การวิเคราะห์แบบพร้อมกันของสารปฏิชีวนะตกค้างในเมทริกซ์อาหารด้วยอันตรกิริยาชอบน้ำ
ควบคู่กับแทนเดมแมสสเปกโทรเมตรี

นางสาว ชญาดา เชี่ยวชาญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี   ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

SIMULTANEOUS ANALYSIS OF ANTIBIOTIC RESIDUES IN FOOD MATRICES USING HYDROPHILIC INTERACTION COUPLED WITH TANDEM MASS SPECTROMETRY

Miss Chayada Chiaochan

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Chemistry

Department of Chemistry

Faculty of Science

Chulalongkorn University

Academic Year 2009

Thesis Title   Simultaneous analysis of antibiotic residues in food matrices using hydrophilic interaction coupled with tandem mass spectrometry

By   Miss Chayada Chiaochan

Field of Study   Chemistry

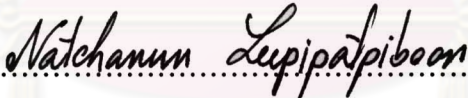Thesis Advisor   Assistant Professor Natchanun Leepipatpiboon, Dr.,rer.nat

---

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

……………………………………Dean of the Faculty of Science

(Professor Supot Hannongbua, Dr.,rer.nat)

THESIS COMMITTEE

………………………………………….. Chairman

(Associate Professor Sirirat Kokpol, Ph.D.)

…………………………………………. Thesis Advisor

(Assistant Professor Natchanun Leepipatpiboon, Dr.,rer.nat)

…………………………………………….. Examiner

(Puttaruksa Varanusupakul, Ph.D.)

…………………………………………….. External Examiner

(Assistant Professor Panthira Ketkaew, Ph.D.)

ชญาดา เชี่ยวชาญ : การวิเคราะห์แบบพร้อมกันของสารปฏิชีวนะตกค้างในเมทริกซ์อาหารด้วยอันตร กิริยาชอบน้ำควบคู่กับแทนเดมแมสสเปกโทรเมตรี. (SIMULTANEOUS ANALYSIS OF ANTIBIOTIC RESIDUES IN FOOD MATRICES USING HYDROPHILIC INTERACTION COUPLED WITH TANDEM MASS SPECTROMETRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. ณัฐชนัญ ลีพิพัฒน์ไพบูลย์, 147 หน้า.

งานวิจัยนี้เป็นการพัฒนาวิธีการวิเคราะห์สารปฏิชีวนะตกค้างหลายชนิดพร้อมกันใน 8 กลุ่มได้แก่ กลุ่ม ซัลโฟนาไมด์ 4 ชนิด, ควิโนโลน 4 ชนิด, แมคโครไลด์ 4 ชนิด, เททราซัยคลิน 3 ชนิด, อะมิโนไกลโคไซด์ 3 ชนิด, เพนนิซิลิน 3 ชนิด, ลินโคซาไมด์ 2 ชนิด และสารแอมโพเลียม รวมทั้งสิ้น 24 ชนิด ในเมทริกซ์อาหาร โดยวิธีการเตรียมตัวอย่างประกอบด้วย การสกัดด้วยสารสารละลายผสมของ 2% กรดไตรคลอโรอะซิติกกับ อะ ซิโตไนไตรล์ ในอัตราส่วนเท่ากัน กำจัดไขมันจากสารตัวอย่างด้วยเฮกเซน จากนั้นเจือจางสารละลายตัวอย่าง และทำการแยกสารและตรวจวัดด้วยเทคนิคการแยกแบบอันตรกิริยาชอบน้ำลิควิดโครมาโทกราฟี- แทนเดม แมสสเปกโทรเมตรี แบบ electrospray ionization โดยตรวจวัดในรูปของประจุบวก เทคนิคอันตรกิริยา ชอบน้ำเป็นเทคนิคที่สามารถเหนี่ยวรั้งสารที่มีสภาพขั้วสูงและช่วยเพิ่มสภาพไวการวิเคราะห์ได้สูงกว่าเทคนิคการ แยกแบบ รีเวิร์สเฟส และ ไอออนแพร์ การรวมกันของวิธีการสกัดและเทคนิคการแยกที่มีประสิทธิภาพ ทำให้ สามารถตรวจวัดทั้งชนิดและปริมาณของสารปฏิชีวนะที่มีสภาพขั้วต่างกันอย่างมากได้ในครั้งเดียว นอกจากนี้ได้ ศึกษาถึงตัวแปรที่มีผลต่อประสิทธิภาพของการสกัด, การแยก และสภาพไวการวิเคราะห์ งานวิจัยที่นำเสนอนี้ได้ ทำการตรวจสอบความใช้ได้ของวิธีการตามข้อกำหนดของสหภาพยุโรป ค่าร้อยละการคืนกลับของสารปฏิชีวนะ ทุกชนิดในตัวอย่างเนื้อไก่ที่ระดับความเข้มข้น MRL อยู่ในช่วง 57 ถึง 99 โดยมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ (n = 10) อยู่ในเกณฑ์ที่ยอมรับได้ (2-14%) เมื่อเทียบกับค่าที่คำนวณได้จาก Horwitz equation และมี ขีดจำกัดต่ำสุดของการวัดเท่ากับ 0.1 ถึง 20 ไมโครกรัมต่อกิโลกรัม, ค่าสัมประสิทธิ์สหสัมพันธ์ของสารทุกชนิด ในช่วงความเข้มข้น 0.25 MRL- 2 MRL (กลุ่มซัลโฟนาไมด์ 0.05 MRL-MRL, เอ็นโรฟลอกซาซิน และ ไซโปรฟลอกซาซิน 0.1 MRL-MRL) มากกว่า 0.99 และได้นำไปประยุกต์ใช้กับตัวอย่างจริง ได้แก่ ไก่, กุ้ง และไข่ไก่ ซึ่งตรวจพบสารเอ็นโรฟลอกซาซินในเนื้อไก่ที่ความเข้มข้น 6.5 ไมโครกรัมต่อกิโลกรัม แสดงถึง ความเหมาะสมของการนำงานวิจัยนี้ไปประยุกต์ใช้กับงานวิเคราะห์ประจำในห้องปฏิบัติการที่ต้องการตรวจ วิเคราะห์สารปฏิชีวนะต่างกลุ่มกันจากเมทริกซ์อาหารหลายชนิดได้อย่างมีประสิทธิภาพ

ภาควิชา	เคมี	ลายมือชื่อนิสิต ชญาดา เชี่ยวชาญ
สาขาวิชา	เคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก
ปีการศึกษา	2552	

5072615223 : MAJOR CHEMISTRY
KEYWORDS : HYDROPHILIC INTERACTION CHROMATOGRAPHY / ANTIBIOTIC RESIDUES / TANDEM MASS SPECTROMETRY / FOOD MATRICES

CHAYADA CHIAOCHAN : SIMULTANEOUS ANALYSIS OF ANTIBIOTIC RESIDUES IN FOOD MATRICES USING HYDROPHILIC INTERACTION COUPLED WITH TANDEM MASS SPECTROMETRY. THESIS ADVISOR : ASST. PROF.NATCHANUN LEEPIPATPIBOON,

Dr.rer.nat., 147 pp.

In this study, simple and sensitive method for simultaneous analysis of twenty four antibiotics belong to eight important classes : four sulfonamides, four quinolones, four macrolides, three tetracyclines, three aminoglycosides, three penicillins, two lincosamides, and amprolium in food was developed. The method involved a liquid extraction with the mixture of acetonitrile and 2% trichloroacetic acid in equal ratio, and removed fat with hexane before dilution of an extract with 10% formic acid : acetonitrile (9:1), and followed by hydrophilic interaction liquid chromatography (HILIC)-electrospray ionization tandem mass spectrometry in positive ion mode. The combination of effective extraction procedure and HILIC separation technique allowed quantification and confirmation of compounds with different polarity in a single chromatographic analysis. Several parameters affecting on the extraction efficiency, separation and sensitivity were optimized. The validation method has been performed in chicken matrix according to European Union Decision 2002/657/EC. Good performance data were obtained for recovery, precision, calibration curve, specificity and limit of detection. The average recoveries for all targeted analytes at MRLs concentration level were obtained in the range of 57 to 99 % with satisfactory %RSD of 2-14 (n=10) and also lower than acceptable value calculated from Horwitz equation. The limits of detection were 0.1-20 µg/kg for all compounds. Good linearity covered the concentration range of 0.25 MRL-2 MRL (sulfonamides ranged from 0.05 MRL-MRL, enrofloxacin and ciprofloxacin ranged from 0.1 MRL-MRL) with coefficient of determination higher than 0.99. The optimized method has been successfully applied to real chicken samples which can be detected enrofloxacin at 6.5 µg/kg. The method can also be employed for shrimp and egg samples analysis in routine laboratory for a different class of antibiotic residues from various matrices.

Department : _____Chemistry_____   Student's Signature _Chayada Chiaochan_

Field of Study : _____Chemistry_____   Advisor's Signature _Natchanun Leepipatpiboon_

Academic Year : _____2009_____

ACKNOWLEDGEMENTS

CONTENTS

LIST OF TABLES

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

FIGURES PAGE

LIST OF ABBREVIATIONS

AGs	aminoglycosides
AMOX	amoxiciilin
AMP	ampicillin
AMPR	amprolium
APCI	atmospheric pressure chemical ionization
ºC	degree Celsius
C.E.	collision energy
CIP	ciprofloxacin
CTC	chlortetracycline
C.V.	cone voltage
DAD	diode array detector
DAN	danofloxacin
DSTR	dihydrostreptomycin
ENR	enrofloxacin
ERY	erythromycin
ESI	electrospray ionization
eV	electronvolt
FLD	fluorescence detector
g	gram
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
IEC	ion exchange chromatography
IPC	ion pair chromatography
kV	kilovolt
L/h	liter per hour

LAs	lincosamides
LIN	lincomycin
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LODs	limit of detection
LOQs	limit of quantification
M	molar
mg/kg	milligram per kilogram
m/z	mass per charge ratio
min	minute
mL	milliliter
mL/min	milliliter per minute
MCs	macrolides
mm	millimeter
MRLs	maximum residue limits
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS, MS^n	tandem mass spectrometry
NPLC	normal phase liquid chromatography
OTC	oxytetracycline
OXO	oxolinic acid
PCs	penicillins
PEN G	penicillin G
PIR	pirlimycin
Qs	quinolones
%	percentage
R^2	correlation coefficient
RPLC	reversed phase liquid chromatography

RSD	relative standard deviation
S/N	signal to noise ratio
SAs	sulfonamides
SDMX	sulfadimethoxine
SDZ	sulfadiazine
sec	second
SMZ	sulfamethazine
SPEC	spectinomycin
SPI	spiramycin
STR	streptomycin
STZ	sulfathiazole
TC	tetracycline
TCA	trichloroacetic acid
TCs	tetracyclines
TIL	tilmicosin
TOF	time of flight mass analyzer
TYL	tylosin
UPLC	ultra performance liquid chromatography
UV	ultraviolet
v/v	volume by volume
V	volt
µg/kg	microgram per kilogram

CHAPTER I

INTRODUCTION

1.1 Problem Definition

Nowadays, a wide variety of veterinary drugs are commonly available for use in modern animal agriculture around the world in order to treat or prevent diseases. Moreover, they can also be illegally used as a growth promoter. In higher usages with harmful concentration, these drugs can leave residues in edible tissue and poses a potential risk to health. This is due to the increasing incidence of microbial resistance and the risk of allergic reactions in some hypersensitive individuals which may compromise the human immune system. In addition, the daily consumption of food with low levels of antibiotics can lead to possible long term carcinogenic and neurotoxicologic effects. Ensuing the safety of food for human consumption, many countries including European Union (EU) and US Food and Drug Administration (FDA), have to monitor for the presence of these compound residues in food, which may cause adverse toxic effects on consumers health. *(1, 2)*

In Thailand, chicken is an important food product. Chilled and frozen chicken is mainly exported to the EU, Japan and Hong Kong. Since the international trading block, due to the detection of nitrofurans in Thai chicken, preventive action for exported products has become an important issue. Therefore, the availability of simple and reliable systems for the detection of antibiotic residues is an essential tool in assuring the safety of food products.

Traditionally, screening methods for veterinary drugs are based on microbiological and immunological assays. These methods provide only semi-quantitative measurements and incomplete data. They often lack the specificity and precision required for modern regulatory purpose. As the antibiotic residues in food are present in very small quantities, the screening procedure should be sensitive, accurate, reliable and rapid. Therefore, higher sensitivity detection analytical methods are required.

Consequently, chromatographic techniques allow quantitative multi-residue determinations and compound identification are based on different retention times. Liquid chromatography (LC) is the method of choice for antibiotics analysis which are rather polar, non-volatile and sometimes thermolabile. There are several developments

in chemical residue techniques using instrumentation of relatively low selectivity such as high performance liquid chromatography (HPLC) with ultraviolet (UV), diode array (DAD), fluorescence (FLD) and refractive index detection (RI). Due to the lack of selectivity in detection step, these methods require highly selective sample preparation which often includes a lengthy clean up procedure. The result from this analysis needs to be confirmed by selective and sensitive chemical ones.

The liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) has become the most powerful instrument in the determination antibiotic residues in food by combining analyte separation with structural information. However, only a few multi-class liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the determination of veterinary drugs residues in food have been presented.

The main difficulties encountered during the analytes extraction and clean up of the extracts, the extraction solvent must be chosen on the basis of the chemical properties of the different classes of target compounds. Thus the clean up optimization, the acceptable recovery and specificity should be considered.

Furthermore, chromatographic separation is one of the important factors for multi-class analysis, because of reversed phase liquid chromatography (RPLC) which has been widely used to separate various analytes, sometimes unable to retain the polar and hydrophilic compounds. Therefore, the ion pair chromatography can only be used for strong hydrophility of analytes but this technique is not ideal for electrospray mass spectrometric detection due to the sensitivity reducing from signal suppression.

Hydrophilic interaction liquid chromatography (HILIC) is a latest useful separation technique alternative to reversed phase and ion pair chromatography for highly polar substances. This technique uses a polar stationary phase and high organic content in the mobile phase which may be able to enhanced detection sensitivity when used in conjunction with mass spectrometry. *(3)*

In this study, the new development and validation of a sensitive multi-residue method for seven important classes including amprolium in chicken muscle is presented.

1.2 Regulation of Drug Residues in Food

For ensuring the safety of food for human consumption, the EU has strictly regulated controls on the use of veterinary drugs, particularly in animal species suitable for consumption. The regulation regarding the control of veterinary drug residues is given in Council Directive 96/23/EC *(4)* with detailed procedures for EU member states to set up national monitoring plans, including details on sampling procedures. For any type of animal or food, there are two main groups of substances that must be monitored: unauthorized substances which belong to Group A, and substances with established maximum residue limits (MRLs) which belong to group B. Criteria to defined the performance expected of both screening and confirmatory methods for residues have been establish in Commission Decision 2002/657/EC. *(5)* The EU council regulation 2377/90/EC lays down the community procedure for the establishment of MRLs of veterinary medicinal products in foodstuffs of animal origin. *(6)*

The definition of maximum residue limit according to this regulation means the maximum concentration of residue resulting from the use of a veterinary medicinal product (expressed in mg/kg or μg/kg on a fresh weight basis) which may be accepted by the community to be legally permitted or recognized as acceptable in food.

For some substances the MRLs laid down in community legislation are expressed in form of sum-MRLs *(7)*. There are two different cases to be distinguished: independent substances like sulfonamides and substances with their metabolites which occur in particular ratios and these ratios are not known for all species and matrix-combinations. These guidelines indicate the technical procedure which can be applied for both cases.

On the basis of Commission Decision 2002/657/EC, when MS detection is performed by fragmentography, the pseudomolecular ion shall preferably be selected as precursor ion and a system of identification points (IPs) shall be used to interpret the data. For the confirmation of Group A substances, a minimum of 4 IPs is required and for the Group B substances as targeted compounds in this study, there is a minimum of 3 IPs. Number of IPs, useful for the confirmation of an analyte and also depends on the type of mass analyzer and its resolution grade. A triple quadrupole is a low resolution mass spectrometer which currently provides the best performances in quantitative determination, when working in multireaction mornitoring (MRM) mode. In this case, 1 IPs is earned for the precursor ion and 1.5 IPs for each product ion. As each ion should

only be counted once, the selection of two MRM transitions allows the earning of 4 IPs, ensuing group A and B compounds confirmation.

The established MRLs for studied veterinary drugs in chicken by the EU are listed in Table 1.1.

Table 1.1 Maximum residue limits (MRLs) of veterinary drugs in chicken by the European Union (EU)

No.	Compounds	MRL (µg/kg)	Class
1	Amoxicillin	50	Penicillin
2	Ampicillin	50	Penicillin
3	Penicillin G	50	Penicillin
4	Tilmicosin	50	Macrolides
5	Sulfamethazine	100	Sulfonamides
6	Sulfadiazine	100	Sulfonamides
7	Sulfadimethoxine	100	Sulfonamides
8	Sulfathiazole	100	Sulfonamides
9	Ciprofloxacin	100	Quinolones
10	Enrofloxacin	100	Quinolones
11	Oxolinic acid	100	Quinolones
12	Oxytetracycline	100	Tetracyclines
13	Chlortetracycline	100	Tetracyclines
14	Tetracycline	100	Tetracyclines
15	Tylosin	100	Macrolides
16	Lincomycin	100	Lincosamides
17	Pirlimycin	100	Lincosamides
18	Danofloxacin	200	Quinolones
19	Erythromycin	200	Macrolides
20	Spiramycin	200	Macrolides
21	Amprolium	200	Coccidiostats
22	Spectinomycin	300	Aminoglycosides
23	Streptomycin	500	Aminoglycosides
24	Dihydrostreptomycin	500	Aminoglycosides

1.3 The important classes of veterinary drugs.

1.3.1 Aminoglycosides

Aminoglycosides (AGs) are active against a broad spectrum of gram-positive and gram-negative bacteria. Streptomycin (STR) and dihydrostreptomycin (DSTR) are commonly used aminoglycosides in food animal production. They exert their antibacterial effect by binding to the 30S ribosome, which disrupt bacterial protein synthesis. Aminoglycosides are very polar molecule and lack chromophores and fluorophores. The chemical structures of AGs are based on an aminocyclitol ring connected to two or more amino sugars in a glycoside linkage (Figure 1.1).
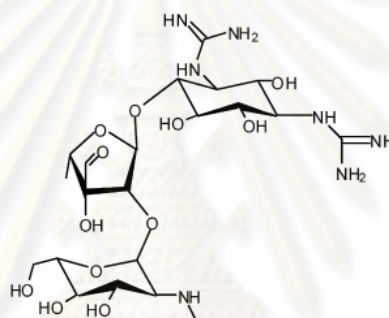
Figure 1.1 The representative chemical structure of AGs (STR)

1.3.2 ß-Lactams

There are three classes of ß-Lactams antibiotics which get their name from the ß-Lactams ring characteristic of their structure: penicillins (PCs), subdivided in more subgroups: cephalosporins and monolactams. The ß-Lactams are compounds with limited stability because of the presence of the four-term ring in their structure. Penicillins are widely used in veterinary medicine for preventing and treating bacterial infections.
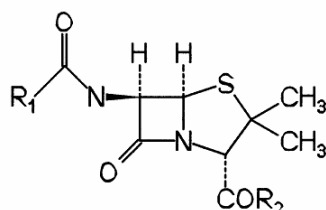
Figure 1.2 The basis chemical structure of PCs

1.3.3 Macrolides

Macrolides (MCs) are macrocyclic lactones isolated first from streptomyces SSP. The chemical structures of macrolides consist of 12-, 14- or 16- membered macrocyclic lactone to which sugar moieties, including amino and deoxy sugars are attached (Figure 1.3). Macrolides are an important class of antibiotics which are widely used in veterinary practice to treat respiratory diseases and enteric infections in cattle, sheep, swine and poultry or as feed additives to promote growth.
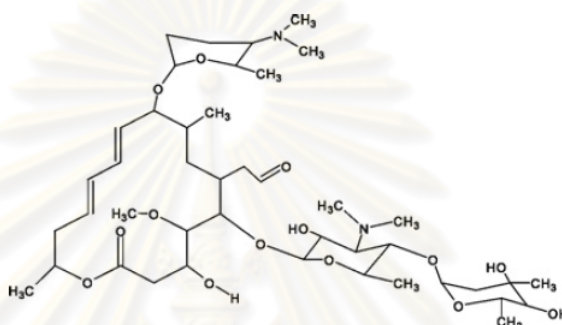
Figure 1.3 The representative chemical structure of MCs (SPI)

1.3.4 Tetracyclines

Tetractclines (TCs) are broad spectrum antibiotic against gram-positive and gram-negative bacterias. They are widely used in veterinary medicine for preventing and treating several diseases as well as for promoting growth in cattle and poultry. The basic structure of TCs consists of a hydronaphtacene framework containing four fused rings.
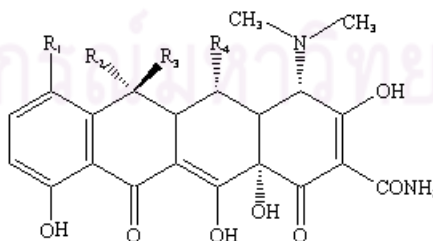
Figure 1.4 The basis chemical structure of TCs

1.3.5 Quinolones

Quinolones (Qs) are a group of relatively new antibiotics synthesized from 3-quinolonecarboxylic acid. Qs which are widely used in food production are of concern because of the recent evidence that these may lead to the development of bacterial resistance important in other human drugs.
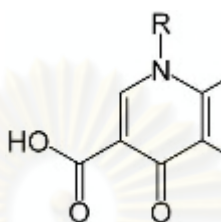
Figure 1.5 The basis chemical structure of Qs

1.3.6 Sulfonamides

Sulfonamides (SAs) comprise of a large number of synthetic bacteriostatic compounds. They act by competing with *p*-aminobenzoic acid in the enzymatic synthesis of dihydropholic acid. This leads to a decreased availability of the reduced folates, which are essential in the synthesis of nucleic acids. Many SAs are widely used in veterinary medicine. Analysis of SAs in foodstuff is particular concern because of the potential carcinogenic character.
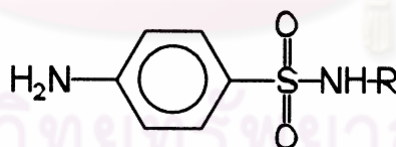
Figure 1.6 The basis chemical structure of SAs

1.3.7 Lincosamides

Lincosamides (LAs) are derived from an amino acid and a sulfur-containing octose, a synthetic monosaccharide containing eight carbon atoms in a molecule. The mechanism of action of lincosamides is reversible binding to the 50S ribosomal subunit and resultant suppression of protein synthesis.
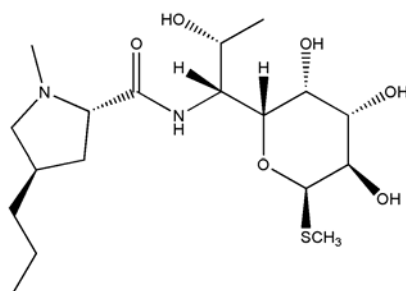
Figure 1.7 The representative chemical structure of LAs (LIN)

1.3.8 Coccidiostats

Coccidiostats are compounds that are widely used to prevent and treat coccidiosis, a contagious amoebic disease affecting livestock, particularly poultry that is associated with warm and humid conditions. The disease is carried by unicellular organisms belonging to the genus *Eimeria* in the class *Sporozoa*. Amprolium (AMPR) is a coccidiostats which is used for the treatment and prevention of coccidiosis in chicken.
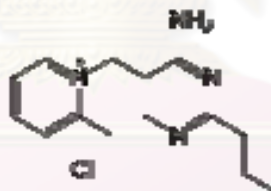
Figure 1.8 The chemical structure of AMPR

1.4 Literature Reviews

Controlling the presence of antibiotic residues in various foods and food products, screening methods based on microbiological and immunological assays have been more commonly used for the detection of antibiotic residues because they are easily performed and inexpensive. However they are lack of specificity and precision. Another drawback is only one or a few classes of antibiotics can be detected.*(8-10)* Several papers have been reported the development of analytical tools to detect antibiotics in food, most of them using chromatographic techniques such as TLC *(11)*, LC-UV *(12)*, LC-FLD *(13, 14)*, LC-MS *(15)* and LC-MS/MS. *(16)*

In 2001, KAO et al. reported the use of solid phase extraction (SPE) as C18 for extraction of 13 veterinary drugs in chicken and swine muscle. The analytes were determined by HPLC equipped with a photodiode array using a Luna-C18 column and gradient elution of phosphate buffer and acetonitrile. This method could detect SAs residual at 1.23 mg/kg in chicken sampled from local market with recoveries ranging from 72-97%. However, the positive results reported by the method which using LC coupled with UV and FLD needed to be confirmed by selective and sensitive tools. Therefore, these conventional detection techniques have been replaced by mass spectrometry. *(17)*

In 2006, the multi-class method for simultaneously detecting 18 compounds of different classes in shrimp was presented using SPE (HLB) for extraction and LC coupled to quadrupole ion trap mass spectrometry OIT-MS for detection. The LC column used is Waters YMC, with acetonitrile/water and formic acid as the mobile phase. The various type of SPE were tested in this article, the SPE-HLB gave the best overall performance. *(18)*

Although, the SPE is the selective partition for the analytes and can eliminate the interference which leads to signal suppression, but there are some drawbacks. For screening of multi-class antibiotics method, the selectivity of SPE is a disadvantage. In addition, the SPE procedure is time consuming and the selection of a solid phase or a solvent can be complicated. For these results, use of SPE can only cover a few different classes of veterinary drugs, therefore it is not ideal for wide range multi-class analysis.

A simple and rapid method of using single extraction, a suitable extraction solvent with versatile properties and able to extract as many drug as possible, had to be found. In the publication, proposed by Yamada et al. *(19)*, 130 different antibiotic residues were extracted by acetonitrile/methanol (95/5). This is the proper solvent for most compounds; unfortunately it is not optimal for tetracyclines and aminoglycosides. Another development for single extraction, published by K. Granelli and C. Branzell which described an ESI-LC-MS/MS method for screening 19 compounds from 5 different classed antibiotics in porcine and bovine muscle. The method used 70% methanol and followed by diluting with water prior to LC/MS/MS analysis. *(20)* The selected extraction solvent gave a satisfactory recovery for all different classes of compounds. However, AGs were not included because of their high polarity. Few years later, K. Granelli and co-workers continue this work as method confirmation. *(21)* In

both reports, the authors achieved the separation of 19 antibiotic compounds on a Genesis C18, a gradient containing 0.2% formic acid with 0.1 mM oxalic acid and acetonitrile was applied. The proposed method is sufficiently good enough to be used for simultaneous quantification and confirmation.

The paper described by J. Chico et al. who were using the same extraction method, employed ultra high pressure liquid chromatography (UPLC) – tandem mass spectrometry, allowing both quantification and confirmation in a single anlysis. The column used was C18 Acquity UPLC BEH from Waters, with a gradient mixing of water and acetonitrile containing formic acid. This article method was designed to cover screening, quantification and confirmation functions with a simplify extraction method in order to achieve high sample throughput. *(22)*

The method of using liquid chromatography coupled to time of flight mass spectrometry (LC-TOF) for the quantitative analysis of about 100 veterinary drugs was published. The samples were extracted using bi-polarity extraction. The separation of veterinary drugs was performed on a HSS T3 UPLC column, is based on high resolution column which provides strong retention for polar analyte. Due to legislative reasons, the authors claimed that the method can not be used for confirmation. *(23)*

The method is based on pressurized liquid extraction (PLE) for multi-class analysis of 31 antimicrobials in meat sample was proposed by V. Carretero et al. The separation was achieved on XTerra MS C18 LC column from Waters. This publication was able to identify and quantify the antibiotic residues present in the various incurred sample. This method proved that, ENR and its metabolite CIP are often present in bovine sample. Average recoveries of this procedure ranged from 75-99%. *(24)*

D.A. Bohm et al. presented the multi-method for 47 antibiotics in milk sample analysis. In sample preparation process, the volume of Trichloroacetic acid (TCA) solution which used for protein precipitation was optimized. The results indicated that, 100 µL of 20% TCA found to be suitable in the proposed method.*(25)*

In 2009, G. Stubbings and T. Bigwood validated an LC-MS/MS method for the determination of veterinary drug residues in chicken muscle using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach for sample preparation. A Synergi Fusion-RP column was applied for the LC separation. This stationary phase is stable under 100% aqueous condition and can give the adequate retention of the most polar analytes. *(26)*

Recently, the only multi-class method reported by Y. A. Hammel et al. can cover a lot of the main classes of antibiotics including the aminoglycosides group which have extreme polarity. The authors described the method development for simultaneous analysis of 42 antibiotic residues in honey, using four subsequence liquid - liquid extractions (LLE) before LC-ESI-MS/MS analysis. The HPLC separation was achieved on Zorbax SB-C18 revered phase with nonafluoropentanoic acid (NFPA) as ion pairing reagent in the mobile phase. However, at the targeted concentration level of 20 μg/kg, the method worked well for 37 analytes from 42 monitored compounds. *(27)*

1.5 Purpose of The Study

At present, the most reliable and acceptable chromatographic technique used for confirmation of veterinary drug residues in food and foodstuffs, is the LC-MS/MS. The combination of LC and MS/MS allows both quantification and identification simultaneously. The requirement for quantitative results with high selectivity and specificity at trace level in food matrices need for a powerful analytical technique, therefore tandem MS detection completely fulfills these criteria.

For the most part, from the presented literature reviews, various methods have been successful in multi-residue analysis of antibiotic residues in foods using LC-MS/MS. However, most reports still do not cover the wide range polarity of compounds, especially AGs group (the extremely polar substance) due to the limit of chromatographic separation or sample extraction used. Therefore method development for various drug classes covering AGs with a single extraction method and chromatographic analysis is the one challenge in this field.

The aim of this study was to develop a new and simple method for simultaneous analysis of multi-class antibiotic residues using hydrophilic interaction chromatography (HILIC) and tandem mass spectrometry in food sample with single extraction and chromatographic analysis. Antibiotics used in this study cover of seven important drug classes and one coccidiostat :

1. Sulfonamides : sulfadiazine, sulfathiazole, sulfamethazine, sulfadimethoxine
2. Tetracyclines : tetracycline, chlortetracycline, oxytetracycline
3. Macrolide : erythromycin, tilmicosin, tylosin, spiramycin
4. Penicillins : amoxicillin, ampicillin, penicillin G
5. Quinolones : ciprofloxacin, danofloxacin, enrofloxacin, oxolinic acid

6. Lincosamides : lincomycin, pirlimycin
7. Aminoglycosides : spectinomycin, streptomycin, dihydrostreptomycin
8. Coccidiostat : amprolium

As described in the 'Problem Definition' section, chicken sample was selected for the representative of food matrix in this study. An attempt to determine the residue of different classes of antibiotics with highest effective method, sample preparation, chromatographic separation and MS/MS condition were optimized. This procedure was validated according to the EU requirements (2002/657/EC) for the determination of multi-class antibiotics at the levels regulated by the EU.

CHAPTER II

THEORY

2.1 Liquid Chromatography *(28, 29)*

Liquid chromatography (LC) is a popular technique of chromatographic separation which is based on the difference in the surface interaction of the compound between two phases, these are called stationary a phase and a mobile phase. High performance liquid chromatography (HPLC) is the term commonly used to describe liquid chromatography. The liquid mobile phase is constantly pumped through a column as a stationary phase which contains fine spherical solid particles. Basically, a HPLC system consist of five component parts. A schematic diagram of a typical HPLC instrument is shown in Figure 2.1.

2.1.1 Pump

The purpose of the pump is to deliver mobile phase solvents from their reservoir. There are a number of different types of pumps that can provide the pressure and flow rate required. Most commercial HPLC pumps are based on a reciprocating piston design. A driven motor pulls the piston back and forth in the pump head. This pump can provide stable flow rates and enable a constant flow of the mobile phase.

Most separations can be done using isocratic elution, which is the use of a single solvent system that does not change during the analysis. For more complex analyzes, gradient elution is required. Gradient elution is done by gradually strengthening the mobile phase composition throughout the separation. Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower and taller peaks for most components. This also improves as the peak shape for tailed peaks as the increasing concentration of the organic eluent pushes the tailing part of the peak forward.

