CHAPTER I

INTRODUCTION

1.1 Problem Definition

In modern animal agriculture, antibiotics are widely used for therapeutic and non-therapeutic purposes with worldwide large-scale consumption. The residual problem of antibiotics as veterinary drugs for food-producing animals is of particularly concern and it is increasing consumer awareness of food safety. Due to the application of antibiotics as feed additives for treatment, prophylactic, and even growth promoter, these drugs can leave residues in edible tissue or transfer to aquatic environment, which can lead to health problems. Even at low concentration, continuous consumption of drug-residue containing meat or water can cause allergic reactions, which are related to the human immune system. Besides all, antibiotic residues may induce resistance of bacteria from promoting bacterial biological mutation and DNA exchange. These new resistant strains of bacteria can transfer from animal to human and, therefore, pose a threat to human health via three ways (i.e., food, working with animal, and environment).

For non-therapeutic purpose, antibiotics are extensively used as feed additives in cattle, swine, sheep, and poultry in low dosage levels to promote growth and prevent infection. As growth-promoter, antibiotics in feed help animals gain weight more efficiently by controlling bacteria that can interfere with animal ability to absorb nutrients. Animals become healthier, grow faster and stronger, and fewer die from disease. In contrast, healthy animals raised on factory farms are regularly fed low dosage levels of antibiotics for extended periods of time, in order to promote faster growth and compensate for overcrowded and unsanitary conditions that may bring on sickness, especially in industrial-scale factory farms. The overuse amount of antibiotics can leave a residue in animal and contaminate in aquatic environment.

In the U.S., it has been reported that meat producer used nearly 25 million pounds or estimate 70% of all antibiotics non-therapeutically in food-producing animal, which are mainly swine, cattle, and poultry. *(1)* To regulate drugs residues, The European Union

(EU) has taken actions in legislation of antibiotic use in feeds and banned antibiotics as growth promoters. Legislation regarding the control of antibiotic residues in live animals and animal products is given in Council Directive 96/23/EC including the prohibition of the use of growth promoting agents. *(2)* Moreover, EU has set the maximum residue limits (MRLs) given in Council Regulation 2377/90 for the use of veterinary drugs in food animal species *(3)* and the method and performance criteria are described in Commission Decision 2002/657/EC. *(4)*

As antibiotic residues in animal foodstuff can also accumulate in every part of the food-chain and endanger human, antibiotics are presently considered as serious emerging contaminants. Antibiotics imply a wide range of substances including natural, semi-synthetic, and synthetic compounds. Classes of antibiotics can be divided by chemical structure or mechanism of action such as macrolides, sulfonamides, tetracyclines, quinolones, β-lactams, aminoglycosides, and others. Macrolide is a one of the most important antibacterial class that has a critical residue problem because of its efficiency against diseases produced by gram-positive bacteria and *Mycoplasma* species in multiple animal species. Poultry is one target of food-producing group that is well known to experience macrolide antibiotic residue in many parts. In 2003, Interscience Conference on Antimicrobial Agents and Chemotherapy scientists reported about the risk of humans acquiring resistant bacteria by eating meat or poultry from animals treated with macrolides that leads to failure in using antibiotic treatment for bee sting. *(5)* Hence, the EU regulates residual macrolides in bovine, porcine, and poultry by setting MRLs as shown in Table 1.1.

Table 1.1 Maximum Residue Limits (MRLs) of macrolide antibiotics in food-producing animal *(3)*.

Macrolide	**MRLs (μg/kg)**
erythromycin	40 – 200
spiramycin	200 – 400
tilmicosin	50 – 1000
tylosin	50 – 200
josamycin	200 – 400
tulathromycin	100 – 3000
tylvalosin	50

However, low amounts of antibiotic residue combined with the complexity of sample matrix lead to difficulties in analysis resulting in a strong need to provide suitable techniques for their determination. Sample preparation step is a powerful tool in solving these analysis problems. Extraction, enrichment, and clean-up are necessary sample preparation processes to improve antibiotic detection in order to follow EU legislation criteria.

