easy adaptability to triple quardrupole analysis.
- Good sensitivity, femtomole to low picomole sensitivity is typical.

Disadvantages:

- Electrospray is not applicable to non-polar or low-polarity compounds.
- The mass spectrum produced from an analyte, in terms of the m/z range of the ions observed and their relative intensities, depends upon the experimental condition.
- Suppression effects may be observed and the direct analysis of mixture is not always possible.
- Electrospray produce intact molecular species and structural information from cone voltage fragmentation but these spectra may not always easily interpretable.
- Simultaneous mixture analysis can be poor.

2.2.2.7 Atmospheric Pressure Chemical Ionization (APCI) Interface

APCI has many common features with ESI and thermospray, but the differences between the techniques are the methods of droplet generation and the mechanism of subsequent ion formation.
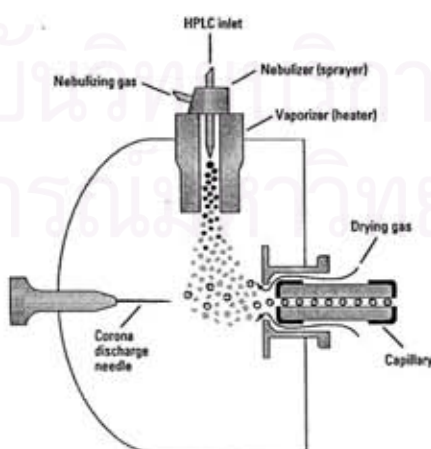
Figure 2.10 Atmospheric pressure chemical ionization (APCI) interface.

The stream of liquid emerging from an HPLC column dispersed into small droplets by a coaxial nebulizing gas, is passed through a heated nebulizer (350-500 °C) where the droplets are both generated and desolvated. The spray formed then passes through a heated region where the vapor is dried. The neutral species produced are then ionized by a corona discharge electrode (3-6 kV). The electric field at the tip of electrode ionizes the gas surrounding it and these solvent ions interact with the analytes in the gas phase at atmospheric pressure. The analyte ions formed are extracted into the MS with a curtain of drying gas to reduce the background cluster ions from the solvent.

The reagent species in the positive-ion mode are protonated solvent ions or proton transfer. While deprotonated solvent ions, O_2^-, its hydrates and clusters may be observed in negative-ion mode. APCI is a soft ionization leading to molecular species with little or no fragmentation, $[M+H]^+$ and $[M-H]^-$. However, APCI leads to the formation of ion cluster involving solvent molecules.

APCI ionizes most efficiently compounds with low to moderate polarities and should not be too thermal labile, such as pesticides, drugs, steroids and PAHs. Moreover, this technique is capable of dealing with high flow rates (0.5-2.0 mL/min), large sample volumes and wide range of buffers.

2.2.3 Mass Analyzer [73-74, 77, 79]

After the ions are formed in the source region they accelerate into the "mass separation device" or "mass analyzer". The mass analyzer separates these ions of different m/z ratios, determines these m/z values and then measures the relative intensities of each group of ions. Generally, the difference between various types of mass spectrometer lies in the manner in which such field is used to effect the separation. However, the selection of a mass analyzer depends upon the resolution, mass range, scan rate, and detection limits required for the application.

Resolution or resolution power refers to the ability of mass spectrometer to distinguish between closely different m/z ratios. Resolution is important because it is contributes to the accuracy and sensitivity of the analytes to be achieved. The most common definition of resolution is given by;

$$R \;=\; m/\Delta m$$

R is the resolution, m is the m/z to be measured, and Δm is the difference between this and the ion from which it is to be separated.

2.2.3.1　Qradrupole Mass Analyzer

The quadrupole mass analyzer uses radio frequency (RF) and direct current (DC) voltages for the separation of ions. It consists of four cylindrical rods arranged in a square array, as shown in Figure 2.11. The opposite pairs are connected electrically and a voltage, consisting of both RF and DC components, is applied, with the RF components on the two pair of rod being 180° out-of-phase. Application of voltages on these pairs of rods creates a hyperbolic field within the rods, resulting in a force on the ions. A specific value of these voltages, ions of a particular m/z follow a stable trajectory through the rods and reach to the detector. A mass spectrum is therefore produced by varying the RF and DC voltages to bring ions of increasing or decreasing m/z ratios to the detector.

However, the combination of both RF and DC yields a stability window, so called a "stable region" as shown in Figure 2.12. All ions with an m/z value that fits with points on this line within a stable region that is stable and move through the rods to the detector.
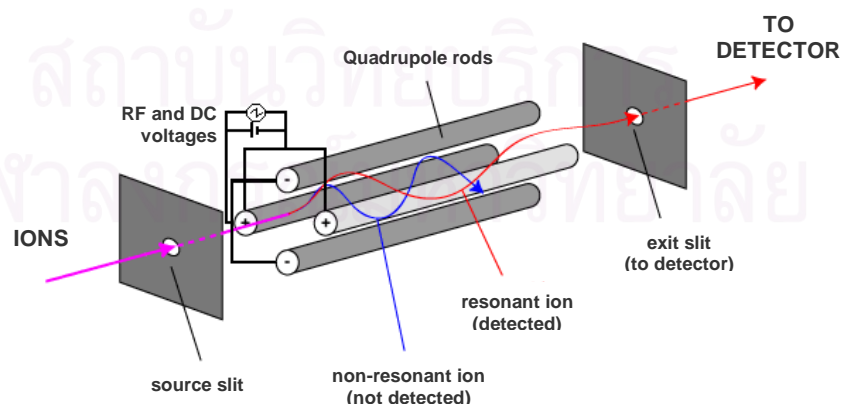
Figure 2.11 Schematic of a quadrupole mass analyzer.
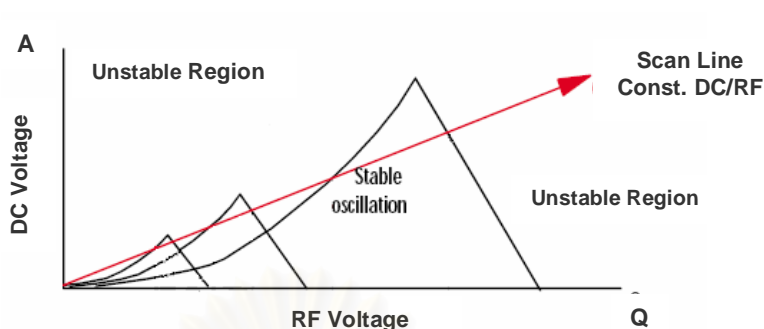
Figure 2.12 Schematic of stability diagram.

A mass spectrum is therefore produced by varying the RF and DC voltages in a systematic way to bring ions of increasing or decreasing m/z ratios to the detector.

Advantages:

- Simple and fast scanning method.
- Uses low potential, make it tolerant of relatively high operating pressures.
- Low cost and easy to couple multiple quadrupoles to other MS analyzers.

Disadvantages:

- Low resolution systems, typically 1 Da.
- Mass range limited to approximately m/z 4000.

2.2.3.2 Quadrupole Ion Trap Mass Analyzer

The quadrupole ion-trap consists of a ring electrode, the top and bottom rods form end-caps above and below the ring electrodes. After ions are introduced into the ion-trap, ions of a m/z with frequencies corresponding to the applied RF voltage become unstable and are ejected through the end-caps toward the detector. By varying the RF voltage, a complete mass spectrum may be obtained.

2.2.3.3 Time-of-Flight (TOF) Mass Analyzer

TOF analysis is based on accelerating a set of ions to a detector with the same amount of energy. Ions have the same enrgy but a different mass reach to the detector at different times. Smaller ions reach the detector first because of their

greater velocity and the larger ions take a longer time, thus the m/z can be determine by their arrival times.

2.2.3.4 Magnetic-Sector Mass Analyzer

A moving charges are accelerated from the source region passing through a magnetic field will experience a force and travel in a circular motion with a radius of curvature. The radius of this motion depends upon the momentum of ion, the charge of ion, and the magnetic field strength. Only ions of a certain m/z can reach the detector at any given magnetic field. The mass spectrum is scanned by changing the magnetic field or the acceleration voltage to transmit different m/z ions.

Double-sector instrument is the combination of magnetic mass analyzer with an electrostatic analyzer, so called "double-focusing mass analyzers". It used to improve the resolution of instrument.

2.2.3.5 Fourier Transform-Ion Cyclotron Resonance (FT-ICR) Mass Analyzer

FT-ICR uses a superconducting magnet to trap ions in a small sample cell. When a RF potential is applied to the emitter plates, ions with the corresponding angular frequency will absorb energy and generate a time dependent current. If scanning the frequency, the receiver plates pick up the signals of all the ions present in the cell. Mass spectrum is obtained by using the Fourier Transform to convert these signals from the time domain to the frequency domain and is then converted to corresponding m/z values.

2.2.4 Ion Detectors [73-74, 79]

After the selected ions pass through the mass analyzer, ions come to the ion detector. The detector allows a MS to generate a signal (electric current) from incident ions that is proportional to their abundance.

2.2.4.1 Faraday Cup

A faraday cup operates based on the change in charge on a metal plate, which results in a flow of electrons which creates an electric current. When an ion

strikes the dynode surface of the faraday cup, which is connected to a resistor, several secondary electrons are induced, ejected and temporarily displaced. The resulting neutralization of the charge on the ions (temporary emission of electrons) leads to flow of current and provides a small amplification of signal.

2.2.4.2 Electron Multiplier

An electron multiplier consists of a series of dynodes maintain at ever-increasing potentials. When a rapidly moving ion strike on the dynode surface the emission of electrons occurs. These secondary electrons are accelerated by an electric potential and then attached to the next dynode where more secondary electrons are generated, ultimately resulting in a cascade of electrons. These provide current gain in order of 10^6.

2.2.4.3 Photomultiplier Conversion Dynode

The photomultiplier conversion dynode detector is similar to an electron multiplier where the ions initially strike a dynode, again releasing secondary electrons. These cascades of electrons are accelerated onto a phosphorus screen, which emits photons once an electron strikes. These photons are then detected by a photomultiplier tube and the signal transformed into an electric current.

2.2.4.4 High-Energy Dynode Detector

The high-energy dynode (HED) uses an electrostatic field prior to the electron multiplier. Once an ion passes through the field, it is accelerated to strike a conversion dynode, resulting in the emission of electrons. The secondary electrons are attached into the electron multiplier, thus producing the cascade of electrons. HED serve to increase ion energy and therefore the signal intensity, which results in greater sensitivity.

2.2.4.5 Array Detector

An array detector consists of a group of detectors in a linear array. These arrays allows for a group of ions (having different m/z) to be detected simultaneously

after passing through a magnetic sector analyzer. The m/z of ions is determined by where it strikes the detector.

2.2.5 Operating Modes of Mass Analyzers for Data Acquisition [73-74]

2.2.5.1 Mass Scaning Mode

The mass spectrometer is set up to scan all m/z over a selected mass range and repetitively for an appropriate period of time. The upper mass limit being defined by the highest molecular weight of the analyte and the lower limit by the background ions. A spectrum is stored and sum of the intensities of the ions present, called total-ion-current (TIC), is computed and stored at the end of each scan. Thus, a single mass spectrum provides complete analytical information for a sample as well as the fundamental piece of information on which the subsequent spectral analysis is based on, the total-ion-current trace.

2.2.5.2 Selected Ion Monitoring (SIM) Mode

From TIC mentioned above, a mass spectrum typically contains a large number of ions and all of these could not be used for quantitation purposes. As long as the identity of the analyte is assured, the signal being measured is derived from the desired analyte and not from interfering compounds. The acquired data can be obtained from some of selected m/z but not of a full-scan spectrum. This technique is known as selected-ion monitoring (SIM), selected-ion recording (SIR), or multiple-ion detection (MID).

In SIM mode, the amount of time spent monitoring each m/z is increased because a small number of ions are selected to scan and many ions of each m/z are used in the analysis, hence resulting in a proportional increase in sensitivity. If compounds other than the target analyte are present but these do not produce ions at the same m/z value as the analyte of interest, they are not important in the analysis. These enhanced the selectivity and sensitivity of the detector.

However, the general parameters for selection of ions in SIM mode are following:
- In LC-MS, the combination of retention time and a single ion may be considered adequate, especially if the molecular ion generated by a soft ionization technique.

- The selected ions should represent the analyte characteristic under investigation.
- The selected ions should be intense to confer sensitivity on the analysis.
- The selected ions should have a high m/z values to reduce the present of background interference.

2.3   Sample Preparation Techniques [80, 81]

A major problem in chromatography is the analysis of analytes in complicated sample matrices such as food, biological matrices, and water. Some matrices contain interfering substances which can affect chromatographic performance by masking the peaks of interest. To eliminate these problems, sample must be treated before injection. Sample preparation prior to HPLC analysis is certainly important steps to consider especially in trace or ultratrace analysis. Because direct introduction of most samples into an instruments are not possible. The analytes have to be extracted into a solution form before analyze by an instrument. However, sample preparation is a technique used to clean-up a sample, to concentrate or enriched sample, and to improve its detection efficiency. Sample preparation consist of several processes depending on the nature of sample, the matrix, and the concentration level at which the analysis needs to be carried out. For instance, trace analysis requires more stringent sample preparation than major component analysis.

Several reported methods for antibiotics and antibacterials analysis revealed several common steps. It is generally agreed that one of the first and the most difficult extraction steps required for antibiotics or any drugs from the biomatrices. This is a prerequisite for all analytical methodologies, though the degree to which it is done varies widely. Screening methodologies require only a minimal extraction and clean-up procedure. Quantitative and confirmative methodologies such as chromatographic or spectrometric assays, particularly of residues in tissue matrices, require extensive treatment of extracts before they are ready for analysis.

In general, sample preparation for most analytical techniques has three objectives:
1. To provide the sample component of interest in a solution form.
2. To provide the analytes free from interfering matrix.

3. To provide the analytes at a concentration appropriate for detection or measurement.

   To accomplish these goals, a sample or a representative portion is prepared via traditional extraction methods. Usually such methods are used in combinations of multiple steps to form a sample preparation protocol.

   This section describes the common utilized techniques for the extraction and clean-up of antibiotics and antibacterials from biomatrices such as centrifugation or ultracentrifugation, precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE).

2.3.1 Centrifugation or Ultracentrifugation

   Centrifugation is one way to isolate or purify macromolecules. Molecules or particles are subjected to a centrifugal force. Since different size particles or molecules will differ in sedimentation velocity, this quality can be use to separate them apart. Therefore, this technique is commonly used to separate fractions in biomatrices. Ultracentrifugation is centrifugation at high speed, up to 80,000 rpm.

2.3.2 Precipitation

   The precipitation of protein-free solutions is important for successful HPLC analysis of blood and tissue extracts. Organic solvents miscible with water and the lower solubility of proteins can be used to precipitate them from aqueous solution. The precipitated proteins are removed by centrifugation. The supernatant is injected directly or after concentration by solvent evaporation. It is important to use a protein-precipitating solvent in which the analyte is highly soluble because some compounds may adsorb or co-precipitate with the protein.

2.3.3 Liquid-Liquid Extraction (LLE)

   Liquid-liquid partitioning in LLE methodologies are utilized for the separation of the compounds of interest from other matrix components. A compound is partitioned between two immiscible liquid phases so as to bring about a favorable extraction of the analytes or contaminants from one phase to another. The extractions are repeated to maximally extract the compounds or contaminants from one phase to

another in order to isolate the agent of interest in a cleaner extract for subsequent analysis.

LLE is very useful, but it has certain limitations. The extracting solvents are limited to those that are water immiscible (for aqueous samples). Emulsion tends to form when the solvents are shaken, and relatively large volumes of solvents are used which generates a substantial waste disposal problem. The operations are often manually performed and may require a back extraction.

2.3.4 Solid-Phase Extraction (SPE) [80-81]

Solid-Phase extraction (SPE) is an extraction technique based on the selective partitioning of one or more components between two phases, one of which is a solid sorbent and the second phase typically is a liquid. SPE is a short tube packed with an appropriate sorbents that are selective with the analyte components. SPE refers to the nonequilibrium, exhaustive removal of chemical constituents from a flowing liquid sample via retention on a contained solid sorbent and subsequent recovery of selected constituents by elution from the sorbent with an appropriate solvent. SPE is sometimes referred to as *digital chromatography,* indicating the all-or-nothing extremes in the sorptive nature of these sorbents.

By careful selection of a solid phase and a solvent, it is possible to achieve total retention of an analyte by driving the equilibrium toward the solid phase or total elution by forcing the equilibrium to the liquid phase. The choice of the adsorbent depends on the nature of the analyte molecules and the matrices.

2.3.4.1 Sorbents in SPE

Appropriate SPE sorbent selection is critical to obtaining efficient SPE recovery of interested components from liquids. Successful SPE has two major requirements: (1) a high, reproducible percentage of the analytical solutes must be taken up by the solid extractant; and (2) the solutes must then be easily and completely eluted from the solid particles. The sorption process must be reversible. In addition to reversible sorption, SPE sorbents should be porous with large surface areas, be free of leachable impurities, exhibit stability toward the sample matrix and

the elution solvents, and have good surface contact with the sample solution. SPE sorbents can be categorized as general purpose, class specific, or compound specific.

(1) Polar sorbent

Polar sorbent or normal phase SPE procedures typically involve a polar stationary phase and a polar analyte, a mid-to-non-polar matrix. The most common polar sorbents are silica $(SiO_2)_x$, alumina (Al_2O_3), magnesium silicate ($MgSiO_3$ or Florisil), and the bonded silica sorbents in which silica is reacted with highly polar functional groups to produce aminopropyl, cyanopropyl, and diol-modified silica sorbents. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. As with typical normal phase silicas, these packing can be used to adsorb polar compounds from non-polar matrices. The interactions between solute and sorbent are controlled by strong polar forces including hydrogen bonding, dipole-dipole interactions, π-π interactions, and induced dipole-dipole interactions.

(2) Bonded silica sorbents

Chemically bonded silica sorbents are commonly used solid phase for SPE. Bonded stationary phases are prepared by "grafting" organic nonpolar, polar, or ionic ligands (denoted R) to a silica particle via covalent reaction with the silanol groups on its surface. The importance of this advancement to solid-phase extraction was the ability to produce highly hydrophobic phases that were more attractive to organic solutes in aqueous solution than any other sorbents. Reversed-phase bonded silica sorbents having alkyl groups covalently bonded to the silica gel backbone interact primarily with analytes via van der Waals forces.

Bonded-phase sorbents are stable to aqueous solvent over a pH range of 1-8.5, above which the silica backbone begins to dissolve and below which the Si-C bond is attacked. Generally, the most common bonded-phase sorbents are based on chemical reaction between silica and organosilanes via the silanol groups on the silica surface to produce chemically stable Si-O-Si covalent linkages to the silica backbone. Nonpolar, polar, or ionic bonded phase can be prepared by varying the nature of the organic moiety bonded to the silica surface.

Bonded phases can be obtained as monomeric or polymeric coverage of an organic ligand group, R, on the silica surface depending on a monofunctional (R_3SiX)

or a trifunctional (RSiX$_3$) reactant is used. The organosilane contains a reactive group, X, that interact chemically with the silanol groups on the silica surface. Typically, the reactant is an organochloro- or organoalkoxysilane in which the X is chloro, methoxy, or ethoxy.

(a)   (b)
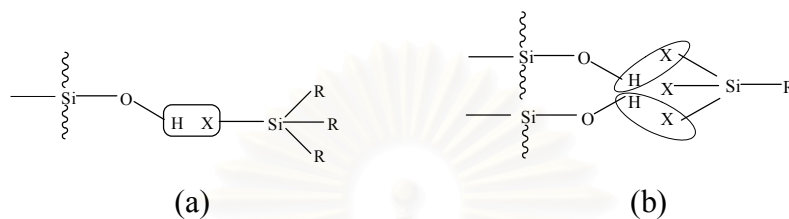
Figure 2.13 Reaction of (a) monofunctional or (b) trifunctional organosilane with silanol groups on the silica surface.

The ligand group, R, of bonded phase produced for reversed-phase application includes hydrophobic, aromatic phenyl, aliphatic alkyl groups, such as octadecyl (C$_{18}$), octyl (C$_8$), ethyl (C$_2$), or cyclohexyl, covalently bonded to the silica gel backbone. Cyanoproply or diol hydrophilic functional groups bonded to the silica sorbent result in polar sorbents. Ionic functional groups (carboxylic acid, sulfonic acid, aminopropyl, or quaternary amines) can also be bonded to the silica sorbent to produce ion-exchange sorbents.

(3)   Graphitized carbon sorbents

Graphitized carbon sorbents are useful for the extraction of very polar, extremely water soluble organic compounds from aqueous samples. Two types of graphitized carbon sorbents, graphitized carbon black (GCBs) and porous graphitized carbons (PGCs), are available for SPE applications.

GCBs do not have micropores and are composed of a homogeneous surface array of graphitized carbon atoms. Polar absorption sites arise from surface oxygen complexes interact strongly with polar compounds. GCBs have the potential for simultaneous extraction of neutral, basic, and acidic compounds. PGCs sorbents are macroporous materials with more highly homogeneous hydrophobic surfaces than GCBs sorbents.

(4)   Apolar polymeric resins

Synthetic styrene-divinylbenzene and other polymers were developed in the late 1960s. Highly cross-linked polystyrene- divinylbenzene (PS-DVB) as shown in Figure 2.14, apolar polymeric resins were produced in a more purified form in 1990s.   The enhanced performance of PS-DVB resins is due to their highly hydrophobic character and greater surface area as compared to the bonded silica sorbents.   The strong sorption properties of PS-DVB resins may arise from the aromatic, polymeric structure.   It can interact with aromatic analytes via π-π interactions.   Because PS-DVB sorbents are highly hydrophobic, they are less selective and exhibit low retention of polar analytes.
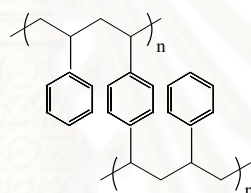
Figure 2.14 Cross-linked styrene-divinylbenzene copolymer.

Polymeric organic sorbents can be used at virtually any pH, 2 to 12 or 0 to 14, increasing the potential to analyze simultaneously multiresidue samples containing acidic, basic, and neutral compounds.   These sorbents can be more retentive than the bonded silica sorbents. Polymeric sorbents have been shown to be capable of retaining chemicals in their ionized form even at neutral pH. Moreover, polymeric sorbents contain no silanol groups and thereby avoid the problems caused by residual silanol groups when bonded silica sorbents are used.

(5)   Functionalized polymeric resins

To eliminate the problem of the highly active sites found on silica sorbents, a completely organic material is used.   This can be accomplished by polymerizing a compound such as styrene or methyl methacrylate.   The cross linking results in spherical beads which are suitable for SPE.   The high degree of hydrophobicity of these polymeric materials generally gives them a large capacity. The proper choice of the main monomer and/or cross linking agent can moderate the

overall hydrophobicity of the completely organic sorbent. However, another method of controlling the relative hydrophobicity is to mix the monomer (co-monomers).

Adding polar functional groups to cross-linked apolar polymeric resins by covalent chemical modification has developed for generation of SPE sorbents suitable for recovery of polar compounds. Hydrophilic functional groups such as acetyl, benzoyl, hydroxymethyl and sulfonate have been chemically introduced into the backbone of PS-DVB copolymers.

However, multiresidue or multiclass environmental samples may contain analytes that range from hydrophilic to hydrophobic extremes, making it difficult to recover all components, effectively from a single extraction. Generation of a macroporous copolymer consisting of two monomers, divinylbenzene (lipophilic) and *N*-vinylpyrrolidone (hydrophilic), produced a hydrophilically-lipophilically balanced SPE sorbent as known "Oasis® HLB" polymer. It provides superior reversed-phase capacity with a special "polar-hook" for enhanced retention of polar analytes. This sorbent has both the lypophilic characters that are specific for apolar compounds and has a trace of hydrophilic characters that simultaneously improve the retention capacities for polar compounds. Moreover, the wetting characteristics of this sorbent make it good for mass transfer.

The main advantages of polymeric materials lie in their ability to withstand pH extremes not achievable with silica-based sorbents. The functionalized polymer materials offer modified non-polar properties, e.g. Oasis® and Bond Elut™ PPL. These polymers are less sensitive to drying out after conditioning and showed enhanced retention of high polar analytes. Apolar polymeric sorbents improve wettability and surface contact between the aqueous sample and the sorbent surface.

N-Vinylpyrrolidone   Divinylbenzene
Hydrophilic-Lipophilic Balance
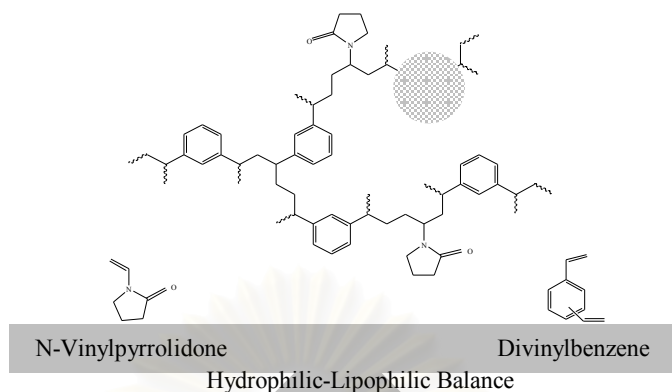
Figure 2.15 Structure of Oasis® HLB solid-phase extraction [poly(divinylbenzene-
co-*N*-vinylpyrrolidone)].

(6)   Ion-exchange sorbents

SPE sorbents for ion-exchange are based on either apolar polymeric resins
or bonded silica sorbents. Ion-exchange sorbents contain ionized functional groups
(primary/secondary amines or carboxylic acids) or associates with the oppositely
charged counterion through an electrostatic or ionic bond. If the sorbent contains a
positively charged functional group and the exchangeable counterion on the analyte is
negatively charged, called *"anion exchange"*. Conversely, if the sorbent surface is
negatively charged and the exchangeable counterion on the analyte is positively
charged, it is called *"cation exchange"*. The analyte retention is affected by the ionic
strength of the sample matrix because other ions present will compete with the analyte
of interest for retention by ion-exchange mechanisms.

(7)   Mixed-mode sorbents

A mixed-mode sorbent is designed chemically to have multiple retentive
sites on an individual particle. These sites exploit different retention mechanisms by
chemically incorporating different ligands on the same sorbent. For example,
sorbents contain hydrophobic alkyl chains and cation-exchange sites on the same
sorbent particle. Mixed-mode sorbents exploit interaction with different functional
groups on a single analyte or different functional groups on multiple analytes.
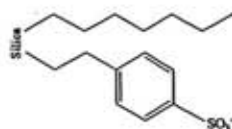
Figure 2.16 A mixed-mode sorbent consisting of silica modified with C_8 chains and strong cation-exchange sites.

2.3.4.2 Strategies of SPE

The concept of SPE is used to separate a compound of interest from impurities in different processes. The two major processes for isolating the desired substances are:

1. *Unretained process*: the desired analyte passes directly through the tube and the matrix interferences are retained or bound within the SPE media. This strategy is usually chosen when the desired component is present in high concentration.

2. *Retained process*: The mixture of the desired analyte and matrix interference are passed through the SPE column under gravity. The desired analyte is immobilized in the SPE media and the interferences can be washed through the column. In the final stage, a selective solvent is added to the SPE column and the desired analyte is eluted. This process can be referred to as "catch and release" SPE. This strategy is usually chosen when the components of interest are present at low levels, or multiple components of widely differing polarities need to be isolated. This strategy may be used for trace enrichment of extremely low level compounds and concentration of dilute sample.
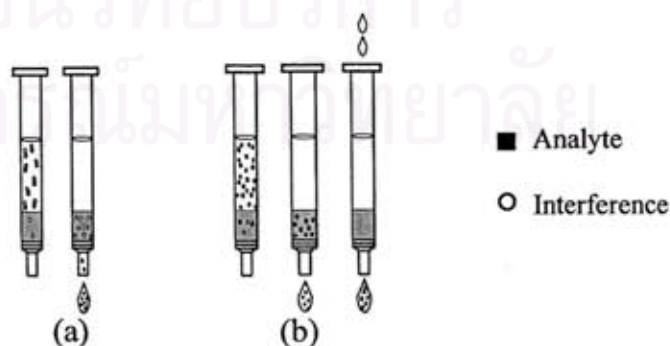
Figure 2.17 Strategies of SPE (a) unretained process and (b) retained process.

A complex matrix may be treated by both elution strategies to isolate different target analytes.

CHAPTER III

EXPERIMENTAL

3.1 Instrument and Apparatus

3.1.1   High Performance Liquid Chromatography (HPLC): A module 1100^{TM} consists of automatic degasser, quaternary pump, autosampler and column thermostat, Agilent Technologies, Palo Alto, U.S.A.

3.1.2   Mass spectrometry detector (MSD): A module 1100 with electrospray ionization interface, Agilent Technologies, Palo Alto, U.S.A.

3.1.3   LC/MS Grade Nitrogen Generators: Models 75-72-K727, Parker Hannifin Corporation, Haverhill, MA.

3.1.4   Air pump for N_2 generator, Model PAC-10, Gast®, Michigan, U.S.A.

3.1.5   Nitrogen Liquid PCC, S size, 180 liter, pressure 350 psi, TIG, Bangplee, Samutplakarn, Thailand.

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

3.1.7   HPLC column: SymmetryShield™ RP18, 150 × 2.1 mm I.D., 3.5 μm, Waters Corporation, Milford, MA, U.S.A.

3.1.8   A SymmetryShield™ Sentry™ guard column with cartridge holder, Waters Corporation, Milford, MA, U.S.A.

3.1.9   A glass filter holder set (300 mL funnel, 1 L flask, glass base with tube cap, and 47 mm spring clamp) for HPLC mobile phase filtration, Millipore, Billerica, MA, U.S.A.

3.1.10 Vacuum pump with pressure regulator, Model DOA-P504-BN, Gast®, Michigan, U.S.A.

3.1.11 Vortex mixer, Model G-5605, Scientific Industries, Bohemia, New York, U.S.A.

3.1.12 Nitrogen gas (99.99% purity), TIG, Bangplee, Samutplakarn, Thailand.

3.1.13   Centrifuge, CENTAURA 2, MSE, MSB020.C×1.5, SANYO, Loughborough, U.K.

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

3.1.15   A Rotary evaporator: consist of BUCHI heating bath B-490, BUCHI rotavapor R-200, SIBATA and circulating aspirator WI-20, Büchi, Flawil, Switzerland.

3.1.16   Micropipetts 0.1-10, 10-100, 100-1,000 µL and tips, Eppendorf, Hamburg, Germany.

3.1.17   Filter membrane 47 mm, 0.45 µm, type Teflon and Nylon, Agilent Technologies, Palo Alto, USA.

3.1.18   Syring filters, Nylon 13 mm, 0.45 µm, Agilent Technologies, Palo Alto, U.S.A.

3.1.19   HPLC amber vials 2 mL with PTFE caps, Agilent Technologies, Palo Alto, U.S.A.

3.1.20   Round bottle flasks 50 and 100 mL.

3.1.21   Volumetric flasks 50.00, 100.00, 250.00 mL.

3.1.22   Beakers10, 25, 50, 100, 150, 250,600 mL.

3.1.23   Graduated cylinders 10.0, 25.0, 50.0, 100.0 mL.

3.1.24   Separatory funnel 250 mL.

3.1.25   Solid phase extraction cartridges:
- Oasis® HLB 500 mg, 6 mL, PN 186000115, Waters Corporation, Milford, MA, U.S.A.
- Bond Elut C_{18} 500 mg, 3 mL, PN 12102028, Varian, Palo Alto, U.S.A.
- Bond Elut PSA 500 mg, 3 mL, PN 12102042, Varian, Palo Alto, U.S.A.
- Carbograph 300 mg, 6 mL, Alltech, Deerfield, IL, U.S.A..

3.1.26   BAKER spe-24G glass vacuum processor, J. T. Baker Chemical Company, Deventer, Holland.

       All glasswares were washed sequentially with detergent and follow by rinsed with deionized water, 1 N HCl, and methanol before use.

3.2 Chemicals

3.2.1 Standard Compounds

Sulfadiazine (SDZ, CAS no. 68-35-9, purity 99%), Sulfathiazole (STZ, CAS no. 72-14-0), Sulfamethazine (SMZ, CAS no. 57-68-1), Sulfamethoxypyridazine (SMP, CAS no. 80-35-3), Sulfamethoxazole (SMX, CAS no. 723-46-6), Sulfadimethoxine (SDM, CAS no. 122-11-2), Chlortetracycline Hydrochloride (CTC, CAS no. 64-72-2, purity 80%), Oxytetracycline Hydrochloride (OTC, CAS No. 2058-46-0, purity 95%), Tetracycline Hydrochloride (TC, CAS No. 64-75-5, purity 95%), and Pyrimethamine (PYR, CAS No. 58-14-0) were supplied by Sigma Aldrich (St. Louis, U.S.A.).

3.2.2 Organic Solvents

Acetonitrile was ultra-residues analytical grade, methanol and 2-propanol were HPLC grade all of these were purchased from J. T. Baker Chemical Company, Deventer, Holland. Chloroform was supplied by KANTO Chemical, Tokyo, Japan.

3.2.3 Other Chemicals

Oxalic acid dihydrate, citric acid monohydrate, ethylenediaminetetraacetic acid disodium salt dehydrate (Na_2EDTA), succinic acid, trifluoroacetic acid were analytical grade supplied by Fluka Chemica, Buchs, Switzerland. Tricholroacetic acid and ammonium hydroxide (NH_4OH) solution were purchased from J. T. Baker Chemical Company, Deventer, Holland. *di*-Sodium hydrogen phosphate dodecahydrate, sodium hydroxide pellet and acetic acid solution were purchased from E. Merck, Darmstadt, Germany. Hydrochloric acid (HCl) and formic acid were purchased from Fisher Scientific, Leicestershire, U.K.

3.3 Preparation of Standard Solutions

3.3.1 The Standard Stock Solutions

Individual standard solution containing 1,000 ppm was prepared by weighing 0.0250 g of each standard and dissolving them with methanol in 25.0 mL

volumetric flasks. Then, each standard stock solution was transferred to an amber glass bottle with Teflon screw cap and stored at -18 ℃ in the refrigerator for maximum period of 2 months.

3.3.2 The Diluted Standard Stock Solutions

Individual standard solution containing 100 ppm was prepared by diluting 1,000 μL of standard stock solutions (section 3.3.1) and made volume to 10.0 mL with acetonitrile in volumetric flasks. Then, each diluted standard stock solution was transferred to an amber glass bottle with Teflon screw cap and stored at -18 ℃ in the refrigerator for maximum period of 5 days.

3.3.3 The Standard Solutions for Flow Injection Analysis (FIA)

Individual standard solution containing 5.00 ppm was prepared by pipetting 100 μL of diluted standard solutions in section 3.3.2 and diluting to 2.00 mL with acetonitrile in an amber vial. These standards were prepared daily and stored at 4 ℃ in the refrigerator before use.

3.3.4 The Mixture Standard Solutions

The mixture of 10 standard solutions at 1.00 ppm were prepared by pipetting 100 μL of each diluted stock solution into an amber glass bottle and then diluting to 10.0 mL with acetonitrile. These standards were prepared daily and stored at 4 ℃ in a refrigerator before use.

The working standard solutions for preparation of calibration curves were prepared from this solution.

3.4 The Optimum Instrumental Analysis Conditions

In this research, the LC-MS instrument was supported from Agilent Technologies, Palo Alto, U.S.A. Liquid chromatography is an Agilent 1100^{TM} series, with a solvent degassing unit, a quaternary pump, an automatic sample injection, and column thermostat. MSD is an Agilent 1100^{TM} SL equipped with ionization source,

single quadrupole, and HED detector. In addition, our laboratory has a facility to operate both ESI and APCI ionization sources.

To achieve optimal mass spectrometric detection, the sensitivities of SAs, TCs, and PYR using ESI and APCI were compared. The optimum mobile phase types, fragmentor voltage, and capillary voltage are determined.

3.4.1 Optimization of ESI Parameters

To optimize the ESI condition, parameters that influence the ionization efficiency of the analytes were investigated. The mobile phase types, fragmentor voltages, and capillary voltage were evaluated by flow injection analysis (FIA). FIA is a direct injection of standard solution into MS without the analytical column connected and detected in full-scan ion detection mode.

3.4.1.1 The Selection of Mobile Phase Type and Fragmentor Voltage

The suitability of the mobile phase compositions and the fragmentor voltage are of paramount importance because they must promote full ionization of the analyte, and provide acceptable desolvation efficiency to charged species prior to the MS analysis.

Throughout the investigation, other MS parameters were fixed at values high enough to provide good sensitivities and structural information. Preliminary, LC-MS conditions using FIA in this study are described in Table 3.1.

Table 3.1 LC-MS conditions used for the selection of mobile phase type and fragmentor voltage using FIA for ESI Optimization.

Parameters	Conditions
Moblie phase*	1. 1% acetic acid: acetonitrile (50:50)
	2. 20 mM ammonium acetate buffer: acetonitrile (50:50)
	3. 1% formic acid: acetonitrile (50:50)
	4. 10 mM ammonium formate buffer: acetonitrile (50:50)
	5. 1 mM oxalic acid: acetonitrile (50:50)
Analyte concentration	5.00 ppm
Injection volume	10 µL
Flow rate	0.50 mL/min
Nebulizer gas pressure	Nitrogen, 35 psi
Drying gas flow	Nitrogen, 10 L/min at 300 °C
Capillary voltage	3,000 V
Mass range	Full-scan mode with positive ion detection between 50-500 amu
Fragmentor voltage*	Scanned 40-250 V, step size 30 V

* Black highlights refer to the varied parameters.

To verify the usefulness of the chosen mobile phase and fragmentor voltage for an optimal ESI-MS response, the intensity of each compound was measured versus the fragmentor voltage in each of the mobile phase type.

3.4.1.2 The Selection of Capillary Voltage

The capillary voltage was applied to the entrance of the capillary that influenced the transmission efficiency of ions. In this study, a suitable capillary

voltage was selected by using the preliminary LC-MS conditions as described in Table 3.2.

Table 3.2 LC-MS conditions used for the selection of capillary voltage using FIA for ESI optimization.

Parameters	Conditions
Moblie phase	1 mM oxalic acid: acetonitrile (50:50)
Analyte concentration	5.00 ppm
Injection volume	10 μL
Flow rate	0.50 mL/min
Nebulizer gas pressure	Nitrogen, 35 psi
Drying gas flow	Nitrogen,10 L/min at 300 °C
Capillary voltage*	Varied between 2,000-4,000 V
Mass range	Full-scan mode with positive ion detection between 50-500 amu
Fragmentor voltage	130 V

* Black highlight refers to the varied parameter.

The intensity of each compound was measured versus the capillary voltage values.