2.1.2 Sample Introduction (Injector)

The purpose of the sample introduction system is to apply the sample extract onto the column in a narrow band. The most widely used method of sample introduction in HPLC is based on a sampling loop. The loop injector, is merely a convenient way of introducing a liquid sample into a flowing liquid stream and consists of a loop of a nominal volume into which a sample is introduced by using a conventional syringe. While the loop is being filled, the mobile phase is pumped, at the desired flow rate, through the valve to the column to keep the column in equilibrium with the mobile phase and maintain chromatographic performance. When injection is required, a rotating switch is moved and the flow is diverted through the loop, thus flushing it's the contents into the top of the column.

2.1.3 Mobile Phase

In HPLC, the relative interaction of an analyte with both the mobile and stationary phases determines its retention characteristics. Hence, it is the varying degrees of interaction of different analytes with the mobile and stationary phases. HPLC requires a mobile phase in which the analytes are soluble. It is not always possible to achieve an adequate separation by using a single solvent as a mobile phase, therefore, the mixtures of solvents are often used. A separation involving a mobile phase of constant composition is called isocratic elution, where the composition of the mobile phase is changed it is called gradient elution. Buffers are used in HPLC to control the degree of ionization of the analyte and thus the tailing of response and the reproducibility of retention.

2.1.4 Stationary Phase

The column is a very important part of the HPLC instrument as the separation occurs here. The most widely used columns contain a chemically modified silica stationary phase, in which the chemical modification determines the polarity of the column. A very popular stationary phase is C18 alkyl group which is bonded to the silica surface and employed to the reversed phase system. However, the reversed phase separation can not retain the highly polar and hydrophilic compounds, the HILIC technique, columns contain a stationary phase which is hydrophilic and quite often also

charged. The hydrophilic compounds can interact and separate on (in?) the column. Further details of this separation mode are discussed in section 2.2.5.

The accumulation of strongly retained material on the HPLC column can reduced its lifetime. By modifying the packing surface, this retained material can cause shifts in peak retention, loss of resolution and efficiency, as well as degradation of the peak shape. The way to protect an analytical column is to stall a guard column between the injection valve and the analytical column.

2.1.5 Detector

An appropriate HPLC detector should have the ability to sense the presence of compounds and send its corresponding electrical signal to a computer data system. The choice of detector depends upon the characteristics and concentration of the compounds which need to be separated and analyzed. A number are in routine method use, including the ultraviolet (UV), fluorescence, electrochemical, conductivity, reflective index and mass spectrometer detectors, however each has particular advantages and disadvantages.
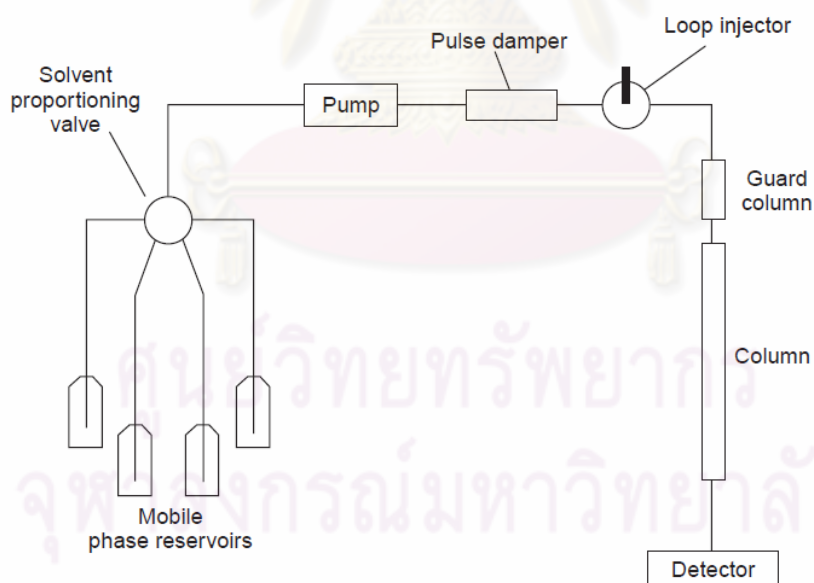
Figure 2.1 Schematic diagram of a typical HPLC instrument *(28)*

2.2 HPLC Separation Mode

In general, primary characteristics of chemical compounds can be used to create HPLC separation including polarity, electrical charge and molecular size. For multi-class, multi-residue analysis with wide range polarity, the common separation mode base on polarity need to be considered.

2.2.1 Normal Phase Liquid Chromatography (NPLC)

In this separation, the stationary phase is more polar than the mobile phase which usually is a mixture of organic solvents without water. The column packing are normally silica or a polar boned phase such as amino, cyano and diol. Retention in NPLC increases as the polarity of the mobile phase decreases. However, this mode is not suitable for the LC-MS analysis due to non aqueous eluents used for NPLC that are not compatible with the electrospray process.

2.2.2 Reversed Phase Liquid Chromatography (RPLC)

RPLC is widely used with the most applications, the stationary phase is less polar than the mobile phase. Mixtures of water or aqueous and organic solvents are used to elute analyte from a reverse phase column. The solvent has to be miscible with water, the most common organic solvents used are acetonotrile, methanol and tetrahydrofuran. Hydrophobic compounds preferably interact with the stationary phase, rather than remaining dissolved in the aqueous phase.

2.2.3 Ion Pair Chromatography (IPC)

Ion pair chromatography, the stationary phase is non polar and the retention of the elute molecules can occur either by ion pair formation in the mobile phase, partitioning of the complex between the mobile and non polar stationary phase. Or, by dynamic ion exchange which involves an ion pair formation between the eluate and the counterion adsorptively bound to the stationary phase. However IPC is not ideal for electrospray mass spectrometric detection because the sensitivity of mass spectrometry is reduced.

2.2.4 Ion Exchange Chromatography (IEC)

Stationary phases for ion exchange chromatography are characterized by the nature and strength of acidic and basic functions on their surface and the type of ion that they attract and retain. Cation exchange is used to retain and separate positively charged ions on negative surface. Conversely, anion exchange is used to retain and separate negatively charged ions on positive surface. With each type of ion exchange, there are at least two general approaches for separation and elution.

2.2.5 Hydrophilic interaction liquid chromatography (HILIC) *(3, 30, 31)*

Hydrophilic interaction chromatography (HILIC) is a chromatographic technique that has been used to improve retention of very polar analytes. HILIC is a version of normal phase liquid chromatography. The separation is achieved by utilizing high organic solvent and low aqueous as a mobile phase. The name was suggested by Alpert. *(32)* The stationary phase of HILIC is polar such as silica, amino, diol and zwitterionic. A wide range of applications are amino acids, peptides, carbohydrates, counter ion and veterinary drugs. *(33-36)*

The mechanism for HILIC, already suggested by Alpert *(32)* was a partitioning between the bulk eluent and water rich layer.

Despite the complexity of the mechanism, the technique is simple in practice and provides many advantages which can be summarized as follows;

- Good peak shape can be obtained for basis compounds

- Mass spectrometer sensitivity is enhanced due to the high organic content in the mobile phase and also provides the high efficiency of spraying and desolvation techniques.

- Direct injection can often be due to without evaporation and reconstitution step.

- The order elution of analytes is generally the opposite to RP separation, giving useful alternative selectivity.

- Good retention time of polar compounds is obtained in HILIC, whereas very poor retention is often obtained from RPLC.

- Higher flow rates are possible due to the high organic content of typical mobile phases.

2.2.5.1 The Zwitterionic Stationary Phase *(37)*

The ZIC-HILIC is one type of zwitterions materials as used for stationary phase in this analysis. This column has sulfobetaine functional group covalently attached to 3.5 μm particle size silica in conventional dimensions from capillaries to preparative scale. The column can provide a sensitivity benefiting from both hydrophilic and weak electrostatic interactions, while maintaining a low eluent strength making the column an ideal choice for LC-MS analysis.
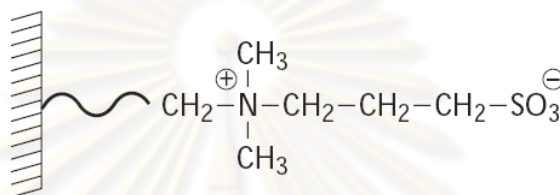
Figure 2.2 The functional group of the ZIC®-HILIC stationary phase *(37)*

2.2.5.2 HILIC retention characteristics

Under HILIC conditions, a water enriched liquid layer is established within the stationary phase. The separation is achieved by partitioning of solutes from the eluent into this hydrophilic environment. A process that is typically exothermic. Hence, both hydrogen bonding, the extent of which depends on the acidity or basicity of the solutes and dipole-dipole interactions, which depend the dipole moments and polarizability of the solutes, are factors governing retention. The primary reaction of HILIC stationary phase is thus to bind water. However, with any of the charged HILIC stationary phases available, the retention will also be influenced by electrostatic interactions as illustrated in Figure 2.3 for a zwitterionic stationary phase.

.
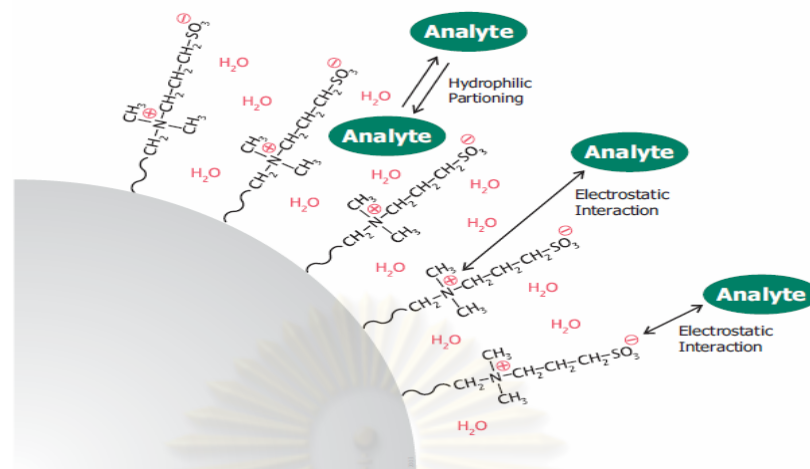
Figure 2.3 Schematic illustration of the processes causing retention on the ZIC®-HILIC stationary phase *(37)*

Although it is of limited importance for the primary HILIC retention, charged stationary phases adds second a very significant dimension of selectivity due to the opportunities of electrostatic interactions with the analytes. The downside of electrostatic interactions is the need of salts or buffers in the mobile phase to disrupt these interactions for the analyte elution. Higher buffer concentrations may be negative to MS detection sensitivity. With zwitterionic stationary phases the electrostatic forces of each charge are partly counterbalanced by the proximity of an ion with opposite charge, the combiled overall effect is weaker electrostatic interactions.

Weak electrostatic interactions lead to lower eluent buffer concentrations which are preferable for high sensitivity MS detection. Neutral HILIC stationary phases typically require lower buffer concentrations, but lack the selectivity benefits of charged stationary phases. The charge density of weak ion-exchangers used as HILIC stationary phase such as silica and amino phases is pH dependent. Hence, the strength of a solutes electrostatic interaction with such materials will have a complex dependence on both the ionization of the analyte and of the stationary phase. For pH independent materials, as the permanent zwitterionic stationary phases, the optimization of the mobile phase pH is solely dictated by the analytes.

2.3 Mass Spectrometry (MS) *(29, 38)*

For confirmation of veterinary drugs in foodstuff, Public Health Agencies in many countries relies on detection by mass spectrometry (MS). The Commission Decision 93/256/EEC states that, methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods. In general, chromatography can be regarded as the separation of the components of a mixture to allow for identification and quantification of all of them. Identification is initially carried out on the retention characteristics; therefore 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.

2.3.1 Ionization Methods

Two ionization interfaces, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), are available with most LC-MS instruments. Choice of an LC-MS interface for a particular application depends on polarity and molecular mass of the analyte.

2.3.1.1 Atmospheric Pressure Chemical Ionization (APCI)

APCI is the chemical ionization of compounds in an ion source operated at atmospheric pressure conditions. In APCI, the reagent ions for the ionization of analyte species are generally created by means of corona discharge. The ionization mechanisms in APCI are identical to those found in conventional medium-pressure CI. Positive ion formation can be achieved by proton transfer, adduct formation, or charge exchange reactions while in negative ion mode, ions are formed due to proton transfer, anion attachment, or electron capture reactions. For the coupling of LC to the APCI system, a heated nebulizer inlet system is needed.
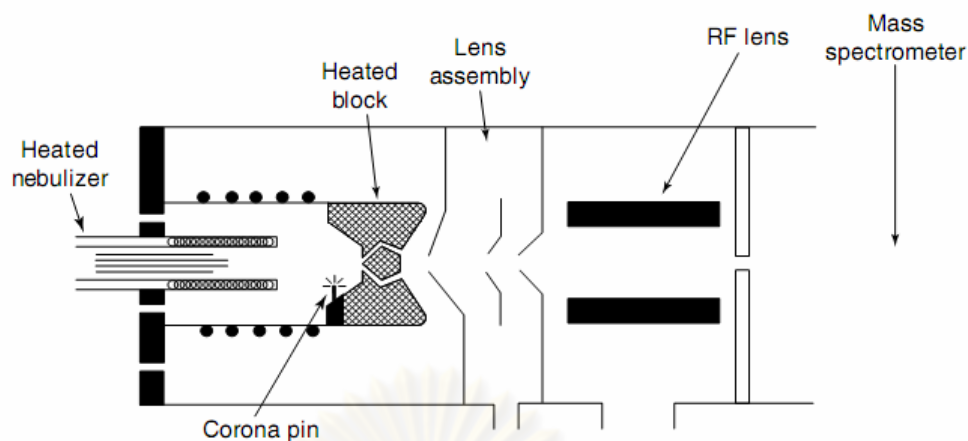
Figure 2.4 Schematic of the atmospheric pressure chemical ionization process *(29)*

2.3.1.2 Electrospray Ionization (ESI)

ESI is a soft ionization technique where a liquid, in which the analytes of interest have been dissolved, is passed through a capillary at atmospheric pressure and maintained at high voltage. The liquid stream breaks up with the formation of highly charged droplets which are desolvated as they pass through the atmospheric-pressure region at the source towards a counter electrode. Desolvation is assisted by a stream of a drying gas, which is usually nitrogen, being continually passed into the spraying region and initial production of small droplets. For this reason, a mobile phase with high surface tension or high viscosity should be avoided. The application of higher voltages to the electrospray needle will result in the production of smaller droplets but will ultimately lead to the formation of a high voltage discharge rather than the formation of droplets. This parameter should be optimized for a particular mobile phase during the instrumental set up procedure prior to analysis. This technique can be analyzed by a wide variety of mass analyzers, including quadrupole, ion trap and time of flight.
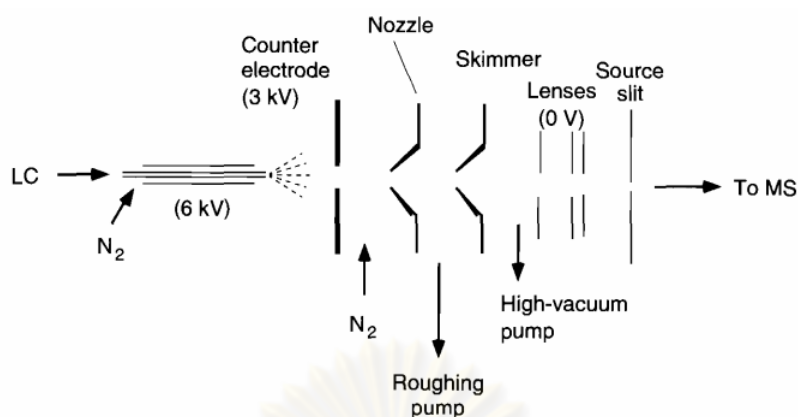
Figure 2.5 Schematic of the atmospheric pressure electrospray ionization process *(39)*

For the selection of ESI or APCI, the decision of whether positive or negative mode works better is obvious at the chemically extreme ends. Bases are best detected as cations M+H in positive mode and acids as anions M-H in negative mode. Another major difference between APCI and ESI can be found in LC flow-rates that are used. APCI is a technique with optimal performance at high flow rate about 1 ml/min or higher. Lower flow-rates can also be used. However, when flow-rates are too low, the stability of the corona discharge may become problematic.

2.3.2 Mass Analyzer

The mass analyzer separates ions by their mass to charge ratio (m/z) in space or in time. After ions are formed in the ion source region, they are accelerated into the mass analyzer. The mechanism is performed with electric and magnetic fields, sometimes including RF fields. There should be some ion focusing device to prevent the spread of ions from the ion source. The selection of the mass analyzer depends on the resolution, mass range, scan rate and detection limits required for the application. Each analyzer has different operating characteristics, and an additional instrument. In hyphenated LC-MS, quadrupole, quadrupole ion trap and time-of-flight (TOF) are widely used mass analyzer. These techniques are considered as an ion transmission system.

2.3.2.1 The Quadrupole Mass Analyzer

The most popular mass analyzer used in tandem mass systems is the quadrupole mass spectrometer which can provide MS/MS spectra. In the mass spectrometer, the quadrupole analyzer consists of four parallel metal rods or electrodes which must be precisely straight and parallel. Two parallel rods are connected to direct current (DC), while the others are connected to radio frequency (RF). When the beam of ions directed axially between the quadrupole, both DC and RF are chosen to filter ions according to their m/z, only ions of selected m/z or resonance ions pass through quadrupole analyzer.
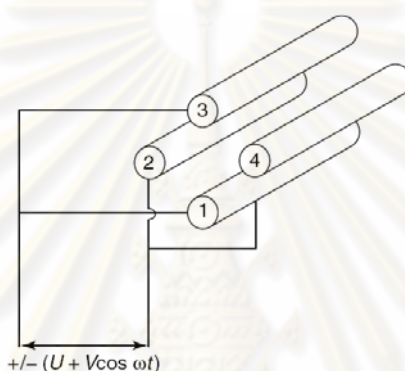
Figure 2.6 Schematic of the quadrupole mass analyzer *(29)*

2.3.2.2 The 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 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.
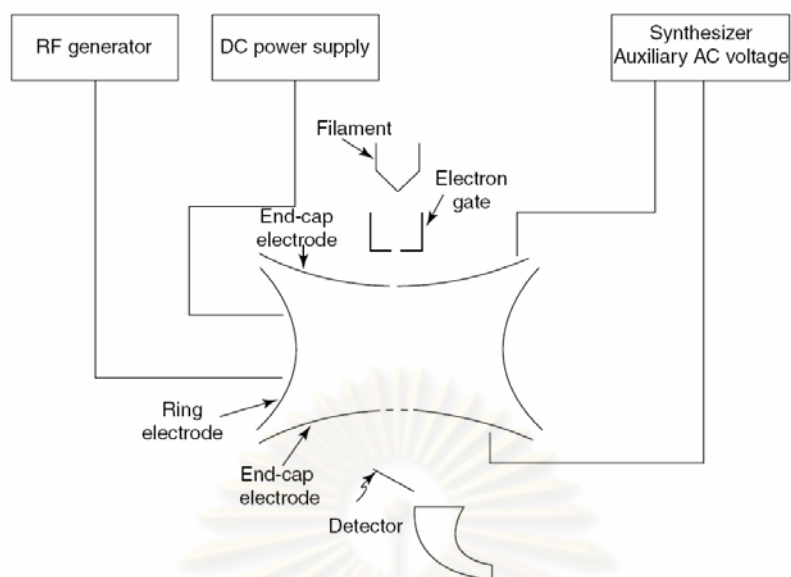
Figure 2.7 Schematic of the quadrupole ion trap mass analyzer *(29)*

2.3.2.3 Time of Flight (TOF) Mass Analyzer

Time of Flight (TOF) analysis is based on accelerating a set of ions to a detector with the same amount of energy. Ions have the same energy 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.

The resolution of a TOF analyzer is dependent upon the ability to measure the very small differences in time required for ions of similar m/z to reach the detector. Increasing the distance that the ions travel between source and detector, i.e., increasing the length of flight tube would accentuate any such small time-differences. The implication of such an increase is that the instrument would be physically larger and this goes against the current trend towards the miniaturization of all analytical equipment.
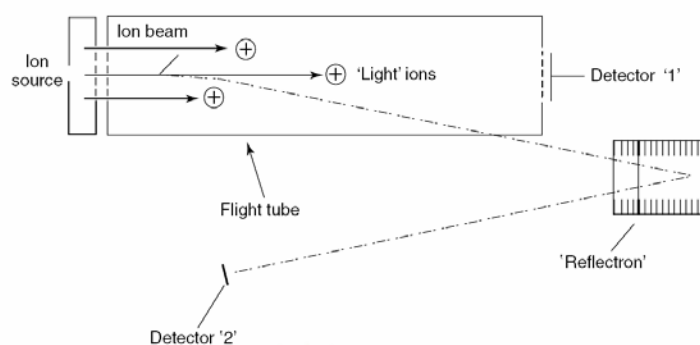
Figure 2.8 Schematic of the Time-of-Fligth (TOF) mass analyzer *(29)*

2.3.3 Detector

The detector is used to measure the ions leaving from the mass analyzer by converting the ions into an electrical current or other forms of signal, processing and recording into mass spectrum. A detector is selected by speed, dynamic range, gain and geometry. Most detectors currently used to amplify the ion signal are electron multiplier tube (Figure 2.9) and photo multiplier tube (Figure 2.10). Electron multiplier tube offers electron from surface of tube for analyte ions. The entrance of tube is held with potential charge opposite from the analyte ions. Analyte ions are attracted to the entrance of tube and collide with tube surface, then the inner surface coated with electron-emissive material releases electrons. These electrons are accelerated to hit another portion of tube by electrostatic force and surface loses more electrons in every collision. Amplified electrons are counted by an electrical circuit and displayed as signal intensity. The photo multiplier tube comprises a photocathode and a series of dynodes. In the high voltage tube, incident photon strikes the photo cathode and emits electrons due to the photoelectric effect. These electrons are accelerated towards a series of additional electrodes called dynodes, the amount of electrons is increased at every collision. This creates an amplified signal that is finally collected and measured at the anode.
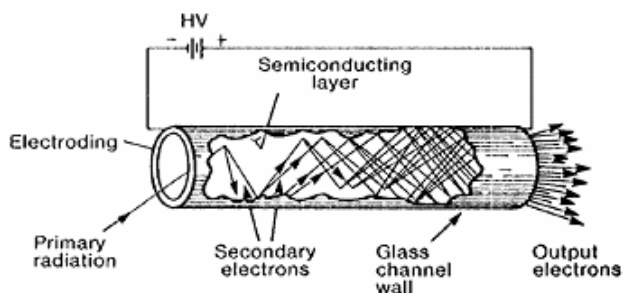
Figure 2.9 Electron multiplier tube *(40)*
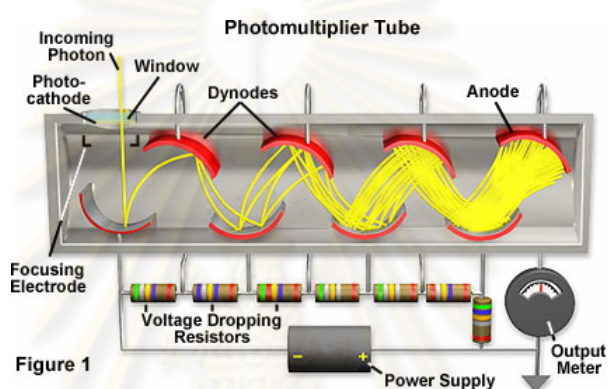
Figure 2.10 Photo multiplier tube *(41)*

2.3.4 **Tandem Mass Spectrometry (MS/MS)**

Tandem mass spectrometry (MS/MS) is a term which covers a number of techniques in which one stage of mass spectrometry, not necessarily the first, is used to isolate an ion of interest. The second stage is then used to probe the relationship of this ion with other form which it may have been generated or which it may generate on decomposition. These two stages of mass spectrometry are related in specific ways in order to provide the desired analytical information. There are a large number of different MS/MS experiments that can be carried out but the four most popular are the product-ion scan, the precursor-ion scan, the constant-neutral-loss scan and selected decomposition monitoring.

The triple quadrupole is probably the most widely used MS/MS instrument. The hardware, as the name suggests, consists of three sets of quadrupole rods in series

(Figure 2.11) The second set of rods is not used as mass separation device but as a collision cell, where fragmentation of ions transmitted by the first of quadrupole rods is carried out, and as a device for focusing any product ions into the third set of quadrupole rods. Both sets of rods may be controlled to allow the transmission of ions of single m/z ratio or a range of m/z values to give the desired analytical information.
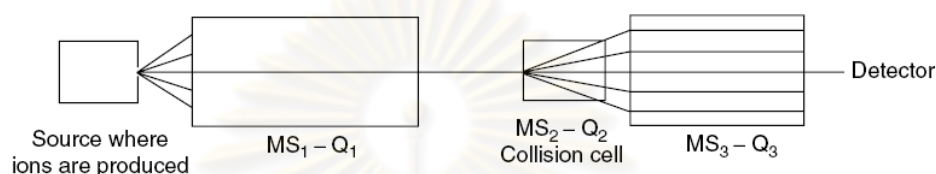
Figure 2.11 Schematic of the triple quadrupole mass spectrometer *(29)*

Ion trap mass spectrometer is capable of performing tandem mass spectrometry (MS/MS), in which a certain ion is selected for fragmentation. This can help to identify particular elements in a molecule. During fragmentation, bonds in the molecule break, thereby producing fragment ions that are characteristic for certain chemical moieties. In some cases, highly labile bonds are present and fragmentation will yield only little compositional information. In that case it is possible to perform sequential fragmentation (MS^n), which enables isolation and subsequent fragmentation of fragment ions. It is possible to break a molecule down to tiny pieces yielding more detailed information on the molecule structure.

In contrast to an ion trap, a time of flight (TOF) is not capable of performing tandem mass spectrometry. On the other hand, it can determine molecular masses of ionized compounds with much higher accuracy than the ion trap. Accurate mass determination can also aid in resolving the elemental composition of an unknown compound.

2.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) *(42)*

The use of the hyphenated technique liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the analysis of food extracted provides important advantages because of the combination of the separation capabilities of LC and the power of MS/MS as an identification and confirmation method. Analysis of complex mixture such as extracts of food products which requires highly selective analytical methods to identify and quantify targeted compounds. HPLC with its wide range of applicability offers the best choice of separation method. Recently, developments in the coupling of HPLC and MS/MS have overcome major analytical problems.

CHAPTER III

EXPERIMENTAL

3.1 Instrumental and Apparatus

3.1.1 Ultra Performance Liquid Chromatography (UPLC): Water Acquity UPLC™ consists of an automatic degasser, a binary pump, an autosampler and a column thermostat, Waters, Corporation, MA, USA.

3.1.2 Mass spectrometry detector (MSD): Micromass Quattro Premier™ XE benchtop tandem quadrupole mass spectrometer using an atmospheric pressure electrospray ionization (AP-ESI) interface and MassLynx 4.1 software processing, Waters Corporation, MA, USA.

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

3.1.4 HPLC column: ZIC®-HILIC, zwitterionic silica-based, 100 x 2.1 mm I. D., 3.5 μm, Sequant, Merck.

3.1.5 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.

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

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

3.1.8 Nitrogen gas, ultra high purity grade (99.999% purity), Chatakorn lab center CO., LTD, Bangkok, Thailand.

3.1.9 Argon gas, ultra high purity grade (99.999% purity), Chatakorn lab center CO., LTD, Bangkok, Thailand.

3.1.10 Centrifuge, Beckman Coulter, Krefeld, Germany.

3.1.11 Microcentrifuge, Microfuge[®]18, Beckman.

3.1.12 Microtubes, 2.0 ml clear MCT-200-C, Axygen, California USA.

3.1.13 pH meter, Model HM- 20S, TOA electronic Ltd., Japan.

3.1.14 Micropipetts   0.1-10, 10-100, 100-1000 µL and tips, Eppendorf, Hamburg, Germany.

3.1.15 Filter membrane 47 mm, 0.2 µm, type Nylon, Whatman International Ltd.,Maidstone, England.

3.1.16 Syringe filters, 13 mm, 0.2 µm, Chrom Tech, MN, U.S.A.

3.1.17 HPLC amber vials 2 mL , Agilent Technologies, CA, U.S.A.

3.1.18 preslit cap, La-Pha-Pack[®]GmbH, Germany

3.1.19 Volumetric flasks 10.00, 50.00, 100.00 mL.

3.1.20 Beakers 100, 250, 1000 mL.

3.1.21 Graduated cylinders 10.0, 50.0, 100.0 mL.

3.1.22 Spatulas

3.1.23 Stirring rod

All experimental glasswares were washed sequentially with detergent and follow by rinsed with deionized water and acetone before used.

3.2 Chemicals

3.2.1 Standard Compounds

Enrofloxacin (ENR), ciprofloxacin (CIP), oxolinic acid (OXO), oxytetracycline (OTC), chlortetracycline (CTC), tetracycline (TC), ampicillin (AMP), penicillin G (PEN G), spiramycin (SPI), tilmicosin (TIL), tylosin (TYL), erythromycin (ERY), spectinomycin (SPEC), lincomycin (LIN), amprolium (AMPR), and sulfadimethoxine (SDMX) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Streptomycin (STR) and dihydrostreptomycin (DSTR) were purchased from Fluka (Buchs, Switzerland). Amoxicillin (AMOX), sulfadiazine (SDZ), sulfathiazole (STZ) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Danofloxacin (DAN) was from Riedel-de Haën (Seelze, Germany). Sulfamethazine (SMZ) was obtained from Wako Chemicals (VA, USA). Pirlimycin (PIR) was from Pfizer (NY, USA).

3.2.2 Organic Solvents and other chemicals

Acetonitrile HPLC grade for sample preparation and LC/MS grade for analysis were purchased from J.T. Baker (Deventer, The Netherlands), ammonium formate and formic acid were supplied by Fluka (Buchs, Switzerland), trichloroacetic acid was from Fisher scientific (Leicestershire, UK), hexane was obtained from Kanto Chemical (Tokyo, Japan). A Milli-Q water purification system from Millipore (Billerica, MA, USA) with 18.2 MΩ/cm resistivity was used.

3.3 Preparation of Standard Solutions

3.3.1 Preparation of stock standard solutions

Individual standard solution containing 100 mg/L was prepared by weighing 0.0100 g of each standard materials and dissolving them in 100.00 mL volumetric flasks with acetonitrile for SMZ, SDZ, SDMX, STZ, TIL, TYL, ERY, SPI, OXO, CIP, ENR and DAN, methanol for TC, OTC, CTC, PEN G and AMPR. Then, each standard stock solution was transferred to an amber glass bottle with Teflon screw cap and stored at -20 °C in the freezer. For SPEC, STR, DSTR, AMOX and AMP were prepare with water and stored at 4 °C in refrigerator until use.

3.3.2 Preparation of mixture standard solutions

The mixture of 24 standard solutions at 1.00 mg/L was prepared by pipetting 100 µL of each stock standard solutions, as detailed in section 3.3.1 and made volume to 10.00 mL with 50% acetonitrile in volumetric flask. These standards were prepared daily and stored at 4 °C in refrigerator until use.

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

3.3.3 The Standard Solutions for tuning

An individual standard solution containing 10.0 mg/L was prepared by pipetting 100 µL of stock standard solutions, as detailed in section 3.3.1 and diluting to 1.0 mL with 50% acetonitrile in an amber vial. These standards were prepared daily and stored at 4 °C in refrigerator until use.

3.4 The Optimum Instrumental Analysis Conditions

In this research, the studied antibiotics were measured on the LC-MS/MS system consisting of an Waters, Acquity UPLC™ coupled to Micromass Quattro Premier™ XE benchtop tandem quadrupole masss spectrometer (Milford, MA, USA). Electrospray ionization (ESI) was used as an ionization source in the positive mode.

3.4.1 MS/MS optimization

Optimized conditions for the tandem mass spectrometer were performed using the capillary voltage 1.0 kV, with the source temperature 120 °C, desolvation temperature 350 °C, extractor voltage 3 V, cone gas (nitrogen) flow 50 L/h, desolvation gas (nitrogen) flow 1000 L/h, and the argon was used as the collision gas at 3.5×10^{-3} mbar. Instrument control and data processing was evaluated using the MassLynx 4.1 software package from Micromass™ (Waters, MA,USA).

Each antibiotic standard tuning solution was directly injected into the electrospray source by syringe pump. Full scan and collision activated dissociation tests were operated to set up an appropriate multiple reactions monitoring (MRM) with the two most sensitive and stable transitions used in both quantification and confirmation purpose of all compounds.