The conventional sample preparation techniques, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) still have some drawbacks. LLE is considered as time-consuming, multi-stage operation, and requires large volume of toxic organic solvent. Even though SPE eliminates LLE disadvantage in case of shorter sample preparation time, less organic solvent usages, and easier operation, SPE requires extra step for evaporation, additional device cost, and provides low preconcentration of analytes. It is difficult to determine macrolides with conventional methods because of their similar structures and low-level residues. Therefore, a simple, low-cost, high enrichment, sensitive, and selective method should be developed for macrolide antibiotics residue determination in food-producing animal and water samples.

1.2 Macrolide Antibiotics

Since the discovery in the 1950s, macrolide antibiotics are used for a variety of applications in both human and animal foodstuffs (poultry, cattle, sheep, swine, fish, and companion animals). Macrolides are delivered to the different animals by various routes of administration such as feed, water, injection, tablet, and others. This antibiotic class is used to treat infections of the respiratory tract and genital and gastrointestinal tissue infections because these compounds are biologically active against living microorganisms. Macrolide common mechanism of action is the inhibition of bacterial protein synthesis with the activity against gram-positive bacteria and *Mycoplasma* species. Consequently, macrolides are important in maintaining a healthy livestock and poultry.

1.2.1 Structure and chemistry

Macrolide are characterized by a macrocyclic lactone ring containing 14, 15, or 16 atoms with sugars linked via glycosidic bonds. Macrolide antibiotics are further classified into three groups based on the number of atoms in the lactone ring as described in Table 1.2. Macrolide compounds are produced semi-synthetically or naturally by microorganism. Macrolide are mainly produced by various *Streptomyces* organisms except rosaramicin and mirosamicin, which are isolated from *Micromonospora* species.

Table 1.2 Macrolide antibiotic compounds classification *(6)*.

No. of atom in lactone ring		
14-membered macrolides	**15-membered macrolides**	**16-membered macrolides**
Erythromycin	Azithromycin	Leucomycin
Oleandomycin	Tulathromycin	Josamycin
Clarithromycin		Kitasamycin
Dirithromycin		Rokitamycin
Roxithromycin		Rosaramicin
Flurithromycin		Mirosamicin
		Spiramycin
		Tilmicosin
		Tylosin
		Tylvalosin

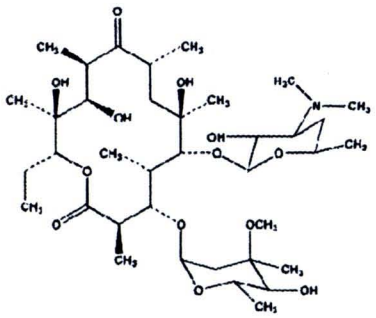
In the group of 14-membered macrolides, the most important compound is erythromycin, a fermentation product produced from *Saccharopolyspora erythraea*. It has been extensively used in many different chemical forms (e.g., free base, salts, and ester) and formulations. It has also been frequently utilized as the chemical starting material for many 14-membered semi-synthetic derivatives, such as clarithromycin, roxithromycin, dirithromycin, and flurithromycin. Another major semi-synthetic derivative is azithromycin, a 15-membered Macrolide, which consists of a heterocyclic nitrogen, is produced from ring expansion process. Although these semi-synthetic derivatives share many common attributes with erythromycin, their individual structural features may also perform some significant difference in their various antimicrobial

activities and biological features. The second largest family is 16-membered macrolide, which is usually divided into two principal sub-families based on differences in the substitution pattern of their structures. Tylosin is the prototype of one sub-family that includes its semi-synthetic compound, tilmicosin. Leucomycin is the other sub-family, which has a unique feature of a second amino sugar in its skeleton. 16-membered macrolides also exhibit their common characteristics with their individual bioactivities. Macrolide compound structures are shown is Figure 1.1. From their structures, macrolides are lipophilic molecules, they are soluble in methanol and are unstable in acid solution. Macrolides are weak bases with pKa values ranging from 7.4 to 9.2.