3.4.2 Optimization of APCI Parameters

In APCI mode, the parameters influencing the ionization efficiency of the analyte solutions, such as mobile phase type and fragmentor voltage were investigated in this study. APCI parameters were optimized using FIA analysis in positive ion detection mode. To estimate the best mobile phase and suitable fragmentor voltage for optimum APCI response, the preliminary LC-MS conditions used in this study are described in Table 3.3.

Table 3.3 LC-MS conditions used for the selection of mobile phase type and fragmentor voltage using FIA for APCI optimization.

Parameters	Conditions
Moblie phase*	1. 0.01% trifluoroacetic acid: acetonitrile (50:50, v/v)
	2. 0.03% heptafluorobutyric acid: acetonitrile (50:50,v/v)
	3. 1 mM oxalic acid: acetonitrile (50:50, v/v)
Analyte concentration	5.00 ppm
Injection volume	10 μL
Flow rate	0.90 mL/min
Nebulizer gas pressure	Nitrogen, 35 psi
Drying gas flow	Nitrogen, 10 L/min at 300 °C
Vaporizer temperature	300 °C
Corona current	10 μA.
Capillary voltage	3,000 V
Mass range	Full-scan mode with positive ion detection between 50-500 amu
Fragmentor voltage*	Scanned 40-250 V, step size 30 V

* Black highlights refer to the varied parameters.

3.5 The Study of HPLC Conditions

The HPLC conditions for separation a mixture of 10 standard solutions were developed by varying the analytical columns and mobile phase strength. To achieve the optimum HPLC conditions, the preliminary chromatographic conditions used in this study are shown in Table 3.4.

The optimum HPLC condition was tested with a standard mixture solution and spiked standard solutions.

Table 3.4 Chromatographic conditions used for the HPLC optimization.

Parameters	Conditions
Analytical column	1. Hypersil BDS C18 (2.1×150 mm, i.d. 3.0 μm)
	2. SymmetryShield™ RP18
	(2.1×150 mm, i.d. 3.5 μm)
Mobile phase	A: 1 mM oxalic acid in 5 % acetonitrle-water
	B: 1 mM oxalic acid in 95 % acetonitrle-water
Flow rate	0.30 mL/min
Analyte concentration	500 ppb
Injection volume	5 μL
Column temperature	Left 35 ºC and
	Right 40 ºC
Detector	ESI-MS, positive ion detection
	(results from section 3.4)
Mass range	SIM mode

* Black highlights refer to the varied parameters.

3.6 The Study of Selectivity Evaluation of LC-ESI-MS Condition

The procedures for the study of selectivity of standard SAs, TCs, and PYR can be described as follows:

3.6.1 The mixture of all standards in acetonitrile solution at 100 ppb was injected into LC-MS under the optimum conditions.

3.6.2 The results of selectivity were reported by peak retention time and ions characteristic of each compound.

3.7 Comparison of Extraction Methods

From the literature reviews, various extraction methods have been used to extract SAs, TCs, and PYR from samples such as animal tissues, eggs, milk, and waters. Each method was speciated by groups of residues and confirmatory techniques. In order to develop a method that is capable for the analysis of all ten analytes, our preliminary experiments were conducted to assess the extraction efficiency of previously published method. Seven published procedures for antibiotic residues in biological matrices as described in APPENDIX B were compared in this study.

Method 1   Determination of sulfamethazine in milk using liquid-liquid extraction and analyzed by HPLC [11].

Method 2   Determination of sulfonamides in milk and egg using Carbograph 4 SPE cartridge and analyzed by LC-MS [22].

Method 3   Determination of oxytetracycline, tetracycline, chlortetracycline, and doxycycline in bovine milk and muscle using Oasis$^®$ HLB cartridge and analyzed by LC-MS [59].

Method 4   Determination of tetracyclines in bovine tissues using Bond Elut C$_{18}$ SPE cartridge and analyzed by LC-MS/MS [50].

Method 5   Determination of tetracycline residues in shrimp and whole milk using Oasis$^®$ HLB cartridge and analyzed by LC-UV-MS [60].

Method 6   Adapted from the method 5.

Method 7   Determination of sulfonamide and tetracycline in ground water and surface water using Oasis$^®$ HLB cartridge and analyzed by LC-MS [73].

Method 8   Determination of sulfonamides in animal liver using Bond Elut PSA and analyzed by LC-UV-MS [20].

In this work, eight extraction methods were compared by spiked standards mixture solution into milk sample at spiking level 10.0 and 20.0 ppb and extracted by the procedures described separately. The extract solutions were injected into

LC-MS and analyzed under the optimum LC-ESI-MS conditions. The results of each method were compared and reported as percent recoveries of each analyte.

3.8   Extraction Method Development

From the basis extraction method obtained from section 3.7, some extraction procedures were developed to provide the optimum conditions for simultaneous extraction of SAs, TCs, and PYR residues in milk in this study.

SPE washing step is significance for removing major interferences from the adsorbed compounds. The generic Oasis[®] HLB method (one-dimension, 1D) mainly recommends controlling methanol strength as eluent. The method suggests using of 5% methanol as the wash solvent and absolute methanol for the elution. The drawback of this non-specific method is the limitation of selective removal of contaminants that interfere with the quantitative analysis at trace level.

Therefore, a more selective method (2D-method) with two wash steps was recommended to the SPE method as described by Waters Corporation. This selective method recommended simply manipulating the pH of wash solvent that helps isolate the analytes of interest from the complex sample matrix and provide a cleaner extract solution. However, the influence of pH on the analytes retention depends on the nature of the compounds, acid or base. Therefore, different SPE methods were studied after loading of sample solution onto the Oasis[®] HLB sorbents:

- 1D-SPE method: the sorbents were washed with 5% methanol in water and then eluted with absolute methanol.

- 2D-SPE method: the sorbents were washed with 5% methanol in water followed by 5% methanol containing 2% ammonium hydroxide (base-modified methanol) and then eluted with absolute methanol followed by 95% methanol containing 2% acetic acid.

- 2D-SPE method: the sorbents were washed with 5% methanol in water followed by 5% methanol containing 2% acetic acid (acid-modified methanol) and then eluted with absolute methanol followed by 95% methanol containing 2% ammonium hydroxide.

Generally, the methanol strength in wash steps needs to be manipulated to selectively isolate the target analytes from the milk matrix. But the analyte retention decrease with increasing of methanol strength especially TCs, therefore, 5% methanol was chosen as the wash solvent for improving analytes retentions.

3.8.1   Effect of Base Modified Methanol of SPE Wash Solvent

The effect of SPE wash solvent to provide the cleaner extract was studied to determine the optimal SPE conditions. The experimental procedures to study the based modified methanol of SPE wash solvent on the percent recoveries of each analyte can be described as follows:

3.8.1.1   Sample blank was prepared by weighing 5.00 g of milk sample into a plastic centrifuge tube.

3.8.1.2   Added 2 mL of 20% trichloroacetic acid into milk sample and vortexed for 2 min.

3.8.1.3   Added 20 mL of McIlvain buffer and vortexed for 3 min.

3.8.1.4   The mixture solution was centrifuged at 3,800 rpm for 20 min.

3.8.1.5   The Oasis$^®$ HLB cartridge was pre-conditioned by washing sequentially with 5 mL of methanol, 5 mL of 0.5 N HCl, and 5 mL of deionized water.

3.8.1.6   The supernatant in step 3.8.1.4 was transferred to the Oasis$^®$ HLB cartridge which connected to a vacuum manifold.

3.8.1.7   After sample loading, the cartridge was washed sequentially with 5 mL of 5% methanol in water and 5 mL of 2% NH$_4$OH in 5%methanol.

3.8.1.8   The cartridge was allowed to dry by drawing air through for 5 min using a pump.

3.8.1.9   The analytes were eluted sequentially with 10 mL absolute methanol and 10 mL of 2% acetic acid in 5%methanol.

3.8.1.10   The solvent was removed by rotary evaporator at 33 °C.

3.8.1.11   The residue was evaporated to dryness under a flow of nitrogen.

3.8.1.12   The dried residue was re-dissolved in 1.00 mL of mobile phase and filtered through 0.45 μm syringe filter for LC-ESI-MS analysis.

3.8.1.13   Spiked samples were prepared by adding the standards mixture solution into 5.00 g milk sample at 10.0 and 20.0 ppb concentration levels.

3.8.1.14 Each spiked sample in step 3.8.1.13 was extracted following the procedure described in step 3.8.1.2 - 3.8.1.12.

3.8.1.15 The final concentrations were calculated and reported as percent recoveries of analyte.

3.8.2   Effect of Sample Treatment

Generally, the residual drugs are homogeneously mixed with milk. Therefore, the treatment procedure used to provide the homogeneous mix between spiked standard solution and milk sample are considered. It may affect the phase separation efficiency of residual drugs from milk matrices.   In this study, the homogenization of milk sample was tested to improve the recoveries of the studied compounds.

The experimental procedures for the study of sample treatment procedure on the percent recoveries of each compound can be described as follows:

3.8.2.1 Sample blank was prepared by weighing 5.00 g of milk sample into a plastic centrifuge tube.

3.8.2.2 Added sequentially 2 mL of 20% trichloroacetic acid and 20 mL of McIlvain buffer into milk sample.

3.8.2.3 The sample solution was homogenized for 5 min at low speed.

3.8.2.4 The mixture solution was centrifuged at 3,800 rpm for 20 min.

3.8.2.5 Pre-conditioned the Oasis$^\circledR$ HLB cartridge by washing sequentially with 5 mL of methanol, 5 mL of 0.5 N HCl, and 5 mL of deionized water.

3.8.2.6 Transferred the supernatant in step 3.8.2.4 to the Oasis$^\circledR$ HLB cartridge which connected to a vacuum manifold.

3.8.2.7 After sample loading, the cartridge was washed sequentially with 5 mL of 5% methanol in water and 5 mL of 2% NH_4OH in 5%methanol.

3.8.2.8 The cartridge was allowed to dry by drawing air through for 5 min using a pump.

3.8.2.9 The analytes were eluted sequentially with 10 mL of absolute methanol and 10 mL of 2% acetic acid in 5%methanol.

3.8.2.10 The solvent was removed by rotary evaporator at 33 °C.

3.8.2.11 The residue was evaporated to dryness under a flow of nitrogen.

3.8.2.12 The dried residue was re-dissolved in 1.00 mL of mobile phase and filtered through 0.45 µm syringe filter for LC-ESI-MS analysis.

3.8.2.13 Spiked samples were prepared by adding the standards mixture solution into 5.00 g milk sample at 10.0 and 20.0 ppb concentration levels.

3.8.2.14 Each spiked sample in step 3.8.2.13 was extracted following the procedure described in step 3.8.2.2–3.8.2.12.

3.8.2.15 The final concentrations were calculated and reported as percent recoveries of analyte.

3.8.3 Effect of Solvent Evaporation

Because the vigorous condition for solvent removal of rotary evaporation under a vacuum may cause the decomposition of analytes. Therefore, a mild condition used for solvent removal such as nitrogen (N_2) evaporation is considered.

The experimental procedures for the study of solvent evaporation on the percent recoveries of each analyte can be described as follows:

3.8.3.1 Sample blank was prepared following the procedure described in step 3.8.1.1-3.8.1.9.

3.8.3.2 The solvent was removed to dryness under a flow of nitrogen gas and the temperature of analyte solution was controlled at 33 °C using water bath.

3.8.3.3 The dried residue was re-dissolved in 1.00 mL of mobile phase and filtered through 0.45 µm syringe filter for LC-ESI-MS analysis.

3.8.3.4 Spiked samples were prepared by adding the standards mixture solution into 5.00 g milk sample at 10.0 and 20.0 ppb concentration levels.

3.8.3.5 Each spiked sample in step 3.8.3.4 was extracted following the procedure described in step 3.8.3.1–3.8.3.3.

3.8.3.6 The final concentrations were calculated and reported as percent recoveries of analyte.

3.8.4 Effect of Sample pH

Because of the studied compounds have different chemical properties. The pH of sample is the most influential variable during sample extraction and has the biggest impact to the analytes retention on the SPE sorbents. Therefore, the influence

of the pH of a milk sample on the extraction efficiency of SAs, TCs, and PYR are considered. The pH values of the milk samples were adjusted in order to achieve the acceptable extraction efficiency.

The experimental procedures for the study of sample pH on the percent recoveries of each analyte can be described as follows:

3.8.4.1   Sample blank was prepared following the procedure described in step 3.8.1.1-3.8.1.3.

3.8.4.2   The sample solution was adjusted to pH 4.0 with 1.0 M NaOH solution and vortexed for 1 min.

3.8.4.3   The mixture solution was centrifuged at 3,800 rpm for 20 min.

3.8.4.4   The Oasis$^®$ HLB cartridge was pre-conditioned by washing sequentially with 5 mL of methanol, 5 mL of 0.5 N HCl, and 5 mL of deionized water.

3.8.4.5   The supernatant was transferred to the Oasis$^®$ HLB cartridge which connected to vacuum manifold.

3.8.4.6   After sample loading, the cartridge was washed sequentially with 5 mL of 5% methanol in water and 5 mL of 2% NH_4OH in 5%methanol.

3.8.4.7   The cartridge was allowed to dry by drawing air through for 5 min using a pump.

3.8.4.8   The analytes were eluted sequentially with 10 mL absolute methanol and 10 mL of 2% acetic acid in 5%methanol.

3.8.4.9   The solvent was removed by rotary evaporator at 33 °C.

3.8.4.10 The residue was evaporated to dryness under a flow of nitrogen.

3.8.4.11 The dried residue was re-dissolved in 1.00 mL of mobile phase and filtered through 0.45 μm syringe filter for LC-ESI-MS analysis.

3.8.4.12 Spiked samples were prepared by adding the standards mixture solution into 5.00 g milk sample at 10.0 and 20.0 ppb concentration levels.

3.8.4.13 Each spiked sample in step 3.8.4.12 was extracted following the procedure described in step 3.8.4.1–3.8.4.11.

3.8.4.14 The final concentrations were calculated and reported as analyte percent recoveries.

3.8.5 Effect of Acid Modified Methanol of SPE Wash Solvent

As previously described, a more selective method (2D-method) with two wash steps was recommended to the SPE method. The effect of acid modified methanol of SPE wash solvent ability to provide the cleaner extraction was studied to determine the optimum SPE conditions. The experimental procedures for the study of acid modified methanol of SPE wash solvent on the percent recoveries of each analyte can be described as follows:

3.8.5.1 Sample blank was prepared following the procedure as described in step 3.8.4.1-3.8.4.5.

3.8.5.2 After sample loading, the cartridge was washed sequentially with 5 mL of 5% methanol in water and 5 mL of 2% acetic acid in 5%methanol.

3.8.5.3 The cartridge was allowed to dry by drawing air through for 5 min using a pump.

3.8.5.4 The analytes were eluted sequentially with 10 mL of absolute methanol and 10 mL of 2% NH_4OH in 5%methanol.

3.8.5.5 The solvent was removed by rotary evaporator at 33 °C.

3.8.5.6 The residue was evaporated to dryness under a flow of nitrogen.

3.8.5.7 The dried residue was re-dissolved in 1.00 mL of mobile phase and filtered through 0.45 µm syringe filter for LC-ESI-MS analysis.

3.8.5.8 Spiked samples were prepared by adding the standards mixture solution into 5.00 g milk sample at 10.0 and 20.0 ppb concentration levels.

3.8.5.9 Each spiked sample in step 3.8.5.8 was extracted following the procedure described in step 3.8.5.1–3.8.5.7.

3.8.5.10 The final concentrations were calculated and reported as percent recoveries of analyte.

3.8.6 Optimization of Sample pH

The adjustment of sample pH proved to have an affect on the analyte recoveries. Therefore, the optimum sample pH was studied to provide the best extraction efficiency.

The experimental procedures for the study of sample pH on the percent recoveries of each analyte can be described as follows:

3.8.6.1 Sample blank were prepared following the procedures described in step 3.8.5.1-3.8.5.7.

3.8.6.2 Spiked samples at pH 4.0 were prepared by weighing 5.00 g of milk sample into a plastic centrifuge tubes.

3.8.6.3 Added the standards mixture solution into the milk samples at 10.0 and 20.0 ppb concentration levels.

3.8.6.4 Added 2 mL of 20% trichloroacetic acid into spiked sample and vortexed for 2 min.

3.8.6.5 Added 20 mL of McIlvain buffer and vortexed for 3 min.

3.8.6.6 The sample solution was adjusted to pH 4.0 with 1.0 M NaOH solution and vortexed for 1 min.

3.8.6.7 The mixture solution was centrifuged at 3,800 rpm for 20 min.

3.8.6.8 The Oasis® HLB cartridge was pre-conditioned by washing sequentially with 5 mL of methanol, 5 mL of 0.5 N HCl, and 5 mL of deionized water.

3.8.6.9 Transferred the supernatant in step 3.8.6.7 to the Oasis® HLB cartridge which connected to vacuum manifold.

3.8.6.10 After sample loading, the cartridge was washed sequentially with 5 mL of 5% methanol in water and 5 mL of 2% acetic acid in 5%methanol.

3.8.6.11 The cartridge was allowed to dry by drawing air through for 5 min using a pump.

3.8.6.12 The analytes were eluted sequentially with 10 mL absolute methanol and 10 mL of 2% NH_4OH in 5%methanol.

3.8.6.13 The solvent was removed by rotary evaporator at 33 °C.

3.8.6.14 The residue was evaporated to dryness under a flow of nitrogen.

3.8.6.15 The dried residue was re-dissolved in 1.00 mL of mobile phase and filtered through 0.45 µm syringe filter for LC-ESI-MS analysis.

3.8.6.16 Spiked samples at pH 4.5 were prepared as same as step 3.8.6.2–3.8.6.15 except the sample solutions were adjusted to pH 4.5 with 1.0 M NaOH solution.

3.8.6.17 Spiked samples at pH 5.0 were prepared as same as step 3.8.6.2–3.8.6.15 except the sample solutions were adjusted to pH 5.0 with 1.0 M NaOH solution.

3.8.6.18 The final concentrations were calculated and reported as percent recoveries of analyte at each sample pH value.

3.9 The Study of Selectivity Evaluation of LC-ESI-MS Condition in Milk Matrix

The matrix sample was studied using a pasteurized milk matrix as a representative of all samples. The experimental procedures for the study of selectivity of standard SAs, TCs, and PYR in a milk matrix can be described as follows:

3.9.1 <u>Blank Sample</u> was prepared by weighing 5.00 g of milk sample into a plastic centrifuge tube.

3.9.2 Added 2 mL of 20% trichloroacetic acid and vortexed for 2 min.

3.9.3 Added 20 mL of McIlvain buffer and vortexed for 3 min.

3.9.4 The sample solution was adjusted to pH 4.5 with 1.0 M NaOH solution and vortexed for 1 min.

3.9.5 The mixture solution was centrifuged at 3,800 rpm for 20 min.

3.9.6 The Oasis$^®$ HLB cartridge was pre-conditioned by washing sequentially with 5 mL of methanol, 5 mL of 0.5 N HCl, and 5 mL of deionized water.

3.9.7 The supernatant in step 3.9.5 was transferred to the Oasis$^®$ HLB cartridge which connected to vacuum manifold.

3.9.8 After sample loading, the cartridge was washed sequentially with 5 mL of 5% methanol in water and 5 mL of 2% acetic acid in 5%methanol.

3.9.9 The cartridge was allowed to dry by drawing air through for 5 min using a pump.

3.9.10 The analytes were eluted sequentially with 10 mL absolute methanol and 10 mL of 2% NH$_4$OH in 5%methanol.

3.9.11 The solvent was removed by rotary evaporator at 33 °C.

3.9.12 The residue was evaporated to dryness under a flow of nitrogen.

3.9.13 The dried residue was re-dissolved in 1.00 mL of mobile phase and filtered through 0.45 µm syringe filter.

3.9.14 The blank milk extract was injected into LC-ESI-MS under the optimized conditions.

3.9.15 The mixture of all standards solution was spiked into the blank milk extract obtained from step 3.9.13 at 100 ppb concentration level.

3.9.16 The solution was injected into LC-ESI-MS under the optimized conditions.

3.9.17 The study results were reported as peak retention time and mass ions characteristic (m/z) of each peak.

3.10 The Study of Method Quantitation Limit (MQL)

The method quantitation limits were determined by analyzing a sample containing low concentration of analytes that provided peaks at signal-to-noise ratio equal to 10. The analyses were done in triplicate analyses and the average result was reported. The experimental procedure can be described as follows:

3.10.1 <u>Blank sample</u> was prepared by the extraction procedures described in step 3.9.1–3.9.13.

3.10.2 <u>Spiked sample</u> was prepared by spiking standards mixture solution into 5.00 g milk sample and extracted in the same way as blank sample.

3.10.3 The blank and spiked samples were analyzed under the optimum LC-ESI-MS conditions. The peak signals of each compound were measured from the chromatograms.

3.10.4 The MQL of each compound was obtained from the concentration that gave peak height at 10 times above the baseline.

3.11 The Study of Method Detection Limit (MDL)

The method detection limits were determined by analyzing samples containing low concentration of analytes that provided a peak at a height of signal-to-noise ratio equal to 3. The MDL value was obtained from triplicate analyses. The experimental procedure can be described as follows:

3.11.1 <u>Blank sample</u> was prepared by using the extraction procedures described in step 3.9.1–3.9.13.

3.11.2 <u>Spiked sample</u> was prepared by spiking standards mixture solution into 5.00 g milk sample and extracted in the same way as blank sample.

3.11.3   The blank and spiked samples were analyzed under the optimum LC-ESI-MS conditions. The peak signals of each compound were measured from the chromatograms.

3.11.4   The MDL of each compound was obtained from the concentration that gave peak height at 3 times over the baseline.

3.12   The Study of Standard Calibration Curves

The experimental procedures to study the calibration curves of standard SAs, TCs, and PYR can be described as follows:

3.12.1   The concentration of standards mixture in acetonitrile solution: 1.00, 3.00, 5.00, 10.0, 50.0, 100, 150, 200, 250, and 300 ppb were injected respectively into LC-ESI-MS under the optimum conditions. Each concentration level was analyzed in duplicate.

3.12.2   The relationship between concentrations and peak area of each compound were plotted.

3.12.3   The results of this study were reported in form of slope, intercept, and correlation coefficient (R^2) values of each compound.

3.13   The Study of Matrix-Matched Calibration Curves

The matrix solution (blank solution) was prepared following the extraction procedures in section 3.9 (3.9.1-3.9.13). The experimental procedures to study the matrix-matched calibration curve of standard SAs, TCs, and PYR can be described as follows:

3.13.1   The mixture of all standards solution at concentration level 1.00, 3.00, 5.00, 10.0, 50.0, 100, 150, 200, 250, and 300 ppb were prepared using the blank milk extract as diluting solvent instead of acetonitrile.

3.13.2   Each concentration level was injected respectively into LC-ESI-MS under the optimum conditions and analyzed in duplicate.

3.13.3   The relationship between concentrations and peak area of each compound were plotted.

3.13.4 The results of this study were reported in form of slope, intercept, and correlation coefficient (R^2) values of each compound.

3.14 The Study of Method Precision

In this study, precision of the method was divided into two categories according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH): repeatability or intra-assay precision, and intermediate precision [83]. Intra-assay precision was obtained by repeatedly analyzing aliquots of a homogeneous sample in the same day. Intermediate precision referred to the results from the same laboratory and equipment, but performed on different days.

The study of method precision was carried out by extraction of spiked milk sample at two concentration levels, MQL and 5-fold MQL, as shown in Table 3.5.

For intra-assay precision study, the extraction at each concentration level was consecutively repeated six times within the same day and the extraction at each concentration level was repeated on two different days for intermediate precision study.

Table 3.5 The concentration of standards solution at MQL and 5-fold MQL levels.

No.	Compounds	Concentration level (ppb)	
		MQL	5-fold MQL
1	Oxetetracycline	1.00	5.00
2	Tetracycline	2.00	10.0
3	Sulfadiazine	1.00	5.00
4	Sulfathiazole	4.00	20.0
5	Pyrimethamine	1.00	5.00
6	Chlortetracycline	9.00	45.0
7	Sulfamethazine	5.00	25.0
8	Sulfamethoxypyridazine	3.00	15.0
9	Sulfamethoxazole	3.00	15.0
10	Sulfadimethoxine	1.00	5.00

The experimental procedures for the study of method precision can be described as follows:

3.14.1 Intra-Assay Precision

3.14.1.1 <u>At MQL level</u>, six spiked samples were prepared by pipetting a standards mixture solution at MQL concentration level into 5.00 g each of milk sample.

3.14.1.2 Each spiked sample in step 3.14.1.1 was extracted following the procedures in step 3.9.1-3.9.13.

3.14.1.3 The analyte sample was injected respectively into LC-ESI-MS under the optimum conditions and analyzed in duplicate.

3.14.1.4 The final concentration of each compound was calculated using the linear equation and showed the recovery as a percentage of the original spiked concentration.

3.14.1.5 The intra-assay precision of this method was calculated and reported in form of percent relative standard deviation (%RSD) of each compound.

3.14.1.6 At 5-fold MQL level, six spiked samples were prepared by spiking a standards mixture solution at 5-MQL concentration level to 5.00 g of milk aliquot.

3.14.1.7 The extraction and calculation were similar to the step 3.14.1.2 - 3.14.1.5.

3.14.2   Intermediate Precision

Similar to the procedure described in section 3.14.1, the extraction at MQL and 5-fold MQL concentration levels were repeated on another one different days. The summary of the results are reported as percent recoveries and percent relative standard deviations (%RSD) of each compound.

3.15   The Study of Method Accuracy

The accuracy was performed by spiking the standards mixture solution into milk sample at two concentration levels, MQL and 5-fold MQL, and extracted following the extraction procedures described in section 3.9 on two different days. The accuracy of the method was determined by the mean of the percentage recovery of each compound.

3.16   The Study of Method Robustness

Robustness related to the capacity of a method to remain unaffected by small but deliberated variations introduced in method parameters. The method relevant factors were identified as shown in Table 3.6. These factors were varied around the values in the method to reflect changes likely to arise in different test

environments.   In order to study the simultaneous variation of these factors, the robustness testing was tested using a Plackett-Burman experimental design.   The experimental plans and variation of each factor are illustrated in Table 3.7.

Table 3.6 Experimental factors studied in the robustness testing.

No	Factors		Normal conditions		Varied conditions
1	pH of sample solution	(A)	pH 4.5	(a)	No adjustment
2	Time for vortex	(B)	2 min	(b)	1 min
3	Time for centrifugation	(C)	15 min	(c)	10 min
4	Concentration of acetic acid in 5% methanol	(D)	2 %	(d)	3 %
5	Concentration of NH_4OH in 5% methanol	(E)	2 %	(e)	3 %
6	Time for SPE air-dried	(F)	5 min	(f)	3 min
7	Evaporation temperature	(G)	33 °C	(g)	40 °C

Table 3.7 Experimental plans using a Plackett-Burman experimental design for robustness test involving seven factors described in Table 3.6.

Factors	Experimental number							
	1	2	3	4	5	6	7	8
1	A	A	A	A	*a*	*a*	*a*	*a*
2	B	B	*b*	*b*	B	B	*b*	*b*
3	C	*c*	C	*c*	C	*c*	C	*c*
4	D	D	*d*	*d*	*d*	*d*	D	D
5	E	*e*	E	*e*	*e*	E	*e*	E
6	F	*f*	*f*	F	F	*f*	*f*	F
7	G	*g*	*g*	G	*g*	G	G	*g*
Observed result	s	t	u	v	w	x	y	z

   The experimental procedures for the study of method robustness can be described as follows:

3.16.1 Eight spiked samples were prepared by adding a mixture of standard solution at 5-fold MQL into 5.00 g milk sample.

3.16.2 Each spiked sample in step 3.16.1 was extracted following the procedures described in section 3.9, but the seven extraction factors were changed in each experiment following the experimental plans as illustrated in Table 3.7.

3.16.3 The analyte sample was injected respectively into LC-ESI-MS under the optimum conditions and analyzed in duplicate.

3.16.4   The final concentrations of each compound were calculated using the linear regression from the matrix-matched calibration curves and reported on the percent recoveries of each compound.

3.16.5   The different of a particular factor (D_x) is estimated by subtracting the mean of the results obtained with the factors of the varied value, from the mean of the results obtained at the normal value.

For example, the different of factor **1** (pH of sample solution) was calculated as follows:

$$D_1 \quad = \quad \frac{(s+t+u+v)}{4} - \frac{(w+x+y+z)}{4}$$

3.16.6   The absolute different values of each factor, $|D_x|$, were compared with normal procedure using statistical student *t*-test at 95% confidence level.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The Optimum Instrumental Analysis Conditions

From the experimental conditions, the results of optimum instrumental analysis conditions can be described as follows:

4.1.1 Optimization of ESI Parameters

4.1.1.1 The Selection of Mobile Phase Type and Fragmentor Voltage

In ESI mode, the selection of mobile phase is of paramount importance because it must offer an acceptable compromise between analyte ionizations and efficient desolvation of charged species in the MS detector. Because the ability to produce ions by ESI depends on the solution chemistry, ionization processes occurs directly from solution. The choice of solvent, therefore, has an effect on the ESI responses.

Among the various types of mobile phases studied, 10 mM ammoniumformate buffer and 1 mM oxalic acid showed increasing in sensitivity better than the others. All compounds exhibited the protonated ion, $[M+H]^+$, as the predominant ions. To establish the optimum mobile phase type for the analysis of SAs, TCs, and PYR, the response intensity of each compounds versus the mobile phase types are shown in Figure 4.1. It can be remarked that the maximum sensitivities of all compounds are observed when using 1 mM oxalic acid: acetonitrile (50:50, v/v) as the mobile phase.

The fragmentor voltage is an important parameter in ESI mode, and should be considered. Fragmentor voltage is applied to the exit of the capillary and affected the fragmentation of sample ions. In general, the optimal fragmentor voltage is compound dependent. As shown in Figure 4.1, the fragmentor voltage at 130 V provides maximum sensitivities for all compounds with positive ESI under all

conditions applied except PYR. At 130 V, the studied compounds show the responses higher than the other voltages of about 31.75%.

Therefore, to compromise for sufficient sensitivity and moderate fragmentation of most compounds, the fragmentor voltage was set at 130 V used 1 mM oxalic acid: acetonitrile (50:50, v/v) as mobile phase for ESI-MS parameters.
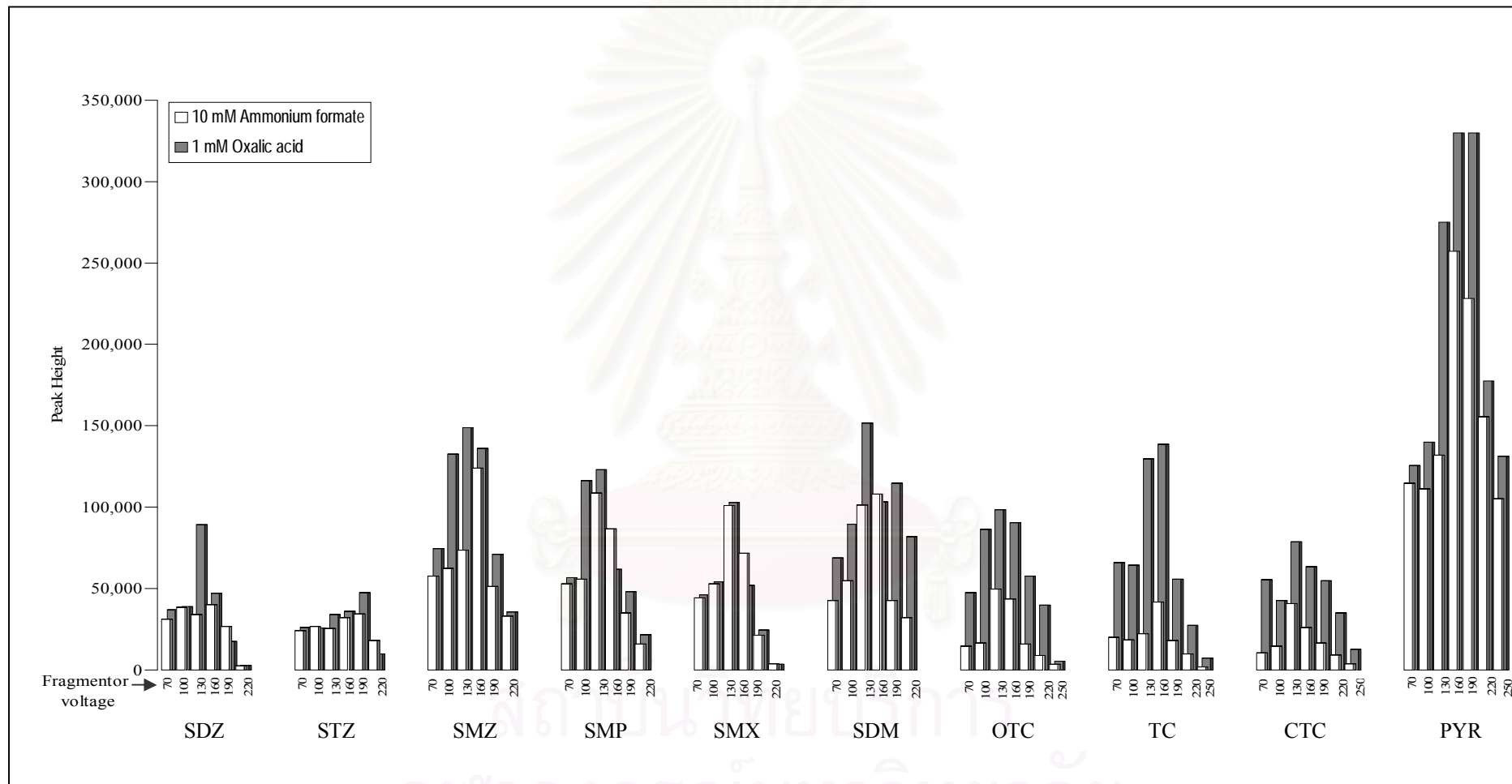
Figure 4.1 The effect of mobile phase types (1 mM oxalic acid and 10 mM ammoniumformate buffer) and fragmentor voltages on the sensitivity of SAs, TCs, and PYR at concentration 5.00 ppm.

4.1.1.2 The Selection of Capillary Voltage

The capillary voltage is applied to the entrance of the capillary that is connected to the spray chamber and influences the transmission efficiency of the ions through the capillary sampling orifice.

By varying the capillary voltages, the optimum value for the analysis of SAs, TCs, and PYR in positive ion mode using 1 mM oxalic acid: acetonitrile (50:50, v/v) as the mobile phase was determined to be 3,000 V as illustrated in Figure 4.2. At this capillary voltage, all analyte ions can be passed through the capillary and provide good sensitivities. This voltage does not exceed the instrument at operation range too long. The optimum voltage is dependent on the ions generated and charged aerosol characteristics.
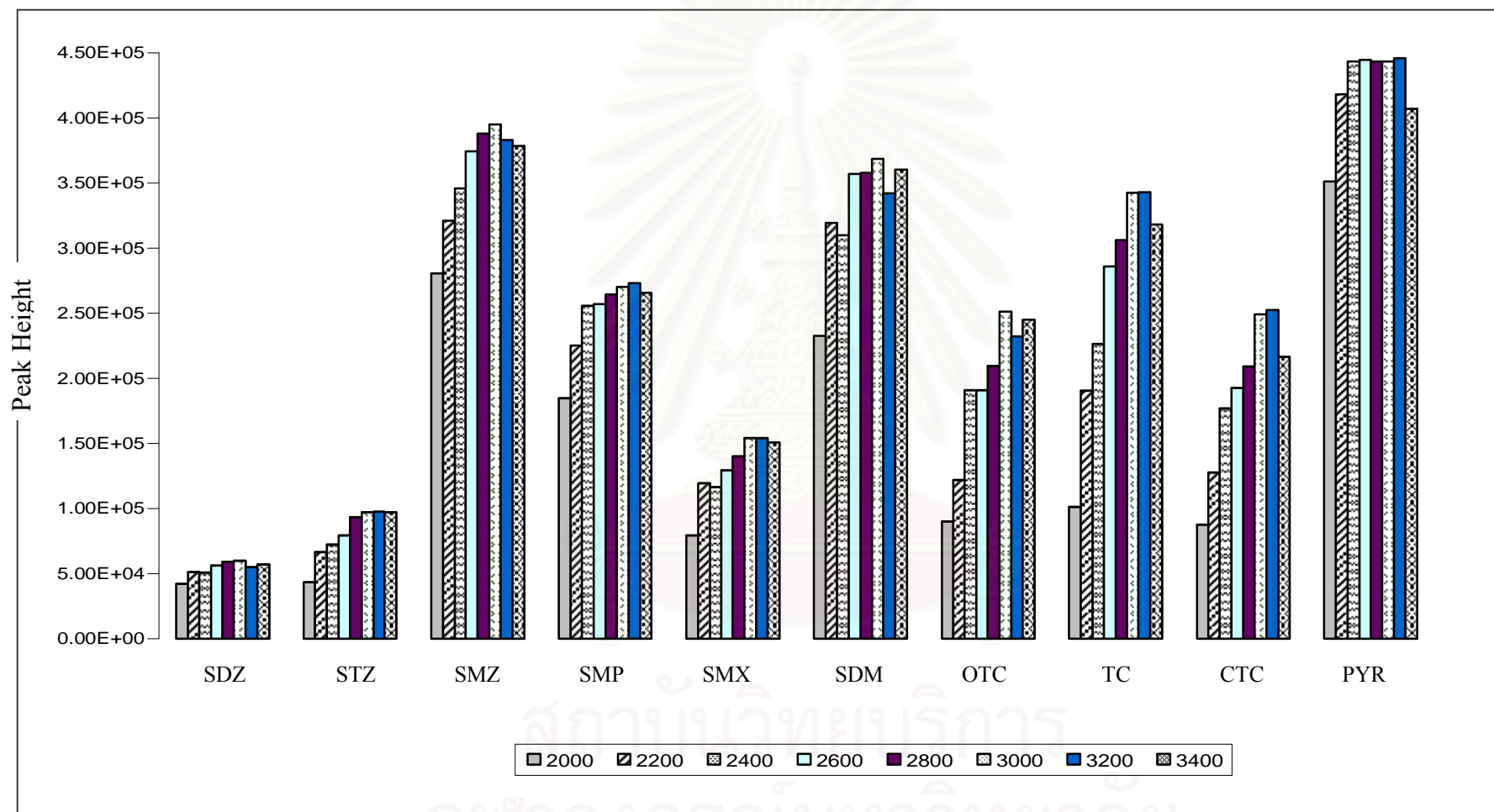
Figure 4.2 The effect of capillary voltages (2,000-3,400 V) on the sensitivity of SAs, TCs, and PYR at concentration 5.00 ppm using 1 mM oxalic acid: acetonitrile (50:50, v/v) as the mobile phase.