3.4.2 LC optimization

In the LC system, chromatographic separation was performed on on a zwitterionic ZIC®-HILIC column (100 mm x 2.1 mm; 3.5 µm particle size) from SeQuant (Umea, Sweden), the column temperature was 40 °C. The flow rate was set at 0.2 mL/min and the injection volume was 10 µL. The following mobile phase gradient was applied: 50 mM ammonium formate, pH 2.5 (mobile phase A); and acetonitrile (mobile phase B). The separation of 24 studied compounds was achieved within 10 minutes in the following gradient program is shown in Table 3.1.

Table 3.1 Optimized gradient elution profile for HILIC separation

Time (min)	initial	1.50	4.00	6.00	10.00	11.00	15.00
% A	10.0	10.0	60.0	90.0	90.0	10.0	10.0
% B	90.0	90.0	40.0	10.0	10.0	90.0	90.0

3.5 Extraction method optimization

For animal muscle, as much fat as possible was removed from the chicken sample. The sample was cut into small pieces and homogenized. In development and optimization of simultaneous extraction for different classes of compounds, the extraction solution was selected in accordance with the physical and chemical properties of the analytes.

3.5.1 The procedure of extraction solution optimization

In this experiment, the study of trichloroacetic acid concentration as the extraction solution on the percent recoveries of each compound can be described as follows :

3.5.1.1   A <u>blank sample</u> was prepared by weighing 5.00 g of chicken sample into the polypropylene centrifuge tube.

3.5.1.2   A 10 mL mixture of 2% (w/v) trichloroacetic acid and acetonitrile (1: 1) was added into chicken and vortexed for 30 sec.

3.5.1.3   The mixture was then mechanically shaken for 10 min.

3.5.1.4   The mixture solution was centrifuged at 3,400 rpm for 5 min.

3.5.1.5   5 mL of hexane was added into the mixture solution to remove fat from the chicken sample.

3.5.1.6   The sample solution was vortexed for 1 min and centrifuged at 3,400 rpm for 5 min.

3.5.1.7   The hexane layer was discarded.

3.5.1.8   The sample solution 200 μL was diluted to 1 mL with 10% formic acid : acetonitrile (1:9, v/v).

3.5.1.9   The sample was filtered through a 0.2 μm nylon membrane syringe filter prior to LC-MS/MS analysis.

3.5.1.10 <u>Spike samples</u> were prepared by adding the standard mixture solution into the 5.00 g chicken sample at MRLs concentration level.

3.5.1.11 Each spiked sample in step 3.5.1.10 was extracted following the same procedure described through 3.5.1.1-3.5.1.9.

3.5.1.12 The final concentration was calculated and reported as percent recovery of analyte.

The black highlight refers to the varied parameters.

3.5.2   The procedure of dilution ratio optimization

In this experiment, the study of dilution ratio (sample solution: dilution solution) on the percent recoveries of each compound can be described as follows:

3.5.2.1   A <u>blank sample</u> was prepared by weighing 5.00 g of chicken sample into the polypropylene centrifuge tube.

3.5.2.2   A 10 mL mixture of 2% (w/v) trichloroacetic acid and acetonitrile (1: 1) was added into chicken and vortexed for 30 sec.

3.5.2.3   The mixture was then mechanically shaken for 10 min.

3.5.2.4   The mixture solution was centrifuged at 3,400 rpm for 5 min.

3.5.2.5   5 mL of hexane was added into the mixture solution to remove fat from chicken sample.

3.5.2.6   The sample solution was vortexed for 1 min and centrifuged at 3,400 rpm for 5 min.

3.5.2.7   The hexane layer was discarded.

3.5.2.8   The sample solution 200 µL was diluted to 1 mL with 10% formic acid : acetonitrile (1:9, v/v).

3.5.2.9   The sample was filtered through a 0.2 µm nylon membrane syringe filter prior to LC-MS/MS analysis.

3.5.2.10   <u>Spike samples</u> were prepared by adding the standards mixture solution into 5.00 g chicken sample at MRLs concentration level.

3.5.2.11   Each spiked sample in step 3.5.1.10 was extracted following the procedure described in 3.5.1.1-3.5.1.9.

3.5.2.12   The final concentration was calculated and reported as percent recovery of analyte.

The black highlight refers to the varied parameters.

3.6 Method validation *(5, 43)*

Validation of the presented method in this research has been performed according to the requirements defined by the guidelines of the EU Commission Decision 2002/657/EC *(5)*, which establishes the performance criteria for the analytical residue method.

3.6.1 Standard calibration curve

The standard calibration curves were prepared by using the matrix matched standard, adding the appropriate amounts of the antibiotics into blank chicken sample extracts. The concentration ranges for each compound correspond to the MRLs with 10 calibration level. Each level was prepared in triplicate.

3.6.2 Linearity

The linearity of a test procedure is its ability (within a given range) to obtain test results proportional to the concentration of analyte in the sample. Linearity of this method was obtained from the standard calibration curve of all analytes. Correlation coefficient (R^2) represents the linearity of the proposed method. Under optimized LC-MS/MS conditions, the linearity was performed over a concentration ranged of 0.5-100 µg/L with three replicates of each level. The calibration curves were plotted as concentration over peak area of each analyte. The slope, y-intercept and correlation coefficient (R^2) of all antibiotics are shown in Table 4.3.

3.6.3 Specificity

To verify the absence of interfering substance around the retention time of the analytes, by analyzing 20 blank chicken samples. The samples were confirmed to be free of target compound residues by LC-MS/MS after sample preparation which used the developed procedure.

3.6.4 Accuracy

The method accuracy refers to the closeness of agreement between the observed results from method and the true value of the analyte in the sample. The recovery experiments were carried out at three concentration levels in independent sample at 0.5, 1.0, 1.5 times MRLs concentration level in 10 replicates for each level.

3.6.5 Precision

The precision is the closeness of agreement between independent test result obtained under same condition. The two categories of precision are intra-assay precision and intermediate precision. The intra-assay precision is the precision derived from repeated tests on the same method with single analytical run, while the intermediate precision is the precision acquired from the repeated test on the same method with different operators or different times. In this work, the intra-assay precision was calculated from the analysis of 10 blank chicken samples fortified with all analytes at each of the three specified fortification levels (0.5 MRL, MRL and 1.5 MRL level). Within laboratory precision was obtained by following the same protocol but performing the analyzes in three different days with two specified fortification levels (0.5 MRL and MRL level).

Ten replicate sample determinations were made together with a simple statistical assessment of the results including the percent of relative standard deviations (% RSD). The % RSD obtained from the results of one analytical day refers to intra-assay precision, whereas intermediate precision was reported as the % RSD from the results of three analytical days.

3.6.6 Limit of detections (LODs) and limit of quantifications (LOQs)

LOD refers to the method lowest concentration of analyte detected, while LOQ is the lowest concentration of analyte which can be quatitatively determined. The LOD was calculated at a signal to noise (S/N) ratio of 3 and the LOQ value was calculated by using a S/N ratio of 10. In this work, LODs and LOQs were obtained by the transition with highest S/N ratio in MRM mode.

3.6.7 Decision limit (CCα)

In the case of substances an with established limit, CCα can be defined in two different ways, first by using the calibration curve procedure according to ISO 11843 (here referred to as critical value of the net state variable). In this case blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the samples and identify the analytes. After identification, plot the signal against the add concentration. The corresponding concentration at the permitted limit plus 1.64 times the standard deviation of the within-laboratory reproducibility equals the decision limit (α = 5%) or by analyzing at least 20 blank materials per matrix fortified with the analyte at the permitted limit. The value of the permitted limit plus 1.64 times the corresponding standard deviation equal the decision limit (α = 5%).

3.6.8 Detection capability (CCβ)

In the case of substances an with established limit, CCβ can be defined in two different ways, first by using the calibration curve procedure according to ISO 11843 (here referred to as minimum detectable value of the net state variable). In this case representative blank material shall be used, which is fortified around the permitted limit in equidistant steps. Analyse the sample and identify the analytes. Calculate the standard deviation of the mean measured content at the decision limit. The corresponding concentration at the value of decision limit plus 1.64 times the standard deviation of the within laboratory reproducibility equals the detection capability (β = 5%) or by analyzing at least 20 blank materials per matrix fortified with the analytes at the decision limit. The value of the decision limit plus 1.64 times the corresponding standard deviation equals detection capability (β = 5%).

3.6.9 Validation of substances for which a sum MRL is established

For SAs and ENR, MRL for the sum of the residues of all substances belonging to the SAs group and substance with its metabolites, respectively according to the requirements of SANCO/2726/2004rev.4 have to be fulfilled. Therefore it was necessary to validate at low concentrations. In this work, the spike level of 0.1, 0.2 and 0.4 times MRLs for SAs and 0.2, 0.5, 1.0 times MRLs for ENR and CIP were chosen.

3.7 Application to real sample

The developed method was applied to analysis in real chicken sample. The 40 chicken samples taken in various local fresh market and supermarket, Bangkok, Thailand and analyzing under the optimized condition.

CHAPTER IV

RESULT AND DISCUSSION

4.1 The Optimization of MS/MS Conditions

From the experimental conditions, the results of optimum instrumental analysis conditions are detailed as follows:

4.1.1 Optimization of ESI-MS/MS parameters

The important parameters of the MS/MS system were optimized to achieved the maximum sensitivity by the manual tuning of the standard substances using syringe infusion pump in the positive ESI mode. The most intensive transition of the compounds are selected for the quantification and the second transition for the confirmation of the compounds. All compounds produced the protonated ion, $[M+H]^+$ as precursor ion except for SPEC and AMPR which produced an intensive water adduct $[M+H_2O+H]^+$ and protonated molecules with a loss of chloride ion $[M-Cl+H]^+$, respectively. The adduct of SPEC is a very sensitive and stable which produced the higher response than the pseudo-molecular ion. The cone voltage was adjusted to its highest signal at the first quadrupole of the mass spectrometer. After that the product ion spectra was recorded at different values of collision energies to find the two most intense transitions for each compound. Then set up an appropriate MRM method, to assess the best signal sensitivity of each analyte, adequately long dwell times are required. Table 4.1 shows MS/MS transitions for quantification and confirmation as well as cone voltages and collision energy values optimized for each of the selected compounds.

Table 4.1 MS/MS parameters for the selected antibiotics

Analyte	M.W.	t_R	C.V. (V)	Quantification transition	C.E. (eV)	Confirmation transition	C.E. (eV)
AMOX	365.4	4.81	20	366.25 > 208.15	13	366.25 > 349.20	10
AMP	349.4	4.75	25	350.23 > 105.90	20	350.23 > 192.10	15
PEN G	334.4	1.5	45	335.32 > 217.24	15	335.32 > 90.91	35
TIL	869.1	4.88	55	869.53 > 174.39	55	869.53 > 696.51	50
SMZ	278.3	1.47	35	279.09 > 186.02	20	279.09 > 124.07	30
SDZ	250.3	1.55	25	251.10 > 155.91	15	251.10 > 107.84	25
SDNX	310.3	1.42	40	311.08 > 156.16	25	311.08 > 108.16	35
STZ	255.3	1.76	30	256.09 > 107.87	25	256.09 > 155.93	15
CIP	331.3	4.69	37	332.22 > 314.22	23	332.22 > 245.22	25
ENR	359.4	4.55	30	360.14 > 245.09	35	360.14 > 316.00	26
OXO	261.2	1.5	32	262.10 > 244.13	20	262.10 > 216.02	35
OTC	460.4	4.89	27	461.19 > 426.18	20	461.19 > 443.21	13
CTC	478.9	4.62	30	479.11 > 462.07	18	479.11 > 444.16	20
TC	444.4	4.82	25	445.25 > 410.45	20	445.25 > 154.15	25
TYL	916.1	4.37	57	916.48 > 174.19	40	916.48 > 772.94	35
LIN	406.5	4.85	40	407.16 > 126.09	30	407.16 > 359.21	20
PIR	410.1	4.71	35	411.17 > 111.82	27	411.17 > 363.15	18
DAN	357.3	4.65	35	358.15 > 82.22	45	358.15 > 340.00	35
ERY	733.9	4.44	25	734.57 > 576.38	25	734.57 > 522.34	25
SPI	843.1	4.97	30	843.51 > 174.10	45	843.51 > 101.07	58
AMPR	278.8	5.41	20	243.11 > 150.05	15	243.11 > 122.03	25
SPEC	332.3	6.33	35	351.21 > 333.27	20	351.21 > 97.75	30
STR	581.6	7.65	65	582.14 > 263.27	30	582.14 > 246.14	40
DSTR	583.6	7.57	60	584.14 > 263.18	30	584.14 > 246.15	40

4.1.2   Mass fragmentation pathways of targeted compound

The mass fragmentation pathways of 24 compounds were studied using ESI, the soft ionization technique which provides the molecular ions as the precursor ions. At suitable fragmentor voltage, common fragmentation ions of targeted analytes which have already been discussed in previous studies *(19, 22, 42, 44-49)* are observed. The results obtained in this work agree with those papers and that the mass spectral data can be used to determine each class of compound for unambiguous identification**.**

4.1.2.1 Aminoglycosides

For STR and DSTR, the fragment pathway of m/z is 246 as glycosidic cleavage and neutral loss as m/z 263 was investigated. In case of SPEC, it was demonstrated that the SPEC gave abundant product ion at m/z 333 by loss of the H_2O and the m/z 98 which derived from m/z 333.

4.1.2.2 Sulfonamides

For SAs, in case of SDZ, SDMX and STZ two characteristic fragment ions with m/z 156 and 108 were observed. The former corresponds to the common molecular fragment for all sulfonamides which is *p*-aminobenzenesulfonic acid moiety generated from $[M-RNH_2]^+$, while m/z 108 corresponding to $[M-RNH_2-SO]^+$ fragment. However, the case of SMZ provides the other fragment ions that are used as ions characteristic of this compound. It produced the ion at m/z 186 and 124 corresponding to $[M-H_2NPh]^+$ and $[RNH_2+2H]^+$ fragment, respectively.

4.1.2.3 Quinolones

For Qs, OXO which have no piperazinyl ring therefore showed less fragmentation. The loss of H_2O $[M-H_2O+H]^+$ was the most intense fragment observed as well as for DAN and CIP and also the fragment ion at m/z 206 for OXO, m/z 82 for DAN and m/z 245 for CIP.

4.1.2.4 Macrolides

The basic structure of MCs consist of 12-, 14- or 16-membered macrocyclic lactone to which several amino and/or neutral sugars are bound. The sugar moieties can form product ions which are illustrated by the presence of ions at m/z 174 corresponding to the sugar moieties [o-desosamine+H]$^+$ for TIL, TYL and SPI.

4.1.2.5 Penicillins

This class contain a side chain attached to the 6-aminopenicillanic acid nuclei. All of the PCs studied share a fragment of m/z 160 corresponding to the thiazolidinic ring. Also characteristic is the presence of the ion formed due to the loss of this fragment [M+H-159].

4.1.2.6 Tetracyclines

TCs have a structure formed by an octahydrotetracene-2-carboxamide skeleton. The characteristic fragmentation patterns for the tetracyclines are the loss of water or ammonia. In the case of TC, the abundance of a fragment of m/z 154 has also been founds in this study.

4.1.2.7 Lincosamides

Identity of LIN was confirmed by the presence of two fragments at m/z 126 and 359 from the precursor ion which corresponding to the 3-propyl-N-methylpyrrolidine ion and the loss of the thiomethanol molecule, respectively.

4.1.2.8 Amprolium

The precursor ion for AMPR was m/z 243 which was the cationic fraction of the molecule without chloride. The most abundant product ion for AMPR was [$C_8H_{12}N_3+$] at m/z 150 which derived from the cleavage of the bond between the carbon and the nitrogen of the pyridine ring.
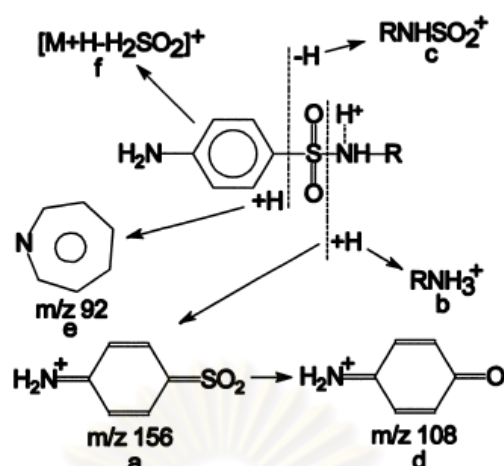
Figure 4.1 Example of fragmentation pathway of some targeted compounds *(50)*

The main advantages of tandem MS/MS detection are the reduction of background noise and providing better selectivity. The sensitivity and confidence level of compound detection can be improved. Therefore, the combination of liquid chromatography and tandem mass spectrometry (LC-MS/MS) is preferred for analysis of samples with complex matrices. In this study, the hyphenated technique was selected for the determination of multi-class antibiotic residues in chicken at trace level.

For the multi-residue LC-MS/MS method, time windows during an LC analysis, while maintaining an optimum number of data points are required to improve precision of peak area and sensitivity. The optimized MRM time window for this method is shown in Figure 4.2.
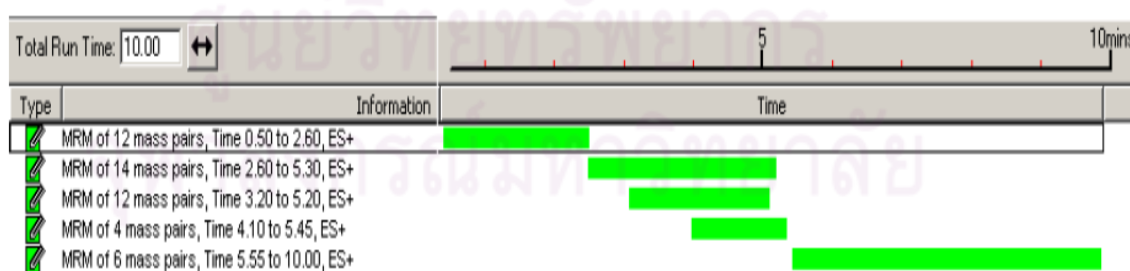
Figure. 4.2 Time window used to perform multi residues analysis with a inter-channel delay of 0.02 sec and inter-scan delay of 0.02 sec.

One advantage of LC-MS/MS, apart from the high sensitivity and specificity of the technique, is the possibility of significantly reducing chromatographic run times. The

mass spectrometer separately measures the characteristic ions of each analyte. Although some studied compounds could not be separated completely at baseline resolution, the complete separation of compounds was achieved with mass transitions. Multiple Reaction Monitoring (MRM) is a tandem mass spectrometric technique that allows the monitoring of specific Collision Induced Dissociation (CID) reactions. The MRM chromatogram of all targeted compounds are shown in Figure 4.9.

4.2 Optimization of chromatographic conditions

Although complete separation is not necessary for the selective MS/MS detection, the chromatographic technique which generally improves sensitivity and reduces ion suppression effect is required. Acetonitrile is now considered to be the best organic mobile phase solvent for HILIC separation and was applied to this work. In order to obtain sufficient retention times, the use of a buffer in eluent or acidification of the mobile phase was recommended, even though it caused the reduction of signal intensities due to ion suppression effects in the MS interface. For the LC-MS/MS analysis, volatile compounds such as ammonium formate, ammonium acetate or formic acid were preferred as mobile phase additives. In this study, the optimization of chromatographic conditions is performed in order to enhance the sensitivity of MS detection. Various parameters which effect the chromatographic separation and MS detection were also investigated.

4.2.1 Selection of HILIC stationary phase

The first step was the selection of the analytical column between Acquity UPLC™ BEH HILIC and ZIC®-HILIC column which contain unbounded silica and sulfobetaine functional group as stationary phase, respectively. The obtained results show that the ZIC®-HILIC column gives higher efficiency than the other and its efficiency is provided a better peak shape for the TCs group and greater sensitivity for the AGs. The ZIC®-HILIC stationary phase has permanent zwitterionic groups that contain both positive and negative charges covalently attached to porous silica with the overall charge being neutral. This material provides a unique environment, not only particularly capable of solvating polar and charged compounds, but also offering the possibility of weak electrostatic interaction with analytes carrying either positive or

negative charges. The retention thus generally increase with hydrophilicity and with charge of the analyte. For these reason, the ZIC®-HILIC was selected for this study.

The ZIC®-HILIC column is a suitable stationary phase for HILIC mode separation of multi-class antibiotic. By taking advantage of the weak electrostatic interactions between the analytes and the overall neutral zwitterionic stationary phase and performing proper tuning of the mobile phase with respect to ionic strength, pH, buffer salt, the column exhibits a unique selectivity in the analysis of a wide range of compounds.

4.2.2 Optimization of mobile phase gradient

Mobile phase strength is an important parameter in HILIC separation. In this study, the retention behaviors of antibiotics on a ZIC®-HILIC column were investigated. The analyte retention times were observed to be inversely proportional to the water content in the eluent and to increase with the polarity of the solute , due to in HILIC, water is a stronger eluting solvent while acetonitrile is a weaker one.

In order to shorten the chromatographic analysis time and separation of the analytes which have a wide range of polarity, a gradient program from 90% acetonitrile to 90% aqueous phase was performed (as show in Table 4.2). The separation order opposite with the reversed phase due to in HILIC, hydrophilic, polar and charged compounds are retained preferentially compared with hydrophobic neutral compounds.

4.2.3 Selection of mobile phase pH

Generally, mobile phase pH is an important parameter in the HILIC separation. Neutral or acidic mobile phase pH is normally used due to the instability of silica based columns at high pH. In this study, the effect of buffer pH in the range from 2.5, 3.0 to 4.0 on separation was investigated. The retention time of all target compounds had no significant difference in the varied pH range. However, the buffer pH can affect to the solute ionization in the mobile phase which showed highest sensitivity of AGs at pH 2.5. Therefore, the used of buffer pH 2.5 was satisfactory for further optimization.

4.2.4 Selection of ionic strength (buffer concentration)

Suitable buffer types for HILIC separations are formate and acetate, due to their excellent solubility even in very high concentrations of organic solvent. A buffer concentration in the range from 5-20 mM is recommended for most analytes. *(37)* In this study, the effect of ionic strength on retention time was examined at pH 2.5 with ammonium formate concentration at 10 mM, 50 mM and 100 mM. From the result, as ionic strength increased, the retention time decreased significantly for STR and DSTR due to higher salt concentration in the eluent would weaken the electrostatic interaction between the protonated basic compounds and surface silinol groups on silica by competing with these active silinol sites. Furthermore, the effect of ionic strength can be affected to the sensitivity of the analyte, 100 mM buffer concentration showed signal suppression more than 10 mM and 50 mM. The ionic strength of 50 mM was selected for further development based on the compromising between retention time and sensitivity effect.

Table 4.2 The HPLC chromatographic condition using ZIC®-HILIC column for the analysis of antibiotics.

Parameters	Conditions	
Analytical column	ZIC®-HILIC column (2.1x100 mm, i.d. 3.5 µm)	
Mobile phase	A: 50 mM ammonium formate pH 2.5	
	B: acetonitrile	
Gradient program	Time (min)	%B
	0.0	90.0
	1.50	90.0
	4.0	40.0
	6.0	10.0
	10.0	10.0
	11.0	90.0
	15.0	90.0
Flow rate	0.2 mL/min	
Injection volume	10 µL	
Column temperature	40 °C	
Sample temperature	20 °C	
Detector	Tandem mass spectrometry detector	
	using the MS/MS parameters as showed in Table 4.1	

Due to the interaction between residual silanols in the stationary phase and the positive charge of basic analytes, peak tailing of LIN, PIR and AMPR was observed when using reversed-phase chromatography or on the addition of ion pairing agents. The use of HILIC enables the extension of retention times for polar analytes, providing good peak shapes and also enhancing the sensitivity of these veterinary drugs, as shown in Figure 4.3. The chromatogram of some representative veterinary drugs at 0.01 mg/kg using the HILIC mode displays higher sensitivity of approximately tenfold in comparison to the ion pairing mode and reversed phase at a concentration of 0.10 mg/kg.
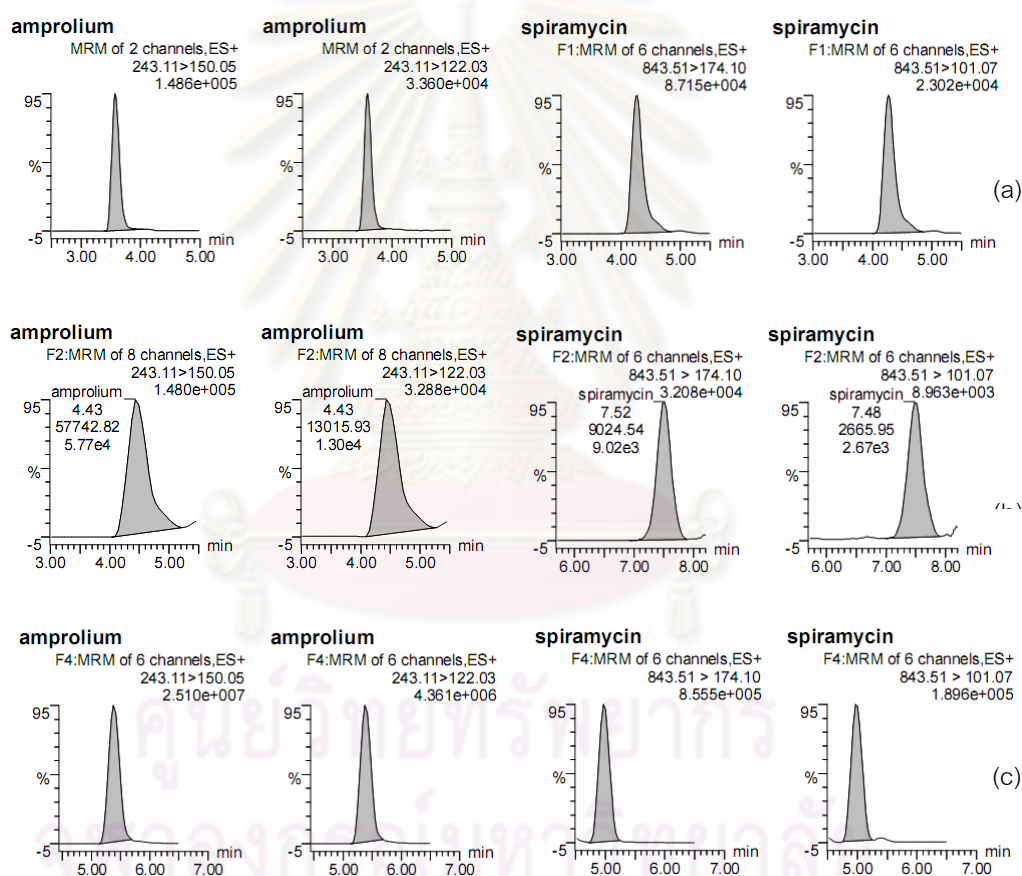
Figure 4.3 Comparison of LC-MS/MS chromatogram between HILIC and other separation modes : (a) standard 0.10 mg/kg in reversed phase, (b) standard 0.10 mg/kg in ion pair and (c) standard 0.01 mg/kg in HILIC

4.3 Optimization of the extraction procedure

Sample preparation is often the most difficult part of multi-class antibiotic residues method due to the different chemical and physical properties of the compounds which have to be extracted simultaneously. Single aqueous solution or acetonitrile are insufficient for multi-class extraction because of the wide ranging polarity of the target analytes. Therefore, a mixture of aqueous and acetonitrile was studied at a ratio of 1:1 in order to achieve reasonable recoveries for all compounds. This ratio was further optimized in the following experiments.

4.3.1 Result of extraction solution

In general, for the extraction of antibiotic residues in biological sample such as muscle, tissue and milk, proteins must be removed from the sample because they can interfere with the extraction, chromatography and detection steps. Precipitating the proteins with an organic solvent or in combination with a strong acid such as trichloroacetic acid (TCA) is usually accomplished in food sample. *(37-39)* TCA can be used as protein precipitating agent and also as an extraction solution in this study. In a previous paper *(37)*, 5% TCA was selected as the best option of extraction solvent providing good recovery for AGs group obtained from spiked pork muscle sample. In this experiment, the concentration of TCA was varied from 1.0, 2.0, 3.0, 4.0 up to 5.0%. Figure 4.4 shows the effect of various concentration of TCA on % recovery and indicated that there was a gradual increase in recovery for SPEC, STR and DSTR at increasing TCA concentration levels. 5 % TCA was proved to be effective for the AGs group as proposed in that paper. However, the recovery of ERY decreased significantly and some target compounds were observed to increase signal suppression which showed a lower response area over 2% of TCA concentration (This data is shown in Figure 4.5). Therefore 2 % TCA which provide good recovery for all compounds was selected as the extracting aqueous solution in the next development.
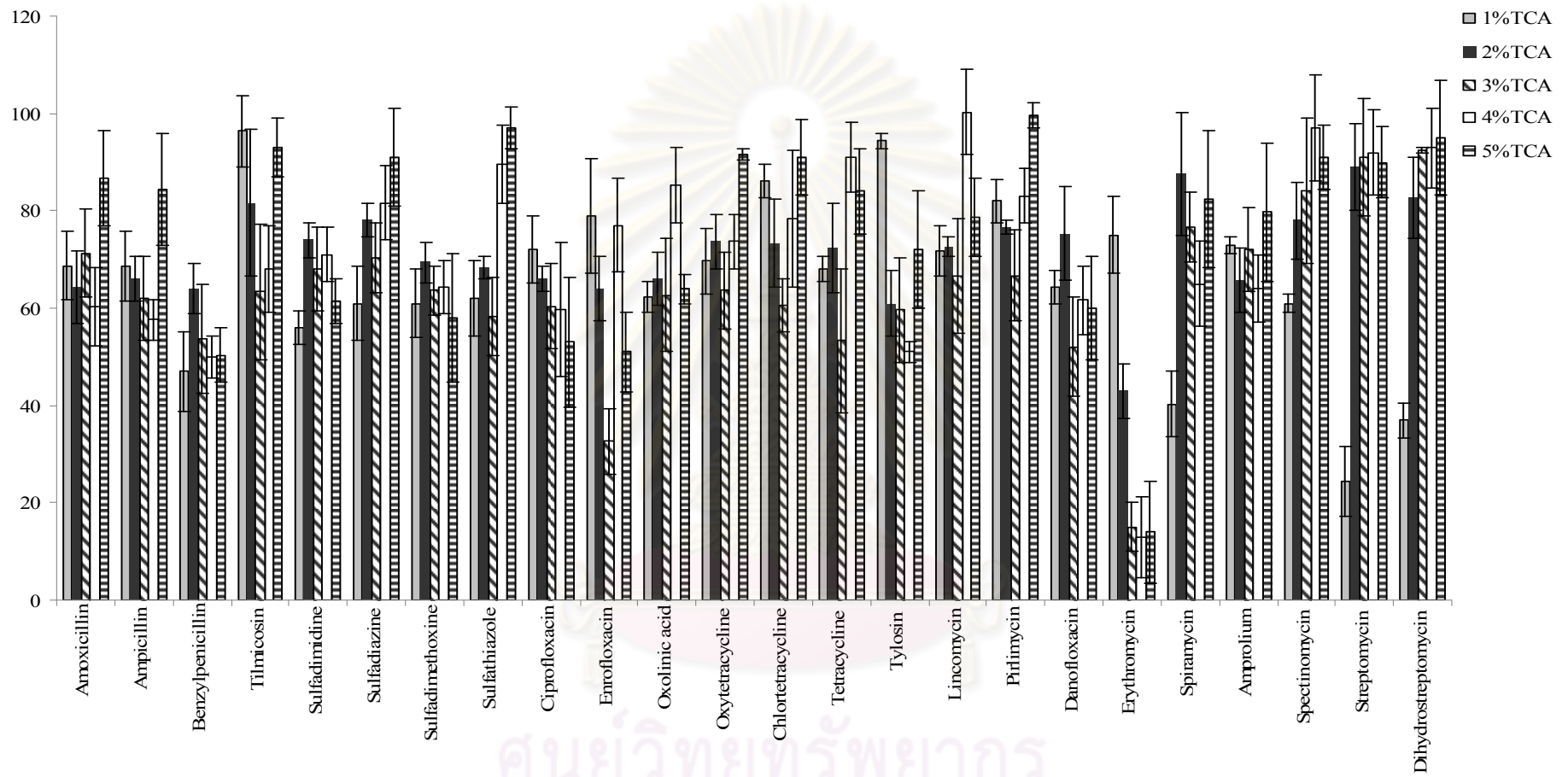
Figure 4.4 The plot of relationship between the percent recoveries of each compound and various concentrations of TCA (n=3)
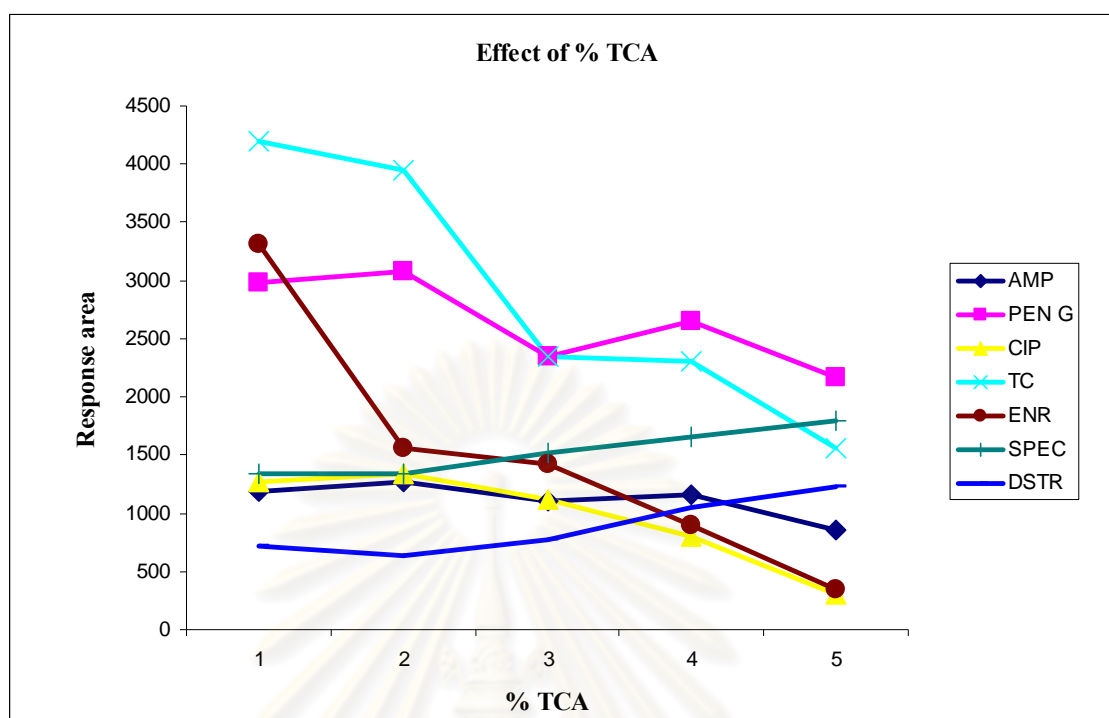
Figure 4.5 The effect of TCA concentration for some selected compounds