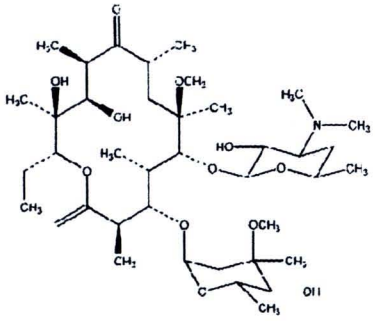
1.2.2 Mechanism of action

All macrolide antibiotics display antibacterial properties and are active against gram-positive and some gram-negative bacteria, and are particularly useful in the treatment of *Mycoplasmas*, *Haemophilus influenzae*, *Chlamydia* species, and *Rickettsia*. Macrolide antibiotics exhibited their antibacterial activity ribosomes. The macrolide mechanism of action inhibits the bacterial protein synthesis via reversibly binding to the 50s ribosomal subunit of bacterial ribosome. A general diagram of macrolide inhibition of bacterial protein synthesis within the ribosome is illustrated in Figure 1.2. There are four modes of macrolide inhibition of protein synthesis: 1) Inhibition of the progression of the initial peptide chain during early steps of translation; 2) Promotion of peptidyl tRNA dissociation from the ribosome; 3) Inhibition of peptide bond formation; and 4) Interference with 50S subunit assembly. All of these mechanisms have some relationship with the location of the macrolide binding site on the ribosome. With macrolide binding, tRNA cannot bind with mRNA and then amino acid of tRNA cannot form peptide bond with another tRNA that inhibits protein production at ribosome of bacteria.

