DEVELOPMENT OF HPLC-MS/MS METHOD FOR SIMULTANEOUS QUANTIFICATION OF VETERINARY ANTIBIOTICS AND HORMONES IN SOIL AND BIOSOLIDS

HO YU BIN

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By

HO YU BIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilments of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated to my parents

Ho Chee Weng and Yeo Bee Chuen

who brought me into this world, taught me the best knowledge, guided and supported me along the way to successful.

To my brother **Ho Yu En** and my fiancé **Thai Hoeng**

who encouraged and supported me during my study.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chair: Professor Mohamad Pauzi Zakaria, PhD

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Repeated applications of animal manure as fertilizer are normal agricultural practices that may release veterinary antibiotics and hormone into the environment from treated animals. The occurrence of veterinary antibiotics and hormone residues in the environment is of concern because of the development of antibiotic-resistant bacteria and its ecotoxicological behavior to plants and animals. To date, the occurrence of veterinary antibiotics and hormone in the Malaysian agricultural field has never been reported. The lack of data could be attributed to the absence of universal method for the analysis of veterinary pharmaceutical residues in complex solid environmental matrices. Therefore, a reliable, simple, fast and cost-effective method was developed in order to quantify nine antibiotics (doxycycline, enrofloxacin, erythromycin, flumequine, norfloxacin, sulfadiazine, tilmicosin, trimethoprim, tylosin) and one hormone (progesterone) in soil, broiler manure and manure compost. Generally, this method has shorter time of analysis, lower cost of solid phase extraction (SPE), better recoveries, lower method detection limit (MDL) and method quantification limit (MQL) values as compared to USEPA Method 1694, Kim and Carlson (2007) and Blackwell et al, (2004). The current method was compared to the USEPA Method 1694 where the method has the closest target analytes and sample matrices. The time of extraction has reduced from >125 mins to >63 mins, the time of liquid chromatography analysis has reduced from 61 mins to 25 min and the cost of SPE cartridge has reduced from RM70.00 to RM10.30 per sample when compared to the USEPA Method 1694. The developed method was based on ultrasonic extraction with MeOH:ACN:EDTA:McIlvaine buffer (pH 4) (30:20:25:25), SPE using HLB (3cc/60mg) cartridge, followed by instrumental analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with 25 mins total run time. The developed method was validated and tested on soil, broiler manure and manure compost samples and showed that the method is able to simultaneously detect and quantify the target analytes with good selectivity and sensitivity. The overall method performance was good for the majority of the analytes, with recoveries greater than 80% for most of the analytes in each type of sample matrix. MDL and MQL were achieved at as low as 0.5 and 2 μg/kg dry weight (DW) for trimethoprim. Tilmicosin was first time reported in environmental matrices. The developed method was then applied on broiler manure samples and its relative manure amended agricultural soil samples collected in Selangor, Negeri Sembilan and Melaka to identify and quantify veterinary antibiotic and hormone residues in the environment. The broiler manure samples were found to be contaminated with at least seven target analytes, which are doxycyline, enrofloxacin, flumequine, norfloxacin, trimethoprim, tylosin and progesterone. The maximum concentration of antibiotic detected in broiler manure was 78516.1 μg/kg DW for doxycycline. For manure amended agricultural soil samples, doxycycline, enrofloxacin, flumequine and trimethoprim residues were detected in every soil samples. The maximum concentration of antibiotic detected in soil was 1331.4 μg/kg DW for flumequine. The results showed that the method can potentially be adopted for the analysis of veterinary antibiotic and hormone wastes in solid environmental matrices.

Keywords: Veterinary antibiotics, hormone, HPLC-MS/MS, soil, manure

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN KAEDAH HPLC-MS/MS UNTUK KUANTIFIKASI SERENTAK ANTIBIOTIK DAN HORMON VETERINAR DALAM TANAH DAN BIO-PEPEJAL