4.3.2 Result of sample dilution

Based on the liquid chromatography theory, the ratio of sample diluents should be similar to the initial mixture of the LC gradient program in order to produce a good peak shape. Dilution of the small sample reduced the signal suppression on the injection caused by the matrix effect. In this experiment, SAs and OXO have relatively short retention time on HILIC. However, no interference peaks or co-eluting substances were found and it was possible to obtain a relatively high sensitivity of chromatographic signal. This was partially caused by high percentage of the organic solvent at these elution times. In contrast, the STR and DHTR antibiotics (having the longest retention time) showed peak signals which were affected by high content of the aqueous mobile phase, causing a loss of sensitivity, and produced a poorer response. The middle range eluting compounds had to be diluted, as the matrix effect caused significant ion suppression on the ESI source. In order to optimize the method, several ratios/mixtures of the sample solution and the sample diluent were compared. This parameter should be optimized to reduce matrix effect and eliminate the solvent effect

which induced non-reproducibility of peak retention and area, which was observed from LIN, PIR and TIL. The effect of dilution ratio on response area is shown in Figure 4.6 and the solvent effect is shown in Figure 4.7.
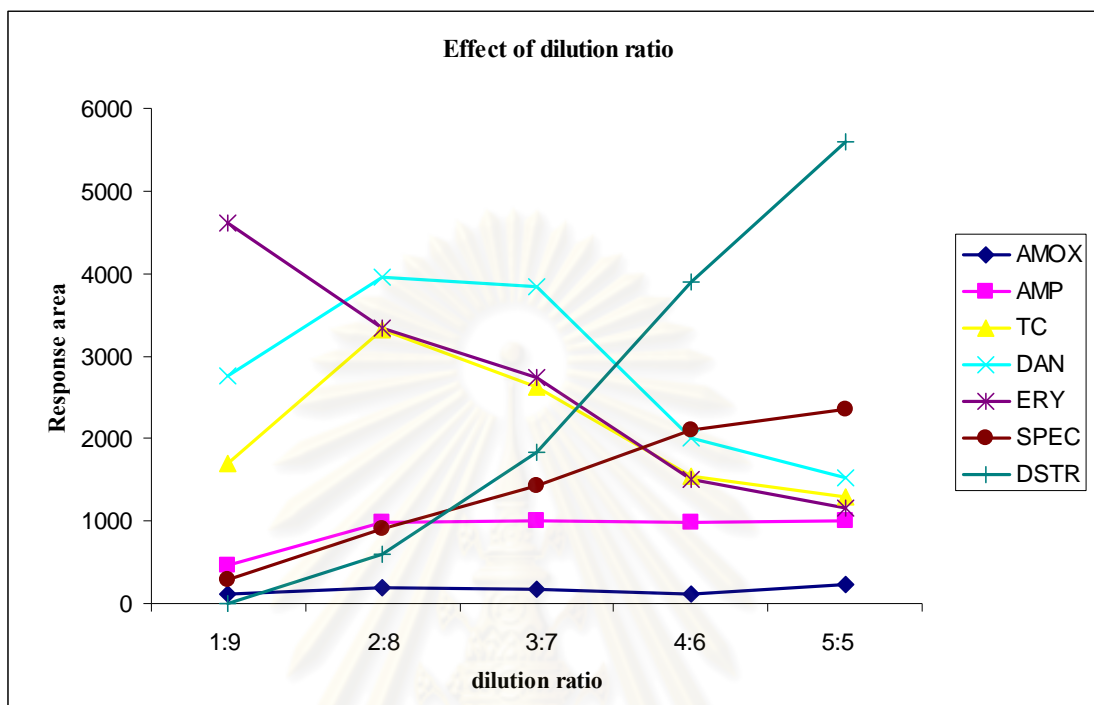
Figure 4.6   The effect of dilution ratio for some selected compounds

Figure 4.6 shows the results, at the ratio 2:8 was found to be most suitable to use in this experiment as considered from the response area.
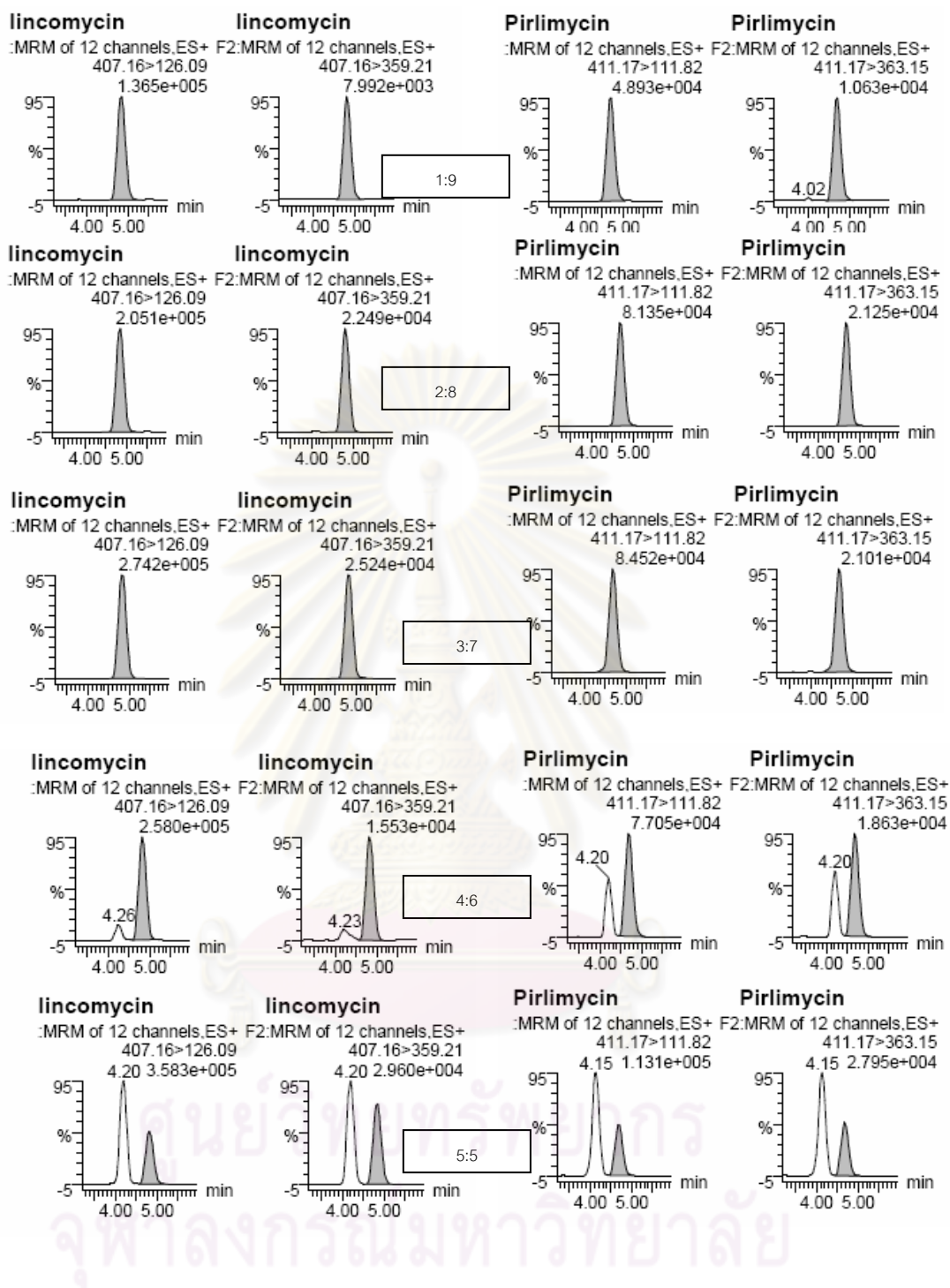
Figure 4.7 The chromatogram of LIN and PIR obtained from the various ratio between the sample solution and sample diluent.

From the chromatograms, the results indicated that the higher ratio of sample diluents will produce a split of peak for LIN and PIR. At the ratio of 3:7, the split of peak was not observed but the method calibration curve was not linear. Therefore, a ratio of 2:8 was optimized to be an appropriate value for a reasonable compromise between linearity and peak split, and also eliminated a reconstitution step in sample preparation.

4.4 Validation results

The developed method was validated in the chicken muscle matrix according to the European Commission Decision 2002/657/EC in terms of the following performance parameters : linearity, specificity, accuracy, intraday and interday precision, decision limit (CCα), and detection capability (CCß).

4.4.1 Method linearity

In LC-MS/MS analysis of food, the MS response obtained from an analyte can differ significantly from that same analyte in matrix. Matrix effects result from co-eluting matrix components that compete for ionization capacity. This competition will vary among samples, causing significantly errors in the accuracy and precision of biological sample analysis. Therefore, matrix-matched standard calibration curves of all compounds are used for quantitative analysis instead of standard calibration curves throughout this study.

Method linearity was assessed by performing calibration curves using chicken samples spiked (matrix-matched calibration) with the selected antibiotics in the range of 1 to 100 µg/kg, corresponding to the MRL. For sulfonamides, the MRL is laid down as a sum of the residues of all substances (belonging to the sulfonamides group), following requirements of SANCO/2726/2004rev.4. *(7)* It is recommended to analyze the samples at spike level which is lower than the MRL, therefore the calibration curve of this group was included 0.5 µgkg^{-1} . The calibration curves were constructed using a peak area based on ten concentration level. Each point was determined in triplicate. The average values were used to constructed calibration curves by plotting the corresponding peak area with analyte concentration. The linear regression plots are

shown in APPENDIX B. The regression data and the correlation coefficient (R^2) are summarized in Table 4.3.

Table 4.3 Linear least-squares regression coefficients of antibiotics standard in matrix solution

No.	Compounds	Slope	y-Intercept	R^2
1	Amoxicillin	58.4	16.5	0.9942
2	Ampicillin	434.6	376.9	0.9973
3	Penicillin G	765.8	190.4	0.9989
4	Tilmicosin	1307.8	194.0	0.9973
5	Sulfamethazine	13812.2	8048.1	0.997
6	Sulfadiazine	10653.6	5796.9	0.9987
7	Sulfadimethoxine	14875.8	7958.5	0.9938
8	Sulfathiazole	132889.9	67758.7	0.9971
9	Ciprofloxacin	644.9	-13.2	0.994
10	Enrofloxacin	454.8	430.6	0.9945
11	Oxolinic acid	50284.9	-1815.6	0.9986
12	Oxytetracycline	581.8	-117.2	0.9972
13	Chlortetracycline	322.0	163.6	0.9973
14	Tetracycline	896.0	-39.0	0.9985
15	Tylosin	1448.5	-448.0	0.9991
16	Lincomycin	6776.3	-1385.1	0.9989
17	Pirlimycin	3214.4	-162.9	0.9988
18	Danofloxacin	472.1	-410.2	0.9953
19	Erythromycin	295.27	508.38	0.9955
20	Spiramycin	1018.8	-411.6	0.997
21	Amprolium	604.3	-797.1	0.9956
22	Spectinomycin	55.5	210.6	0.9974
23	Streptomycin	7.5	34.9	0.9979
24	Dihydrostreptomycin	21.1	27.8	0.9988

As shown in APPENDIX B, the standard calibration curves for all compounds are linear in the studied concentration range 0.5-100 µg/kg. The calibration curves were prepared using at least 10 concentration levels and triplicate analysis and all fit well with the linear model. The correlation coefficients (R^2) varied from 0.9940-0.9991 linearly in detector response and were all acceptable for quantitative analysis. The R^2 value of TYL was found to be the highest (0.9991) and the lowest (0.9940) for CIP. Furthermore, the sensitivity of each analyte which 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, STZ has the highest sensitivity (slope = 132,889.9), while STR has the lowest sensitivity (slope = 7.5).

4.4.2 Method specificity

The specificity was assessed by analyzing blank chicken samples. The absence of background peaks, above a signal-to-noise ratio of 3, at the retention time of the target compounds showed that the method is free of endogenous interferences. The chromatogram of blank chicken sample is shown in Figure 4.8.
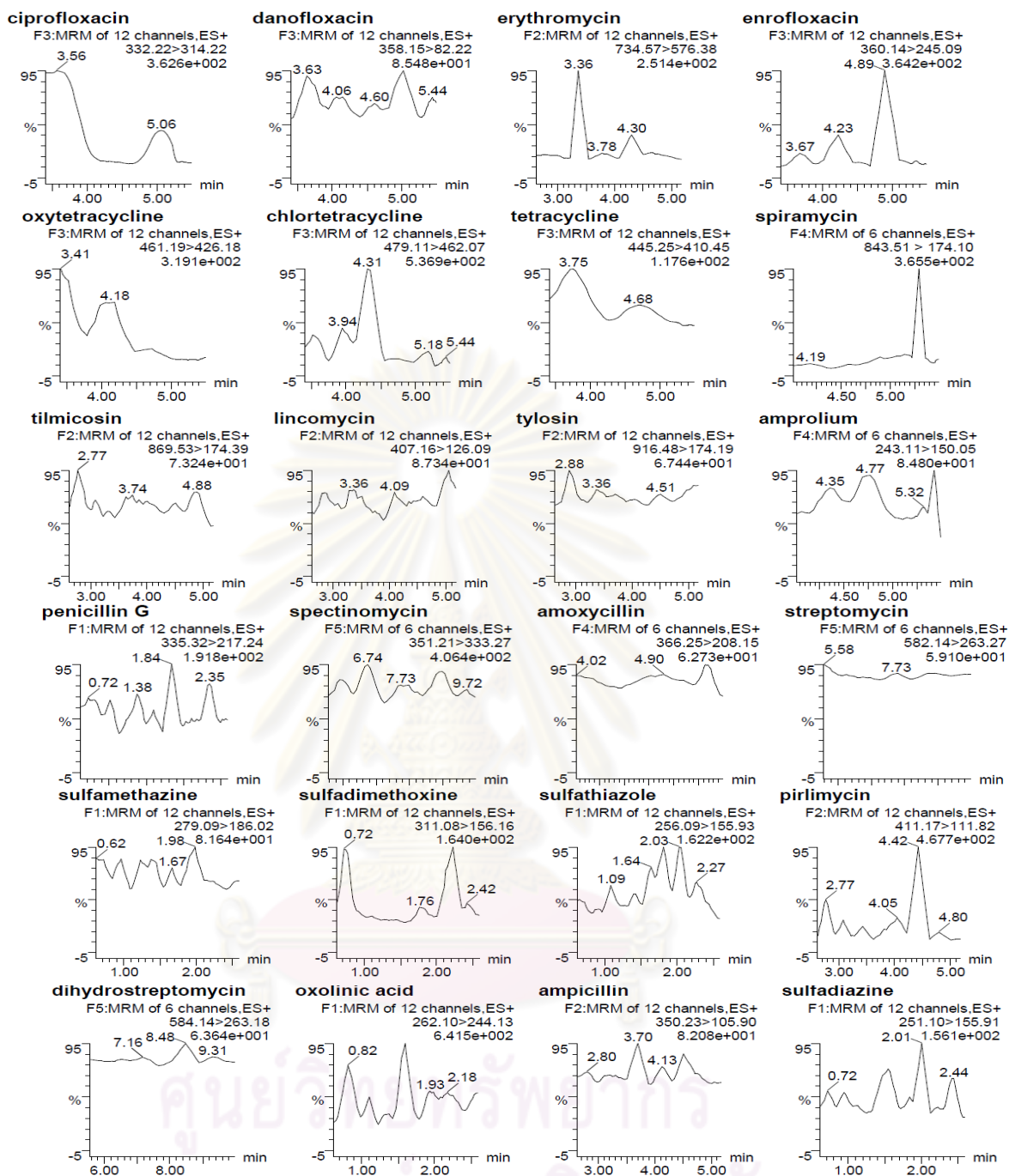
Figure 4.8 Chromatogram of blank chicken sample
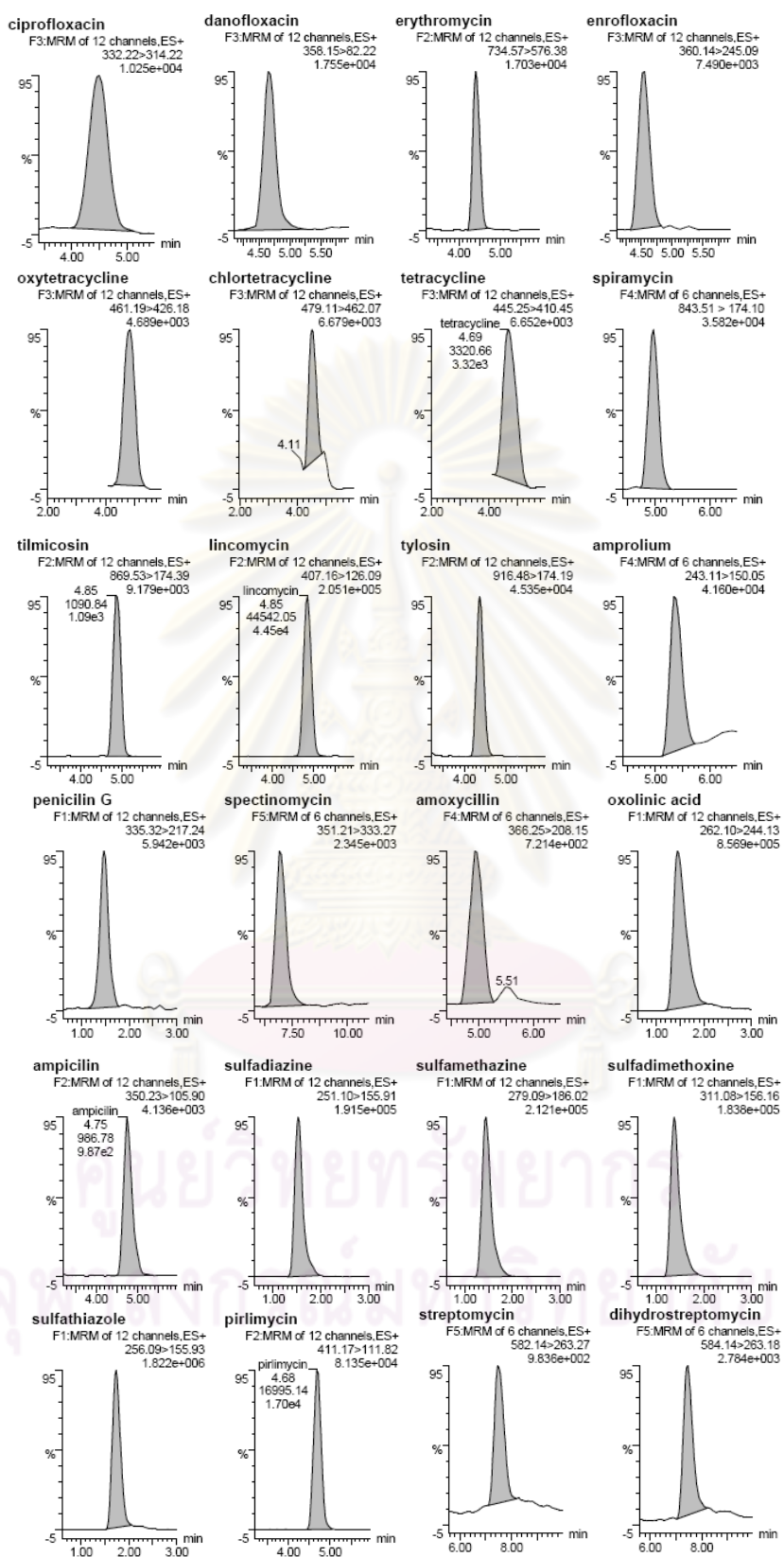
Figure 4.9 MRM chromatogram of spiked chicken sample at MRLs concentration level

4.4.3 Method accuracy

Accuracy is the closeness of agreement between a test result and the accepted reference value. To determine the accuracy of the method, chicken samples were fortified at three concentration levels (0.5, 1, 1.5 times MRL) in ten replicates for each concentration level. After sample preparation and LC-MS/MS analysis, the accuracy was expressed as recovery (%). All recovery are determined by comparing the peak areas obtained from fortified samples with the peak areas resulting from direct injection of the matrix matched standards. The result of method accuracy on the percent recoveries of each compound are shown in Table 4.4.

Table 4.4 Recovery (%) of all antibiotics at 0.5 MRLs, MRLs and 1.5 MRLs levels for spiked chicken sample (n=10)

No.	Compounds	% Recovery ± % RSD		
		0.5 MRLs	MRLs	1.5 MRLs
1	AMOX	68 ± 14	76 ± 14	68 ± 14
2	AMP	73 ± 15	71 ± 7	79 ± 7
3	PEN G	64 ± 6	70 ± 5	87 ± 5
4	TIL	85 ± 10	81 ± 6	84 ± 5
5	SMZ	61 ± 11	66 ± 3	68 ± 8
6	SDZ	65 ± 8	70 ± 4	72 ± 7
7	SDMX	60 ± 11	66 ± 4	66 ± 6
8	STZ	68 ± 10	73 ± 6	66 ± 7
9	CIP	85 ± 6	84 ± 10	84 ± 5
10	ENR	77 ± 6	93 ± 6	92 ± 10
11	OXO	72 ± 6	68 ± 5	99 ± 4
12	OTC	70 ± 14	73 ± 6	93 ± 4
13	CTC	79 ± 12	80 ± 4	89 ± 6
14	TC	83 ± 10	83 ± 4	83 ± 5
15	TYL	80 ± 4	75 ± 4	80 ± 7
16	LIN	75 ± 4	71 ± 2	98 ± 3
17	PIR	71 ± 3	73 ± 5	90 ± 4
18	DAN	82 ± 8	86 ± 9	93 ± 6
19	ERY	53 ± 6	57 ± 4	55 ± 7
20	SPI	75 ± 7	67 ± 7	65 ± 7
21	AMPR	77 ± 7	85 ± 9	91 ± 4
22	SPEC	83 ± 6	77 ± 6	88 ± 6
23	STR	83 ± 12	74 ± 9	84 ± 3
24	DSTR	82 ± 12	72 ± 8	84 ± 9

Recovery of the spiked chicken matrix at 0.5 MRLs level (20-200 µg/kg) ranged from 53 to 85%, 57 to 93% at MRLs level (40-400 µg/kg) and 55 to 99% at 1.5 MRLs level (60-600 µg/kg). These recovery values of most compound are accepted by the Codex Alimentarius Commission that recommend the acceptable recovery values of the method at ppb concentration level ranging between 70-110%. *(51)* Exception for AMOX, PEN G, SMZ, SDZ, SDMX, STZ and ERY at 0.5 MRL concentration level, for SMZ, SDMX, OXO, ERY, and SPI at MRL concentration level, for AMOX, SMZ, SDMX, STZ, ERY and SPI at 1.5 MRL concentration level which are slightly lower than the value obtained by the Codex. The results obtained from the above studies

indicate that the developed extraction method in this research provided good precision and accuracy for the analysis of these antibiotic residues in chicken.

4.4.4 Method Precision

The precision of the method was determined from repeatability and within laboratory reproducibility. Repeatability was evaluated at three different concentration levels (0.5, 1, and 1.5 times MRLs)

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. 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 assessed using the modified Horwitz equation. *(52)*

Horwitz equation

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

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

C =   Mass fraction: for 100% (pure material),

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 of most routine method of analysis.

From the Horwitz equation, the predicted present RSD values of all analytes at 0.5, 1.0, 1.5 times MRL levels are illustrated in Table 4.5.

Table 4.5 Predicted RSD (%) of all selected antibiotics compounds at 0.5 MRLs, MRLs and 1.5 MRLs concentration levels.

No.	Compounds	RSD (%) from Horwitz equation		
		0.5 MRLs	MRL	1.5 MRLs
1	AMOX	19.02	17.14	16.12
2	AMP	19.02	17.14	16.12
3	PEN G	19.02	17.14	16.12
4	TIL	19.02	17.14	16.12
5	SMZ	19.02	17.14	16.12
6	SDZ	19.02	17.14	16.12
7	SDMX	19.02	17.14	16.12
8	STZ	19.02	17.14	16.12
9	CIP	19.02	17.14	16.12
10	ENR	19.02	17.14	16.12
11	OXO	17.14	15.44	14.53
12	OTC	17.14	15.44	14.53
13	CTC	17.14	15.44	14.53
14	TC	17.14	15.44	14.53
15	TYL	17.14	15.44	14.53
16	LIN	17.14	15.44	14.53
17	PIR	17.14	15.44	14.53
18	DAN	14.93	13.45	12.66
19	ERY	14.93	13.45	12.66
20	SPI	14.93	13.45	12.66
21	AMPR	14.93	13.45	12.66
22	SPEC	14.93	13.45	12.66
23	STR	13.45	12.12	11.40
24	DSTR	13.45	12.12	11.40

4.4.4.1   Result of Method Precision at 0.5 MRLs level

The precision of this method is a measurement of the closeness expected between the replicate tests which results under the optimal conditions. Method precision at 0.5 MRLs level was studied by repeating the analysis both the same day and on three different days. The results of the method precision are summarized in Table 4.6- 4.9.

Table 4.6 Recovery (%) and RSD (%) of spiked chicken matrix at 0.5 MRLs concentration level on the first day (n=10)

No.	Compounds	Recovery (%)										Mean	RSD (%)
		1	2	3	4	5	6	7	8	9	10		
1	AMOX	71	70	75	69	64	66	88	60	57	56	68	14
2	AMP	81	80	83	86	69	61	62	60	62	82	73	15
3	PEN G	60	59	62	69	62	68	65	65	68	62	64	6
4	TIL	95	78	86	99	73	88	81	86	76	84	85	10
5	SMZ	58	54	69	73	55	56	64	63	62	54	61	11
6	SDZ	60	63	76	62	62	61	73	67	62	62	65	8
7	SDMX	57	60	69	74	53	53	64	60	57	56	60	11
8	STZ	62	68	68	80	69	64	61	70	75	66	68	8
9	CIP	96	71	88	92	79	78	91	89	91	76	85	10
10	ENR	74	73	76	81	78	83	79	82	71	72	77	6
11	OXO	68	73	68	78	71	73	68	67	74	77	72	6
12	OTC	74	80	79	84	64	57	62	58	74	69	70	14
13	CTC	67	65	74	84	72	79	78	79	95	92	79	12
14	TC	94	100	76	85	74	81	75	79	84	77	83	10
15	TYL	84	83	80	82	80	81	76	78	76	76	80	4
16	LIN	75	76	76	78	72	79	76	70	76	71	75	4
17	PIR	70	73	69	70	70	73	75	70	69	73	71	3
18	DAN	84	90	87	74	84	86	82	83	69	80	82	8
19	ERY	50	51	52	52	49	59	56	55	50	51	53	6
20	SPI	80	68	71	79	70	74	73	78	71	84	75	7
21	AMPR	71	81	79	84	72	80	70	69	81	78	77	7
22	SPEC	78	83	87	77	89	88	76	83	86	83	83	6
23	STR	70	85	88	77	96	93	74	90	92	68	83	12
24	DSTR	82	90	66	79	78	99	92	84	86	68	82	12

Table 4.7 Recovery (%) and RSD (%) of spiked chicken matrix at 0.5 MRLs concentration level on the second day (n=10)

No.	Compounds	Recovery (%)										Mean	RSD (%)
		1	2	3	4	5	6	7	8	9	10		
1	AMOX	84	55	62	65	66	80	88	55	67	87	71	18
2	AMP	62	65	61	63	64	66	60	59	59	66	63	4
3	PEN G	58	65	64	64	60	60	65	69	57	51	61	8
4	TIL	70	93	98	85	82	79	90	61	100	95	85	15
5	SMZ	64	65	66	70	68	59	63	58	68	84	67	11
6	SDZ	75	74	75	78	78	57	58	55	62	90	70	16
7	SDMX	66	65	67	69	67	58	62	52	57	80	64	12
8	STZ	75	80	84	82	79	72	68	69	61	62	73	11
9	CIP	96	70	63	102	86	73	88	93	89	90	85	15
10	ENR	76	94	85	76	100	84	67	77	72	70	80	13
11	OXO	69	67	70	71	71	61	62	56	59	84	67	12
12	OTC	70	81	77	70	73	85	79	71	64	82	75	9
13	CTC	81	84	78	62	77	52	62	60	69	75	70	15
14	TC	87	86	99	77	77	72	79	78	75	76	81	10
15	TYL	97	96	79	90	98	91	83	71	77	80	86	11
16	LIN	72	71	70	72	69	69	70	71	72	70	71	2
17	PIR	78	70	83	82	82	73	79	72	83	82	78	6
18	DAN	81	78	77	81	69	70	74	66	75	85	76	8
19	ERY	66	60	65	53	62	48	56	52	66	49	58	12
20	SPI	87	70	78	63	84	71	88	87	83	70	78	11
21	AMPR	63	67	78	75	79	70	68	68	62	73	70	8
22	SPEC	75	72	76	70	72	81	77	84	87	84	78	8
23	STR	92	86	70	89	72	75	68	81	71	70	77	11
24	DSTR	84	93	75	81	80	97	78	85	87	68	83	10

ศูนย์วิทยทรัพยากร

จุฬาลงกรณ์มหาวิทยาลัย

Table 4.8 Recovery (%) and RSD (%) of spiked chicken matrix at 0.5 MRLs concentration level on the third day (n=10)

No.	Compounds	Recovery (%)										Mean	RSD (%)
		1	2	3	4	5	6	7	8	9	10		
1	AMOX	71	60	54	63	75	65	77	65	75	55	66	13
2	AMP	78	77	65	71	77	80	87	85	70	72	76	9
3	PEN G	62	65	62	63	61	56	59	52	60	55	60	7
4	TIL	75	81	72	75	80	89	64	94	68	77	78	12
5	SMZ	60	65	69	56	57	55	66	66	65	66	63	8
6	SDZ	64	64	69	57	60	63	68	65	64	64	64	5
7	SDMX	58	60	56	57	65	66	62	62	63	63	61	5
8	STZ	69	71	69	68	64	63	67	62	66	67	67	4
9	CIP	76	98	83	81	77	100	102	95	80	84	88	11
10	ENR	85	84	62	81	80	96	81	92	95	94	85	12
11	OXO	64	67	69	65	63	63	71	70	71	73	68	5
12	OTC	58	60	58	60	57	65	59	73	61	67	62	8
13	CTC	72	79	67	55	67	68	77	71	63	71	69	10
14	TC	92	89	75	73	72	79	76	79	73	81	79	9
15	TYL	78	80	79	81	82	79	85	79	86	87	82	4
16	LIN	73	74	74	71	83	69	74	82	79	81	76	6
17	PIR	82	73	74	83	74	70	72	72	76	75	75	6
18	DAN	85	90	81	79	83	93	87	81	91	90	86	6
19	ERY	64	67	54	62	60	55	49	52	54	53	57	10
20	SPI	69	68	72	80	71	66	80	66	68	66	71	8
21	AMPR	72	82	73	62	80	68	73	71	74	73	73	8
22	SPEC	72	83	69	83	84	77	82	89	86	83	81	8
23	STR	70	72	87	75	70	74	72	89	79	76	76	9
24	DSTR	83	86	80	87	87	79	83	87	84	76	83	5

Table 4.9 Overall RSD (%) of spiked chicken matrix at 0.5 MRLs level (n=3)

No.	Compounds	RSD (%)				F-value	
		Day 1	Day 2	Day 3	Overall	Fcalculated	Fcritical
1	AMOX	14	18	13	15	0.58	3.35
2	AMP	15	4	9	13	8.96	
3	PEN G	6	8	7	7	2.78	
4	TIL	10	15	12	13	1.82	
5	SMZ	11	11	8	10	2.12	
6	SDZ	8	16	5	12	2.03	
7	SDMX	11	12	5	10	1.12	
8	STZ	8	11	4	9	3.25	
9	CIP	10	15	11	12	0.20	
10	ENR	6	13	12	11	2.12	
11	OXO	6	12	5	8	2.10	
12	OTC	14	9	8	13	8.53	
13	CTC	12	15	10	14	3.21	
14	TC	10	10	9	10	0.53	
15	TYL	4	11	4	8	3.14	
16	LIN	4	2	6	5	7.20	
17	PIR	3	6	6	6	8.25	
18	DAN	8	8	6	9	8.31	
19	ERY	6	12	10	11	2.55	
20	SPI	7	11	8	10	3.11	
21	AMPR	7	8	8	8	3.07	
22	SPEC	6	8	8	7	2.14	
23	STR	12	11	9	11	1.83	
24	DSTR	12	10	5	9	0.03	

(1) **Intra assay precision** (0.5 MRLs level)

In this study, the precision of the method was expressed as the percentage relative standard deviation (% RSD). On the basis of the Horwitz equation and taking into account the concentration of the analytes at the 0.5 MRLs concentration level measured the acceptable RSD (%) range between 13.45-19.02 %. The obtained RSD (%) values in Table 4.6-4.8 were clearly illustrated that, this method is sufficiently precise at the concentration level of analytes being measured within the same day.

