Automated High-throughput Analysis of Multi-class Multi-residue Pharmaceutical Drugs in Animal Tissue Using Solid Phase Microextraction

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Chemistry

Waterloo, Ontario, Canada, 2020

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Author’s Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The use of veterinary pharmaceuticals in today´s intensive farming of food-producing animals is essential for the humane treatment of animals and as well as for the prevention of outbreaks of infectious diseases. Improper administration of these drugs may lead to excessive levels of drug residues in the edible tissues of treated animals, posing a potentially serious risk to human health. Accordingly, monitoring of veterinary drug residues is an integral component of food safety programs worldwide. Reliable analytical methods play a crucial role in the endeavor to enforce regulations surrounding the use of veterinary drugs in food-producing animals.

Recent advancements in liquid chromatography (LC) and mass spectrometry (MS) have facilitated the development of multi-class, multi-residue analytical methods capable of screening a large number of analytes in a single analytical run. However, due to the complexity of biological tissues, the large number of veterinary drugs to be monitored, and the wide variation in their physico-chemical properties, sample preparation constitute a major bottleneck of their analytical workflow. Sample preparation is critical in relation to sample turnaround and the reliability of the analytical outcome. In this context, an ideal sample preparation method for multi-class multi-residue analysis should offer enough non-selectivity so as to cover a wide range of polarities and thus allow extraction of as many analytes as possible, while at the same time delivering a high degree of sample clean-up so as to reduce matrix effects. Solid phase microextraction (SPME) has been demonstrated as a suitable sample preparation tool that allows isolation/enrichment of multiple analytes in addition to excellent sample clean-up.

The main objective of this thesis was to develop automated high-throughput analytical methods for quantitative analysis of multi-class multi-residue veterinary drugs in meat using liquid chromatography and mass spectrometric techniques.

The introductory chapter offers an extensive review of veterinary drug classes used in food animal production and the current analytical methodologies used for their analysis. In addition, chapter 1 offers an extensive review of the most commonly used generic sample preparation techniques. The experimental section of this thesis is constituted of four chapters, 2-5. Chapter 2 describes the development and validation of an automated high-throughput direct-immersion SPME method for the quantitative analysis of 77 veterinary drugs belonging to more than 12 classes of drug compounds in homogenized tissue from chicken. In chapter 3, the scope of the SPME method presented in chapter 2 was expanded to a new matrix (beef tissue), and for detection of an additional 25 analytes. Moreover, the work also encompassed a comparison of the SPME method to two well-documented sample preparation procedures, namely solvent extraction (SE) and the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method. Chapter 4 and 5 present works aimed to increase efficiency and sample throughput through the development of rapid screening methods that involve the direct coupling of SPME to mass spectrometry. For this purpose, two different ambient ionization mass spectrometry techniques were studied, namely coated blade spray (CBS) and direct analysis in real time (DART). Finally, chapter 6 summarizes the main findings of this work and provides future directions to be considered with regards to the applicability of SPME towards multi-residue analysis of animal tissue.

Acknowledgements

I would like to express my sincere thanks to my supervisor, Professor Janusz Pawliszyn, for giving me the opportunity to work within his research group, and for his guidance, endless support, and continuous motivation to explore and implement new ideas.

I would also like to thank my advisory committee members, Professor Michael Chong and Professor Wojciech Gabryelski for providing their advice, effort, and time to review this thesis and throughout my studies and thesis preparation. I would also like to thank Professor Tadeusz Gorecki who, although could not attend my defense due to being on Sabbatical, was part of my PhD advisory committee.

I also would like to extend my gratitude to my external examiner, Dr. David Borts and my internal/external examiner, Professor Mark Servos who agreed to provide their valuable time and feedback required for the evaluation and examination of my thesis.

I also extend my sincerest appreciation and gratitude to all the industrial research collaborators from Perkin Elmer for financial support and their scientists for sharing their knowledge during the last four years.

I would also like to give special thanks to members from our university support staff including Harmen Vander Heide, Andrew Dube, and Hiruy Haile, from the University of Waterloo Science Shop, Krunomir Dvorski from the Science Electronics shop, and Catherine Van Esch and Kim Rawson from the graduate administrative support, for their assistance and outstanding support throughout my studies.

I would like to give a special thanks to all my colleagues within Professor Pawlisyzn's Research Group who assisted me professionally throughout those years and for their support and friendship, particularly Varoon Singh, Jonathan Grandy, Daniel Rickert, and Ezel Boyaci. I would also like to thank my co-authors, Emanuela Gionfriddo, German Augusto Gómez-Ríos, Joao Raul Belinato, and Vinicius Acquaro for their support.

I would like to express my deepest gratitude to my mother for her love, endless support, motivation, and encouragement to pursue my dreams, to my sisters, Elham, Hanan, and Mona, and to my brothers Mohammed, Imad, Yasser, Younes, and Mounir for their continuous love and support.

Lastly, I owe my deepest gratitude to my husband Mahmoud, my sons Omar and Khaled, and my daughter Ola for their love and support.

Dedication

I dedicate this thesis to my biggest cheerleader, from thousands of miles away, my beloved mother. Thanks!

I also dedicate it to the memory of my father, who would have been overjoyed to see me accomplish this goal.

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List of Abbreviations and Symbols

a	Time constant
A	Surface area
b	Thickness of the coating
δ	Thickness of the boundary layer
ACE	Acetone
AGP	Antibacterial growth promoter
AIBN	Azobisisobutyronitrile
C_{18}	Octadecyl
C_e^{∞}	Equilibrium concentration of analyte on the coating
C_s^{∞}	Equilibrium concentration of analyte in sample
DART	Direct analysis in real
DI	Direct immersion
DMF	Dimethylformamide
d-SPE	Dispersive solid phase extraction
ESI	Electrospray ionization
EU	European Union
FE-SEM	Field emission scanning electron microscopy
GC	Gas Chromatography
HLB	Hydrophilic-lipophilic balance
HRMS	High resolution MS
LC	Liquid chromatography
log P	Logarithmic octanol-water partition coefficients
LOQ	Limit of quantitation
ME	Matrix effects
MeCN	Acetonitrile
MeOH	Methanol

MMM	Multi-residue multi-class methods
MRL	Maximum residue level
MS	Mass spectrometry
MS/MS	Tandem MS
NSAIDs	Non-steroidal anti-inflammatory drugs
PAN	Polyacrylonitrile
PBS	Phosphate-buffered saline solution
PDMS	Polydimethylsiloxane
PP	Polypropylene
PS-DVB	Polystyrene-divinylbenzene
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RSDs	Relative standard deviation
SLMD	Simplex-lattice mixture design
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
TFME	Thin-film microextraction (interchangeable with TF-SPME)
TF-SPME	Thin-film solid phase microextraction (interchangeable with TF-SPME)
TOF	Time-of-flight

Chapter 1
Veterinary Drugs and Food Safety

1.1 Introduction

Food safety is of vital interest to consumers worldwide, a fact that is reflected in its frequent emergence as a prominent topic of discussion in the media.[1] The topic of food safety is especially relevant to foodstuffs derived from animals, which play a major role in providing sufficient quantities of essential nutrients needed to sustain life and promote good health, such as proteins with high biological value, group B vitamins, minerals, and other important trace elements.[2,3] On a global level, livestock products alone supply an estimated 28% of the protein consumed by humans.[3] With the growth of the global population, there is an ever-growing demand for increased food production. In order to maximize production and profits, the animal food industry has adopted intensive production practices, including high stock density grazing, housing, and transportation. While these intensive farming practices have significantly increased the production of food worldwide, such practices are burdened by increased animal vulnerability to stress and disease − a factor that is largely associated with housing and transporting animals in high densities.[1,4] Consequently, veterinary dugs (VDs) are widely administered to food-producing animals as a means to reduce animal stress, as well as prevent and treat diseases.[5,6] In addition, certain veterinary drugs are used in animal feed to promote growth and maximize production output.[7–9]

