Development and application of dispersive liquid-liquid microextraction for the determination of tetracyclines in meat by liquid chromatography tandem mass spectrometry

BY

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FEBRUARY 2016

CERTIFICATION

As the candidate’s supervisors, we have approved this dissertation for submission.

Signature ----------

Date ----------

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Date ----------

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Declaration

I hereby declare that the dissertation submitted for the degree of Master of Science in Chemistry to the University of South Africa is my work except where due reference is made by means of citations, acknowledgements and a comprehensive list of references. This work was carried out at Botswana National Veterinary Laboratory between March 2012 and March 2015 and has not been submitted wholly or in part for the award of a degree or diploma to this or any institution of higher learning.

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Sandy Oshi Squizer Mookantsa (Student ID: 50810162) Date: 07/08/2015

Abstract

An environmentally friendly, rapid and cost effective analytical procedure based on dispersive liquid-liquid microextraction was developed for the determination of six tetracyclines (TCs) in meat destined for human consumption. Meat extracts were analyzed for TCs using a sensitive and selective analytical technique, liquid chromatography tandem mass spectrometry. Various influencing factors on the extraction, separation and determination of TCs such as pH of mobile phases, type and volume of disperser solvent, type and volume of extraction solvent and sample pH were optimized. Validation parameters such as calibration function, limit of detection (LOD), limit of quantification (LOQ), detection capability (CCα), decision limit (CCβ), accuracy and precision were established according to EU commission decision 2002/657/EC. Linearity in the range of 25-200 µg kg^{-1} was obtained with regression coefficients ranging from 0.9991 to 0.9998. Recoveries of spiked blank muscle samples at three levels (i.e. 50, 100 and 150 µg kg^{-1}) ranged from 80 to 101% and reproducibility was between 2 and 7%. The LODs and LOQs ranged from 2.22 to 3.59 µg kg^{-1} and from 7.38 to 11.49 µg kg^{-1} respectively. The CCα ranged from 105 to 111 µg kg^{-1} while CCβ ranged from 107 to 122 µg kg^{-1}. The proposed method compared well with the dispersive solid phase extraction method and was successfully applied to the determination of TCs in meat samples. Some of the thirty bovine muscle samples obtained from local abattoirs and butcheries were found to contain two tetracycline antibiotics residues (chlortetracycline and oxytetracycline) with oxytetracycline being the most commonly detected. The concentration levels of the TC residues detected in the eleven bovine muscle samples were between 12.4

and 68.9 µg kg^{-1}, levels that are lower than the European Union set maximum residue level (MRL) of 100 µg kg^{-1} hence the meat was fit for human consumption.

Keywords: Dispersive liquid liquid microextraction, sample preparation, liquid chromatography mass spectrometry, dispersive solid phase microextraction, tetracycline, meat.

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I gratefully acknowledge the Botswana National Veterinary Laboratory for research facilities and University of South Africa for financial support. I will forever be indebted to these two institutions. I would also like to thank my workmates, fellow postgraduate students and friends for their support and encouragement during my studies.

I am most grateful to my parents and siblings for their constant support and advice in all areas of my life. Finally and most importantly, my deepest gratitude goes to my wife Seedu and daughters Lesika 'Lizzy' and Larona 'Lala' for their motivation and greatest support. I dedicate this dissertation to you and my late sister Maungo Bone Mookantsa. The Lord is highly praised for the successful completion of my Master of Science studies.

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List of Abbreviations, Symbols and Scientific Units

Abbreviations

AR	Analytical Reagent
CD	Commission Decision
DLLME	Dispersive Liquid-Liquid Microextraction
dSPE	Dispersive Solid Phase Extraction
DSPME	Dispersive Solid Phase Microextraction
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
GCMS	Gas Chromatography Mass Spectrometry
HF-LPME	Hollow Fibre-Liquid Phase Microextraction
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
LPME	Liquid Phase Microextraction
MRL	Maximum Residue Limit
MS	Mass Spectrometry
SDME	Single Drop-Liquid Phase Microextraction

SIM	Selected ion monitoring
SRM	Selected reaction monitoring
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
TLC	Thin Layer Chromatography
TM	Trademark
UK	United Kingdom
US	United States

Scientific units and symbols

g	gram
mg	milligram
mm	millimeter
mL	milliliter
kg	kilogram
rpm	revolutions per minute
µm	micrometer
µL	microliter
µg	microgram
ºC	degrees Celsius

CHAPTER ONE

INTRODUCTION

1.1 Background of the research

Over the last two decades, modern and intensive farming in Botswana has made it possible to increase the meat production capacity at a lower cost per head. Also the introduction of farmers` financial aid programs such as young farmers` fund and livestock management and infrastructure development (LIMID) has led to increased population of livestock. These improvements have had many drawbacks to livestock, such as increased vulnerability to diseases and stress [1-4]. Diseases and stress cause high mortality and poor quality meat to food producing animals thus causing non-negligible financial losses for farmers, who are therefore led to use medicinal preparations (veterinary drugs) to fight diseases and stress. However, all drugs that are administered to food producing animals may lead to residues in the edible tissues, milk, eggs and their products. The presence of these residues may pose potential health risks, including allergic reactions, direct toxic effects, cancer and a change in the resistance of bacteria exposed to antibiotics [4-10].

To safeguard human health, many countries in the world that include, the European Union (EU), Japan, Canada, Australia and United States (US) have established safe maximum residue limits (MRLs) or tolerance levels for residues of veterinary drugs in animal tissues entering the human food chain to ensure that human food is entirely free from potentially harmful residues [11-17].

Beef export to European Union is the backbone of economy of Botswana after diamonds. To support beef export to EU market and protect public health, residue

monitoring is carried out in meat and meat products destined for export to EU market and public consumption. For this purpose, Botswana National Veterinary Laboratory (BNVL) is mandated with analysis of residues of veterinary drugs, prohibited substances such as anabolic steroids and environmental contaminants (pesticides and heavy metals) in beef and beef products.

Laboratories involved in food safety control, need to analyze a large number of residues and contaminants in different food commodities. In the framework of analyzing animal tissues destined for human consumption, it is indispensable to develop methods for identifying and quantifying all such substances unequivocally. Several techniques have been used to analyze these compounds, notably TLC, ELISA, GC, GC-MS, HPLC and LC-MS/MS [3-4,18-29]. The ever-increasing demand for determining compounds at very low concentration levels in samples with complex matrices requires the use of analytical techniques, which are characterized by high sensitivity such as LC-MS/MS. In order to take advantage of the multi-residue analysis capability of the LC-MS/MS technique, sample preparation methods should be incorporated to effectively extract a broad range of compounds from food samples, which is in fact a challenge. The different physico-chemical characteristics of the target analytes, as well as the presence of high concentrations of fat and proteins in the food matrices complicate sample extraction and clean-up. Therefore, dispersive liquid-liquid microextraction was developed in this study to overcome some of the limitations of traditional or commonly used sample extraction and clean-up methods (liquid-liquid extraction and solid phase extraction). It is a fast growing method due to its simplicity of operation, low cost, high recovery, rapidity and use of small volume of organic solvents. The method that was first reported by Rezaee and

co-workers in 2006 [24] has been applied successfully and widely for the extraction of target analytes from aqueous samples which are less complicated. Unfortunately, extracting analytes using DLLME from biological samples is not that simple. Thus biological solid matrix samples such as muscles present a challenge in that there is need to first extract the analyte from a complex matrix prior to applying DLLME [25-27]. There are various factors that affect the extraction efficiencies and good chromatographic behaviour of compounds of interest (tetracyclines) such as pH of the aqueous solution, extraction time, extraction and disperser solvent type and their volumes that require to be optimized [24, 28].

1.2 Aim and Objectives

1.2.1 Aim

The aim of this study was to develop a rapid, cost effective and 'greener' sample preparation method (DLLME) for analysis of tetracycline residues in meat by LC-MS/MS and to determine their prevalence below and or above MRLs.

1.2.2 Specific objectives

(i) To develop a DLLME method and optimise parameters affecting extraction efficiency for analysis of tetracyclines in meat samples.

(ii) To validate the DLLME method for analysis of tetracycline residues in meat samples according to EU Commission Decision 2002/657/EC.

(iii) To compare the proposed DLLME method with a South Africa National Accreditation System (SANAS) accredited dSPE method.

(iv) To apply the validated DLLME method in the determination of tetracycline residues in real meat samples by LC-MS/MS.

1.2.3 Scientific contributions

(i) Conference Poster Presentation

S.O.S. Mookantsa, M.M. Nindi *"Multiclass-multiresidue determination of sedatives and tetracyclines in kidney samples using DLLME and ESI-LC-MSMS"*, 7th to 12th July 2013, 12th International Chemistry Conference Africa (ICCA) Pretoria, South Africa.

(ii) Publication

S.O.S. Mookantsa, S. Dube, M.M. Nindi. Development and application of a dispersive liquid-liquid microextraction method for the determination of tetracyclines in beef by liquid chromatography mass spectrometry. *Talanta*. 2016; 148: 321–328.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and use of tetracycline antibiotics

Antibiotics are natural or semi-synthetic chemicals that interfere with the existence of pathogens by several action mechanisms while synthetic ones are termed 'antibacterials' [29]. The use of antibiotics in veterinary medicine goes back to the 1950s with the use of oxytetracycline (Terramycin^{TM}) and chlortetracycline (Aureomycin^{TM}) as feed additives [30-31]. Common tetracycline compounds, their trade names and discovery dates are listed in Table 2.1 below. Nowadays, many classes of antibiotics and antibacterials (tetracyclines, cephalosporins, sulphonamides, nitroimidazoles, macrolides, nitrofurans, penicillins, amphenicols, lincosamides, aminoglycosides and quinolones) are widely used for preventing and treating enteric bacterial infections in human and food producing animals [32-34]. Antibiotics are used for enhancing feed efficiency as well as for promoting growth in food producing animals [19]. Moreover, some of the antibiotics can be added directly to food, mainly to milk to prolong its freshness. Tetracyclines merit a special attention among other veterinary/antibiotic drugs as they are the most widely used class of antibiotics mainly because they have a broad- spectrum activity, are inexpensive, readily available (can be bought over the counter) and are easy to administer. In the 1980s, it was estimated that at least 60% of all animals used for food were exposed to antibiotics at some point in their lives [29]. With current intense animal husbandry practices, this figure is most probably higher.

Table 2.1: List of common tetracycline compounds, their trade names and discovery dates [31]

Generic Name	Chemical Name	Trade Name	Year of discovery	Therapeutic administration
Chlortetra-cycline	7-Chlortetracycline	Aureomycin	1948	Oral
Oxytetracy-cline	5-Hydroxytetracycline	Terramycin	1948	Oral and Parenteral
Tetracycline	Tetracycline	Achromycin	1953	Oral
Demeclocy-cline	6-Demethyl-7-Chlortetracycline	Declomycin	1957	Oral
Rolietracy-cline	2-N-Pyrrolidinomethyl-Tetracycline	Reverin	1958	Oral
Limecycline	2-N-Lysinomethyl-Tetracycline	Tetralysal	1961	Oral and Parenteral
Clomocy-cline	N-Methylol-7-Chlortetracycline	Megaclor	1963	Oral
Methacy-cline	6-Methylene-5-Hydroxytetracycline	Rondomycin	1965	Oral
Doxycycline	6-Deoxy-5-Hydroxytetracycline	Vibramycin	1967	Oral and Parenteral
Minocycline	7-Dimethylamino-6-Demethyl-6-Deoxytetracycine	Minocin	1972	Oral and Parenteral

2.2 Physico-chemical properties of tetracyclines

Tetracyclines are amphoteric molecules with multiple ionizable functional groups and have complicated ring structures. The rings are lettered A to D from right to left, and the numbers begin at the bottom of ring A as shown in Figure 2.1. Substitutions may occur in positions C1 through C12 [35]. Three macroscopic pKa values have been reported for TCs, corresponding to the tricarbonylamide (C1, C2, C3), phenolic diketone (C10, C11, C12), and dimethylamine groups (C4), respectively [35-38]. Table 2.2 shows structures and physico-chemical properties of tetracycline compounds studied whereas Figure 2.2 illustrates functional groups of tetracyclines and their corresponding pKa values. A Tetracycline molecule can behave as a

cation, a neutral/zwitterion, an anion or a dianion depending on the pH of the sample extract or solution as shown in Figure 2.3 below [38].

The stability of TCs is poor under strongly acidic and alkaline conditions but they are generally more stable in acidic than in alkaline conditions [37]. Reversible formation of epimers takes place at positions C4 to form 4-epi-TCs in weak acids (pH 3) and to anhydro-TC under strong acidic conditions (Figure 2.1) [36-38].

The presence of a hydroxyl group at C6 favours dehydration and aromatization of the C-ring of tetracycline leading to anhydro-TC at very low pH. The epimerization of anhydro-TC and the dehydration of the epi-TC lead to the formation of 4-epianhydrotetracycline [35-39]. At alkaline pH, TCs that have a hydroxyl group at C6 can form their respective iso-TCs, via a nucleophilic attack of the C6 hydroxyl group at the C11 carbonyl carbon. Among TCs, CTC is especially prone to this irreversible transformation to yield iso-CTC quickly [38-40]. Due to the additional hydroxyl group at the C5 position, OTC can transform irreversibly to α-apo-OTC and β-apo-OTC in acidic conditions via a nucleophillic attack of this –OH group at C12 [41]. Tetracyclines have a strong tendency to complex with metal ions. Previous studies have reported that the A ring and the BCD rings are probable metal complexation sites of TCs [39-44]. However, none of these tetracycline antibacterial agents undergoes metabolism within the animal body with the exception of tetracycline, about 5% of which is excreted as the metabolite 4-epitetracycline [36-38].

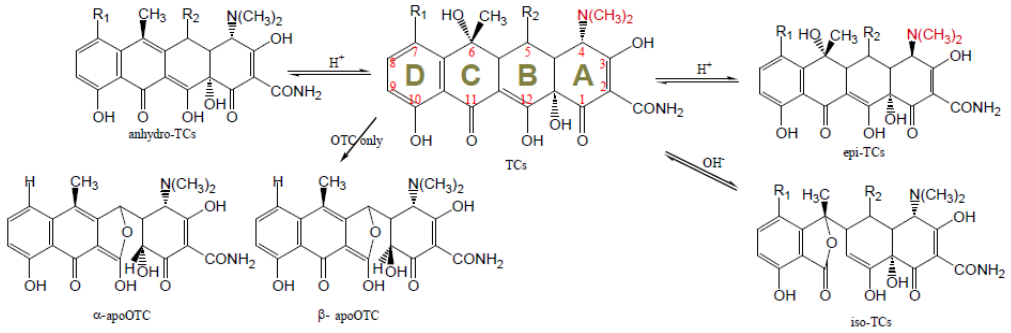
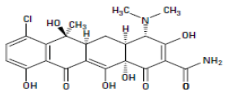
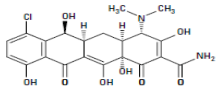
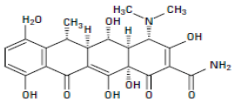
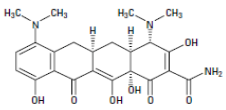
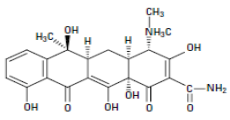
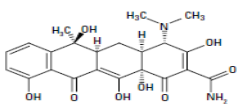
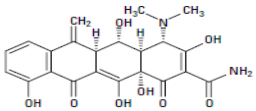


Figure 2.1: Structure and reported transformation products of TCs [43]

Table 2.2: Structures and physico-chemical properties of tetracycline compounds studied [45]

Tetracycline	**Structure**	**CAS number**	**MW**	**pKa**
Chlortetracycline		64-72-2	478.1143	3.3, 7.4, 9.3
Demeclocycline		64-73-3	464.0986	3.3, 7.2, 9.3
Doxycycline		24390-14-5	444.533	3.1, 7.7, 9.3
Minocycline		13614-98-7	457.1849	3.3, 7.2, 9.3
Oxytetracycline		2058-46-0	460.1482	3.3, 7.3, 9.1
Tetracycline		64-75-5	444.1533	3.3, 7.7, 9.7
Methacycline		3963-95-9	442.1376	3.5, 7.6, 9.3

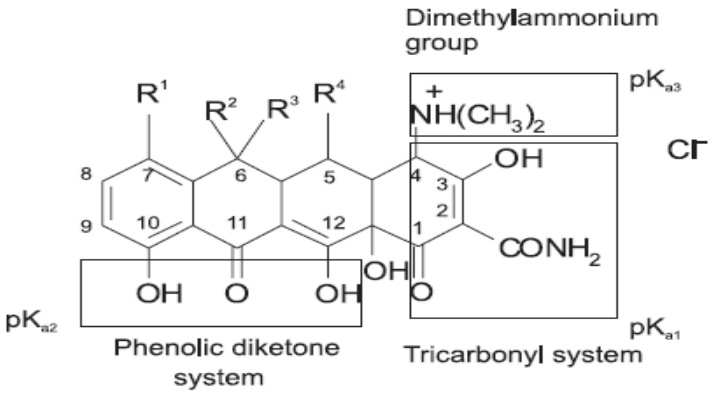


Figure 2.2: Functional groups of tetracyclines and their pKa values [44]

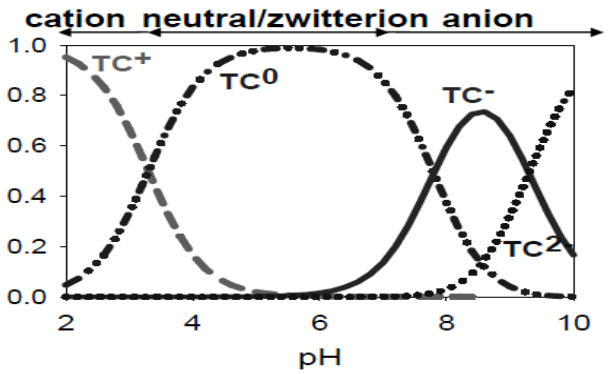


Figure 2.3: Speciation of TC molecule at pH 2-10 [43]

2.3 Health effects of tetracyclines on human beings

Tetracyclines (TCs) produced by streptomyces spp. are a wide family of antibiotics used in human and veterinary medicine that have a broad-spectrum activity against most Gram-positive and Gram-negative bacteria including some anaerobes. Tetracyclines are actively transported into the cells of susceptible bacteria where they bind to the 30S ribosomal sub-particle. In this way, protein synthesis is inhibited, which explains their bacteriostatic effect [1, 46-48]. Oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) are four members of this antibiotic group that are commonly used in food producing animals. When tetracycline drugs are administered by laymen, as in most cases, correct dosages as

well as withdrawal periods before slaughter are unlikely to be observed. This raises the possibility that residues of TCs may remain in animal tissues intended for human consumption. The failure to follow good veterinary practices can lead to unsafe residue levels in different tissues, with potential adverse effects on human health. Improper dosages of tetracycline drugs especially subtherapeutic doses may lead to the emergence of resistant bacteria. Humans may become resistant to tetracyclines and other antibiotic drugs. Resistant strains of *Staphylococci, Coliforms, Bacilli, heumococci, Haemolytic streptococci, haemophilus influenczae* and *Clostridium welchii* have been reported [29,46]. Human health problems resulting from intake of subchronic exposure levels of TCs include gastrointestinal disturbances, poor foetal development, hypersensitivity and other toxic effects. Tetracyclines in meat potentially may stain teeth of young children. In addition to immediate adverse effects, there are also long-term effects to the exposure of low levels of antibiotic residues that are still unknown [29-30, 49-51].