Based on the experimental results, the optimum ESI conditions for the analysis of SAs, TCs, and PYR are concluded in Table 4.1.

Table 4.1 The optimum ESI-MS conditions for the analysis of SAs, TCs, and PYR.

ESI parameters	Conditions
Detection mode	Positive ion detection
Mobile phase	1 mM oxalic acid: acetonitrile (50:50, v/v)
Drying gas flow	10.0 L/min
Drying gas temperature	300 ˚C
Nebulizer gas pressure	35 psi
Capillary voltage	3,000 V
Fragmentor voltage	130 V

4.1.2   Optimization of APCI Parameters

4.1.2.1   The Selection of Mobile Phase Type and Fragmentor Voltage

Similarly in ESI mode, three different types of mobile phase and variations of fragmentor voltages were tested for APCI optimization in order to obtain a good sensitivity of all compounds. To establish the optimum mobile phase types for the analysis of SAs, TCs, and PYR, the intensity of studied compounds versus mobile phase types are shown in Figure 4.3.

The better sensitivities of most compounds were achieved by using 1 mM oxalic acid: acetonitrile as the mobile phase under the studied conditions. As considered in fragmentor voltage, most of the analytes good sensitivities and structural information is at 130 V.
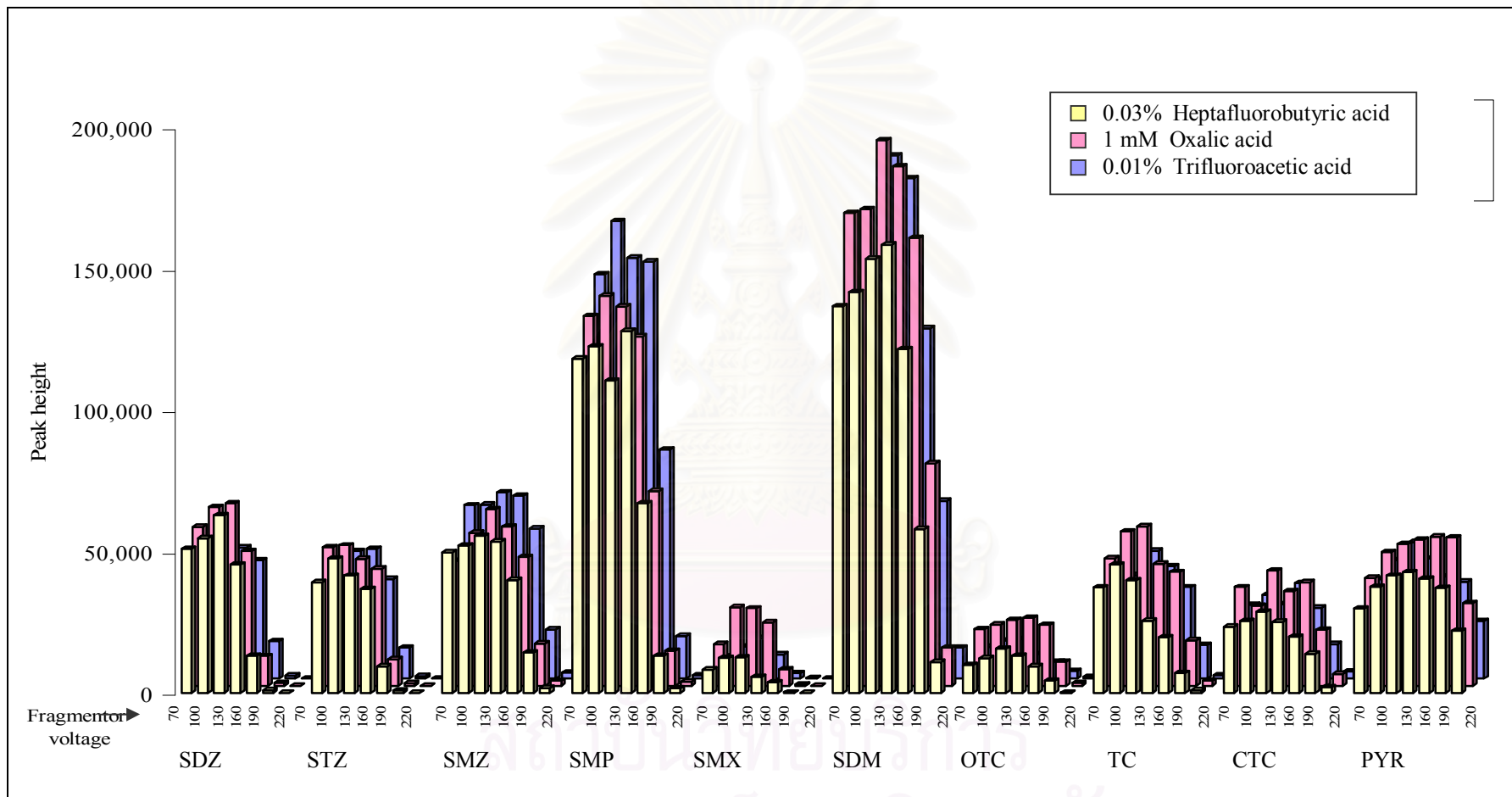
Figure 4.3   The effect of mobile phase types (0.03% heptafluorobutyric acid, 1 mM oxalic acid and 0.01% trifluoroacetic acid)

and fragmentor voltages on the sensitivities of SAs, TCs, and PYR at concentration 5.00 ppm.

4.1.3   Comparison of ESI and APCI

Because the studied compounds can be detected by both ESI and APCI, the sensitivities of both ionization techniques in positive ion mode are compared as shown in Figure 4.4.

From the results, ESI provided greater sensitivity of all compounds than APCI. It is possible that the high vaporizer temperature when using APCI may cause the decomposition of analyte compounds before transfer into mass analyzer, resulting in the lower sensitivities. On the contrary, the mechanism of ESI is a charging of droplets containing analyte and the formation of ions as the charge density on the surface of a droplet increases as the desolvation progresses. The sample can be directly ionized in the liquid phase at quasi-ambient temperature; in agreement with the nature of studied compounds that are slightly polar compounds and intrinsically charged in mobile phase solution. This leads to minimizing the decomposition of analyte compounds and allows it to be detected with a high sensitivity using ESI interface. Furthermore, ESI is selected as the ionization technique due to its ruggedness, ease of use and low maintenance.

Therefore, ESI mode is used for the analysis of SAs, TCs, and PYR residues in milk sample throughout this study.
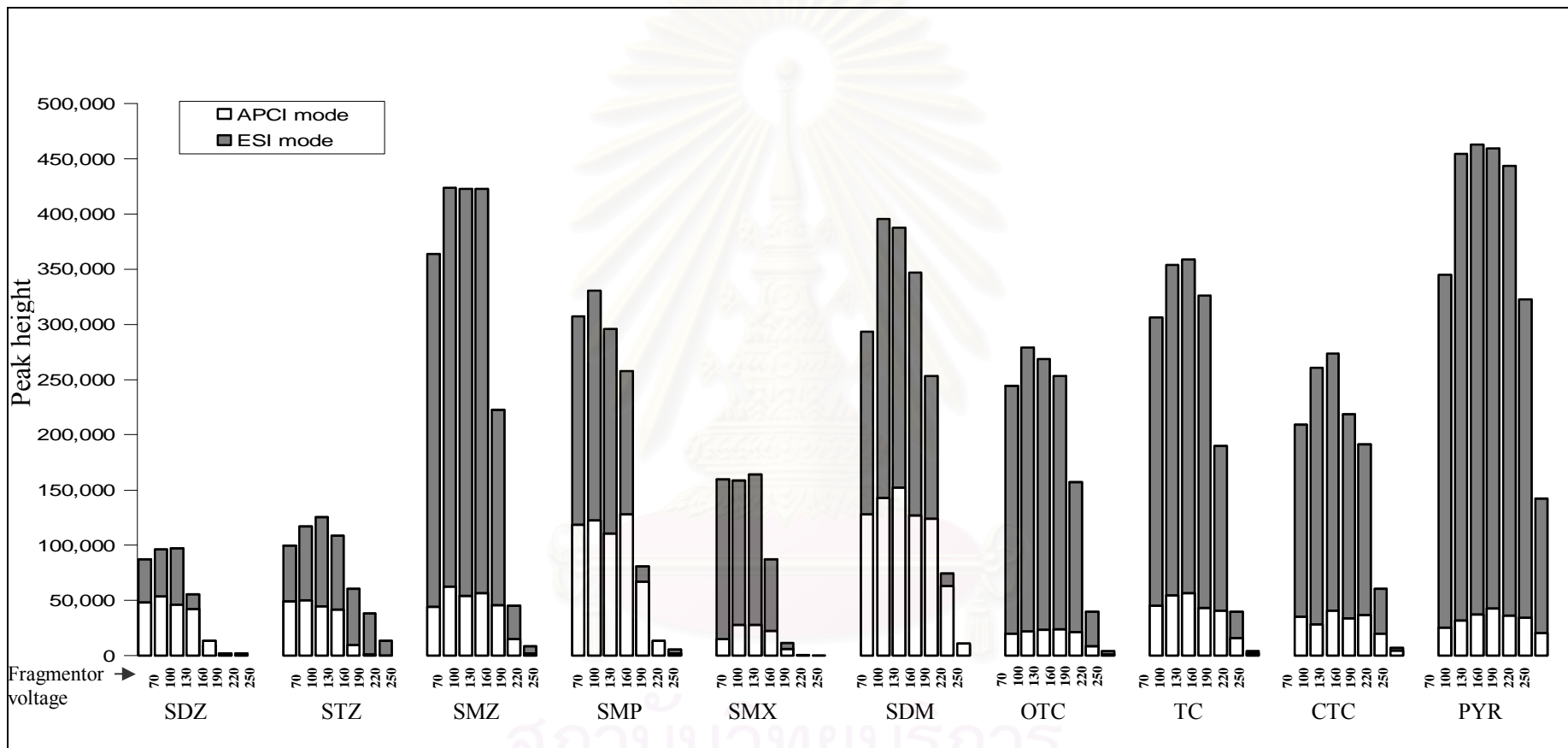
Figure 4.4 Comparison of the sensitivities of SAs, TCs, and PYR at 5.00 ppm between APCI and ESI mode by FIA. 1 mM oxalic acid:
acetonitrile (50:50, v/v) used as the mobile phase.

Based on the above results, the optimum MS conditions for the analysis of SAs, TCs, and PYR throughout this research are concluded in Table 4.2.

Table 4.2 The optimum MS conditions used for the analysis of SAs, TCs, and PYR..

Parameters	Conditions
Ionization	**ESI**, positive ion detection
Mobile phase	1 mM oxalic acid: acetonitrile (50:50, v/v)
Nebulizer gas pressure	Nitrogen, 35 psi
Drying gas flow	Nitrogen, 10.0 L/min
Drying gas temperature	300 ˚C
Capillary voltage	3,000 V
Fragmentor voltage	130 V

4.2 Mass Spectra of Sulfonamides, Tetracyclines, and Pyrimethamine

ESI is the soft-ionization technique and provides the molecular ions as the predominant ions. At suitable fragmentor voltage, common fragmentation ions are observed and the mass spectral data can be used to determine each class of compound for unequivocal identification.

4.2.1 Sulfonamides

From the full-scan MS spectra, all SAs exhibited the protonated molecular ion, $[M+H]^+$, as the base peak that is defined as 100 % relative abundance. The general fragmentation pathway proposed for sulfonamide group is shown in Figure 4.5.
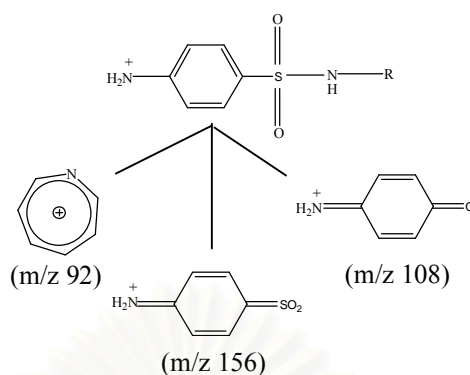
Figure 4.5 Fragmentation pathway of protonated sulfonamide.

The CID mass spectra of $[M+H]^+$ generated a series of group-specific ions at m/z 156, 108, and 92, as used for identifying SAs in complex matrices. The product ion at m/z 156 is the *p*-aminobenzenesulfonic acid moiety generated from $[M-RNH_2]^+$, while m/z 108 and 92 corresponding to $[M-RNH_2-SO]^+$ and $[M-RNH_2-SO_2]^+$ fragment, respectively.

However, SAs such as SMZ and SMP provide the other fragment ions that are used as ions characteristic of these compounds. From the molecular structure, SMZ produced the ion at m/z 124 corresponding to $[RNH_2+2H]^+$ as same as in SMP which m/z 126 is match to $[RNH_2+2H]^+$.

4.2.2 Tetracyclines

For TC, CTC, and OTC, the $[M+H]^+$ ions are detected as the base peak with a relative abundance of 100%. Their mass spectra showed fragment ions generated by loss of NH_3 from the carboxyamide group and subsequent loss of H_2O for those TCs processing a hydroxyl group at C_6 position, as shown in Figure 4.6. Both of these losses agree with the findings of this compound group.

	R_1	R_2	R_3	R_4
TC	H	CH_3	OH	H
CTC	Cl	CH_3	OH	H
OTC	H	CH_3	OH	OH
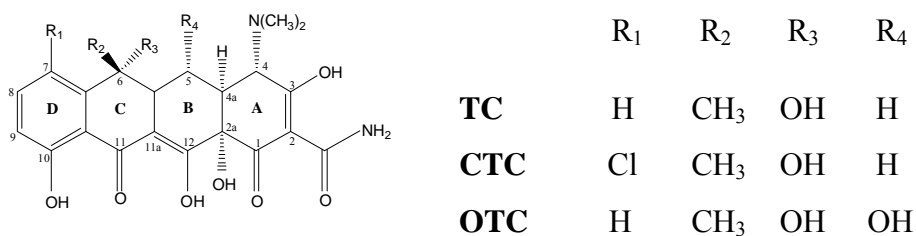
Figure 4.6 Chemical structure of tetracycline group.

From the mass spectra of tetracyclines group, the $[M+H-NH_3]^+$ ion corresponds to m/z 428, 462, and 444 for TC, CTC, and OTC, respectively. The $[M+H-NH_3-H_2O]^+$ provides the ions at m/z 410, 444, and 426 for TC, CTC, and OTC, respectively. Because the chlorine atom has two isotopes, ^{35}Cl and ^{37}Cl, the presence of two different mass units of chloride ion will be detected. Therefore, the occurrence of one chlorine atom in CTC structure is clearly evident by the relative abundance of the $[M+H]^+$ (m/z 479) and $[M+2+H]^+$ (m/z 481) ions reflect the abundance of ^{35}Cl and ^{37}Cl with a ratio of 3:1. Therefore, the product ion at m/z 481 can be used to confirm the existing of CTC in milk samples.

4.2.3 Pyrimethamine
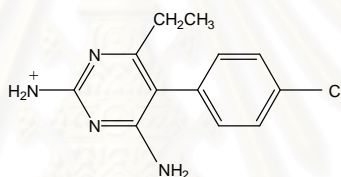
Figure 4.7 Chemical structure protonated pyrimethamine.

The positive ion mass spectra of PYR show a protonated molecular ion, $[M+H]^+$, at m/z 249 as the base peak (100% relative abundance) but it does not provide the other fragment ions at fragmentor voltage 130 V. However, the occurrence of one chlorine atom in PYR, the $[M+2+H]^+$ ion corresponds to m/z 251 can be used to confirm the residue of PYR in milk samples.

Table 4.3 Summary of mass ions characteristic and their relative abundance of SAs, TCs, and PYR obtained from FIA-ESI-MS in positive ion detection mode, fragmentor voltage 130 V.

Compounds	MW.	Compound-specific ions, m/z *(relative abundance)*	
		Target ions [M+H]$^+$	Fragment ions
SDZ	250	251 *(100)*	156 *(36.6)*, 108 *(13.4)*, 92 *(12.2)*
STZ	255	256 *(100)*	156 *(57.8)*, 108 *(45.8)*, 92 *(56.2)*
SMZ	278	279 *(100)*	156 *(13.2)*, 124 *(32.0)*, 108 *(8.4)*, 92 *(7.6)*
SMP	280	281 *(100)*	156 *(63.9)*, 126 *(65.5)*, 108 *(43.2)*, 92 *(42.5)*
SMX	253	254 *(100)*	156 *(31.4)*, 108 *(12.5)*, 92 *(10.6)*
SDM	310	311 *(100)*	156 *(25.5)*, 108 *(14.3)*, 92 *(4.3)*
TC	444	445 *(100)*	428 *(43.8)*, 410 *(47.4)*
CTC	478	479 *(100)*	462 *(12.4)*, 444 *(9.0)*, 481* *(39.5)*
OTC	460	461 *(100)*	444 *(42.4)*, 426 *(33.7)*
PYR	248	249 *(100)*	233 *(12.4)*, 251* *(35.4)*

* ^{37}Cl isotope.

4.3 Ions Characteristic

In this study, the MS detection operated in the positive ion mode and analyte compounds were detected using SIM mode. SIM mode is potentially selective identification and quantitation for trace analysis. From their mass spectra, the two characteristic ions of each compound are selected for use in SIM mode as illustrated in Table 4.4. Since SIM mode is used for identification and simultaneous

quantitation of analyte compounds, and it improved the detection sensitivity (signal-to-noise ratio) to permit a suitable quantitation analysis in the biological matrices.

The predominant ion of each compound is the protonated molecular ion, $[M+H]^+$ because it showed the base peak ion under the given conditions. The qualifying ions of each compound were selected from their fragment ions, corresponding to the molecular structure as described in section 4.2. Moreover, $[M+2+H]^+$ ion can be used as the qualifying ion for CTC and PYR, because these compounds have one chlorine atom in their structure that has the isotope ratio of ^{35}Cl and ^{37}Cl at 3:1.

Table 4.4 Chemical structures and selected ions characteristic used for the LC-ESI-MS of the SAs, TCs, and PYR analysis.
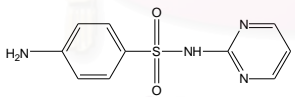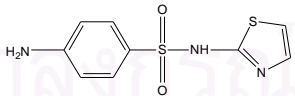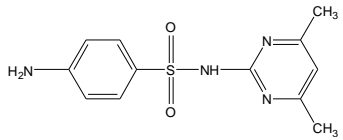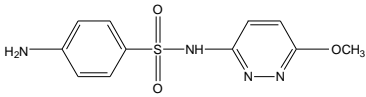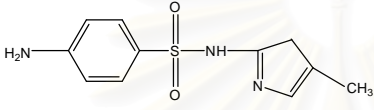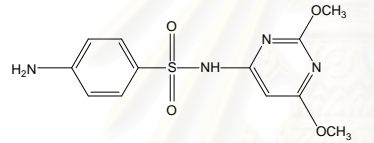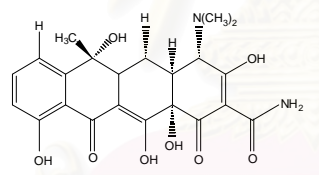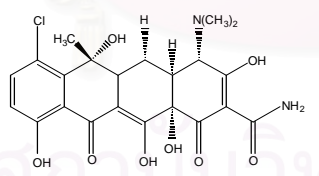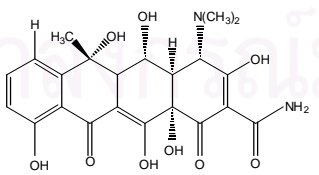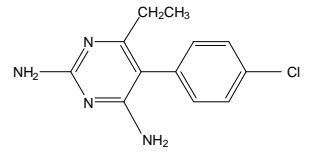
Compounds	Molecular structures	MW.	Ions characteristic, m/z (relative abundance)		
			Target ions $[M+H]^+$	Qualify ions	
SDZ		250	251 (100)	156 (36.6)	$[M-RNH_2]^+$
STZ		255	256 (100)	108 (45.8)	$[M-RNH_2-SO]^+$
SMZ		278	279 (100)	124 (32.0)	$[RNH_2+2H]^+$
SMP		280	281 (100)	126 (65.5)	$[RNH_2+2H]^+$

Table 4.4 *(continued).*

Compounds	Molecular structures	MW.	Ions characteristic, m/z *(relative abundance)*		
			Target ions $[M+H]^+$	Qualify ions	
SMX		253	254 *(100)*	156 *(31.4)*	$[M-RNH_2]^+$
SDM		310	311 *(100)*	156 *(25.5)*	$[M-RNH_2]^+$
TC		444	445 *(100)*	410 *(47.4)*	$[M+H-NH_3-H_2O]^+$
CTC		478	479 *(100)*	481* *(39.5)*	$[M+H+2]^+$
OTC		460	461 *(100)*	426 *(33.7)*	$[M+H-NH_3-H_2O]^+$
PYR		248	249 *(100)*	251* *(35.4)*	$[M+2+H]^+$

* ^{37}Cl isotope.

4.4 HPLC Method Development

To optimize the HPLC conditions for the separation of all studied compounds, the mixture of standards solution was injected to HPLC using the ESI-MS as a detector. Preliminarily, the mixture solution was tested on first column using water-acetonitrile (1mM oxalic acid) as the mobile phase. By varying the mobile strength, it could not separate three pairs of SMZ-SMP, SMX-OTC and TC-SDM at baseline resolution and long time analysis (about 45 minute). Moreover, the high background noise and drifting of baseline were observed, which made the sensitivities of analyte peaks very low and difficult for integration.

However, SymmetryShield™ RP18 column was used to improve the separation of all analytes. After adjusting the proportion of mobile phase in gradient mode, it could separate all of the analytes in 12 minutes. The result of separation conditions is shown in Figure 4.8 in the order of analytes elution. The optimum conditions of HPLC for the separation of all analytes are reported in Table 4.5. The effects of background noise and baseline drift were lower than obtained from the first column, which leads to the increase in sensitivity of all analytes. This result can be described as the SymmetryShield™ RP18 column embedded with polar group, which literally shields the silica's residual surface silanols from highly basic analytes. The embedded polar group, which close proximity to the silica surface, further reduces the acidity of the surface silanol, and then exhibits less retention of basic compounds than conventional C_{18} columns.

Table 4.5 The HPLC chromatographic conditions using SymmetryShield™ RP18 column for the analysis of SAs, TCs, and PYR.

Parameters	Conditions
Analytical column	SymmetryShield™ RP18 column (2.1×150 mm, i.d. 3.5 µm)
Mobile phase	A: 1 mM oxalic acid in 5 % acetonitrle-water B: 1 mM oxalic acid in 95 % acetonitrle-water

Gradient program

Time (min)	% B
0.0	8.0
2.0	8.0
5.0	45.0
9.0	47.0
12.0	55.0
15.0	95.0
21.0	95.0
21.0	8.0
32.0	8.0

Parameters	Conditions
Flow rate	0.30 mL/min
Injection volume	5 µL
Column temperature	Left 35 ℃ and Right 40 ℃
Detector	Mass spectrometry detector using the MS parameters as illustrated in Table 4.2 and 4.3
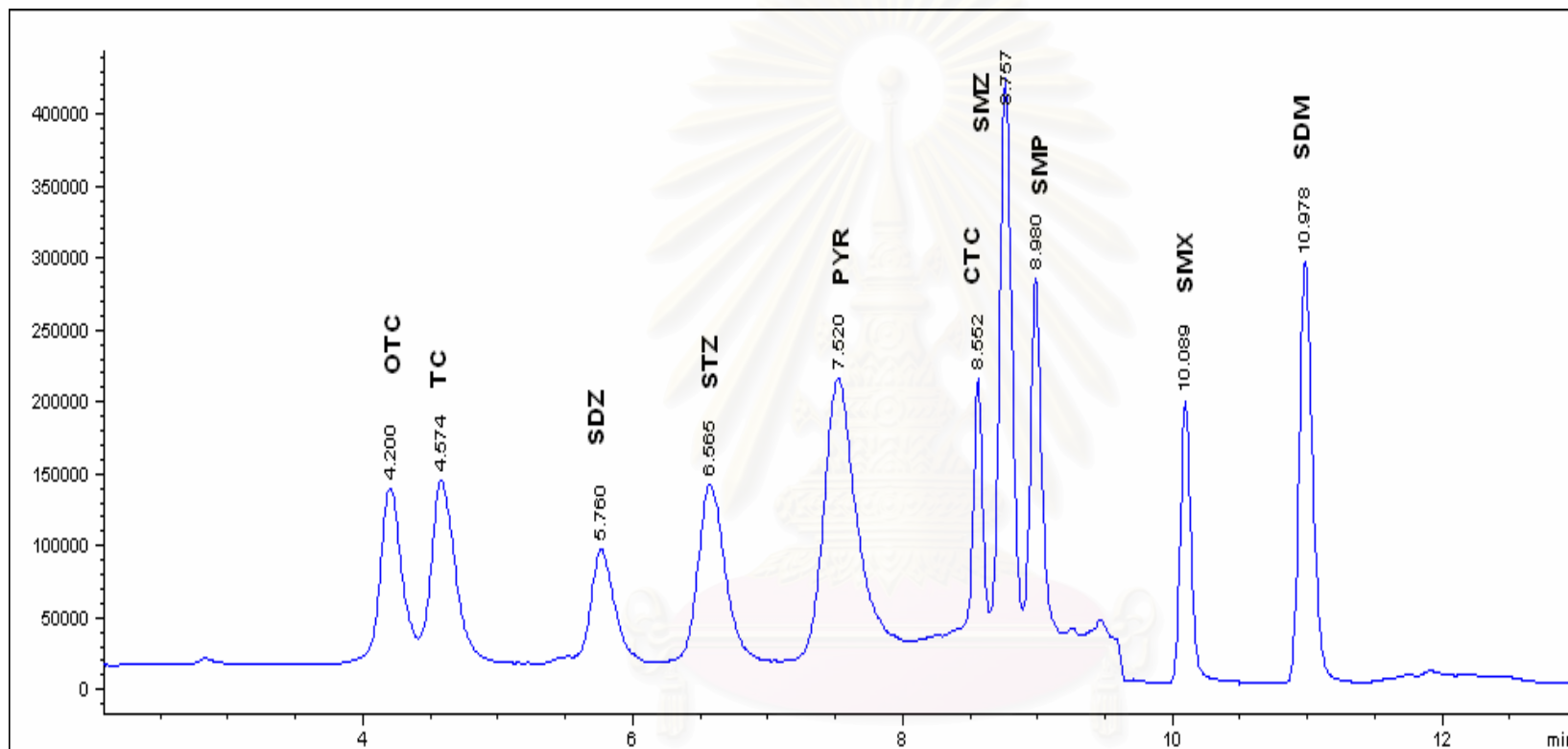
Figure 4.8 Total-ion chromatogram of SIM mode of a standards mixture 500 ppb using the optimum LC-ESI-MS conditions
listed in Table 4.5.
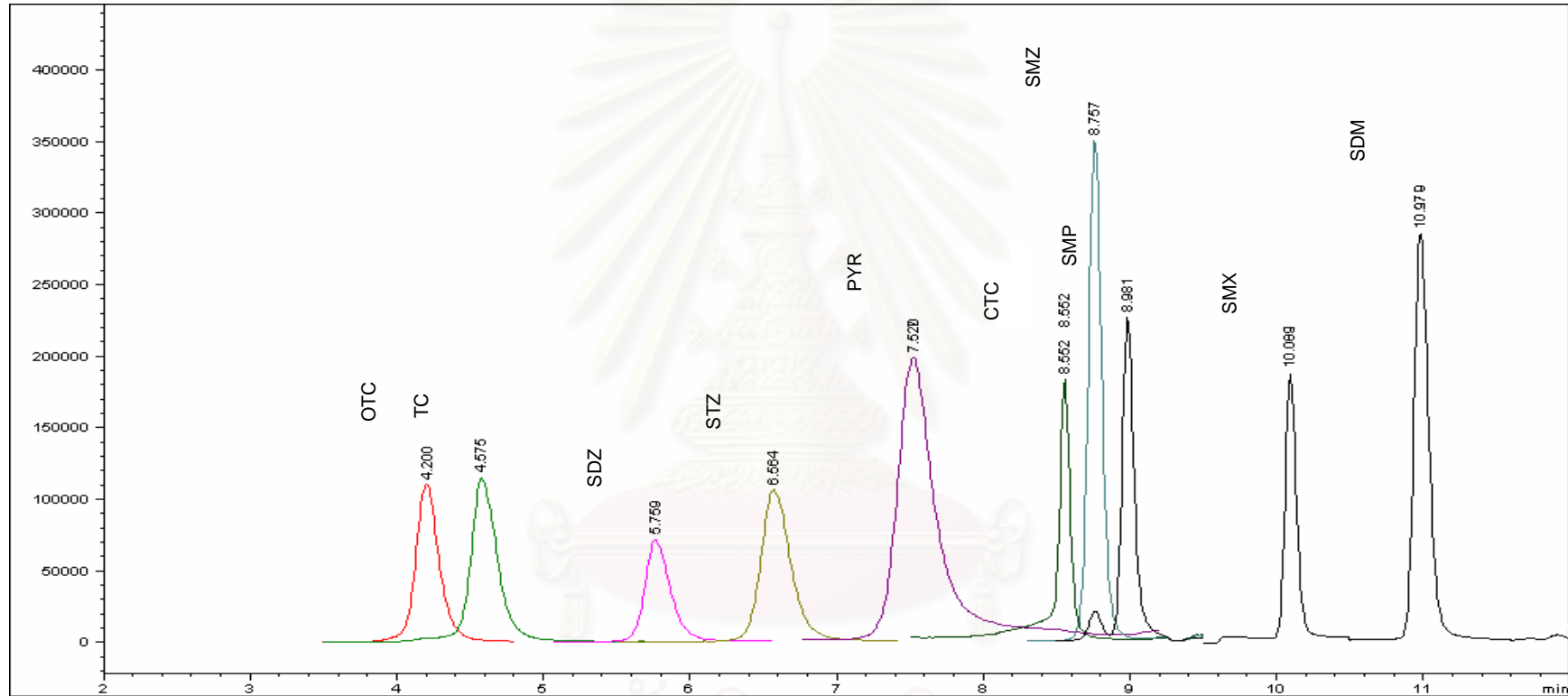
Figure 4.9 Extracted-ion chromatogram of SIM mode of a standards mixture 500 ppb using the optimum LC-ESI-MS conditions listed in Table 4.5.

Although CTC, SMZ, and SMP could not be separated completely at baseline resolution, the extraction of ion from the ions characteristic of each compound allowed these analyte peaks to be completely separated and easily analyzed quantitatively analysis. The extracted ion chromatogram of SAs, TCs, and PYR is shown in Figure 4.9.

To increase the signals detectability of each compound, ion signals were acquired using the time-scheduled multiple-ion SIM mode as listed in Table 4.6.

Table 4.6 Time-scheduled multiple-ion SIM conditions for analysis SAs, TCs, and PYR.

No.	Compounds	Retention window (min)	Selected ion monitoring (m/z)
1	Oxytetracycline		**461**[a] *(100)*, 426[b] *(33.7)*
2	Tetracycline		**445**[a] *(100)*, 410[b] *(47.4)*
3	Sulfadiazine		**251**[a] *(100)*, 156[b] *(36.6)*
4	Sulfathiazole	2.0 – 9.40	**256**[a] *(100)*, 108[b] *(45.8)*
5	Pyrimethamine		**249**[a] *(100)*, 251[b] *(35.4)*
6	Chlortetracycline		**479**[a] *(100)*, 481[b] *(39.5)*
7	Sulfamethazine		**279**[a] *(100)*, 124[b] *(32.0)*
8	Sulfamethoxypyridazine		**281**[a] *(100)*, 126[b] *(65.5)*
9	Sulfamethoxazole	9.40 – 10.40	**254**[a] *(100)*, 156[b] *(31.4)*
10	Sulfadimethoxine	10.40 – 12.00	**311**[a] *(100)*, 156[b] *(25.2)*

[a] Base peak ion.   [b] Qualify ion.

By using time-scheduled multiple-ion SIM mode, the mass range for ions detection was narrowed and the detection cycle was increased that lead to increases in detectability and signal sensitivity.

4.5   Result of LC-MS Selectivity Evaluation

The selectivity of an analytical method was a parameter used to differentiate and identify each of the analyte components. The analyte compounds were identified by chromatographic retention times, and the ion characteristics of each compound can be used to identify the existence of analytes.

In this study, the selectivity of LC-MS can be determined using the retention time (t_R) and ion characteristics (m/z) of each analyte peak under the optimum conditions in Table 4.5. Table 4.7 shows the results of selectivity data in the order of analytes elution.

Table 4.7 Retention times (t_R) and ions characteristics (m/z) of standard SAs, TCs, and PYR in acetonitrile solution (n=10).

No.	Compounds	Retention time (min)	Selected ions monitoring (m/z)
1	Oxytetracycline	4.195 ± 0.009	**461**[a] *(100)*, 426[b] *(33.7)*
2	Tetracycline	4.580 ± 0.010	**445**[a] *(100)*, 410[b] *(47.4)*
3	Sulfadiazine	5.761 ± 0.008	**251**[a] *(100)*, 156[b] *(36.6)*
4	Sulfathiazole	6.563 ± 0.009	**256**[a] *(100)*, 108[b] *(45.8)*
5	Pyrimethamine	7.515 ± 0.019	**249**[a] *(100)*, 251[b] *(35.4)*
6	Chlortetracycline	8.551 ± 0.003	**479**[a] *(100)*, 481[b] *(39.5)*
7	Sulfamethazine	8.756 ± 0.003	**279**[a] *(100)*, 124[b] *(32.0)*
8	Sulfamethoxypyridazine	8.979 ± 0.003	**281**[a] *(100)*, 126[b] *(65.5)*
9	Sulfamethoxazole	10.088 ± 0.002	**254**[a] *(100)*, 156[b] *(31.4)*
10	Sulfadimethoxine	10.977 ± 0.002	**311**[a] *(100)*, 156[b] *(25.2)*

[a] Base peak ion.   [b] Qualify ion.

From Table 4.6, the main ion of each compound **(boldface)** was the protonated molecular ion, $[M+H]^+$, as shown the base peak. The other ions were the qualifying ions, which were obtained from the fragmentation profiles of each compound, and were used to confirm the qualitative identification of suspected analyte peaks.

Because the analyte compounds have significantly different molecular weights, the mass spectra information allows a greater confidence in compound identification and allows for distinguishing each analyte from the matrix interference. Moreover, the ability of LC-MS to extract ion chromatogram from mass spectral data, allows the co-eluting components to not be the cause of ion-suppression in the ion information process, therefore, a good selectivity of each analyte was observed.
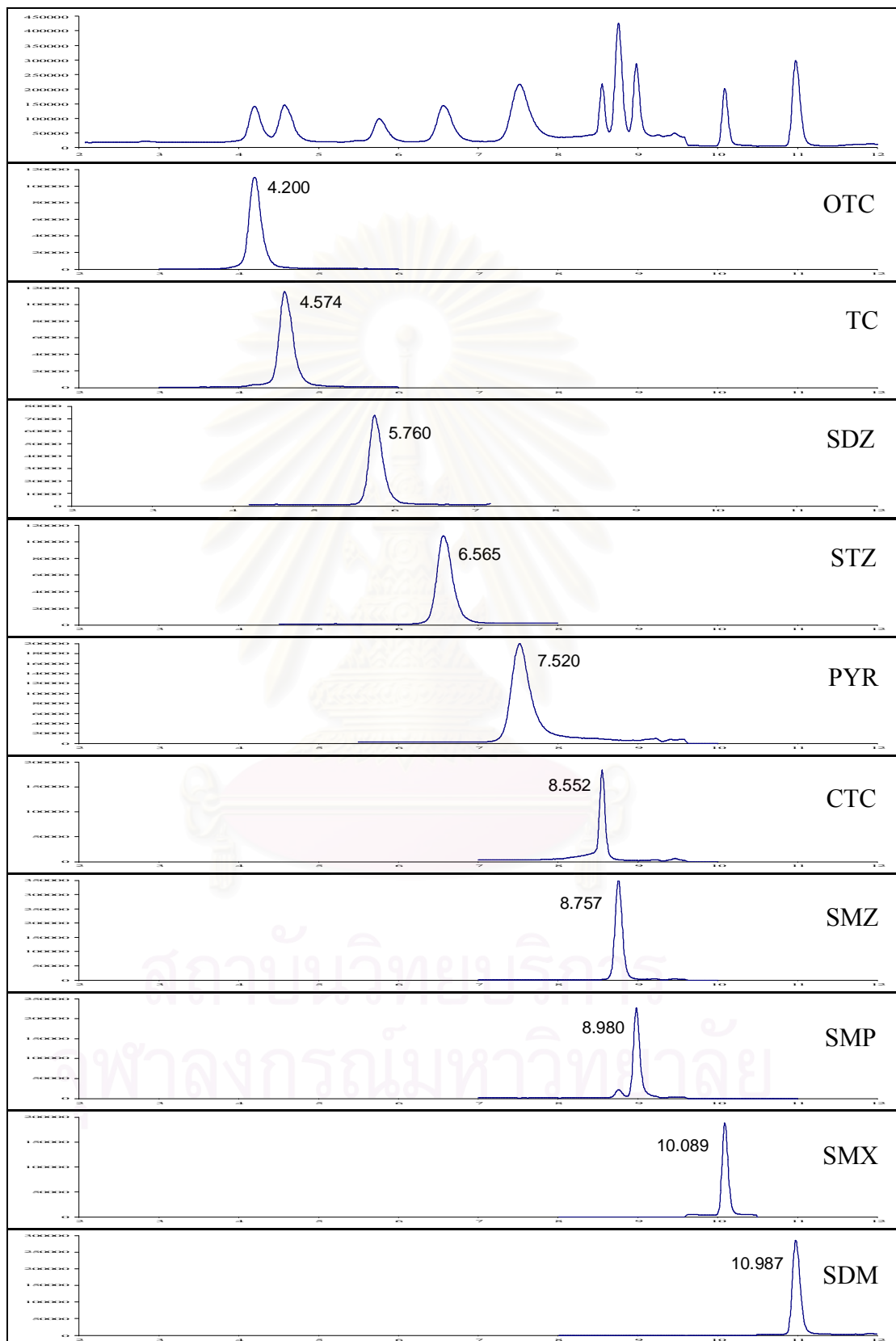
Figure 4.10 Extracted-ion chromatogram of SIM mode of standard SAs, TCs, and

PYR in acetonitrile solution at 100 ppb

4.6 Result of The Extraction Methods Comparison

The difference in chemical properties such as solubility and pK_a of SAs, TCs, and PYR made it very difficult to simultaneous extract these compounds from milk matrices. From the selected methods, each method was speciated in groups of drug residues in matrices. These methods were tested to simultaneous extract the studied compounds. Comparisons of the efficiency of extraction methods discuss in percent recoveries of each compound are summarized in Table 4.8-4.9.