Despite the obvious benefits of using VDs in animal production, malpractices such as improper dosage, fraudulent administration, or failure to keep the withdrawal period of approved drugs can lead to excessive residues being present in edible tissues of animals treated with VDs.[10]

Here, a residue is defined as the trace of a given substance that remains present in matrices derived from a given animal after administration of said substance to the animal.[11] At excessive levels, most drug residues pose a potential risk to human health, and may lead to serious allergic, toxic, or carcinogenic reactions.[7,12–19] One of the greatest concerns associated with the use of VDs is related to the misuse of antibiotics, which has been linked to the emergence of antibiotic-resistant strains of bacteria.[19–22] Antibiotic resistance is an increasing global concern that requires immediate actions across governments and international organizations.[23,24]

Other effects of VD use in food producing animals that may be considered of some importance are related to the quality of the resulting meat. Lone et al. demonstrated that while meat derived from animals treated with hormones or growth promoters tends to be leaner, it is also less tender due to the accumulation of connective tissue and the higher rate of collagen cross-linking.[25] Likewise, meat derived from animals treated with a class of drugs called thiouracils, which cause water retention in animals, tends to become dry upon cooking due to the retained water becoming rapidly lost during its preparation.[26,27]

The presence of chemical residues in animals can also be a consequence of environmental contamination and naturally occurring toxicants in foods.[7,28,29]

1.2 Classification of veterinary drugs

In Canada, as per the Food and Drugs Act (R.S.C., 1985, c. F-27), a drug is defined as any substance or mixture of substances used for any of the following purposes:[30]

1. diagnosis, treatment, mitigation or prevention of a disease, disorder, or abnormal physiological state, or its symptoms in human beings or animals,
2. restoring, correcting or modifying organic functions in human beings or animals, or
3. disinfection in premises in which food is manufactured, prepared or kept.

Several classes of drugs, with varying chemical structures and properties, are currently used worldwide for treatment of food-producing animals.[22] For the purpose of this thesis, the only classes that will be discussed are those that comprise the drugs of concern listed in the official multi-class multi-residue method developed by the United States Department of Agriculture (USDA)-Food Safety and Inspection Service (FSIS) for screening and confirmation of animal drug residues in bovine, poultry, porcine, caprine, and ovine kidney and muscle tissue as well as equine and fish muscle tissues.[31,32] The following subsections provide brief descriptions of these classes.

1.2.1 Antibiotics

By strict definition, the term antibiotic refers to a substance produced by a microorganism which at a low concentration acts against competing microorganisms but with little or no damage to the host. An antimicrobial is any substance of natural, semi-synthetic, or synthetic origin which at a low concentration acts against the growth of competing microorganisms but causes little or no damage to the host. Nowadays, both terms are used interchangeably.[19] In the same vein, while the term antimicrobial refers to all substances that act against all types of micro-organisms, such as bacteria, fungi, and protozoa, the term antibacterial is a sub-class of antimicrobials which act against bacteria only. Since antibacterials comprise the largest and most widely studied class of

antimicrobials, the term antibacterial is also often used interchangeably with the term antimicrobial.[33]

While the use of antibiotics is necessary to prevent or treat infections in animals, antibiotics have been widely exploited for economic reasons due to their growth-promoting effects in animals when used in sub-therapeutic doses.[34,35] Antibiotics that have growth promotion effects are called antibacterial growth promoters (AGP), a characteristic that can be exploited for weight gain. The practice of using AGPs for growth promotion is prohibited in Canada, USA and the European Union (EU).[19]

While antibiotic resistance does occur naturally, there is general consensus in the scientific and governmental communities that the abuse and misuse of antibiotics is largely responsible for accelerating the process.[22] Anti-microbial resistance happens when microorganisms exposed to antimicrobial drugs change and ultimately become resistant to antibiotics used in human medicine. This concern about antibiotic resistance has led to a ban on the use of antimicrobials in animal feed as growth-promoting substances in most countries.[36]

Currently, while hundreds of antibiotics are commercially available for human and animal treatment, only few of them are approved for use in food-producing animals, mainly due to concerns over the transfer of antibacterial resistance from animals to humans.[19] The antibiotics investigated in this thesis comprise the following classes:

1.2.1.1 β-Lactams

The world's first antibiotic, a β-lactam called penicillin, was first discovered in 1928 by Alexander Fleming.[33] While the antibacterial properties of mold had been known previously, penicillin was not known until Fleming identified the Penicillium strain as the active substance.[37] Penicillin, however, was not recognized as a life-saving drug until a decade later, when Florey and Chain developed a method to purify and concentrate penicillin, and test its effects on laboratory mice.[19] β-Lactams, divided into two major classes, the cephalosporins and penicillins, have at their basic structure a four-membered lactam ring, known as a β-lactam ring, and a variable side chain that accounts for the major differences in their chemical and pharmacological properties.

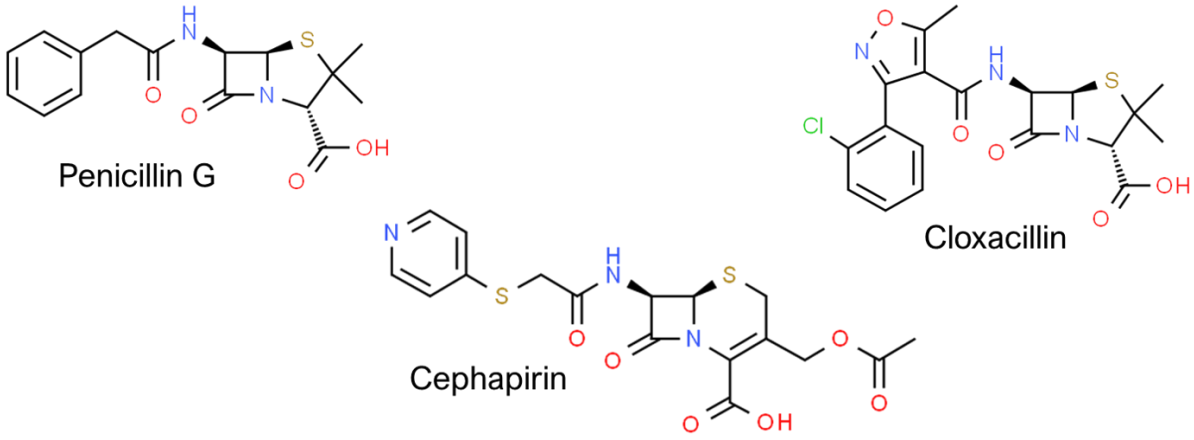


Figure 1.1 Molecular structures of penicillin G, cloxacillin, and cephapirin. Structures were obtained from chemspider.com.

The primary distinguishing structural difference between penicillins and cephalosporins is the thiazolidine ring attached to the lactam ring. In penicillins, such as penicillin G, the lactam ring is fused to a five-member thiazolidine ring, while in cephalosporins such as cephapirin shown in figure 1.1, it is fused to a six-member ring. The β-lactam ring is responsible for antibacterial

activity as well as for reduced stability in alcohols and acidic solutions. β-lactams are also thermolabile.[38–40] Representative compounds for β-lactams are shown in Figure 1.1.

1.2.1.2 Fluoroquinolones

Fluoroquinolones are synthetic antibiotics derived from 3-quinolinecarboxylic acid. The main difference between older classic quinolones and the recently introduced fluoroquinolones is that the latter contain a fluorine atom at the C-6 position and a piperazinyl group at the C-7 position. These two structural differences increase antibacterial activity against Gram-positive and Gram-negative bacteria, respectively, endowing fluoroquinolones with a broad spectrum of antibacterial activity.[38] Fluoroquinolones are widely used in human and veterinary medicine for the treatment of a variety of illnesses, and are largely prescribed to treat gastrointestinal and respiratory infections.[39,41] Their widespread use in food-producing animals is of great concern due to recent evidence linking increased bacterial resistance in humans to the use of these antibiotics in food-producing animals.[42,43] Representative fluoroquinolones, namely ciprofloxacin, enrofloxacin, and sarafloxacin, are shown in Figure 1.2.