2.4 Legislations and residue monitoring of tetracyclines

In order to safeguard human health, the World Health Organisation (WHO) and Food and Agricultural Organisation (FAO) have set standards for acceptable daily intake of antibiotics. In the frame of its policy on consumer health protection, the European Union (EU) established maximum residue levels (MRLs) for various classes of antibiotics among which are tetracyclines, in different animal tissues [12, 14-15, 17, 52]. Tetracyclines fall in group B1 (antimicrobial) substances. The acceptable MRLs for all tetracyclines are set at 100 µg kg^{-1} for muscle, 300 µg kg^{-1} for liver and 600 µg kg^{-1} for kidney for all food-producing animals, in order to protect humans from

exposure to TCs in edible tissues of animal origin. Withdrawal periods of 5-20 days before slaughter are recommended for food-producing animals, depending on the species and nature of the food product [5-6, 10-11, 51-53]. The EU is the largest market for most African countries for beef. This implies that all countries that export or wish to export beef to the EU market must comply with the EU regulations on residue monitoring.

2.4.1 EU criteria for testing methods

The EU criteria for testing methods formed the basis for this work because most African countries, including Botswana export beef to the European Union market which has proven to be the largest and most lucrative beef market. The testing laboratories of the exporting countries are therefore expected to adopt the EU directive for their sampling, testing and detecting. A system of identification points is used to qualify mass spectrometry methods for identifying veterinary drugs residues [54] as illustrated in Table 2.3 below. For the confirmation of substances listed in Group A of Annex I of Directive 96/23/EC a minimum of 4 identification points is required and for the confirmation of substances listed in Group B of Annex I of Directive 96/23/EC a minimum of 3 identification points are required [54, 55].

Table 2.3: Number of identification points collected by each analytical technique [54]

Technique(s)	Number of ions to be monitored	Identification points
GC-MS LC-MS	1 precursor and 1 product	2
GC-MS/MS LC-MS/MS	1 precursor and 2 product ions	4
GC-MS/MS LC-MS/MS	2 precursor ions, each with 1 product ion	5

When mass spectrometric determination is performed by techniques such as selected ion monitoring (SIM), selected reaction monitoring (SRM) or full scan, the molecular ions that are usually monitored are either of the selected diagnostic ions (the molecular ion, characteristic adducts of the molecular ion, characteristic fragment ions and all their isotope ions). The signal-to-noise ratio for each diagnostic ion should be greater than 3:1 [54].

2.4.2 Analytical Performance Limits

Results in veterinary drug residue analysis are reported with criteria laid down in Commission Decision (CD) 2002/657/EC [54]. Validation of an analytical method must demonstrate key characteristics laid down in this commission. The key characteristics are selectivity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, ruggedness, stability and performance limits e.g. decision limit (CCα) and detection capability (CCβ) [54-58]. The methods must be able to detect analytes at or below maximum residue limit (MRL) or minimum required performance limit (MRPL). The MRL is the maximum level or maximum tolerance for substances established in Community legislation i.e. EU

legislation. The MRPL is the lowest concentration of analyte which is expected to be detected in the case of screening method or its identity confirmed in case of confirmatory methods [52, 56-57].

The LOD of a method is the lowest analyte concentration that produces a response detectable above the noise level of the instrument, typically three times the noise level. By this LOD criterion, it is taken that at the 0.05% level, an impurity will have S/N ≥ 3. However, it is very important that the LOD should be determined theoretically by using regression data [58]. The LOQ is strictly the lowest concentration of analyte that can be determined with an acceptable level of repeatability precision and trueness. It is also defined by various conventions to be the concentration of analyte corresponding to the sample blank value plus 5, 6 or 10 standard deviations of the blank mean. The LOQ is an indicative value and should not be used in decision making [58].

Analytical performance limits (CCα and CCβ) are used to decide whether the sample is compliant or non-compliant in veterinary drug residue analysis. If the amount of analyte detected in a sample is at set CCα then the sample is regarded as non-compliant according to CD 2002/657/EC and only if the true concentration in the sample is above or equal to CCβ then one can be statistically confident that the residue is over MRL. For permitted drugs CCα is estimated with 95% statistical confidence and for unauthorized drugs CCα is estimated with 99% statistical confidence. CCβ is estimated with 95% statistical confidence in both instances [54, 56]. Figure 2.4 relates the two performance characteristics (CCα and CCβ).

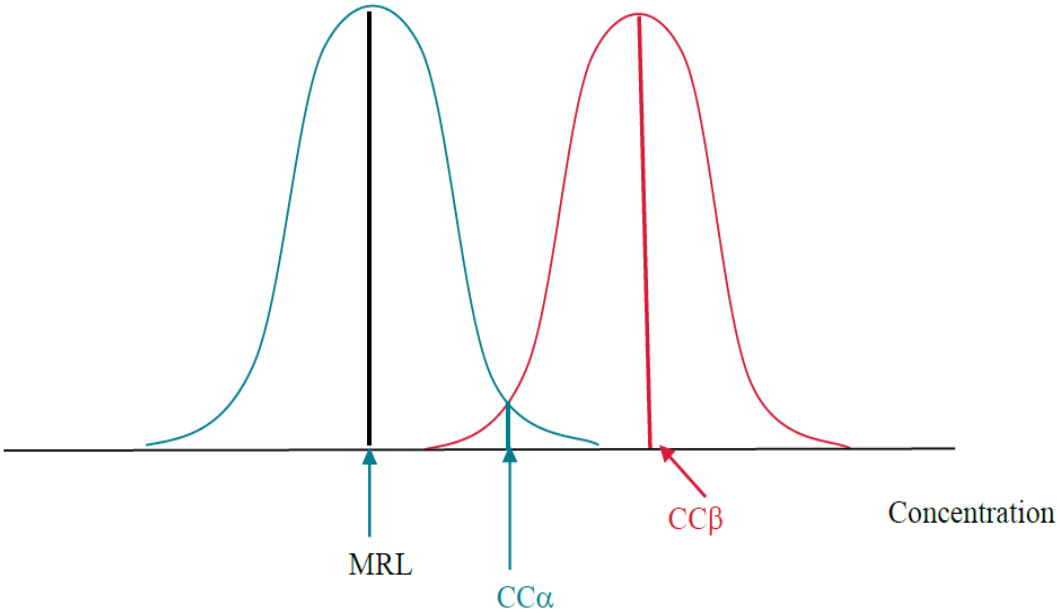


Figure 2.4: Relationship between MRL, CCα and CCβ [54]

2.5 Sample preparation procedures for analysis of tetracycline residues

Food samples present an enormous challenge in analytical laboratories performing numerous analyses in their efforts to determine residues of veterinary drugs at trace levels to satisfy regulations in EU, USA and Japan. In addition, biological samples are normally a challenge and may require further clean-up to reduce interferences and/or eliminate impurities, which might damage columns or affect the accuracy of results. The most common techniques used for sample clean-up in determination of veterinary drug residues are liquid-liquid extraction (LLE) [59-61] and solid phase extraction (SPE) [62-66]. These traditional sample extraction and clean-up methods are labour intensive, time consuming and use large volumes of organic solvents that are environmentally unfriendly, carcinogenic and toxic. These consuming and laborious techniques have a negative impact on laboratory throughput with some laboratories only managing to process less than 50 samples per day.

Sample preparation procedures have undergone numerous changes in the past few years. Miniaturized sample preparation methods have been the focus in order to overcome the limitations of the traditional sample preparations [67-73]. These miniaturized sample extraction and pre-concentration procedures include solid phase microextraction [74-75], dispersive solid phase microextraction [76], single-drop liquid-phase microextraction [77], hollow fibre - liquid phase microextraction [78-81] and dispersive liquid-liquid microextraction [24-28, 82-86]. A general trend at present is not only to improve the analytical performance of these techniques but also to endeavor to satisfy the requirements of green chemistry, that is, to minimize the amounts of hazardous reagents consumed and waste generated.

2.5.1 Dispersive solid phase microextraction

Dispersive solid phase microextraction (DSPME) method is similar to dispersive solid phase extraction (dSPE) method which has been successfully used to clean-up samples for the analysis of pesticides residues in various matrices and veterinary drug residues in animal tissues. However, in DSPME the dispersive sorbents are not only used for clean-up but also for trapping the analytes out of the solution and then transferring them to a smaller volume of a secondary desorption solvent after discarding most of liquid and drying [76,87]. The DSPME method is simple, fast and inexpensive and has been used for sample extraction in the analysis of tetracyclines mostly in water samples [76, 87]. Analytes were detected with LOD of 0.7-3.5 ng mL^{-1} and 7.9-35.3 ng mL^{-1} in water and milk, respectively. Average recoveries ranged from 97.1 to 104.1% [76]. The limitation that comes with this method is that it uses about 5 mL of acetonitrile, which is a considerable high volume of organic solvent.

2.5.2 Cloud point extraction

Cloud point extraction (CPE) method which was introduced in 1976 by Watanabe and co-workers [88] is a miniaturized liquid-phase microextraction and pre-concentration technique based upon the unique properties (i.e. solubilization and phase separation [clouding] ability) of aqueous solutions of neutral surfactant micellar systems [88-89]. The CPE methods exploit a special property of most non-ionic surfactants that form micelles in aqueous solution, which is that they become turbid when heated to the appropriate cloud point temperature. Above the cloud point temperature, the micellar solution separates into a small, surfactant rich phase and a larger diluted aqueous phase. In the aqueous phase, the surfactant concentration remains near critical micellar concentration. Any analyte solubilized in the hydrophobic core of the micelle in the unheated solution, will be concentrated in the surfactant-rich phase following the cloud point extraction [88-91]. Figure 2.5 illustrate the schematic procedure of the CPE technique. The method was initially introduced for the pre-concentration of inorganic analytes such (metals) [88] but has since been exploited as a primary isolation step for the purification of proteins and as an extraction and pre-concentration method for the determination of organic and veterinary drug analytes [92]. To our knowledge, this technique has not yet been applied to tetracycline antibiotics but to fluoroquinolones antibiotics in water samples [92]. The reported CPE method showed good analytical features with linearity for four fluoroquinolones analytes in a wide range of 0.045–0.90 µg mL^{-1} and coefficients of determination of no less than 0.9901. The limits of detection ranged from 0.007 to 0.013 µg mL^{-1} and average recoveries ranged from 83.0% to 96.7%

[92]. One of the disadvantages of the CPE method is its limited applications to solid samples.

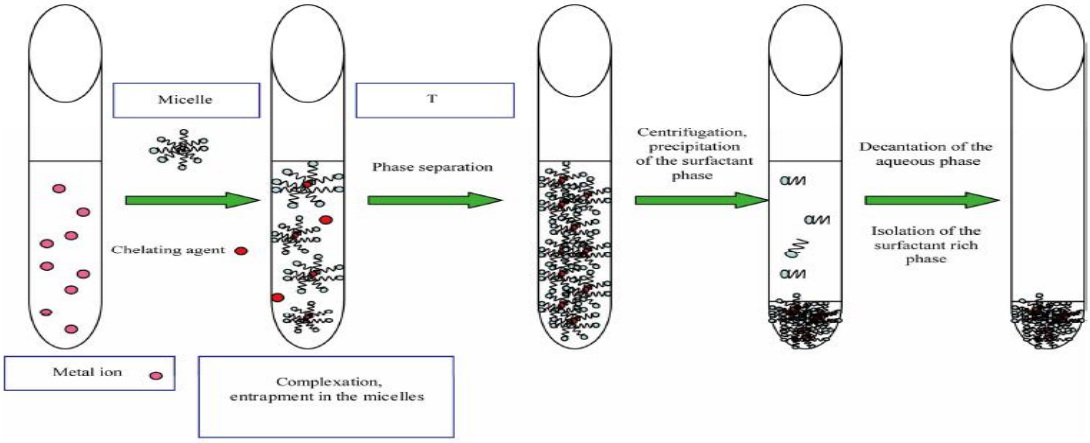


Figure 2.5: Schematic procedure of the CPE method [90]

2.5.3 Single-drop liquid-phase microextraction (SDME)

Single-drop liquid phase microextraction (SDME) is a sample preparation method which uses a single droplet for extraction purposes and was first recommended in the mid 1990s [93]. The SDME technique employing a microsyringe is shown in Figure 2.6 below. The syringe needle is used to pierce the septum of a closed container. When the tip of the needle is in the desired position (in the aqueous phase or in the headspace) a hanging droplet of solvent is exposed to the matrix by depressing the plunger of the syringe. After extraction is completed, the droplet is withdrawn into the syringe barrel by lifting the plunger [70-71, 77, 93-95]. The extracted samples can then be submitted to the instrument for analysis. In addition to the advantage of using small volume of organic solvent, in this technique, analytes with high partition coefficient can reach high concentrations, since they are transferred by diffusion from a significant volume of sample (1-5 mL) to a small

micro-extract (5-50 µL) [77, 87, 94-96]. Despite its simplicity, easy implementation and low cost, SDME technique has some limitations; i) direct immersion requires careful and intricate manual operation because of problems of drop dislodgement and instability, ii) complex matrices requires a pre-treatment or extra filtration step. Since its inception, different modes of SDME have been developed, in order to improve its extraction efficiency, such as direct SDME, headspace SDME and continuous-flow SDME [70-71, 77, 87, 94-96]. Direct SDME has extensively been used for the direct extraction of pesticides residues from aqueous samples [70-71, 77, 87, 95]. Application of SDME in veterinary drugs was reported for monensin with limits of quantification in surface water, soil, and human urine of 6.7, 12.4 and 7.8 ng mL^{-1}, respectively [96].

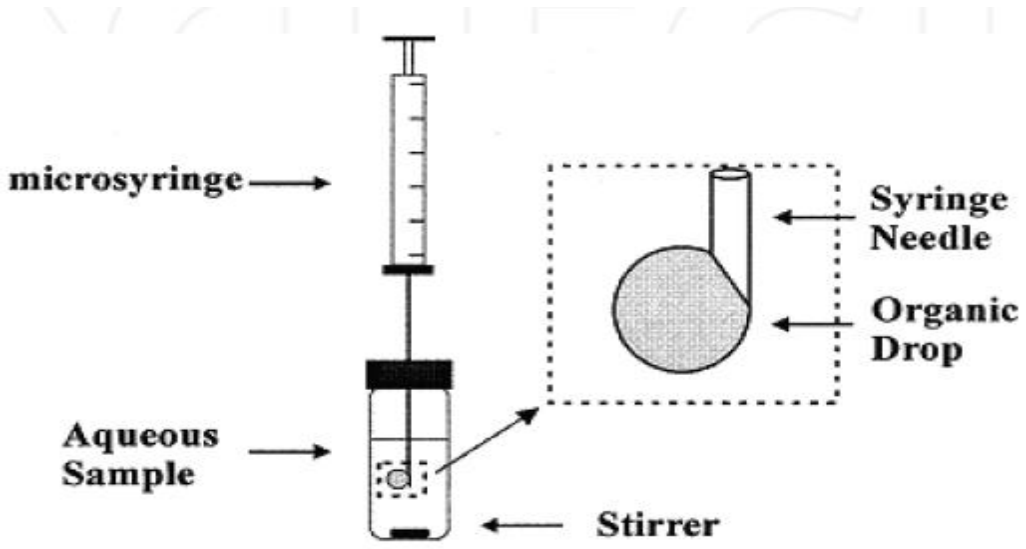


Figure 2.6: Schematic illustration of the SDME procedure [94]

2.5.4 Hollow- fibre liquid- phase microextraction

Hollow-fiber liquid-phase microextraction (HF-LPME) was introduced in 1999 by Pedersen-Bjergaard and Rasmussen to improve the stability and reliability of SDME techniques [97]. This method uses single, low cost, disposable porous hollow-fibre

(HF) made of propylene [97]. A short piece of the porous HF is closed at the bottom or u-shaped and both ends connected to guiding tubes. Prior to extraction, the HF is dipped in a water immiscible organic solvent for a few seconds. The organic solvent is immediately immobilized in the pores of the HF by capillary forces, forming a supported liquid membrane (SLM). The internal lumen of the HF is then filled with an acceptor solution, and the hollow fibre is placed in the sample. With strong agitation or stirring of the sample, target analytes are extracted from the sample, through SLM, and into the acceptor solution. After a certain period of time, the acceptor solution is removed from the HF, and injected in analytical instrument for analysis [71, 78-81, 95, 97-100]. Figure 2.7 below illustrates the principle of the HF-LPME method.