Table 4.8 Percent recoveries of spiked milk sample at spiking level 10.0 ppb using different extraction methods.

No.	Com-pounds	% Recovery							
		Method 1	Method 2	Method 3	Method 4	Method 5	Method 6	Method 7	Method 8
1	TC	ND	25.99	70.18	ND	31.06	26.85	38.43	478.06
2	CTC	ND	31.47	69.12	9.06	36.48	34.90	61.12	ND
3	OTC	ND	19.79	76.11	ND	40.84	32.34	65.13	314.54
4	SDZ	27.06	ND	74.85	ND	36.75	ND	63.65	231.20
5	STZ	11.58	ND	55.21	ND	29.63	ND	52.47	110.59
6	SMZ	44.75	25.28	51.45	ND	35.23	30.42	44.15	116.21
7	SMP	37.18	25.13	48.78	ND	33.15	29.56	35.93	114.90
8	SMX	25.66	21.86	35.80	ND	33.39	21.39	54.63	123.86
9	SDM	43.10	26.35	41.87	1.69	21.37	23.50	42.49	154.41
10	PYR	40.94	31.83	52.78	ND	25.69	19.55	26.11	53.37

* ND = not detectable

Table 4.9 Percent recoveries of spiked milk sample at spiking level 20.0 ppb using different extraction methods.

No.	Compounds	% Recovery							
		Method 1	Method 2	Method 3	Method 4	Method 5	Method 6	Method 7	Method 8
1	TC	ND	32.25	95.93	ND	33.52	28.05	64.25	380.67
2	CTC	ND	32.43	90.42	4.93	45.17	30.96	82.87	ND
3	OTC	ND	31.44	97.11	ND	41.25	32.63	68.25	6.81
4	SDZ	23.54	ND	79.99	ND	39.53	3.24	63.52	203.98
5	STZ	4.54	ND	59.45	ND	30.29	4.46	60.25	93.87
6	SMZ	48.55	30.54	49.36	ND	42.38	30.88	62.23	201.72
7	SMP	34.71	29.90	38.50	ND	38.43	29.66	48.33	238.95
8	SMX	24.44	22.77	59.86	ND	32.43	26.12	52.99	205.28
9	SDM	45.44	30.94	59.63	1.60	25.07	26.97	37.35	166.31
10	PYR	47.32	44.86	75.59	ND	28.32	19.89	66.97	53.19

*ND = not detectable

%Recovery of all compounds was significantly differed in each extraction method. From the data obtained, it was found that method 3 showed the best compromise of the percent recoveries of all compounds more than the other methods. It is possible that Oasis[®] HLB cartridge is a polymer mixed-mode SPE that was designed to simultaneous retains both hydrophilic and hydrophobic compounds, including the studied compounds in this research. The Oasis[®] HLB cartridge does not contain silanol groups on the sorbents. This encourages metal ions to bind, which leads to the irreversible binding of the studied compounds, because TCs are

chemically preferred when forming metal complexes. Therefore, multi-residue extraction using this SPE sorbent helps to improve the recovery of all compounds.

Moreover, McIlvain buffer (sodium-EDTA buffer), was added to milk samples prior to chelate metals, which may be present in the solution or adsorbed on the surface of the sorbents. These reasons resulted in high percent recoveries of most analyte compounds observed.

However, low values of percent recoveries obtained from methods 1, 2, and 4-8 may cause the sorbent properties of each SPE, which can not simultaneously retain all compounds on the cartridge. Moreover, the extraction conditions used in those methods may provide the unsuitable form of the studied compounds, which can be retained on the sorbents. Many steps in extraction procedures can cause the interested compounds to be lost and percent recoveries to be low.

As a result, we will use method 3 as a basis for the extraction method for multi-residue extraction of SAs, TCs, and PYR in milk samples. The experimental procedure of the basis extraction method is described in Figure 4.11. However, some extraction procedures of the basis method were adapted to provide the best extraction efficiency.

Milk 5.00 g was placed in a plastic centrifuge tube

Added 2 mL of 20% trichloroacetic acid and the mixture was shaken

Added 20 mL of McIlvain buffer and the mixture was centrifuged at 3,800 rpm for 20 min

The supernatant was transferred to a Oasis® HLB cartridge which was previously preconditioned with 3 mL methanol and 2 mL of deionized water

After sample loading, the cartridge was washed with 2 mL of 5% methanol in water

The analytes were eluted with 3 mL of absolute methanol

The solvent was evaporated to dryness under a flow of nitrogen

Re-dissolved the residue with 1.00 mL of methanol and filtered through a 0.45 µm syringe filter for LC-ESI-MS analysis

Figure 4.11 Schematic of the extraction procedures used as a basis method for simultaneous extraction of SAs, TCs, and PYR in this study.

4.7 Extraction Method Development

From the results of the basis extraction method, the recoveries of the SAs, TCs, and PYR ranged from 35.80-76.11% at a spiking level of 10.0 ppb, and 38.50-97.11% at a spiking level of 20.0 ppb. The extraction efficiencies of TCs were within an acceptable range, but SAs and PYR were slightly low. Thus, these results are

caused from the SPE extraction steps. To improve the recovery of all compounds, the SPE extraction steps were considered.

4.7.1   Result of Base Modified SPE Wash Solvent

Generally, the SPE washing step is important in order to remove the interferences from the sorbents. If the suitable SPE wash solvent was used, a cleaner extract solution was achieved and percent recoveries were improved. In this study, a cleaner extract was obtained by simply manipulating the pH values of additional wash solvent. The results of the base modified wash solvent on the percent recoveries of each compound are illustrated in Table 4.10. The plot of the relationship between the percent recoveries of each compound and SPE wash solvents are shown in Figure 4.12-4.13.

Table 4.10 The results of base modified SPE wash solvent on the percent recoveries of SAs, TCs, and PYR at spiking level 10.0 and 20.0 ppb.

No.	Compounds	% Recovery			
		Without wash with 2% NH$_4$OH in 5% methanol		Wash with 2% NH$_4$OH in 5% methanol	
		Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb
1	TC	70.18	85.93	94.76	93.91
2	CTC	69.12	78.42	89.31	98.73
3	OTC	76.11	97.11	63.34	68.21
4	SDZ	74.85	79.99	33.81	45.01
5	STZ	55.21	59.45	83.43	85.56
6	SMZ	51.45	49.36	44.09	39.72
7	SMP	48.78	45.59	43.01	40.61
8	SMX	35.8	59.86	102.13	104.84
9	SDM	41.87	59.63	68.79	74.39
10	PYR	52.78	75.59	74.13	93.90
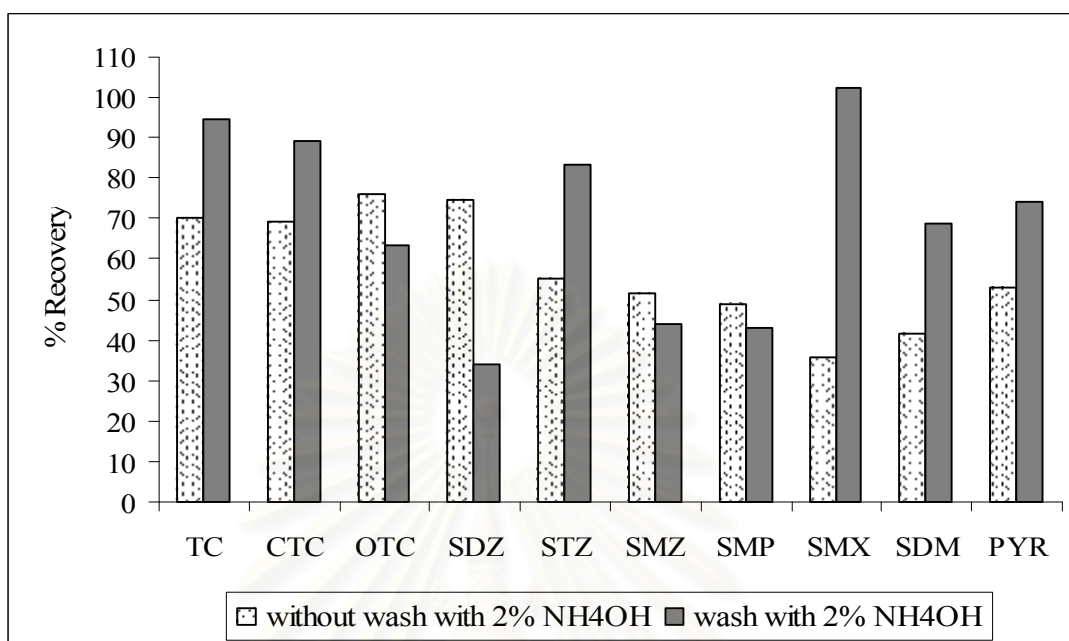
Figure 4.12 The plot of relationship between the percent recoveries of each compound and base modified SPE wash solvent at spiking level 10.0 ppb.

Figure 4.13 The plot of relationship between the percent recoveries of each compound and base modified SPE wash solvent at spiking level 20.0 ppb.

When 2% NH$_4$OH in 5% methanol was used in the SPE washing step, the recoveries were between 33-102% for six SAs, 63-94% for three TCs, and 74% for PYR at spiking level 10.0 ppb. At 20.0 ppb spiking level, the recoveries ranged from 45-104 % for SAs, 68-93 % for TCs, and 93 % for PYR. It can be seen from the data that the recoveries of all analytes increased except OTC, SDZ, SMZ, and SMP.

%Recoveries of all compounds increased when washed sequentially the SPE with 5% methanol followed by 2% NH$_4$OH in 5% methanol. This is because 5% methanol is used in removing neutral polar interferences, and base modified organic solvents can help to remove acidic polar interferences, that may be bound with analyte molecules and retained on the SPE packing. By wash the SPE cartridge in two steps, it helps to reduce matrix interferences more than just the basis method and increase the retention of analyte molecules. As a result, percent recovery of the extraction was improved.

4.7.2   Result of Sample Treatment

The recoveries of the studied compounds except OTC, SDZ, SMZ, and SMP were increased when using the base modified organic solvent in the SPE washing step. The recoveries of SDZ, SMZ, and SMP are usually low (less than 50%). Therefore, the preparation of the milk sample before being transferred to SPE cartridge was considered, because drug residues in milk may bind strongly with protein and can not be isolated by using 20 % trichloroacetic acid and vortex mixing. Sonication and homogenization of the milk samples coupled with acid protein-precipitation, may help to disturb the interaction of protein interferences-analytes molecule and improve the recovery of extraction.

The results of the sample treatment procedures on the percent recoveries of each compound are illustrated in Table 4.11. The plot of the relationship between the percent recoveries of each compound and sample treatment is shown in Figure 4.14-4.15.

Table 4.11 The results of sample treatment procedure on the percent recoveries of SAs, TCs, and PYR at spiking level 10.0 and 20.0 ppb.

No.	Compounds	% Recovery					
		Vortex		Sonication		Homogenization	
		Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb
1	TC	94.76	93.91	ND	38.68	ND	49.42
2	CTC	89.31	98.73	ND	68.82	ND	79.69
3	OTC	63.34	68.21	44.07	44.64	53.34	69.48
4	SDZ	33.81	45.01	ND	8.87	ND	36.21
5	STZ	83.43	85.56	ND	55.96	68.43	70.32
6	SMZ	44.09	39.72	37.75	55.18	68.73	65.47
7	SMP	43.01	40.61	36.10	49.40	43.66	46.64
8	SMX	102.13	104.84	23.20	40.35	17.40	54.74
9	SDM	68.79	74.39	42.63	58.76	48.65	61.17
10	PYR	74.13	93.90	66.99	68.48	60.71	64.03

* ND = not detectable

Figure 4.14 The plot of relationship between the percent recoveries of each compound and sample treatment procedures at spiking level 10.0 ppb.
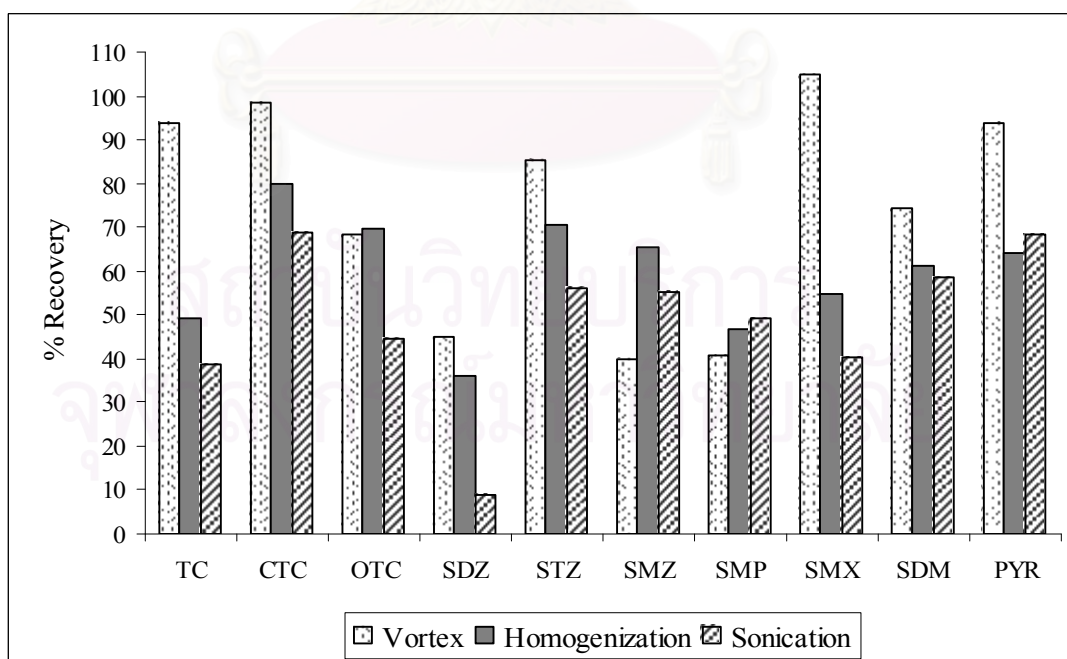
Figure 4.15 The plot of relationship between the percent recoveries of each compound and sample treatment procedures at spiking level 20.0 ppb.

When the milk sample was treated by homogenization, the recoveries of all studied compounds were greater than treatment by sonication except in PYR. The reason for this is the vibration of water molecules generates heat energy that causes an increase in temperature of the solution during sonication process. This results in the decomposition of the analyte compounds. However, the highest of the overall recoveries of all studied compounds were obtained from treatment by vortex mixer rather than homogenization. This is because the vortex is a mild condition for mixing milk samples and extraction of solvents. This procedure was simple to perform. Therefore, vortex mixing was used as the treatment procedure for milk samples throughout this study.

4.7.3 Result of Solvent Evaporation

The procedure required for removal of all solvent from the eluate to dryness was essential for drug residues analysis. A couple methods were studied because an unsuitable evaporation procedure can cause the loss of the studied compounds. This effect was studied by comparing the differences between rotary evaporation under vacuum system and nitrogen evaporation. The results of solvent evaporation procedure on the percent recoveries of each compound are illustrated in Table 4.12. The plot of relationship between the %recoveries of each compound and solvent evaporations are shown in Figure 4.16-4.17.

Table 4.12 The results of solvent evaporations on the percent recoveries of SAs, TCs, and PYR at spiking level 10.0 and 20.0 ppb.

No.	Compounds	% Recovery			
		Rotary evaporation at 33 °C		Nitrogen evaporation at 33 °C	
		Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb
1	TC	72.52	74.49	79.72	80.09
2	CTC	68.77	71.83	66.34	50.14
3	OTC	45.38	66.10	79.56	82.48
4	SDZ	35.91	34.35	23.39	21.67
5	STZ	54.00	60.35	67.90	69.45
6	SMZ	41.72	48.37	50.87	54.58
7	SMP	43.11	51.13	53.18	60.60
8	SMX	64.98	60.04	62.27	63.85
9	SDM	55.80	54.99	58.41	67.77
10	PYR	80.25	79.72	70.80	71.62
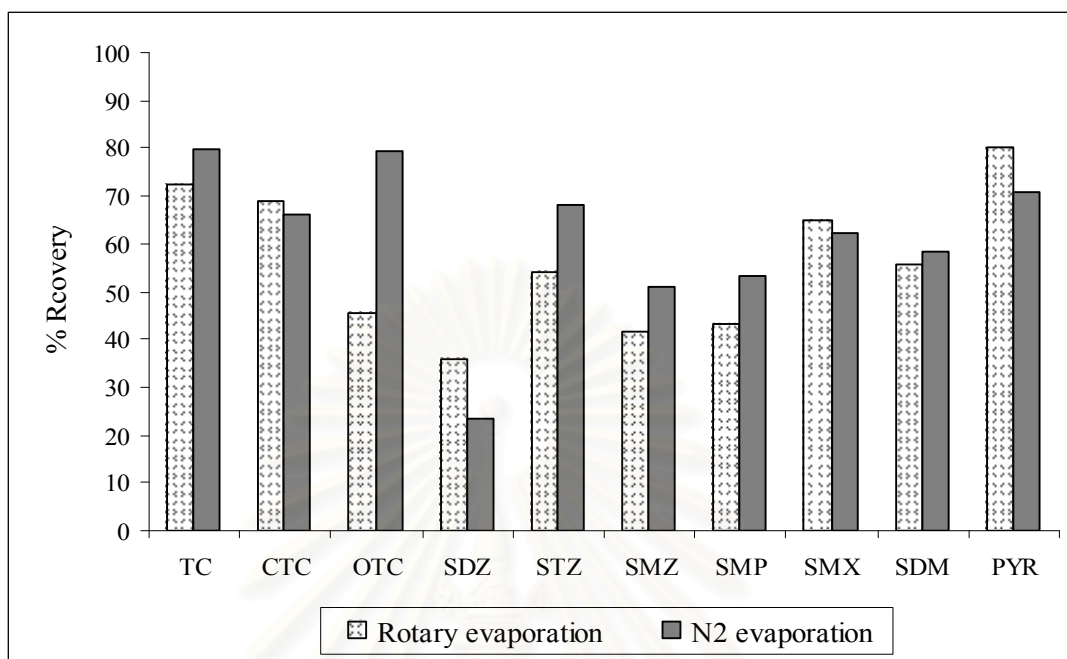
Figure 4.16 The plot of relationship between the percent recoveries of each compound and solvent evaporations at spiking level 10.0 ppb.
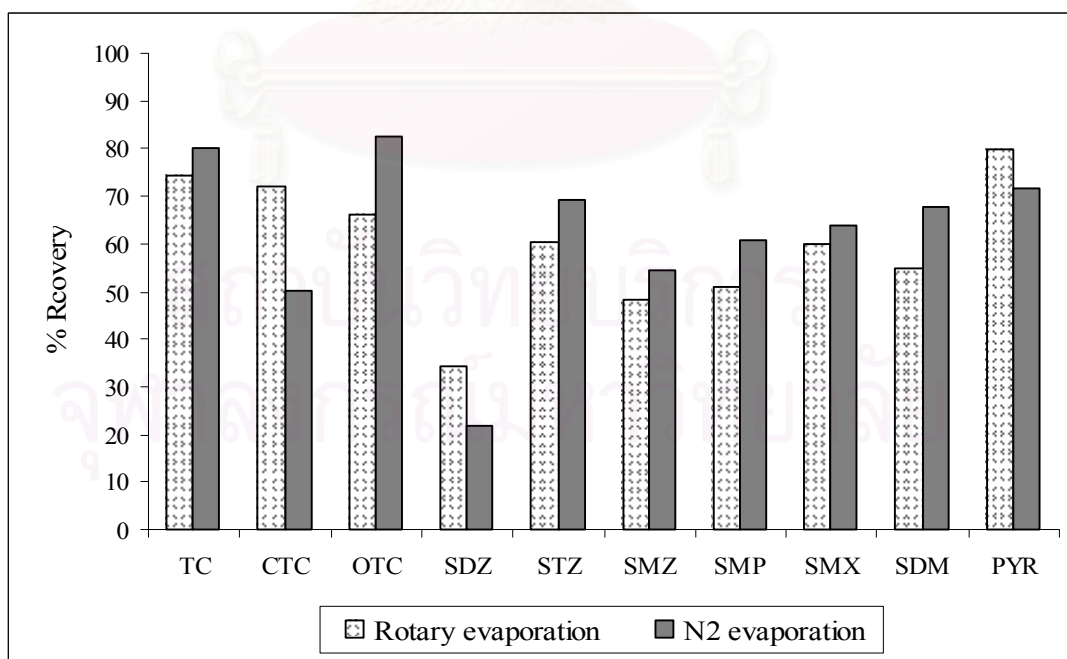
Figure 4.17 The plot of relationship between the percent recoveries of each compound and solvent evaporations at spiking level 20.0 ppb.

The results showed that solvent removal by nitrogen evaporation affected the loss of analyte compounds less than rotary evaporation. This is due to nitrogen evaporation is a mild condition and nitrogen gas does not react with the studied compounds, which would cause analytes to decompose. However, it takes a very long time for the solvent to dry and the high cost of nitrogen gas, makes it not suitable for use in routine analysis. Therefore, rotary evaporation under vacuum system at 33°C was selected for solvent removal throughout this study.

4.7.4 Result of Sample pH

Base modified SPE wash solvent can improve the recoveries of all studied compounds but the recoveries of SDZ, SMZ, and SMP are usually low (less than 50%). By considering the difference in chemical properties of SAs, TCs, and PYR, the pH of the sample solution prior to transfer to an Oasis® HLB cartridge may affect the retention of the analytes on SPE sorbents. The results of sample pH on the percent recoveries of each compound are illustrated in Table 4.13. The plot of relationship between the %recoveries of each compound and sample pH are shown in Figure 4.18-4.19.

Table 4.13 The results of sample pH on the percent recoveries of SAs, TCs, and PYR
at spiking level 10.0 and 20.0 ppb.

No.	Compounds	% Recovery			
		No adjusted pH		Adjusted to pH 4.0	
		Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb
1	TC	80.46	79.97	44.14	54.12
2	CTC	52.13	78.39	61.79	75.25
3	OTC	69.38	68.94	72.00	85.05
4	SDZ	31.59	39.20	34.93	24.69
5	STZ	57.31	60.43	70.61	68.88
6	SMZ	63.75	61.15	55.88	66.96
7	SMP	43.01	40.61	57.51	61.86
8	SMX	65.48	68.93	66.92	69.61
9	SDM	58.60	52.94	71.53	80.39
10	PYR	80.80	97.68	79.38	91.06
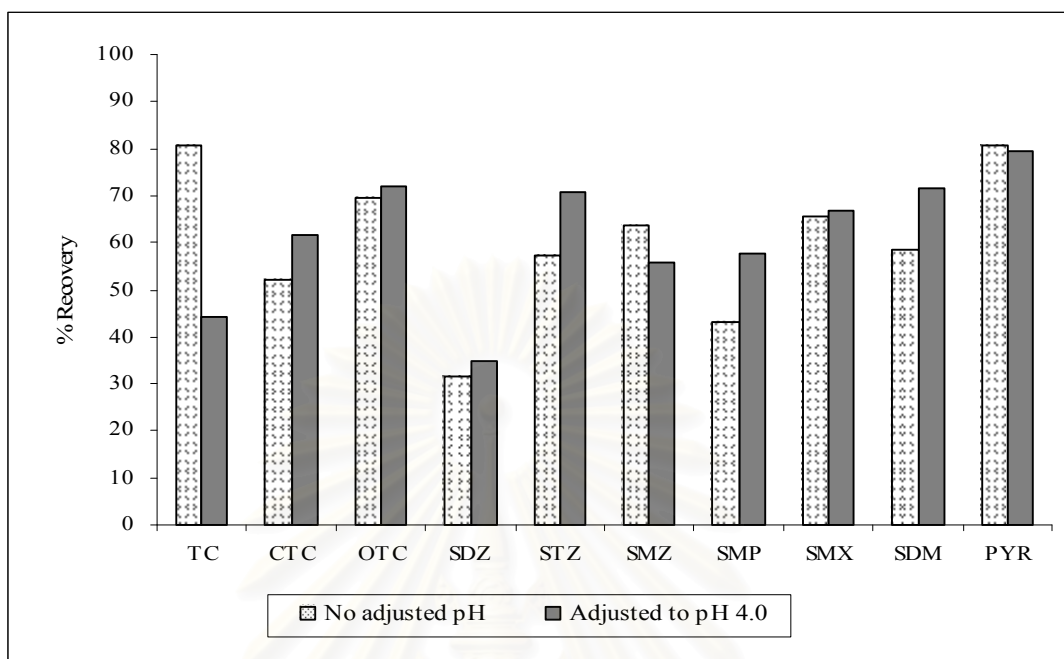
Figure 4.18 The plot of relationship between the percent recoveries of each compound and sample pH at spiking level 10.0 ppb.
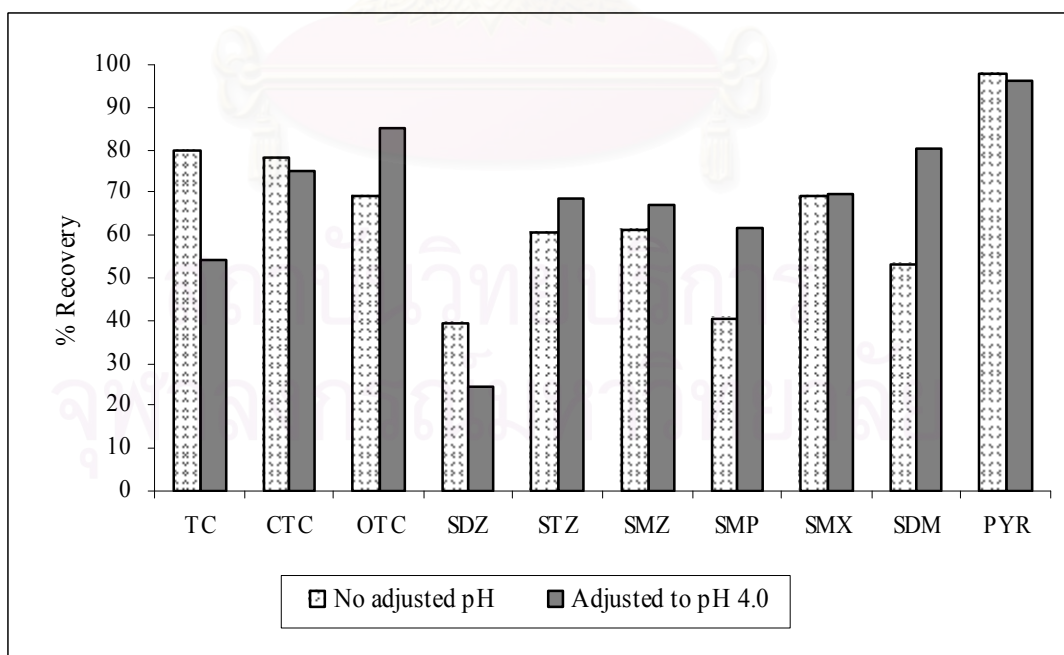
Figure 4.19 The plot of relationship between the percent recoveries of each compound and sample pH at spiking level 20.0 ppb.

As a result, overall recoveries of the studied compounds were improved especially for SMZ and SMP (recoveries >50%) when the pH of sample solution was adjusted to 4.0 prior to passing it through the Oasis® HLB cartridge. This was not the case for TC and SDZ. Without pH adjustment, the pH of the sample solution was around 3.6. Because pKa_1 of sulfonamides range between 5-8 and 2-2.5 of pKa_2, sulfonamides are positively charged at low pH due to the protonation of amino group in their structures and negatively charged at high pH. At pH ~4.0, sulfonamides are neutral and have reasonably high retention on the SPE packing, resulting in the high recovery of extraction achieved. Interestingly, the percent recovery of PYR showed no strong pH dependence.

4.7.5 Result of Acid Modified SPE Wash Solvent

Base and acid modified organic solvents were selected to remove the different matrix interferences. Base modified organic solvent was specified for eliminating acidic polar interferences while basic polar interferences can not be removed from the SPE sorbents. In this study, the additional acid modified organic solvent in SPE washing steps was considered just as in Section 4.7.1. The results of the acid modified wash solvent on the percent recoveries of each compound are illustrated in Table 4.14. The plot of relationship between the %recoveries of each compound and SPE wash solvents are shown in Figure 4.20-4.21.

Table 4.14 The results of acid modified wash solvent on the percent recoveries of
SAs, TCs, and PYR at spiking level 10.0 and 20.0 ppb.

No.	Compounds	% Recovery			
		Wash with 2% NH$_4$OH in 5% methanol		Wash with 2% Acetic acid in 5% methanol	
		Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb
1	TC	44.14	54.12	81.13	84.37
2	CTC	61.79	75.25	87.47	92.03
3	OTC	72.00	85.05	92.33	93.43
4	SDZ	34.93	24.69	60.98	67.34
5	STZ	70.61	68.88	62.88	68.81
6	SMZ	55.88	66.96	75.82	79.98
7	SMP	57.51	61.86	72.80	79.72
8	SMX	66.92	69.61	81.46	89.31
9	SDM	71.53	80.39	73.04	79.87
10	PYR	79.38	91.06	91.66	90.86
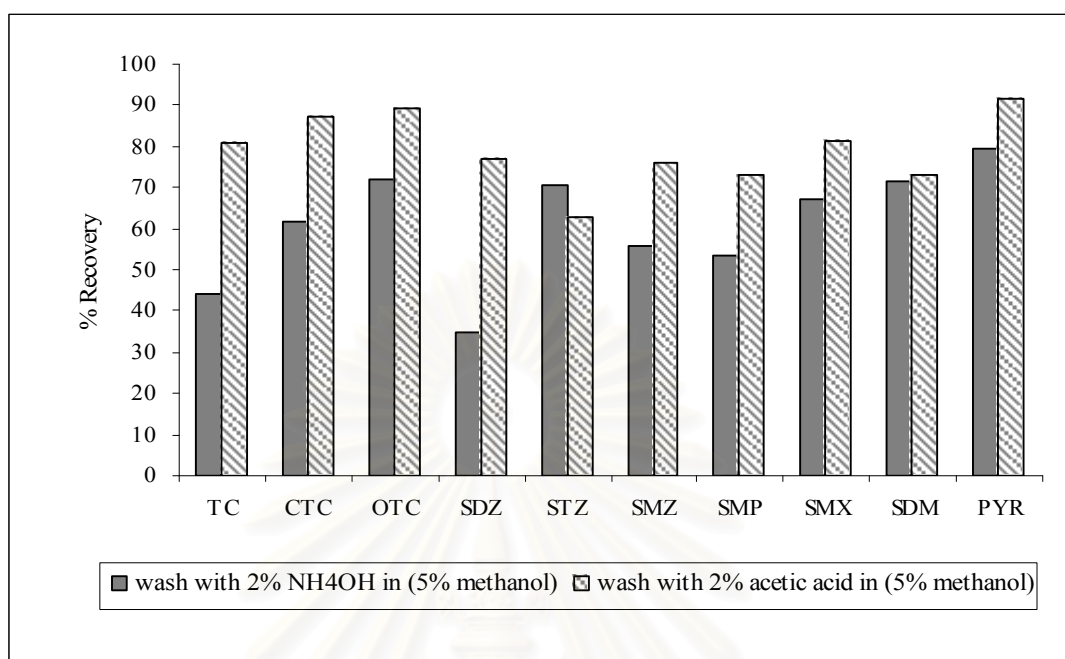
Figure 4.20 The plot of relationship between the percent recoveries of each compound and SPE wash solvents at spiking level 10.0 ppb.
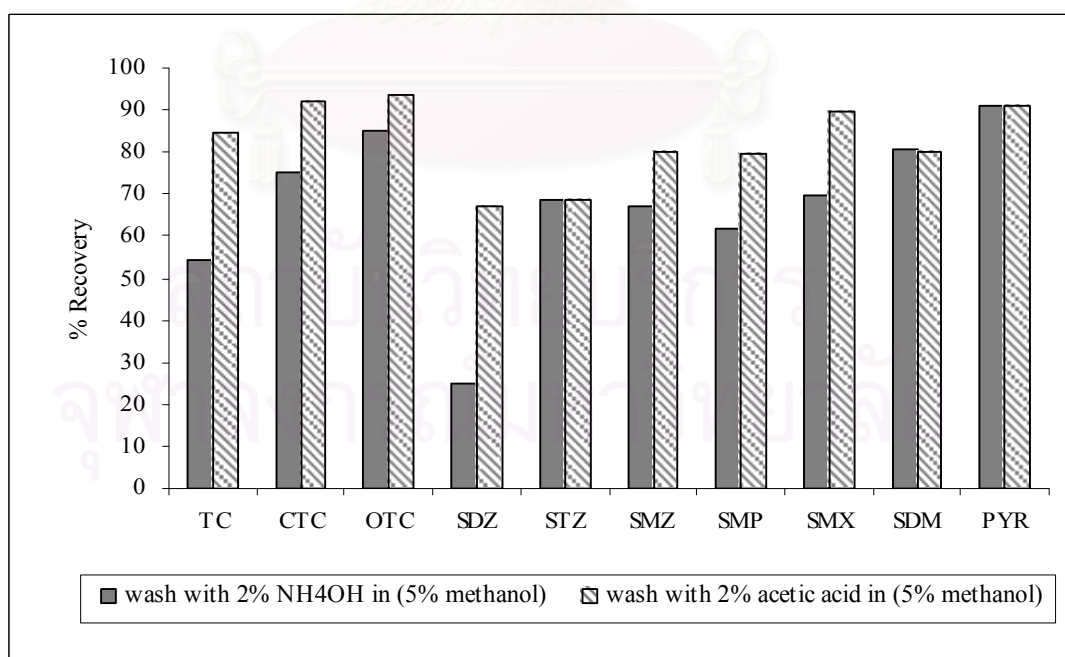
Figure 4.21 The plot of relationship between the percent recoveries of each compound and SPE wash solvents at spiking level 20.0 ppb.

It was found that when the SPE cartridge was sequentially washed with 5% methanol in water and 2% acetic acid in 5% methanol, overall recoveries of the analytes increased, especially SDZ (recovery > 60%). It is possible that acid modified organic solvent was more selective in removing both neutral and basic polar interferences such as protein that bound with analyte molecules and were retained on the SPE sorbents. Moreover, acetic acid is a weak acid that may not hydrolyze the analyte molecules when passing through the SPE cartridge. By was additionally with 2% acetic acid in 5% methanol, a cleaner extract was achieved and %recovery of the extraction was improved (Figure 4.20 to 4.22).
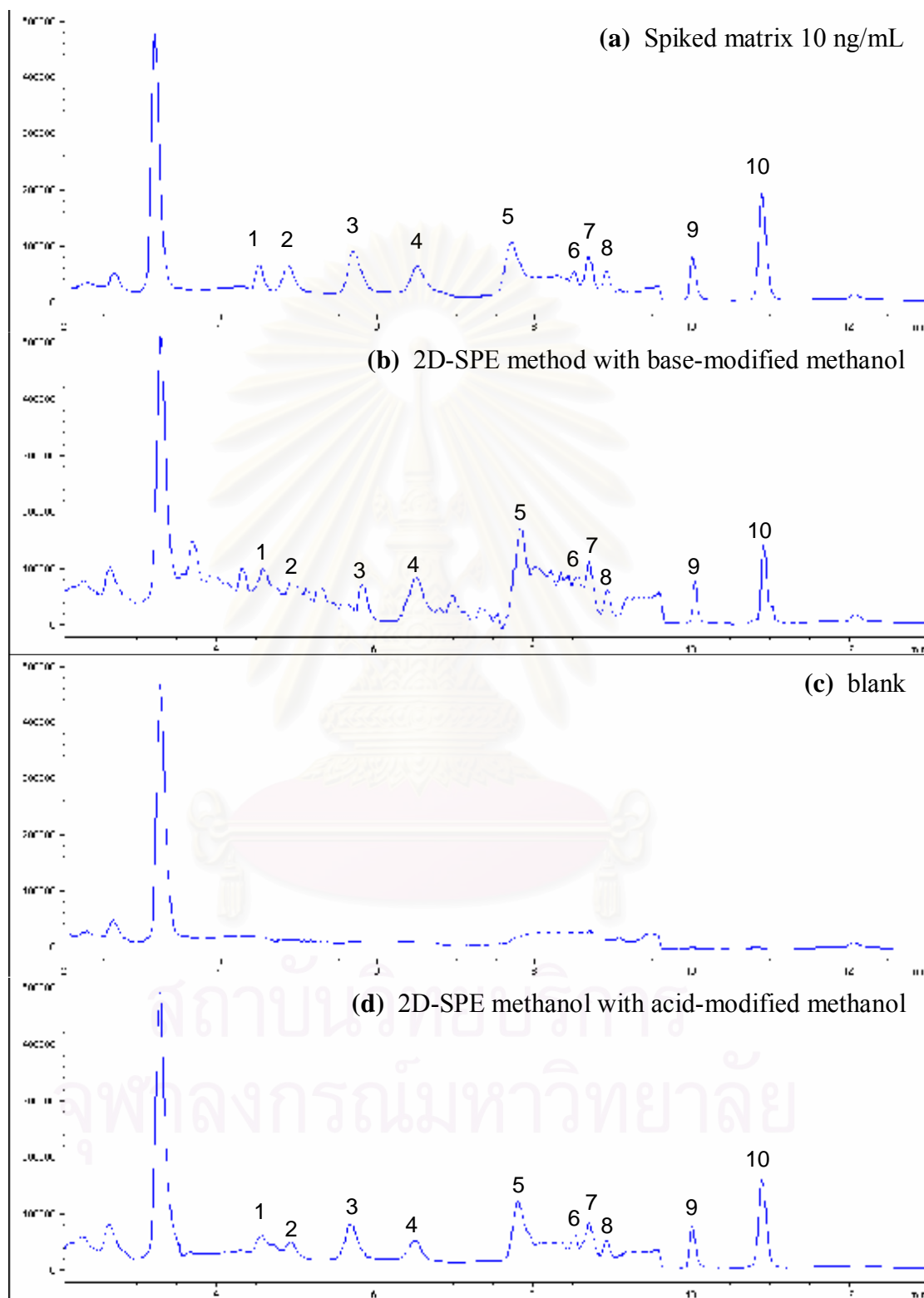
Figure 4.22 Total-ion SIM chromatograms of milk extracts at 10 ng/mL using LC-
ESI-MS conditions listed in Table 4.2, 4.3, and 4.5. 1: OTC, 2: TC, 3:
SDZ, 4: STZ, 5: PYR, 6: CTC, 7: SMZ, 8: SMP, 9: SMX, and 10:
SDM.

4.7.6   Optimization of Sample pH

As shown in section 4.7.4, the pH of the sample solution proved to be the most influentially variable, for the retention of analytes on the cartridge packing. Suitable samples of pH prior to be transferred through the SPE cartridge were considered. The results of pH values of sample solutions on the percent recoveries of each compound are illustrated in Table 4.15. The plot of relationship between the percent recoveries of each compound and pH values are shown in Figure 4.23-4.24.