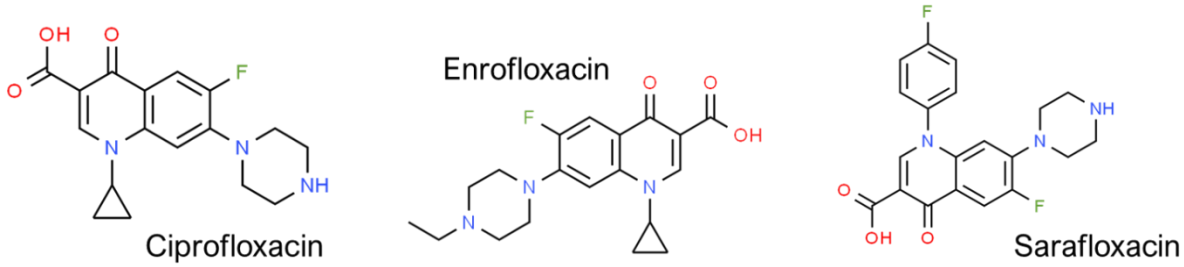


Figure 1.2 Molecular structures of ciprofloxacin, danofloxacin, and enrofloxacin. Structures were obtained from chemspider.com

1.2.1.3 Macrolides and lincosamides

Macrolides are basic macrocyclic compounds that have a common 14-, 16-, or 17- membered macrocyclic lactone ring linked by glycoside bonding to one or more molecules of deoxy sugars.[38,39] They are widely used in veterinary medicine to treat respiratory diseases, as well as feed additives to promote growth.[40] Erythromycin, shown in Figure 1.3, is an example of a 14-membered ring. Macrolides are reported to be unstable in both acidic and basic aqueous solutions. Lincosamides constitute a small group of antibiotics that includes compounds such as lincomycin and clindamycin, shown in Figure 1.3. Lincomycin was the first member of the lincosamide class that was reported in the literature.[44] They mainly consist of three components: an amino acid and a sugar (lincosamine), that are connected by an amide bond.[45] Both macrolides and lincosamides target the bacterial ribosome and inhibit protein synthesis.[38]

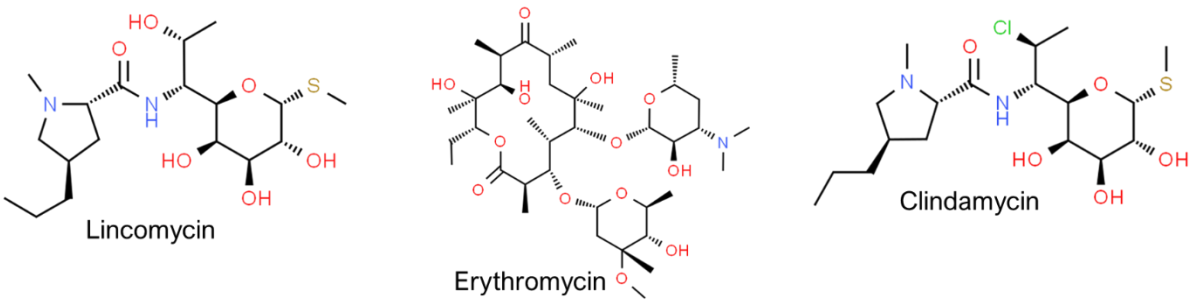


Figure 1.3 Molecular structures of lincomycin, erythromycin, and clindamycin. Structures were obtained from chemspider.com

1.2.1.4 Phenicols

Phenicols such as chloramphenicol, florfenicol, and thiamphenicol, shown in Figure 1.4, are very potent broad-spectrum antibiotics. Phenicols possess chemical structures that makes them

active against a variety of pathogens through interaction with their ribosomes, resulting in inhibition of protein synthesis.[38,39] The *p*-nitro group attached to the benzene ring in chloramphenicol has been associated with the causation of irreversible dose-independent aplastic anemia in some humans, and as a result, chloramphenicol use in food-producing animals has been banned in Canada, USA, and the EU.[16,46,47] Thiamphenicol and florfenicol, which are structurally similar but lack the *p*-nitro group, have been introduced as substitutes for chloramphenicol.[38] Because they lack the p-nitro moiety, neither thiamphenicol nor florfenicol are associated with dose-independent aplastic anemia in humans or any other species, but both are associated with dose-dependent bone marrow suppression in humans.[19]

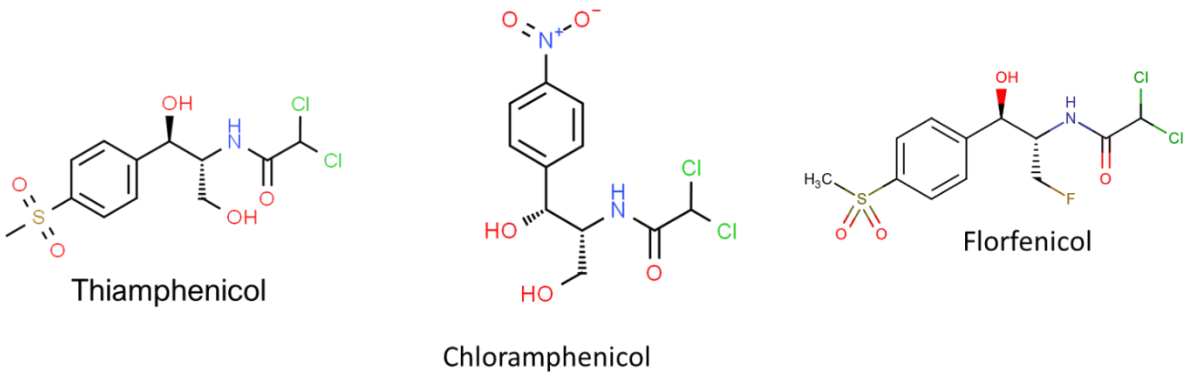


Figure 1.4 Molecular structures of thiamphenicol, chloramphenicol, and florfenicol. Structures were obtained from chemspider.com

1.2.1.5 Sulfonamides

While much of the credit is given to penicillin as the first antibiotic, the first commercially available synthetic antibiotic belongs to the sulfonamide group,[48] which is comprised of broad-spectrum synthetic antibiotics that are active against gram-positive and gram-negative bacteria.[2,49] They share a common chemical nucleus that comes from sulfanilamide, which contains the

structural requirements for antibacterial activity. The sulfonamides of this group differ in the radical attached to the amido group, or occasionally, in the substituent on the amino group.[50] Representative compounds are presented in Figure 1.5. Sulfonamides have amphoteric properties imparted by the weak basic anilinic nitrogen and the weakly acidic sulfonamide group present in their structure.[38,39] Several sulfonamides are usually combined in one preparation and administered to animals to reduce toxicity and cover a wider activity range. Sulfonamides are potentially carcinogenic and highly susceptible to antibiotic resistance in humans. Their antibacterial activity has been greatly diminished due to the extensive resistance that has developed throughout their over 70 years of use.[40]