Oleh

HO YU BIN

Julai 2012

Pengerusi: Profesor Mohamad Pauzi bin Zakaria, PhD

Fakulti: Fakulti Pengajian Alam Sekitar

Aplikasi berulangan najis haiwan sebagai baja di tanah pertanian adalah amalan biasa pertanian yang boleh membebaskan sisa farmaseutikal veterinar ke alam sekitar melalui haiwan yang dirawat. Kejadian sisa antibiotik dan hormon veterinar di alam sekitar amat membimbangkan kerana pembangunan bakteria tahan antibiotik dan tingkah laku eko-tosikologikal kepada tumbuh-tumbuhan dan haiwan. Sehingga kini, kejadian antibiotik veterinar dan hormon di tanah pertanian Malaysia tidak pernah dilaporkan. Kekurangan data mungkin disebabkan oleh tiada kaedah yang sejagat untuk analisis sisa antibiotik dan hormon dalam matriks pepejal alam sekitar yang kompleks. Oleh itu, satu kaedah yang boleh dipercayai, mudah, cepat dan kos efektif telah dibangunkan untuk kuantifikasi sembilan antibiotik (doxycycline, enrofloxacin, erythromycin, flumequine, norfloxacin, sulfadiazine, tilmicosin, trimethoprim, tylosin) dan satu hormon (progesteron) dalam najis haiwan, tanah, dan kompos najis. Secara umumnya, kaedah ini mempunyai masa analisis yang lebih singkat, kos pengekstrakan fasa pepejal (PFP) yang lebih rendah, kadar perolehan yang lebih baik, had pengesanan kaedah (HPK) dan had kuantifikasi kaedah (HKK) yang lebih rendah berbanding dengan Kaedah USEPA 1694, Kim dan Carlson (2007) dan Blackwell et al, (2004). Kaedah ini mempunyai analit sasaran dan matriks sampel yang paling dekat dengan Kaedah USEPA 1694. Masa pengekstrakan telah dikurangkan daripada> 125 minit kepada > 63 minit, masa analisis kromatografi cair telah dikurangkan daripada 61 minit kepada 25 minit dan kos PFP kartrij telah dikurangkan daripada RM70.00 kepada RM10.30 per sampel berbanding dengan Kaedah USEPA 1694. Kaedah yang dibangunkan adalah berdasarkan pengekstrakan ultrasonik dengan MeOH: ACN: EDTA: McIlvaine penampan (pH 4) (30:20:25:25), PFP menggunakan kartrij HLB (3cc/60mg) diikuti oleh analisis menggunakan kromatografi cair berprestasi tinggi-tandem jisim spektrometri (KCBT-JS/JS) dengan 25 minit jumlah masa analisis. Kaedah ini telah disahkan dan diuji ke atas tanah, najis dan sampel kompos najis dan ia menunjukkan bahawa kaedah ini mampu untuk mengesan dan kuantifikasi analit sasaran secara serentak dengan pemilihan dan kepekaan yang baik. Prestasi keseluruhan kaedah adalah baik untuk analisis majoriti analit, dengan kadar perolehan lebih daripada 80% untuk majority analit dalam setiap jenis sampel matrik. HPK dan HKK telah dicapai serendah 0.5 dan 2 µg/kg berat kering (BK) untuk

trimethoprim. Tilmicosin adalah kali pertama dilaporkan dalam matriks alam sekitar. Kaedah ini kemudiannya diaplikasikan pada sampel najis ayam daging dan tanah pertanian yang diambil di Selangor, Negeri Sembilan dan Melaka untuk mengenal pasti dan menentukan sisa antibiotik dan hormon veterinar dalam alam sekitar. Sampel najis ayam daging didapati tercemar dengan sekurang-kurangnya tujuh analit sasaran, iaitu doxycyline, enrofloxacin, flumequine, norfloxacin, trimethoprim, tylosin dan progesteron. Kepekatan maksimum antibiotik yang dikesan dalam najis ayam daging ialah doxycycline iaitu sebanyak 78516.1 µg/kg BK. Untuk sampel tanah pertanian, sisa doxycycline, enrofloxacin, flumequine dan trimethoprim telah dikesan dalam setiap sampel tanah. Kepekatan maksimum yang dikesan di dalam tanah adalah flumequine iaitu sebanyak 1331.4 µg/kg BK. Hasil kajian menunjukkan bahawa kaedah ini berpotensi untuk analisis sisa antibiotik dan hormon veterinar dalam matriks pepejal alam sekitar.