14-membered macrolides

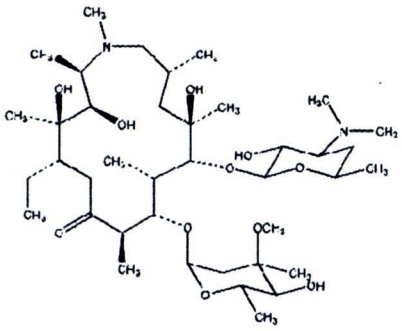


Erythromycin

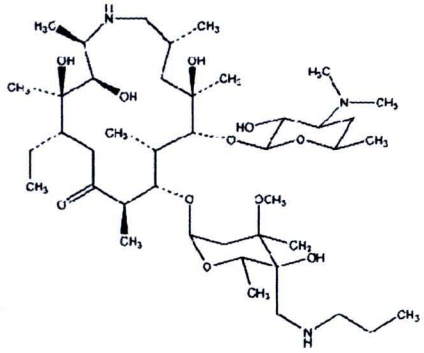


Clarithromycin

15-membered macrolides

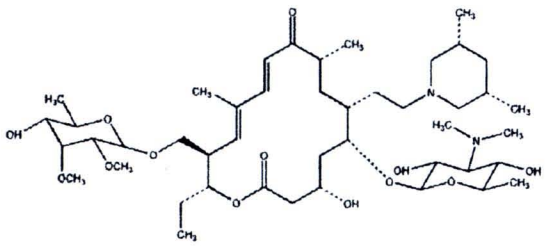


Azithromycin

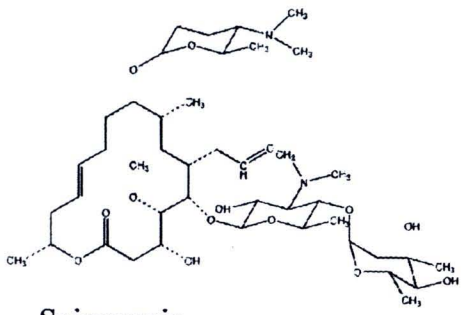


Tulathromycin

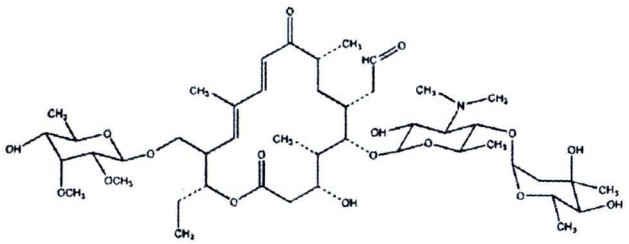
16-membered macrolides



Tilmicosin



Spiramycin



Tylosin

Figure 1.1 Some macrolide antibiotic chemical structures

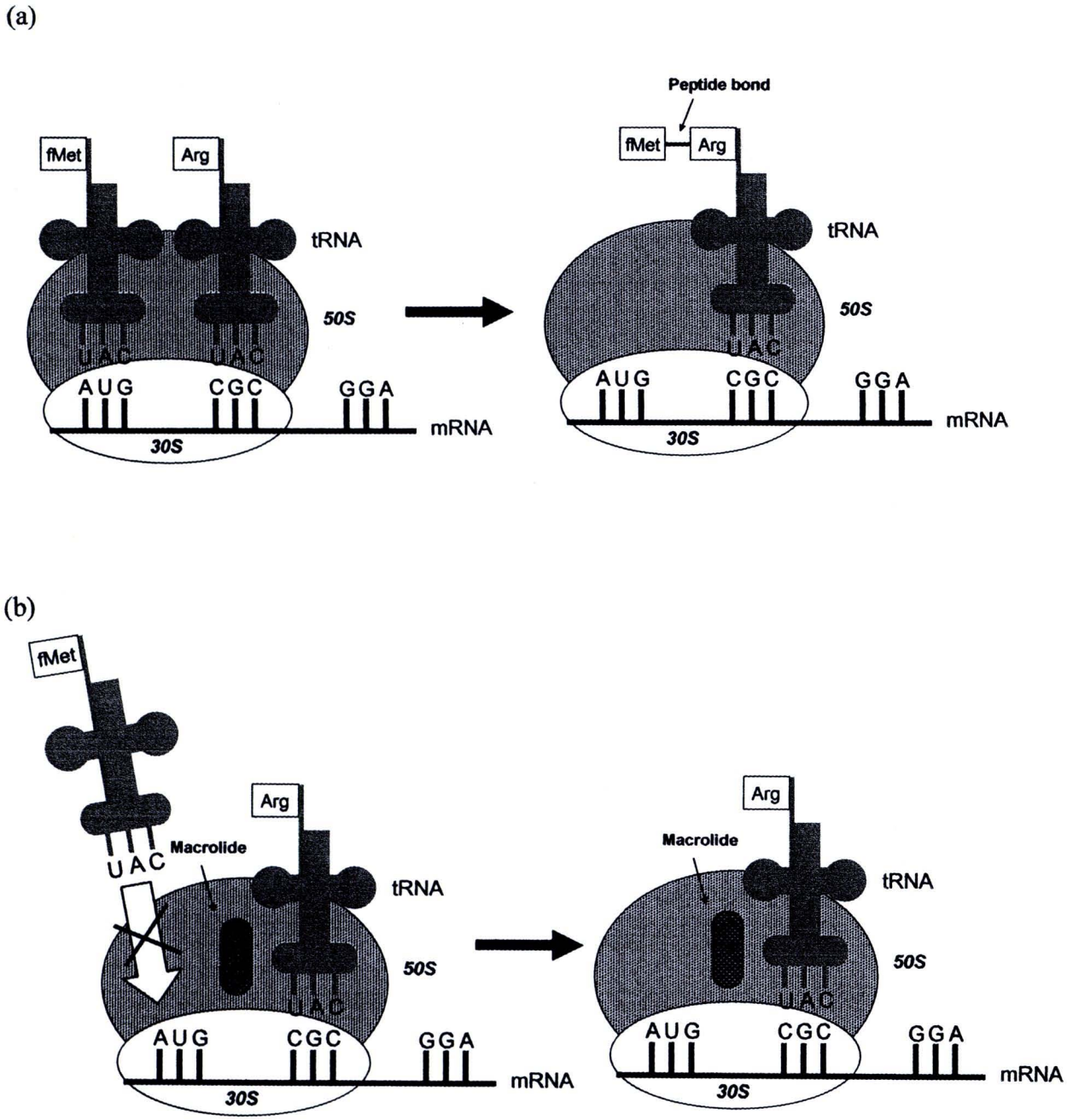


Figure 1.2 Diagram of macrolide inhibition of protein synthesis within the bacterial ribosome (adapted from *(7)*) a) without macrolide b) with macrolide.