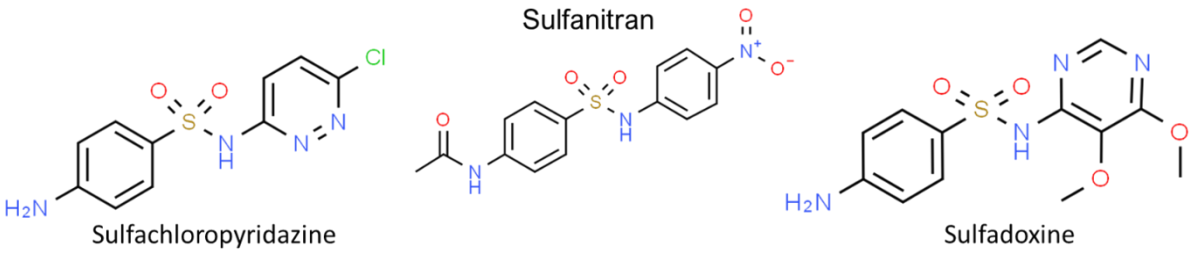


Figure 1.5 Molecular structures of sulfachloropyridazine, sulfanitran, and sulfadoxine. Structures were obtained from chemspider.com

1.2.1.6 Tetracyclines

Tetracyclines are broad-spectrum antibiotics that are widely used in veterinary medicine against both Gram-positive and Gram-negative bacteria by inhibiting their protein biosynthesis. Because tetracyclines are relatively inexpensive, they are also widely used for cost-effective prophylactic and therapeutic treatment and also as growth-promoting substances in both cattle and poultry.[19,38,51]

From an analytical perspective, tetracyclines are not stable in bases, and they can decompose rapidly under the influence of light and atmospheric oxygen. They are also susceptible to configurational degradation to their 4-epimers in aqueous solutions and during sample preparation. They also have the ability to chelate with metal ions and interact with silanol groups.[52,53] Representative tetracyclines are shown in Figure 1.6.

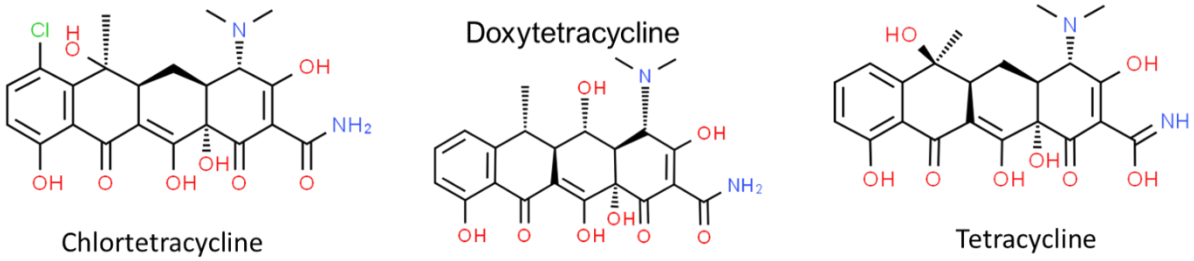


Figure 1.6 Molecular structures of chlortetracycline, doxycycline, and tetracycline. Structures were obtained from chemspider.com

1.2.2 Anthelmintics

The term anthelmintic is derived from the Greek words *anti* - 'against', and *helminth* – 'intestinal worm'; therefore, anthelmintics are a group of antiparasitic drugs that are active against internal parasites without causing significant damage to the host.[39] However, they have also been exploited for other uses due to their ability to increases milk yield in lactating animals.[13] Levamisole, albendazole, cambendazole, fenbendazole, oxfendazole, thiabendazole, and ivermectin are the most frequently used of these drugs.[54] One of the main concerns arising from the use of anthelmintics in food-producing animals is the emergence of resistance in humans, particularly as most anthelmintics are thermally stable and can withstand conventional cooking and fermentation processes of milk. Representative structures are shown in Figure 1.7.

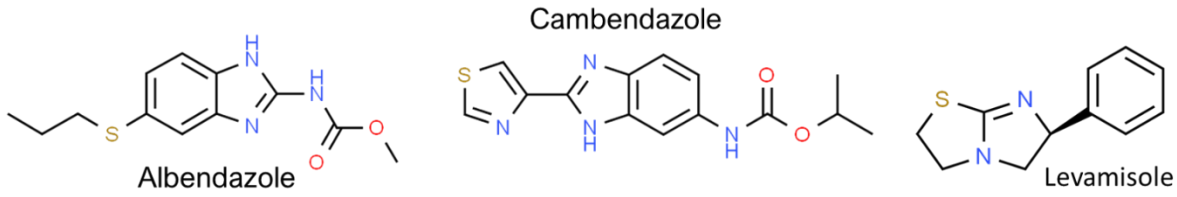


Figure 1.7 Molecular structures of Albendazole, cambendazole, and levamisole. Structures were obtained from chemspider.com

1.2.3 Non-steroidal anti-inflammatories

Non-steroidal anti-inflammatory drugs (NSAIDs) have analgesic and fever-reducing effects at low dosages, and anti-inflammatory effects at higher doses.[55] The term "non-steroidal" is used to distinguish these drugs from steroids, which are also prescribed for their anti-inflammatory effects. While NSAIDS are often chemically unrelated, most of them are organic acids consisting of a carboxylic and/or phenolic functional group.[13,39,55] While a wide variety of NSAIDs are available for human and veterinary use, only a few are licensed or commonly used in food-producing animals due to concerns over animal safety, lack of efficacy, or drug residues. The most common NSAIDs administered to food producing animals include ketoprofen, flunixin, and meloxicam, which are shown in Fig 1.8.

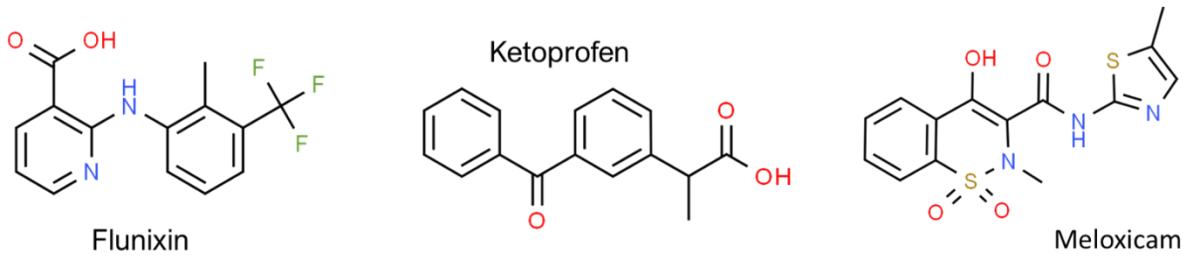


Figure 1.8 Molecular structures of flunixin, ketoprofen, and meloxicam. Structures were obtained from chemspider.com

1.2.4 β-agonists

β-Agonists are synthetically produced phenethanolamines that have been used in medicine for more than three decades as bronchodilating agents for the treatment of asthma and chronic obstructive pulmonary diseases due to their relaxing effect on muscles.[12] Their ability to increase lean body mass in animals by increasing meat-to-fat ratios has been demonstrated in several studies.[12,39] This characteristic has led to the illicit exploitation of these compounds to enhance leanness in livestock.

Serious concerns over the misuse of β-agonists began to emerge in the early 1990s after reports of several human intoxication incidents worldwide. The first outbreak was reported in France, where 22 people were poisoned as a result of consumption of veal liver contaminated with clenbuterol residues. Within the next two years, 232 cases were reported in Spain, where people complained of a variety of symptoms, including racing heart, dizziness, nausea, headaches, and peripheral tremors following ingestion of veal liver and possibly veal tongue.[16,56] Similar incidents were also reported in Italy, Hong Kong, and Portugal.[16] As a result, an absolute ban was placed on β-agonists in food-producing animals in the EU, Russia, and China.[57–59] However, two β-agonists are still approved for use for this purpose in food-producing animals within the US and Canada. They are administered to enhance feed efficiency and produce leaner meat, namely ractopamine for use in swine, turkeys and cattle, and zilpaterol for use in cattle only.[3,60,61] The beef hormone dispute between the EU and the USA and Canada is considered one of the longest-standing trade disputes due to the use of β-agonists and hormones, ractopamine in particular.[58,62] In Canada, the only approved β-agonist currently in use is ractopamine, for cattle use only. The manufacturer of

zilpaterol removed it from the Canadian market due to animal welfare problems.[3] The molecular structures of clenbuterol, ractopamine, and salbutamol are shown in Fig 1.9.