Kata kunci: antibiotik veterinar, hormon, KCBT-JS/JS, najis, tanah

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ACN	Acetonitrile
ANADA	Abbreviated new animal drug application
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric pressure ionization
ASE	Accelerated Solvent Extraction
C/N	Carbon to nitrogen ratio
Ca	Calcium
CAD	Collisionally activated dissociation
CAFOs	Concentrated animal feeding operations
CAS	Chemical Abstract Service
Cd	Cadmium
C.E.	Collision Energy
CEC	Cation exchange capacity
Cr	Chromium
Cu	Copper
CYP	Cytochrome P450 aromatase
DCM	Dichloromethane

DMCS	Dimethylchlorosilane
DW	Dry weight
E1	Estrone
EDCs	Endocrine disrupting compounds
EDTA	Ethylenediamine-tetraacetic acid
ESI	Electrospray Ionization
EU	European Union
FDA	US Food and Drug Administration
Fe	Iron
GC	Gas chromatography
GnRH	Gonadotrophin releasing hormones
GPS	Global Positioning System
H_2O	water
H_3PO_4	Ortho-phosphoric acid
HCG	Human chorionic gonadothrophin
HCl	Hydrochloric acid
HFBA	Heptafluorobutyric acid
HGPs	Hormonal growth promotants
HLB	Hydrophilic-lipophilic-balanced
HNO_3	Nitric acid

HPLC	High performance liquid chromatography
HPLC-FLD	High performance liquid chromatography-postcolumn fluorescence derivatization
HPLC-MS	High performance liquid chromatography-mass spectrometry
HPLC-MS/MS	High performance liquid chromatography -tandem mass spectrometry
HPLC-UV	performance liquid chromatography-ultraviolet detector
ICH	International Conference on Harmonisation
IDL	Instrumental Detection Limit
IQL	Instrumental Quantification Limit
IS	Internal Standard
IUPAC	International Union for Pure and Applied Chemistry
K_d	Soil partition coefficient
K_{oc}	Organic carbon partition coefficient
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
Log P	Octanol-water partition coefficient
µg/g	Microgram per gram
µg/kg	Microgram per kg
µg/L	Microgram per liter

M	Molar
m/z	Mass to charge ratio
MAE	Microwave assisted extraction
MCX	Mixed-mode cation exchange
MDL	Method Detection Limit
MeOH	Methanol
MeOH	Methanol
mg/kg	milligram per kg
mg/L	milligram per liter
Min	Minute
mM	millimolar
MQL	Method quantification limit
MRLs	Maximum residual limits
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
M_w	Molecular weight
N_2	Nitrogen
Na	Sodium
Na_2HPO_4	Disodium hydrogen phosphate
NADA	New animal drug application

ng/g	nanogram per gram
ng/L	nanogram per liter
ng/mL	nanogram per milliliter
Ni	Nickel
NPCB	National Pharmaceutical Control Bureau
PAHs	Poly aromatic hydrocarbons
Pb	Lead
PCBs	Polychlorinated biphenyls
pK_a	Acid dissociation constant
PLE	Pressurized liquid extraction
PPCPs	Pharmaceutical and personal care products
Q1	First quadrupole
Q3	Third quadrupole
QC	Quality control
QqQ	Triple quadrupole analyzer
R^2	Coefficient of determination
rpm	Revolutions per minute
RRF	Relative Response Factor
RRT	Relative retention time
RSD	Relative standard deviation

RT	Retention time
S/N	Signal to noise ratio
SAX	Strong anion exchange
SCX	Strong cation exchange
SD	Standard Deviation
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
STPs	Sewage Treatment Plants
TK	Total potassium
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorous
TrBA	Tributylamine
USDA	United States Department of Agricutural
USEPA	United States Environmental Protection Agency
USP	United States Pharmacopeia
UV	Ultraviolet
VMP	Veterinary medicinal product
Zn	Zinc

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The agricultural sector occupies a major portion of Malaysia's economy; the production of broiler chickens raised for human consumption comprises of 48% compared with other livestock (MOA, 2010). To maintain economic viability, large agribusiness companies began contracting with farmers. This arrangement offered a guaranteed price to the farmer and a controlled and stable animal food-producing environment for the agribusiness (Sarmah et al., 2006).

Malaysia's poultry industry has advanced to the integrated production system of broilers, which produce enormous amounts of chicken manure. Broiler chickens, feed, manure, urine, dead animals, and production operations are all gathered on a small land area. According to the Department of Veterinary Services Malaysia, the population of broiler has increased from 121.1 million birds in year 2005 to 158.7 million birds in year 2010. The production of broilers in Malaysia has generated RM 4,369 million income in year 2005 and increase to RM 5,776 million in year 2010 (Figure 1.1). Problems may come to surface with this continuously growing number of broiler chickens. This is a global phenomenon, especially for countries that rely on agricultural income as their source of economy. Integrated production systems of broilers are classified as concentrated animal feeding operations (CAFOs). CAFOs is a regulatory concept for large animal feeding operations, which take advantage of the cost effectiveness of large facilities (Shore and Pruden, 2009). The United States Environmental Protection Agency (USEPA) defines CAFOs as agricultural operations where animals are kept and raised in confined situations (USEPA, 2007b).