The HF-LPME method has two configurations; two-phase and three-phase systems. In a two-phase mode, the analytes are extracted from the aqueous sample into an organic solvent immobilized in the pores and the lumen of HF. This mode is well suited for analytes with high solubility in non-polar organic solvents. In a three-phase configuration, the analytes are extracted from an aqueous sample through the thin film of the organic solvent into an aqueous acceptor solution [95, 97-100]. The thin film of organic phase serves as a barrier between the donor phase and acceptor phase. This mode is suited for acidic or basic analytes with ionizable functionalities, where the analyte is in neutral form in the donor phase. The major advantages of HF-LPME are the high enrichment, high degree of sample clean-up and low solvent consumption. Like all other sample preparation methods, HF-LPME has some limitations. HF-LPME procedure is typically slow, extraction times range from 15 to 45 minutes and target analytes may partly be trapped in the SLM [71, 78-81, 95].

Another disadvantage is the lack of commercially available equipment and HF although commercially available, they have to be cut manually to appropriate length and sealed [100]. Using microextraction method some researchers have reported the determination of multi-classes of antibiotics in water samples with LODs ranging from 10 to 250 ng L^{-1}. Relative recoveries were between 79 and 118% with up to 156 times enrichment [101].

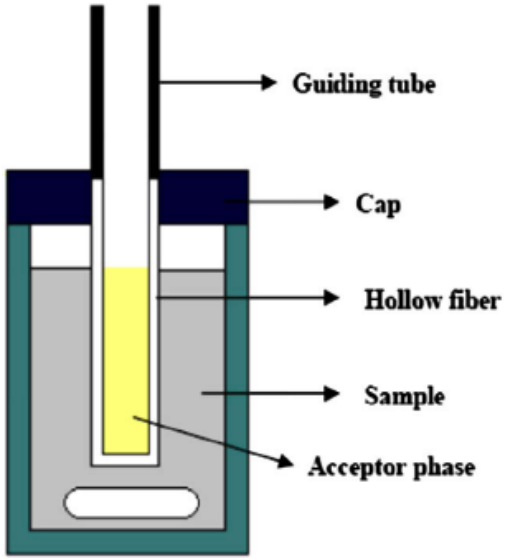


Figure 2.7: Illustration of the HF-LPME principle [95].

2.5.5 Dispersive liquid-liquid microextraction (DLLME)

Dispersive liquid-liquid microextraction method which was first reported by Rezaee et al. in 2006, was developed to overcome some of the limitations of liquid phase microextraction and clean-up methods described above [24]. It is a fast growing method due to its simplicity of operation, low cost, high recovery, rapidity and use of small volume of organic solvents. The DLLME method is a ternary solvent, miniaturised liquid-liquid extraction method using microliter volumes of extraction

solvent, which is based on the equilibrium distribution process of the target analytes between sample solution and extraction solvent [24-28]. A mixture of a water-immiscible extraction solvent and water-miscible disperser solvent is rapidly injected into the aqueous sample by a syringe. The mixture is then gently shaken and a cloudy solution containing fine droplets of extraction solvent dispersed entirely in the aqueous phase is formed. The analytes in the sample are extracted into the fine droplets, which are further separated by centrifugation, and the enriched analytes in the sedimented phase are determined by either chromatographic or spectrometric methods [24-28, 82-86]. Figure 2.8 below illustrates the procedure for the DLLME method.

As with any sample preparation technique, one must still optimise the DLLME method. Rezaee et al. and Kozani et al. have studied the factors affecting analytical performance of DLLME method. They reported that the sample pH, type and volume of extraction and disperser solvents as well as the extraction time affect the DLLME method performance [24, 28]. Since its inception, DLLME has found several applications in the analysis of pesticides, metallic elements and veterinary drugs in samples with relatively simple matrix, such as aqueous samples [24, 28, 72, 85, 98]. However, publications have recently appeared devoted to analysis of other sample matrices such as foods, environmental and biological samples [26, 27, 84, 86-87].

When compared with other liquid–phase microextraction techniques, DLLME is more advantageous in terms of short total time, low cost and feasibility [73]. The DLLME method compared to cloud point extraction methods that used the same techniques revealed that both techniques have similar efficiency in terms of sensitivity and

recovery [73]. However, DLLME is faster than CPE because the latter sometimes needs heating aqueous solutions for long periods to achieve cloud point temperature. Furthermore, extraction efficiency of CPE may decrease in the presence of more than 3% of water-miscible organic solvents such as THF due to dissolution of the surfactant-rich phase and decreasing the volume of this phase [73, 102]. On the other hand, comparison of DLLME with HF-LPME revealed that both techniques provide high enrichment, with easy clean-up and low solvent consumption, however, HF-LPME requires long extraction times [73, 98].

Applications of the DLLME method in tap water, environmental water and egg samples for the determination of tetracycline antibiotics have been reported [103-105]. However, ionic liquids were used as extraction solvents in these methods. Validation/performance parameters of the methods obtained under optimum conditions are reported in Table 2.4 below. To the best of our knowledge, this is the first time that DLLME has been used in the determination of tetracycline antibiotic residues in tissue samples (muscle) [106].

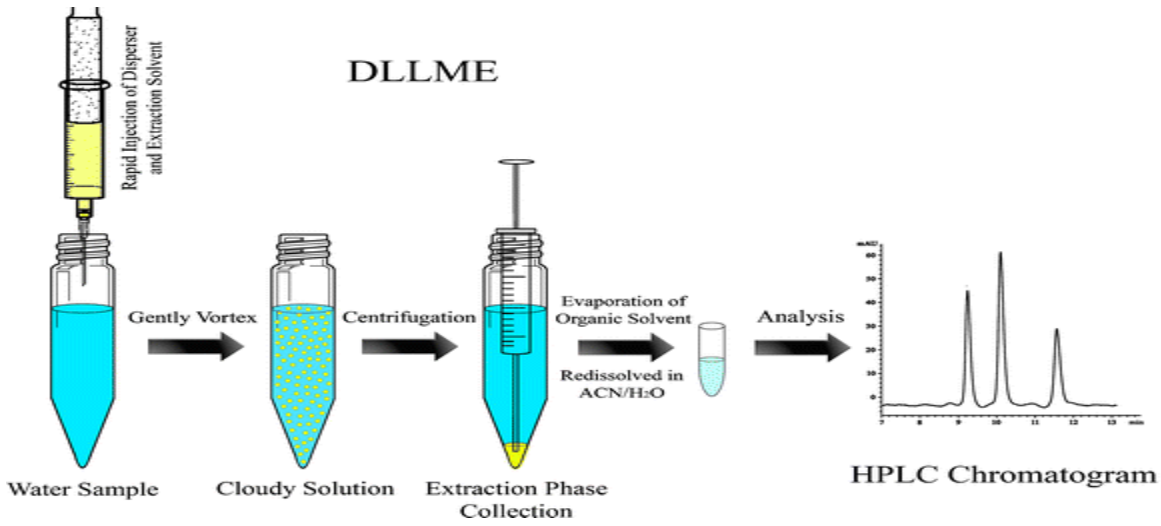


Figure 2.8: Schematic diagram of DLLME procedure [98]

Table 2.4: Validation/ performance parameters of some of the DLLME methods applied to tetracycline antibiotics.

Matrix	Analytical method	LOD µg L^{-1}	LOQ µg L^{-1}	Linear range (µg L^{-1})	R^2	Recovery (%)	EF	Ref.
Tap water	HPLC	9.7-11.4	-	10-500	0.998-0.999	62.6-109.6	-	103
River water	UPLC	0.031-0.079	0.10-0.26	0.1-200	0.992-0.999	64.1-103.1	-	104
Fishpond water	UPLC	0.031-0.079	0.10-0.26	0.1-200	0.992-0.999	61.4-99.2	-	104
Hog leachate	UPLC	0.031-0.079	0.10-0.26	0.1-200	0.992-0.999	60.7-92.6	-	104
Egg	HPLC	2.0-12.0	-	-	-	58.6-95.3	12-44	105
Muscle	LC-MS	2.2-7.4	7.3-11.5	25-200	0.9994-0.9998	80-101	-	106

N.B: Units for LOD, LOQ and linear range for egg and muscle are µg kg^{-1}.

2.6 Analytical methods used for analysis of tetracyclines

In the framework of analyzing animal tissues destined for human consumption, it is indispensable to develop methods for identifying and quantifying all such substances unequivocally. Commission Decision 2002/657/EC implementing Council Directive 96/23/EC establishes criteria for testing methods that should be met by techniques that are used to analyze substances in meat and meat products to ensure the quality and comparability of analytical results generated by official laboratories [54]. Several techniques have been used to analyze these veterinary drug residues, notably TLC, ELISA, GC, GC-MS and HPLC [18-22]. The disadvantages of some of these methods are that some require extensive sample clean-up [19-21], the use of expensive apparatus [20-22], can only process a limited number of samples [18],

lack specificity and sensitivity [18-20]. Although GC-MS is a specific and sensitive technique, the handling and maintenance of the instrument that include frequent cleaning of the ion source is very demanding and time consuming. Moreover sample preparation is very laborious and includes procedures of sample extraction, analyte derivatisation and long run times with a typical sample throughput of less than 50 samples per day [21]. Routine laboratories for control of veterinary drug residues in food producing animals have to analyze a large number of samples frequently, handling different families of compounds. Coupling HPLC to mass spectrometer has improved the LC technique tremendously allowing for its applications to endogenous components such as proteins, peptides, carbohydrates, DNA, and drugs or metabolites. Furthermore, powerful new technologies of ion- separation (tandem MS, Time of-Flight-MS, Ion-Trap MS) substantially increased the capabilities of MS analyzers with respect to specificity, universality, sensitivity, short run times due to their compatibility with short columns and the fact that analytes do not have to be fully resolved or separated to be identified and quantified as required in other analytical methods. These advantages are the reason for the widespread use of MS techniques over other analytical methods in routine laboratory analysis [3-4, 29, 31, 45, 59-61, 63-66].

2.6.1 Liquid Chromatography Mass Spectrometry

Liquid chromatography-mass spectrometry is a hyphenated technique, which combines the separating power of high performance liquid chromatography with the detection power of mass spectrometry.

2.6.1.1 High Performance Liquid Chromatography

High performance liquid chromatography is the most widely used separation technique applied in analysis of veterinary drug residues in food samples. Several papers dealing with determination of tetracyclines in water, biological and food samples by conventional liquid chromatography coupled to ultraviolet (UV) or diode-array (DAD) and or fluorescence detectors have been published [22, 36, 62, 76, 79, 103-105]. Tetracyclines were detected at a range of 0.7-35 ppb (ng mL^{-1} or ng g^{-1}) in various. These conventional LC techniques are limited in terms of specificity and sensitivity.

2.6.1.2 Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical technique used to detect and determine the amount of a given analyte in less than nanogram quantities. MS is also used to determine the elemental composition and some aspect of the molecular structure of an analyte. MS utilizes the principle of separating ions on the basis of *m/z* values. Ions pass through the mass analyzer one at a time to reach the detector resulting in a mass spectrum [107-108]. Liquid chromatography coupled to mass spectrometry detectors (LC-MS or LC-MS/MS) is a fast growing and reliable technique used to overcome the above mentioned limitations of the HPLC analytical instrument for analysis of veterinary drugs (tetracycline antibiotics) in any matrix [1, 4, 7, 29, 45, 47-48, 50, 60, 64]. The major advantage of LC-MS/MS is that it can simultaneously determine many classes of veterinary drugs at very short run times since analytes do not have to be fully resolved or separated to be identified and quantified. Another interesting feature of this technique is the ability to fragment and

isolate ions several times in succession before the final mass spectrum is obtained, resulting in tandem (MS^n) capabilities [109]. The tandem capabilities are important for providing structural information of a compound by fragmenting the ions isolated during the first experiment (MS scan), and/or for achieving better selectivity and sensitivity for quantitative analysis by selecting representative ion transitions using both the first and second analyzers. Using this technique, very low detection limits of 0.01- 0.25 ng mL^{-1} (ppb) have been reported for simultaneous determination of multiple classes of antibiotics (tetracyclines, macrolides, sulfonamides and quinolones) [101]. The use of multi reaction monitoring (MRM) as a tool in liquid chromatography-mass spectrometry is known to enhance the sensitivity of LC-MS/MS technique.

2.7 Current research work

In this work, a rapid, cheap and simple 'green chemistry' extraction method based on DLLME was developed and validated for the analysis of tetracycline residues in bovine muscle samples to determine their prevalence below and or above MRLs using a liquid chromatography tandem mass spectrometry. The LC-MS/MS instrument was chosen over other analytical techniques for the analysis of tetracyclines in meat for its major advantages of sensitivity, selectivity and capability to simultaneously determine many analytes at very short run times since analytes do not have to be fully resolved or separated to be identified and quantified.

CHAPTER THREE

EXPERIMENTAL

3.1 Reagents, Chemicals and Materials

Acetonitrile, methanol and formic acid were of LC-MS grade from Fluka (Steinheim, Germany) while acetone, chloroform, dichloromethane, trichloromethane, trifluoroacetic anhydride and tetrachloroethylene were of HPLC grade from Merck (Darmstadt, Germany). Sodium hydroxide pellets, sodium chloride, trisodium citrate dihydrate and magnesium sulphate anhydrous were of analytical grade from Merck (Darmstadt, Germany). Chlortetracycline hydrochloride (97.8%), oxytetracycline hydrochloride (95%), tetracycline hydrochloride (95%), doxycycline hyclate (97%), demeclocycline hydrochloride (98%), minocycline hydrochloride and methacycline hydrochloride (95%) analytical standards were from Sigma-Aldrich (Steinheim, Germany). Ultra high purity water (UHP) (18.2 MΩ. cm resistivity) was obtained from the Milli-Q purification system from Millipore (Bedford, MA, United States). The Bond ElutC_{18} sorbent was from Agilent Technologies (Santa Clara, USA) and 0.45 µm PVDF membrane filters were from Pall Corporation (New York, USA).

3.2 Instrumentation

3.2.1 Mass Spectrometer

An Applied Biosystems 4000 Qtrap mass spectrometer was from Applied Biosystems/ABSciex (Pty) LTD (Darmstadt, Germany) and was used for all mass

spectral measurements. The mass spectrometer was equipped with electrospray (ESI) interface operating in a positive ion mode. ESI parameters were optimized for each tetracycline by direct infusion of individual standard solution into the mass spectrometer. The mass spectrometer parameters were set as follows; ion-source temperature (500ºC), ion-source gas 1 (50 psi), ion-source gas 2 (40 psi), curtain gas (25 psi) and collision gas (medium). It was operated in the selective reaction monitoring (SRM) mode to confirm the identity of tetracyclines. This was achieved by selecting specific precursor-to-product ion for each tetracycline and quantifying using the most abundant transition. Figure 3.1 shows a liquid chromatography tandem mass spectrometer (LC-MS/MS) analytical instrument used in this work.