1.2.3 Mechanism of resistance

Resistance of macrolides can occur by target site modification, drug inactivation, or drug efflux out of the bacteria cell. Organisms that develop resistance to one macrolide antibiotic may also be resistant to other macrolide antibiotics. Therefore, certain peptides can bind with 50s subunit and continue their protein synthesis processes, which leads to a reduction of antibiotic activities. These macrolide resistance genetics are capable of being transmitted from gram-positive to gram-negative bacteria and vice versa. *(8)* Many of the macrolide-resistance genes have become physically linked to other drug resistance symptoms and result in other drug resistance abilities to other antibiotic classes. Furthermore, the danger of drug resistance also influences human health because of the transportation of these antibiotic-resistance genes from bacteria to human through food, environment, and working with animals that contain resistant bacteria. When human were treated with the antibiotics, drug resistance gene that accumulated in body are affected to the effectiveness of drug in treatment diseases.

1.2.4 Growth promoters

Non-therapeutic applications of antibiotics are growth promotion and disease prevention, whereas most of the concern about human health consequences of antimicrobial use has focused on growth promotion rather than disease prevention purpose because of the economic profits. Macrolide is one antibiotic class commonly added in low doses to the feed of farm animals to improve their growth performance for significant economic benefits such as weight gain and improved feed efficiency. This increasing growth rate depends on the hygiene level on the farm, the age of the animal, and the influence of feed additives. Healthy food-producing animals raised on factory farms such as swine, cattle, and poultry are regularly fed low dosage levels of macrolide antibiotics for extended periods of time, in order to promote faster growth and compensate for overcrowded and unsanitary conditions that may bring on sickness, especially in industrial-scale factory farms. Unfortunately, the use of low dosages of antibiotics over an extended period is one of the best ways to promote the development of antibiotic-resistant bacteria and induce human health at risk. As a result, EU has prohibited the use of antibiotics as growth promoting agents but there still is misuse of antibiotic applications for those purposes.

1.3 Literature review

In 1990, EU has set legislation and the Maximum Residue Limits (MRLs) of the use of veterinary drugs in food animal species. *(3)* There are several works that attempt to determine the residue of antibiotics with highest effective analysis methods.

Traditionally, screening methods for antibiotic, including macrolides, are based on microbiological and immunological assays (ELISA) but they often lack selectivity and precision for regulatory purposes. Therefore, in ELISA it is difficult to confirming what kinds of residual antibiotics are found in the animal tissue. To overcome these problems, chromatographic methods have been utilized for many macrolide determinations. Liquid chromatography is common coupled with spectrophotometric detections, such as UV and diode array detector (DAD), to determine macrolide antibiotics in food-producing animal.

High-Performance Liquid Chromatography (HPLC) with UV detection was used to determine five macrolides in swine, cattle, and chicken meat by Horie et al. *(9)* The samples were submitted to LLE using a mixture of 0.3% metaphosphoric acid and methanol followed by a SPE clean-up on Bond Elut SCX cartridges. The separation of the macrolides was performed on a C18 column using a gradient elution with a mixture of phosphate buffer and acetonitrile. The macrolide determination was monitored at two different wavelengths, 232 and 287 nm. The recoveries ranged from 70.8 and 90.4% and the detection limits (LODs) were estimated to 50 μg/kg for each macrolides.