Table 4.15 The results of sample pH on the percent recoveries of SAs, TCs, and PYR at spiking level 10.0, and 20.0 ppb.

| No.	Compounds	% Recovery							
		No adjusted pH		Adjusted to pH 4.0		**Adjusted to pH 4.5**		Adjusted to pH 5.0	
		Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb	Spiking level 10.0 ppb	Spiking level 20.0 ppb
1	TC	70.63	79.54	81.13	84.37	**80.60**	**80.66**	64.33	92.52
2	CTC	73.79	82.99	87.47	92.03	**81.41**	**70.39**	38.23	55.60
3	OTC	58.03	62.26	89.33	93.43	**71.52**	**72.58**	13.81	45.52
4	SDZ	62.02	70.59	76.98	67.34	**97.96**	**96.65**	99.79	103.56
5	STZ	67.70	62.34	62.88	68.81	**74.56**	**75.55**	63.58	56.73
6	SMZ	68.98	64.26	75.82	79.98	**96.42**	**97.80**	87.93	85.49
7	SMP	76.59	78.10	72.80	79.72	**86.21**	**88.70**	84.74	84.56
8	SMX	107.63	114.11	81.46	89.31	**95.70**	**100.89**	88.15	102.39
9	SDM	102.69	100.07	73.04	79.87	**91.64**	**88.60**	79.60	82.08
10	PYR	86.59	85.90	91.66	90.86	**105.66**	**99.28**	105.98	104.29
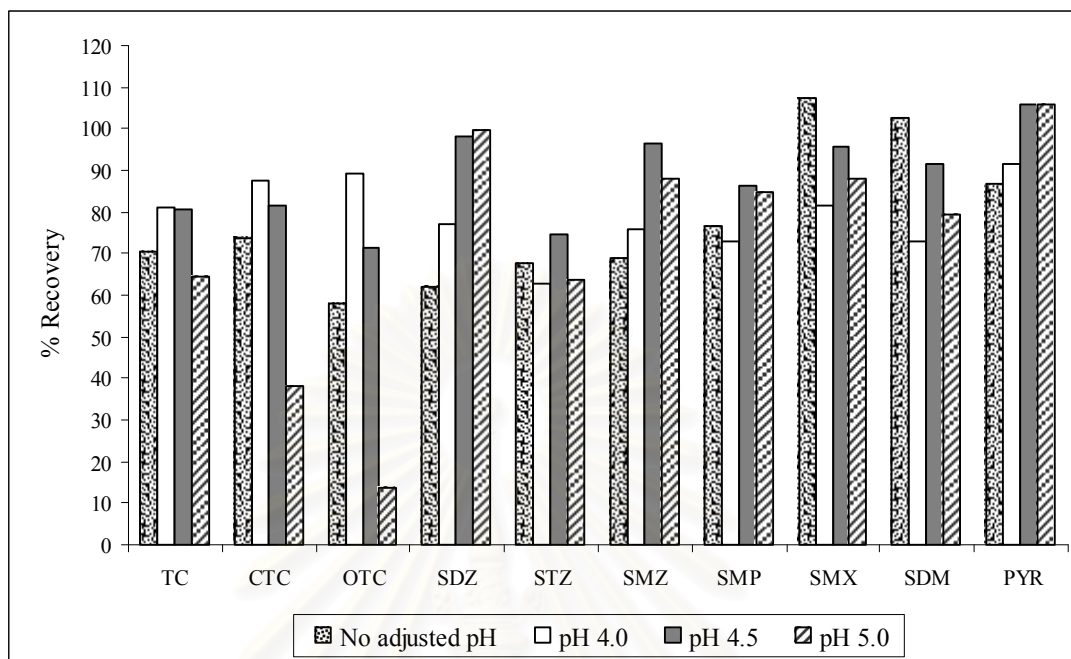
Figure 4.23 The plot of relationship between the percent recoveries of each compound and sample pH at spiking level 10.0 ppb.

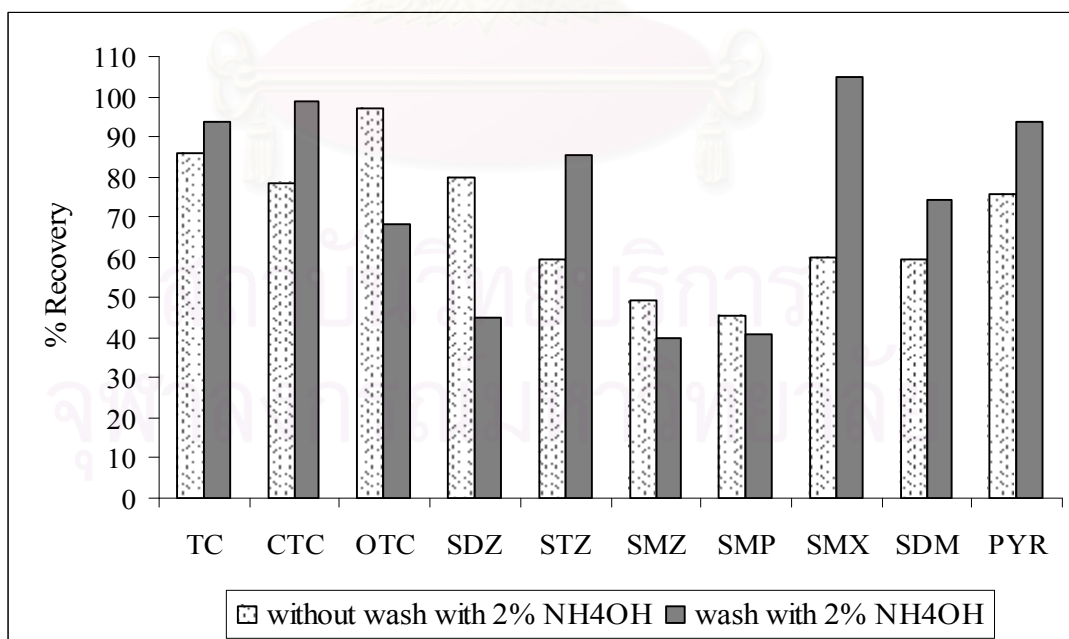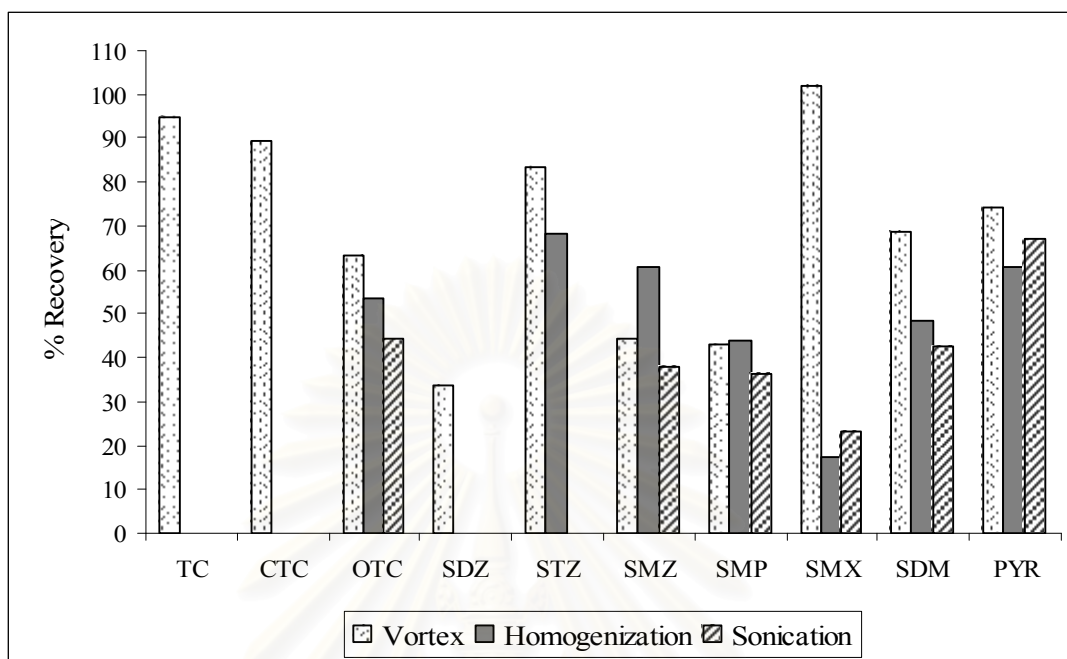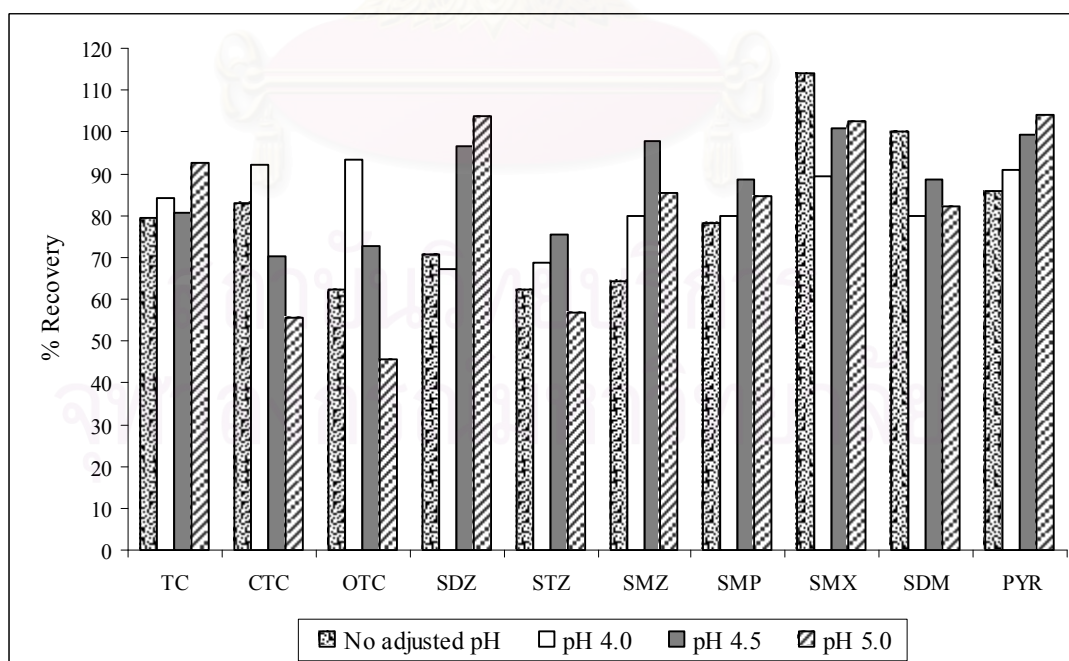**Figure 4.24** The plot of relationship between the percent recoveries of each compound and sample pH at spiking level 20.0 ppb.

Based on the results, the highest recoveries for all analytes increased as the pH of the sample solution increased. The recoveries of most of the studied compounds reached their greatest values at pH 4.5. At pH 4.5, all analytes are in neutral form and can retain with high efficiency both hydrophilic and hydrophobic compounds on the Oasis® HLB packing. Even if the extraction at pH 4.5 yielded a slightly low percent recovery of CTC and OTC, this pH value is compromised for simultaneous extraction of six SAs, three TCs, and PYR. The percent recoveries of most compounds ranged from 71.52–105.66 at spiking level 10.0 ppb and 70.39–100.89 at spiking level 20.0 ppb. Thus these percent recoveries are considered to be acceptable with in the range of AOAC Peer-verified methods [82].

Therefore, the adjustment of sample solution to pH 4.5 with 1.0 M sodium hydroxide solution, followed by isolation on Oasis® HLB cartridge was selected as the best procedure for extraction for multi-residue of the studied compounds in milk samples throughout this research.

4.8   Result of Selectivity Evaluation in Milk Matrix

The matrix acts as the direct or indirect interference in signal due to the presence of unintended analytes or other interfering substances in the sample. The matrices are derived from various physical and chemical processes and may be difficult or impossible to decrease detector responses or affect the retention time of standard compounds. Therefore, the selectivity of an analytical method may differ between the analytes prepared in pure organic solvent and those in matrix solution. As in section 4.5, the results of the matrix effect on the selectivity of LC-MS conditions in matrix solution are shown in Table 4.16 in order of analyte elutions.

Table 4.16 Retention times (t_R) and ion characteristic (m/z) of standard SAs, TCs, and PYR in matrix solution (n=10).

No.	Compounds	Retention time (min)	Selected ions monitoring (m/z)
1	Oxytetracycline	4.597 ± 0.020	**461**, 426
2	Tetracycline	4.982 ± 0.021	**445**, 410
3	Sulfadiazine	5.788 ± 0.012	**251**, 156
4	Sulfathiazole	6.597 ± 0.014	**256**, 108
5	Pyrimethamine	7.935 ± 0.008	**249**, 251
6	Chlortetracycline	8.572 ± 0.003	**479**, 481
7	Sulfamethazine	8.765 ± 0.005	**279**, 124
8	Sulfamethoxypyridazine	8.987 ± 0.003	**281**, 126
9	Sulfamethoxazole	10.087 ± 0.003	**254**, 156
10	Sulfadimethoxine	10.976 ± 0.005	**311**, 156

* Boldfaces refer to target ions.

From Table 4.16, the main ion of each compound **(boldface)** was the protonated molecular ion, $[M+H]^+$, as shown the base peak. The other ions were the qualifying ions, which were obtained from the fragmentation profiles of each compound, and were used to confirm the qualitative identification of suspected analyte peaks. The different in retentime time (t_R) and mass ions characteristic of all compounds were used to differentiate these compounds from the matrix interferences.
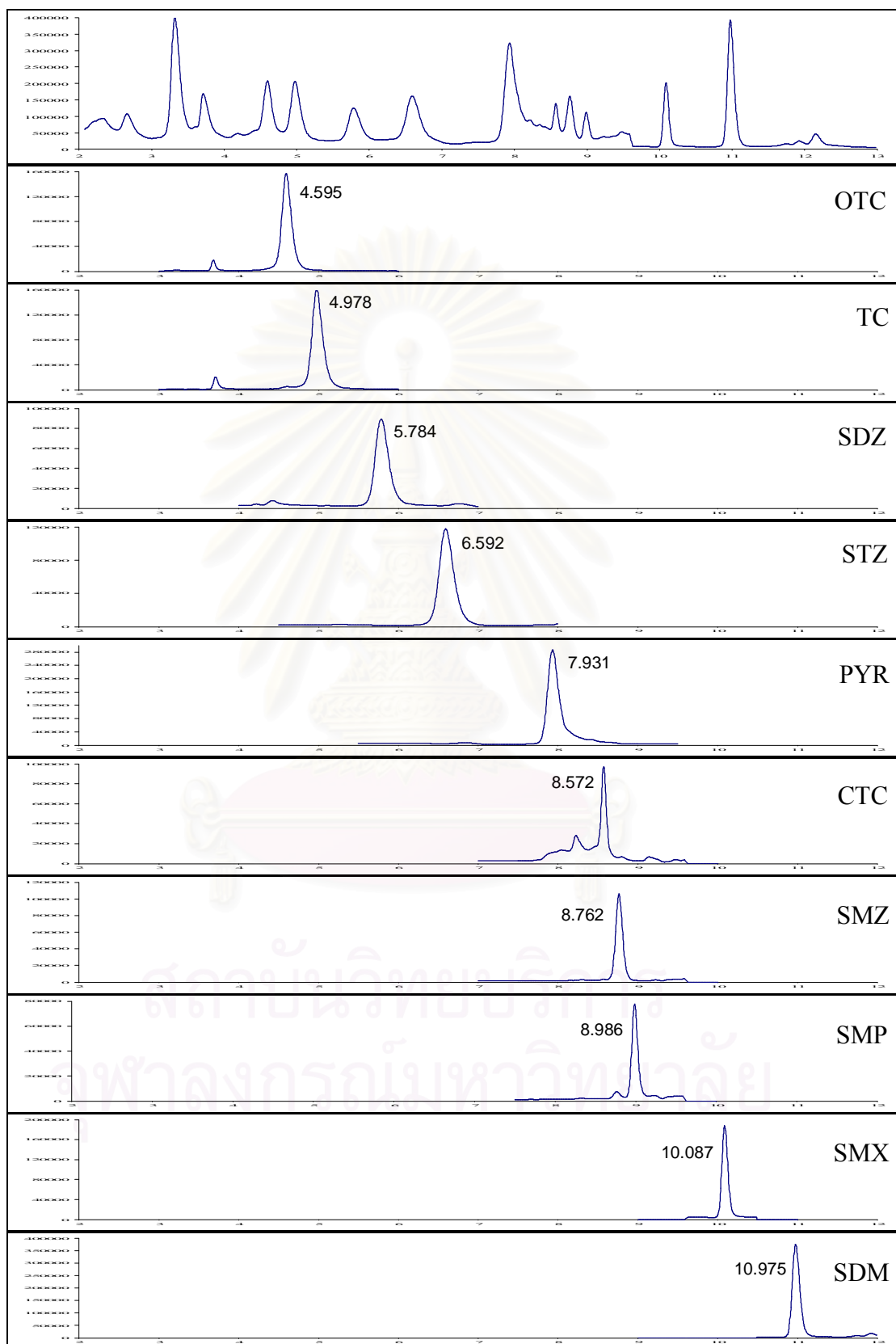
Figure 4.25 Extracted-ion chromatogram of SIM mode of standard SAs, TCs, and
PYR at 100 ppb in milk matrix.

4.9 Method Detection Limit (MDL) and Method Quantitation Limit (MQL)

The method quantitation limit (MQL) is defined as the lowest concentration of analytes that can be quantified with an acceptable level of uncertainty at signal-to-noise-ratio equal to 10 after passing through all sample preparation steps.

Method detection limit (MDL) is defined as the lowest concentration of the analytes that can be reliably detected at signal-to-noise-ratio equal to 3 after passing through all sample preparation steps.

The MDL and MQL values of all analyte compounds are summarized in Table 4.17.

Table 4.17 Method detection limits (MDLs) and method quantitation limits (MQLs) of sulfonamides, tetracyclines, and pyrimethamine in milk matrix.

No.	Compounds	Method Detection Limit (ppb)	Method Quantitation Limit (ppb)
1	Oxytetracycline	0.65	1.03
2	Tetracycline	0.67	1.43
3	Sulfadiazine	0.75	1.12
4	Sulfathiazole	1.27	4.16
5	Pyrimethamine	0.51	0.97
6	Chlortetracycline	2.64	8.64
7	Sulfamethazine	1.47	5.10
8	Sulfamethoxypyridazine	0.87	3.00
9	Sulfamethoxazole	0.84	2.68
10	Sulfadimethoxine	0.48	1.06

The results show that, the high sensitivity compounds had low MDL and MQL values, while the lower sensitivity compounds such as chlortetracycline had higher MDL and MQL values.   However, the high MDL and MQL values of chlortetracycline may have been caused from the mobile phase gradient program that led to the baseline drift and system noise.   Moreover, the presence of co-elute caused an interference near the analyte peak, resulting in the low sensitivity of chlortetracycline and slightly higher MDL and MQL values.

4.10   Preparation of Calibration Curves

4.10.1   The Standard Calibration Curves

Based on the EU regulation of SAs and TCs, MRL values did not exceed 100 ppb.   A mixture of 10 standard analytes in acetonitrile solution covering the concentration range of 1.00–300 ppb were measured and plotted by peak area versus analytes concentration.

The regression plots illustrating the relationship between peak areas and analyte concentrations are shown in APPENDIX E.   The results of the correlation coefficient (R^2) and regression data in the order of analyte elutions are summarized in Table 4.18.

Table 4.18 Linear least-squares regression coefficients of standard SAs, TCs, and PYR in acetonitrile solution.

No.	Compounds	Slope	y-Intercept	R^2
1	Oxytetracycline	7,237.4	12,745	0.9985
2	Tetracycline	7,711.5	-4,674.3	0.9983
3	Sulfadiazine	11,061	14,238	0.9998
4	Sulfathiazole	12,504	23,940	0.9996
5	Pyrimethamine	28,398	-20,417	0.9996
6	Chlortetracycline	6,800.2	-12,734	0.9974
7	Sulfamethazine	21,471	42,573	0.9971
8	Sulfamethoxypyridazine	15,875	63,679	0.9987
9	Sulfamethoxazole	7,193.4	15,674	0.9995
10	Sulfadimethoxine	18,398	36,037	0.9994

As shown in APPENDIX E, the standard calibration curves of all compounds are linear across the studied concentration range. The calibration curves that were prepared using at least 7 concentration levels and duplicate analysis, all fit well with the linear model. The corresponding least-squares regression correlation coefficients (R^2) described in Table 4.18 are greater than 0.9900, which was acceptable for quantitative analysis. The R^2 value of SDZ was found to be the highest (0.9998) and the lowest (0.9971) for SMZ. Furthermore, the sensitivity of each analyte that best shows the detector response is indicated by the slope values. The compound with the higher slope value is the greater of the detector response and higher sensitivity. In this study, PYR has the highest sensitivity (slope = 28,398), while CTC has the lowest sensitivity (slope = 6,800.2).

4.10.2 The Matrix-Matched Calibration Curves

LC-MS analysis of biological samples can cause signal suppression, evaluated background, and other negative matrix effects. Thus the response of some determination systems to certain analytes may be affected by the presence of co-extractives from the sample (matrix). These matrix effects, which are derived from various physical and chemical processes, may be difficult or impossible to eliminate [5]. Therefore, information about the strength of matrix effects and chromatographic efficiency is important in acheiving optimum sensitivity for method development. Moreover, matrix effects are considered for most method validations if they influence the reproducibility or assay linearity. Quantification based on MS technique is strongly influenced by ionization suppression caused by matrix co-extractants, therefore, the matrix effects must be evaluated prior to method quantification.

To estimate the matrix effects, a more reliable calibration curve may be obtained with matrix-matched calibration when techniques or equipment that are potential prone to the effect are used. The matrix calibration curve should be prepared using the same biological matrix as the samples of the same concentration levels in the standard calibration curves in acetonitrile solution. Matrix-matched calibration may compensate for matrix effects [5]. These calibration curves are made so that the ionization efficiency and background are similar when applied in the assay quantification.

The regression plots showing the relationship between peak areas and analytes concentration are shown in APPENDIX E. The results of the correlation coefficient (R^2) and regression data, in order of analytes elution, are summarized in Table 4.19.

Table 4.19 Linear least-squares regression coefficients of standard SAs, TCs, and
PYR in matrix solution.

No.	Compounds	Slope	y-Intercept	R^2
1	Oxytetracycline	7,417.7	30,235	0.9989
2	Tetracycline	8,827.3	20,018	0.9983
3	Sulfadiazine	10,431	148,950	0.9996
4	Sulfathiazole	11,982	19,574	0.9998
5	Pyrimethamine	22,737	12,011	0.9990
6	Chlortetracycline	2,982	8,495.6	0.9970
7	Sulfamethazine	6,900.4	-54,983	0.9988
8	Sulfamethoxypyridazine	3,279.9	-10,473	0.9990
9	Sulfamethoxazole	5,498.3	67,661	0.9972
10	Sulfadimethoxine	13,299	-28,966	0.9980

As shown in APPENDIX E, the calibration curves for all compounds are
linear on the studied concentration range (1.00-300 ppb). The calibration curves were
prepared using at least 7 concentration levels and duplicate analysis, all fit well with
the linear model. The correlation coefficients (R^2) varied from 0.9970-0.9998 linearly
in detector response and were all acceptable for quantitative analysis. The R^2 value of
STZ is the highest (0.9998) and the lowest (0.9970) for CTC. The slope values are all
different indicating a difference in detector response of different analytes.   The
detector response of PYR is the highest (slope = 22,737) which shows the higher
sensitivity, while CTC shows the lowest value (slope = 2,982).

4.11 Result of Matrix Effect Study

From Section 4.10.1 and 4.10.2, peak areas of analyte compounds obtained from the matrix calibration curves are not equal to the standard calibration curves. Most matrixes resulted in an increase of signal intensities, however, some resulted in total suppression of the MS signal. To understand the potential effect of milk matrix on the analysis, the results of the standard calibration curve and matrix calibration curve were compared by using a statistical tool, the Paired *t*-test.

By comparing the two calibration curves, the results indicated in the *t*-values at 95% confident limit as shown in Table 4.20.

Table 4.20 Calculated |*t*|-values of two tailed paired *t*-test at 95% confident limit.

No.	Compounds	Concentration (ppb)	Peak area		Paired *t*-test	
			Standard solution	Matrix standard	$|t|_{calculated}$	$t_{critical}$
1	Oxytetracycline	3.00	30646	30099	3.64	2.31
		5.00	34948	48160		
		10.0	72248	86342		
		50.0	359396	425289		
		100	778986	792062		
		150	1084705	1179260		
		200	1492087	1548473		
		250	1776290	1855990		
		300	2153800	2228570		
2	Tetracycline	3.00	26195	28209	3.60	2.31
		5.00	30499	39838		
		10.0	79479	92270		

Table 4.20 *(continued).*

No.	Compounds	Concentration (ppb)	Peak area		Paired *t*-test	
			Standard solution	Matrix standard	$\|t\|_{\text{calculated}}$	t_{critical}
		50.0	325431	501629		
		100	827578	921301		
		150	1147960	1372423		
		200	1552000	1839723		
		250	1872807	2190830		
		300	2331833	2636143		
3	Sulfadiazine	1.00	10061	133019	2.37	2.26
		3.00	34358	154084		
		5.00	60387	182088		
		10.0	115025	269220		
		50.0	594193	706219		
		100	1133537	1214900		
		150	1691987	1732077		
		200	2254633	2250963		
		250	2759843	2732697		
		300	3312663	3265193		
4	Sulfathiazole	5.0	68987	63903	3.62	2.36
		10.0	130973	140802		
		50.0	691310	653805		
		100	1310427	1235237		
		150	1927437	1819793		

Table 4.20 *(continued).*

No.	Compounds	Concentration (ppb)	Peak area		Paired *t*-test	
			Standard solution	Matrix standard	$|t|_{calculated}$	$t_{critical}$
		200	2533260	2381587		
		250	3141700	3015633		
		300	3744370	3623670		
5	Pyrimethamine	1.00	16987	103741	2.82	2.26
		3.00	70650	130668		
		5.00	127182	159825		
		10.0	247461	268695		
		50.0	1433237	1110587		
		100	2683144	2161163		
		150	4287657	3319870		
		200	5744883	4484620		
		250	7109913	5759793		
		300	8431957	6926953		
6	Chlortetracycline	10.0	63664	48589	3.62	2.45
		50.0	287018	151092		
		100	718549	287683		
		150	958651	443341		
		200	1338010	621393		
		250	1689513	731679		
		300	2045120	925328		

Table 4.20 *(continued).*

No.	Compounds	Concentration (ppb)	Peak area		Paired *t*-test	
			Standard solution	Matrix standard	$|t|_{\text{calculated}}$	t_{critical}
7	Sulfamethazine	5.00	127405	11279	3.64	2.36
		10.0	258414	35786		
		50.0	1256283	254367		
		100	2296763	611064		
		150	3310763	980965		
		200	4300957	1295103		
		250	5560520	1688173		
		300	6467033	2032343		
8	Sulfamethoxypyri-dazine	3.00	64344	8899	3.36	2.31
		5.00	104097	14449		
		10.0	207028	26229		
		50.0	937432	152562		
		100	1750607	302700		
		150	2518280	464679		
		200	3252503	635631		
		250	3944223	814174		
		300	4809027	989297		
9	Sulfamethoxazole	3.00	29817	50156	2.37	2.31
		5.00	43577	62019		
		10.0	82281	108232		
		50.0	386582	389994		

Table 4.20 *(continued).*

No.	Compounds	Concentration (ppb)	Peak area		Paired *t*-test	
			Standard solution	Matrix standard	$|t|_{calculated}$	$t_{critical}$
		100	740980	663295		
		150	1122340	914227		
		200	1481587	1164887		
		250	1799467	1448247		
		300	2150447	1681123		
10	Sulfadimethoxine	1.00	24166	12370	3.34	2.26
		3.00	67928	16513		
		5.00	124028	49037		
		10.0	229904	141969		
		50.0	1021947	651496		
		100	1874773	1261582		
		150	2820417	1919039		
		200	3616990	2516699		
		250	4718700	3255726		
		300	5528997	4102587		

After the statistical data treatments of peak area values were determined by the Paired *t*-test at 95% confident limit, the calculated $|t|$-values of all compounds were higher than the critical *t*-values. These values obtained are significantly different. It was concluded that the milk matrices significantly affect the signal of analyte compounds in this study. When milk was analyzed, the analytes were co-eluted with other matrix components, thus any ion suppression effect occurring during the detection steps made quantitative analysis unreliable.

Moreover, the retention times of all of the compounds spiked in milk matricx (Table 4.6) were identical to those obtained from acetonitrile (Table 4.6) except for OTC, TC, and PYR as shown in Figure 4.26. The shift of retention time of OTC, TC, and PYR in spiked matrix may have been caused from the endogenous components in the milk and the chemicals from the reagents in the sample preparation such as the McIlvain buffer that used a metal chelating agent which is known to affect retention times. Chelate metals from the McIlvain buffer may be present in solution or absorbed on the surface of sorbents in an analytical column, thus prevent the binding of metals by TC, OTC, and PYR. Therefore, the retention powers of TC, OTC, and PYR on the analytical column are higher, resulting in the increase of retention times.

Therefore, matrix-matched calibration curves of all compounds are used for quantitative analysis instead of standard calibration curves throughout this study.

Figure 4.26 Total-ion SIM chromatogram of SAs, TCs, and PYR spiked in acetonitrile solution and milk extract at 100 ppb.
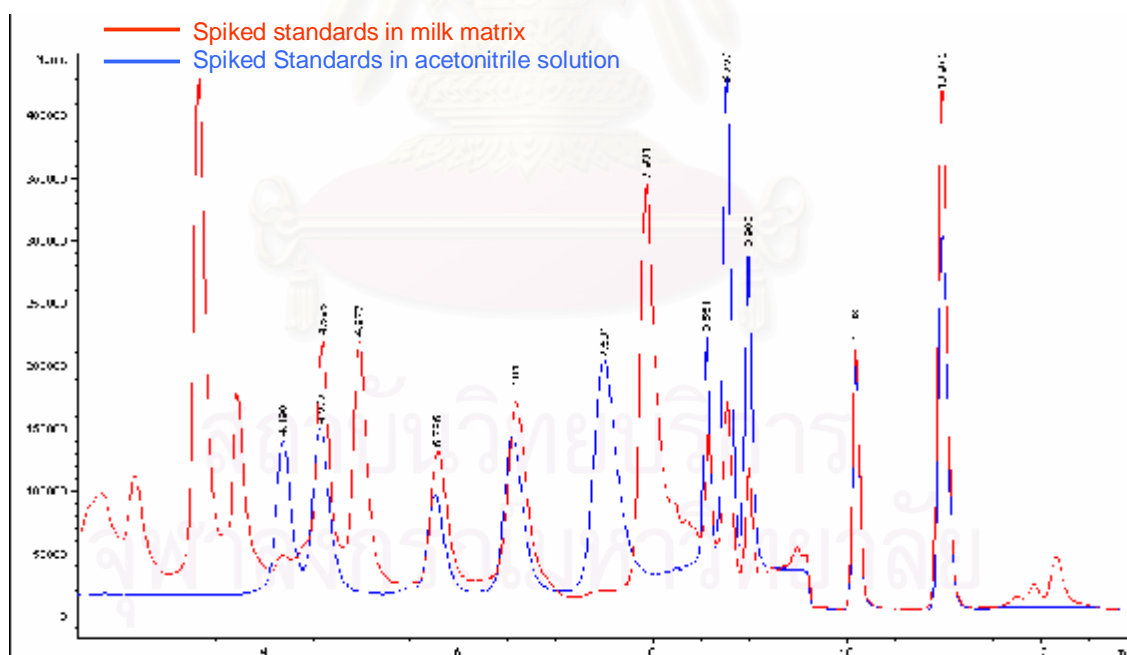
4.12   Method Precision

The milk matrix proved to be the most influential to the analysis of six SAs, three TCs, and PYR. Therefore, a study of method precision was carried out in the milk matrix. Method precision was evaluated at two different concentration levels above six replicates; method quantitation limit (MQL) and 5-fold MQL.

Generally, precision is usually stated in terms of standard deviation or relative standard deviation (RSD). Both repeatability and reproducibility are generally dependent on analyte concentration, and should be determined at a number of concentrations and if relevant, the relationship between precision and analyte concentration should be established [85]. Relative standard deviation may be useful in this case because concentration has been factored out and is constant over the range of interest provided. The acceptability of the precision values should be based on the modified Horwitz equation.

Horwitz equation [84, 85]:

$$RSD_r \ = \ 0.67 \times 2^{(1 - 0.5 \, \log C)}$$

where, RSD_r =   the relative standard deviation calculated from results generated
                under repeatability conditions (within-laboratory).

C   =   mass fraction: for 100% (pure materials), C = 1.00
                          for 1 μg/g (ppm), C = 0.000001.

This is a generalized precision equation which has been found to be independent of the analyte and matrix, and is solely dependent on the concentration for most routine methods of analysis.

From the Horwitz equation, the predicted percent RSD values of all analytes at MQL and 5-MQL studied concentration levels are illustrated in Table 4.21.

Table 4.21 Predicted % RSD of SAs, TCs, and PYR at MQL and 5-MQL
concentration levels.

Compounds	Predicted % RSD[*]	
	MQL	5-MQL
OTC	30.19	23.69
TC	28.73	22.55
SDZ	29.81	23.40
STZ	24.47	19.20
PYR	30.46	23.91
CTC	21.92	17.20
SMZ	23.73	18.62
SMP	25.70	20.17
SMX	26.14	20.52
SDM	30.06	23.59

* Predicted % RSD is calculated from Horwitz equation.

4.12.1   Result of Method Precision at MQL Level

The precision of this method is a measure of the closeness expected
between the replicate tests which results under the optimal conditions.   Method
precision at MQL level was studied by repeating the analysis both on the same day
and on two different days.   The results of the method precision are summarized in
Table 4.22-4.24.

Table 4.22 % Recoveries and % RSD of spiked milk matrix at **MQL** level on the
first day (n=6).

No.	Compounds	%Recovery						Mean	%RSD
		1	2	3	4	5	6		
1	OTC	79.69	84.14	78.93	81.41	77.54	72.95	79.11±3.78	4.78
2	TC	78.31	81.82	87.90	75.03	74.97	88.39	83.07±6.03	7.44
3	SDZ	71.78	69.62	81.59	76.38	74.64	68.43	75.41±4.37	5.80
4	STZ	97.37	84.92	95.89	80.46	93.52	81.73	88.98±7.49	8.42
5	PYR	78.23	67.93	75.65	74.46	66.78	75.58	73.11±4.64	6.34
6	CTC	74.73	80.46	79.94	67.14	78.78	81.46	79.09±5.40	7.01
7	SMZ	99.18	92.13	95.90	95.28	93.91	98.23	96.77±2.63	2.75
8	SMP	86.57	84.83	82.91	79.83	76.41	80.68	81.87±3.66	4.47
9	SMX	85.78	84.65	88.45	89.12	90.46	93.55	88.67±3.22	3.63
10	SDM	76.83	78.49	79.15	74.64	68.36	76.82	75.75±3.93	5.19

Table 4.23 % Recoveries and % RSD of spiked milk matrix at **MQL** level on the second day (n=6).

No.	Compounds	%Recovery						Mean	%RSD
		1	2	3	4	5	6		
1	OTC	76.62	74.71	78.24	82.53	81.61	87.31	81.17±4.58	5.71
2	TC	77.88	71.38	82.55	78.29	85.66	88.71	80.75±6.21	7.69
3	SDZ	77.27	78.46	72.24	82.10	80.98	83.15	79.03±3.99	5.05
4	STZ	94.60	97.86	92.68	85.44	90.63	92.27	92.25±4.15	4.50
5	PYR	66.14	70.94	69.65	74.72	72.87	71.14	70.91±2.92	4.12
6	CTC	80.42	84.68	85.97	77.89	75.30	87.94	72.03±4.95	6.03
7	SMZ	96.63	95.92	95.64	90.83	86.47	84.71	91.70±5.19	5.66
8	SMP	84.59	93.60	83.80	86.73	91.76	83.46	87.32±4.34	4.97
9	SMX	84.46	85.04	88.43	83.35	81.61	85.42	84.72±2.28	2.69
10	SDM	80.94	77.40	73.25	71.84	77.24	79.51	76.70±3.10	4.60

Table 4.24 Overall % recovery and % RSD of Spiked Milk Matrix at **MQL level**
        (n=2).

No.	Compounds	% Recovery		Mean	% RSD	F-test[*]	
		1	2			$F_{calculated}$	$F_{critical}$
1	OTC	79.11	80.17	79.64±0.75	0.94	0.19	4.69
2	TC	81.07	80.75	80.91±0.23	0.28	0.01	
3	SDZ	75.41	79.03	77.22±2.56	3.52	2.25	
4	STZ	88.98	92.25	90.61±2.31	2.55	0.87	
5	PYR	73.11	70.91	72.01±1.55	2.16	0.96	
6	CTC	77.09	82.03	79.59±3.50	4.40	2.73	
7	SMZ	95.77	91.70	93.74±2.88	3.07	2.93	
8	SMP	81.87	87.32	84.60±3.85	4.56	<u>5.51</u>	
9	SMX	88.67	84.72	86.69±2.79	3.22	<u>6.02</u>	
10	SDM	75.72	76.70	76.21±0.69	0.91	0.21	

* *F*-values were calculated from one-way ANOVA at 95% confidential limit, n = 2.

(1) **Intra-assay precision**

In this study, the precision of the method was expressed as the percent relative standard deviation (%RSD). On the basis of the Horwitz equation, taking into account the concentration of the analytes at the MQL levels measured the acceptable RSD range between 21.92-30.46 %. The experimentally determined %RSD values on the first day varied from 2.75-8.42, and 4.12-7.69 on the second day. Both these ranges were below and complied with the %RSD predicted by Horwitz. The results demonstrated that this method is sufficiently precise at the concentration level of analytes being measured within the same day.

(2) **Intermediate precision**

The precision of the method on different days (Table 4.24), %RSD$_{overall}$ at 1.00-9.00 ppb ranged between 0.28-4.56. From one-way ANOVA at 95% confident limit, critical F-value is 4.41 (n = 2). The calculated F-values of all analytes are less than critical F-value except SMP and SMX, but these values are slightly different from the critical F-value. The result indicates that a fairly good intermediate precision of this method is observed at the level of analytes being measured.

4.12.2   Result of Method Precision at 5-fold MQL Level

Method precision at 5-fold MQL level was studied by repeated the analysis both within one day and on different days. The results of method precision are summarized in Table 4.25-4.27.