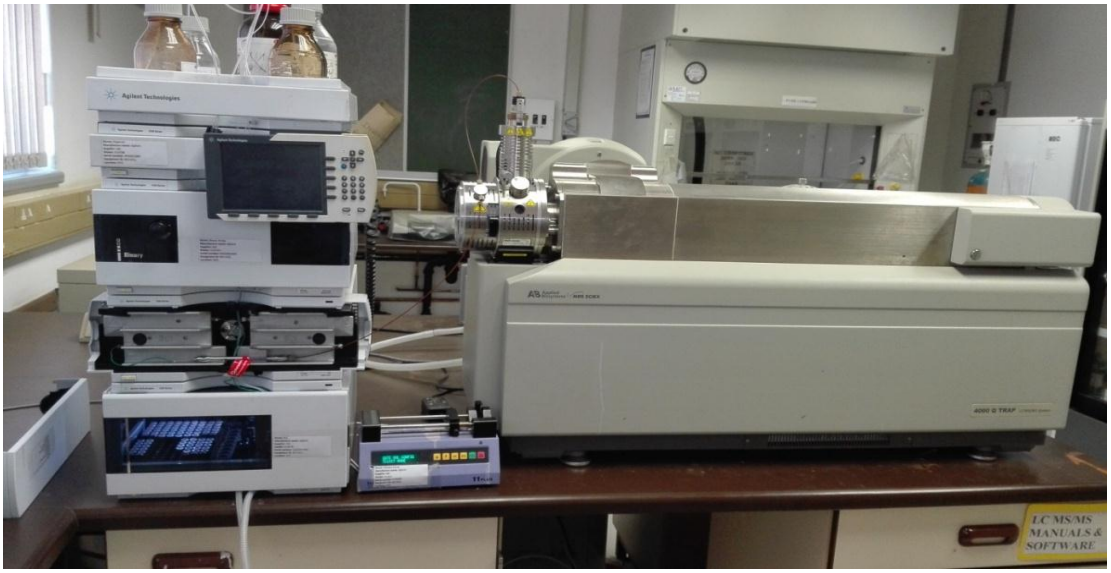


Figure 3.1: Picture of LC-MS/MS instrument used in this study

3.2.2 High pressure liquid chromatography

For the chromatographic separation an Agilent 1200 series HPLC instrument from Agilent Technologies (Waldbronn, Germany) was used. The instrument consisted of

an auto-sampler, thermostated column compartment and a quaternary pump. The separations of tetracycline compounds were performed on a Kinetex 2.6 µm XB-C_{18} 100 Å, 100 mm x 4.6 mm column from Phenomenex (Aschaffenburg, Germany). An Analyst 1.5 software was used to control the LC-MS instrument and for data acquisition.

Several other small pieces of equipment were used for sample preparation. A Heraeus Megafuge centrifuge was from Thermo Scientific (Steingrund, Germany), Ultra Turrax T25 homogeniser was from Optolabor (Atlanta, United State) and TurbovapLV concentrator workstation was from Caliper Life Sciences (Hopkinton, Mass, United State). The multireax vortex mixer was obtained from Gilson (Middleton, United Kingdom).

3.3 Procedures

3.3.1 Preparation of standard solutions

Stock standard solutions of every tetracycline compound were prepared separately in methanol at a concentration of 1 mg mL^{-1}. The stock solutions were prepared by accurately weighing an amount corresponding to 20-30 mg after correcting for purity, water of hydration and the fact that some standards were salts. The weighed amount was then quantitatively transferred into a 25 mL volumetric flask, dissolved and diluted to the mark with methanol. The solutions were stored at -20ºC. A mixed working standard solution, with all the compounds, was prepared by transferring 100 µL aliquots of each stock standard solution (1 mg mL^{-1}) into a 100 mL volumetric flask and making up to the volume with methanol such that the final concentration

was 1.0 µg mL^{-1}. The internal standard (methacycline) was prepared separately but in the same way as the mixed standard solution. The standard solutions were stored at a temperature of 2-8ºC until ready for analysis. Calibration standards were freshly prepared with every run by fortifying 1.0 g of blank ground muscle samples with appropriate volumes of the 1.0 µg mL^{-1} mixture of tetracycline standard solution and 50 µL internal standard. A total of three spiked samples at each of the eight concentration levels in the range of 25 - 200 µg kg^{-1} were taken through the entire DLLME procedure and used to construct a matrix matched calibration curve.

3.3.2 MS experiments

In setting up an LC-MS method, a significant number of conditions and parameters need to be taken into account and optimized. Ion source parameters and collision energies were optimized by continuously infusing a dilute solution of each of the seven tetracycline analytes in methanol and ammonium formate mixture using the Q1 scan type to determine the maximum signal intensity for each ion. This was followed by running MRM scan of a mixture of the seven compounds to obtain the most intense precursor/product ion pair for each compound with the corresponding optimal ion optics values. The optimized MS compound dependent parameters {Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Collision Cell Exit Potential (CXP)} and ion source parameters (Gases, Temperature and Ion Spray Voltage) were used to create MS method/project that was used for subsequent experiments in this study.

3.3.3 HPLC separation

A 10 µL of a mixture of the seven tetracycline compounds was injected and an elution gradient of mobile phase (MP) A (0.2% formic acid in UHP water) and mobile phase B (0.2% formic acid in acetonitrile) was developed to separate the tetracycline analytes. The six tetracycline compounds and internal standard were separated using a Kinetex 2.6 µ XB-C_{18} 100 Å, 100 mm x 4.6 mm column. The column was maintained at 40ºC at a flow rate of 550 µL min^{-1} and the injection volume was 20 µL. The gradient started with 90% MP A followed by a linear decrease to 65% MP A in 2 min and was maintained for 7 min. Mobile phase A was then changed back to the initial percentage and held for 1 min until the next injection. Table 3.1 below shows the gradient elution program used to achieve separation of tetracyclines.

Table 3.1 Gradient Elution program used to achieve separation of seven TCs; A= 0.2% Formic Acid in water, B= 0.2% Formic acid in acetonitrile.

Total time (min)	Flow rate (µl/min)	A%	B%
0.00	550	90.0	10.0
1.00	550	90.0	10.0
3.00	550	65.0	35.0
8.00	550	90.0	10.0
9.0	550	90.0	90.0

3.3.4 Sample collection and storage

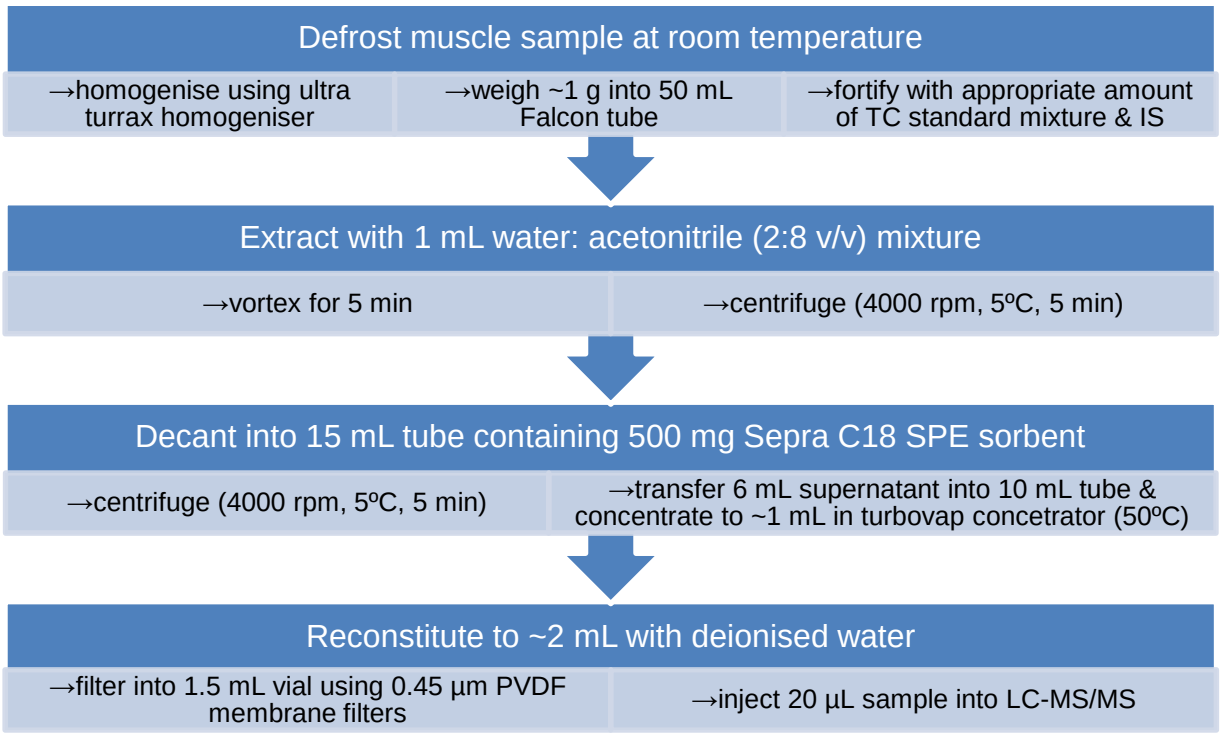
Sampling was carried out according to the Commission Decision 96/23/EC of 29 April 1996 that lays down detailed rules on official sampling for the monitoring of certain substances and residues thereof in live and slaughtered animals [53]. The EU regulation prescribes residue monitoring of tetracyclines in kidney, liver and

muscle. Furthermore some studies have shown that the tetracycline drugs are distributed among these tissues/organs in the animal body [1, 19, 47, 48]. Bovine muscle was chosen in this case because of ease of access and lower MRL which is compatible with the sensitive LC-MS/MS instrument used in this study. The samples were collected from Botswana national abattoirs and stored in a freezer at -20 ± 3ºC to preserve them before analysis.

3.3.5 Dispersive Solid Phase Extraction procedure

The samples were removed from the freezer on the day of analysis, defrosted at room temperature and homogenized using a homogenizer. The homogenized muscle samples were weighed (1.00±0.005 g) into a 50 mL Falcon tubes. A 2:8 (v/v) mixture of water and acetonitrile (10 mL) was added to the samples. Magnesium sulphate and sodium chloride, 0.5 g each were then added to displace the extraction equilibrium towards the organic phase. The extracts were vortexed for 10 minutes and then centrifuged (5°C, 4000 rpm, and 10 minutes). The supernatants were decanted into a 15 mL tube containing 500 mg Sepra C_{18} SPE sorbent, vortex mixed for 5 minutes and then centrifuged (5°C, 4000 rpm) for 5 minutes. Aliquots (6 mL) of the resulting supernatants were transferred into graduated tubes and the contents were evaporated down to near dryness under a stream of N_2 flow in a turbovap concentrator at 40°C. The volume of the samples were then adjusted to 1 mL with water and filtered using 0.45 µm PVDF membrane filters. The filtered (20 µL) samples were injected into LC-MS/MS instrument for analysis. Table 3.2 below shows a flowchart for the dispersive solid phase extraction method.

Table 3.2: Flowchart for dispersive solid phase extraction procedure



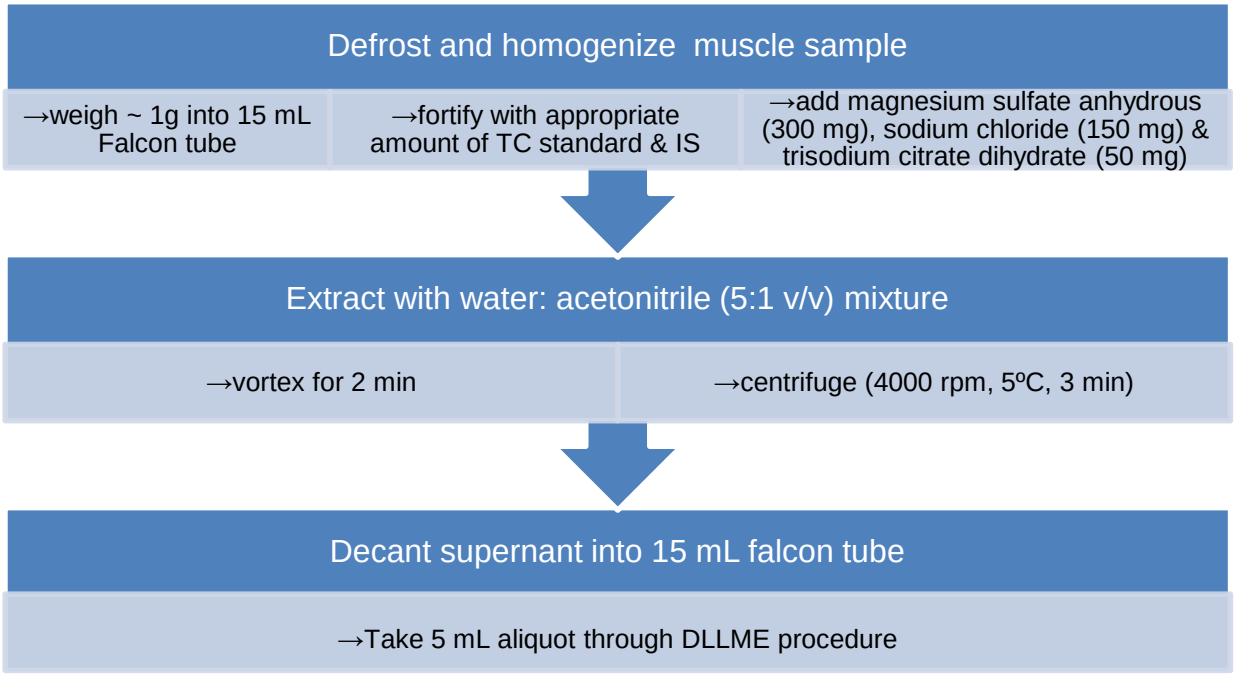
3.3.6 DLLME method development

3.3.6.1 DLLME method sample pre-treatment

When DLLME is applied to solid biological samples there is a need to first extract the analyte from a complex matrix such as muscle prior to applying DLLME procedure [26, 27, 106] as outlined in Table 3.3 below. The muscle samples were removed from the freezer on the day of analysis, defrosted at room temperature and homogenized. The homogenized samples were weighed (1.00±0.005 g) into a 15 mL Falcon tubes. The samples were treated with 6 mL of a 5:1 (v/v) mixture of water and acetonitrile. A 500 mg mixture of salts, i.e., magnesium sulfate anhydrous, sodium chloride and trisodium citrate dihydrate (3:1.5:0.5) was added to each

sample and vortex mixed for 2 minutes. The samples were then centrifuged (5°C, 4000 rpm) for 5 minutes. The supernatants were decanted into a 15 mL tube and then taken through the DLLME procedure.

Table 3.3: Flowchart for DLLME sample pre-treatment procedure

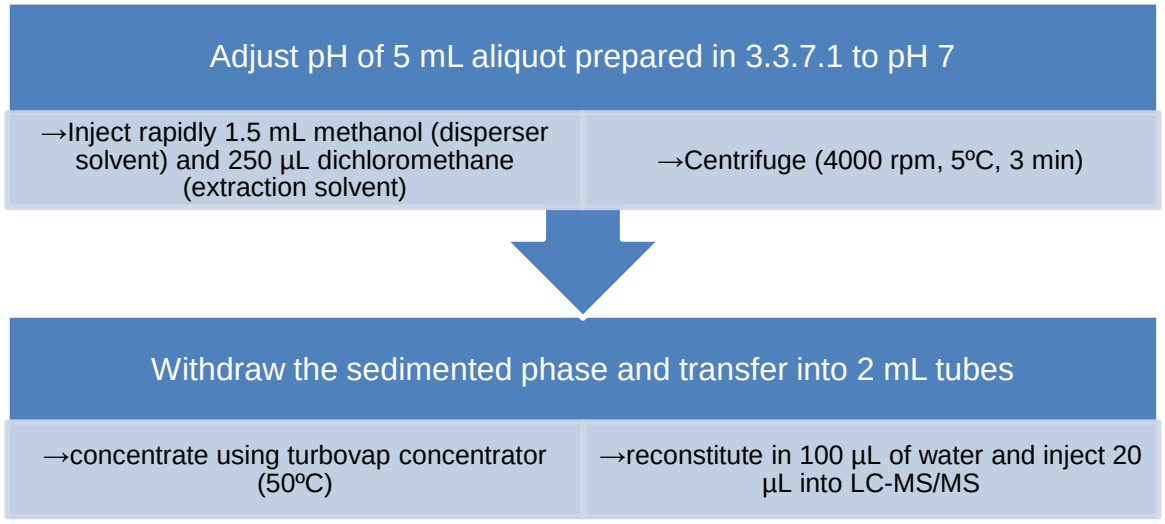


3.3.6.2 DLLME method sample preparation procedure

The pH of 1 mL aliquots of the supernatants prepared in Section 3.3.6.1 above were adjusted to pH 7 using sodium hydroxide solution and formic acid. For the DLLME procedure, 1.5 mL methanol (disperser solvent) and 250 µL chloroform (extracting solvent) were injected rapidly into the sample solutions contained in conical test tubes. Immediately after injection, cloudy solutions were formed which were then centrifuged for 5 min at 4000 rpm. The sedimented phases were withdrawn with micro syringes and transferred into 2 mL centrifuge tubes for evaporation of the

solvents using turbovap concentrator. The residues were reconstituted in 100 µL of water and 20 µL injected into the LC-MS/MS for analysis. It should be noted that the conditions described above were optimum. Thus disperser solvents (acetone, methanol and acetonitrile) and extraction solvents (chloroform, dichloromethane, trichloromethane and tetrachloroethylene) were investigated. The sample preparation procedure for DLLME method is presented in Table 3.4 below.

Table 3.4: Flow chart for the DLLME procedure



3.3.6.3 Procedure for Optimization of DLLME parameters

The influences of pH of the sample extract on the recovery of tetracycline analytes were investigated at a pH range of 2-12 by using hydrochloric acid and sodium hydroxide solution to adjust the pH while keeping other parameters constant (i.e. acetonitrile (1 mL) and dichloromethane (250 µL) as disperser and extraction solvents respectively).

Methanol, acetonitrile and acetone were tested as disperser solvents and the effect of these solvents on the performance of DLLME was investigated. The experiments were performed using sample extract at a constant pH of 7 and 250 µL of chloroform with 1.0 mL aliquot of each of acetone, methanol and acetonitrile. The DLLME procedure was followed and sedimented phases were dissolved in water and injected on LC-MS/MS.