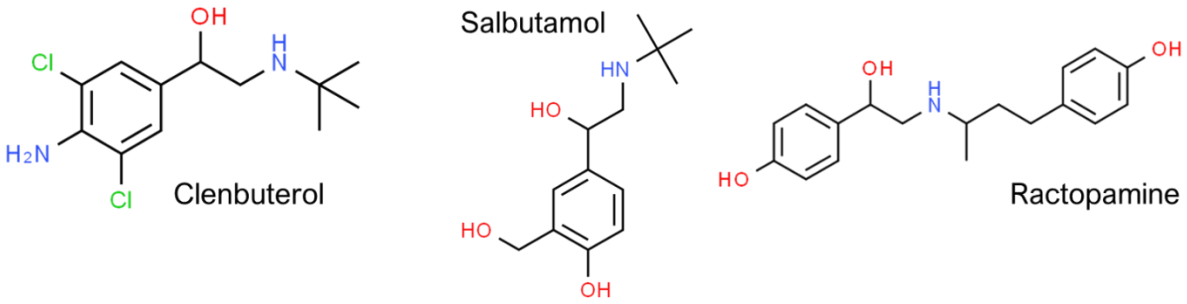


Figure 1.9 Molecular structures of clenbuterol, salbutamol, and ractopamine. Structures were obtained from chemspider.com

1.2.5 Coccidiostats

Coccidiostats or anticoccidial agents are used to treat coccidiosis, a highly contagious infection caused by single-cell protozoan parasites that affect the intestinal tract.[63] These parasites are prevalent in the warm humid conditions normally observed in high density rearing environments in which animals such as pigs and poultry are intensively reared.[39,64,65] Coccidiosis costs the Canadian poultry industry in excess of $50 million annually despite an annual expenditure of more than $85 million on preventive drugs.[66] To counteract this threat, most intensively reared animals are given coccidiostats as feeds additives for the whole or the majority of their growing periods, with a withdrawal of medication time of 1–5 days prior to slaughtering.[39] Whilst acute toxicity in humans has never been reported, coccidiostats such as 5-nitroimidazoles have been found to display mutagenic and carcinogenic properties, and as a result have been banned for use in food animals in the United States, Canada, and the EU.[67–69] Representative 5-

nitroimidazole compounds, namely dimetridazole, ronidazole, and ipronidazole, are shown in Figure 1.10.

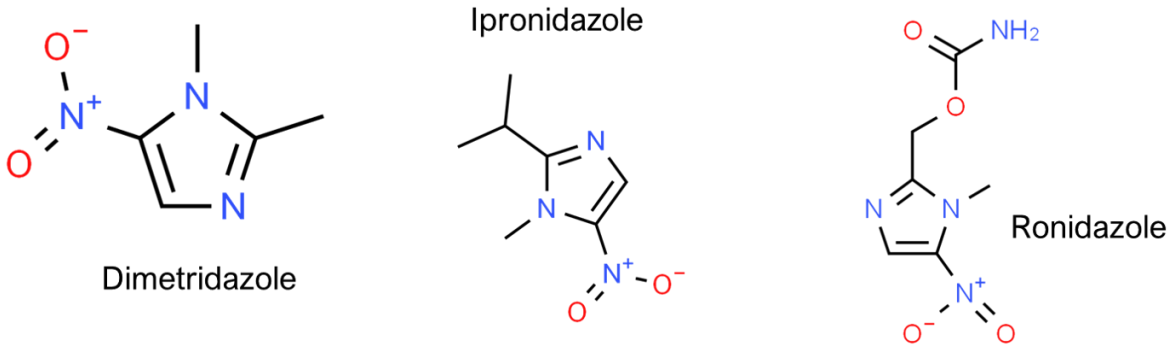


Figure 1.10 Molecular structures of dimetridazole, ipronidazole, and ronidazole. Structures were obtained from chemspider.com

1.2.6 Sedatives and Tranquilizers

Food animals raised in intensive farming units are prone to stress due to overcrowding, especially during loading and transportation to the slaughterhouse. Stress in food-producing animals reduces the quality of their meat and may even lead to death of animals as a result of a heart attack.[39,40,54] Sedatives and tranquilizers are often used to induce calmness and reduce anxiety as a means to manage stress and minimize death and injury of food animals. It has also been reported that tranquilizers have been used as feed additives to enhance growth rates and improve milk yield due to their ability to slow down metabolic processes and reduce animal activity.[70] On the basis of their chemical structure, sedatives and tranquilizers are classified into four families: butyrophenones, phenothiazines, benzodiazepines, and imidazopyridines. Major members of the tranquilizers–sedatives group include phenothiazine-type drugs such as acepromazine and

chlorpromazine, and butyrophenone-type drugs such as azaperone. The molecular structures of these compounds are presented in Figure.1.11.

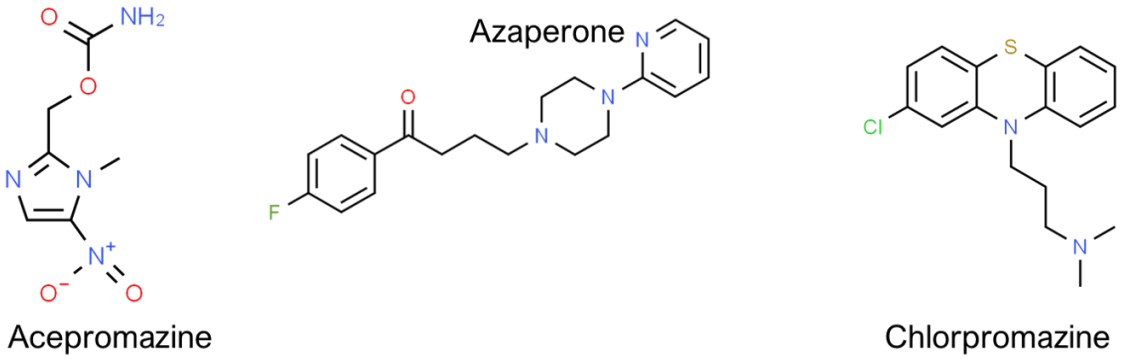


Figure 1.11 Molecular structures of acepromazine, azaperone, and Chlorpromazine. Structures were obtained from chemspider.com

1.2.7 Thyreostats

Thyreostatics or antithyroid agents inhibit the synthesis of thyroid hormones, which regulate metabolism and as a result moderate animal weight.[12,39] The use of these drugs also result in considerable animal weight gain due to increased water retention in edible tissues and accumulation in the gastrointestinal tract.[71,72] This class of drugs can be exploited as growth promoters as well as utilized fraudulently to fatten animals prior to slaughter. In addition to the negative effects on the quality of meat, thyreostats are potentially harmful to humans due to their teratogenic and carcinogenic properties.[73] The most powerful and most frequently abused thyreostatic agents in cattle are thiouracil and its analogues, methylthiouracil, propyl thiouracil, and phenyl thiouracil (shown in Figure 1.12). From an analytical perspective, extraction and

analysis of these molecules pose a challenge due to their high polarity which affects their retention in reverse phase LC and their small molecular weight that makes them susceptible to chemical noise in the mass spectrum.[74]