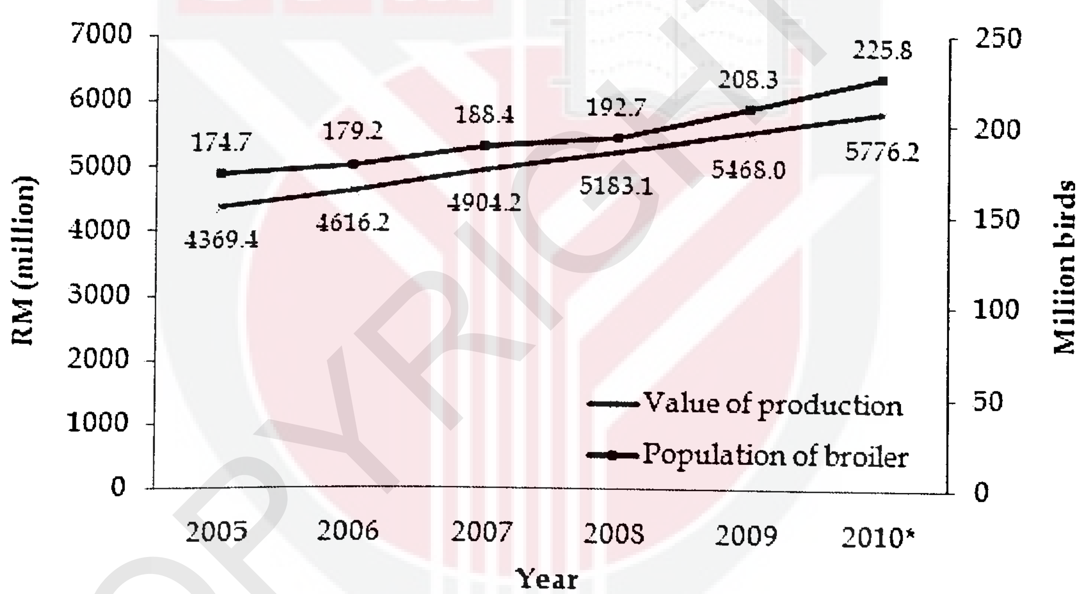

Figure 1.1 Population of broiler and value of production of broiler in Malaysia from year 2005 to 2010 (DVS, 2010). The number given in year *2010 represents the estimated value from Department of Veterinary Services Malaysia.

To date, only conventional "priority" pollutants in effluent discharged from sewages are regulated, such as inorganic compounds, nitrates and heavy metals. Recently, attention has turned to a new group of pollutants released from the CAFOs that cause negative effects to the environment even in very low concentrations (μg/L or ng/L). These micropollutants are known as the "emerging pollutants" and are not included in the legislation. Three groups of compounds discharged from CAFOs are identified as "emerging pollutants", namely, the steroid hormones, antibiotics, pesticides and insecticides (Laurence Shore and Pruden, 2009).

Antibiotics are often used in animal husbandry in large quantities. Antibiotics exhibit antimicrobial activity in animals in four ways: therapy, metaphylaxis, prophylaxis, and growth promotion (Schwarz et al., 2001). Since antibiotics can improve growth and feed efficiency, the number of days broilers need to reach market weight is reduced. Most antibiotics used in the animal food-producing industry are poorly absorbed in the gut of the animal, resulting in as much as 30–90% of the parent compound release into the environment via excetion from animal body (Sarmah et al., 2006). Some see this as a public health concern, believing such drugs could then enter the ground or surface water, or be taken up by plants and thus contribute to antibiotic resistance development and/or produce adverse reactions in those with antibiotic allergies (Venglovsky et al., 2009).

The pathway of veterinary pharmaceuticals entering the environment is different from human pharmaceuticals. Human pharmaceuticals are discharged into the environment mainly through sewage treatment plants (STPs); however, veterinary pharmaceuticals could enter the environment not only through direct application in aquaculture, wash off from topical treatments and livestock treatment plants, but also through manure application (Hamscher and Hartung, 2008). Once a veterinary pharmaceutical enters the environment, its behaviour is affected by its physical properties including water solubility, lipophilicity, volatility and sorption potential. Therefore, investigation of antibiotic pollution in chicken manure has special importance because they constitute a major source of hazardous unmetabolized antibiotics to the environment via fertilization of agricultural soil. Occurrence of veterinary antibiotics and hormone residuals in the environment is of concern due to the emergence and development of antibiotic resistance in pathogenic bacteria, and the ecotoxicological behaviour of these compounds. Besides, steroid hormones are potential endocrine disrupting compounds (EDCs); when released into the environment they cause the feminization of aquatic organisms (Saaristo et al., 2009).