Few years later, Leal et al. were using the same extraction method, employed both LC-UV and LC-DAD detection for the determination of macrolide antibiotics in spiked chicken muscle. *(10)* In LC-UV, the authors achieved the separation of seven macrolides on a C18 reversed phase column using a binary gradient elution of phosphate buffer (mobile phase A), and a phosphate buffer and acetonitrile mixture (mobile phase B). The method was also based on UV detection at different wavelengths and could determine five compounds from the seven tested macrolides in chicken poultry with spiked below their MRLs. The authors tested two different UV detection systems based on absorption, wavelength-programming, and multi-wavelength detection, it was found that the latter system is more suitable. For macrolides determination with LC-DAD, the proposed method was not sensitive enough for

determining some macrolides at the MRL values because of the lack of suitable chromophore groups in macrolide chemical structures. At three spiking levels, recoveries between 60 and 80% were gained. It was found that DAD has an additional advantage over UV system in case of the confirmation of identical analytes by spectra of the eluting peaks.

For confirmatory purpose, mass spectrometry (MS) is the preferred detection system for analyte identification rather than DAD. Due to its high specificity and sensitivity, LC-MS has been widely applied in antibiotic determination, especially in animal tissues. The LC-MS detector can reach low detection limit to determine of all macrolides in their MRLs. Codony et al. determined seven macrolides in poultry muscle with LC-MS using electrospray ionization (ESI). *(11)* The samples were treated like in the previous study, extraction with meta-phosphoric acid followed by clean-up with SPE cartridge. The separation was performed on a C18 column applying a gradient elution with a mobile phase consisting of a mixture of 0.02% aqueous trifluoroacetic acid and acetonitrile. LC-ESI-MS was operated in positive mode and each compound was monitored with selected ion monitoring (SIM) mode for quantification purposes. Recovery ranged from 56 and 93% with RSD lower than 12%. The proposed method was successfully applied for determination macrolides below the MRLs. However, there still are drawbacks of this method due to the concern about the number of analysis ions required for confirmatory purposes according to EU legislation.

Tandem mass spectrometry (MS/MS) overcomes this problem by providing abundant ions for quantitative and qualitative information. LC-MS/MS allows separation and detection compounds that have the same molecular mass but different product ions. For this reason, macrolide antibiotic class, which consists of many compounds with similar structures, can utilize LC-MS/MS for determination. Wang et al. have developed a method for determination of five macrolide antibiotics in honey with the comparison between LC–ESI–MS and LC–ESI–MS/MS systems. *(12)* The samples were extracted with phosphate buffer adjusted to pH 8.0, and then submitted to SPE on Oasis HLB cartridges and filtered before injection into the system. The separation of the macrolides was carried out on a C18 column using a gradient elution with a mixture of acetonitrile, 1% formic acid and water as mobile phase. For LC–ESI–MS, the obtained recoveries were between 97.8 and 109.3% with R.S.D. below 12% and detection limits below 1

μg/kg. For LC–ESI–MS/MS, recoveries ranged from 98.3 to 114.6% with R.S.D. below 13% and the detection limits were between 0.01 and 0.07 μg/kg. This work proved that the sensitivity of MS/MS is higher than single MS system and allows detection of macrolide antibiotics in ng/kg level. LC coupled with MS is proved that it is a necessary tool in many applications for determination the low-level residues.

In water sample analysis, macrolides antibiotics are considered as serious emerging contaminant in every parts of aquatic resource and have several researches in the determination of macrolide antibiotics in diverse water samples with LC-MS and LC-MS/MS detection. Mcardell et al. define seven macrolide antibiotics in wastewater and river samples via filtered sample and clean-up with solid-phase extraction (SPE) followed by both LC-MS and LC-MS/MS detection. *(13)* They claimed that macrolides are mainly contaminated in surface water. This method showed the low detection limit ranged from 0.06 to 0.33 μg/L with the acceptable ranges of relative standard deviation. With single LC-MS detection system, Abuin et al. can determine five macrolide antibiotics in natural water sample with detection limit in very low μg/L and satisfactory recovery ranged from 85 to 115 %. *(14)* The water sample was filtered and clean-up with the same process like previous work. This way to prepared sample is traditional mode in the application with water sample. Therefore, the difficulty between water sample and food-producing animal analysis is the sample preparation process. The animal matrices are complicated sample because of their components and required more preparation step than water sample which required only filtration or some clean-up steps.