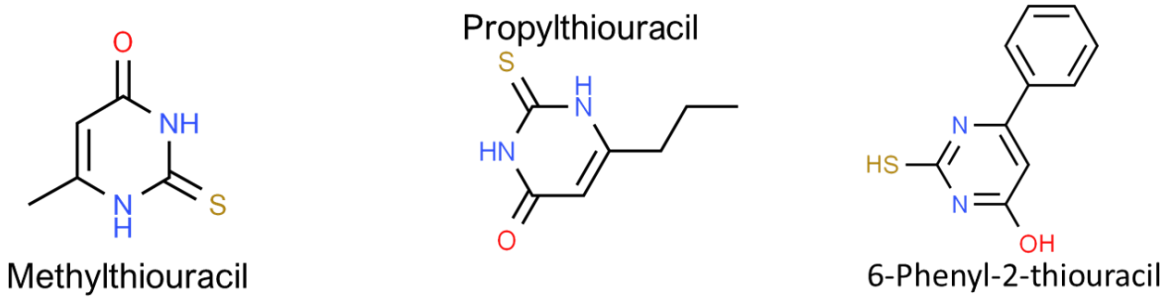


Figure 1.12 Molecular structures of methylthiouracil, propylthiouracil, and 6-phenyl-2-thiouracil. Structures were obtained from chemspider.com

1.2.8 Others

Veterinary drugs listed in the FSIS-USDA method that do not fall into any of the previous categories include carbadox and its metabolite 2-quinoxalinecarboxylic acid, bacitracin, novobiocin, virginiamycin, and melengesterol acetate.

Carbadox is an antimicrobial agent used to control swine dysentery and bacterial swine enteritis. Due to its growth promotion effects, it has been exploited as an animal feed additive.[39] Since carbadox is a suspected carcinogen, it has been banned in Canada and the EU; however, the use of carbadox is still approved in pigs in the US for up to 42 days before slaughter.[75,76]

Bacitracin is a cyclic polypeptide antibiotic that is active against Gram-positive bacteria by affecting protein synthesis.[77] Since it has growth promotion effects, it is considered as an AGP that can be exploited for weight gain. Novobiocin is a narrow-spectrum coumarin-derived AGP

frequently used in combination with penicillin for treatment of bovine mastitis.[39] Virginiamycin is a mixture of macrocyclic lactones with a peptide part, collectively called peptolides. It is also considered an AGP, and has been reported to increase egg production in laying hens.[39] Canada and the US are required to certify that meat products intended for export to the EU are free from AGPs.[77] Melengesterol acetate is a synthetic steroidal hormone administered as a feed additive to heifers intended for slaughter. While it is widely used in the US, it is not approved for use in the EU. In Canada, similar to all hormones and growth promoters, the only approved use for this drug is in the production of beef cattle. It is illegal to administer melengesterol acetate to dairy cows to increase milk production, or to any other food animal species.[78,79]

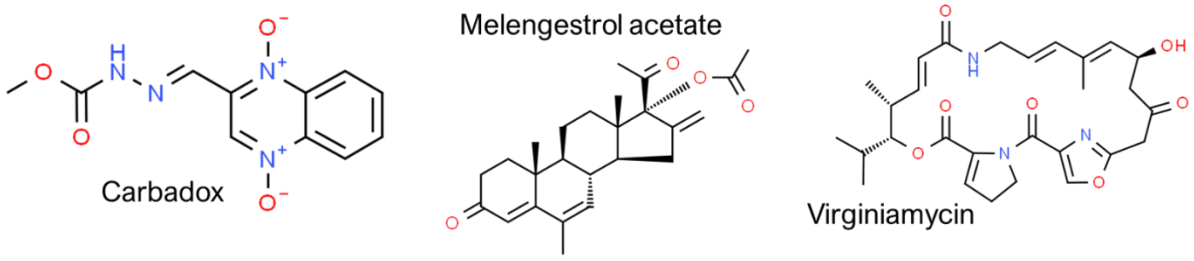


Figure 1.13 Molecular structures of carbadox, melengestrol acetate, and virginiamycin. Structures were obtained from chemspider.com

1.3 Regulatory framework

Since the use of veterinary drugs in food producing animals is essential to the maintenance of a sustainable animal food industry, developed countries have established several measures and extensive regulatory controls to prevent veterinary drug exploitation in the food-animal industry and safeguard consumer health. In Canada, Health Canada through the Veterinary Drugs Directorate (VDD) establishes maximum residue limits (MRL) for veterinary drugs upon risk

assessments of veterinary drugs under the Food and Drugs Act (R.S.C., 1985, c. F-27).[80] Risk assessments are conducted by performing the following six steps:[81]

1. Review of metabolism studies of veterinary drugs conducted in food-producing animals to determine the length of time it takes for a given drug and its metabolites to be excreted, as well as their amounts.

2. Review of comparative metabolism studies conducted in laboratory test animals to ensure that similar patterns of metabolism exist in these animals.

3. Toxicity of the substance is determined by toxicity/carcinogenicity testing. This information allows VDD scientists to determine the Acceptable Daily Intake (ADI) quantity, which is defined as the safe quantity of a given substance that can be consumed by humans on a daily basis over a lifetime without posing a threat to their health.

4. After determining the ADI, safe concentrations for total residue levels are established by ensuring that consumption of edible tissues does not exceed the ADI. A maximum residue limit (MRL) is then set for the concentration of the marker residue that will ensure that humans are not exposed to residues levels above the safe concentrations for the total residues. MRL is defined by Health Canada as the maximum amount of residue that could safely remain in the edible tissue of the treated animal.[82] At its MRL level or below, a residue is considered to pose no adverse health effects if ingested daily by humans over a lifetime.

5. The analytical methodology developed for the marker residue in food is evaluated.

6. A withdrawal period is then established based on residue depletion data for the specific residue in the target tissue.

Similarly, in the U.S.A., the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) establishes tolerances for veterinary drugs under the Federal Food, Drug, and Cosmetic Act (FFDCA).[83,84] In the EU, the use of veterinary drugs is regulated through Council Regulation 2377/90/EC, which also describes the procedure for the establishment of MRLs.[54] In China, the ministry of agriculture issues maximum residue levels for veterinary drugs in food of animal origin.[22]

At the international level, MRLs are established by the Codex Alimentarius Commission (Codex), a joint initiative between the Food and Agricultural Organization and the World Health Organization.[85] MRLs for veterinary drugs developed by Codex play an important role as trading standards to facilitate fair practices and ensure the quality of imported and exported meat.[13,18,86]

Consumers rely on regulatory authorities to ensure that foods derived from animals treated with veterinary drug products are safe for consumption.

Monitoring of veterinary drug residues in foodstuffs is necessary to ensure that MRLs are not breached, and that only approved drugs are used. In Canada and the USA, the Canadian Food Inspection Agency (CFIA) and FSIS are respectively responsible for monitoring residues of drugs and chemicals in animal food, and overseeing the implementation of legislative frameworks to

ensure that food derived from animals treated with veterinary drugs does not contain residues that pose a health hazard to consumers.[2,18,83,84]

1.4 Analytical approaches for multi-residue veterinary drugs

Analysis of veterinary drug residues in animal-tissue samples is a challenging task due to a variety of factors, including the complexity of the matrix, the large number of analytes with a wide range of physico-chemical properties that must be taken into consideration, the often very low concentration levels of said compounds that the method must be able to detect, and the large number of samples that must be analyzed. As a result, high-throughput multi-residue multi-class methods (MMM) i.e., methods capable of analyzing residues of multiple compounds belonging to different classes in a single run, are preferred by regulatory agencies and food testing laboratories due to their time-saving abilities and cost-effectiveness.[32,38,84,87,88]

In Canada and the EU there is no obligation to use standardized methods for residue analysis, as long as established performance characteristics are met by the methods used. This approach offers a significant advantage in that it allows development and introduction of new analytical methods.[73]