Veterinary antibiotics and hormone usually occur as a wide range of pollutants from different chemical classes, with different physical and chemical properties. Analysis of this wide range of compounds in complex sample matrices such as soil, animal manure and compost is more challenging than in water samples. The extraction methods of these pharmaceuticals from soil, manure and compost are mainly based on ultrasonication and solid phase extraction (SPE) (Blackwell et al., 2004; Karcı and Balcıoğlu, 2009). Others are based on liquid-liquid extraction (LLE)

(Martínez-Carballo et al., 2007) and pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE) (Jacobsen et al., 2004). High performance liquid chromatography in tandem with mass spectrometry (HPLC-MS/MS) is often employed for the environmental analysis of veterinary antibiotics due to their low limit of detection (LOD) (Martínez-Carballo et al., 2007; Aust et al., 2008). However, high performance liquid chromatography in combination with ultraviolet detector (HPLC-UV) or high performance liquid chromatography with postcolumn fluorescence derivatization (HPLC-FLD) is still used due to their relatively low cost (Blackwell et al., 2004; Malintan and Mohd, 2006; Karcı and Balcıoğlu, 2009).

To date, there is neither a list of veterinary antibiotics and hormone priority pollutants nor a comprehensive method for antibiotics and hormones analysis for soil, animal manure and compost samples. Since broiler manure is a major source of veterinary of antibiotics in agricultural fields, thus the veterinary antibiotics and hormone composition, and their fate in manure-amended soil needs to be given special attention. This study is extensively designed to accommodate different classes of antibiotics and hormone for the very first time and the compilation of these compounds is unique for our country.

1.2 Problem Statement

Currently, there is no legislation for the limits of pharmaceuticals in animal manure and soil in any country. Livestock producers have "blindly" used veterinary antibiotics as supplements in animal feed to increase weight gain and prevent disease among their livestock. These chemicals are introduced increasingly without realising the consequences for the environment, as well as direct and indirect effects for human health. Early research and studies have focussed extensively on the aquatic environment and especially on contaminants being released from pharmaceutical and personal care products (PPCPs) (Boyd et al., 2003; Vanderford et al., 2003; Ellis, 2006; Vanderford and Snyder, 2006; Kim et al., 2007; Al-Odaini et al., 2010). Up till now, only a few studies have investigated the occurrence of veterinary antibiotics in animal manure and manure amended agricultural soil (De Liguoro et al., 2003; Blackwell et al., 2004; Martínez-Carballo et al., 2007; Aust et al., 2008; Karcı and Balcıoğlu, 2009). However, they feature limited classes of pharmaceuticals.

To date, scientists have reported the occurrence of PPCPs in Malaysian waters (Al-Odaini et al., 2010) as well as the veterinary antibiotics in swine wastewater (Malintan and Mohd, 2006). The occurrence and fate of veterinary antibiotics and hormone in Malaysian agricultural soil have not been documented. Previous analytical methods for antibiotics and hormones analysis in soil and biosolid samples required complex extraction and analysis methods, hence resulted in higher cost. Thus, a multi-residue analytical method was developed in order to fill the gap of knowledge of the occurrences of these compounds in Malaysian agricultural field. The developed method was subsequently applied in chicken manure and manure amended agricultural soil to investigate the level of veterinary antibiotics and hormone pollution in Malaysian terrestrial environment. The same method was applied in manure compost to study the evolution of veterinary antibiotics and hormone in a laboratory composting experiment.

Therefore, the present study is able to answer the following questions:

i. Is there any available method for the simultaneous determination and quantification of nine veterinary antibiotics and one hormone in broiler chickens manure and manure amended agricultural soil samples?
ii. What is the level of antibiotics and hormone pollution in manure and agricultural soil in Malaysia?
iii. Can veterinary antibiotics and hormone lead to the contamination of agricultural soil via fertilization of animal manure?