(2)   **Intermediate precision** (0.5 MRLs level)

The intermediate precision of the method on three different days at 0.5 MRLs level as shown in Table 4.9, ranged between 5-15%. From the statistical analysis (a one way analysis of variance (ANOVA) at 95% confident limit) illustrated that in most cases, no significant difference between RSD (%) values except for AMP, DAN, LIN, OTC and PIR at 0.5 MRLs level. However, the RSD (%) values of these compounds were still lower than the acceptable values calculated by Horwitz's equation. Therefore the proposed method has reliable intermediate precision at the level of analytes being measured.

4.4.4.2   Result of Method Precision at MRLs level

Method precision at MRLs concentration level was studied by repeating the analysis both within one day and on three different days. The obtained results of method precision are summarized in Tables 4.10-4.13.

Table 4.10 Recovery (%) and RSD (%) of spiked chicken matrix at MRLs concentration level on the first day (n=10)

No.	Compounds	Recovery (%)										Mean	RSD (%)
		1	2	3	4	5	6	7	8	9	10		
1	AMOX	69	70	80	70	88	66	88	77	94	62	76	14
2	AMP	67	81	77	70	69	69	64	73	65	71	71	7
3	PEN G	67	65	67	68	69	73	71	76	72	69	70	5
4	TIL	88	81	76	82	81	82	88	72	81	83	81	6
5	SMZ	67	67	70	68	65	67	63	68	64	65	66	3
6	SDZ	72	76	73	70	67	68	68	73	67	70	70	4
7	SDMX	67	68	70	69	65	64	64	64	63	67	66	4
8	STZ	71	79	71	79	68	69	78	75	68	68	73	6
9	CIP	83	79	80	95	77	88	74	93	96	74	84	10
10	ENR	92	84	92	100	99	95	97	95	88	87	93	6
11	OXO	69	75	70	69	68	65	66	66	65	62	68	5
12	OTC	69	71	76	74	71	81	79	73	71	68	73	6
13	CTC	84	81	82	79	78	75	84	78	82	76	80	4
14	TC	82	85	79	82	81	83	81	85	90	78	83	4
15	TYL	74	76	79	72	73	73	72	72	77	79	75	4
16	LIN	71	73	69	70	70	71	73	68	73	72	71	2
17	PIR	74	80	78	70	70	76	69	70	75	72	73	5
18	DAN	97	86	83	84	90	80	73	95	92	79	86	9
19	ERY	56	58	59	58	56	55	55	61	55	60	57	4
20	SPI	67	68	65	75	67	62	69	74	62	64	67	7
21	AMPR	81	86	89	92	71	90	93	82	90	73	85	9
22	SPEC	86	78	75	72	74	73	79	81	82	74	77	6
23	STR	75	60	75	70	68	76	75	83	79	80	74	9
24	DSTR	70	69	70	71	68	75	88	69	68	71	72	8

Table 4.11 Recovery (%) and RSD (%) of spiked chicken matrix at MRLs concentration level on the second day (n=10)

No.	Compounds	Recovery (%)										Mean	RSD (%)
		1	2	3	4	5	6	7	8	9	10		
1	AMOX	99	83	80	84	77	63	76	84	63	67	78	14
2	AMP	66	65	65	67	69	64	76	66	72	65	68	6
3	PEN G	62	63	64	64	60	60	65	62	69	60	63	4
4	TIL	99	97	92	88	102	80	61	104	85	89	90	14
5	SMZ	68	74	70	76	74	63	63	62	63	69	68	8
6	SDZ	75	79	77	80	76	64	60	60	60	72	70	12
7	SDMX	72	75	72	74	71	62	61	58	61	64	67	10
8	STZ	69	73	82	83	75	72	77	68	70	74	74	7
9	CIP	74	82	77	76	77	83	74	71	78	73	77	5
10	ENR	84	79	89	84	94	83	95	97	85	83	87	7
11	OXO	71	74	72	75	75	64	61	59	61	70	68	9
12	OTC	67	65	77	64	67	70	76	70	69	65	69	6
13	CTC	75	76	75	73	79	73	75	80	76	81	76	4
14	TC	80	74	86	87	88	83	71	80	80	66	80	9
15	TYL	75	72	71	70	74	71	72	68	71	72	72	3
16	LIN	65	68	69	77	77	66	67	66	63	66	68	7
17	PIR	75	71	70	72	71	72	70	69	71	73	71	2
18	DAN	88	75	82	70	63	89	79	69	88	75	78	12
19	ERY	53	54	51	58	52	53	51	53	54	51	53	4
20	SPI	78	73	86	88	75	78	83	67	70	85	78	9
21	AMPR	67	73	75	87	68	66	71	80	75	72	73	9
22	SPEC	82	73	72	77	80	74	81	71	78	73	76	5
23	STR	66	70	71	64	68	69	64	73	71	74	69	5
24	DSTR	69	70	74	68	75	76	86	69	71	66	72	8

Table 4.12 Recovery (%) and RSD (%) of spiked chicken matrix at MRLs concentration level on the third day (n=10)

| No. | Compounds | Recovery (%) | | | | | | | | | | Mean | RSD (%) |
		1	2	3	4	5	6	7	8	9	10		
1	AMOX	67	85	86	64	82	92	69	77	72	77	77	12
2	AMP	75	73	68	82	70	74	76	71	82	62	73	8
3	PEN G	70	62	63	60	69	75	65	65	61	68	66	7
4	TIL	97	85	79	89	105	92	98	94	84	84	91	9
5	SMZ	64	70	74	66	73	72	73	69	75	72	71	5
6	SDZ	70	65	68	63	65	61	66	64	66	68	66	4
7	SDMX	67	69	62	68	74	67	72	72	73	69	69	5
8	STZ	66	64	69	71	68	72	71	69	67	66	68	4
9	CIP	88	73	70	93	77	82	74	70	82	68	78	11
10	ENR	94	90	99	97	81	97	101	98	96	83	94	7
11	OXO	67	66	67	70	68	66	72	72	71	73	69	4
12	OTC	72	67	65	71	71	72	73	71	75	65	70	5
13	CTC	82	71	87	89	84	71	86	86	87	71	81	9
14	TC	67	71	68	85	74	66	78	84	71	63	73	10
15	TYL	70	74	71	72	71	69	74	70	70	74	72	3
16	LIN	84	64	80	83	80	71	78	70	80	82	77	9
17	PIR	68	78	74	76	70	75	71	79	82	83	76	7
18	DAN	87	95	85	80	90	70	80	84	89	75	84	9
19	ERY	61	53	54	51	56	51	58	51	52	55	54	6
20	SPI	76	81	83	66	72	68	69	70	68	69	72	8
21	AMPR	87	74	79	92	74	87	92	87	74	80	83	9
22	SPEC	75	85	73	89	78	87	79	82	85	77	81	7
23	STR	66	75	85	65	74	77	76	80	83	69	75	9
24	DSTR	73	70	74	73	78	87	74	89	88	77	78	9

Table 4.13 Overall RSD (%) of spiked chicken matrix at MRLs level (n=3)

No.	Compounds	RSD (%)				F-value	
		Day 1	Day 2	Day 3	Overall	$F_{calculated}$	$F_{critical}$
1	AMOX	14	14	12	13	0.03	3.35
2	AMP	7	6	8	8	3.18	
3	PEN G	5	4	7	7	8.58	
4	TIL	6	14	9	11	3.16	
5	SMZ	3	8	5	6	3.26	
6	SDZ	4	12	4	8	2.62	
7	SDMX	4	10	5	7	1.37	
8	STZ	6	7	4	7	5.22	
9	CIP	10	5	11	10	3.02	
10	ENR	6	7	7	7	3.2	
11	OXO	5	9	4	6	0.37	
12	OTC	6	6	5	6	2.98	
13	CTC	4	4	9	7	2.83	
14	TC	4	9	10	9	6.39	
15	TYL	4	3	3	4	6.43	
16	LIN	2	7	9	8	8.79	
17	PIR	5	2	7	5	3.11	
18	DAN	9	12	9	10	2.68	
19	ERY	4	4	6	6	7.16	
20	SPI	7	9	8	10	8.69	
21	AMPR	9	9	9	11	1.27	
22	SPEC	6	5	7	6	2.92	
23	STR	9	5	9	9	3.08	
24	DSTR	8	8	9	9	3.19	

(1)   **Intra assay precision** (MRLs level)

In this study, the precision of the method was expressed as the percentage relative standard deviation (% RSD). On the basis of the Horwitz equation, taking into account the concentration of the analytes at the MRL concentration level measuring the acceptable RSD (%) range between 12.12-17.14%. The obtained RSD (%) values in Table 4.10-4.12 have clearly illustrated that this method in sufficiently precise at the concentration level of analytes being measured within the same day.

(2)   **Intermediate precision** (MRLs level)

The intermediate precision of the method on three different days at MRLs level as shown in Table 4.13, ranged between 5-13%. From the statistical analysis (a one way analysis of variance (ANOVA) at 95% confident limit) illustrated that in most cases, no significant difference between RSD values except for PEN G, ERY, LIN, SPI, STZ, TC and TYL at MRL level. However, the RSD (%) values of these compounds were still lower than the acceptable values calculated by Horwitz's equation. Therefore the proposed method has reliable intermediate precision at the level of analytes being measured.

4.4.5 Identification and confirmation

As stated in Commission Decision 2002/657/EC *(5)*, a minimum of three identification points (IPs) are required for group B substance (as all studied compounds) which have established MRLs. In this method, all analytes were measured in the multiple reaction monitoring mode to fulfill the EU identification point concept. Two diagnostic daughter ion transitions (quantifier ion and qualifier ion) were monitored and the ratio between the monitored fragment ions is calculated and compared with the ratio obtained from the matrix-matched standard. The detection of two transition products scores four identification points (one point for the precursor ion and two times 1.5 points for the daughter ions). Therefore this developed method is adequate for the identification and confirmation.

4.4.6 Limit of detection (LODs) and Limit of quantification (LOQs)

LODs and LOQs were calculated by analyzing blank sample spiked at 0.5 MRLs level, and they were determined as the lowest concentrations of the analyte for which the signal to noise ratios were 3 and 10 respectively. LODs ranged from 0.1 to 20 µg/kg and LOQs from 0.3 to 60 µg/kg which are lower than the MRLs established by the EU, despite of the dilution of the extract. The method LODs and LOQs are expressed in Table 4.14.

Table 4.14 The limit of detection (LODs) and limit of quantifications (LOQs) of selected antibiotics

No.	Compounds	LODs (µg/kg)	LOQs (µg/kg)
1	AMOX	2.5	8.5
2	AMP	1.5	5.0
3	PEN G	1.5	5.0
4	TIL	0.5	1.7
5	SMZ	0.1	0.3
6	SDZ	0.1	0.3
7	SDMX	0.1	0.3
8	STZ	0.1	0.3
9	CIP	1.5	5.0
10	ENR	0.5	1.7
11	OXO	3.0	10
12	OTC	3.0	10
13	CTC	1.0	3.0
14	TC	1.0	3.0
15	TYL	1.5	5.0
16	LIN	0.5	1.8
17	PIR	0.2	0.7
18	DAN	0.2	0.7
19	ERY	1.5	5.0
20	SPI	4.0	13
21	AMPR	8.0	25
22	SPEC	10	30
23	STR	20	60
24	DSTR	20	60

4.4.7 Decision limit (CCα) and detection capability (CCß)

The European Decision no.657/2002/EC *(5)* concerning the performance of analytical methods and the interpretation of result, recommends to calculate two statistical limits, CCα and CCß which allow to assessment of the critical concentrations above, in which the method reliably distinguishes and quantifies a substance taking into account the variability of the method and the statistical risk of making a wrong decision.

The decision limit (CCα) is defined as the concentration value above which it can be concluded that a sample is non compliant with an error probability α. For compounds with established MRL (as all studied compounds), CCα were calculated as the MRL plus 1.64 times the corresponding standard deviations, when analyzing 20 blank chicken samples spiked at the MRL.

The detection capability (CCß), for compounds with an established MRL, is defined as the concentration value at which the method can be detect compliant concentration limits with an error probability of ß. In this case it was calculated as the decision limit plus 1.64 times the corresponding standard deviations when analyzing 20 blank chicken samples spiked at the CCα level. Table 4.15 summarized the obtained CCα and CCß values.

Table 4.15 The decision limit (CCα) and detection capability (CCß) of selected antibiotics

| No.	Compounds	CCα (µg/kg)	CCß (µg/kg)
1	AMOX	67.5	81.1
2	AMP	57.8	73.1
3	PEN G	57.5	64.6
4	TIL	66.8	84.1
5	SMZ	106.6	117.2
6	SDZ	110.0	117.0
7	SDMX	107.7	115.4
8	STZ	108.0	116.9
9	CIP	112.2	135.7
10	ENR	110.3	123.7
11	OXO	108.2	116.3
12	OTC	107.8	120.2
13	CTC	105.7	120.0
14	TC	109.4	125.4
15	TYL	104.7	108.5
16	LIN	106.2	125.1
17	PIR	105.0	119.6
18	DAN	212.5	231.9
19	ERY	205.0	210.8
20	SPI	213.3	226.0
21	AMPR	214.8	232.6
22	SPEC	307.0	319.5
23	STR	509.5	524.9
24	DSTR	509.4	521.6

4.5 Matrix effect

When the biological samples such as milk, kidney or muscle which normally rich in protein and lipid components are analyzed by LC-MS or LC-MS/MS for the presence of drug residues, especially in the ESI mode, can greatly affect the analyte signals. It is well known that ESI mass spectrometry is trendy to signal suppression or enhancement effects. Numerous co-eluting matrix components have an influence on the ionization efficiency of the analyte and can adversely affect the reproducibility and accuracy of the method, particularly when external standard calibration curves are used

for quantification. Therefore, matrix effects have to be investigated during method development. The matrix effects depend on the type of matrix as described by Becker et al. *(16)*, the authors demonstrated the significant difference of the effect in various matrices. Experiments examining the significance of the suppression effect from biological matrices have been highlighted in previous papers *(16,53)*, using statistical evaluations such as the t-test at 95% confidence level. These studies have concluded that the matrix effect is a major factor contributing to LC-MS quantification.

In this study, prior to the analysis of chicken samples, the matrix effect on the chromatographic determination was investigated by comparing the response obtained from a standard solution in pure solvent and from matrix-matched standards which were prepared by using the blank samples. It is observed that the chicken extract matrix led to alternation in the chromatogram, including variation in peak intensity for some peak, mainly Qs and TCs group. To minimize variation due to this effect, the calibration curve should be carried out by using matrix-matched standard or standard addition, in this case, the former was chosen since standard addition is more tedious for a large number of samples.

4.6 Application to real sample

To prove the applicability of the developed multi-class method when applied in real samples, more than 30 chicken samples (from local markets and supermarkets) were analyzed. Out of these samples, only ENR (at 6.5 µg/kg) was detected. The confirmatory on ion ratio of analyte in incurred sample was within the range of the permitted tolerance when compared to the ion ratio in the matrix matched standard, according to the European Commission Decision 2002/657/EC. *(5)* In addition, the retention time of the analyte in positive samples was identical, within instrumental variations, to the retention time of the analyte in matrix matched standard as illustrated in Table 4.16. The chromatogram detected in chicken sample is shown in Figure 4.10.
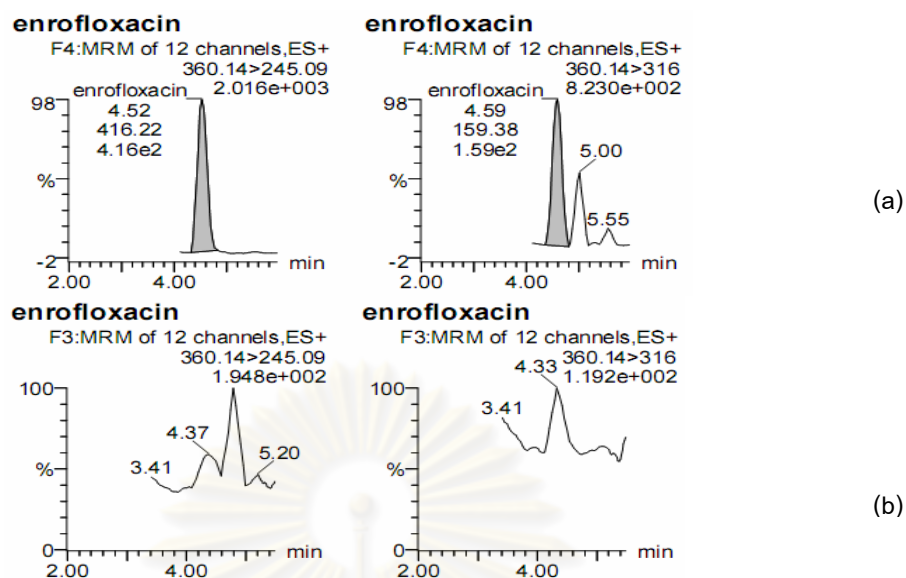
Figure 4.10 The LC-MS/MS chromatogram obtained from the real sample analysis, (a) the incurred chicken sample with ENR content calculated at 6.5 µg/kg and (b) the blank chicken sample.

**Table 4.16** Confirmatory LC-MS/MS analysis of incurred chicken sample

| Sample	Detected analyte	Product ion	Incurred samples		Matrix-matched standard	
			Ion ratio	t_R (min)	Ion ratio	t_R (min)
Chicken	Enrofloxacin	316/245	0.39	4.52	0.36	4.52

4.7 Application to egg and shrimp matrix

4.7.1 Egg sample

After the optimum extraction procedure and LC-MS/MS condition for simultaneous analysis of multi-class antibiotics were obtained, the method was validated. This procedure was also applied to the egg sample anlysis. For evaluation the method performance in term of accuracy and precision, the fortified egg sample at 0.5MRL and MRL concentration level with 5 replicate for each level were analyzed.
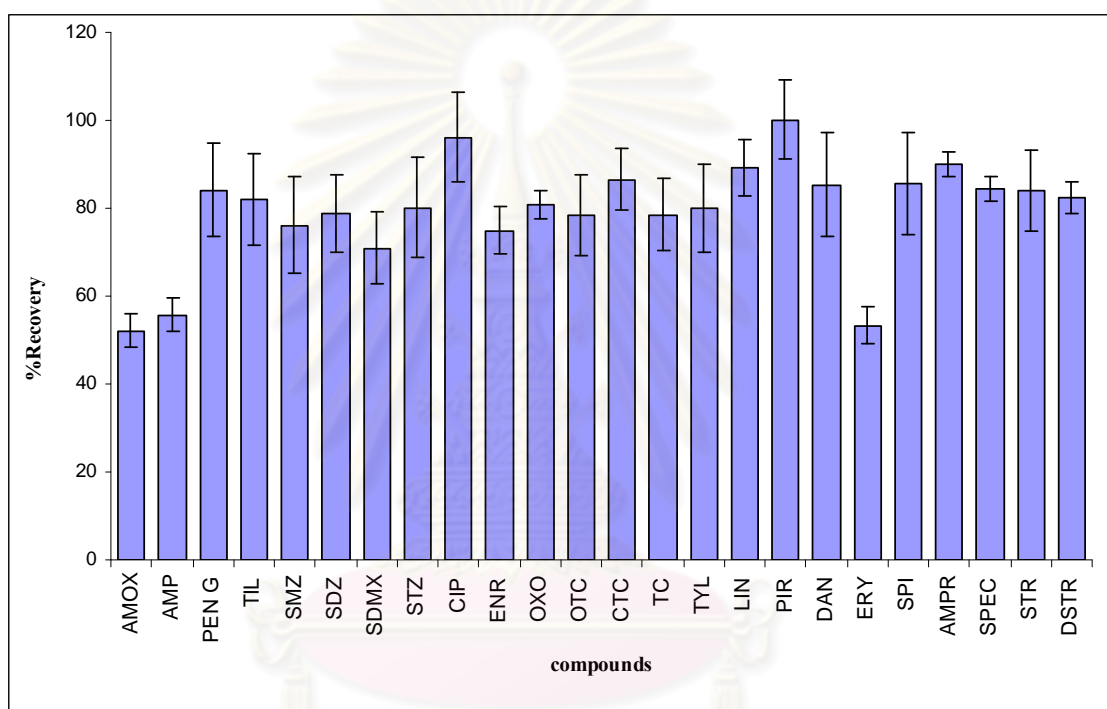
Figure 4.11 The recovery (%) of antibiotics in egg sample at 0.5 MRLs (n=5)
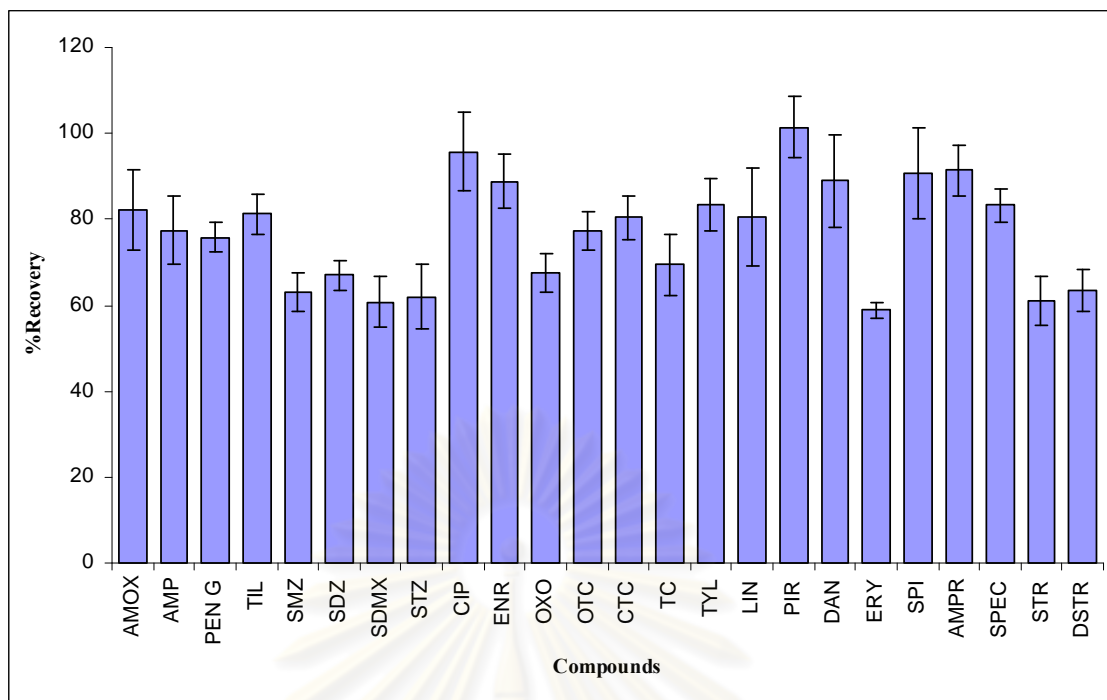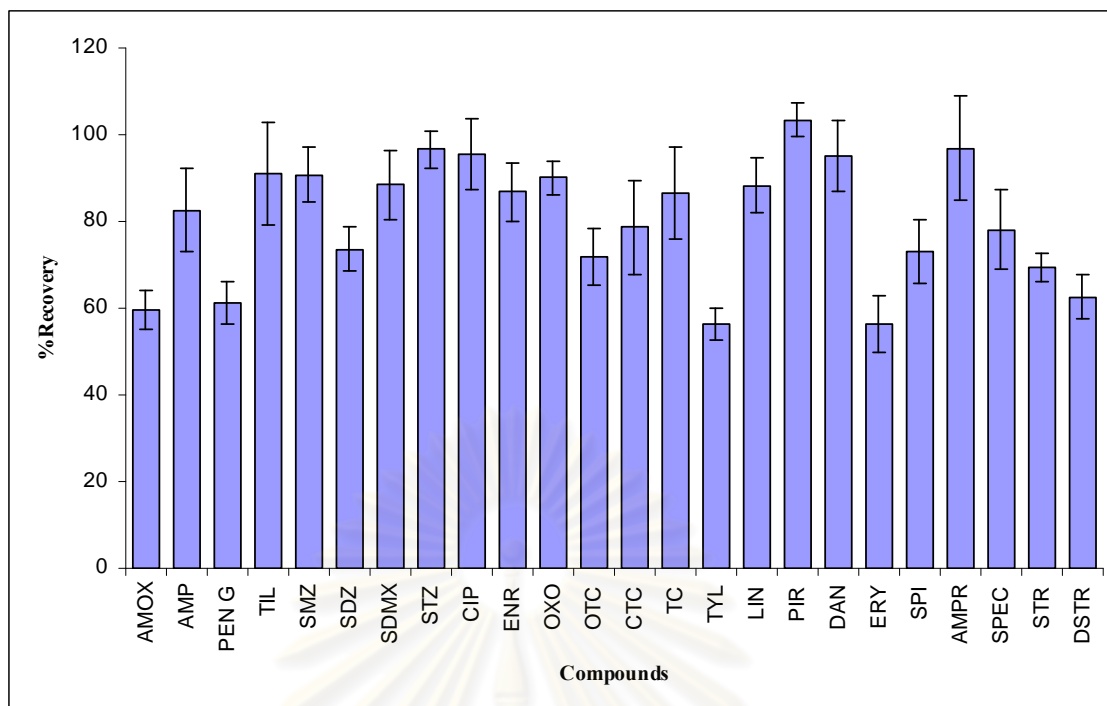
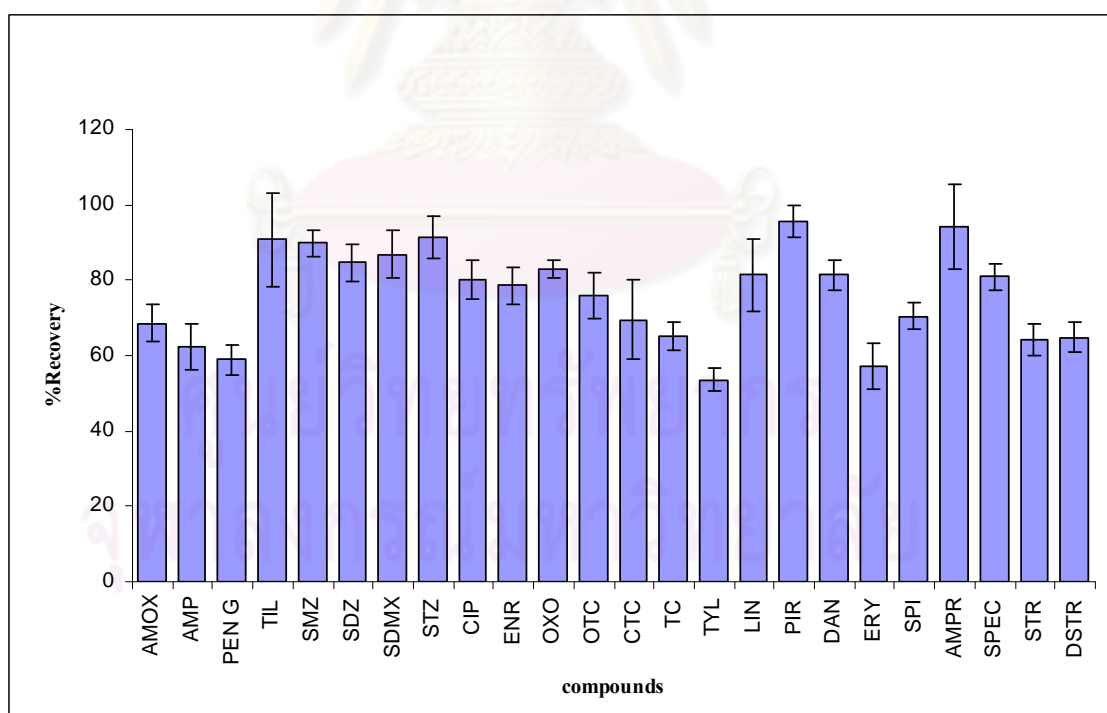**Figure 4.12** The recovery (%) of antibiotics in egg sample at MRLs (n=5)

From the results, recovery of the spiked egg matrix at 0.5 MRL level (20-200 μg/kg) ranged from 52 to 100% with %RSD ranged from 3-12% , 59 to 101 with %RSD ranged from 2-11% at MRL level (40-400 μg/kg). These recovery values of most compounds are accepted by the Codex Alimentarius Commission that recommends the acceptable recovery values of the method at a ppb concentration level range between 70-110 %. Exception for AMOX, AMP and ERY at 0.5 MRL level and ERY, SMZ, SDZ, SDMX, STZ, OXO, TC, STR and DSTR at MRL level which are slightly lower than the value obtained by the Codex. The %RSD of all compounds were lower than the critical values calculated by the Horwitz equation. The results obtained from the above studies indicate that the developed extraction method in this research provided good precision and accuracy for the analysis of these analytes residue in egg.

4.7.2 Shrimp sample

After the optimum extraction procedure and LC-MS/MS condition for simultaneous analysis of multi-class antibiotics were obtained, the method was validated. This method also applied to the shrimp sample. For evaluation of the method performance in term of accuracy and precision, the fortified shrimp sample at 0.5MRL and MRL concentration level with 5 replicate for each level were analyzed.