In 2012, the FSIS started employing MMMs as a means to analyze more compounds per sample and reduce the overall amount of required samples. Despite the continual increase of VD residues, the use of MMMs has helped expand the total number of samples per animal population within a given time frame, a factor which has helped increase the probability of detecting a residue

violation to 99% if the violation rate is equal to or greater than 1% of the population of animals being sampled.[83]

Development of MMMs has been facilitated by the recent major advances in both liquid chromatography (LC) and mass spectrometry (MS) technologies.[89,90] The introduction of ultra-high performance liquid chromatography (UHPLC), featuring sub-2 μm stationary phase particles, and fast MS/MS scanning (<10 ms), has enabled detection of larger numbers of compounds within one run.[6,90] Narrower peaks and consistent retention times afforded by UHPLC enable narrower multiple reaction monitoring (MRM) windows and longer MS/MS dwell times per analyte, and therefore, notable improvements in quantitative and qualitative analytical performance.[5,91]

Currently, LC coupled with electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) in selected reaction monitoring (SRM) acquisition mode is the main analytical platform for MMMs.[92] High resolution MS (HRMS) instruments such as time-of-flight (ToF-MS) and orbitrap have also been successfully used for multi-residue analysis, mainly for screening purposes.[9] Berendsen et al. comprehensively evaluated the use of LC-MS/MS in SRM mode and high-resolution HRMS with respect to the selectivity of 100 veterinary drugs in liver, muscle, and urine extracts, and concluded that MS/MS using SRM mode is more selective than single stage full-scan HRMS monitoring only the molecular ion and allowing a ±5 ppm mass tolerance. The authors concluded that full scan alone is insufficient for confirmatory analysis.[93] However, HRMS can be fit for the purpose for confirmatory analysis when monitoring the molecular ion in full scan and one fragment ion after precursor ion selection.[93] In terms of detectability, Hou et al. compared a MS/MS method with QTOF-MS for multi-class screening of veterinary drugs and pesticides.

The results demonstrated that MS/MS in SRM mode has higher detectability for low amounts of residues (<1 μg·kg−1), whereas QTOF-MS provided comparable results to MS/MS for higher residue concentrations (>10 μg·kg−1).[94] Accordingly, acquisition in SRM mode isolating one precursor ion while monitoring two product ions is considered the benchmark for any reliable confirmation of target analytes and the gold standard for both quantification and screening of veterinary drug residues.[1,5,13,17,22,95]

With that said, and considering the old adage, "you can only find what you are looking for", a major limitation of the MS/MS approach is that it is only capable of detecting targeted drugs, while HRMS have the clear advantage of retrospective data analysis.[93] Moreover, HRMS allows for screening of a theoretically unlimited number of residues, and thus, detection of unknown drugs and low dose cocktails.[73]

1.5 Sample preparation approaches for multi-residue veterinary drugs

Quantification of veterinary drugs in animal tissue samples can be daunting due to the high contents of protein, fat and phospholipids present in this type of complex matrix.[96] The major components of a typical meat sample are water (up to 70%), protein (15–25%), fat (5–25%) and phospholipids (1–3%).[97] Improper sample preparation and clean-up can lead to the presence of interferences from co-eluting components, which adversely affect the signal and compromise the analytical outcome.[40,92,98] Accordingly, a successful outcome for an MMM depends mostly on adopting a sample preparation workflow that minimize interferences from proteins and phospholipids and their adverse effects on chromatographic separation, ionization, and mass

spectrometric analysis.[84] While the modern UHPLC–MS/MS platform is very powerful in terms of detectability and selectivity, it simply cannot replace proper sample preparation.[99–101]

Conventional sample preparation is considered to be the Achilles' heel of any analytical workflow, as it typically takes more than 70% of the total analysis time.[98] Considering the high throughput requirements imposed by MMMs, sample preparation time must be minimized sufficiently so as to render the MMM applicable to fast, high-throughput analysis of extensive quantities of samples within very short timeframes.

Further, an ideal sample preparation procedure for an MMM must be able to not only address an increasing number of analytes with a wide range of physical and chemical properties, but must also be applicable to different types of matrices.[96,102] Therefore, sample preparation procedures that are very generic, non-selective, and allow for sufficient recovery of as many analytes as possible from different matrices are ideally suited for this type of application.[22,90]

In this context, and since this thesis is mainly focused on multi-class multi-residue veterinary drug methods in animal tissue, the scope of the following sub-sections will only discuss generic sample preparation procedures for LC-MS based analysis.

1.5.1 Solvent extraction without clean-up

Solvent extraction (SE) or liquid extraction of homogenized tissues is the first and most essential sample preparation step in conventional LC-MS based analysis of VD residue in tissue samples. Prior to extraction of residues from tissue samples, a homogenization step is carried out using a blade blender or processor to create a uniform sample and assist in the release of any

residues. Cryogenic sample comminution with dry ice or under liquid nitrogen has been demonstrated to achieve much better sample homogeneity as it transforms the sample to a uniform fine powder and also reduces the degradation of labile analytes.[103]

SE only, i.e., without any further clean-up steps, is considered the fastest, simplest, and most economic extraction method among conventional methods.[13] Accordingly, it is frequently reported in the literature as a method of choice.[91,104] An ideal generic extraction solvent for this method should offer efficient extraction of as many target analytes as possible while minimizing the extraction of matrix constituents so as to prevent excessive matrix effects (MEs).[90] The selection of a suitable solvent therefore depends not only on the analytes under study, but also on the matrix. SE is often performed with organic solvents such as acetonitrile, methanol, acetone, or ethyl acetate, since such solvents allow simultaneous precipitation of proteins and extraction of a wide range of target analytes.[91,104] Mol et al. investigated the performance of acetonitrile (MeCN), methanol (MeOH) and acetone (ACE) as extraction solvents for extraction of 86 veterinary drug residues belonging to several drug classes in milk, muscle, egg, honey, and feed matrices.[102] The authors found that MeOH performed worst in term of MEs, which led to ion suppression during detection, probably due to inadequate protein precipitation. While acetone was found to yield the highest recoveries across all tested matrices, it delivered a sub-optimal performance compared to MeCN in terms of MEs. Further, the authors determined that solvent suitability was mostly dependent on the matrix under study, and MeCN was selected as the solvent of choice for extraction of veterinary drugs from muscle samples.

Similarly, Chen et al. performed a comparison of three organic solvents, MeCN, MeOH, and ethyl acetate, using ultrasound-assisted extraction (UAE) for screening of multi-residue veterinary drugs in food-animal tissue, milk, and eggs.[88] The authors reached a similar conclusion to Mol et al. that MeCN offered the best extraction efficiency of analytes and least MEs compared to MeOH and ethyl acetate, as the latter solvents tend to extract too many matrix components, complicating analysis or any further clean-up procedures.[88,99] MeCN in general is the most common generic extraction solvent used in tissue analysis due to its ability to precipitate proteins which is considered the first clean-up step for tissue analysis.[90,99,105] However, MeCN does not sufficiently extract polar analytes such as tetracyclines and penicillins.[32,88,91,106] To enhance extraction of polar analytes, a mixture of MeCN and water is usually employed as extraction solvent. Higher MeCN fractions decreases the extraction efficiency of the more polar analytes, while higher fractions of water lead to higher co-extraction of the matrix components.[106,107] Chen at al. tested different ratios of MeCN:water (90:10, 80:20, and 70:30), and found (90:10, v/v) to provide the highest recovery rate of analytes under investigation. Yet, scientists from the USDA selected 4:1 MeCN/water as their extraction solvent for the current MMM used for extraction of bovine muscle after testing different ratios.[9,32,84,91]

Following SE and prior to instrumental analysis, centrifugation and sometimes ultra-filtration are applied to assist in separating the supernatant from the tissue and protein precipitates.[96,102] However, many reports established that ultra-filtration tends to not only selectively adsorb certain analytes and affect recoveries, but also has the potential to introduce interferences.[32,91,99,107,108] While protein precipitation and centrifugation remove most of the

protein interferences present in animal tissue, fat and phospholipids, which can introduce matrix effects, remain dissolved in MeCN extracts. To address this drawback, defatting of the extracted solvent with hexane has been utilized in different works.[84,89,109] However, the use of hexane was found to cause losses of less hydrophilic compounds. Furthermore, final extracts were found to contain trace amounts of hexane, which could adversely impact chromatography.[32]

To increase the detectability of MMM and enhance chromatography, sample extracts are often evaporated to near dryness and then reconstituted with a solvent compatible with the mobile phase used in the LC method.[84,104,109]

Assisted SE techniques such as pressurized liquid extraction (PLE) or supercritical fluids, which utilize high pressure to heat water above its atmospheric boiling point instead of utilizing an organic solvent, have been applied to improve extraction efficiency and reduce the use of organic solvents.[90,96,110] However, the high temperatures used in PLE render such techniques incompatible with analytes with low thermal stability. For example, degradation of macrolides has been observed with PLE at temperatures above 100 °C.