Among the extraction solvents with density higher than water (mainly chlorinated solvents), dichloromethane (CH_2Cl_2) ρ = 1.33 g/cm^3, chloroform ($CHCl_3$) ρ = 1.48 g/cm^3, tetrachloroethylene ρ = 1.62 g/cm^3 ($Cl_2C=CCl_2$) and trichloroethylene ρ = 1.46 g/cm^3 (C_2HCl_3) were studied. In this experiment, 250 µL of each of the extraction solvents and 1 mL of methanol were added to a series of sample extracts (at pH 7). DLLME procedure was followed and the extracts injected on LC-MS/MS.

The effect of disperser solvent volume was investigated using different volumes of methanol (0.5, 1.0, 1.5, 2.0 and 2.5 mL) at constant volume of dichloromethane (250 µL) and pH 7 of the sample extract. The extraction solvent volume effect was investigated by keeping the methanol volume at 1.0 mL and sample extracts at pH 7 while varying volumes of dichloromethane from 100 to 300 µL. The sample extracts were taken through the DLLME procedure and analyzed as described above.

3.4 Procedure for DLLME method validation

The European Union (EU) Commission Decision 2002/657/EC directive that defines performance criteria for validation of analytical methods was used for DLLME

method validation. Several validation parameters such as linearity, recovery, precision, limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ) were determined under optimum conditions. Methacycline was used as an internal standard (IS). An equal volume (50 µL) of the IS was added to all samples and the ratio of peak area of the analytes to peak area of internal standard was used in the quantification of tetracyclines in all DLLME method validation experiments.

The validated DLLME method was applied to thirty bovine meat samples for the determination of tetracycline compounds. The bovine meat samples were obtained from the national abattoirs and local butcheries.

3.4.1 Procedure for linearity

Linearity was determined by fortifying blank muscle samples with tetracycline standard mixture at concentrations corresponding to eight calibration levels in the range of 25-200 µg kg^{-1}. A total of three spiked samples at each spiking level were taken through the entire DLLME procedure and used to construct a calibration curve.

3.4.2 Procedure for precision batches

For precision batches, four controls for each analytical batch were prepared. Each spiking level was represented by 7 replicates. The samples that were used to prepare the validation batch were the ones which were previously screened and found negative for the target analytes. Samples were spiked at 0 x MRL (blank), 0.5

x MRL (spike 1), 1 x MRL (spike 2) and 1.5 MRL (spike 3). The experiment was repeated on two occasions (two consecutive weeks). The data obtained from precision batches were used to calculate limit of detection, limit of quantification and method performance characteristics (CCα and CCβ) for the DLLME method.

3.4.3 Procedure for recovery studies

Muscle samples were spiked with tetracycline analytes at a range of concentrations (0 MRL, 0.5 MRL, MRL, 1.5 MRL and 2 MRL) known as pre extraction matrix spikes (PrEMS). These spiked samples were taken through the entire DLLME analytical procedure and used to construct a calibration curve. Twenty one (21) blank samples known as post extraction matrix spikes (PoEMS) were also taken through the analytical procedure and were spiked at three concentration levels (0.5 MRL, MRL and 1.5 MRL) after extraction. This procedure was repeated twice to make in total three batches. The PrEMS response curves were used to quantify the samples. The PoEMS were used to estimate overall method percentage recovery, relative standard deviation and reproducibility at each level.

3.5 Procedure for dSPE method verification

In duplicates, 1.0±0.05 g of homogenized subsamples were weighed into 50 mL Falcon tubes. Negative controls and pre-extracted matrix samples were fortified with appropriate volumes (0 x MRL, 0.5 x MRL, 1x MRL and 1.5 MRL) of spiking standard solution. A minimum of four calibration levels were used, with a duplicate PrEMS at each level. An equal volume (50 µL) of internal standard (methacycline) was added

to all samples and controls. For each PrEMS extract, additional negative control samples were taken through the dSPE procedure for preparation of equivalent post extracted matrix samples (PoEMS). To prepare PoEMS, spiking standards were added to samples labelled PoEMS to match the PrEMS prepared at the beginning of sample preparation procedure. The supernatants were filtered through 0.45 µm syringe filters into sample vials and the extracts injected onto the LC-MS/MS.

The validated DLLME method was applied to thirty bovine meat real samples for the determination of tetracycline compounds. The bovine meat samples were obtained from the national abattoirs and local butcheries.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 LC-MS/MS experiments

4.1.1 Optimization of Mass Spectrometer Compound Dependent Parameters

The compound dependent mass spectrometer parameters were optimized for each of the seven tetracycline compounds investigated in this study. This was achieved by the infusion of individual standard solutions of the seven tetracycline compounds using the Q1 scan type to determine the maximum signal intensity for each ion. Both ESI+ and ESI⁻ were attempted and it was found that all the seven compounds have maximum signal intensities of parent ions when using ESI+. This was followed by running MRM scan of a mixture of the seven compounds to obtain the most intense precursor/product ion pair for each compound with the corresponding optimal ion optics values. The optimized MS compound dependent parameters (Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE) and Collision Cell Exit Potential (CXP)) and ion source parameters (Gases, Temperature and Ion Spray Voltage) were used to create MS method that was used for subsequent experiments in this study. Using the optimized values ensures there is maximum transmission of ions and thus affords improved sensitivity. The results of the optimized MS compound dependent parameters and ion source parameters are presented in Table 4.1 and 4.2 respectively.

Table 4.1 Optimized mass spectrometer compound dependent parameters for each of the seven tetracycline compounds

Analytes & Transitions	MS Parameters						
	Q1 Mass (Da)	Q3 Mass (Da)	Dwell time (msec)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Chlortetracycline T1	479	443	40	61	10	29	24
Chlortetracycline T2	479	154	40	61	10	41	22
Chlortetracycline T3	479	303	40	61	10	31	16
Demeclocycline T1	466	431	40	31	10	33	36
Demeclocycline T2	466	289	40	31	10	51	14
Demeclocycline T3	466	262	40	31	10	63	12
Doxycycline T1	445	266	40	21	10	51	12
Doxycycline T2	445	320	40	21	10	43	18
Doxycycline T3	445	98	40	21	10	71	6
Methacycline T1	443	200	40	51	10	61	30
Methacycline T2	443	126	40	51	10	61	30
Methacycline T3	443	426	40	51	10	61	30
Minocycline T1	458	336	40	31	10	55	26
Minocycline T2	458	352	40	31	10	49	12
Minocycline T3	458	282	40	31	10	47	12
Tetracycline T1	445	410	40	54	10	33	6
Tetracycline T2	445	427	40	54	10	19	24
Tetracycline T3	445	97	40	54	10	29	6
Oxytetracycline T1	461	426	40	46	10	27	24
Oxytetracycline T2	461	443	40	46	10	19	12
Oxytetracycline T3	461	201	40	46	10	55	14

Table 4.2 Optimized Ion Source and Gas parameters

Source and Gas parameters	Optimal value
Ion Source Voltage (IS)	5500
Temperature (Temp)	500
Ion Source gas 1(GS1)	50
Ion source 2(GS2)	40
Curtain Gas(CUR)	25
Collision Gas (CAD)	Medium

4.1.2 Optimization of the HPLC separation

The optimized chromatographic separation was achieved by changing different mobile phase compositions and flow rates, one parameter at a time and noting the

improvement in the intensity, separation and symmetry of the peaks. The chromatographic run was done within 10 minutes which as a rule of thumb is considered relatively fast and less than 25 minutes which in residue analysis is considered acceptable. The chromatogram of the separated tetracycline compounds are presented in Figure 4.1 respectively. The average retention times and intensities of the tetracycline compounds are as in Table 4.3. Although the last three compounds (chlortetracycline, methacycline and doxycycline) are not well separated, they could still be fully identified and quantified in LC-MS/MS since the technique does not require analytes to be fully resolved or separated to be identified and quantified.

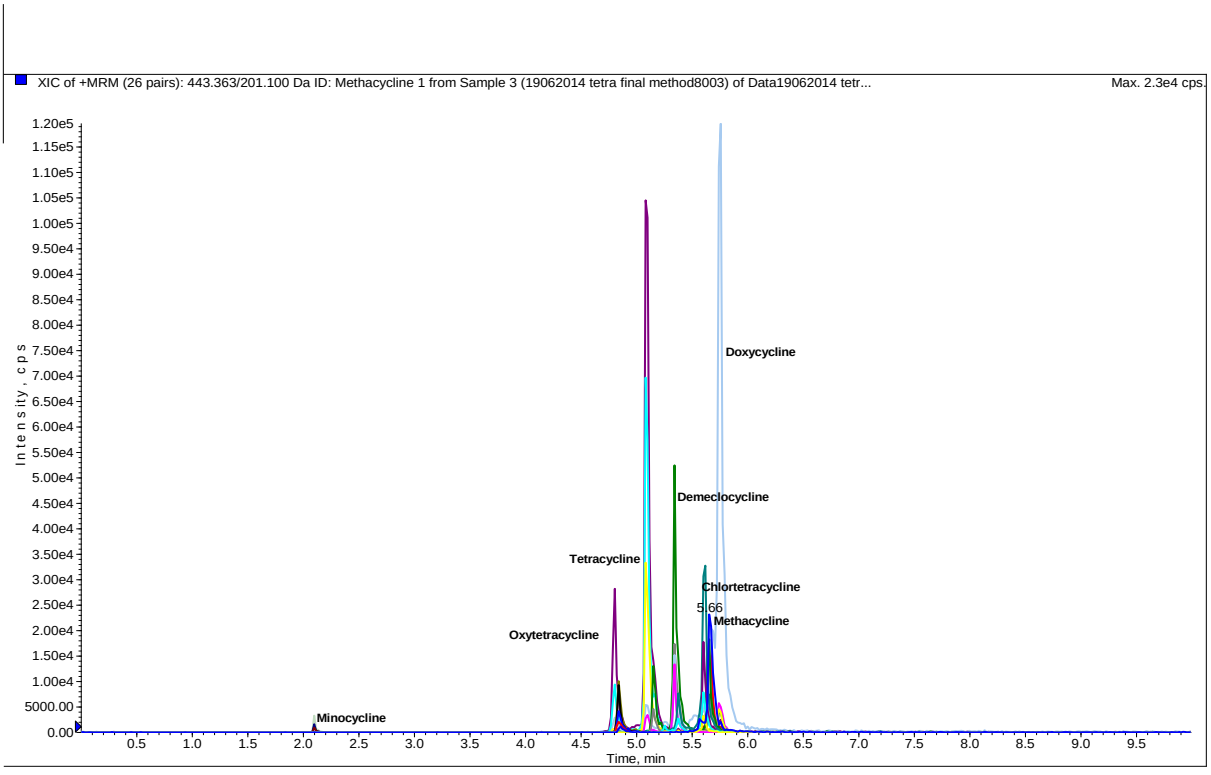


Figure 4.1 Chromatogram of seven TC compounds obtained using TC standard mixture at 100 µg kg^{-1}.

Table 4.3. Average retention times, intensities and parent ions of TC compounds

Compound name	Parent Ion m/z (Da)	Intensity (cps)	Retention time (min)
Minocyclline	221	3.0×10^3	2.10
Oxytetracycline	299	3.0×10^4	4.80
Tetracycline	445	1.05×10^5	5.10
Demeclocycline	341	5.5×10^4	5.40
Chlortetracycline	327	3.5×10^4	5.66
Methacycline	201	2.30×10^4	5.70
Doxycycline	319	1.2×10^5	5.80

4.2 DLLME method development and optimization

Dispersive liquid-liquid microextraction was used as a sample extraction and clean-up method for tetracyclines in bovine muscle samples. Extracting analytes from biological samples using DLLME presents challenges. Unlike in water where the analytes are in an aqueous phase, in biological samples there is a need to first extract analytes of interest from the solid matrix into an aqueous phase prior to application of DLLME procedure which is based on a ternary component solvent system of an aqueous sample, disperser solvent and extraction solvent. During the sample pre-treatment step, salts are added for analyte partitioning, phase separation, buffering and reducing the amount of co-extracted matrix that could hinder the quantitative transference of analytes from the aqueous phase to the organic phase. As with any sample preparation technique, one must still optimize the DLLME method. The sample pH, extraction time, type and volume of extraction and dispersive solvents are important factors to optimize [24, 28].

4.2.1 Optimization of pH

It is very important to optimize the pH of the aqueous solution because it determines the existing state of analytes (Figure 2.3), as well as the extraction efficiency of target compounds [24]. Thus the partitioning of an analyte from an aqueous phase into a hydrophobic organic solvent is greater for a neutral molecule than for an ionized species. Tetracyclines are amphoteric compounds with three functional groups (Figure 2.2) hence they are charged over a wide pH range [35, 39]. An appropriate pH should therefore be selected so that these analytes are extracted from the aqueous phase into the extraction solvent in their neutral form. In this work, the effect of pH on the extractability of tetracyclines was investigated in the range of 2 - 12 using formic acid and sodium hydroxide solution to adjust the pH. Acetonitrile (1 mL) and dichloromethane (250 µL) were used as disperser and extraction solvents respectively. The extracts were then injected onto the LC-MS/MS and data acquired using the Analyst software. Figure 4.2 shows the effect of variation of pH on the extraction efficiencies of tetracyclines. When the pH was in the range of 2-7, the extraction efficiency of all TCs increased gradually, whereas between 7 and 12, it decreased. High extraction efficiencies (70 – 84%) for all TC compounds were observed at pH 7. This is due to the fact that, in acidic solutions (pH 2-6), the chemical structures of TCs would be isomerized to 4-epimers, while in alkaline medium (pH 8-12) TCs are oxidized and in neutral solutions (pH 7), TCs exists as zwitterions (neutral analytes) [35, 39, 42]. So, neutrality (pH 7) was selected as extraction condition and used for subsequent experiments.

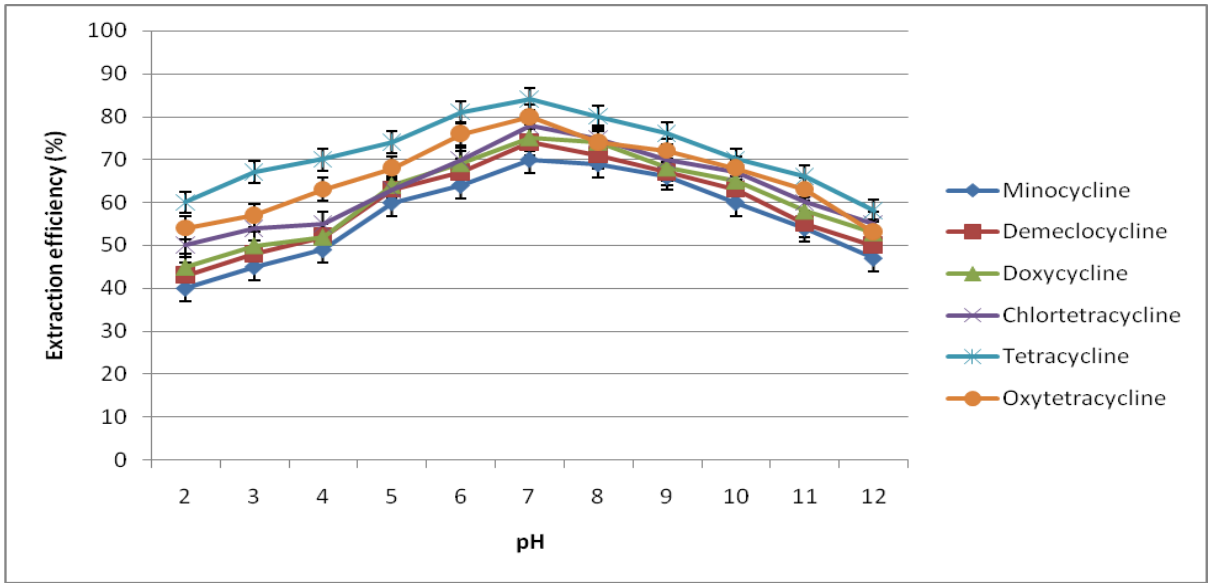


Figure 4.2:Effect of sample pH on extraction efficiencies of tetracyclines in blank muscle samples spiked at 50 µg kg^{-1}. DLLME conditions: sample extract volume, 5 mL; extraction solvent (dichloromethane) volume, 250 µL; disperser solvent (acetonitrile) volume, 1 mL; sample pH range 2-12.

4.2.2 Selection of disperser solvent

The selection of the disperser solvent is also crucial for the DLLME method. The dispersive solvent should be miscible with both water and the extraction solvent and should effectively dissolve the analytes. In this work acetone, acetonitrile and methanol were investigated. A 250 µL volume of dichloromethane and 1 mL of each of the disperser solvents were used for the optimization of disperser solvent. The extracts were then injected onto the LC-MS/MS and data acquired using the Analyst software. Figure 4.3 shows the results of the three disperser solvents investigated while maintaining the pH of the aqueous phase at pH of 7. On the basis of extraction efficiencies, methanol was observed to be the most efficient (efficiencies ranged from 72 to 92%) disperser solvent for all tetracyclines except minocycline which had

higher extraction efficiency (88%) in acetone. This could be due to the high solubility of minocycline in acetone than in methanol. The solubility of TCs decreases in the order of methanol, acetone and acetonitrile [110]. Therefore, methanol was selected as the dispersive solvent for this work.