Sample preparation is a very important and essential step to improved method analytical performance. Many researchers tried to extract and clean-up macrolide antibiotics from complex sample matrices such as animal sample. As previously described, several works initiated the same extraction and deproteinisation procedure with a mixture of meta-phosphoric acid and methanol followed by a partial evaporation of the extract and a final clean-up step on Oasis SPE HLB cartridges. Horie et al. developed a multiresidue method for eight macrolides in meat and fish with single run LC-ESI-MS. *(15)* The authors modified the sample preparation step with optimization the percentage of metaphosphoric acid in order to reduce the degradation of the macrolides in acidic media while keeping the efficiency of the extraction process. 0.2%

metaphosphoric acid in methanol was found to be the appropriate proportion. The detection limit stated in the method was 10 μg/kg for all the target macrolides. With the same SPE process, Berrada et al. applied different extraction procedure from previous work for seven macrolides determination in animal tissues using LC-DAD and LC-ESI-MS. *(16)* EDTA-McIlvaine's buffer was utilized to extract macrolide before SPE steps. Recovery data were satisfactory with values higher than 67% and R.S.Ds were lower than 13% and 15% for intra-day and inter-day assays. The author claimed that this confirmatory method could efficiently determine macrolides in animal sample according to EU regulation 2002/657/EC.

Another type of liquid extraction for determining seven macrolide antibiotics in meat and fish is pressurized liquid extraction (PLE); combines with LC-ESI-MS it was reported by Berrada et al. *(17)* PLE is an accelerated liquid extraction (ASE) procedure, whereby increased temperature for accelerating the extraction kinetics, and extended pressure to keep the solvent below its boiling point. ASE is reported to use the same aqueous and organic solvents as traditional extraction methods. The extracts were completely transferred for further solid-phase extraction, typically using Oasis HLB cartridge. The advantage of using PLE is the online capability, high specificity, and selectivity in extraction.

For the extraction of three macrolides in milk and bovine tissues, Msagati et al. investigated supported liquid membrane (SLM) as sample pre-treatment and clean-up technique. *(18)* In SLM, an organic liquid is immersed in small pores of a polymer support and held by capillary forces. If the organic solvent is immiscible with water, this polymer membrane separated two aqueous phases, feeding and stripping streams. Macrolides were extracted from sample with ACN-isopropyl alcohol (95:5) and then preconcentrated and clean-up with online-SLM. After extraction, macrolides were dried and dissolved in feeding solution, and continually extracted with organic solvent into pores of membrane and passed through to stripping solution with pH adjustment. With LC-ESI-MS system, the macrolides were detected following extraction at concentration levels between 0.01 and 0.08 μg/kg. In SLM, the membrane is reusable and the organic solvent employed is at minimal amount. Even though, SLM is an environmental friendly technique, there may be carry-over effects, online-SLM requires a flow system and it can extract only one sample at a time.

1.4 Purpose of the study

Since macrolide antibiotics were regulated by the EU due to health risk assessment, many researchers were paid attention to find a method that obtains limits of detection below the MRLs. From literature review, macrolide antibiotics in water and food-producing animal were analyzed with various sample preparation and detection techniques. LC-MS and LC-MS/MS have become a common detection technique because of their improved selectivity and high sensitivity. However, despite the high sensitivity of LC coupled with MS system, sample preparation is normally a necessary tool to reach the low limits of detection, which are required in the analysis of antibiotics in food from animal origin and water sample. Most extraction methods of macrolide antibiotics from animal tissue consist of extract and protein precipitation with meta-phosphoric acid in methanol followed by clean-up procedure. However, common extraction processes require an additional step of filtration and evaporation. Traditionally, SPE is the only preconcentration and clean-up method used in the macrolide determination. SPE required sorbent and elution solvent optimization in order to obtain strong interaction with analytes and completely elute all analyte from sorbent. Even if the consumption of organic solvent is relatively low in SPE, high preconcentration of analyte is difficult. In addition, SPE requires an extra step for evaporation and SPE cartridges are expensive.