**Figure 4.13** The recovery (%) of antibiotics in shrimp sample at 0.5 MRLs (n=5)

Figure 4.14   The recovery (%) of antibiotics in shrimp sample at MRLs (n=5)

From the results, recovery of the spiked shrimp matrix at 0.5 MRL level (20-200 µg/kg) ranged from 56 to 103% with %RSD ranged from 3-12% , 59 to 101 with %RSD ranged from 3-12% at MRL level (40-400 µg/kg). These recovery values of most compounds are accepted by the Codex Alimentarius Commission that recommend the acceptable recovery values of the method at ppb concentration level range between 70-110 %. Exception for AMOX, PEN G, TYL, ERY, STR and DSTR at 0.5 MRL and AMOX, AMP, ERY, PEN G, TC, TYL, STR and DSTR at MRL level which are slightly lower than the value obtained by the Codex. The %RSD of all compounds were lower than the critical values calculated by the Horwitz equation. The results obtained from the above studies indicate that the developed extraction method in this research provided good precision and accuracy for the analysis of these analyte residues in shrimp.

The developed method was successful for testing the accuracy from the assigned value in an incurred shrimp test material from National Food Institute   with sample code (NFI –PTC-1/53). The obtained result was 7.45µg/kg, with % RSD was 4.75% (n=2) and % Recovery was 83%.
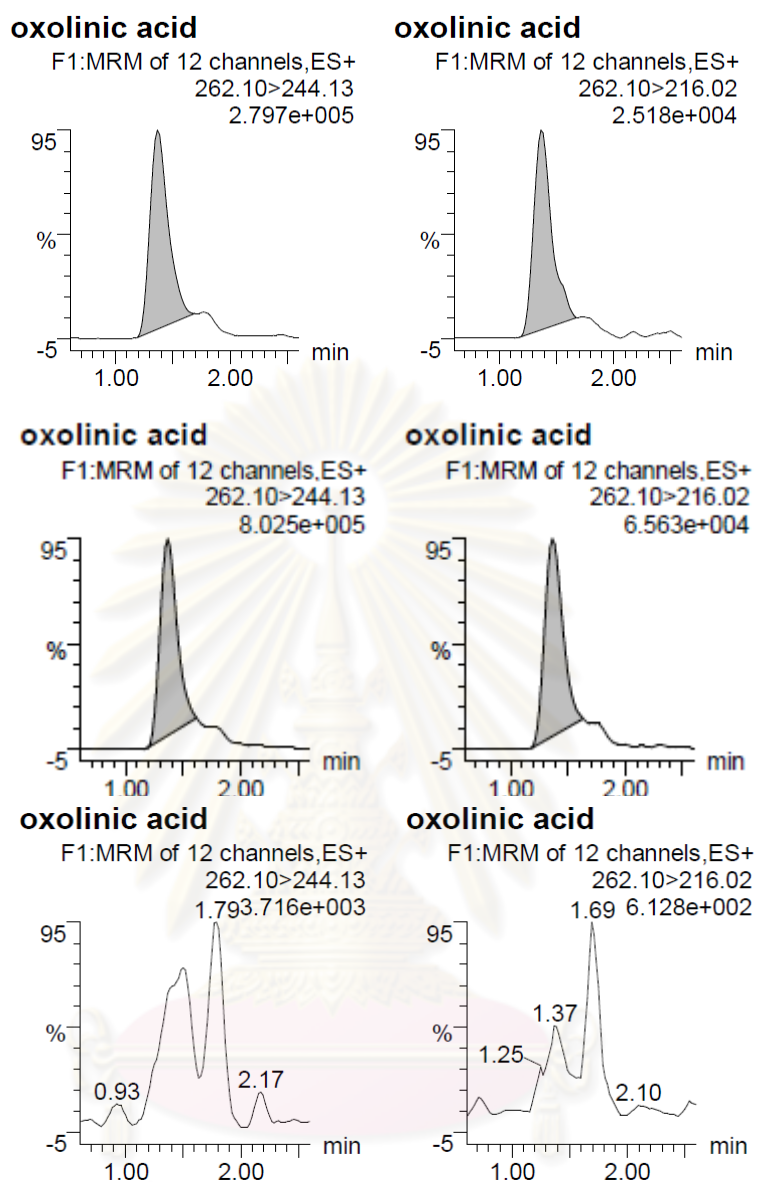
Figure 4.15 The LC-MS/MS chromatogram obtained from the shrimp sample analysis (a) matrix standard, (b) incurred shrimp sample and (c) blank shrimp sample

CHAPTER V

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDY

In this study, the proposed LC-MS/MS method was successfully applied to simultaneous quantitative determination and identification of twenty four antibiotics from seven important classes including amprolium at residue level. The analysis was performed by using optimized condition of liquid chromatography tandem mass spectrometry (LC-MS/MS) with the electrospray ionization (ESI). Hydrophilic interaction liquid chromatography (HILIC) was used for the separation of analytes.

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

Table 5.1 Optimum ESI-MS/MS conditions for analysis of antibiotic compounds

ESI parameters	Conditions
Detection mode	positive
Capillary voltage	1000 V
Source temperature	120 °C
Desolvation temperature	350 °C
Cone gas flow (nitrogen)	50 L/h
Desolvation gas flow (nitrogen)	1000 L/h
Collision gas (argon)	3.5×10^{-3} mbar

The selection of ionization mode and the optimization of various parameters influencing on analyte MS signals, including the specific cone and collision energies for each compound are also carried out. The method was operated in multiple reactions monitoring mode (MRM) with the most two sensitive and stable transitions which used for quantification and confirmation. The MRM transition of all analytes were previously illustrated in Table 4.1.

Separation and selectivity of all antibiotic compounds were achieved by using zwitterionic-HILIC stationary phase with gradient elution. The mobile phase containing of 50 mM ammonium formate pH 2.5 (A) and acetonitrile (B) was applied. The selectivity of LC-ESI-MS/MS method was evaluated by the matching of peak retention time and ion ratio of parent and product ion. Although some compounds have same retention time, it can be confirmed by structural information of each compound. The optimum chromatographic condition in this study is shown in Table 5.2.

Table 5.2 Optimum chromatographic conditions for analysis of antibiotic compounds

Parameters	Conditions
Analytical column	ZIC®-HILIC column
	(2.1x100 mm,i.d. 3.5 μm)
Mobile phase	A: 50 mM ammonium formate pH 2.5
	B: acetonitrile
Flow rate	0.2 mL/min
Injection volume	10 μL
Column temperature	40 °C
Sample temperature	20 °C

The use of HILIC separation technique was successfully completed for retention of polar, moderately polar and extremely polar compounds that allow the method to simultaneously analyze of multi-class antibiotics which cover a wide range of polarity compounds in single chromatographic run.

By development of the extraction methods, this optimal procedure involved liquid extraction with the mixture of aqueous and acetonitrile. The concentration of trichloroacetic acid (TCA) is also investigated. Satisfactory results were obtained by using 2% TCA as an aqueous solution which showed influential in the extraction efficiency and chromatographic separation. The sample extracted was diluted with 10% formic acid:acetonitrile (1:9) in order to reduce signal suppression from the matrix effect with dilution a ratio of 2:8 (sample solution:dilution solution). The optimum procedure for sample preparation is summarized in Figure 5.1

Signal suppression from the matrix effect was observed during LC-MS/MS analysis. To compensate this effect, the matrix matched standards were used in this study for quantification in order to reach high accuracy.

The presented method was validated according to Commission Decision 2002/657/EC. The results showed good linearity over the concentration range of 0.2MRL-2MRL and 0.1MRL-MRL for SMZ, STZ, SDZ, SDMX with correlation coefficient (R^2) better than 0.9900. Table 5.3 shows the characteristic validation data of all studied compounds.

| Weigh 5 g sample into polypropylene centrifuge tube |

↓

| Added 10 mL mixture of 2%TCA and acetonitrile (1:1) |

↓

| Vortex for 30 s and mechanically shaken for 10 min |

↓

| Centrifuged for 5 min at 3400 rpm |

↓

| Added 5 mL of hexane to remove fat from the extract |

↓

| Vortex for 1 min and centrifuge for 5 min at 3400 rpm |

↓

| 200 μL of the extract was diluted to 1 mL with 10% formic acid: ACN (1:9,v/v) |

↓

| Filtered thought 0.2 μm nylon filter prior to LC-MS/MS |

Figure 5.1 Schematic diagram of optimized sample preparation

Table 5.3 Characteristic validation data consists of retention time (t_R), coefficient of determination (R^2), limit of detections (LODs), limit of quantifications (LOQs), decision limit (CCα) and detection capability (CCß).

Compounds	t_R	R^2	CCα	CCß	LODs (µg/kg)	LOQs (µg/kg)
AMOX	4.81	0.9942	67.5	81.1	2.5	8.5
AMP	4.75	0.9973	57.8	73.1	1.5	5.0
PEN G	1.51	0.9989	57.5	64.6	1.5	5.0
TIL	4.88	0.9973	66.8	84.1	0.5	1.7
SMZ	1.47	0.997	106.6	117.2	0.1	0.3
SDZ	1.55	0.9987	110.0	117.0	0.1	0.3
SDMX	1.42	0.9938	107.7	115.4	0.1	0.3
STZ	1.76	0.9971	108.0	116.9	0.1	0.3
CIP	4.69	0.994	112.2	135.7	1.5	5.0
ENR	4.55	0.9945	110.3	123.7	0.5	1.7
OXO	1.50	0.9986	108.2	116.3	3.0	10
OTC	4.89	0.9972	107.8	120.2	3.0	10
CTC	4.62	0.9973	105.7	120.0	1.0	3.0
TC	4.82	0.9985	109.4	125.4	1.0	3.0
TYL	4.37	0.9991	104.7	108.5	1.5	5.0
LIN	4.85	0.9989	106.2	125.1	0.5	1.8
PIR	4.71	0.9988	105.0	119.6	0.2	0.7
DAN	4.65	0.9953	212.5	231.9	0.2	0.7
ERY	4.44	0.9955	205.0	210.8	1.5	5.0
SPI	4.97	0.997	213.3	226.0	4.0	13
AMPR	5.41	0.9956	214.8	232.6	8.0	25
SPEC	6.33	0.9974	307.0	319.5	10	30
STR	7.65	0.9979	509.5	524.9	20	60
DSTR	7.57	0.9988	509.4	521.6	20	60

The method accuracy and precision were evaluated at three concentration levels, 0.5 MRL (20-200 μg/kg), MRL (40-400 μg/kg) and 1.5 MRL (60-600 μg/kg) for intra-assay precisions and 0.5 MRL (20-200 μg/kg), MRL (40-400 μg/kg) for intermediate precision in chicken matrix. In case of SAs, ENR and it metabolite CIP which have the sum MRLs can be done at 0.1 MRL, 0.2 MRL respectively. The intra-assay precision was expressed as relative standard deviation (% RSD) and the value of % RSD for within day precision at 0.5 MRL level ranged from 3 to 15% , at MRL level ranged from 2 to 14 % and at 1.5 MRL ranged from 3-14 %. The satisfactory intermediate precisions of this method on three different days were achieved with 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 indicated good intermediate precision of this method were obtained except for AMP, DAN, LIN, OTC and PIR at the 0.5 MRL level, and for PEN G, ERY, LIN, SPI, STZ, TC and TYL at the 1 MRL level. However, the RSD values of these compounds were still lower than the acceptable values calculated by the Horwitz equation, therefore the proposed method has reliable intermediate repeatability. The mean recovery for most compounds for all level in the range of 53 to 99 %.

A real chicken samples bought from the local market were detected by this newly developed method for enrofloxacin which has a result higher than LOQ (6.5 μg/kg). The positive result satisfied the EU analytical criteria, in terms of correspondence of retention time and ion chromatogram area ratio with the standard. The method can be applied to egg and shrimp matrices, good accuracy and precision values were obtained.

The method also fulfills the criteria of confirmation by using the identification points (IPs) system. This measurement earned four IPs from the precursor ion plus the two product ions.

The developed method is simple and sensitive allowing for simultaneous extraction of 24 antibiotic residues belonging to eight classes: aminoglycosides, penicillins, quinolones, tetracyclines, macrolides, sulfonamides and lincosamides including amprolium, in which different chemical and physical properties in chicken matrix. The method has been tested in egg and shrimp matrices for accuracy and precision. The results illustrated that the proposed study is suitable to apply in routine laboratories where regularly required analysis of multi-class residues from various matrices.

This method can be further applied to compounds with no estabished MRL, and providing a highly sensitive determination of low levels of contaminants. The extended HILIC method could be studied with other polar analytes that are also in critical concern about residues in food such as pesticides.

REFERENCES

[1] Stolker, A.A.M.; and Brinkman, U.A.T. Analytical strategies for residue analysis of veterinary drugs and growth-promoting agent in food-producing animals-a review. <u>Journal of Chromatography A</u> 1067 (2005): 15-53.

[2] Blasco, C.; Torres, C.M.; and Picó, Y. Progress in analysis of residual antibacterials in food. <u>Trends in Analytical Chemistry</u> 26 (2007): 895-913.

[3] Grumbach, E.S.; Wagrowski-Diehl, D.M.; Mazzro, J.R.; Alden, B.; and Iraneta, P.C. Hydrophilic Interaction Chromatography Using Silica Columns for the Retention of Polar Analytes and Enhanced ESI-MS Sensitivity. <u>LCGC North America</u> 22 (2004): 1010-1023.

[4] European Union. <u>EC No.96/23/EC On measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decision 89/187/EEC and 91/664/EEC.</u>, Brussels, 1996.

[5] European Union. <u>EEC No. 2002/657/EC Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.</u>, Brussels, 2002.

[6] European Union. <u>EEC No. 2377/90 Laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin.</u>, Brussels, 1990.

[7] European Commission. <u>Health & Consumer Protection Directorate-General, Guidelines for the Implementation of Decision 2002/657/EC, SANCO/2004/2726rev. 4</u>, 2008.

[8] Myllyniemi, A.L.; Nuotio, L.; Lindfors, E.; Rannikko R.; Niemi A.; and Backman, C. A microbiological six-plate method for the identification of certain antibiotic groups in incurred kidney and muscle samples. <u>The Analyst</u> 126 (2001): 641-646.

[9] Cliquet, P.; Cox, E.; Haasnoot, W.; Schacht, E.; and Goddeeris, B.M. Extraction procedure for sulfachloropyridazine in porcine tissues and detection in a sulfonamide-specific enzyme-linked immunosorbent assay (ELISA). <u>Analytica Chimica Acta</u> 494 (2003): 21-28.

[10] Situ, C.; Grutters, E.; Wichen, P.V.; and Elliott, C.T.A collaborative trial evaluate the performance of a multi-antibiotic enzyme-linked immunosorbent assay for screening five banned antimicrobial growth promoters in animal feedingstuffs. Analytica Chimica Acta 561 (2006): 62-68.

[11] Vincent, U.; Gizzi, G.; Holst, C.V.; Dejong, J.; and Michard J. Validation of an analytical method for the determination of spiramycin, virginiamycin and tylosin in feeding-stuffs by thin-layer chromatography and bio- autography. Food Additive and Contaminants 24 (2007): 351-359.

[12] Leal, C.; Codony, R.; Compano, R.; Granados, M.; and Prat, M.D. Determination of macrolide antibiotics by liquid chromatography. Journal of Chromatography A 910 (2001): 285-290.

[13] Posyniak, A.; Zmudzki, J.; and Mitrowska, K. Dispersive solid-phase extraction for the determination of sulfonamides in chicken muscle by liquid chromatography. Journal of Chromatography A 1087 (2005): 259-264.

[14] Gamba, V.; and Dusi, G. Liquid chromatography with fluorescence detection of amoxicillin and ampicillin in feeds using pre-column derivatization. Analytica Chimica Acta 483 (2003): 69-72.

[15] Cherlet, M.; Schelkens, M.; Croubles, S.; and Backer, P.D. Quantitative multi-residue analysis of tetracyclines and their 4-epimers in pig tissues by high-performance liquid chromatography combined with positive-ion electrospray ionization mass spectrometry. . Analytica Chimica Acta 492 (2003); 199-213.

[16] Becker, M.; Zittlau, E. and Petz, M. Residue analysis of 15 penicillins and cephalosporins in bovine muscle, kidney and milk by liquid chromatography-tandem mass spectrometry. Analytica Chimica Acta 520 (2004): 19-32.

[17] Kao, Y.M.; Chang, M.H.; Cheng, C.C.; and Chou, S.S. Multiresidue determination of veterinary drugs in chicken and swine muscles by high performance liquid chromatography. Journal of Food and drug Analysis 9 (2001): 84-95.

[18] Li, H.; Kijak, P.J.; Turnipseed, S.B.; and Cui, W. Analysis of veterinary drug residues in shrimp : A multi-class method by liquid chromatography-quadrupole ion trap mass spectrometry. Journal of Chromatography B 836 (2006): 22-38.

[19] Yamada, R.; Kozono, M.; Ohmori, T.; Morimatsu, F.; and Kitayama, M. Simultaneous determination of residual veterinary drugs in bovine, porcine and chicken muscle using liquid chromatography couple with electrospray ionization tandem mass spectrometry. Bioscience Biotechnology and Biochemistry 70 (2006): 54-65.

[20] Granelli, K.; and Branzell, C. Rapid multi-residue screening of antibiotics in muscle and kidney by liquid chromatography-electrospray ionization-tandem mass spectrometry. Analytica Chimica Acta 586 (2007): 289-295.

[21] Granelli, K.; Elgerud, C.; Lundström, A.; Ohlsson, A.; and Sjöberg, P. Rapid multi-residue analysis of antibiotics in muscle by liquid chromatography-tandem mass spectrometry. Analytica Chimica Acta 637 (2009): 87-91.

[22] Chico, J.; Rubies, A.; Centrich, F.; Companyo, R.; Prat, M.D.; and Granados, M. High-throghput multiclass method for antibiotic residue analysis by liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1213 (2008): 189-199.

[23] Kaufmann, A.; Butcher, P.; Maden, K.; and Widmer, M. Quantitative multiresidue method for about 100 veterinary drugs in different meat matrices by sub 2-μm particulate high performance liquid chromatography coupled to time of flight mass spectrometry. Journal of Chromatography A 1194 (2008): 66-79.

[24] Carretero, V.; Blasco, C.; and Pico, Y. Multi-class determination of antimicrobials in meat by pressurized liquid extraction and liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 1209 (2008): 162-173.

[25] Bohm, D.A.; Stachel, C.S.; and Gowik, P. Multi-method for the determination of antibiotics of different substance groups in milk and validation in accordance with Commission Decision 2002/657/EC. Journal of Chromatography A 1216 (2009): 8217-8223.

[26] Stubbings, G.; and Bigwood, T. The development and validation of multiclass liquid chromatography tandem mass spectrometry (LC-MS/MS) procedure for the determination of veterinary drug residues in animal tissue using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach. Analytica Chimica Acta 637 (2009): 68-78.

[27] Hammel, Y.; Mohamed, R.; Gremaud, E.; LeBreton, M.; and Guy, P.A. Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography-tandem mass spectrometry. <u>Journal of Chromatography A</u> 1177 (2008): 58-76.

[28] Harvey, D. <u>Modern Analytical Chemistry.</u> 1st ed. United States of America: The McGraw-Hill Companies, Inc., 2000.

[29] Ardrey, R.E. <u>Liquid Chromatography–Mass Spectrometry: An Introduction.</u> England: John Wiley & Sons, 2003.

[30] Ikegami, T.; Tomomatsu, K.; Takubo, H.; Horie, K.; and Tanaka, N. Separation efficiencies in hydrophilic interaction chromatography. <u>Journal of Chromatography A</u> 1184 (2008): 474-503.

[31] McCalley, D.V. Is hydrophilic interaction chromatography with silica columns a viable alternative to reversed phase liquid chromatography for the analysis of ionisable compounds. <u>Journal of Chromatography A</u> 1171 (2007): 46-55.

[32] Alpert, A.J. Hydrophilic interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. <u>Journal of Chromatography</u> 499 (1990): 177-196.

[33] Jiang, W.; Fischer, G.; Girmay, Y.; Irgum, K. Zwitterionic stationary phase with covalently bonded phosphorylcholine type polymer grafts and its applicability to separation of peptides in the hydrophilic interaction liquid chromatography mode. <u>Journal of Chromatography A</u> 1127 (2006): 82-91.

[34] Hao, Z.; Lu, C.Y.; Xiao, B.; Weng, N.; Parker, B.; Knapp, M.; Ho, C.T. Separation of amino acids, peptides and corresponding Amadori compounds on a silica column at elevated temperature. <u>Journal of Chromatography A</u> 1147 (2007): 165-171.

[35] Wang, X.; LI, W.; Rasmussen, H.T. Orthogonal method development using hydrophilic interaction chromatography for the determination of pharmaceuticals and impurities. <u>Journal of Chromatography A</u> 1083 (2005): 58-62.

[36] Oertel, R.; Neumeister, V.; Kirch, W. Hydrophilic interaction chromatography combined with tandem-mass spectrometry to determine six aminoglycosides in serum. <u>Journal of Chromatography A</u> 1058 (2004): 197-201.

[37] SeQuant, <u>A Practical Guide to HILIC</u>. SeQuant, Umeå, Sweden, 2008.

[38] Niessen, W.M.A.; and Tinke A.P. Liquid chromatography-mass spectrometry General principle and instrumentation-a review. <u>Journal of Chromatography A</u> 703 (1995): 37-57.

[39] Settle, F.A.; <u>Handbook of instrumental Techniques of Analytical Chemistry.</u> United States of America: A Simon & Schuster Company, 1997.

[40] NASA. <u>Mass Spectrometer</u>[online]. (n.d.) Available from: http://ael.gsfc.nasa. gov/sa.gov/saturnGCMSMass.shtml [2010, March 5]

[41] Abramowitz, M.; <u>Photomultiplier Tubes.</u>[online] (2004) Available from: http://micro.magnet.fsu.edu/primer/digitalimaging/concepts/photomultipliers.ht ml [2010, March 11]

[42] Petrovic, M.; Hernando, M.D.; Diaz-Cruz, M.S.; Barcelo, D. Liquid chromatography-tandem mass spectrometry for the analysis of pharmaceutical residues in environmental sample-a review. <u>Journal of Chromatography A</u> 1067 (2005): 1-14.

[43] The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics, *EURACHEM Guide*, First internet version, December 1998.

[44] Kaufmann, A.; and Maden, K. Determination of 11 Aminoglycoside in meat and liver by liquid chromatography with tandem mass spectrometry. <u>Journal of AOAC International</u> 88 (2005): 1118-1125.

[45] Holthoon, F.L.; Essers, M. L.; Mulder, P.J.; Stead, S.L.; Caldow, M.; Ashwin, H. M.; and Sharman, M. A generic method for the quantitative analysis of aminoglycosides and spectinomycin in animal tissue using methylated internal standards and liquid chromatography tandem mass spectrometry. <u>Analytica chimica acta</u> 637 (2009): 135-143.

[46] Zhu, W.X.; Yang, J.Z.; Wei, W.; Liu, Y.F.; and Zhang, S.S. Simultaneous determination of 13 aminoglycoside residues in foods of animal origin by liquid chromatography-electrospray ionization tandem mass spectrometry with two consecutive solid-phase extraction steps. <u>Journal of Chromatography A</u> 1207 (2008): 29-37.

[47] Balizs, G.; and Hewitt, A. Determination of veterinary drug residues by liquid chromatography and tandem mass spectrometry. <u>Analytica Chimica Acta</u> 492 (2003): 105-131.

[48] Song, W.; Huang, M.; Rumbeiha, W.; and Li, H. Determination of amprolium, carbadox, monensin and tylosin in surface water by in liquid chromatography tandem mass spectrometry. Rapid Communications in Mass Spectrometry 21 (2007): 1944-1950.

[49] Sin, D. W.M.; Ho, C.; Wong, Y.C.; Ho, S. K.; and Ip, A. C.B. Simultaneous determination of lincomycin and virginiamycin in swine muscle, liver and kidney by liquid chromatography-electrospray ionization tandem mass spectrometry. Analytica Chimica Acta 517 (2004): 39-45.

[50] Niessen, W.M.A. Analysis of antibiotics by liquid chromatography-mass spectrometry-a review. Journal of Chromatography A 812 (1998) 53-75.

[51] FAO/WHO Codex alimentarius commission No.CX/RDVX 03/10 Review of performance-based criteria for methods of analysis for veterinary drug residues in foods Washington, D.C., USA, 2002.

[52] Taverniers, I.; Loose, M.D.; and Bockstaele, E.V. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. Trend in Analytical Chemistry 23 (2004): 535-552.

[53] Koesukwiwat, U.; Jayanta, S. and Leepipatpiboon, N. Validation of a liquid chromatography-mass spectrometry multi-residue method for the simultaneous determination of sulfonamides, tetracyclines, and pyrimethamine in milk. Journal of Chromatography A 1140 (2007): 147-156.
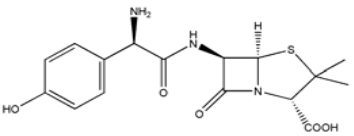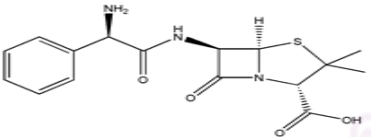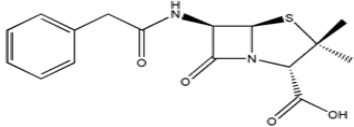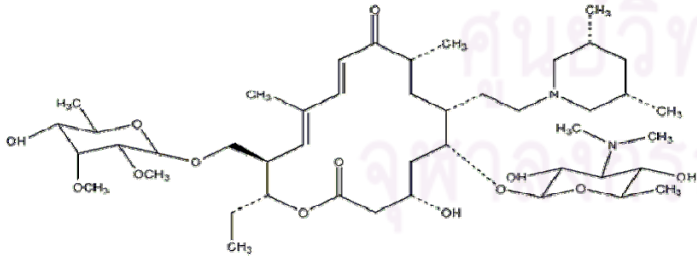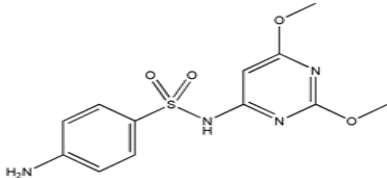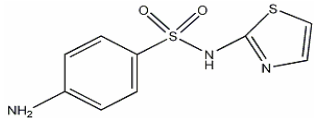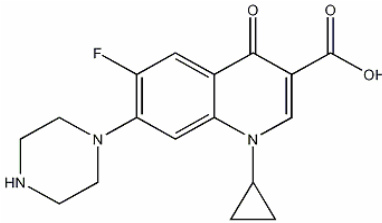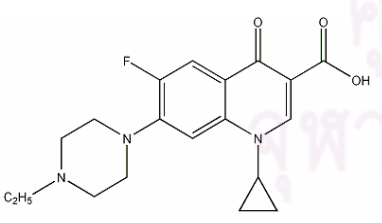
APPENDICES
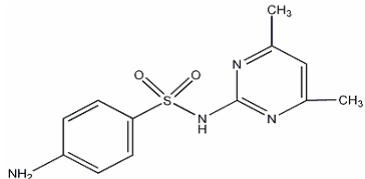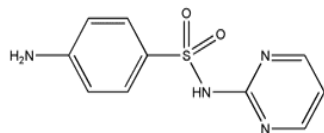
APPENDIX A

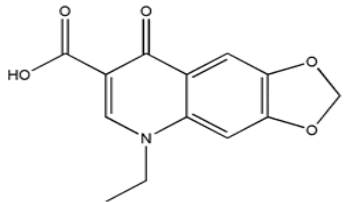**Table A-1** Chemical structures and physicochemical properties of twenty-four targeted compounds
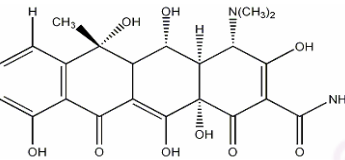
Compound structures	M.W.	pKa	Stability	Toxicological informations
Amoxicillin (AMOX) $C_{16}H_{19}N_3O_5S$	365.4	2.8 7.2	- Light sensitive.	- May cause damage to the following organ: kidneys. - Slightly hazardous in case of skin contact (irritant), of ingestion, of inhalation. - May cause skin irritation (rash) with itching, redness and swelling of skin - May cause eye irritation.
Ampicillin (AMP) $C_{16}H_{19}N_3O_4S$	349.4	2.7 7.3	- Light sensitive.	- May cause sensitisation and irritation by skin contact. - Contact may be irritating to eyes. - May be harmful if ingestion occurs. - The substance or preparation can induce specific

.