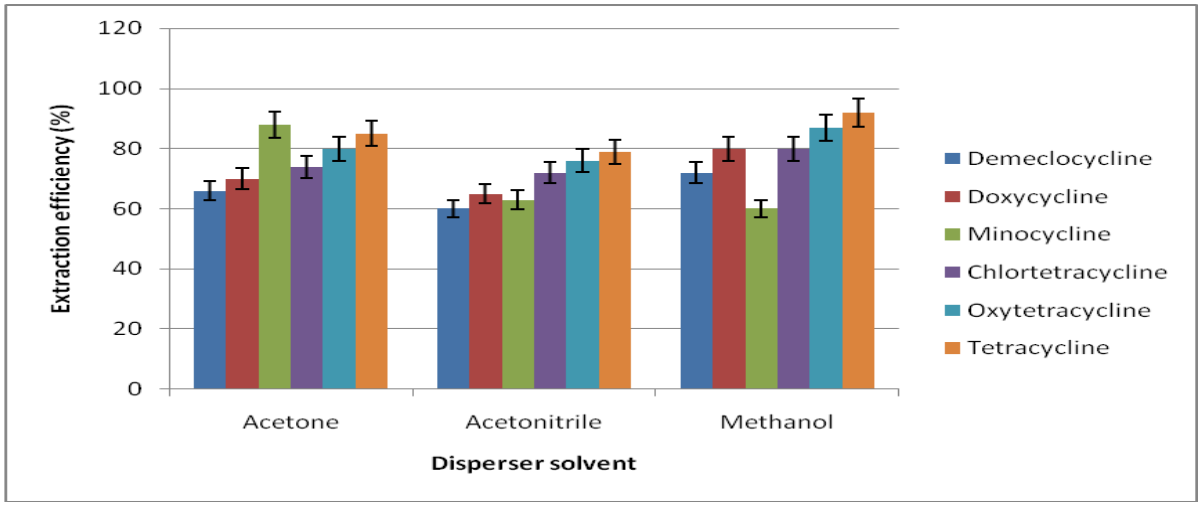


Figure 4.3: Effect of disperser solvent on extraction efficiencies of tetracyclines in blank muscle samples spiked at 50 µg kg^{-1}. DLLME conditions: sample extract volume, 5 mL; sample pH 7; extraction solvent (dichloromethane) volume, 250 µL; disperser solvents, 1 mL of each of acetone, acetonitrile and methanol.

4.2.3: Selection of extraction solvent

The extraction solvent is one of the most important parameters that requires to be optimized for successful use of DLLME in any group of compounds. In DLLME work, organic solvents are selected on the basis of their high density over water, immiscibility in water and extraction efficiency of compounds of interest. Four chlorinated solvents (dichloromethane (CH_2Cl_2) ρ = 1.33 g/cm^3, chloroform ($CHCl_3$) ρ = 1.48 g/cm^3, tetrachloroethylene ρ = 1.62 g/cm^3 ($Cl_2C=CCl_2$), and trichloroethylene ρ = 1.46 g/cm^3 (C_2HCl_3) were investigated for the extraction of tetracyclines. In this

experiment, 250 µL of each of the extraction solvents and 1 mL of methanol were added to a series of sample extracts (pH 7). The extracts were then injected onto the LC-MS/MS and data acquired using the Analyst software. All extraction solvents yielded satisfactory extraction efficiencies ranging from 68 to 98% (Figure 4.4). However, chloroform extracted all TCs better than the other extraction solvents with extraction efficiencies ranging from 75 to 98%. Despite chloroform being the best extraction solvent, dichloromethane which also yielded high efficiencies (74 – 95%) was chosen because it is less toxic than chloroform.

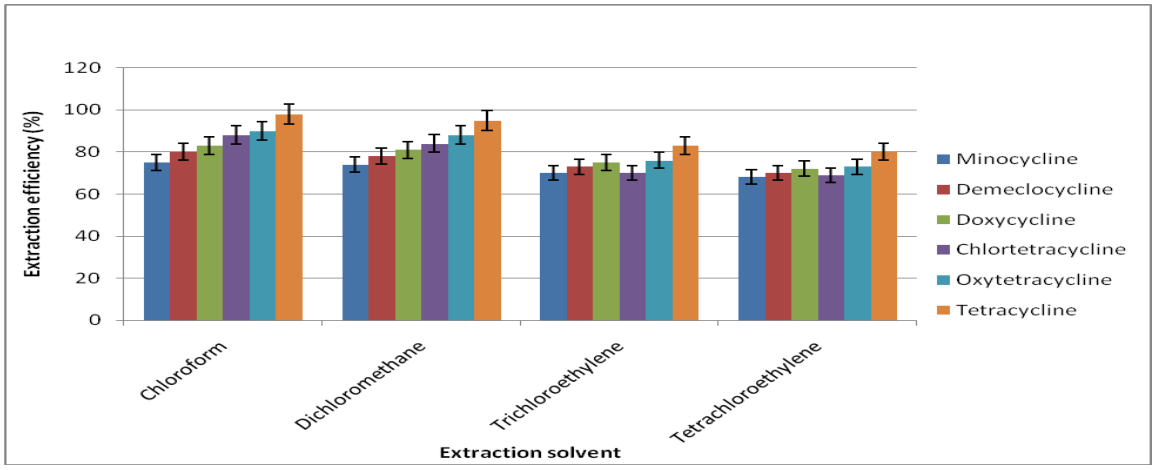


Figure 4.4: Effect of extraction solvent on extraction efficiencies of tetracyclines in blank muscle samples spiked at 50 µ gkg^{-1}. DLLME conditions: sample extract volume, 5 mL; sample pH 7; disperser solvent (methanol) volume, 1 mL; extraction solvents, 250 µL of each of chloroform, dichloromethane, trichloroethylene and tetrachloroethylene.

4.2.4: Optimization of disperser solvent volume

Disperser solvent volume has a significant effect on DLLME sample preparation because it affects the volume of the sedimented phase. The effect of disperser solvent volume was investigated with different volumes of methanol (0.25, 0.5, 1.0,

1.5, 2.0 and 2.5 mL) at constant volume of dichloromethane (250 µL). The extracts were injected onto the LC-MS/MS and data acquired using the Analyst software. It was observed that analytes extraction efficiencies increased with the increase of methanol volume from 0.25 to 1.0 mL and then decreased as methanol volume was increased from 1.5 to 2.5 mL (Figure 4.5). A similar trend has been previously observed by other researchers [24-26, 28]. The decrease in extraction efficiencies at low volumes of methanol was attributed to the fact that a cloudy state was not formed well, thus giving low efficiencies. On the other hand very high methanol volumes increase the solubility of analytes in water thus leading to decreased extraction efficiency due to a decrease in distribution coefficient [24, 28,104]. Based on the results in Figure 4.5 which showed the highest extraction efficiencies (75 – 91%) at a disperser solvent volume of 1.0 mL, this volume was chosen as the optimum for the rest of the work.

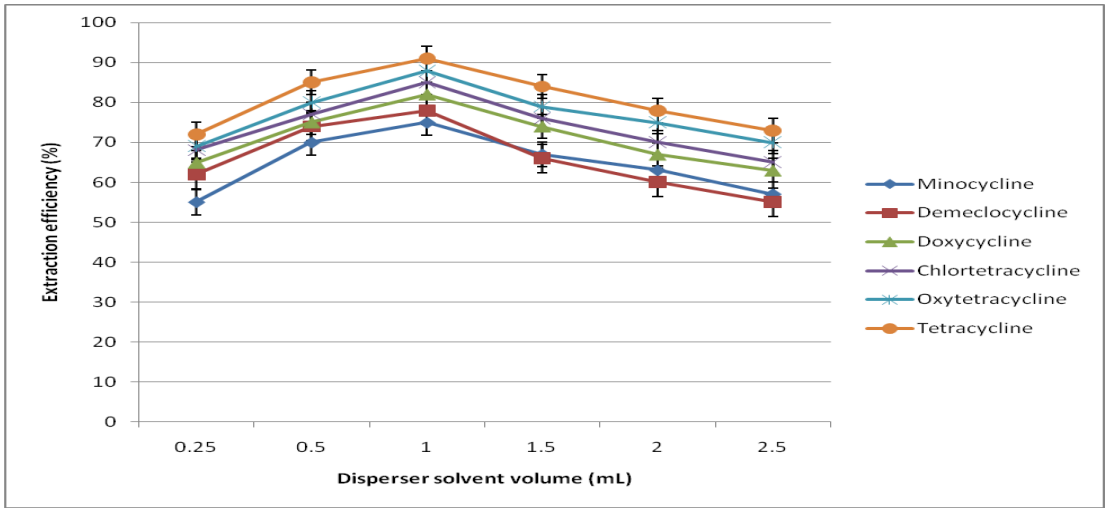


Figure 4.5: Effect of disperser solvent volume on extraction efficiencies of tetracyclines in blank muscle samples spiked at 50 µg kg^{-1}. DLLME conditions: sample extract volume, 5 mL; sample pH 7; extraction solvent (dichloromethane) volume, 250 µL; disperser solvent (methanol) volumes, 0.25-2.5 mL.

4.2.5 Optimization of extraction solvent volume

The volume of the extraction solvent is also one of the factors affecting extraction efficiency and enrichment factors of analytes in DLLME. The effect of extraction solvent volume was investigated by maintaining pH at 7, methanol volume at 1.0 mL while varying volumes of dichloromethane (50 - 300 µL). The extracts were injected onto the LC-MS/MS and data acquired using the Analyst software. Figure 4.6 shows the results of the effect of the variation of dichloromethane volume. The extraction efficiencies increased (45 – 96%) with increase in volume from 50 to 200 µL and then decreased (96 – 52%) as dichloromethane volume was increased to 300 µL. Other researchers have reported that this trend is usually a result of the dilution of the target analytes in the sedimented phase, because the higher the extracting volume, the greater the volume of the sedimented phase [24-26, 28, 104]. Thus, 200 µL of dichloromethane was used for all subsequent DLLME experiments.

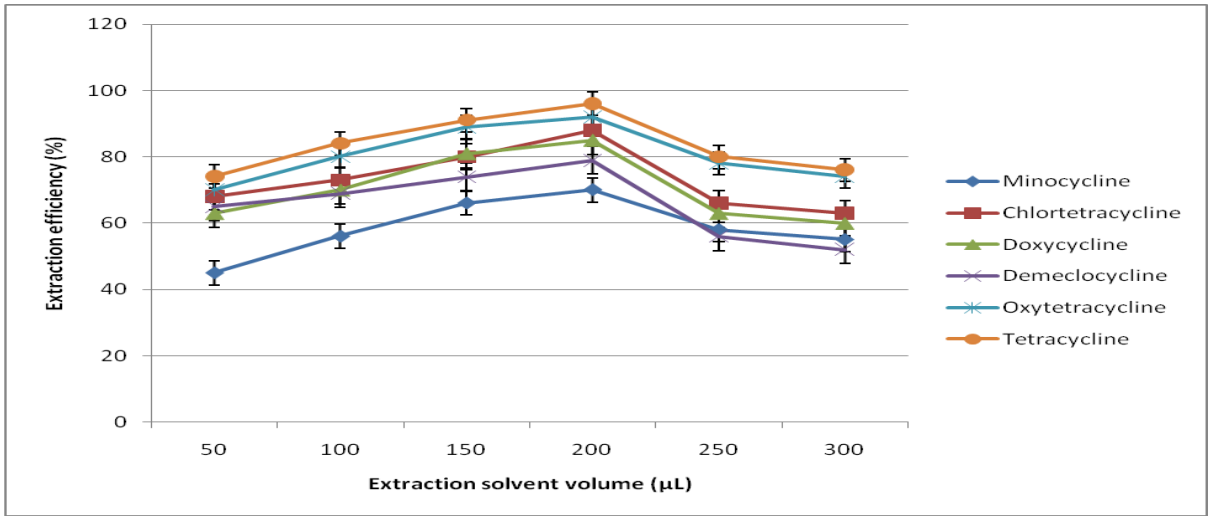


Figure 4.6: Effect of extraction solvent volume on extraction efficiencies of tetracyclines in blank muscle samples spiked at 50 µg kg^{-1}. DLLME conditions: sample extract volume, 5 mL; sample pH 7; disperser solvent (methanol) volume, 1 mL; extraction solvent (dichloromethane) volumes, 50-300 µL.

4.3; Validation of the DLLME method

The European Union (EU) Commission Decision 2002/657/EC Directive that defines performance criteria for validation of analytical methods were used for DLLME method validation [54]. Several validation parameters such as linearity, recovery, precision, limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ) were determined under optimum conditions. Methacycline was used as an internal standard (IS) and the ratio of peak area of the analyte to peak area of internal standard was used in the quantification of tetracyclines. Methacycline was chosen and used as internal standard because unlike other tetracyclines, it was never found in samples tested in our laboratory over the past ten years. This implied that it has not yet been used as an antibiotic locally. In places where it has been proven that it is used as an antibiotic then it would not be recommended for use as an internal standard since this could jeopardize the results.

4.3.1 Linearity

Linearity was determined using pre-extraction matrix spikes which are matrix samples that were fortified with tetracycline standard solutions prior to extraction. It was necessary to use matrix calibration standards to minimize matrix effects and improve the method precision. Linearity in the range of 25 - 200 µg kg^{-1} was obtained with regression coefficients ranging from 0.9994 to 0.9998 as indicated in Table 4.4.

4.3.2 Precision and recovery

A Microsoft Data Analysis toolpak was then performed on the 21 resulting measured concentrations (7 replicates x 3 batches) at each spiking level to determine validation parameters. The validation results are reported below and displayed in Tables 4.4 and Table 4.5. The mean recoveries of spiked blank muscle samples at three levels (i.e. 50, 100 and 150 µg kg^{-1}) ranged from 80 to 101% and the reproducibility was between 2 and 7%. The recoveries are satisfactory as they fall within the acceptable range of 80 – 110% and the method is reproducible (%CV are less than 23) according to the EU Commission Decision 2002/657/EC directive [54].

Table 4.4: Analytical performance parameters for the determination of six tetracycline antibiotics in muscle samples using DLLME method at n = 21.

Analyte	**Linear range (µg kg^{-1})**	**R^2**	**LOD (µg kg^{-1})**	**LOQ (µg kg^{-1})**	**CCα (µg kg^{-1})**	**CCβ (µg kg^{-1})**	**Reproducibility (%CV) at MRL (100 µg kg^{-1})**
Chlortetracycline	25-200	0.9997	2.2	7.3	111	122	7
Demeclocycline	25-200	0.9994	2.9	9.7	110	117	4
Doxycycline	25-200	0.9998	2.2	7.4	110	122	7
Minocycline	25-200	0.9995	7.4	11.5	110	117	4
Oxytetracycline	25-200	0.9996	2.4	8.0	105	107	2
Tetracycline	25-200	0.9997	2.5	8.3	108	114	4

Table 4.5: Recovery studies of tetracycline antibiotics in fortified blank muscle samples using DLLME method

Analyte	**Spiking level (µg kg^{-1})**	**Mean recovery (%) n = 21**	**RSD%**	**CV%**
Chlortetracycline	50	101	9.5	10
	100	95	7.4	7
	150	93	4.3	4
Demeclocycline	50	90	8.9	8
	100	89	6.7	6
	150	80	12.5	10
Doxycycline	50	93	6.4	6
	100	97	7.2	7
	150	101	5.9	6
Minocycline	50	92	8.7	8
	100	87	6.9	6
	150	80	12.5	10
Oxytetracycline	50	103	8.7	9
	100	96	9.4	9
	150	91	7.7	7
Tetracycline	50	101	10.6	11
	100	101	7.9	8
	150	97	8.2	8

4.3.3 Method performance characteristics (CCα and CCβ)

The method performance characteristics (decision limit and detection capacity) were also determined. The decision limit is defined as the limit above which it can be concluded, with an error probability of α, that a sample is non-compliant (higher than the MRL). Detection capability is defined as the smallest content of the substance that may be detected, identified and quantified in a sample with an error probability of β. The decision limit, CCα was calculated as the mean measured concentration at MRL + 1.64 x in-house reproducibility at this concentration and the detection capacity, CCβ was calculated as CCα + 1.64 x the in-house reproducibility at MRL. Table 4.4 shows that the obtained CCα ranged from 105 to 111 µg kg^{-1} while CCβ ranged from 107 to 122 µg kg^{-1}.

4.3.4 Limit of detection and limit of quantification

The LOD and LOQ were calculated from the mean signal of 10 muscle blank samples. Limit of dectection was calculated as the mean of blank concentration plus three times standard deviation of concentration of the 10 blank samples. Limit of quantification was calculated as mean of blank concentration plus ten times standard deviation of concentration of the blank samples. Table 4.4 above shows that LODs and LOQs ranged from 2.2 to 7.4 µg kg^{-1} and from 7.3 to 11.5 µg kg^{-1} respectively.

4.4 Dispersive Solid Phase Extraction method verification

A single factor analysis of variance (ANOVA) from Microsoft Analysis toolpak was performed on the 21 measured concentrations (7 replicates x 3) at each spiking level to obtain verification parameters such as recoveries, repeatability, LOD and LOQ for the dSPE method which are displayed in Table 4.6 below. The average percentage recoveries and reprodubicility (%CV) ranged from 83 - 99% and 4 - 13% respectively showing that the dSPE is accurate and precise according to the Commission Decision 2002/657/EC directive [54].