To detect very low amounts of drug residues in complex matrices, a preconcentration method should be provided and SLM is proved to be an alternative on-line liquid extraction technique to obtain high preconcentration with very low organic solvent consumption. As mentioned, SLM overcomes some SPE drawbacks and polymeric membrane is less expensive than SPE cartridge. However, SLM still has disadvantages by which it remains a carry-over effect, it allows only one sample per extraction, and it includes additional devices for online system.

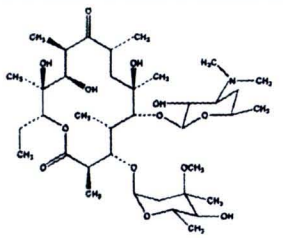
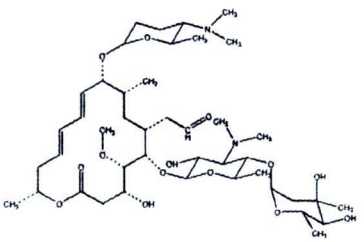
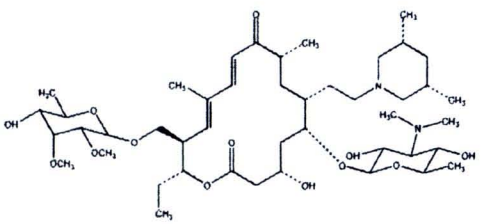
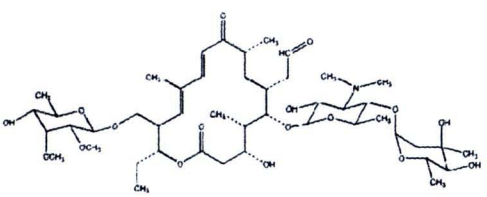
Liquid-phase microextraction (LPME) is termed from the off-line version of SLM and it shares some characteristics with on-line SLM such as the extraction principle, high preconcentration, and clean-up abilities. To overcome on-line SLM drawbacks, LPME plays an important role with regard to carry-over effect, high sample throughput, and almost free of organic solvent use. In addition, the configuration of LPME is generally

simple, inexpensive, and the method is high sensitivity and versatile for various types of samples.

In this work, LPME based on hollow fiber employment, well known as hollow-fiber liquid-phase microextraction (HF-LPME) in three-phase mode was chosen for the determination of four macrolide antibiotis residues in poultry muscle and water. The four macrolides; erythromycin, spiramycin, tilmicosin, and tylosin; are commonly used as veterinary medicine in food-producing animal and easily transfer to aquatic environment. Their residues usually exist in low amounts and induce the difficulty to extract from complex matrices. The structure and property of these four macrolides are shown in Table 1.3.

In HF-LPME, analytes were extracted from aqueous donor or sample solution with organic solvent immersed in the hollow fiber pores and back-extract to aqueous acceptor solution in the hollow fiber lumen. After extraction, the acceptor solution was directly injected to LC-ESI-MS/MS. Because of the difference between volume of donor and acceptor solution, analytes were preconcentrated with good performance. HF-LPME can simultaneous enrich and clean-up analytes from sample matrix. The related parameters were optimized such as the donor pH, the acceptor pH, type of donor and acceptor, organic solvent type, organic solvent composition, immersion time of hollow fiber in organic solvent and extraction time. The optimized HF-LPME method was applied with various extraction methods for extract macrolide antibiotics in poultry muscle obtained from a local market and water sample collected from the river.

Table 1.3 The studied macrolide antibiotics properties *(19,20)*

Analyte	Chemical structure	Chemical formula	Molecular mass (g/mol)	pKa	Log K_{ow}
Erythromycin		$C_{37}H_{67}NO_{13}$	733.93	8.9	3.06
Spiramycin		$C_{43}H_{74}N_2O_{14}$	843.05	7.9	2.49
Tilmicosin		$C_{46}H_{80}N_2O_{13}$	869.13	7.4 8.5	2.60
Tylosin		$C_{46}H_{77}NO_{17}$	916.10	7.1	2.50