Compound structures	M.W.	pKa	Stability	Toxicological informations
Penicillin G (PEN G) $C_{16}H_{18}N_2O_4S$	334.4	2.8	- Hazardous decomposition product	- May cause irritation of eyes. - May cause irritation ,dermatitis - Individuals an allergic reaction. - May be absorbed through uninjured skin.
Tilmicosin (TIL) $C_{46}H_{80}N_2O_{13}$	869.13	7.4 8.5	- Stable under normal temperature	- Increased adrenal and kidney weights, increased cell size in adrenal cortex, mucosal edema of the gallbladder, . - Decreased food consumption and body weight gains, slightly decreased urine pH, occult blood in urine
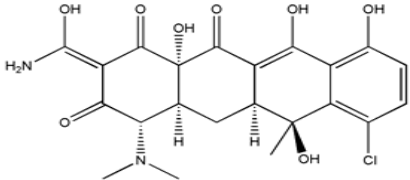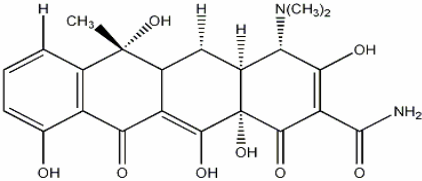
Compound structures	M.W.	pKa	Stability	Toxicological informations
Sulfamethazine (SMZ) $C_{12}H_{14}N_4O_2S$	278.34	0.4 7.0 2.8	- The product is stable.	- Very hazardous in case of ingestion. - Hazardous in case of skin contact (irritant). - Slightly hazardous in case of skin contact
Sulfadiazine (SDZ) $C_{10}H_{10}N_4O_2S$	250.28	0.35 1.6 6.8	- 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.
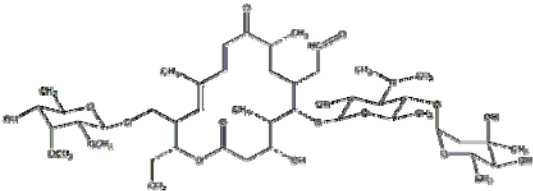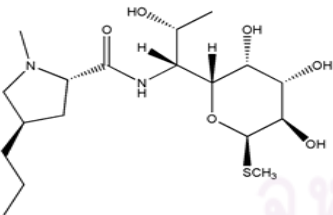
Compound structures	M.W.	pKa	Stability	Toxicological informations
Sulfadimethoxine (SDMX) $C_{12}H_{14}N_4O_4S$	310.33	0.7 2.4 6.5	- Sensitive to temperature	- Harzadous in case of skin contact (irritant), eye contact (irritant), ingestion, inhalation (lung irritant). - Slightly hazardous in case of skin contact (permeator).
Sulfathiazole (STZ) $C_9H_9N_3O_2S_2$	255.32	0.7 2.3 7.8	- Light sensitive.	- Irrtating to eye or if inhaled as dust. - Harmful by ingestion. - May cause nausea, vomiting, dizziness or mental confusion.
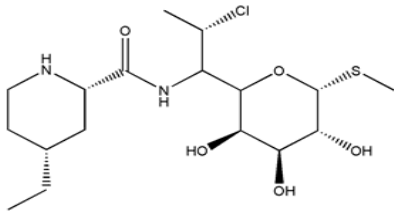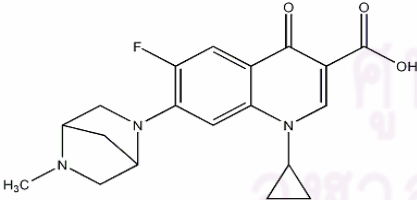
Compound structures	M.W.	pKa	Stability	Toxicological informations
Ciprofloxacin (CIP) $C_{17}H_{13}FN_3O_3$	331.34	5.9 8.2	- The product is stable.	- May cause skin irritation. May cause dermatitis. - May cause eye irritation with itching, buring sensation, tering, decreased vision. - Dust may cause respiratory tract irritation. - May cause gastrointestinal tract irritation with nausea,
Enrofloxacin (ENR) $C_{19}H_{22}FN_3O_3$	359.39	5.9 7.7	- Stable under recommended storage conditions.	- Chronic exposure may cause nausea and vomiting - Symptoms of overexposure may be headache, dizziness, nausea and vomiting.
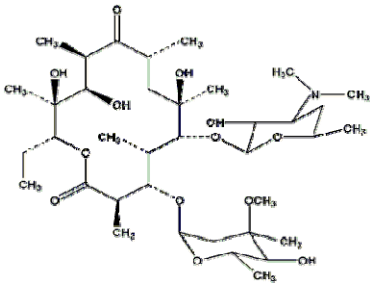
Compound structures	M.W.	pKa	Stability	Toxicological informations
Oxolinic acid (OXO) $C_{13}H_{11}NO_5$	261.23	6.8	- Stable	- May cause skin irritation. - May cause eye irritation. - May cause respiratory tract irritation. - May be harmful if swallowed. - May cause gastrointestinal tract irritation
Oxytetracycline (OTC) $C_{22}H_{24}N_2O_9$	460.43	3.5	- Light sensitive and stable at ambient temperatures.	- Antibiotic substance isolated from the elaboration products of the actinomycete, *Steptomyces rimosus*. - May cause irritation/dryness or defatting of the skin with prolonged contact.
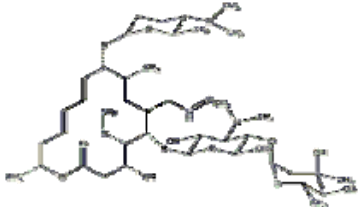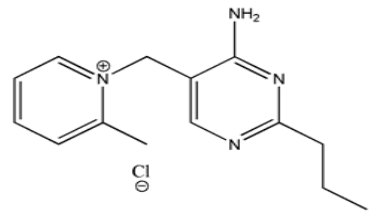
Compound structures	M.W.	pKa	Stability	Toxicological informations
Chlorotetracycline (CTC) $C_{22}H_{23}ClN_2O_8$	478.89	3.3	- Sensitive to light and temperature	- May cause adverse reproductive effects (growth, viability) based on animal data. - Chronic effects on human: excreted into braes milk at low concentrations. - May cause irritation of the digestive tract and mucous membranes, n vomiting, diarrhea.
Tetracycline (TC) $C_{22}H_{24}N_2O_8$	444.43	3.3	- Sensitive to light and temperature	- Antibiotic substance produced by *Steptomyces spp*. - Antibiotic uesd to treat infections with bacteria, mycoplasma. - Toxic to reproductive system, liver. - Hazardous in case of ingestion, inhalation.
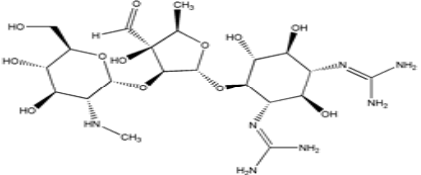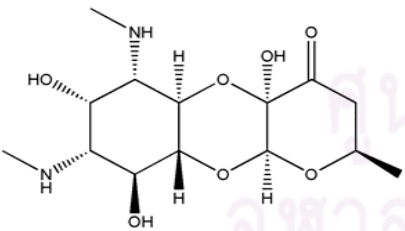
Compound structures	M.W.	pKa	Stability	Toxicological informations
Tylosin (TYL) $C_{46}H_{77}NO_{17}$	916.1	7.1	- The product is stable.	- Chronic effect on humans : the substance is toxic to gastrointestinal tract, upper respiratory tract, skin, central nervous system. - Hazardous in case of ingestion. - Slightly hazardous in case of inhalation.
Lincomycin (LIN) $C_{18}H_{34}N_2O_6S$	406.54		- The product is stable.	- May cause skin irritation and rash. - May cause eye irritation. - May cause respiratory tract irritation with sore throat. - May cause gastrointestinal tract irritation with abdominal cramps or pain nuasea, or vomiting,

Compound structures	M.W.	pKa	Stability	Toxicological informations
Pirlimycin (PIR) $C_{17}H_{37}ClN_2O_5S$	410.96		- Exposure to light.	- Moderately irritating to abraded and intact skin. - Severely irritating to the eye.
Danofloxacin (DAN) $C_{19}H_{20}FN_3O_3$	357.38	6.1 8.6	- Sensitive to light.	- Skin sensitization and/or photosensitization (allergic response after UV exposure)

Compound structures	M.W.	pKa	Stability	Toxicological informations
Erythromycin (ERY) $C_{37}H_{67}NO_{13}$	733.92	8.8	- Stable in dry air. - Slightly hygroscopic; absorbs moisture from air.	- May cause iirritation of the nasal and respiratory passage, causing sore throat, coughng. - May cause allergic respiratory reaction. - May cause iirritation , with redness, itching. - May cause skin sensitization, with skin rash and eruptions on repeat exposure. - May cause gastrointestinal irritation with nausea, vomiting and diarrhea.

Compound strutures	M.W.	pKa	Stability	Toxicological informations
Spiramycin (SPI) $C_{43}H_{74}N_2O_{14}$	843.05	7.9	- Exposure to air or moisture over prolonged periods.	- May include moderate to severe erythema (redness) and moderate edema (raised skin), nausea, vomiting,
Amprolium (AMPR) $C_{14}H_{19}ClN_4$	278.79		- Product is stable.	- Mildly irritating to eyes. - May cause mild irritation of the eye. - May result in allergic respiratory and skin reactions.

Compound strutures	M.W.	pKa	Stability	Toxicological informations
Streptomycin (STR) $C_{21}H_{39}N_7O_{12}$	581.57	8.7	- Stable under normal use and storage conditions.	- Mildly irritating to eyes. - Non-irritating to dermal.
Spectinomycin (SPEC) $C_{14}H_{24}N_2O_7$		7.0 8.7	- Exposure to air or moisture over prolonged periods.	- May include moderate to severe erythema (redness) and moderate edema (raised skin), nausea, vomiting, headache.

Compound structures	M.W.	pKa	Stability	Toxicological informations
Dihydrostreptomycin (DSTR) $C_{21}H_{41}N_7O_{12}$	583.6	7.8	- Stable under normal use and storage conditions.	- May include moderate to severe erythema (redness) and moderate edema (raised skin), nausea, vomiting, headache.
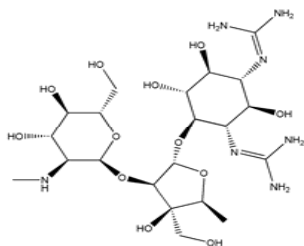
APPENDIX B

B. Matrix matched standard calibration curve
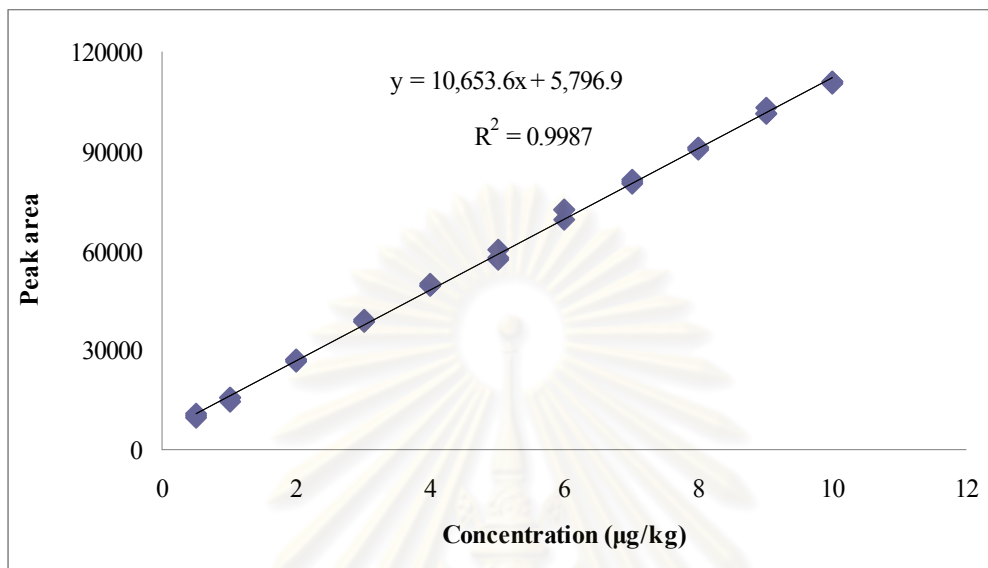
Figure B-1 Matrix matched standard calibration curve of sulfadiazine
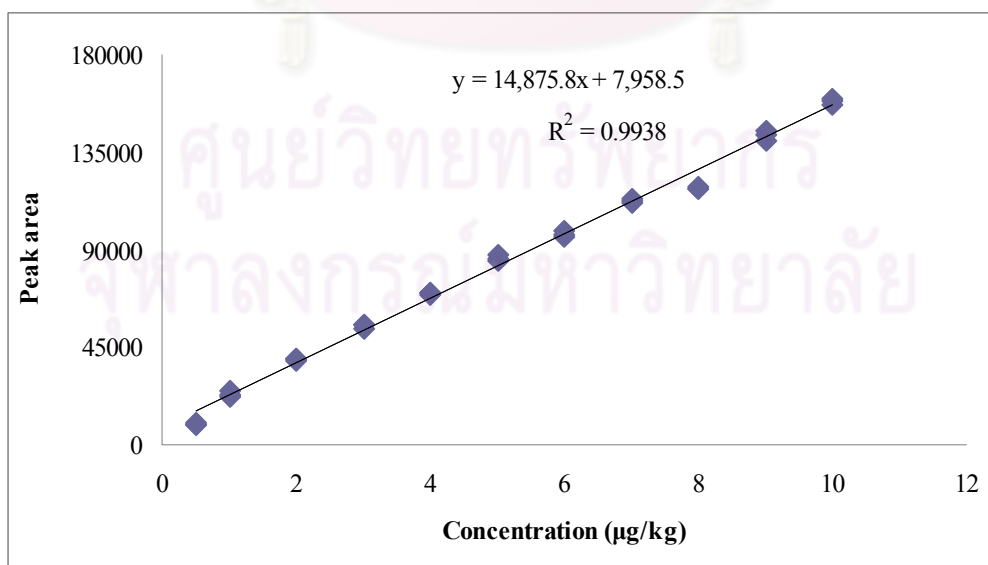
Figure B-2 Matrix matched standard calibration curve of sulfadimethoxine
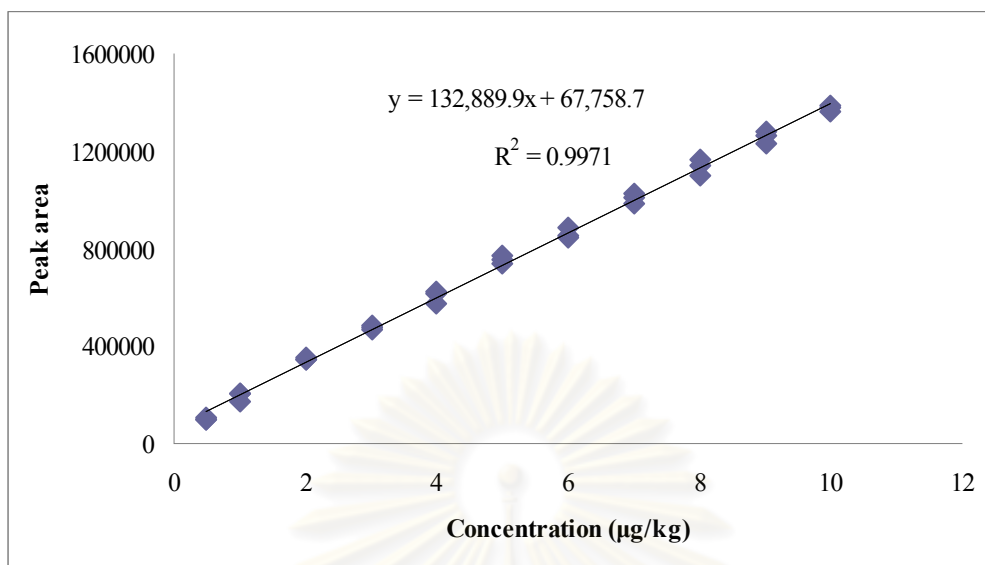
Figure B-3 Matrix matched standard calibration curve of sulfathiazole
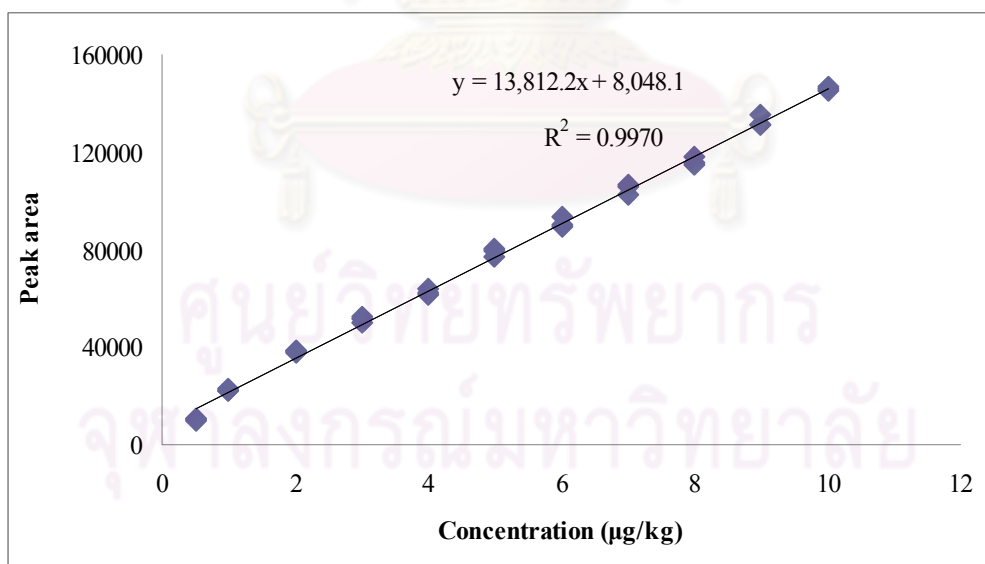
Figure B-4 Matrix matched standard calibration curve of sulfadimidine
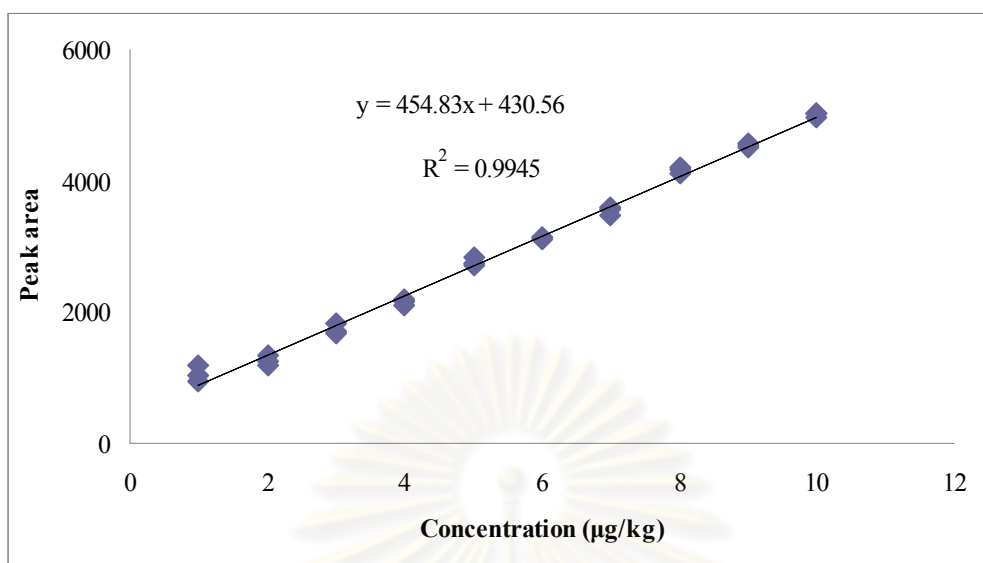
Figure B-5 Matrix matched standard calibration curve of enrofloxacin
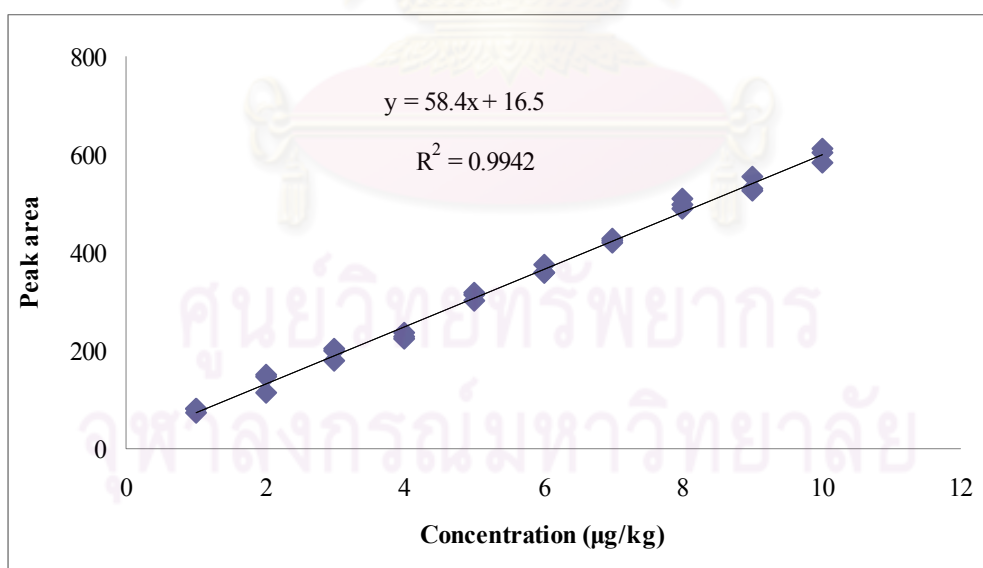
Figure B-6 Matrix matched standard calibration curve of amoxicilin
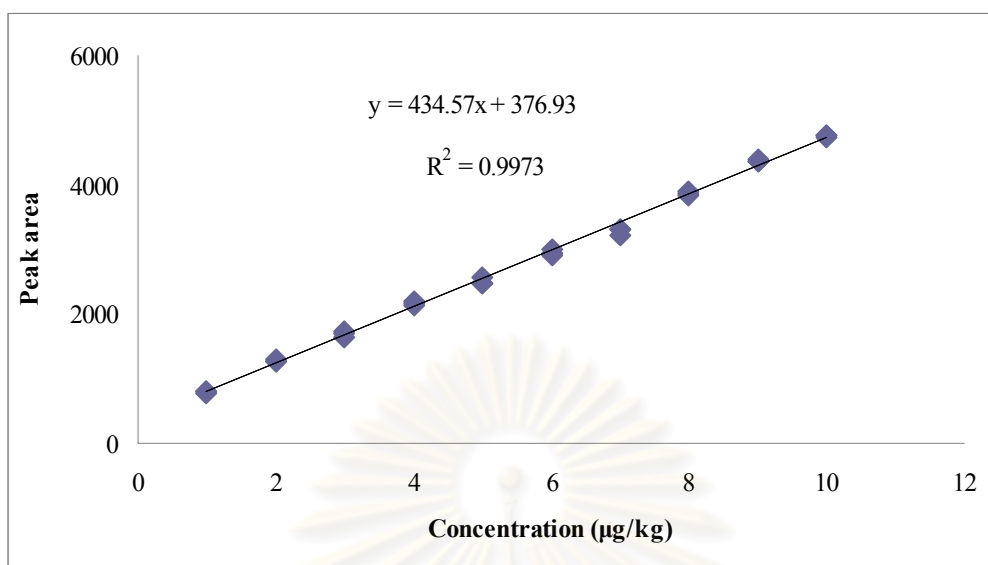
Figure B-7 Matrix matched standard calibration curve of ampicillin
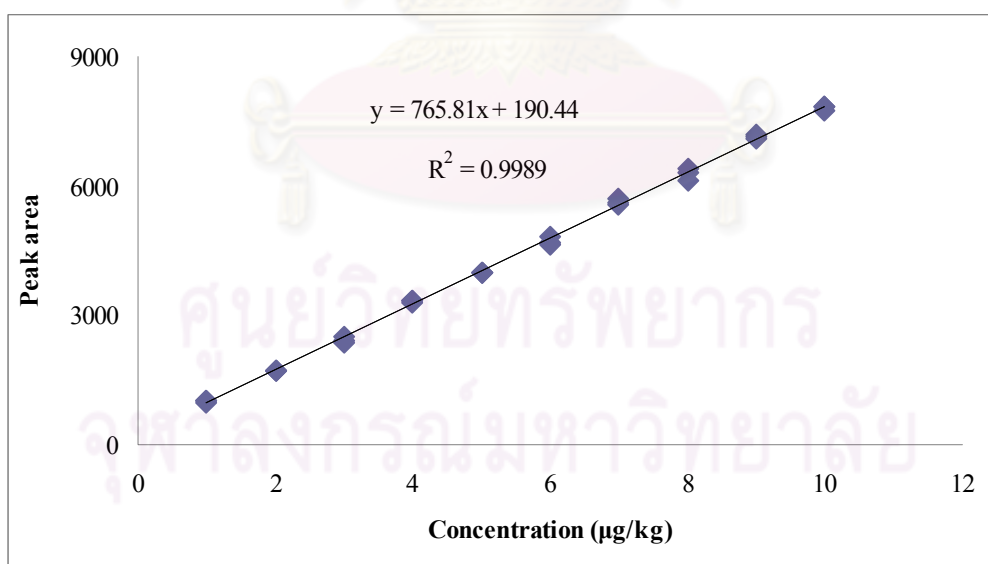
Figure B-8 Matrix matched standard calibration curve of penicillin G
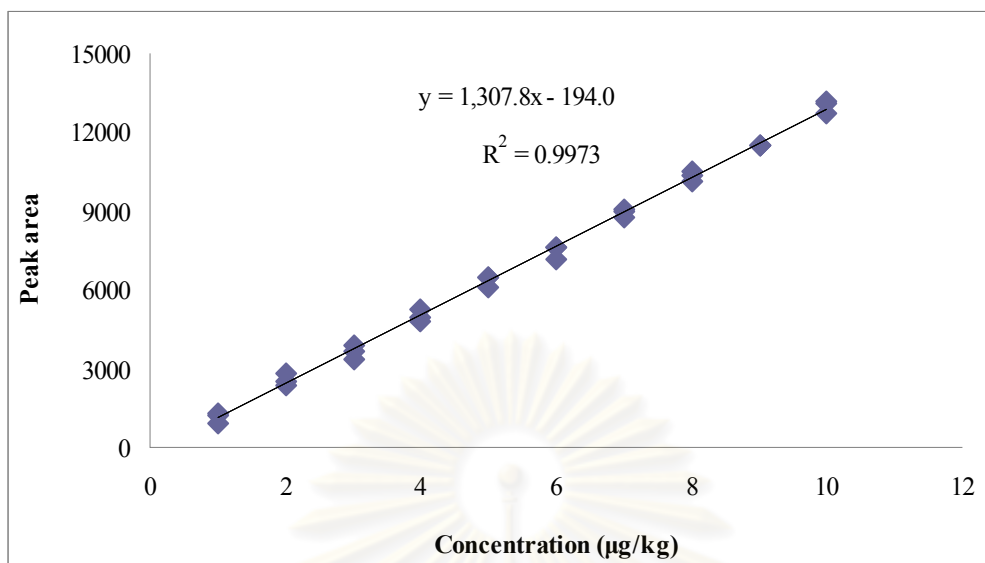
Figure B-9 Matrix matched standard calibration curve of tilmicosin
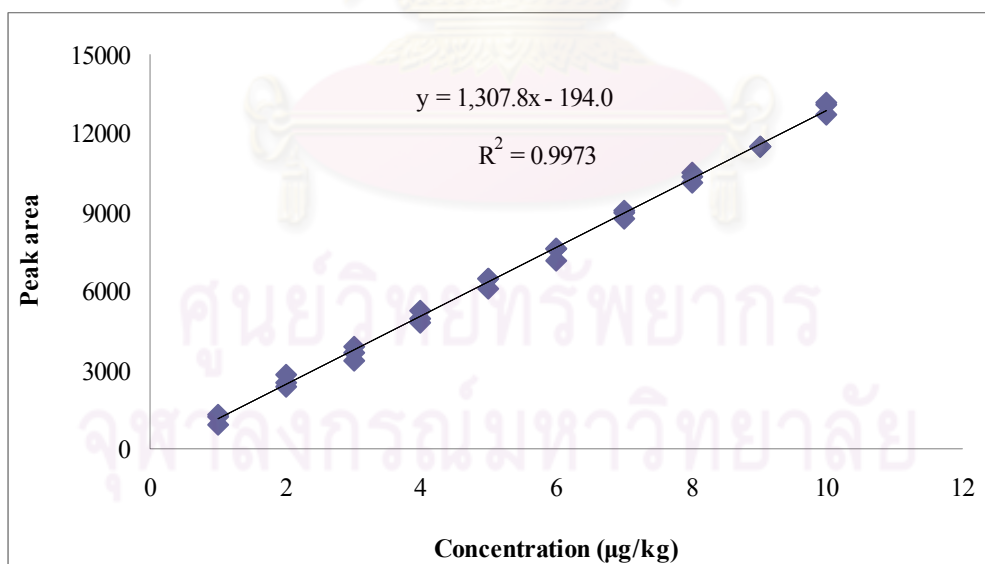
Figure B-10 Matrix matched standard calibration curve of ciprofloxacin
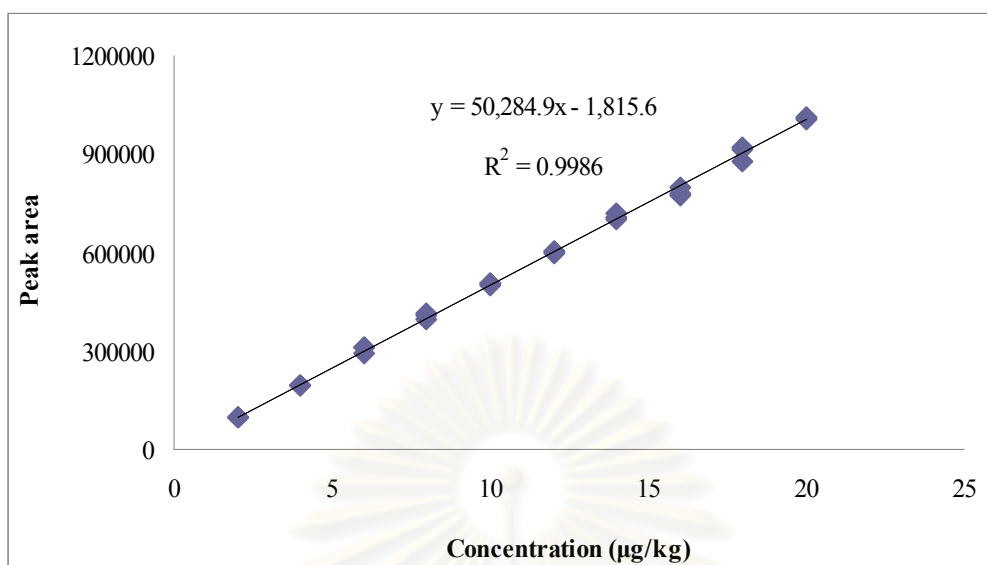
Figure B-11 Matrix matched standard calibration curve of oxolinic acid
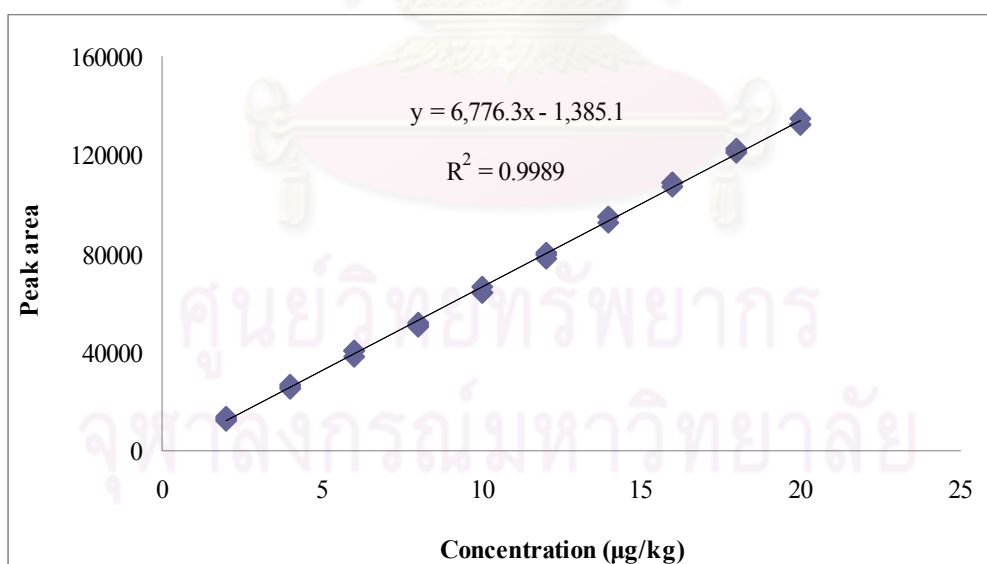
Figure B-12 Matrix matched standard calibration curve of lincomycin
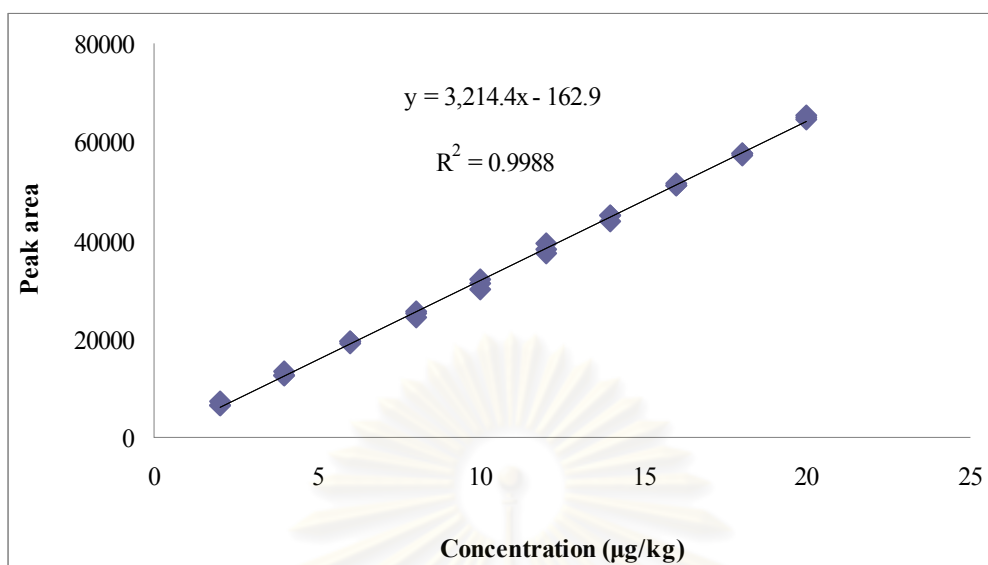
Figure B-13 Matrix matched standard calibration curve of pirlimycin
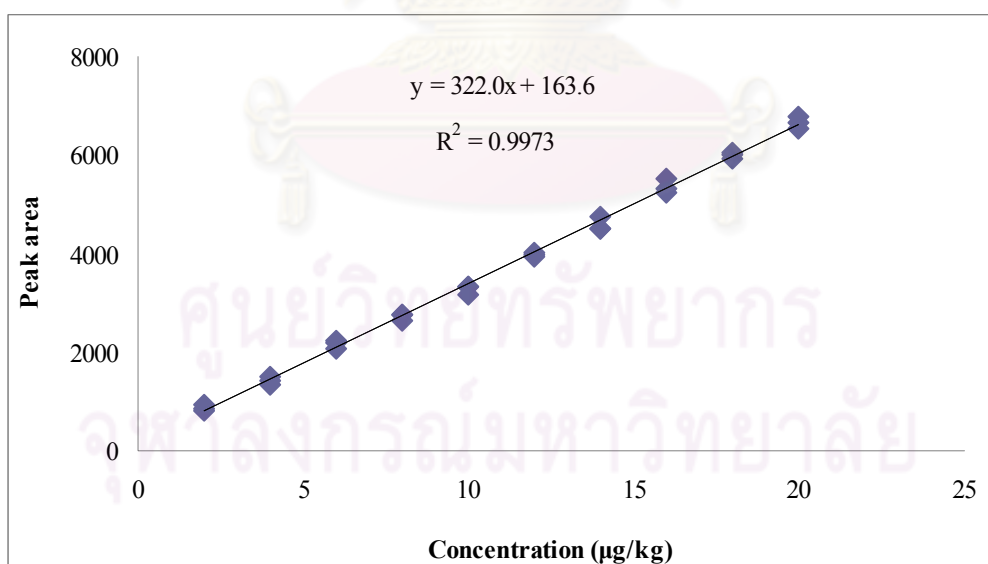
Figure B-14 Matrix matched standard calibration curve of chlortetracycline
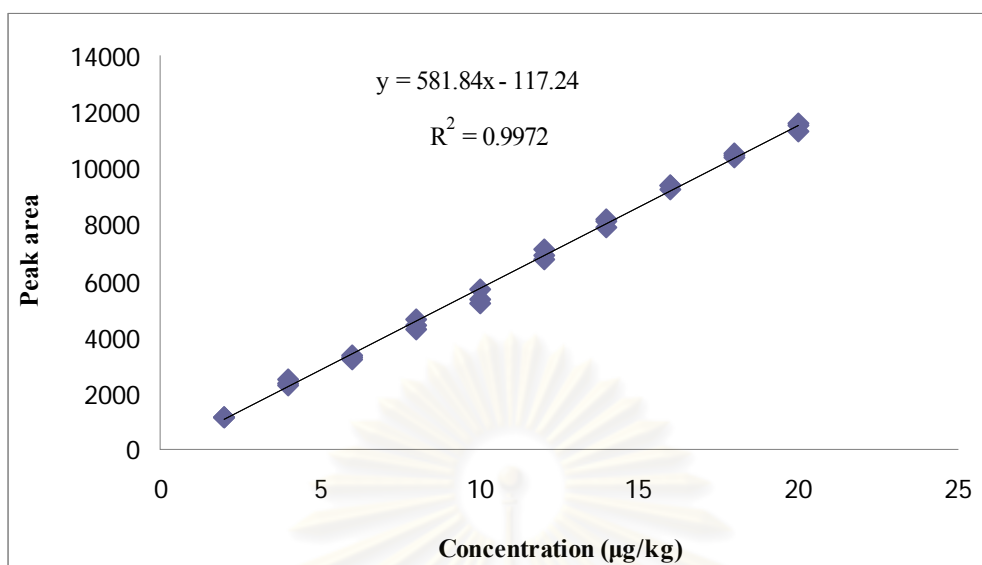
Figure B-15 Matrix matched standard calibration curve of oxytetracycline
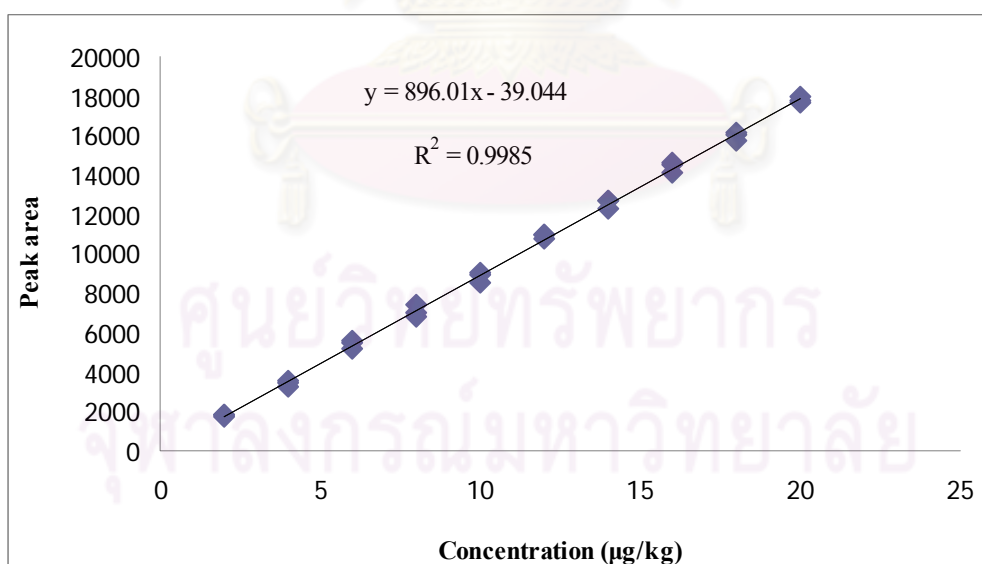
Figure B-16 Matrix matched standard calibration curve of tetracycline
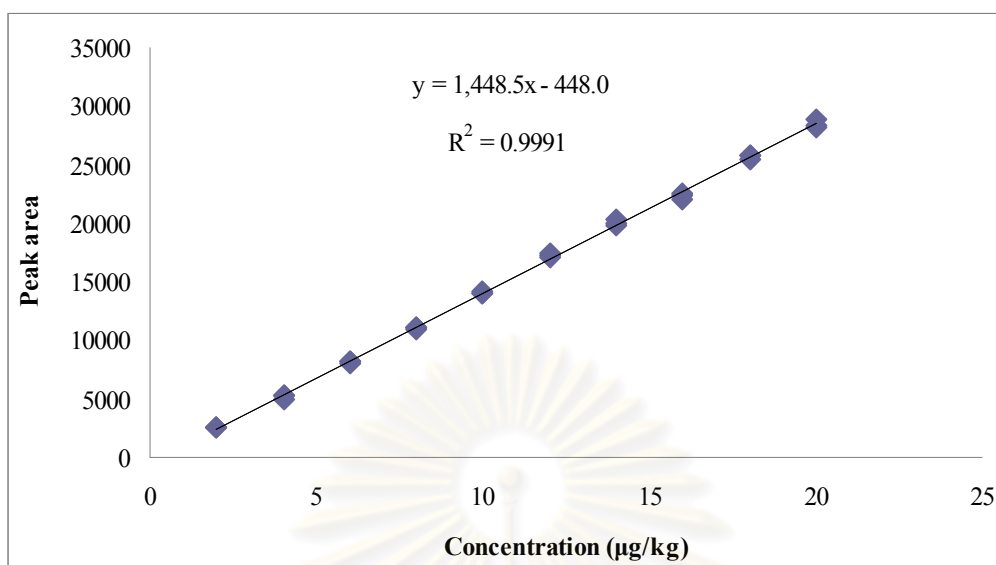
Figure B-17 Matrix matched standard calibration curve of tylosin
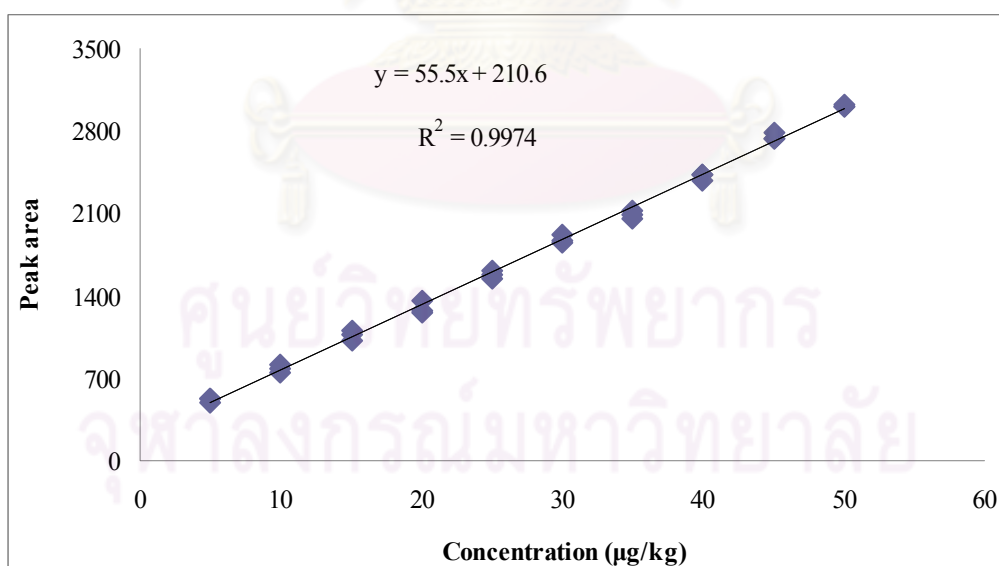
Figure B-18 Matrix matched standard calibration curve of spectinomycin
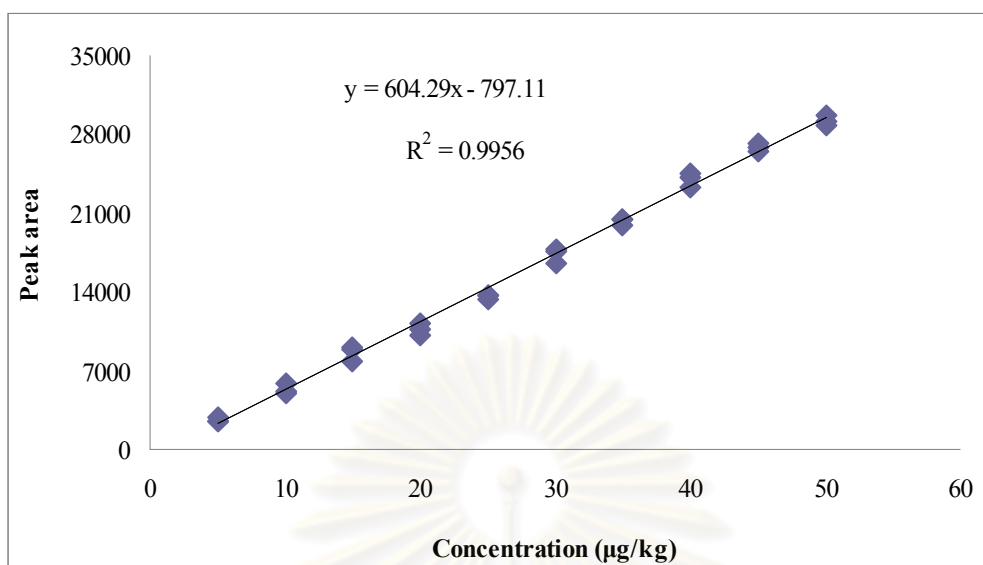
Figure B-19 Matrix matched standard calibration curve of amprolium
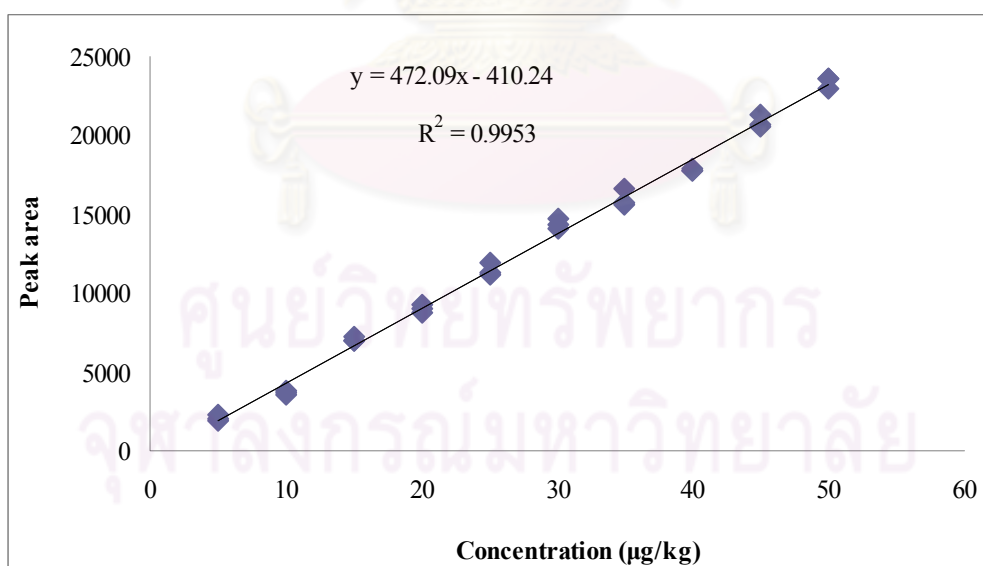
Figure B-20 Matrix matched standard calibration curve of danofloxacin
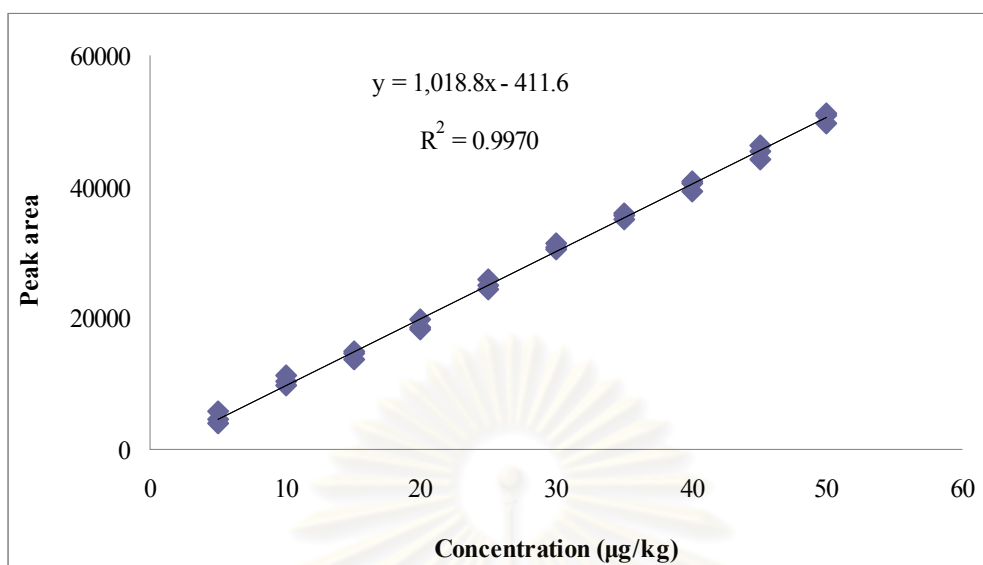
Figure B-21 Matrix matched standard calibration curve of spiramycin
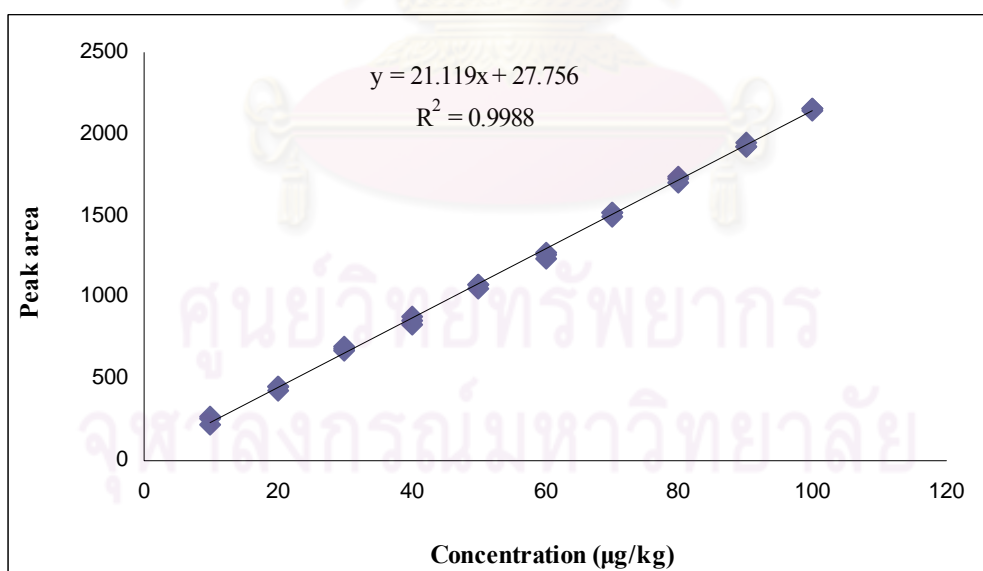
Figure B-22 Matrix matched standard calibration curve of dihydrostreptomycin
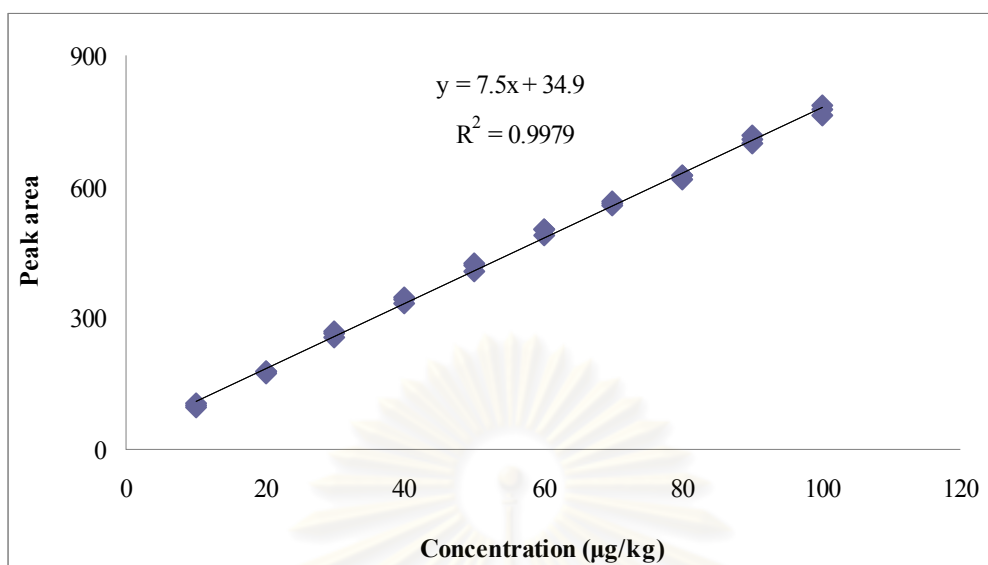
Figure B-23 Matrix matched standard calibration curve of streptomycin
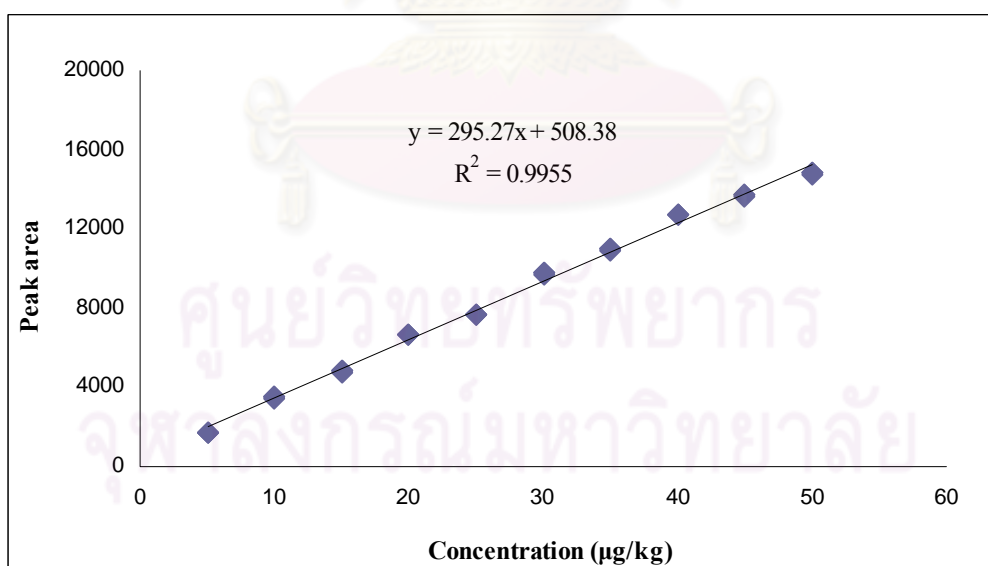
Figure B-24 Matrix matched standard calibration curve of erythromycin