Table 4.6: Recovery studies of tetracycline antibiotics in fortified blank muscle samples using dSPE method

Analyte	**Spiking level (µg kg^{-1})**	**Mean recovery (%) n=21**	**RSD%**	**CV%**
Chlortetracycline	50 100 150	96 94 97	11.94 5.69 8.21	12 6 8
Demeclocycline	50 100 150	88 94 83	9.90 4.98 11.13	9 5 11
Doxycycline	50 100 150	96 94 98	7.45 13.18 6.06	7 13 6
Minocycline	50 100 150	94 90 88	7.90 10.11 8.75	8 10 8
Oxytetracycline	50 100 150	99 98 88	9.72 9.14 5.07	10 9 5
Tetracycline	50 100 150	96 99 98	11.42 6.15 4.24	11 6 4

4.5 Comparison of DLLME and dSPE

The proposed DLLME method was compared with a SANAS accredited method (dSPE) using a paired t-test to find out if there was any significant difference between the two methods. Mean recoveries of tetracycline analytes for the two analytical methods were compared at concentrations of 50, 100 and 150 µg kg^{-1} (i.e. 0.5, 1.0 and 1.5 x MRL). The results in Table 4.7 show that the calculated t-value for all analytes is less than the t-critical value of 4.30 therefore, the results obtained by DLLME and dSPE do not differ significantly in terms of accuracy. Dispersive liquid-liquid microextraction is thus very attractive and a preferred method than dispersive solid phase extraction as it is cheaper, simpler, efficient and greener (DLLME uses

microliter volumes of organic solvents while dSPE uses more than 5 mL volumes of organic solvents). Figure 4.7 below shows chromatograms of spiked meat samples obtained using DLLME and dSPE methods.

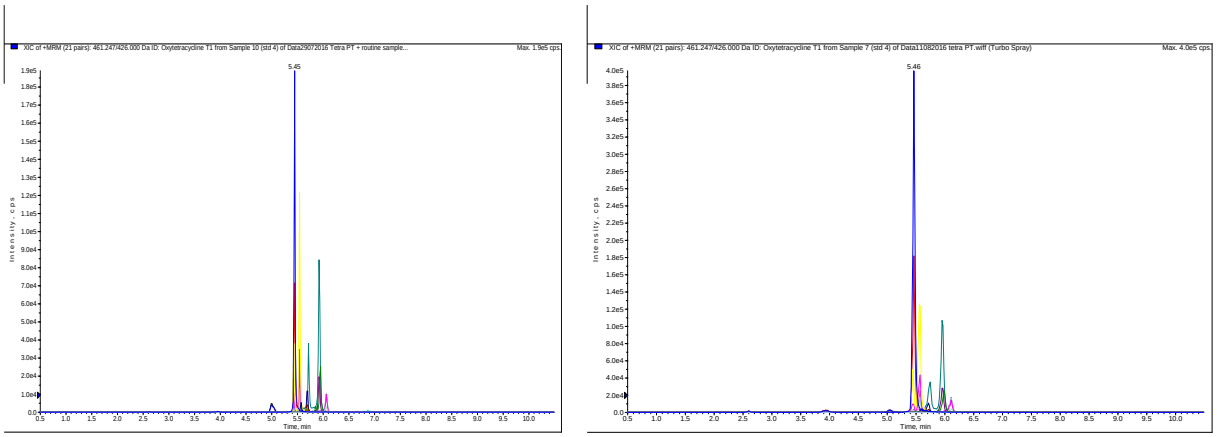


Figure 4.7: Chromatograms of spiked meat samples obtained using DLLME and dSPE respectively

Table 4.7: Comparison of DLLME with dSPE method using a paired t-test; mean recovery values of tetracycline analytes were compared at spiking levels of 50, 100 and 150 µg kg^{-1}.

Analyte	**Method**	**Spiking levels**			**SD of the Difference**	**t-value**	**t-critical value**
		50	**100**	**150**			
chlortetracycline	dSPE	48	94	145.5	4.5	0.45	4.30
	DLLME	50.5	95	139.5			
demeclocycline	dSPE	44	94	124.5	3.3	2.08	4.30
	DLLME	45	89	120			
Doxycycline	dSPE	48	94	147	3.1	-1.57	4.30
	DLLME	46.5	97	151.5			
Minocycline	dSPE	47	90	132	5.86	2.22	4.30
	DLLME	46	87	120			
Oxytetracycline	dSPE	49.5	98	142.5	4.0	1.22	4.30
	DLLME	51.5	96	136.6			
Tetracycline	dSPE	48	99	147	2.18	-1.12	4.30
	DLLME	50.5	101	145.5			

4.6 DLLME application on real meat samples

The proposed method was successfully applied for the determination of tetracyclines in meat samples. Eleven of the thirty bovine muscle samples obtained from the local abattoirs were found to contain two types of tetracycline antibiotic residues (chlortetracycline and oxytetracycline) with oxytetracycline being the most detected. The two tetracyclines were detected probably because they are mostly used and their withdrawal periods were not observed. The concentration levels of the tetracycline residues detected in eleven bovine muscle samples reported in Table 4.8 were between 38.4 and 82.3 µg kg^{-1}, levels that are lower than the European Union set maximum residue level (MRL) of 100 µg kg^{-1}. Therefore, the meat was compliant and fit for human consumption. Figure 4.8 shows chromatogram of a real meat sample.

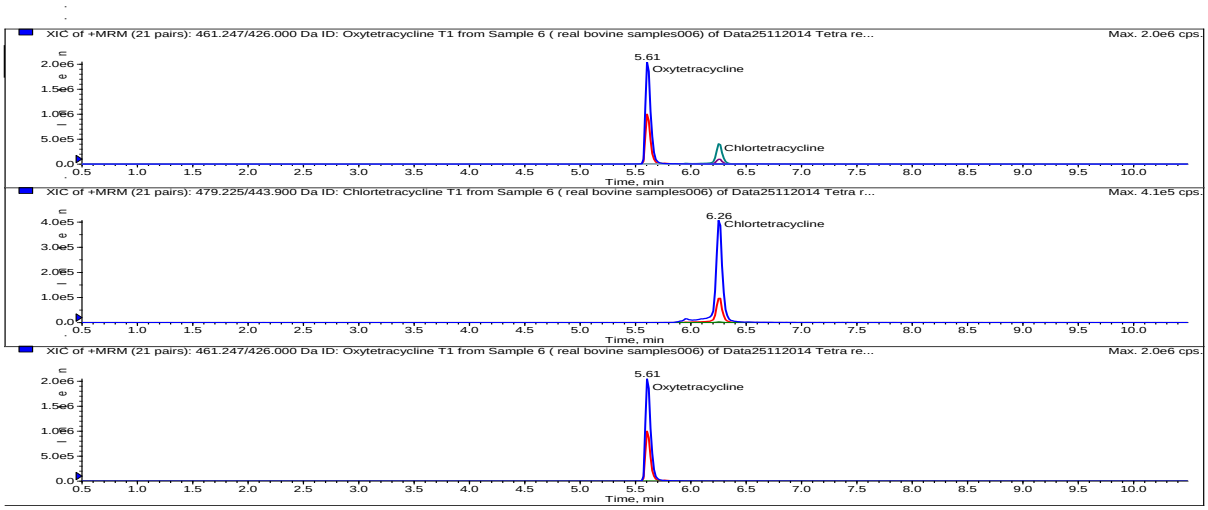


Figure 4.8: Chromatogram of a real meat sample

Table 4.8: Tetracycline levels found in bovine muscle samples from two beef exporting abattoirs and three local butcheries (Botswana)

Sample	**TC ($\mu g\ kg^{-1}$)**	**CTC ($\mu g\ kg^{-1}$)**	**MNC ($\mu g\ kg^{-1}$)**	**DXC ($\mu g\ kg^{-1}$)**	**OTC ($\mu g\ kg^{-1}$)**	**DMC ($\mu g\ kg^{-1}$)**
1	nd	46.3	nd	nd	nd	nd
2	nd	58.4	nd	nd	76.5	nd
3	nd	49.0	nd	nd	67.6	nd
4	nd	nd	nd	nd	58.0	nd
5	nd	nd	nd	nd	74.0	nd
6	nd	73.4	nd	nd	82.3	nd
7	nd	nd	nd	nd	38.4	nd
8	nd	39.0	nd	nd	nd	nd
9	nd	52.6	nd	nd	51.0	nd
10	nd	nd	nd	nd	46.7	nd
11	nd	61.5	nd	nd	42.5	nd

nd = not detected.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

This work was set out to develop a rapid, cost effective and 'green chemistry' method (DLLME) for analysis of tetracycline residues in meat by LC-MS/MS to determine their prevalence below and or above MRLs. An attractive dispersive liquid-liquid microextraction method combined with liquid chromatography tandem mass spectrometry was successfully developed, optimized and validated for the extraction and rapid determination of tetracycline antibiotics in meat samples. The tetracyclines in this study were found to extract well when the sample extract pH was neutral, methanol and dichloromethane were used as disperser and extraction solvents respectively. Under the optimal conditions, validation parameters such as linearity (0.9994 to 0.9998), recoveries (80 to 101%), LODs (2.2 to 7.4 µg kg^{-1}), LOQs (7.3 to 11.5 µg kg^{-1}), CCα (105 to 111 µg kg^{-1}), CCβ (107 to 122 µg kg^{-1}) and reproducibility (2 to 7%) were established, all of which confirmed the applicability of the proposed method to real samples as an extraction and clean-up method. The validated method was used for the quantification of tetracycline residues in meat samples. About 37% of the analyzed samples contained chlortetracycline and oxytetracycline residues at levels that are lower than the EU set MRLs of 100 µg kg^{-1} hence the meat was compliant and fit for human consumption.

The DLLME method was compared with a SANAS accredited dSPE method in terms of accuracy and did not differ significantly therefore it may be used as a routine

method for the determination of tetracyclines in meat samples due to its advantages over other methods. Through this study, it was proven that the DLLME method is not only limited to water samples but can be applied with accuracy to solid biological matrices as well for the determination of veterinary drugs/antibiotics.

5.2 Recommendations

The LODs and LOQs of the method could be further improved by investigating the matrix effects to further reduce ion suppression. The present study indicates the presence of some tetracycline residues in meat from various slaughterhouses in Botswana and as such regulatory authorities should constantly conduct surveillance on withdrawal periods to ensure that they are observed before cattle are slaughtered. The DLLME method can probably be applied with slight modifications for the determination of other drug residues in various matrices. Another recommended modification to this method is to explore the use of ionic liquids to completely eliminate the use of toxic organic solvents and fulfil the requirements of 'green chemistry'.

REFERENCES

1. Bogialli S, Curini R, Di Corcia A, Laganà A, Rizzuti G., A rapid confirmatory method for analyzing tetracycline antibiotics in bovine, swine, and poultry muscle tissues: Matrix solid-phase dispersion with heated water as extractant followed by liquid chromatography-tandem mass spectrometry. *Journal of Agricultural Food Chemistry*. 2006; 54 (5): 1564-1570.

2. Delahaut P, Levaux C, Eloy P, Dubois M., Validation of a method for detecting and quantifying tranquillisers and a [beta]-blocker in pig tissues by liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta*. 2003; 483 (1-2): 335-340.

3. Gentili A, Perret D, Marchese S., Liquid chromatography-tandem mass spectrometry for performing confirmatory analysis of veterinary drugs in animal-food products. *Trends in Analytical Chemistry*. 2005; 24 (7): 704-733.

4. Mastovska K., Multiresidue analysis of antibiotics in food of animal origin using liquid Chromatography–Mass spectrometry. *Methods in Molecular Biology (Clifton, NJ)*. 2011; 747: 267-307.

5. Van Dresser WR, Wilcke JR., Drug residues in food animals. *Journal of American Veterinary Medicine Association*. 1989; 194 (12): 1700-1710.

6. Paige J, Tollefson L, Miller M., Public health impact on drug residues in animal tissues. *Veterinary Human Toxicology*. 1997; 39 (3): 162-169.

7. Niessen W., Analysis of antibiotics by liquid chromatography–mass spectrometry. *Journal of Chromatography A*. 1998; 812 (1): 53-75.

8. Doyle MP, Busta F, Cords BR, Davidson PM, Hawke J, Hurd HS, Isaacson RE, Matthews K, Maurer J, Meng J., *Antimicrobial resistance: Implications for the food system. Comprehensive Reviews in Food Science and Food Safety*. 2006; 5 (3), 71-137.

9. Smith DL, Dushoff J, Morris JG., Agricultural antibiotics and human health - Does antibiotic use in agriculture have a greater impact than hospital use? *PLOS Medicine*. 2005; 2 (8):171-175.

10. Phillips I., Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health. *International Journal of Antimicrobial Agents*. 2007; 30 (2): 101-107.

11. Rambla-Alegre M., Is it really necessary to control the abuse of antibiotics in food-producing animals? *Biochemistry Pharmacology*. 2012; 1: e123.

12. Council regulation (EEC) no. 2377/90 of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Official Journal of the European Communities*. 1990; 224 (18/08): 0001-0008.

13. Joint FAO/WHO Codex Alimentarius Commission. *Codex alimentarius.* Vol 5. Food and Agriculture Organization of the United Nations; 1992.

14. Commission regulation (EC) 1442/95, 1995, of 26 June 1995 amending annexes I, II, III and IV to regulation (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. *Official Journal of the European Communities*. 1995; 143: 26-30.

15. Zhuang W., The comprehensive compilation of national maximum residue limits for pesticides, veterinary drugs in foodstuffs and feedstuffs in the world. *China Foreign Economic Trade Publishing House*. 1995.

16. FDA U., Guidance for industry. *Q9 Quality Risk Management. Rockville, MD*. 2006.

17. European Commission. Commission regulation (EU) no. 37/2010 of 22 december 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of European Union*. 2010; 15: 1-72.

18. Haagsma N, Bathelt E, Engelsma J., Thin-layer chromatographic screening method for the tranquillizers azaperone, propionylpromazine and carazolol in pig tissues. *Journal of Chromatography A*. 1988; 436: 73-79.

19. De Wasch K, Okerman L, De Brabander H, Van Hoof J, Croubels S, De Backer P., Detection of residues of tetracycline antibiotics in pork and chicken meat: Correlation between results of screening and confirmatory tests. *Analyst*. 1998; 123 (12): 2737-2741.

20. Aga D S, Goldfish R, Kulshrestha P., Application of ELISA in determining the fate of tetracyclines in land- applied livestock wastes. *Analyst*. 2003; 128: 658-662.

21. Barker SA, Walker CC., Chromatographic methods for tetracycline analysis in foods. *Journal of Chromatography A.* 1992; 624 (1-2): 195-209.

22. Pena A, Pelantova N, Lino C, Silveira M, Solich P., Validation of an analytical methodology for determination of oxytetracycline and tetracycline residues in honey by HPLC with fluorescence detection. *Journal of Agricultural Food Chemistry*. 2005; 53 (10): 3784-3788.

23. Kodba ZC, Voncina DB., A rapid method for the determination of organochlorinated, pyrethroid pesticides and polychlorobiphenyls in fatty foods using GC with electron capture detection. *Chromatographia*. 2007; 66: 619-624.

24. Rezaee M, Assadi Y, Milani Hosseini MR, Aghaee E, Ahmadi F, Berijani S., Determination of organic compounds in water using dispersive liquid–liquid microextraction. *Journal of Chromatography A*. 2006; 1116 (1-2): 1-9.

25. Rezaee M, Yamani Y, Faraji M., Evolution of dispersive liquid-liquid microextraction method. *Journal of Chromatography A*. 2010; 1217 (16): 2342-2357.

26. Tsai WH, Huang TC, Huang JJ, Hsue YH, Chuang HY., Application of dispersive liquid-liquid microextraction and dispersive micro-solid-phase extraction for the determination of quinolones in swine muscle by high-performance liquid chromatography with diode-array detection. *Analytica Chimica Acta*. 2009; 656 (1-2): 56-62.

27. Moema D, Nindi MM, Dube S., Development of a dispersive liquid-liquid microextration method for the determination of fluoroquinolones in chicken

liver by high performance liquid chromatography. *Analytica Chimica Acta*. 2012; 730 (1): 80-86.

28. Kozani RR, Assadi Y, Shemirani F, Hosseini MRM, Jamali MR., Determination of Trihalomethanes in drinking water by DLLME then GC with electron-capture detection. *Talanta*. 2007; 72: 287-393.

29. Di Corcia A, Nazzari M., Liquid chromatographic–mass spectrometric methods for analyzing antibiotic and antibacterial agents in animal food products. *Journal of Chromatography A*. 2002; 974 (1): 53-89.

30. Marazuela M, Bogialli S., A review of novel strategies of sample preparation for the determination of antibacterial residues in foodstuffs using liquid chromatography-based analytical methods. *Analytica Chimica Acta*. 2009; 645 (1-2): 5-17.

31. Toussaint B, Boardin G, Janosi A, Rodriguez A.R., Validation of a liquid chromatography tandem mass spectrometry method for the simultaneous quantification of 11 (fluoro)quinolones antibiotics in swine kidney. *Journal of Chromatography A.* 2002; 976: 195-206.

32. Kemp I, Gesbert M, Guiltel M, Bennejean G., Quinolones antibiotic agents. *Journal of Veterinary Science.* 1992; 53: 257.

33. Mann D.D, Frame G.M., Pharmacokinetic study of danofloxacin in cattle and swine. *American Journal of Veterinary Research.* 1992; 59: 765-771.

34. Chopra I, Roberts M., Tetracycline antibiotics: mode of action, applications, molecular biology and epidemiology of bacterial resistance. *Microbiology, Molecular Biology Revision.* 2001; 65 (2): 232-260.