Table 4.25 % Recoveries and % RSD of spiked milk matrix at **5-fold MQL** level on the first day (n=6).

No.	Compounds	%Recovery						Mean	%RSD
		1	2	3	4	5	6		
1	OTC	96.77	89.32	80.75	94.25	88.36	88.81	89.71±5.54	6.18
2	TC	101.11	98.68	103.13	90.77	95.75	95.28	97.45±4.46	4.58
3	SDZ	71.83	79.87	75.94	91.57	84.61	91.93	82.63±8.24	9.97
4	STZ	102.51	85.10	94.54	78.72	98.57	89.71	91.53±8.81	9.62
5	PYR	69.85	81.61	86.81	72.20	70.55	70.86	75.31±7.13	9.46
6	CTC	86.88	76.24	86.43	74.21	73.10	78.41	79.21±6.05	7.63
7	SMZ	82.50	77.48	82.33	72.79	83.34	83.51	80.49±4.47	5.56
8	SMP	93.45	88.23	90.68	87.72	98.68	98.17	94.82±4.80	5.17
9	SMX	100.43	99.52	94.73	92.17	97.74	105.68	98.3±4.71	4.79
10	SDM	82.20	79.13	72.17	79.27	74.24	73.40	76.74±4.01	5.22

Table 4.26 % Recoveries and % RSD of spiked milk matrix at **5-fold MQL** level on the second day (n=6).

No.	Compounds	%Recovery						Mean	%RSD
		1	2	3	4	5	6		
1	OTC	89.43	88.91	86.32	90.86	94.57	83.27	88.89±3.86	4.35
2	TC	91.47	95.39	86.29	87.99	95.96	86.85	90.66±4.29	4.73
3	SDZ	84.13	82.72	73.43	77.57	78.84	86.68	80.56±4.85	6.02
4	STZ	88.40	87.53	88.87	76.17	69.54	78.33	81.47±8.00	9.82
5	PYR	78.80	79.78	70.43	82.99	79.94	75.91	77.98±4.34	5.56
6	CTC	94.15	83.53	78.68	78.42	73.98	72.41	82.20±7.88	9.83
7	SMZ	86.25	82.21	88.61	86.40	84.25	82.66	85.56±2.46	2.90
8	SMP	96.88	96.54	101.93	90.50	106.91	93.21	97.66±5.95	6.09
9	SMX	100.43	94.27	93.44	102.37	94.41	93.53	96.41±3.93	4.08
10	SDM	71.31	73.80	79.84	78.72	74.47	81.21	78.56±3.91	5.11

Table 4.27 Overall % recovery and % RSD of spiked milk matrix at **5-fold MQL**
level (n=2).

No.	Compounds	% Recovery		Mean	%RSD	F-values[*]	
		1	2			$F_{calculated}$	$F_{critical}$
1	OTC	89.71	88.89	89.39±0.58	0.65	0.09	4.96
2	TC	97.45	89.66	93.56±551	5.89	<u>7.24</u>	
3	SDZ	82.63	80.56	81.59±1.46	1.79	0.28	
4	STZ	91.53	81.47	86.50±7.11	7.22	4.28	
5	PYR	75.31	77.98	76.64±1.88	2.46	0.61	
6	CTC	79.21	80.20	79.70±0.70	0.87	0.06	
7	SMZ	76.33	85.06	80.69±6.18	7.66	4.81	
8	SMP	92.82	97.66	95.54±3.24	3.59	2.41	
9	SMX	98.38	96.41	97.39±1.39	1.43	0.62	
10	SDM	76.74	76.56	76.68±0.12	0.16	0.01	

* *F*-values were calculated from one-way ANOVA at 95% confidential limit.

(1) Intra-assay precision

The predicted RSD for intra-assay precision range between 17.20-23.91 %
at the 5-MQL levels measured and calculated from the Horwitz equation. The
experimentally determined %RSD values on the first day varied from 4.57-9.97, and
2.90-9.83 on the second day. Both these ranges are all below the %RSD predicted by
the Horwitz equation. The results indicate that the method is sufficiently precise at
the concentration level of analytes being measured within the same day.

(2)   Intermediate precision

The precision of the method on different days as shown in Table 4.27, %RSD$_{overall}$ at 5.00-45.0 ppb varied from 2.90-9.83. The statistics result by one-way ANOVA at 95% confident limit, critical F-value is 4.41 (n = 2). The calculated F-values of all analytes are lower than critical F-value except TC but this value is slightly different from critical F-value. Therefore, the result indicates that a good intermediate precision of this method is observed at the level of analytes being measured.

4.13   Method Accuracy

Accuracy is the measure of exactness of an analytical method or the closeness of agreement between the measured value and the true or accepted value. Accuracy is measured as the percent of analyte recovered by spiking samples with known amounts of standard compounds. In this study, the concentration of analyte standards at MQL and 5-fold MQL levels were used to determine the method accuracy for milk sample. The results of method accuracy on the percent recoveries of each compound are shown in Table 4.28.

Table 4.28 % Recovery of method at MQL and 5-fold MQL levels for spiked milk
sample (n=2).

No.	Compounds	% Recovery	
		MQL level	5-fold MQL level
1	OTC	79.64±0.75	89.39±0.58
2	TC	80.91±0.23	93.56±551
3	SDZ	75.55±4.92	81.59±1.46
4	STZ	90.61±2.31	86.50±7.11
5	PYR	72.01±1.55	76.64±1.88
6	CTC	79.59±3.50	79.70±0.70
7	SMZ	93.74±2.88	80.69±6.18
8	SMP	84.60±3.85	95.54±3.24
9	SMX	85.28±4.80	97.39±1.39
10	SDM	76.21±0.69	76.68±0.12

Recovery of the spiked milk matrix at MQL concentration level (1.00-9.00
ppb) ranged from 72.01 to 93.74 %, and 76.64 to 97.39 % at 5-fold MQL
concentration level (5.00 – 45.0 ppb). These recovery values are accepted by the
AOAC Peer-verified methods that recommend the acceptable recovery values of the
method at ppb concentration level range between 60-115 %. The results obtained
from the above studies indicated that the developed extraction method in this research
provided good precision and accuracy for the analysis of these analytes residue in
milk.

4.14   Method Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small changes, but deliberate, variations in method parameters and provides an indication of its reliability during method usage. Robustness testing was performed in order to obtain information about the critical factors affecting the response of analytes. The robustness of a method can be tested using Plackett-Burman experimental designs in order to study the simultaneous variation of the factors. In this work, the seven factors to investigate were shown in Table 3.3. Robustness of this method was investigated by comparing a domain conditions (A-G) with varied conditions (a-g) and the effect of changing the level for any one factor, Dx_i, of each analytes are summarized in Table 4.29.   The results of statistical evaluation, t-values are illustrated in Table 4.30.

Table 4.29 The calculated difference values (D_x) of each factor studied in the robustness testing.

No.	Compounds	Difference values						
		D_A	D_B	D_C	D_D	D_E	D_F	D_G
1	OTC	28.42	-1.83	-1.42	-1.88	-5.04	-1.50	0.28
2	TC	8.09	-1.36	-0.22	0.35	-7.60	2.00	-4.26
3	SDZ	8.32	1.51	-7.54	-10.06	14.51	8.50	-6.21
4	STZ	7.27	17.26	-12.01	1.66	0.22	12.03	1.28
5	PYR	-4.77	-4.39	1.15	-0.27	-0.95	-0.32	-4.01
6	CTC	8.09	4.53	-2.02	-0.04	5.48	-4.88	2.17
7	SMZ	16.32	1.89	3.03	-7.03	0.34	3.05	-1.12
8	SMP	23.08	-0.05	-5.97	-7.02	9.50	-3.04	0.58
9	SMX	12.72	-0.60	2.13	-1.80	-1.05	2.49	0.36
10	SDM	7.16	3.50	4.76	-6.37	9.04	3.71	-2.57

Table 4.30 The results of statistical $|t|$-values of each analyte at 95% confident level.

No.	Compounds	Statistical $|t|$-values						
		t_A	t_B	t_C	t_D	t_E	t_F	t_G
1	OTC	2.59	0.17	0.13	0.17	0.46	0.14	0.03
2	TC	1.76	0.30	0.05	0.08	1.66	0.44	0.93
3	SDZ	0.94	0.17	0.85	1.13	1.64	0.96	0.70
4	STZ	0.76	1.80	1.25	0.17	0.02	1.25	0.13
5	PYR	1.62	1.49	0.39	0.09	0.32	0.11	1.36
6	CTC	1.76	0.98	0.44	0.01	1.19	1.06	0.47
7	SMZ	2.34	0.27	0.44	1.01	0.05	0.44	0.16
8	SMP	2.28	0.00	0.59	0.69	0.94	0.30	0.06
9	SMX	2.53	0.12	0.42	0.36	0.21	0.49	0.07
10	SDM	1.25	0.61	0.83	1.11	1.58	0.65	0.45

From these experiments, it was observed that all factors have an effect on the percent recovery of analytes, especially the pH of sample solution (factor A). For 7 degree of freedoms, the critical t-value is 2.36 ($P = 0.05$). The calculated t-value at 95% confidence level for comparing reference condition (factor A) with varied condition (factor a) ranged from 0.76-2.56. Because of OTC and SMX showed that the calculated $|t|$-values are greater than critical t-value, it can be determined that the variation of pH in the sample solution has a significant effect on the method performance for simultaneous analysis. By considering the effect of factor B-G found that the calculated $|t|$-values of all analytes are less than critical t-value. As results, it is indicated that the variations of factor B-G do not have a significant effect on the method performance.

From the results, only the factor pH of sample solution is significant for the simultaneous extraction of these analytes. It can be concluded that the simultaneous extraction of these analytes residue in milk should be controlled by the sample solution to pH 4.5 before transferred to Oasis[®] HLB cartridges to improve the analysis efficiently. To reduce the analysis time, the length of time for vortex mixing, centrifugation, and SPE air-dried can be used at 1, 10 and 3 min, respectively. Moreover, the evaporation temperature can be increased to 40 °C without a significant effect to the percent recovery of all analytes.

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

In this study, a new method for simultaneous identification and determination of antibiotics and antibacterials, sulfadiazine (SDZ), sulfathiazole (STZ), sulfamethazine (SMZ), sulfamethoxypyridazine (SMP), sulfamethoxazole (SMX), sulfadimethoxine (SDM), chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TC), and pyrimethamine (PYR), was developed. The analysis was performed by using a reliable liquid chromatography-mass spectrometry (LC-MS) technique. LC-MS, equipped with a single quadrupole was used for analysis throughout this study.

The best MS sensitivity of all target compounds was achieved by using 1 mM oxalic acid: acetonitrile, (50:50, v/v) as the mobile phase and analyzed by electrospray ionization (ESI) technique with positive ion detection mode. The optimum MS conditions for analysis of analyte compounds are illustrated in Table 5.1.

Table 5.1 Optimum electrospray (ESI) conditions for analysis of SAs, TCs, and PYR.

ESI parameters	Conditions
Detection mode	positive
Drying gas flow	Nitrogen, 10.0 L/min
Drying gas temperature	300 ˚C
Nebulizer gas pressure	Nitrogen, 35 psi
Capillary voltage	3,000 V
Fragmentor voltage	130 V

A good separation and selectivity of all studied compounds was achieved on reversed-phase gradient elution with water-acetonitrile (1 mM oxalic acid) as the

mobile phase. The selectivity of LC-ESI-MS method was evaluated by the matching of peak retention time in matrix solution with standard peaks. Although retention time of OTC, TC, and PYR were shifted from the standard peaks, it can be confirmed by the mass spectrum pattern of each peak. Thus, the mass spectrum pattern is the characteristic profile of each analyte that was used to differentiate these compounds from matrix interference.

The quantitative analysis was carried out using selected ion monitoring (SIM) of the base peak ions [M+H]$^+$ (bold text) according to the program shown in Table 5.2 in order of analytes elution. Moreover, simultaneous identification of the presence of target analytes was confirmed using qualifies ions.

Table 5.2 Time-scheduled multiple-ion SIM conditions for detecting SAs, TCs, and PYR.

No.	Compounds	Retention window (min)	Selected ion monitoring (m/z)
1	Oxytetracycline		**461**[a] *(100)*, 426[b] *(33.7)*
2	Tetracycline		**445**[a] *(100)*, 410[b] *(47.4)*
3	Sulfadiazine		**251**[a] *(100)*, 156[b] *(36.6)*
4	Sulfathiazole	2.0 – 9.40	**256**[a] *(100)*, 108[b] *(45.8)*
5	Pyrimethamine		**249**[a] *(100)*, 251[b] *(35.4)*
6	Chlortetracycline		**479**[a] *(100)*, 481[b] *(39.5)*
7	Sulfamethazine		**279**[a] *(100)*, 124[b] *(32.0)*
8	Sulfamethoxypyridazine		**281**[a] *(100)*, 126[b] *(65.5)*
9	Sulfamethoxazole	9.40 – 10.40	**254**[a] *(100)*, 156[b] *(31.4)*
10	Sulfadimethoxine	10.40 – 12.00	**311**[a] *(100)*, 156[b] *(25.2)*

[a] Base peak ion. [b] Qualify ion.

By comparison of the extraction methods, satisfactory results were obtained from the method that used Oasis® HLB solid phase extraction. The extraction method was developed by using a mixed-mode co-polymeric solid-phase extraction cartridge for simultaneous extraction of the analytes from milk samples. The effects of SPE wash solvent and sample pH proved to be the most influential in the extraction efficiency of analytes. The sample preparation procedure was developed using 20% trichloroacetic acid and McIlvain buffer to precipitate protein in milk and to prevent the chelation of analytes by metals. The adjustment of sample solution to pH 4.5 to being prior isolated on the Oasis® HLB cartridge preserved the uncharged form of analytes molecule. The Oasis® HLB cartridge was washed with 5% methanol and 2% acetic acid in 5% methanol to remove basic polar interferences such as protein. The analytes were eluted with methanol and 2% NH_4OH in 5% methanol. These procedures are the optimum conditions and the best results for simultaneous extraction of the studied compounds as residue in milk samples. The developed extraction method used in this study is described in Figure 5.1.

Through consideration of the selectivity of LC-MS conditions in milk matrix and the results of matrix-matched calibration curves, the matrix proved to be influential in the analysis of these analytes. Therefore all analytical validations were carried out in the milk matrix solution. The method detection limits (MDLs) and method quantitation limits (MQLs) ranged from 0.48 to 2.64 ppb and 0.57 to 8.64 ppb, respectively. These MDLs and MQLs values show the capability of the method to detect these drugs at concentration below the MRLs and are adequate for practical analysis in milk sample.

This method showed good linearity ($R^2 > 0.9900$) up to 300 ppb and covered the MRLs values established by EU, U.S. FDA, and Thai's Ministry of Public Health. All matrix-matched calibration curves show good linear relationships with an acceptable range of $R^2 > 0.9900$ over a dynamic range from MQL to 300 ppb for each analyte as shown in Table 5.3.

Milk 5.00 g was placed in a plastic centrifuge tube

↓

Added 2 mL of 20% trichloroacetic acid and vortexed for 2 min

↓

Added 20 mL of McIlvain buffer and vortexed for 3 min

↓

The mixture was adjusted to pH 4.5 with 1.0 M NaOH and vortexed for 1 min

↓

The mixture was centrifuged at 3,800 rpm for 20 min

↓

The Oasis® HLB cartridge was previously pre-conditioned by washing sequentially with 5 mL methanol, 5 mL of 0.5 N HCl, and 5 mL of water

↓

The supernatant was transferred to an Oasis® HLB cartridge which connected to a vacuum manifold

↓

After sample loading, the cartridge was washed sequentially with 5 mL of 5% methanol in water and 5 mL of 2% acetic acid in 5% methanol

↓

The cartridge was allowed to dry by drawing air through for 5 min using vacuum pump

↓

The analytes were eluted sequentially with 10 mL methanol and 10 mL of 2% NH_4OH in 5% methanol

Figure 5.1 Schematic of the developed extraction method for simultaneous extraction of the SAs, TCs, and PYR residue in milk sample.

The solvent was removed by rotary evaporator at 33 °C and
the residue was evaporated to dryness under a flow of nitrogen gas

The dried residue was re-dissolved in 1.00 mL of mobile phase filtered through
0.45 μm syringe filter and analyzed using the optimum LC-MS conditions
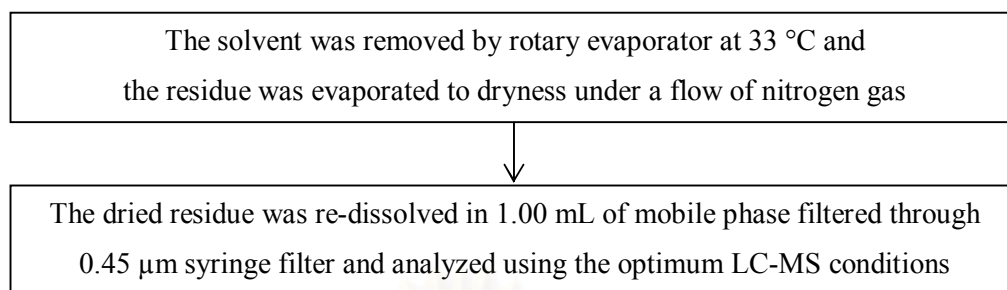
Figure 5.1 *(continued).*

Table 5.3 Characteristic validation data consists of retention time (t_R), correlation coefficient (R^2), method detection limit (MDL), and method quantitation limit (MQL) of each analyte in milk matrix.

No.	Compounds	Retention time (min)	R^2	MDL (ppb)	MQL (ppb)
1	Oxytetracycline	4.597 ± 0.020	0.9989	0.65	1.03
2	Tetracycline	4.982 ± 0.021	0.9984	0.67	1.43
3	Sulfadiazine	5.788 ± 0.012	0.9996	0.75	1.12
4	Sulfathiazole	6.597 ± 0.014	0.9998	1.27	4.16
5	Pyrimethamine	7.935 ± 0.008	0.9991	0.51	0.97
6	Chlortetracycline	8.572 ± 0.003	0.9972	2.64	8.64
7	Sulfamethazine	8.765 ± 0.005	0.9988	1.47	5.10
8	Sulfamethoxypyridazine	8.987 ± 0.003	0.9990	0.87	3.00
9	Sulfamethoxazole	10.087 ± 0.003	0.9972	0.84	2.68
10	Sulfadimethoxine	10.976 ± 0.005	0.9980	0.48	1.06

The method precision and accuracy were evaluated at two concentration levels, MQL (1.00-9.00 ppb) and 5-fold MQL (5.00-45.0 ppb), for both intra-assay

precision and intermediate precision in milk matrix. The satisfactory intra-assay precisions of this method on two days were achieved with relative standard deviation lower than the limited %RSD derived from the Horwitz equation. Statistical *F*-values of most analytes were less than critical *F*-value at 95% confidence level, which indicated the good intermediate precision of this method were observed.

Overall recoveries of all analytes at MQL and 5-fold MQL were greater than 70%. This demonstrated the acceptability of method accuracy according to the AOAC standard at the ppb concentration studied (60-115 %).

Moreover, the developed method was found to be fairly robust with minor changes of seven parameters in the extraction method (pH of sample solution, time for vortex, time for centrifugation, concentration of acetic acid in 5% methanol, concentration of NH_4OH in 5% methanol, time for SPE air-dried, and evaporation temperature). Statistical $|t|$-values at 95% confident limit of all analytes were less than critical *t*-value except OTC and SMX when the change in pH of sample solution was tested. This result shows that the pH value of a sample solution is a critical point in this sample preparation, and should be controlled at pH 4.5 before being isolated on the HLB Oasis cartridge.

This method was developed by using pasteurized milk as the matrix sample. Other samples such as fresh cow's milk, processed milk, and diary milk products would be interesting to apply by this method for simultaneous extraction and determination the residual of sulfonamides, tetracyclines, and pyrimethamine in further work.

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APPENDICES

APPENDIX A

Table A-1 Chemical structures and physicochemical properties of SAs, TCs, and PYR.
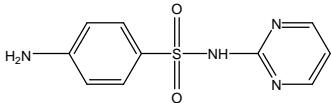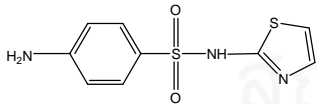
Compound structures		MW.	pKa	Stability	Toxicological informations
Sulfadiazine (SDZ) $C_{10}H_{10}N_4O_2S$		250.28	6.36[a]	Light sensitive	- Extremely hazardous in case of ingestion. - Toxic to blood, kidneys, liver, mucous membranes. - Prolonged exposure to the substance can produce target organs damage. - Human; passes through the placenta, excreted in maternal milk.
Sulfathiazole (STZ) $C_9H_9N_3O_2S_2$		255.32	7.2[a]	Light sensitive	- Irritating to eyes or if inhaled as dust. - Harmful by ingestion. - May cause nausea, vomiting, dizziness or mental confusion.

Table A-1 (*continued*).

Compound structures		MW.	pKa	Stability	Toxicological informations
Sulfamethazine (SMZ) $C_{12}H_{14}N_4O_2S$		278.33	7.59^a	Sensitive to light and temperatures	- Very hazardous in case of ingestion. - Hazardous in case of skin contact (irritant) - Excreted in maternal milk in animal.
Sulfamethoxypyridazine (SMP) $C_{11}H_{12}N_4O_3S$		280.31	6.7^a	- Desiccate - Sensitive to light and temperature	- Very hazardous in case of ingestion. - Hazardous in case of skin contact (irritant), of inhalation. - Human; passes through the placenta, excreted in maternal milk.
Sulfamethoxazole (SMX) $C_{10}H_{11}N_3O_3S$		253.28		Light sensitive and stable at ambient temperatures	- Lead to decreased urine volume. Complete cessation of urine production, dermatitis, rash, and systematic metabolic changes. - Allergy-like reactions may develop.

Table A-1 (*continued*).
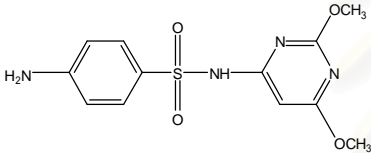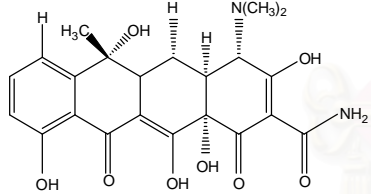
Compound structures		MW.	pKa	Stability	Toxicological informations
Sulfadimethoxine (SDM) $C_{12}H_{14}N_4O_4S$		310.33		- Desiccate - Sensitive to temperature	- Harzadous in case of skin contact (irritant), eye contact (irritant), ingestion, inhalation (lung irritant). - Slightly hazardous in case of skin contact (permeator).
Tetracycline (TC) $C_{22}H_{24}N_2O_8$		444.44	3.3[*]	- Desiccate - Sensitive to light and temperature	- Antibiotic substance produced by *Steptomyces spp.* - Antibiotic used to treat infections with bacteria, mycoplasma. - Toxic to reproductive system, liver. - Hazardous in case of ingestion, inhalation. - Slightly hazardous in case of skin contact (irritant, permeator). - Prolonged exposure to the substance can produce target organs damage. - May affect teeth and bones

Table A-1 (*continued*).

Compound structures	MW.	pKa	Stability	Toxicological informations
Chlortetracycline (CTC) $C_{22}H_{23}ClN_2O_8$	478.89	3.3[a]	- Desiccate - Sensitive to light and temperature	- Antibiotic substance isolated from the substrate of *Steptomyces aureofaciens*. - May cause adverse reproductive effects (growth, viability) based on animal data. - Chronic effects on human: excreted into braes milk at low concentrations. - May cause irritation of the respiratory tract and mucous membranes. - May cause irritation of the digestive tract and mucous membranes, nausea, vomiting diarrhea. - May affect behavior, metabolism, and urinary system.

Table A-1 (*continued*).

Compound structures		MW.	pKa	Stability	Toxicological informations
Oxytetracycline (OTC) $C_{22}H_{24}N_2O_9$		460.44		Light sensitive and stable at ambient temperatures.	- Antibiotic substance isolated from the elaboration products of the actinomycete, *Steptomyces rimosus*. - Board spectrum antibiotic for the treatment and control of a wide variety of bacterial infections - May cause irritation/dryness or defatting of the skin with prolonged contact. - May cause irritation to eyes, nose, and throat following exposure to mists, vapours, and fumes. - May cause gastrointestinal inflammation. - Effects of excessive exposure may include liver damage.

Table A-1 (*continued*).

Compound structures		MW.	pKa	Stability	Toxicological informations
Pyrimethamine (PYR) $C_{12}H_{13}ClN_4$		248.71	7.34	Light sensitive and stable at ambient temperatures.	- Antibacterial, antimalarial, folic acid antagonist. - Toxic to the nervous system, central nervous system (CNS), and muscle tissue. - Very hazardous in case of inhalation (lung irritant). - Hazardous in case of skin contact (irritant). - Prolonged exposure to the substance can produce target organ damage. - Repeated exposure to a highly toxic material may produce general deterioration of health by an accumulation in one or many human organs.
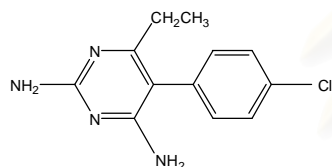
APPENDIX B
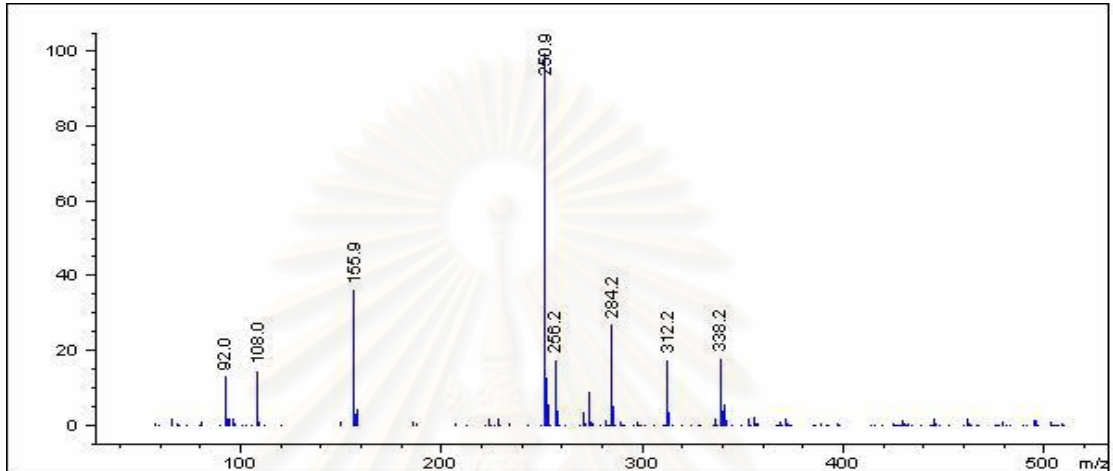
Figure B-1 Mass spectrum of standard sulfadiazine (SDZ) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
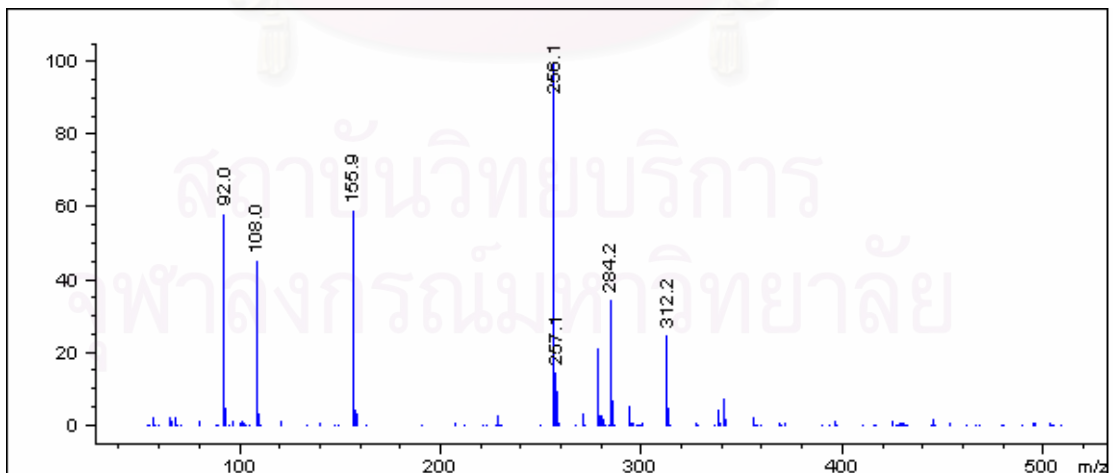
Figure B-2 Mass spectrum of standard sulfathiazole (STZ) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
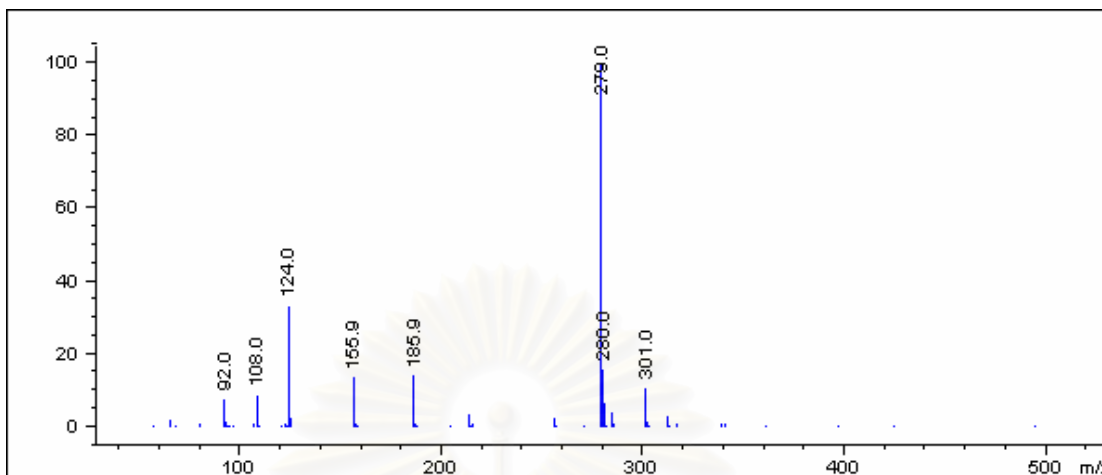
Figure B-3 Mass spectrum of standard sulfamathazine (SMZ) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
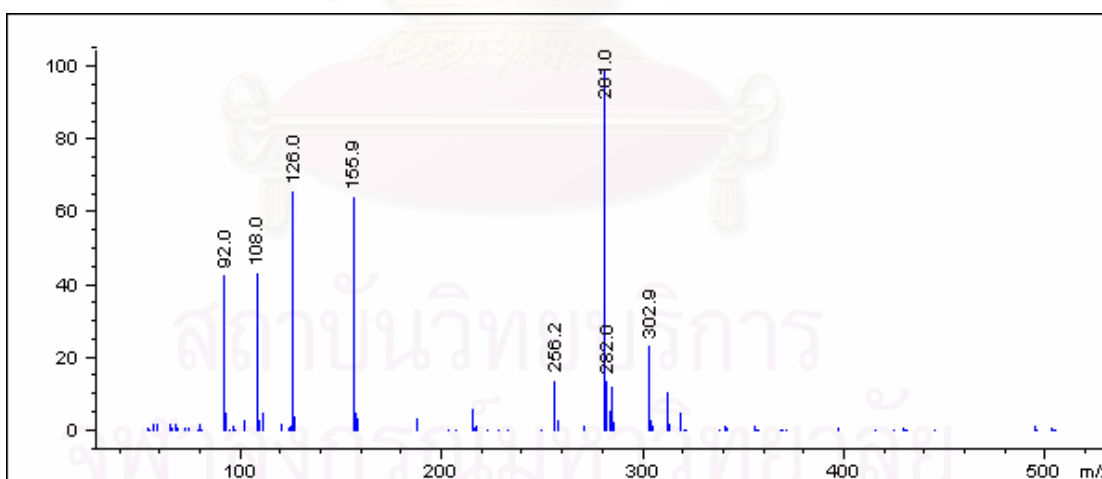
Figure B-4 Mass spectrum of standard sulfamethoxypyridazine (SMP) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
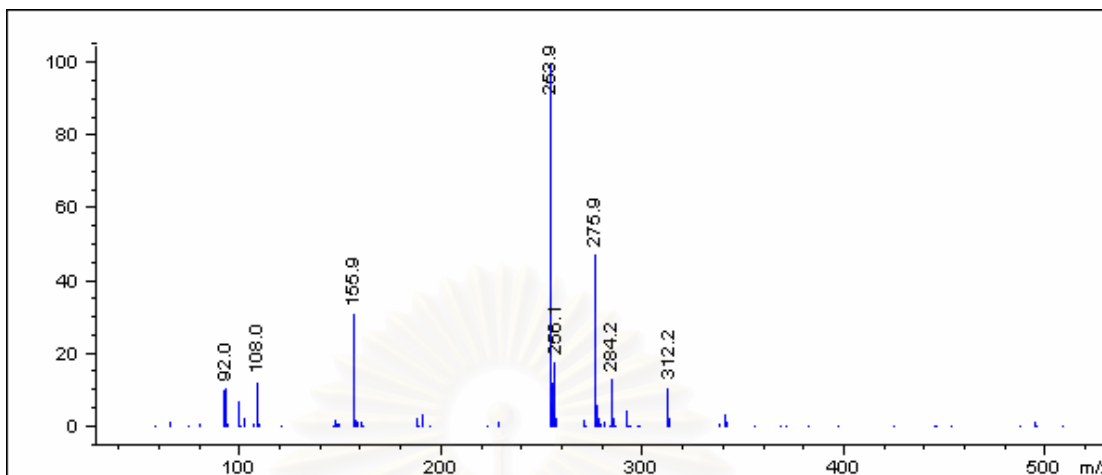
Figure B-5 Mass spectrum of standard sulfamethoxazole (SMX) 5.00 ppm analyzed
by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage
3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
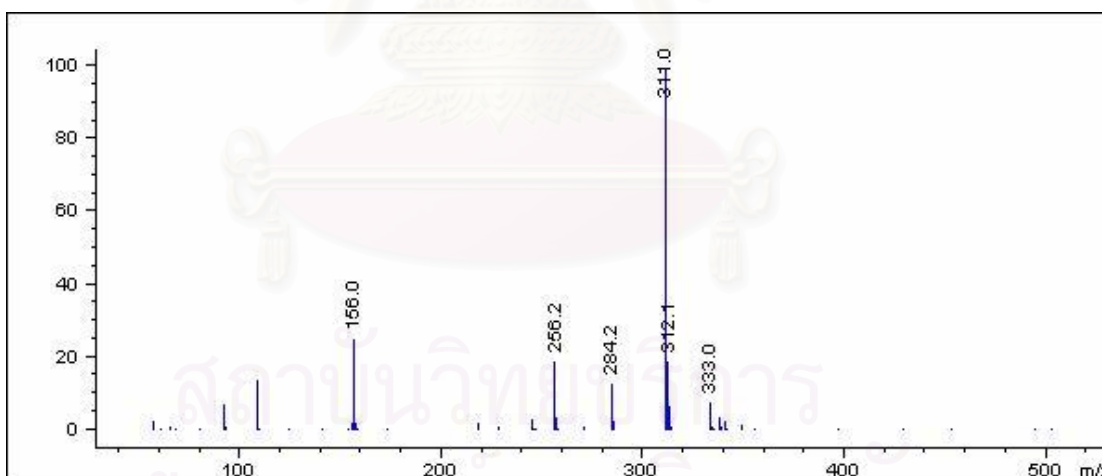
Figure B-6 Mass spectrum of standard sulfadimethoxine (SDM) 5.00 ppm analyzed
by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage
3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
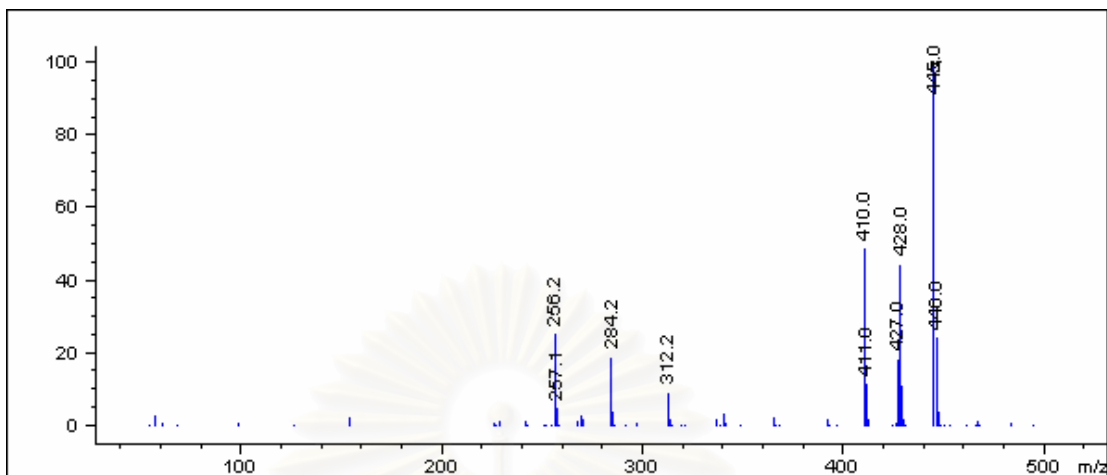
Figure B-7 Mass spectrum of standard tetracycline (TC) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
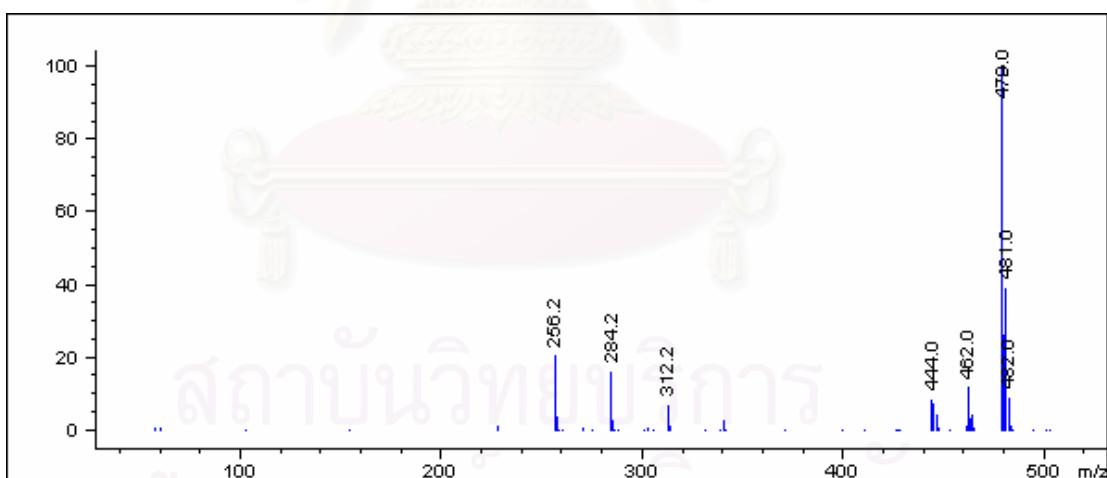
Figure B-8 Mass spectrum of standard chlortetracycline (CTC) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
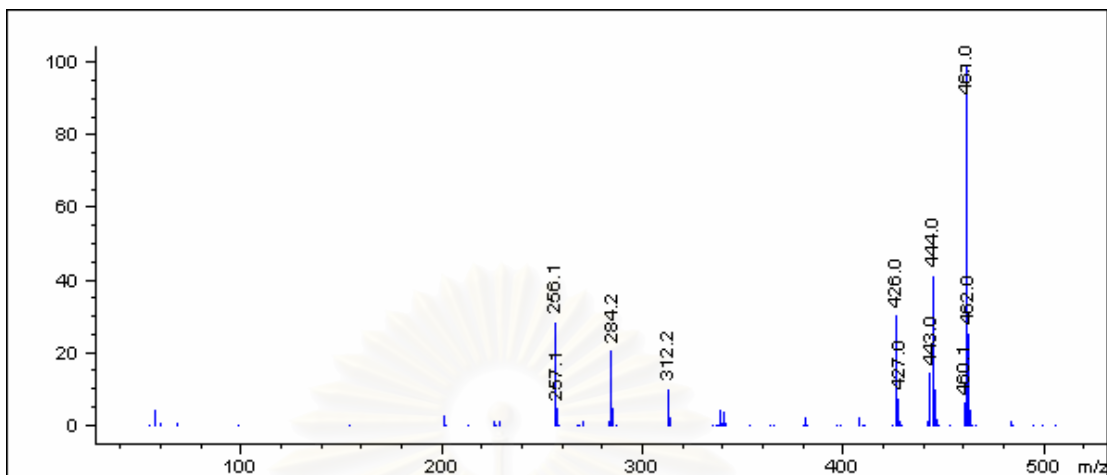
Figure B-9 Mass spectrum of standard oxytetracycline (OTC) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).
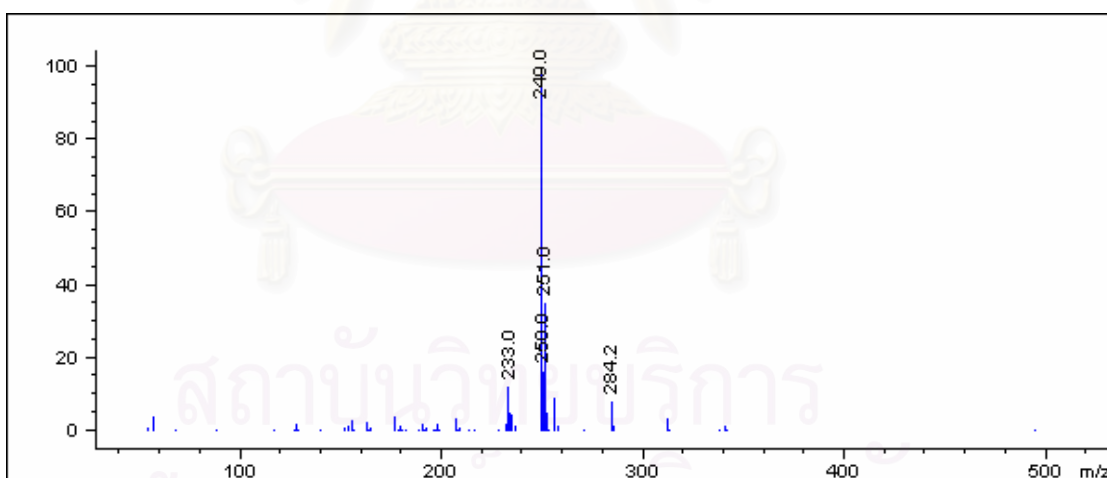
Figure B-10 Mass spectrum of standard pyrimethamine (PYR) 5.00 ppm analyzed by LC-FIA-ESI-MS at fragmentor voltage 130 V and capillary voltage 3,000 V. Mobile phase is a 1mM oxalic acid: ACN (50: 50, v/v).