1.3 Significance of Study

Pollutants such as veterinary antibiotics and hormones that are not covered by environmental legislation have increasingly become important emerging environmental contaminants. By complying with the Food Act 1985, the maximum residual limits (MRLs) in food producing animals does not mean that we are safe from pharmaceutical pollution. There is a growing concern that the antibiotics and steroid hormones discharge from livestock production are contaminating soil, surface and groundwater; contributing to the development of antibiotic resistance in the environment and possesses the potential as EDCs.

To date, there is no data about antibiotics and hormone pollution in the Malaysian agricultural soil. This is the pioneering study on the development of sensitive and selective multi-residue HPLC-MS/MS method for the determination of nine antibiotics and one hormone in chicken manure, land applied manure and manure compost. The occurrence data of antibiotics and hormone in Malaysian agricultural soil in this study is of great importance in awakening the public understanding to the source, transport and effects of these compounds.

Specifically, the significances of the study are listed below:

i. A new method is developed for the simultaneous determination and quantification of nine antibiotics and one hormone in broiler manure, manure amended agricultural soil and manure compost.

ii. The occurrence data in this study can be used to investigate the level of antibiotics and hormone pollution in manure and agricultural soil samples in Malaysia for the very first time.

iii. The findings obtained in this study can be used to demonstrate that veterinary antibiotics can lead to the contamination of agricultural soils via fertilization with animal manure.

iv. This study will produce the baseline data for the occurrence of nine antibiotics (doxycycline, enrofloxacin, erythromycin, flumequine, norfloxacin, sulfadiazine, tilmicosin, trimethoprim, tylosin) and one hormone (progesterone) in manure and agricultural soil samples in Malaysia. Data from this study is anticipated to be an important reference for other studies conducted in the future regarding this issue, especially fate and effects of these compounds to environment. These data are especially significant when considering the lack of information on the presence of antibiotic residues in agricultural fields in Malaysia.

1.4 Objectives of the Study

The overall aim of this study is to develop a multi-residue analytical method for quantifying veterinary antibiotics and hormones in soil and biosolid matrices, based on ultrasonication with buffer, solid phase extraction (SPE) followed by HPLC-MS/MS for the simultaneous analysis of veterinary antibiotics and hormone. The method is validated using relative reference matrices, namely, pre-cleaned soil, pre-cleaned chicken manure and pre-cleaned composts. The validated method is subsequently applied on environmental samples to investigate the occurrences of these pollutants in broiler farms and agricultural fields in Malaysia.

This study embarks on the following specific objectives:

i. To develop and validate the HPLC-MS/MS method for simultaneous determination of nine antibiotics (doxycycline, enrofloxacin, erythhromycin, flumequine, norfloxacin, sulfadiazine, tilmicosin, trimethoprim and tylosin) and one hormone (progesterone) in broiler chicken manure, soil and manure compost at µg/kg level.

ii. To apply the developed method to study the occurences of veterinary antibiotics and hormone residues in broiler chicken manure and manure amended soil samples collected from selected locations in Selangor, Negeri Sembilan and Melaka.

1.5 Thesis Synopsis

This thesis focuses on the development of HPLC-MS/MS method for the detection and quantification of veterinary antibiotics and hormone in broiler manure, soil and manure compost. The following section describes the organization of this thesis and its relative chapters.

Chapter 2 gives a full review of veterinary antibiotics and hormone in CAFOs. The sources, transport pathway and fate of veterinary pharmaceuticals in environment are reviewed in detail. In addition, the effects of veterinary pharmaceuticals enter to the environment are summarized. Previous available analytical methods to determine veterinary pharmaceuticals in soil and biosolid are reviewed. Finally, proper waste management to control the pharmaceuticals from CAFOs is also summarized in this chapter.

Chapter 3 describes in detail the development and optimization of the HPLC-MS/MS analytical method for simultaneous detection and quantification of nine antibiotics and one hormone in soil, manure and manure compost. The optimizations of sample preparation and extraction method are discussed comprehensively.

Chapter 4 discusses the validation of the developed method in environmental samples. The instrumental performance and overall analytical method performance are also discussed.

Chapter 5 discusses the application of the developed method on broiler manure samples and its relative manure amended soil samples. The relationship between the physico-chemical properties of manure and soil samples to the concentration of pharmaceuticals in manure and soil were presented in Pearson correlation matrix. Results of analysis of broiler manure and its relative manure amended soil samples are also discussed.

Chapter 6 summaries the research, discusses the overall findings and the contribution of this research. Recommendations for improving the results of the study and some future directions for the study are also discussed.

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