35. Stephens CR, Murai K, Brunings KJ, Woodward RB., Acidity constants of the tetracycline antibiotics. *Journal of American Chemical Society.* 1956; 78: 4155-4158.

36. Pena A, Carmona A, Barbosa A, Lino C, Silveira I, Castillo B., Determination of tetracyclines and its major degradation product by liquid chromatography with fluorescence detection. *Journal of Pharmaceutical and Biomedical Analysis.* 1998; 18: 839-845.

37. Oka H, Nakagawa H, Haranda KI, Mac Neil JD., Chemical analysis for antibiotic used in Agriculture. *Journal of AOAC International.* 1995; 332-346.

38. Waller CW, Hutching BL, Wolf CF, Goldman AA, Broschard RW, Williams JH., Degradation of aureomycin vi, isoaureomycin and aureomycin. *Journal of American Chemical Society.* 1952; 74: 4981-4981.

39. Baker B, Leyland D., The chemistry of tetracycline antibiotics. *Journal of the Association of Chromatography.* 1983; 24: 30-35.

40. Stephens CR, Conover LH, Pasternack R, Hochstein FA, Moreland WT, Regna PP., Terramycin .12. The Structure of Aureomycin. *Journal of American Chemical Society.* 1954; 76(13): 3568-3575.

41. Arikan OA, Sikora LJ, Mulbry W, Khan SU, Rice C, Foster GD., The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves. *Process Biochemistry*. 2006; 41(7): 1637-1643.

42. Mitscher LA, Slater-Eng B, Sokoloski TD., Circular dichroism measurement of the tetracyclines, 5-hydroxylated derivatives. *Antimicrobial Agents Chemotherapy.* 1972; 2 (2): 66-72.

43. Wan-Ru Chen., Interactions of tetracycline antibiotics with dissolved metal ions and metal oxides. *School of civil and environmental engineering, Georgia Institute of Technology.* 2008.

44. Sauli S, Sauli N, Alsancak G., Determination of protonation constants of some tetracycline antibiotics by potentiometry and liquid chromatography methods in water and acetonitrile-water binary mixture. *Journal of Brazilian Chemical Society.* 2009; 20 (5): 939-946.

45. Fang Y, Zhai H, Zou Y, Zweigenbaum J., Determination of multi-residue tetracyclines and their metabolites in milk by liquid chromatography tandem mass spectrometry. *Agilent Technologies incl.2009, Wilmington, DE 19809. drugs.com.know more. Be sure. Tetracycline. www.agilent.com/chem.*

46. Cristofani E, Antonini C, Tovo G, Fioroni L, Piersanti A, Galarini R., A confirmatory method for the determination of tetracyclines in muscle using high-performance liquid chromatography with diode-array detection. *Analytica Chimica Acta*. 2009; 637(1-2): 40-46.

47. Cherlet M, Schelkens M, Croubels S, De Backer P., 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*. 2003; 492 (1-2): 199-213.

48. Blasco C, Corcia AD, Picó Y., Determination of tetracyclines in multi-species animal tissues by pressurized liquid extraction and liquid chromatography-tandem mass spectrometry. *Food Chemistry*. 2009; 116 (4): 1005-1012.

49. Stolker A, Brinkman U., Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals- a review. *Journal of Chromatography A*. 2005; 1067 (1): 15-53.

50. Kennedy D, McCracken R, Cannavan A, Hewitt S., Use of liquid chromatography–mass spectrometry in the analysis of residues of antibiotics in meat and milk. *Journal of Chromatography A*. 1998; 812 (1): 77-98.

51. Mathers JJ, Flick SC, Cox Jr LA., Longer-duration uses of tetracyclines and penicillins in US food-producing animals: Indications and microbiologic effects. *Environmental International Journal*. 2011; 37 (5): 991-1004.

52. JOSÉ MANUEL BARROSO (COMMISSION, T. P. (2009)) on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, in: COMMISSION, T. E. (Ed.) *COMMISSION REGULATION (EU) No 37/201* (Brussels, THE EUROPEAN COMMISSION).

53. DIRECTIVE HASAT. Council directive 96/23/EC of 29 April 1996 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 decisions 89/187/EEC and 91/664/EEC. *Official Journal European Union*. 1996; 125 (23/05): 0010-0032.

54. European Commission. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, as amended by Decision 2003/181/EC (4). *Official Journal of the European Communities.* 2002: 8-36.

55. Bruno Le Bizec GP, Jean-Philippe A., Options for veterinary drug analysis using mass spectrometry. *Journal of Chromatography A*. 2009; 1216 (16): 8016-34.

56. DIRECTORATE-GENERAL, E. C. H. C. P., Guidelines for the implementation of decision 2002/657/ec 2, in: Commission, e. (ed.) *Sanco/2004/2726-Rev 4-December 2008*. 2008.

57. Pöttering HG., (European Parliament, Kohout PJ (Council regulation (EC) no. 470/2009 of the European parliament and of the council. Strasbourg, *Official Journal of the European Union*. 2009.

58. Eurachem group. The fitness for purpose of analytical methods, a laboratory guide to method validation and related topics. *Eurachem Guide.* 1998.

59. Baulac S, Barron D, Sanz-Nebot V, Barbosa J., Determination of fluoroquinolones in chicken tissues by liquid chromatography coupled with

electrospray ionisation and atmospheric pressure chemical ionisation. *Journal of Separation Science*. 2006; 29 (1): 131-6.

60. Martins-Junior HA, Kussumi TA, Wang AY, Lembre DT., A rapid method to determine antibiotic residues in milk using liquid chromatography coupled to electrospray tandem mass spectrometry. *Journal of Brazilian Chemical Society*. 2007; 18 (2): 255-451.

61. Zenxuan C, Zhang Y, HongFeng P, Xiaowen T, Yiping R., Simultaneous determination of 24 sulfonamides residues in meat by ultra-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A.* 2008; 1200 (2): 144-155.

62. Mulders EJ, Van de lagemaat D., Determination of residues of tetracycline antibiotics in animal tissues by HPLC. *Journal of Pharmaceutical Biomedical Analysis*. 1989; 7 (12): 1829-1835.

63. Wang J., Analysis of macrolide antibiotics using liquid chromatography-mass spectrometry in food, biological and environmental matrices. *Mass Spectrometry Revisions*. 2009; 28 (1): 50-92.

64. Lindsey ME, Meyer MT, Thurman EM., Analysis of trace levels of sulphonamides and tetracycline antimicrobials in ground water and surface using solid-phase extraction and liquid chromatography mass spectrometry. *Analytical Chemistry.* 2001; 74: 4640-4646.

65. Meyer MT, Lee EA, Ferrell GM, Blumgarner JE, Varns J., Evaluation of offline tandem and online solid phase extraction with liquid chromatography electrospray ionisation- mass spectrometry for analysis of antibiotics in

ambient water and comparison to an independent method. *United State Geological Survey Scientific Investigation Report*. 2007; 5021.

66. Ocsana O, Maria-Loredana S, Virginia C, Florina C, Dumitru R., Determination of some frequently used antibiotics in waste waters using solid phase extraction followed by HPLC with diode array and mass spectrometry detection. *Central European Journal of Chemistry.* 2013; 11(8) 1345-1351.

67. Pena-Pereira F, Lavilla I, Bendicho C., Miniatured pre-concentration methods based on liquid-liquid extraction and their application in inorganic ultratrace analysis and speciation. *Spectrochimica Acta, part B.* 2009; 64 (1): 617-628.

68. Neric C, Salafranca J, Aznar M, Battle R., Critical review on recent developments in solventless techniques for extraction of analytes. *Analytical and Bioanalytical Chemistry.* 2009; 393: 809-833.

69. Dadfarnia S, Mohammad HS., Recent development in liquid phase microextraction for determination of trace level concentration of metals-review. *Analytica Chimica Acta.* 2010; 658: 107-119.

70. Rutkowska M, Dubalska K, Konieczka P, Namiesnik J., Microextraction techniques used in the procedures for determining organomercury and organotin compounds in environmental samples. *Molecules.* 2014; 19: 7581-7609.

71. Cunha S, fernandes JO, Beatriz M, Oliveira PP., Current trends in liquid-liquid microextraction for analysis of pesticides residues in food and water. *Department of Bromatology, Faculty of Pharmacy, University of Porto, Portugal.*

72. Andruch V, Balogh IS, Kocurova L, Sandrejova J., Five years of dispersive liquid-liquid microextraction. *Applied Spectroscopy Reviews.* 2013; 48: 161-259.

73. Al-Saidi HM, Emara AA., The recent developments in dispersive liquid-liquid microextraction for pre-concentration and determination of inorganic analytes. *Journal of Chemistry.* 2013; 11(8): 1343-1351.

74. Wen Y, Zhang M, Zhao Q, Feng YQ., Monitoring of five sulfonamide antibacterial residues in milk by in-tube solid-phase microextraction coupled to high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*. 2005; 53 (22): 8468-8473.

75. Menezes-Filho A, Santos FN, Paula-Pereira PA., Development, validation and application of a methodology based on solid-phase microextraction followed by gas chromatography coupled to mass spectrometry (SPME/ GC-MS) for the determination of pesticides residues in mangos. *Talanta.* 2010; 81: 346-354.

76. Tsai WH, Huang TC, Huang JJ, Hsue YH, Chuang HY., Dispersive solid-phase microextraction method for sample extraction in the analysis of four tetracyclines in water and milk samples by high-performance liquid chromatography with diode-array detection. *Journal of Chromatography A*. 2009; 1216 (12): 2263-2269.

77. Gioti EM, Skalkos DC, Fiamegos YC, Stalikas CD., Single-drop liquid-phase microextraction for the determination of hypericin, pseudohypericin and

hyperforin in biological fluids by high performance liquid chromatography. *Journal of Chromatography A*. 2005; 1093 (1): 1-10.

78. Payán MR, López MÁB, Fernández-Torres R, Bernal JLP, Mochón MC., HPLC determination of ibuprofen, diclofenac and salicylic acid using hollow fiber-based liquid phase microextraction (HF-LPME). *Analytica Chimica Acta*. 2009; 653 (2): 184-190.

79. Shariati S, Yamini Y, Esrafili A., Carrier mediated hollow fiber liquid phase microextraction combined with HPLC-UV for pre-concentration and determination of some tetracycline antibiotics. *Journal of Chromatography B*. 2009; 877 (4): 393-400.

80. Msagati T., Hollow fibre supported liquid membrane extraction of diphenylhydramine, chlorpheniramine and ketoprofen in pharmaceutical products. *Bulletin of the Chemical Society of Ethiopia*. 2010; 24 (2).

81. Gure A, Lara FJ, Megersa N, García-Campaña AM, del Olmo-Iruela M., Hollow-Fiber Liquid-Phase Microextraction combined with Capillary HPLC for the selective determination of six sulfonylurea herbicides in environmental waters. *Journal of Separation Science.* 2013; *36*: 3395 - 3401.

82. ZANG XH, WU QH, ZHANG MY, XI GH, WANG Z., Developments of dispersive liquid-liquid microextraction technique. *Chinese Journal of Analytical Chemistry*. 2009; 37 (2): 161-168.

83. Zhang S, Li C, Song S, Feng T, Wang C, Wang Z., Application of dispersive liquid–liquid microextraction combined with sweeping micellar electrokinetic

chromatography for trace analysis of six carbamate pesticides in apples. *Analytical Methods*. 2010; 2 (1): 54-62.

84. Nuhu AA, Basheer C, Saad B., Liquid-phase and dispersive liquid-liquid microextraction techniques with derivatization: Recent applications in bioanalysis. *Journal of Chromatography B*. 2011; 879 (17-18): 1180-1188.

85. Sereshti H, Khojeh V, Samadi S., Optimization of dispersive liquid-liquid microextraction coupled with inductively coupled plasma-optical emission spectrometry with the aid of experimental design for simultaneous determination of heavy metals. *Talanta*. 2010; 83 (3): 885-90.

86. Chen H, Chen H, Ying J, Huang J, Liao L., Dispersive liquid-liquid microextraction followed by high-performance liquid chromatography as an efficient and sensitive technique for simultaneous determination of chloramphenicol and thiamphenicol in honey. *Analytica Chimica Acta*. 2009; 632 (1): 80-85.

87. Tan GH, Chai M., Sample preparation in the analysis of pesticides residue in food by chromatographic techniques. *Pesticides-Strategies for Pesticides Analysis.*

88. Miura J, Ishii H, Watanabe H, Kagaku B., Cloud Point Extraction: Application to heavy metals analysis. *Journal of microchemica Acta*. 1976; 92: 135-139.

89. Paleogos EK, Giokas DL, Karayannis MI., Micelle-mediated separation and cloud-point extraction. *Trends in Analytical Chemistry.* 2005; 24 (5): 426-436.

90. Ojeda CB, Rojas FS, Pavon JMC., Pre-concentration of Cadmium in environmental samples by cloud point extraction and determination by FAAS. *American Journal of Analytical Chemistry.* 2010; 1: 127-134.

91. Carabias AR, Rodriguez GE, Moreno CB, Perez PJL, Garcia PC, Laespada FE., Surfactant cloud point extraction and pre-concentration of organic compounds prior to chromatography and capillary electrophoresis. *Journal of Chromatography A.* 2000; 902: 251-265.

92. Xia Q, Yang Y, Liu M., Vortex-assisted acid induced cloud point extraction coupled with spectrofluorometry for the determination o fluoroquinolones in environmental water samples. *Spectroscopy letters: An International Journal for Rapid Communication.* 2013; 47 (3): 206-213.

93. Liu H, Dasggupta PK., Analytical Chemistry in a drop, solvent extraction in a microdrop. *Analytical Chemistry*. 1996; 68 (11): 1817-2821.

94. Xu L, Basheer C, Lee HK., Developments in single-drop microextraction. *Journal of Chromatography A.* 2007; 1152 (1-2): 184-192.

95. Sarafraz YA, Amiri A., Liquid-phase microextraction. *Trends in Analytical Chemistry.* 2010; 29 (1): 1-14.

96. Sekar R, Wu HF., Quantitative method or analysis of monensin in soil, water and urine by direct combination of single-drop microextraction with atmospheric pressure-assisted laser desorption/ionization mass spectrometry. *Journal of American Chemical Society.* 2006; 78 (18): 6306-6313.

97. Pedersen BS, Rasmussen KE., Liquid-Liquid-liquid microextraction for sample preparation of biological fluids prior to capillary electrophoresis. *Analytical Chemistry.* 1999; 71 (14): 2650-2656.

98. Saraji M, Boroujeni MK, Bidgoli AAH., Comparison of dispersive liquid-liquid microextraction and hollow-fibre liquid-liquid-liquid microextraction for the determination of fentanyl, alfentanil and sufentanil in water and biological fluids by high performance liquid chromatography. *Journal of Analytical and Bioanalytical Chemistry.* 2011; 400 (7): 2149-2158.

99. Shariati S, Yamini Y, Esrafili A., Carrier mediated hollow fiber liquid phase microextraction combined with HPLC-UV for pre-concentration and determination of some tetracycline antibiotics. *Journal of Chromatography B.* 2009; 877 (4): 393-400.

100. Gjelstad A, Pedersen BS., Perspective: Hollow fibre liquid-phase microextraction – principles, performance, applicability, and future directions. *Scientia Chromatographica.* 2013; 5 (3): 181-189.

101. Yudthaorasit S, Chiaochan C, Leepipatpiboon N., Simultaneous determination of multi-class antibiotic residues in water using carrier-mediated hollow-fiber liquid-phase microextraction coupled with ultra-high performance liquid chromatography tandem mass spectrometry. *Microchimica Acta.* 2011; 172 (1): 39-49.

102. Manzoori JL, Ghasem Karim NG., Selective cloud point extraction and preconcentration of trace amounts of silver as a dithizone complex prior to

flame atomic absorption spectrometric determination. *Analytica Chimica Acta.* 2003; 484 (2): 155-161.

103. Du D, Wu Y, Kang D, Wang H, Huang C, Li Y., Determination of tetracyclines in water by ethyl acetate- ionic liquid dispersive liquid-liquid microextraction and high performance liquid chromatography. *Analytical Letters.* 2014; 47 (10): 1783-1795.

104. Hou D, Guan Y, Di X., Temperature-induced ionic liquids dispersive liquid-liquid microextraction of tetracycline antibiotics in environmental water samples assisted by complexation. *Chromatographia*. 2011; 73 (1): 1057-1064.

105. Song J, Zhang ZH, Zhang YQ, Feng C, Wang GN, Wang JP., Ionic liquid dispersive liquid-liquid microextraction combined with high performance liquid chromatography for determination of tetracycline drugs in eggs. *Analytical Methods.* 2014; 6: 6459-6466.

106. S.O.S. Mookantsa, S. Dube, M.M. Nindi. Development and application of a dispersive liquid-liquid microextraction method for the determination of tetracyclines in beef by liquid chromatography mass spectrometry. *Talanta*. 2016; 148: 321–328.

107. Siuzdak G., Mass spectrometry for Biotechnology. *Academic Press.* 1996.

108. Jimenez JL., Mass spectrometry detectors. *University of Colorado-Boulder.* 2007.

109. Jennings KR, Dolnikowski GG., Mass analyzers. *Methods in Enz*ymology. 1990; 193: 37-61.

110. Zhao Y, Wang Y., Measurement and correlation of solubility of tetracycline hydrochloride in six organic solvents. *Journal of Chemical Thermodynamics*. 2013; 57 (1): 9-13.