APPENDIX C

LC-MS Method Information

Method: D:\HPCHEM\1\METHODS\URAIRAT.M of 1/20/2006 1:13:15 PM

1100 Quaternary Pump 1

Control

    Column Flow   :   0.300 ml/min

    Stoptime   :   22.00 min

    Posttime   :   10.00 min

Solvents

    Solvent A   :   92.0 % (1 mM oxalic acid in water-5% ACN)

    Solvent B   :   8.0 % (1 mM oxalic acid in water-95% ACN)

    Solvent C   :   0.0 % (H_2O)

    Solvent A   :   0.0% (ACN)

Time Table

No.	Time (min)	A	B	C	D	Flow Rate ml/min
1	0.00	92.0	8.0	0.0	0.0	0.300
2	2.00	92.0	8.0	0.0	0.0	0.300
3	5.00	55.0	45.0	0.0	0.0	0.300
4	9.00	57.0	43.0	0.0	0.0	0.300
5	12.00	45.0	55.0	0.0	0.0	0.300
6	15.00	5.0	95.0	0.0	0.0	0.300
7	21.00	5.0	95.0	0.0	0.0	0.300
8	22.00	92.0	8.0	0.0	0.0	0.300

PressureLimits

Minimum Pressure	:	0 bar
Maximum Pressure	:	400 bar

Auxiliary

Maximal Flow Ramp	:	100.00 ml/min^2
Primary Channel	:	Auto
Compressibility	:	100*10^-6/bar
Minimal Stroke	:	Auto

Store Parameters

Store Ratio A	:	Yes
Store Ratio B	:	Yes
Store Ratio C	:	Yes
Store Ratio D	:	Yes
Store Flow	:	Yes
Store Pressure	:	Yes

Mass Spectrometer Detector

General Information

Use MSD	:	Enabled
Ionization Mode	:	API-ES
Tune File	:	atunes.tun
StopTime	:	13.00
Time Filter	:	Enabled
Data Storage	:	Condensed
Peakwidth	:	0.08 min
Scan Speed Override	:	Disabled

Signals

[Signal 1]

Polarity	: Positive
Fragmentor Ramp	: Not Applicable

Sim Parameters

Time (min)	Group Name	SIM Ion	Frag-mentor	Gain EMV	SIM Resol.	Actual Dwell
2.00	Cpd.1-8	108.00	130	1.0	Low	37
		124.00	130			
		126.00	130			
		156.00	130			
		249.00	130			
		251.00	130			
		256.00	130			
		279.00	130			
		281.00	130			
		410.00	130			
		426.00	130			
		445.00	130			
		461.00	130			
		479.00	130			
		481.00	130			
9.40	Cpd.9	156.00	130	1.0	Low	289
		254.00	130			
10.40	Cpd.10	156.00	130	1.0	Low	289
		311.00	130			

[Signal 2]

Not Active

[Signal 3]

Not Active

[Signal 4]

Not Active

Spray Chamber

[MSZones]

Gas Temp	:	300 C	maximum 350 C
DryingGas	:	10.0 l/min	maximum 13.0 l/min
Neb Pres	:	35 psig	maximum 60 psig

VCap (Positive)	:	3000 V	
VCap (Negative)	:	0 V	

[Time Table]

Time Table is empty.

FIA Series

FIA Series in this Method   :   Disabled

Time Setting

   Time between Injections   :   0.73 min

   Injection Loop Flush Time   :   0.17 min

....

Agilent 1100 Autosampler 1

....

Injection

   Injection Mode   :   Needle Wash

   Injector volume   :   5.00 ml/min

   Wash Vial   :   81

   Optimization   :   none

Auxiliary

   Drawspeed   :   100 ml/min

   Ejectspeed   :   100 ml/min

   Draw position   :   0.0 mm

Time

   Stoptime   :   As Pump

   Posttime   :   Off

....

Agilent 1100 Column Thermostat 1

....

Temperature settings

   Left temperature   :   35.0 °C

   Right temperature   :   40.0 °C

   Enable analysis   :   when Temp. is within setpoint +/- 0.8 °C

Store left temperature   : Yes

Store right temperature : Yes

Time

 Stoptime   : As pump

 Posttime   : Off

Column Switching Value : Column 1

APPENDIX D

Pipette 10 mL of milk sample into separatory funnel

↓

Add 50 mL of chloroform

↓

The mixture was shaking vigorously for 1 min and vents the excess pressure

↓

Repeat the shaking procedure at one time and let phases separate for 1 min

↓

Repeat the shaking procedure at twice and let phases separate for 5 min

↓

The chloroform phase was draw off from separatory funnel to filter paper and collect them in pear-shaped flask

↓

Rinse filter paper twice with 5 mL portion of chloroform and collect the washings in the same flask

↓

Evaporate chloroform solution to dryness on rotary evaporator at 32 ± 2 °C

Figure D-1 Extraction procedure of <u>method 1</u> followed by *Bacteriological Analytical Manual Online*, chapter 20B, January 2001. (Rapid HPLC determination of sulfamethazine in milk).

```
┌─────────────────────────────────────────────────────────┐
│           Re-dissolve with 5 mL hexane                    │
│  and agitate vigorously on vortex mixer for 1 min         │
└─────────────────────────────────────────────────────────┘
                            │
                            ▼
┌─────────────────────────────────────────────────────────┐
│  Add 1.00 mL of 0.1 M potassium dihydrogen phosphate into hexane solution │
└─────────────────────────────────────────────────────────┘
                            │
                            ▼
┌─────────────────────────────────────────────────────────┐
│  The mixture was agitate vigorously on vortex mixer for 1 min at least 4 times │
└─────────────────────────────────────────────────────────┘
                            │
                            ▼
┌─────────────────────────────────────────────────────────┐
│       Collect the potassium dihydrogen phosphate phase    │
└─────────────────────────────────────────────────────────┘
                            │
                            ▼
┌─────────────────────────────────────────────────────────┐
│   Filtered through 0.45 µm syringe filter for LC-MS analysis │
└─────────────────────────────────────────────────────────┘
```

Figure D-1 (*continued*).

Milk 10 mL was diluted with 100 mL of distilled water

↓

Stirred with magnetic stirrer for 10 min at room temperature

↓

The mixture was passed through the Carbograph 4 cartridge connected with vacuum manifold

↓

The cartridge was pre-condition sequentially with 20 mL of hydrochloric acid solution (pH 2) and 5 mL of distilled water

↓

After sample loading, the cartridge was washed with 25 mL of water and then air-dried by forcing room air for 1 min

↓

The analytes were eluted with 1.5 mL of methanol followed by 6 mL of CH_2Cl_2/methanol (80:20, v/v) solution acidified with 10 mM TFA

↓

The analytes were eluted with 1.5 mL of methanol followed by 6 mL of CH_2Cl_2/methanol (80:20, v/v) solution acidified with 10 mM trifluoroacetic acid

↓

The solvent was evaporated to dryness at 40 ºC under nitrogen stream

↓

Re-dissolve with 1.00 mL of 0.1 M ammonium acetate solution and filtered through 0.45 µm syringe filter for LC-MS analysis

Figure D-2 Extraction procedure of <u>method 2</u> followed by *J. Agric. Food Chem.*, 51, 2003. (A simple and sensitive liquid chromatography-mass spectrometry confirmatory method for analyzing sulfonamide antibacterials in milk and egg).

Milk 5.00 g was placed in a centrifuge tube

Add 2 mL of 20% trichloroacetic acid and the mixture was shaken

Add 20 mL of McIlvain buffer and the mixture was centrifuged
at 4000 rpm for 20 min

The supernatant was applied to a Oasis® HLB cartridge which previously
activated with 3 mL methanol and 2 mL of water

After sample loading, the cartridge was washed with 2 mL of 5%methanol in
water

The analytes were eluted with 3 mL methanol

The solvent was evaporated under a nitrogen stream

Re-dissolved the residue with 1.00 mL of methanol and filtered through
a 0.45 μm syringe filter for LC-MS analysis

Figure D-3 Extraction procedure of method 3 followed by *J. Chromatogr. A*, 987,
2003. (Validation of high-performance liquid chromatography method for the
determination of oxytetracycline, tetracycline, chlortetracycline, and doxycycline
in bovine milk and muscle).

Sample 5.00g was blended with 20 mL of 0.1 M Na2EDTA-McIlvain buffer and centrifuged at 850 rpm for 5 min

↓

The residue was blended and then centrifuged two times with 20 and 10 mL of 0.1 M Na_2EDTA-McIlvain buffer

↓

The supernatants were combined, centrifuged at 850 rpm for 15 min and filtered

↓

The filtrate was applied to a Bond Elut C_{18} SPE cartridge

↓

Bond Elute C_{18} was pre-condition with saturated aqueous Na_2EDTA

↓

After sample loading, the cartridge was washed with 20 mL of distilled water and then air-dried by aspiration for 5 min

↓

The analytes were eluted with 10 mL ethyl acetate followed by 20 mL of methanol-ethyl acetate (5:95, v/v)

↓

The eluate was evaporated to dryness under reduced pressure at 30 ºC

↓

Re-dissolved with 1.00 mL of water and filtered through a 0.45 µm syringe filter for LC-MS analysis

Figure D-4 Extraction procedure of <u>method 4</u> followed by *J. Chromatogr. B*, 693, 1997. (Improvement of chemical analysis of antibiotics XXIII[1]. Identification of residual tetracyclines in bovine tissues by electrospray high-performance liquid-chromatography-tandem mass spectrometry).

```
┌─────────────────────────────────────────────────────────────────────┐
│   Milk 10 mL was extracted with 10 mL of succinic acid (pH 4.0)       │
│              and shake vigorously for 10 s                            │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│  The mixture solution was centrifuged at 3800 rpm for 5 min and the   │
│  supernatant was filtered into a centrifuge tube (first portion)      │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│  The residues of milk was re-extracted with 10 mL of succinic acid    │
│  (pH 4.0), shake vigorously for 10 s, and then centrifuged at         │
│  3800 rpm for 5 min                                                   │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│  The supernatant was filtered and combined with the first portion.    │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│  The supernatant was applied to a Oasis® HLB cartridge which          │
│  pre-conditioned by washing sequentially with 4 mL of methanol,       │
│  4 mL of water, and 4 mL of succinic acid (pH 4.0)                     │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│  After sample loading, the cartridge was washed with 4 mL of          │
│  distilled water and then air-dried by drawing full vacuum for 5 min   │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│           The analytes were eluted with 2 mL of methanol              │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│     The eluate was diluted to 10 mL with 0.01 M oxalic acid           │
└─────────────────────────────────────────────────────────────────────┘
                                 │
                                 ▼
┌─────────────────────────────────────────────────────────────────────┐
│  The analyze solution was filtered through a 0.45 μm syringe filter    │
│  for direct injection and analysis by LC-MS                           │
└─────────────────────────────────────────────────────────────────────┘
```
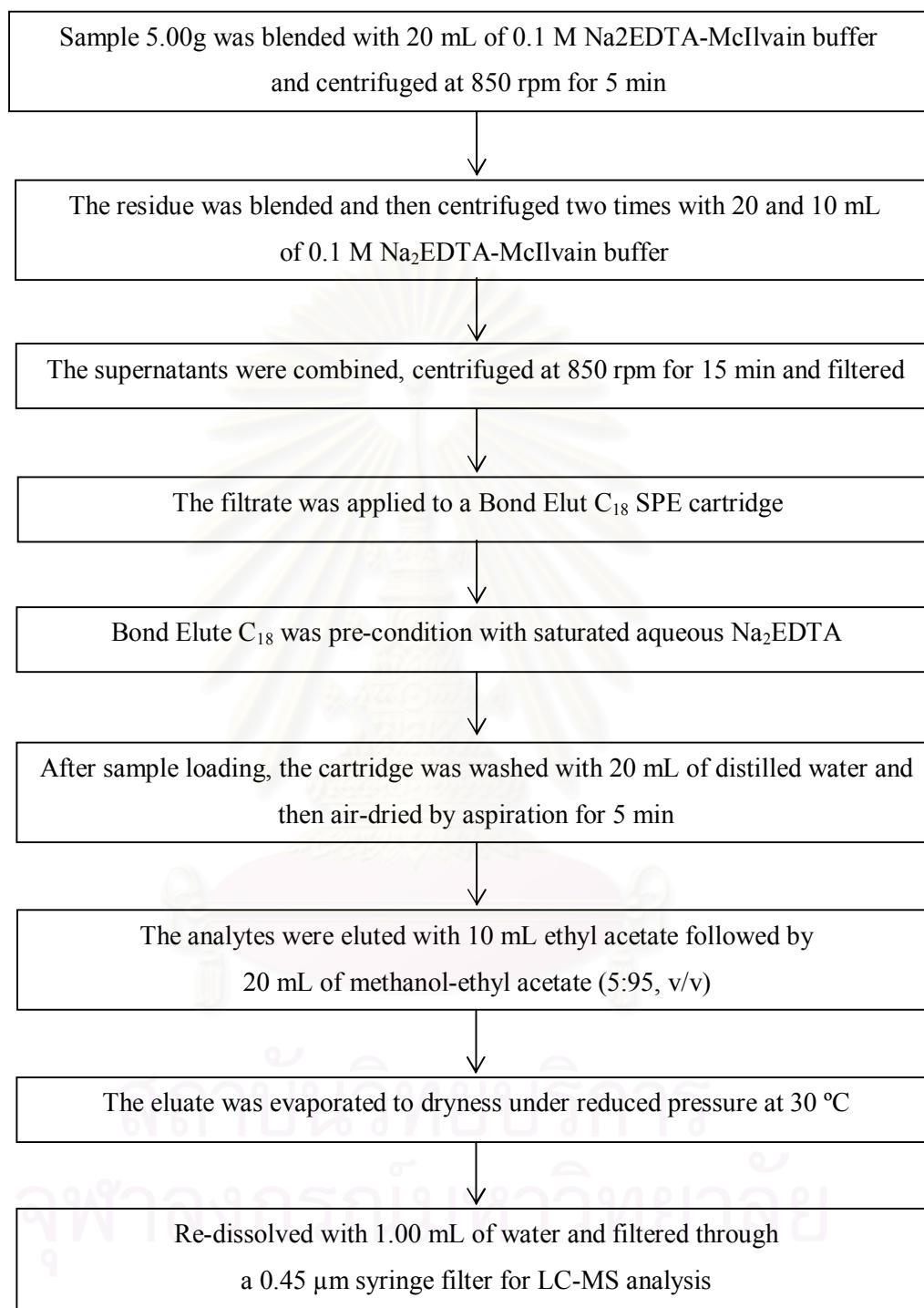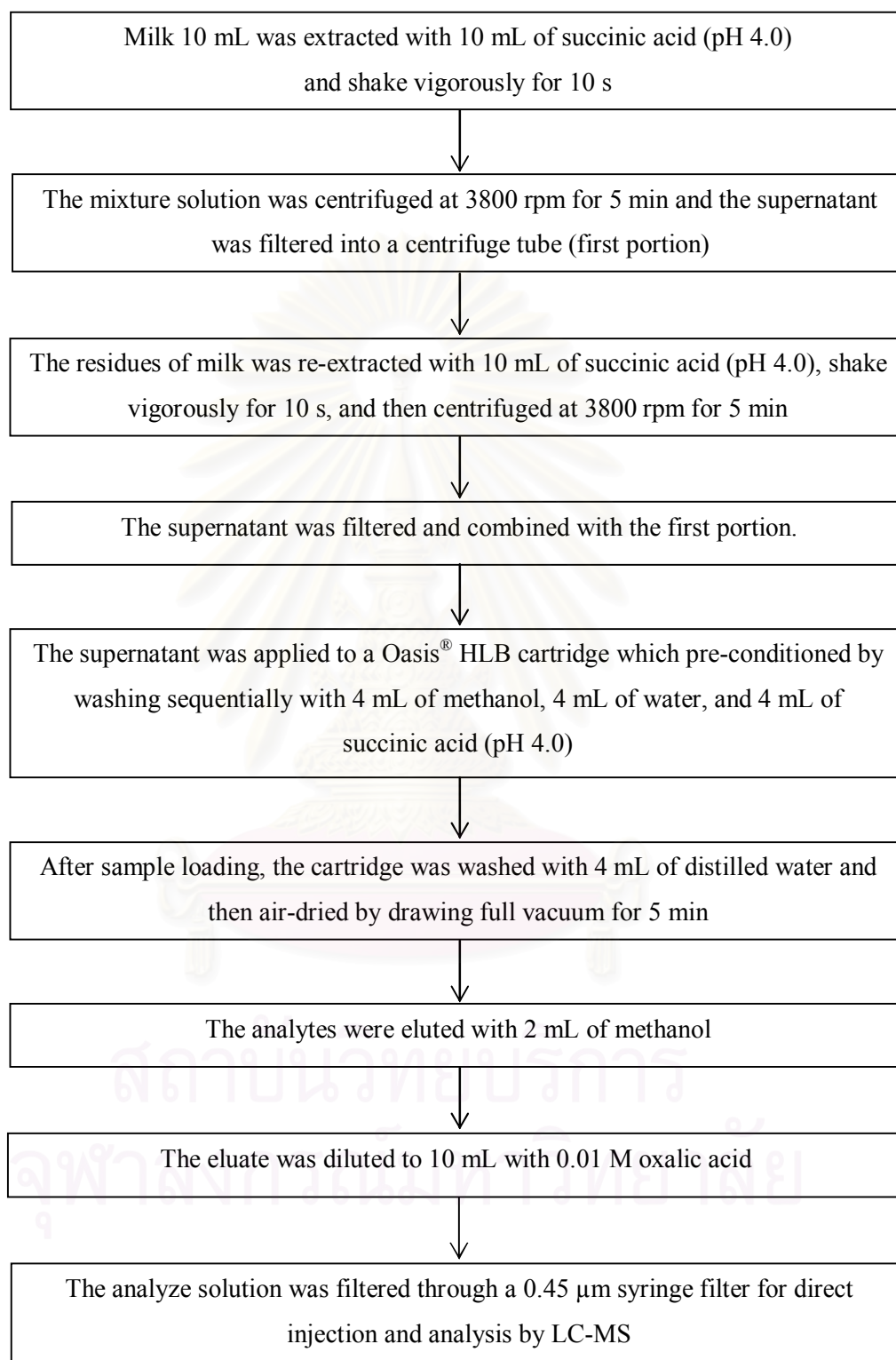
Figure D-5 Extraction procedure of <u>method 5</u> followed by *Analytica Chimica Acta,* 2004. (Determination of tetracycline residues in shrimp and whole milk using liquid chromatography with ultraviolet detection and residue confirmation by mass spectrometry).
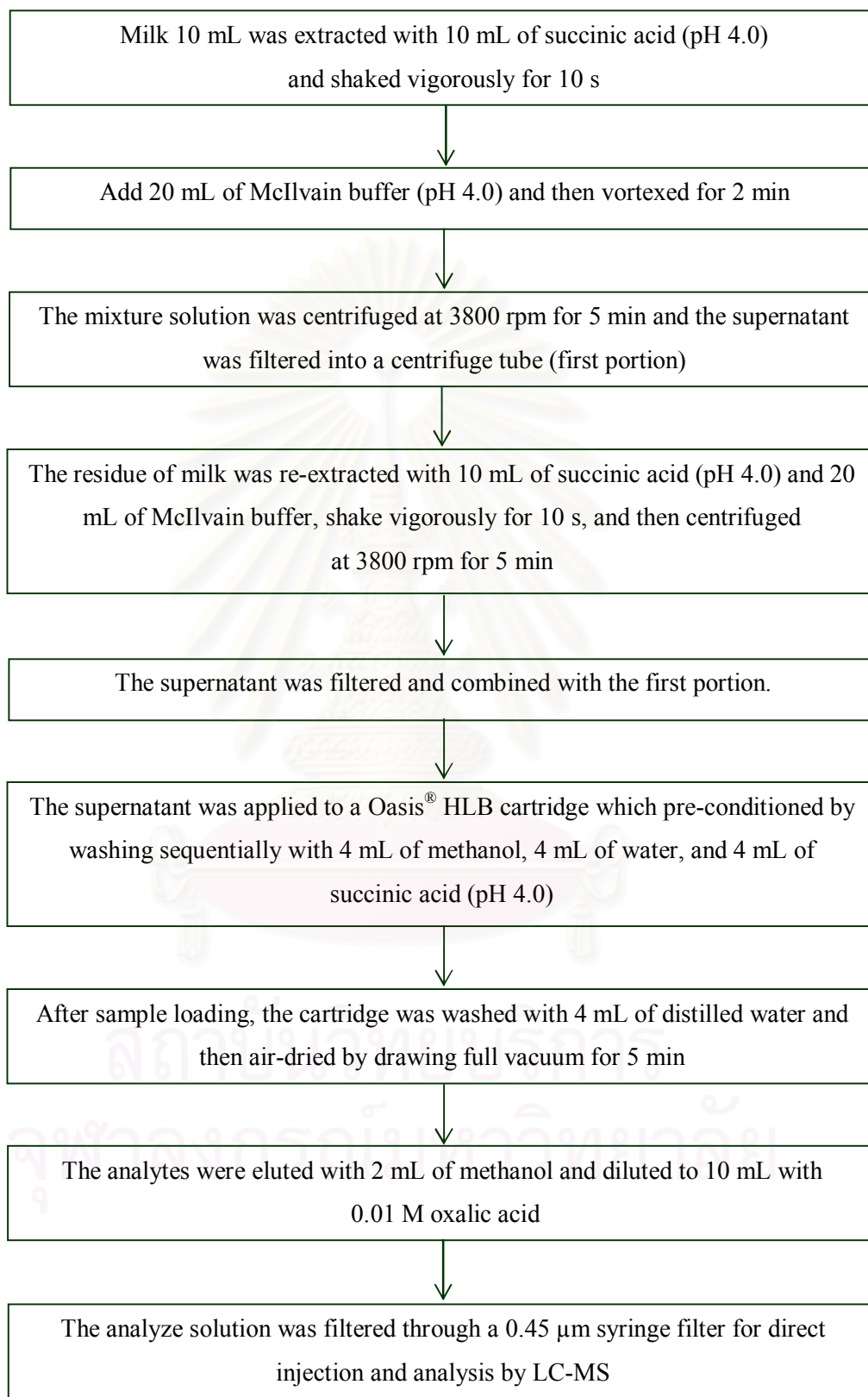
Milk 10 mL was extracted with 10 mL of succinic acid (pH 4.0)
and shaked vigorously for 10 s

Add 20 mL of McIlvain buffer (pH 4.0) and then vortexed for 2 min

The mixture solution was centrifuged at 3800 rpm for 5 min and the supernatant
was filtered into a centrifuge tube (first portion)

The residue of milk was re-extracted with 10 mL of succinic acid (pH 4.0) and 20
mL of McIlvain buffer, shake vigorously for 10 s, and then centrifuged
at 3800 rpm for 5 min

The supernatant was filtered and combined with the first portion.

The supernatant was applied to a Oasis® HLB cartridge which pre-conditioned by
washing sequentially with 4 mL of methanol, 4 mL of water, and 4 mL of
succinic acid (pH 4.0)

After sample loading, the cartridge was washed with 4 mL of distilled water and
then air-dried by drawing full vacuum for 5 min

The analytes were eluted with 2 mL of methanol and diluted to 10 mL with
0.01 M oxalic acid

The analyze solution was filtered through a 0.45 µm syringe filter for direct
injection and analysis by LC-MS

Figure D-6 Extraction procedure of <u>method 6</u> was adapted from method 5.

Sample 5.00 g was extracted with 2 mL of 40% sulfuric acid solution
and then added 1 mL of saturated Na_2EDTA in water

↓

The mixture solution was shaked for 60 min and then centrifuged for 5 min

↓

The supernatant was applied to a Oasis® HLB cartridge

↓

HLB Oasis cartridge was sequentially pre-condition with 3 mL methanol, 3 mL of
0.5 N hydrochloric acid solution, and 3 mL of distilled water

↓

After sample loading, the cartridge was washed with 1 mL of distilled water and
to remove excess Na_2EDTA

↓

The analytes were eluted with 5 mL of methanol

↓

The eluate was evaporated to dryness under a flow of nitrogen gas at 30 ºC

↓

Re-dissolved with 1.00 mL of mobile phase and filtered through
a 0.45 µm syringe filter for LC-MS analysis

Figure D-7 Extraction procedure of <u>method 7</u> followed by *Anal Chem*, 73, 2001.
(Analysis of trace levels of sulfonamide and tetracycline antimicrobials in
groundwater and surface water using solid-phase extraction and liquid
chromatography/mass spectrometry).

Sample 5.00 g and anhydrous NaSO₄ 10.00 g were blended with 20 mL of
ethyl acetate for 2 min

↓

The mixture was centrifuged at 700 rpm for 5 min and the supernatant was
collected in round bottom flask

↓

The solid residue was re-extracted with 20 mL of ethyl acetate and the
supernatant was combined with the first fraction

↓

The combined extracts were evaporated to dryness at 35 ℃ and
re-dissolved in 5 mL of 50% ethyl acetate in *n*-hexane

↓

The solution was applied to a Bond Elut PSA which pre-conditioned
with 5 mL of *n*-hexane

↓

After sample loading, the cartridge was washed with 3 mL of *n*-hexane and
allowed to air-dried by aspiration for 3 min

↓

The analytes were eluted with 5 mL of 20% acetonitrile in
0.05 M ammonium formate

↓

The eluate was evaporated to dryness under a flow of nitrogen gas at 30 ℃

↓

Re-dissolved with 1.00 mL of 20% actonitrile -0.05 M ammonium formate and
filtered through a 0.45 µm syringe filter for LC-MS analysis

Figure D-8 Extraction procedure of <u>method 8</u> followed by *J. Chromatogr. A*, 898,
2000. (Application of ion-exchange cartridge clean-up in food analysis V,
Simultaneous determination of sulphonamide antibacterials in animal liver and
kidney using high-performance liquid chromatography with ultraviolet and mass
spectrometric detection).