APPENDIX C

C. Statistical analysis of twenty-four targeted compounds.

Amoxicillin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	676	67.6	90.04444
Day2	10	709	70.9	162.7667
Day3	10	660	66	68.88889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	124.8667	2	62.43333	0.582219	0.566	3.354131
Within Groups	2895.3	27	107.2333			
Total	3020.167	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	764	76.4	116.0444
Day2	10	776	77.6	124.0444
Day3	10	771	77.1	83.65556

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7.266667	2	3.633333	0.033669	0.967	3.354131
Within Groups	2913.7	27	107.9148			
Total	2920.967	29				

Ampicillin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	726	72.6	114.7111
Day2	10	625	62.5	7.388889
Day3	10	762	76.2	46.84444

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1008.867	2	504.4333	8.957382	0.001	3.354131
Within Groups	1520.5	27	56.31481			
Total	2529.367	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	706	70.6	27.6
Day2	10	675	67.5	14.5
Day3	10	733	73.3	37.12222

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	168.4667	2	84.23333	3.189762	0.057	3.354131
Within Groups	713	27	26.40741			
Total	881.4667	29				

Penicillin G

	0.5
Anova: Single Factor	MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	640	64	12.44444
Day2	10	613	61.3	26.67778
Day3	10	595	59.5	16.27778

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	102.6	2	51.3	2.777978	0.080	3.354131
Within Groups	498.6	27	18.46667			
Total	601.2	29				

Anova: Single Factor	MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	697	69.7	10.9
Day2	10	629	62.9	7.877778
Day3	10	658	65.8	21.95556

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	232.8667	2	116.4333	8.575286	0.001	3.354131
Within Groups	366.6	27	13.57778			
Total	599.4667	29				

Tilmicosin

Anova: Single
Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	846	84.6	66.26667
Day2	10	853	85.3	158.6778
Day3	10	775	77.5	82.05556

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	372.4667	2	186.2333	1.81987	0.181	3.354131
Within Groups	2763	27	102.3333			
Total	3135.467	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	814	81.4	23.15556
Day2	10	897	89.7	160.4556
Day3	10	907	90.7	63.56667

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	521.2667	2	260.6333	3.16331	0.058	3.354131
Within Groups	2224.6	27	82.39259			
Total	2745.867	29				

Sulfamethazine

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	608	60.8	43.28889
Day2	10	665	66.5	52.5
Day3	10	625	62.5	25.16667

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	171.2667	2	85.63333	2.123921	0.139	3.354131
Within Groups	1088.6	27	40.31852			
Total	1259.867	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	664	66.4	4.488889
Day2	10	682	68.2	27.95556
Day3	10	708	70.8	12.62222

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	97.86667	2	48.93333	3.257396	0.054	3.354131
Within Groups	405.6	27	15.02222			
Total	503.4667	29				

Sulfadiazine

Anova: Single
Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	648	64.8	29.95556
Day2	10	702	70.2	132.8444
Day3	10	638	63.8	11.95556

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	237.0667	2	118.5333	2.034842	0.150	3.354131
Within Groups	1572.8	27	58.25185			
Total	1809.867	29				

Anova: Single
Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	704	70.4	9.155556
Day2	10	703	70.3	70.01111
Day3	10	656	65.6	6.933333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	150.4667	2	75.23333	2.62137	0.091	3.354131
Within Groups	774.9	27	28.7			
Total	925.3667	29				

Sulfadimethoxine

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	603	60.3	47.12222
Day2	10	643	64.3	59.56667
Day3	10	612	61.2	11.28889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	88.06667	2	44.03333	1.119702	0.341	3.354131
Within Groups	1061.8	27	39.32593			
Total	1149.867	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	661	66.1	5.877778
Day2	10	670	67	40.66667
Day3	10	693	69.3	12.9

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	54.46667	2	27.23333	1.374393	0.270	3.354131
Within Groups	535	27	19.81481			
Total	589.4667	29				

Sulfathiazole

Anova: Single Factor 0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	683	68.3	33.56667
Day2	10	732	73.2	66.4
Day3	10	666	66.6	8.266667

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	234.8667	2	117.4333	3.255005	0.054	3.354131
Within Groups	974.1	27	36.07778			
Total	1208.967	29				

Anova: Single Factor MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	726	72.6	22.04444
Day2	10	743	74.3	26.23333
Day3	10	683	68.3	6.677778

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	191.2667	2	95.63333	5.220582	0.012	3.354131
Within Groups	494.6	27	18.31852			
Total	685.8667	29				

Ciprofloxacin

| Anova: Single Factor | | 0.5MRL | | |

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	851	85.1	69.87778
Day2	10	850	85	153.1111
Day3	10	876	87.6	100.7111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	43.4	2	21.7	0.201112	0.8190	3.354131
Within Groups	2913.3	27	107.9			
Total	2956.7	29				

| Anova: Single Factor | | MRL | | |

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	839	83.9	72.54444
Day2	10	765	76.5	14.5
Day3	10	777	77.7	69.56667

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	315.466667	2	157.7333	3.021497	0.0654	3.354131
Within Groups	1409.5	27	52.2037			
Total	1724.96667	29				

Enrofloxacin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	769	76.9	18.76667
Day2	10	801	80.1	112.3222
Day3	10	850	85	104.2222

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	332.8667	2	166.4333	2.121872	0.1394	3.354131
Within Groups	2117.8	27	78.43704			
Total	2450.667	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	929	92.9	28.1
Day2	10	873	87.3	37.12222
Day3	10	936	93.6	46.26667

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	238.4667	2	119.2333	3.208391	0.05622	3.354131
Within Groups	1003.4	27	37.16296			
Total	1241.867	29				

Oxolinic acid

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	717	71.7	15.56667
Day2	10	670	67	64.44444
Day3	10	676	67.6	13.6

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	130.8667	2	65.43333	2.096973	0.1424	3.354131
Within Groups	842.5	27	31.2037			
Total	973.3667	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	675	67.5	12.72222
Day2	10	682	68.2	39.73333
Day3	10	692	69.2	7.288889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	14.6	2	7.3	0.366561	0.697	3.354131
Within Groups	537.7	27	19.91481			
Total	552.3	29				

Oxytetracycline

Anova: Single Factor   0.5

MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	701	70.1	91.43333
Day2	10	752	75.2	43.95556
Day3	10	618	61.8	25.51111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	914.8667	2	457.4333	8.5289	0.0013	3.354131
Within Groups	1448.1	27	53.63333			
Total	2362.967	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	733	73.3	18.01111
Day2	10	690	69	20
Day3	10	702	70.2	11.51111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	98.46667	2	49.23333	2.982499	0.0676	3.354131
Within Groups	445.7	27	16.50741			
Total	544.1667	29				

Chlortetracycline

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	785	78.5	95.83333
Day2	10	700	70	112
Day3	10	690	69	46.88889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	545	2	272.5	3.209378	0.056	3.354131
Within Groups	2292.5	27	84.90741			
Total	2837.5	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	799	79.9	10.1
Day2	10	763	76.3	7.788889
Day3	10	814	81.4	54.93333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	137.4	2	68.7	2.83018	0.077	3.354131
Within Groups	655.4	27	24.27407			
Total	792.8	29				

Tetracyclines

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	825	82.5	73.61111
Day2	10	806	80.6	63.37778
Day3	10	789	78.9	46.54444

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	64.86667	2	32.43333	0.530149	0.595	3.354131
Within Groups	1651.8	27	61.17778			
Total	1716.667	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	826	82.6	11.82222
Day2	10	795	79.5	52.05556
Day3	10	727	72.7	56.45556

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	512.8667	2	256.4333	6.393075	0.0053	3.354131
Within Groups	1083	27	40.11111			
Total	1595.867	29				

Tylosin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	796	79.6	8.933333
Day2	10	862	86.2	89.51111
Day3	10	816	81.6	10.71111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	229.0667	2	114.5333	3.147801	0.059	3.354131
Within Groups	982.4	27	36.38519			
Total	1211.467	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	747	74.7	8.011111
Day2	10	716	71.6	3.822222
Day3	10	715	71.5	3.611111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66.2	2	33.1	6.429496	0.005	3.354131
Within Groups	139	27	5.148148			
Total	205.2	29				

Lincomycin

Anova: Single   0.5

Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	749	74.9	8.766667
Day2	10	706	70.6	1.377778
Day3	10	760	76	23.77778

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	162.8667	2	81.43333	7.201769	0.003	3.354131
Within Groups	305.3	27	11.30741			
Total	468.1667	29				

Anova: Single
Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	710	71	3.111111
Day2	10	684	68.4	23.15556
Day3	10	772	77.2	43.51111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	408.8	2	204.4	8.787898	0.001	3.354131
Within Groups	628	27	23.25926			
Total	1036.8	29				

Pirlimycin

Anova: Single   0.5MRL

Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	712	71.2	4.4
Day2	10	784	78.4	24.71111
Day3	10	751	75.1	18.1

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	259.8	2	129.9	8.254413	0.002	3.354131
Within Groups	424.9	27	15.73704			
Total	684.7	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	734	73.4	14.48889
Day2	10	714	71.4	2.933333
Day3	10	756	75.6	25.15556

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	88.267	2	44.13333	3.109603	0.061	3.354131
Within Groups	383.2	27	14.19259			
Total	471.467	29				

Danofloxacin

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	819	81.9	38.98889
Day2	10	756	75.6	36.04444
Day3	10	860	86	24

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	548.8667	2	274.4333	8.313363	0.002	3.354131
Within Groups	891.3	27	33.01111			
Total	1440.167	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	859	85.9	57.87778
Day2	10	778	77.8	80.62222
Day3	10	835	83.5	55.38889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	346.2	2	173.1	2.678338	0.087	3.354131
Within Groups	1745	27	64.62963			
Total	2091.2	29				

Erythromycin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	525	52.5	10.05556
Day2	10	577	57.7	49.12222
Day3	10	570	57	34.44444

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	159.2666667	2	79.63333	2.551745	0.097	3.354131
Within Groups	842.6	27	31.20741			
Total	1001.866667	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	573	57.3	4.9
Day2	10	530	53	4.444444
Day3	10	542	54.2	11.28889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	98.46666667	2	49.23333	7.15832	0.003	3.354131
Within Groups	185.7	27	6.877778			
Total	284.1666667	29				

Spiramycin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	748	74.8	26.84444
Day2	10	781	78.1	80.54444
Day3	10	706	70.6	28.71111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	282.6	2	141.3	3.114622	0.061	3.354131
Within Groups	1224.9	27	45.36667			
Total	1507.5	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	673	67.3	20.01111
Day2	10	783	78.3	50.67778
Day3	10	722	72.2	34.17778

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	607.4	2	303.7	8.688175	0.001	3.354131
Within Groups	943.8	27	34.95556			
Total	1551.2	29				

Amprolium

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	765	76.5	29.61111
Day2	10	703	70.3	34.23333
Day3	10	728	72.8	31.28889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	194.6	2	97.3	3.068325	0.063	3.354131
Within Groups	856.2	27	31.71111			
Total	1050.8	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	14	959.8431	68.56022	1021.693
Day2	10	734	73.4	40.71111
Day3	10	826	82.6	52.93333

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1157.042	2	578.5212	1.269693	0.295	3.304817
Within Groups	14124.8	31	455.6388			
Total	15281.84	33				

Spectinomycin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	830	83	21.77778
Day2	10	778	77.8	34.62222
Day3	10	808	80.8	39.06667

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	136.2667	2	68.13333	2.141061	0.137	3.354131
Within Groups	859.2	27	31.82222			
Total	995.4667	29				

Anova: Single Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	774	77.4	20.93333
Day2	10	761	76.1	16.1
Day3	10	810	81	29.11111

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	128.8667	2	64.43333	2.922392	0.071	3.354131
Within Groups	595.3	27	22.04815			
Total	724.1667	29				

Streptomycin

Anova: Single Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	833	83.3	104.2333
Day2	10	774	77.4	78.71111
Day3	10	764	76.4	45.15556

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	278.0667	2	139.0333	1.828584	0.180	3.354131
Within Groups	2052.9	27	76.03333			
Total	2330.967	29				

Anova: Single
Factor   MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	741	74.1	44.1
Day2	10	690	69	12.22222
Day3	10	750	75	45.77778

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	209.4	2	104.7	3.076396	0.063	3.354131
Within Groups	918.9	27	34.03333			
Total	1128.3	29				

Dihydrostreptomycin

Anova: Single
Factor   0.5MRL

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	824	82.4	105.378
Day2	10	828	82.8	71.5111
Day3	10	832	83.2	14.6222

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.2	2	1.6	0.02506	0.975	3.354131
Within Groups	1723.6	27	63.837			
Total	1726.8	29				

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Day1	10	719	71.9	36.1
Day2	10	724	72.4	33.1556
Day3	10	783	78.3	49.7889

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	253.4	2	126.7	3.19293	0.057	3.354131
Within Groups	1071.4	27	39.6815			
Total	1324.8	29				

VITA

Miss Chayada Chiaochan was born on July 25, 1976 in Prachuapkhirikhan, Thailand. She had graduated a Bachelor's degree of Science in Chemistry from Mahidol University in 1999. Afterwards, she has worked for Overseas Merchandise Inspection Co., Ltd. (OMIC) company since 1999 until now. In 2007, she continued her academic education for Master degree at Department of Chemistry, Faculty of Science, Chulalongkorn University. She had completed the program in April 2010 and received her Master's degree of Science in Analytical Chemistry in July 2010.