APPENDIX E

E.1 Standard Calibration Curves
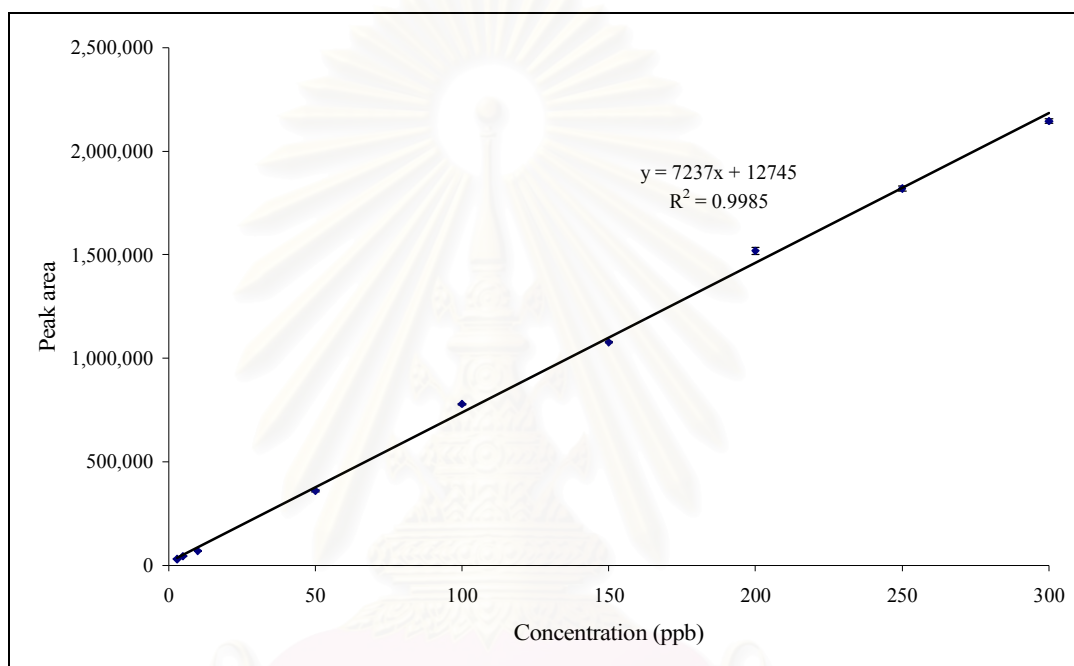
Figure E-1 Standard calibration curve of oxytetracycline (OTC) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
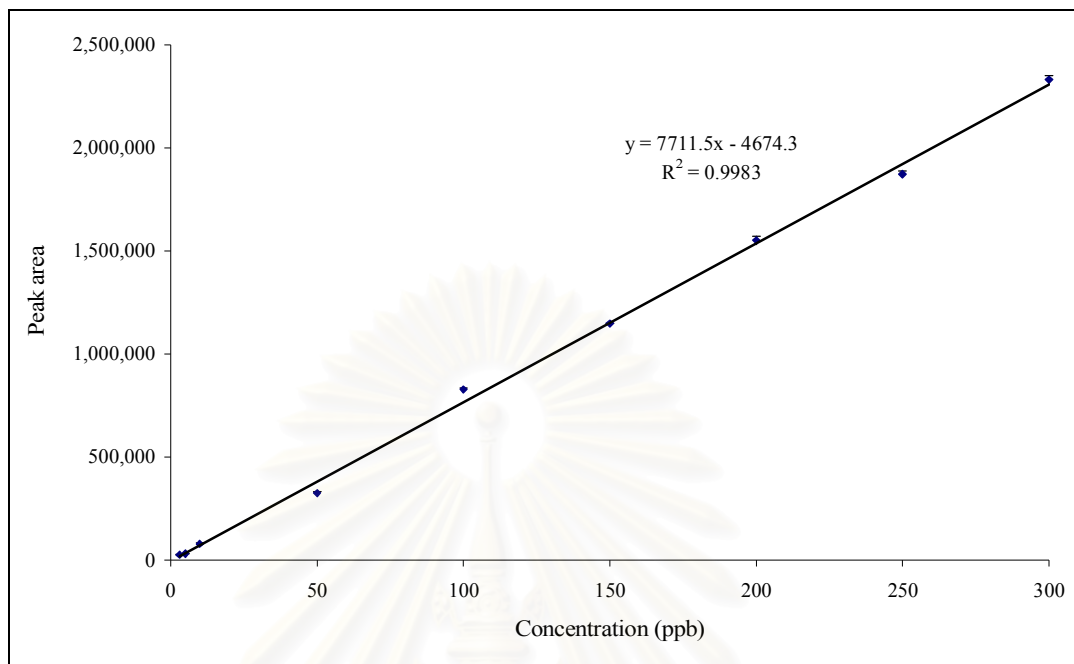
Figure E-2   Standard calibration curve of tetracycline (TC) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
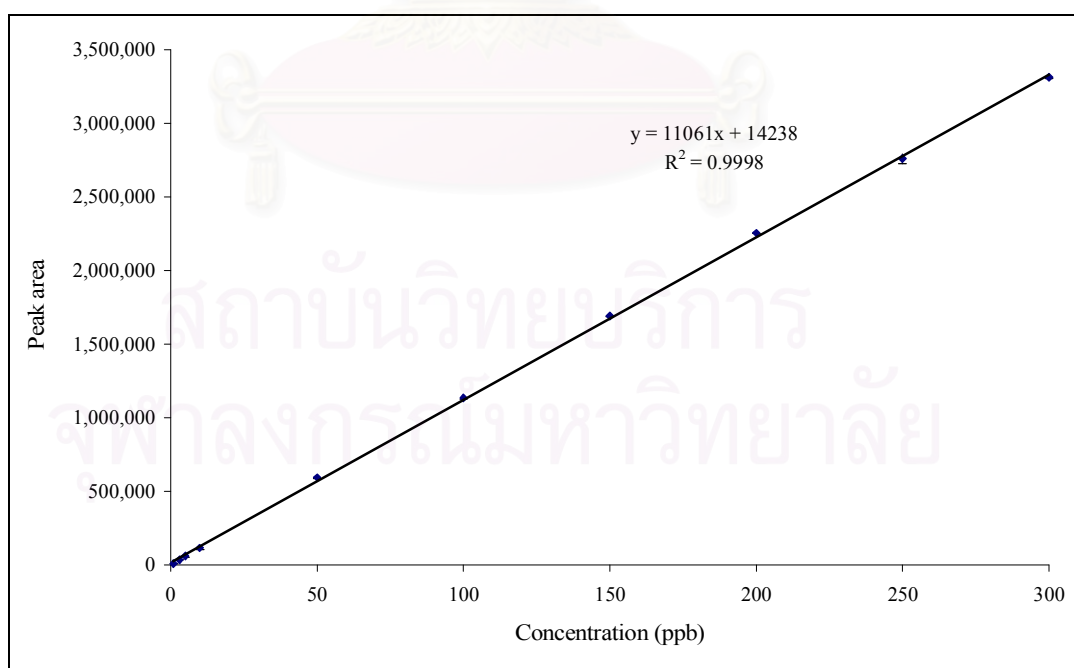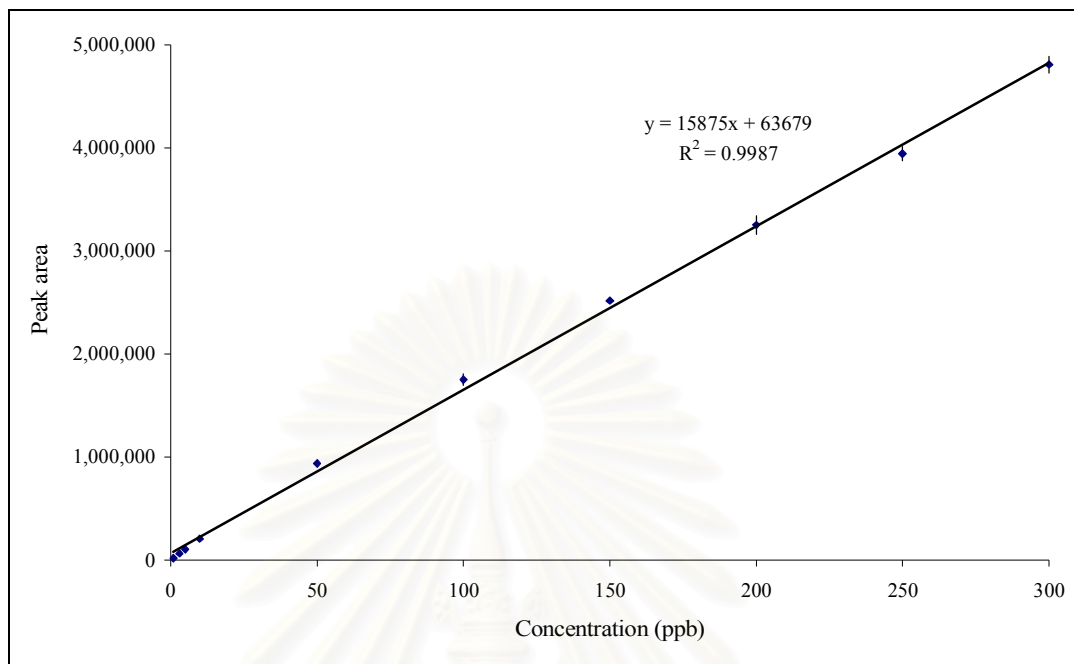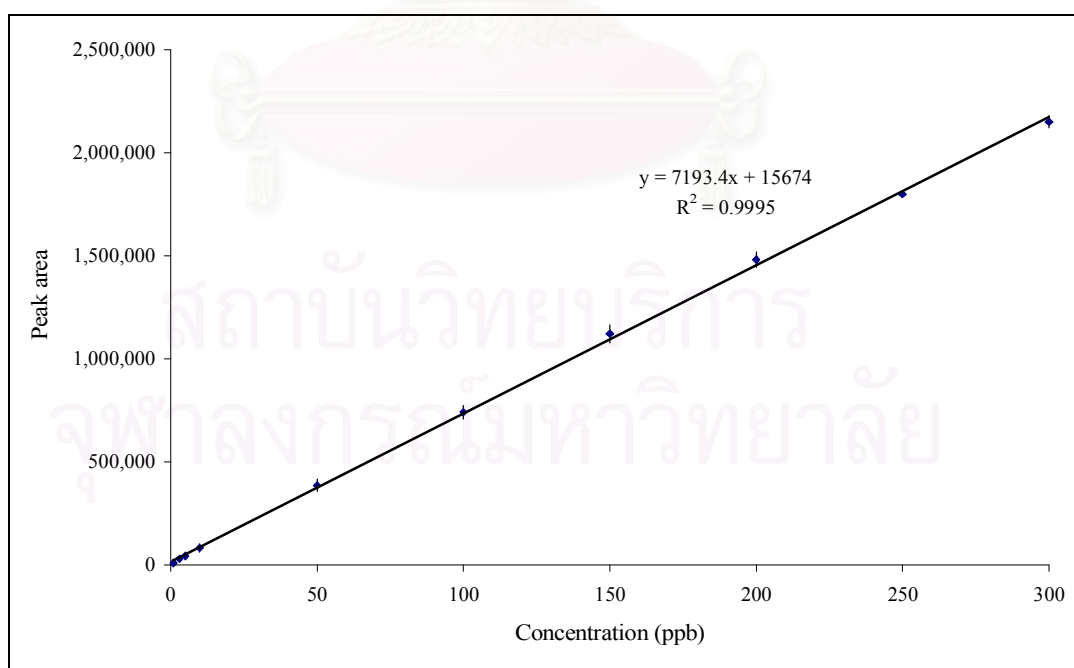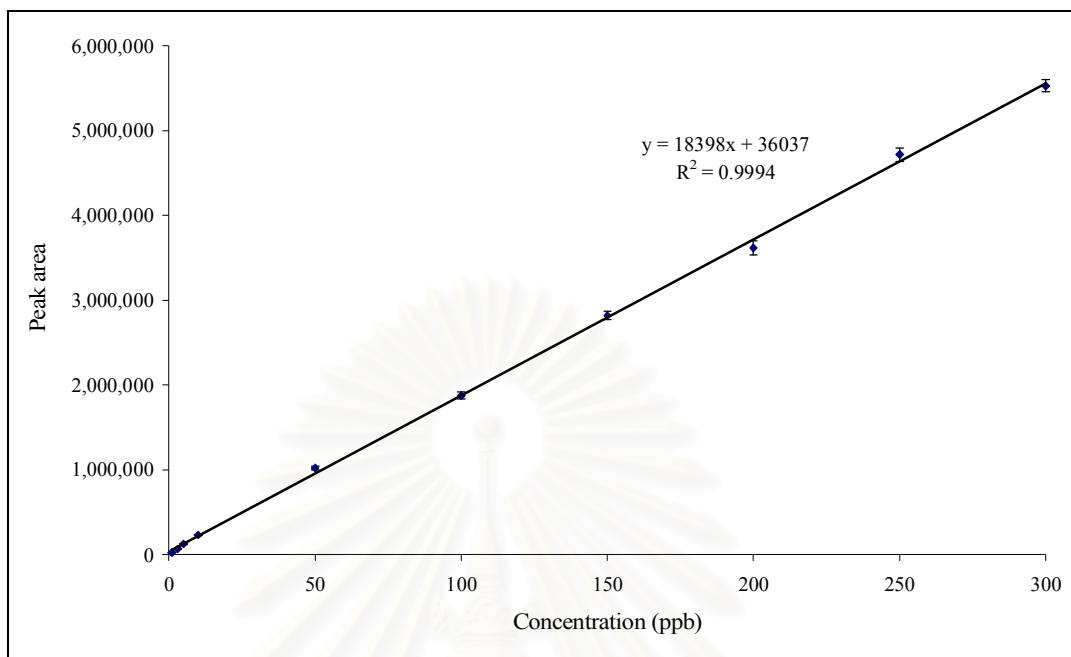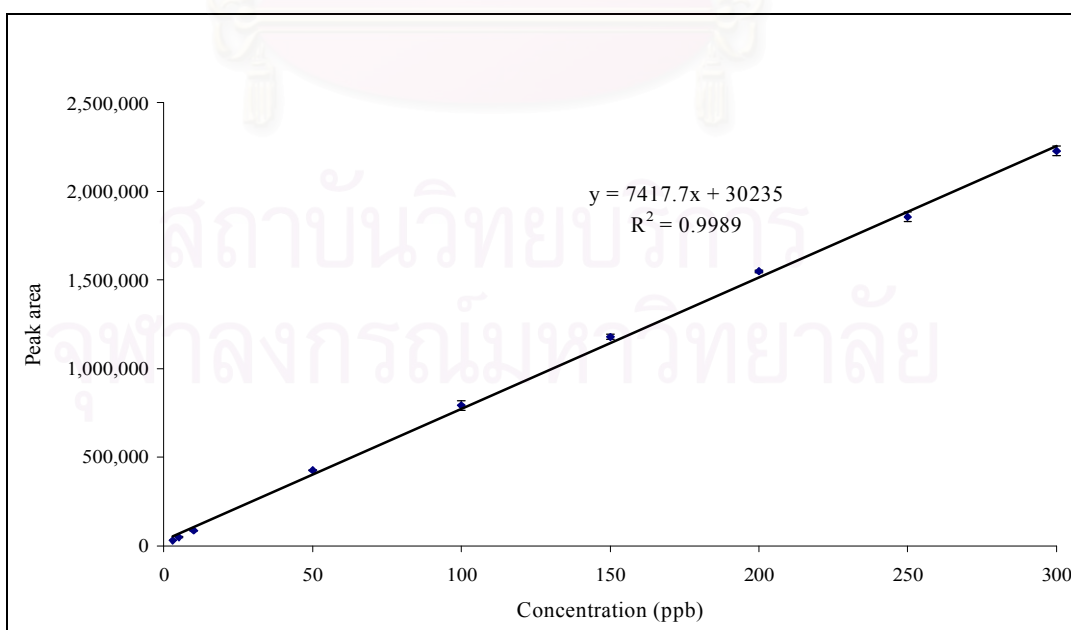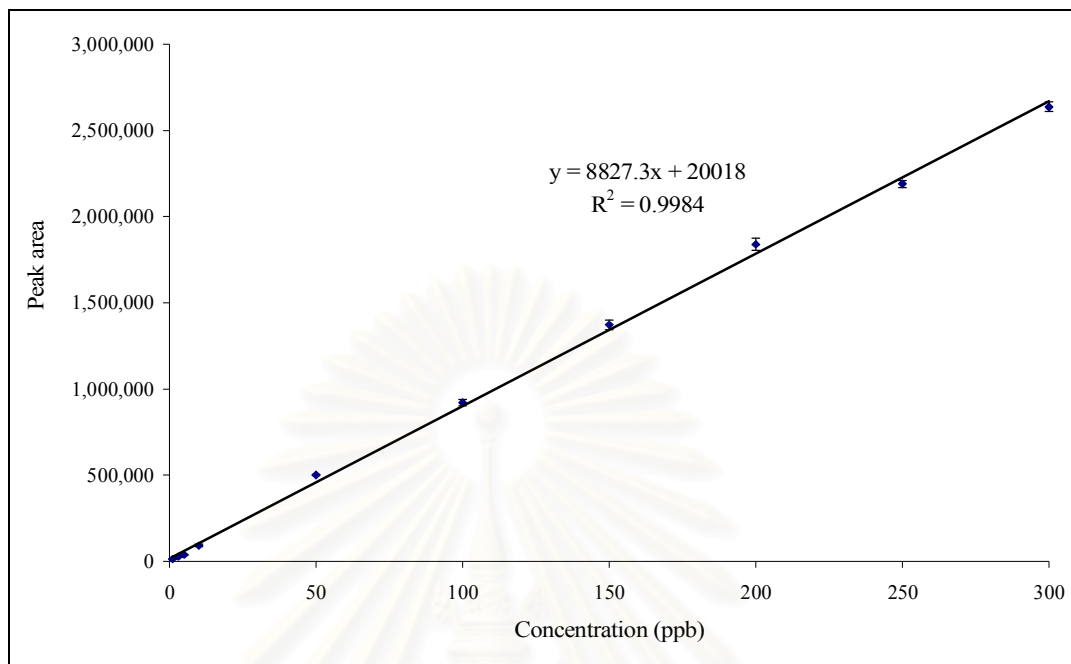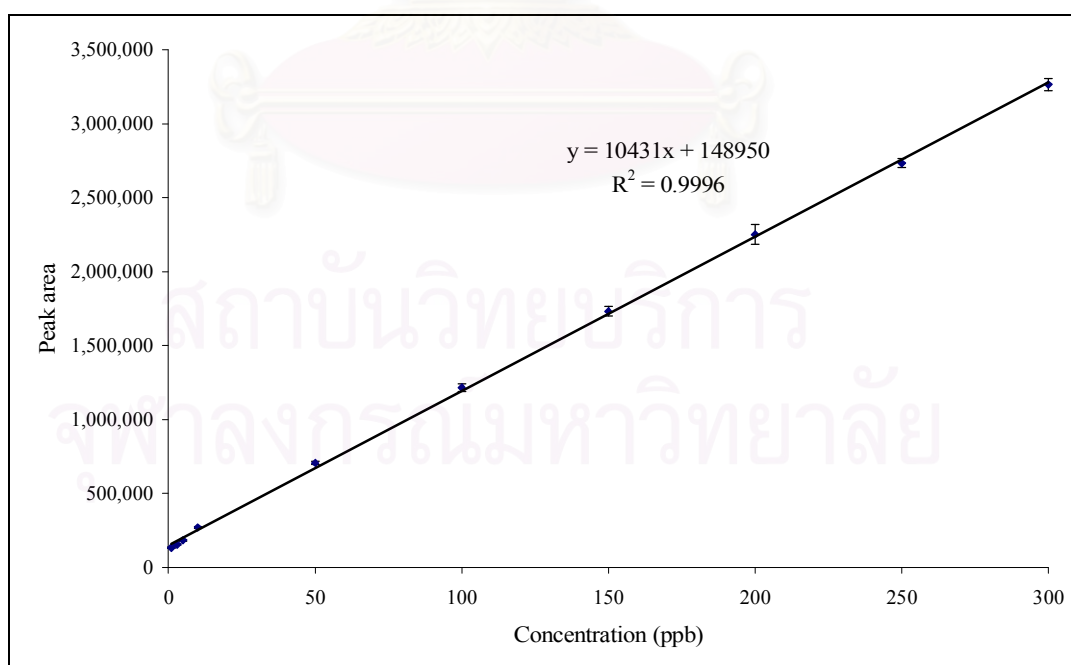

Figure E-3   Standard calibration curve of sulfadiazine (SDZ) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
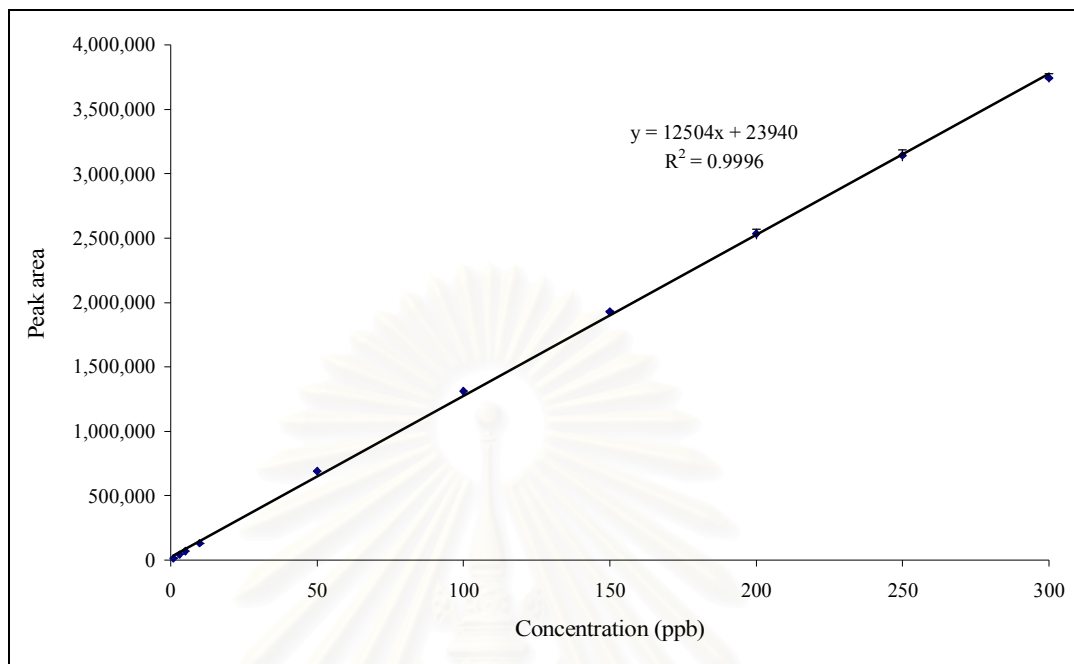
Figure E-4   Standard calibration curve of sulfathiazole (STZ) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
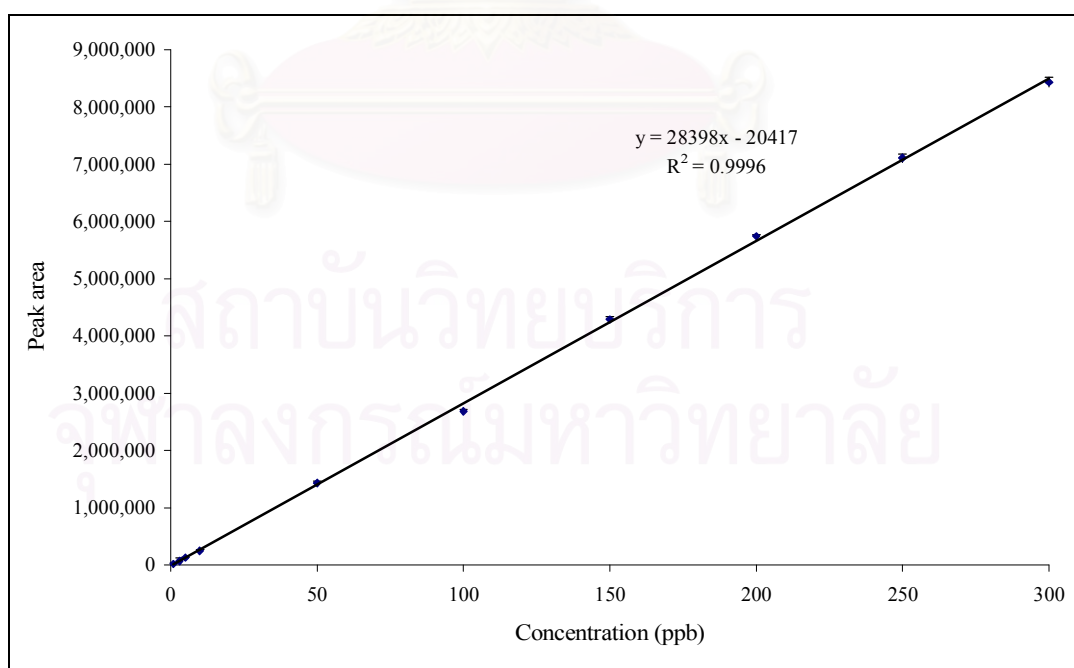
Figure E-5   Standard calibration curve of pyrimethamine (PYR) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
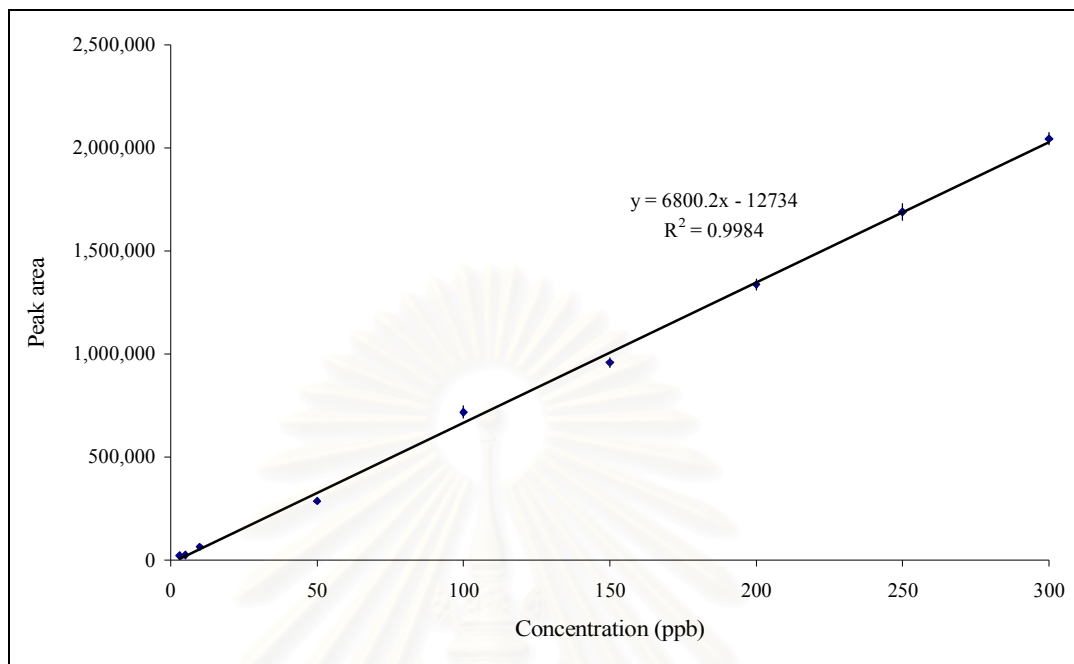
Figure E-6   Standard calibration curve of chlortetracycline (CTC) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
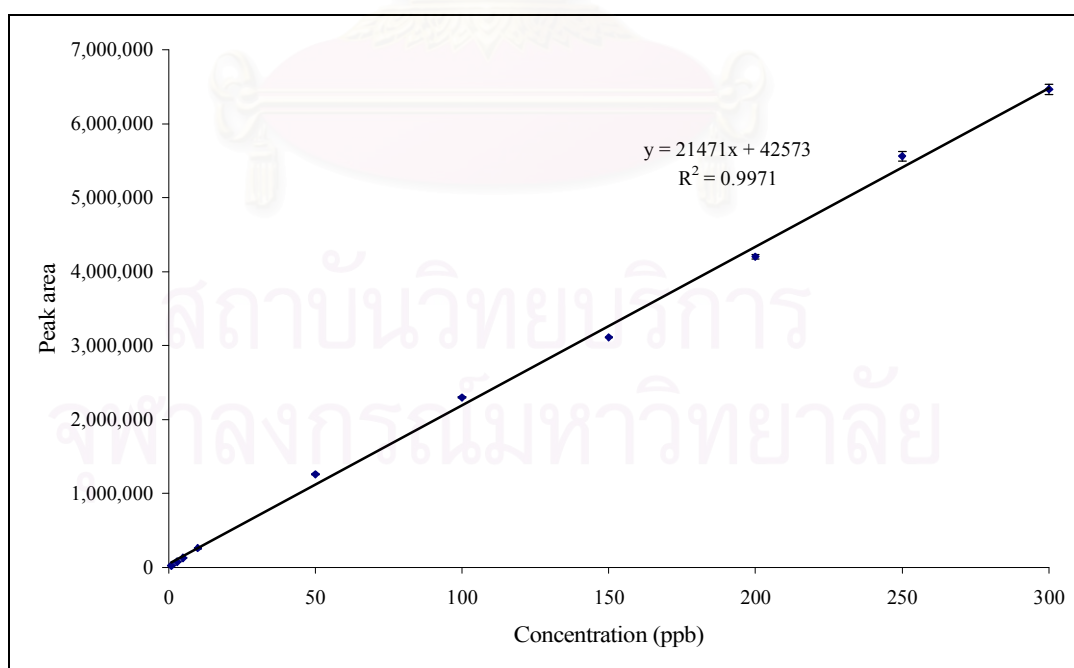
Figure E-7   Standard calibration curve of sulfamethazine (SMZ) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
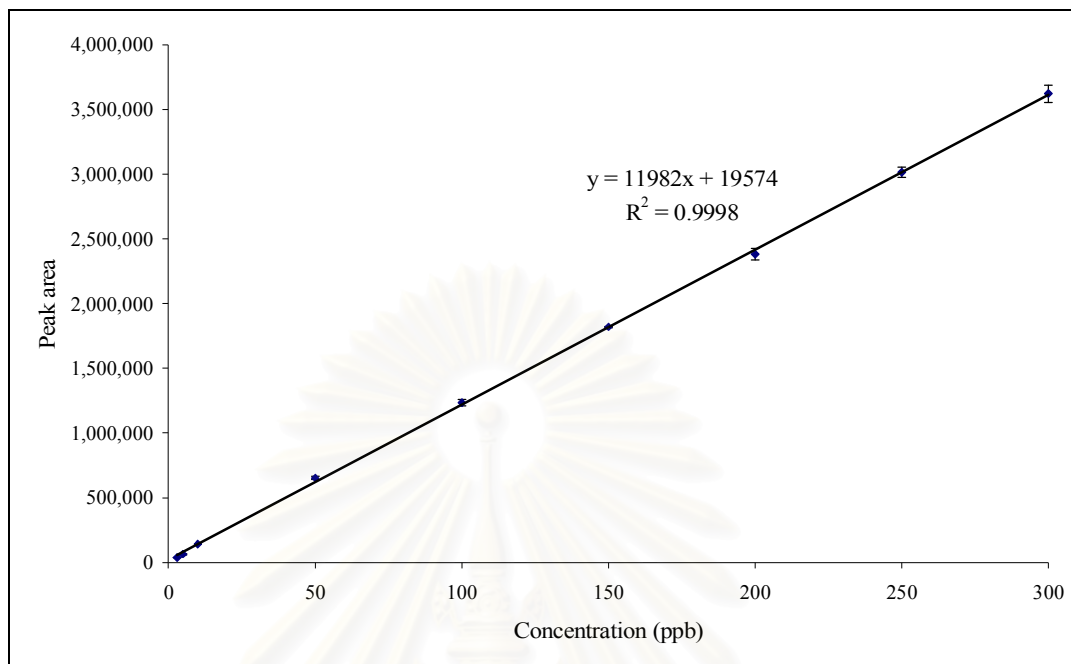
Figure E-8   Standard calibration curve of sulfamethoxypyridazine (SMP) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

Figure E-9   Standard calibration curve of sulfamethoxazole (SMX) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
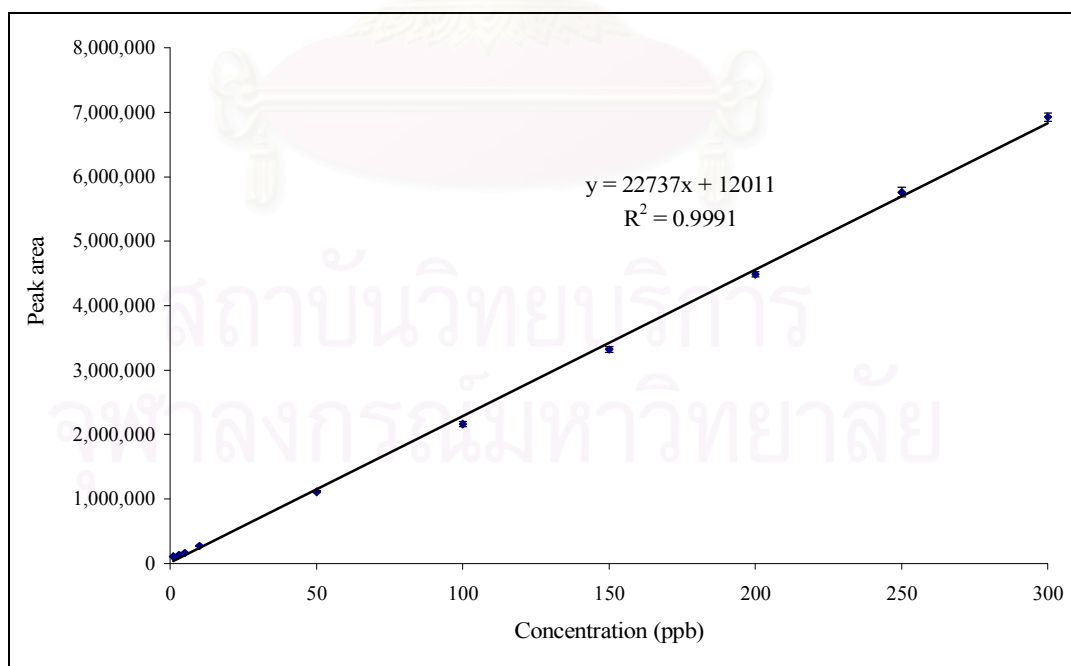
Figure E-10 Standard calibration curve of sulfadimethoxine (SDM) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
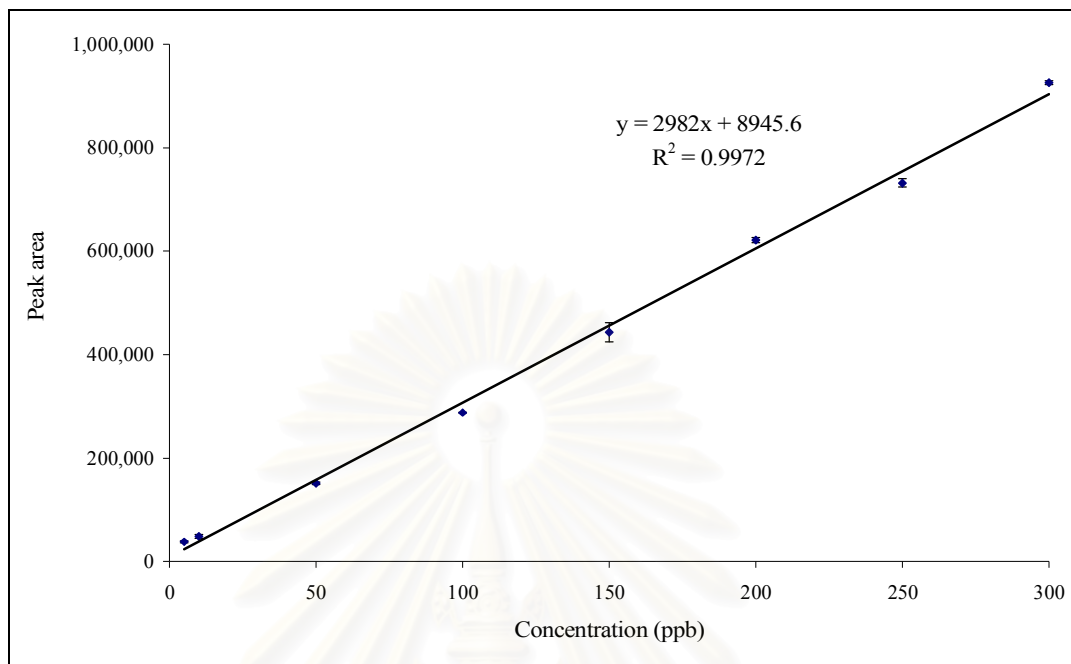
E.2 Matrix-Matched Calibration Curves

Figure E-11 Matrix-matched calibration curve of oxytetracycline (OTC) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

$$y = 8827.3x + 20018$$
$$R^2 = 0.9984$$

Figure E-12 Matrix-matched calibration curve of tetracycline (TC) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

$$y = 10431x + 148950$$
$$R^2 = 0.9996$$

Figure E-13 Matrix-matched calibration curve of sulfadiazine (SDZ) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

Figure E-14 Matrix-matched calibration curve of sulfathiazole (STZ) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

Figure E-15 Matrix-matched calibration curve of pyrimethamine (PYR) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

Figure E-16   Matrix-matched calibration curve of chlortetracycline (CTC) under the
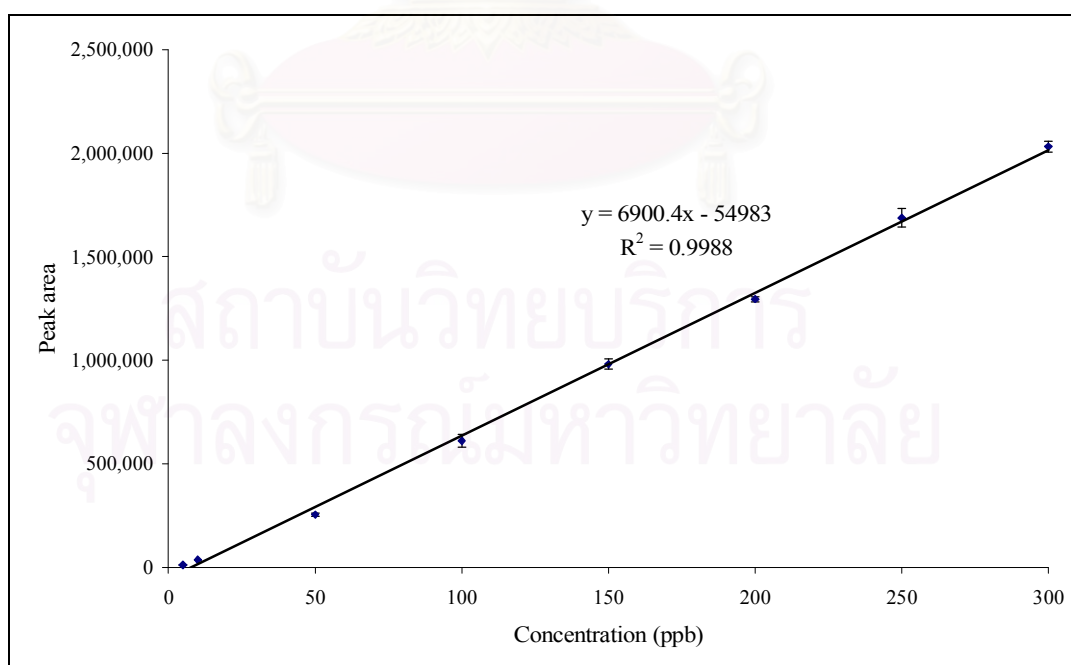optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

Figure E-17   Matrix-matched calibration curve of sulfamethazine (SMZ) under the
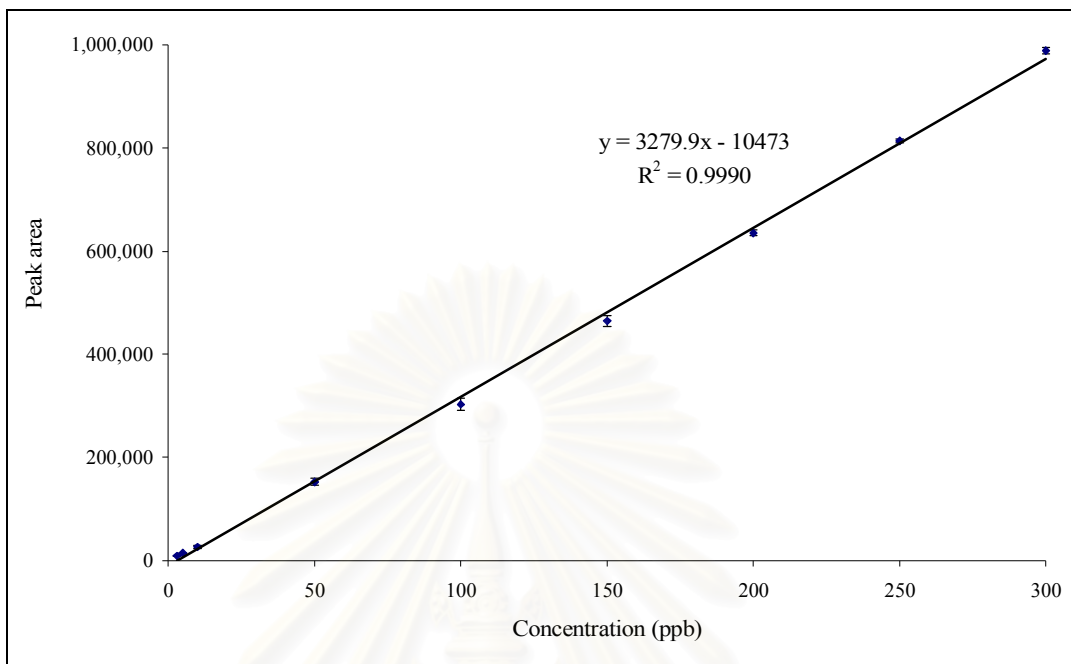optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

Figure E-18 Matrix-matched calibration curve of sulfamethoxypyridazine (SMP) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
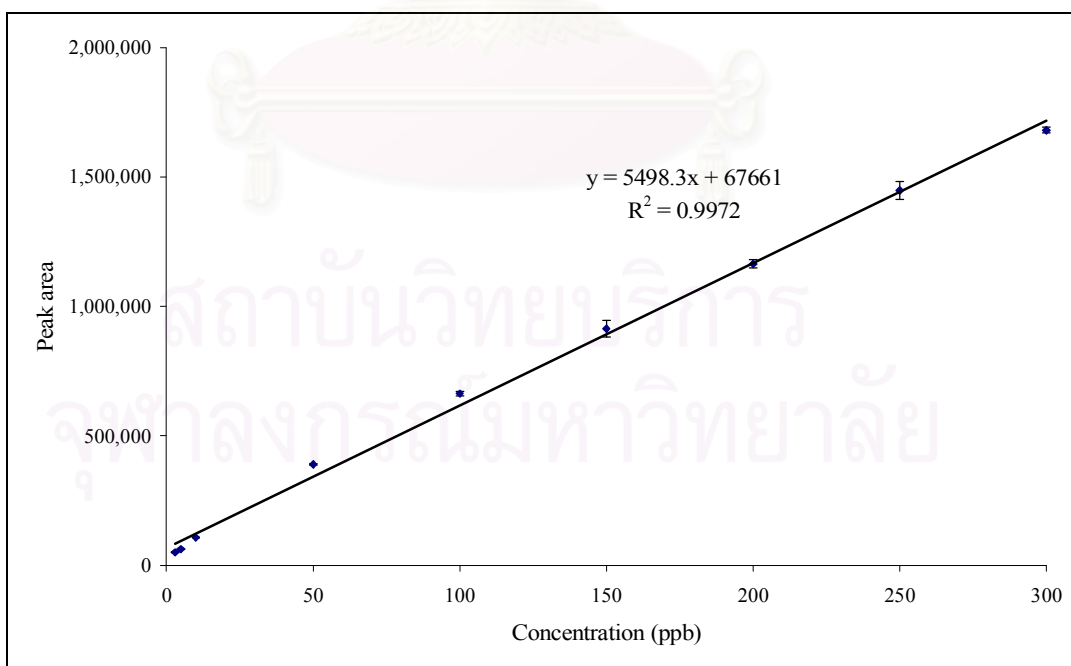

Figure E-19 Matrix-matched calibration curve of sulfamethoxazole (SMX) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.
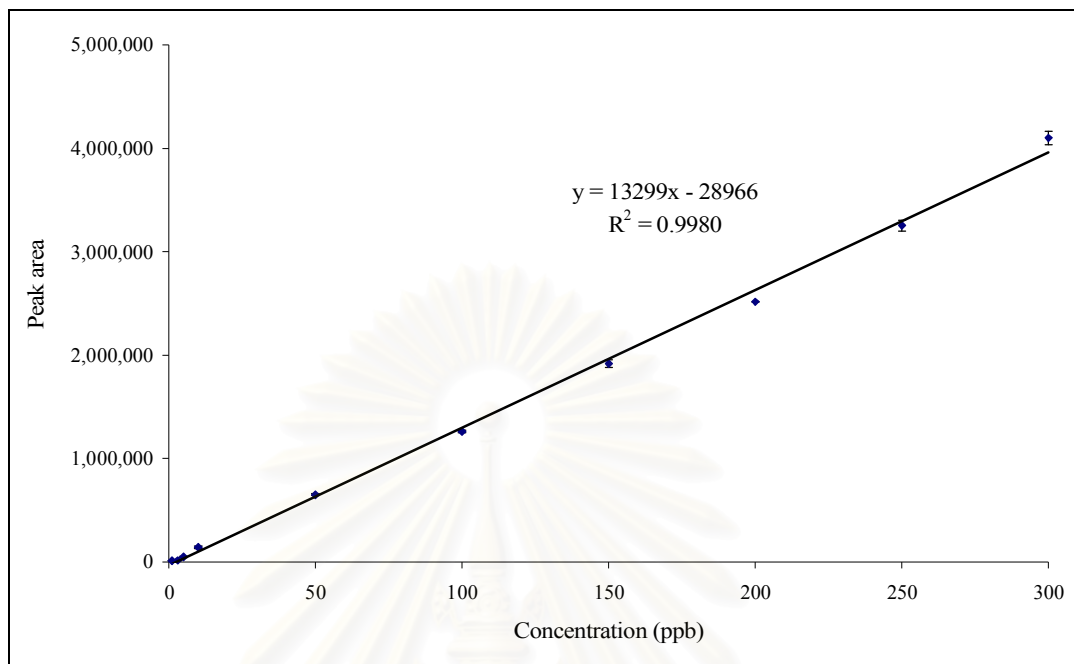
Figure E-20 Matrix-matched calibration curve of sulfadimethoxine (SDM) under the optimum LC-ESI-MS conditions listed in Table 4.2 and 4.5.

VITA

Miss Urairat Koesukwiwat was born on November 17, 1980, in Lopburi, Thailand. She received a Bachelor Science Degree in Chemistry from the Faculty of Science, Chulalongkorn University in 2002. After that, she continued study for a M.Sc. Program in Analytical Chemistry, Department of Chemistry, Faculty of Science, Chulalongkorn University and will received her Master of Science Degree in 2006.