While SE is a simple and cost-effective sample preparation technique, one of the main disadvantages is the use of large amounts of organic solvents and generation of organic waste due to the use of large amounts of organic solvents. Another evident drawback of SE is the occurrence of abundant matrix effects, which compromise detection limits, quantitative aspects, and method selectivity, in addition to increasing instrument maintenance frequency and accordingly, the overall costs associated with analysis.[13,73,90]

1.5.2 Solvent extraction followed with sorbent clean-up

1.5.2.1 Solid Phase Extraction (SPE) in cartridge format

SPE is frequently used in its conventional packed cartridge or column format as a clean-up technique in the analysis of veterinary drugs in animal tissue.[90,96] There are two modes of SPE clean-up:

1. Conventional SPE mode, in which the solvent extract is applied to the sorbent to selectively adsorb and retain the analytes of interest, while allowing matrix components to pass through and be discarded, followed by a weak solvent wash step to remove matrix interferences and subsequent elution of the analytes of interest from the cartridge with a stronger solvent.[88,111,112]
2. Flow-through mode SPE, in which the sample extract is applied to a sorbent in a highly organic solvent, in such a way that the analytes remain dissolved in the solvent. The eluent is immediately collected for further analysis while the matrix components are retained by sorbent.[106,112]

While conventional SPE clean-up is suitable for single compound methods where selective wash and elution steps can be used effectively to separate the target compounds from matrix interferences, its application is not suitable in multi-class methods due to the diverse range of physico-chemical properties of target analytes. For example, reverse phase (RP) sorbents such as C_{18} and C_8, are not suitable for polar compounds due to their selective affinity for hydrophobic compounds.[90,99] Frenich at al. compared the flow-through method to the conventional SPE method,

and concluded that conventional SPE lead to significant losses of many analytes. The authors concluded that the conventional SPE method was not suitable for simultaneous extraction of several classes of veterinary drugs.[112] A similar conclusion was made by Zhao et al.[113] Moreover, Souverain et al found that SPE tends to concentrate not only the analyte but also matrix components, which may significantly increase ionization suppression.[114]

Regardless of the SPE mode used, silica-based sorbents are susceptible to the binding of tetracyclines to free silanol groups, which can lead to significant losses of these analytes during the clean-up step.[115,116] In efforts to eliminate silanol group interactions and extend the scope of SPE sorbents for a broad range of compounds with different physico-chemical properties, polymeric sorbents with both polar and non-polar affinities, such as hydrophilic-lipophilic balance (HLB), were introduced as alternatives to silica-based sorbents. The HLB sorbent consists of a copolymer of the hydrophilic N-vinylpyrrolidone to increase water wettability, and lipophilic divinylbenzenes, which assist in the reversed-phase retention of analytes. Frenich at al. compared C_{18} and HLB sorbents in the flow-through SPE method and found poor recoveries for tetracyclines with C_{18}.[112]

While SPE in flow-through mode might be more suitable for MMM than conventional SPE, both modes share certain limitations, such as loss of analytes due to blocking of sorption sites by fatty components originating from lipid-rich samples, difficulties in achieving consistent flow, and clogging of cartridges, which renders automation difficult.[13,117]

1.5.2.2 Dispersive SPE/QuEChERS

Dispersive SPE (d-SPE) is a sorbent based clean-up approach that involves the use of solid sorbents in dispersed form to bind to matrix components in the sample extracts.[96] It is best known for its use in the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method. To separate the purified solvent extracts from the sorbent material, centrifugation and sometimes filtration prior to instrumental analysis are needed. The QuEChERS approach found popularity over the well-established conventional SPE method due to its quick and easy extractions, which eliminate clogging concerns while maintaining the clean-up advantage.[13,89] For samples of animal origin, which have higher lipid contents, the selected sorbent material is usually a reverse-phase material such as C_{18}. The original QuEChERS method achieves phase separation via addition of inorganic salts; however, use of salt may cause loss of sensitive or polar analytes due to metal chelation, degradation, or limited solubility of polar compounds in MeCN.[113] Scientists from the USDA evaluated several materials for use as clean-up sorbents, including end-capped C_{18}, graphitized carbon black, Z-Sep, and Z-Sep$^+$ (zirconium oxide-based materials from Supelco), a polymeric ENV+ from Biotage, and enhanced-matrix removal of lipids (EMR-L), a new material from Agilent. While the structures of these sorbents were not disclosed, in the case of EMR-L, it is mentioned that the mechanism involves size exclusion and hydrophobic interactions.[9,32,84,109] As a compromise between sample throughput, recovery, and cleanliness of sample extracts, the authors selected C_{18} as the clean-up sorbent for their final method. The authors determined that while the E-MRL method provided cleaner extracts than C_{18}, it was not suitable in terms of throughput and practicality, as the E-MRL method required extra time-sensitive steps to condition and activate the

sorbent, consequently adding cost and time to the already lengthy sample preparation step.[9] However, the authors did not include in their studies an assessment of increased instrument maintenance requirements due to the introduction of dirtier extracts, which in the long run impact downtime and cost. Nevertheless, following further investigation of matrix effects in their latest MMM evaluation, the authors concluded that the sample extract clean-up provided by the d-SPE method step does not merit the extra time and effort, nor the reduction of analytical scope that is incurred. Instead, the authors opted for injecting a small sample equivalent by using a large dilution factor.[91] Similarly, Robert et al., after investigating several SPE clean-up methods and sorbents, skipped the sorbent clean-up step due to the loss of certain analytes and the added time, effort, and cost associated with this step.[104] While large dilution factors were demonstrated to be more efficient than d-SPE in minimizing matrix effects, it can complicate the method's limit detectability.[12]

While the QuEChERS/d-SPE approach offers several advantages over conventional SPE, it is obvious from the abovementioned studies that it does not provide efficient clean-up and thus higher matrix effects in addition to partial loss of the analytes.[9,13,104]

Similar to SE only, both SPE and d-SPE require the use of large sample volumes of toxic organic solvents resulting in large volume of hazardous waste, which make these techniques not only time-consuming, but also environmentally harmful.[13]

Given the challenges and limitations associated with the conventional methods described above, an alternative sample preparation approach is required to address the pressing analytical demand currently observed in this growing field of analysis. An ideal sample preparation for multi-

residue drug analysis in tissue samples should be non-selective, as well as able to isolate and enrich a wide range of analytes from matrix components while using a minimum amount of analytical steps, and generating minimum amounts of solvent waste. Moreover, it should be suitable for automation and high-throughput analysis.

1.6 Solid phase microextraction (SPME)

1.6.1 Theoretical aspects of SPME

Solid-phase microextraction (SPME) is a versatile sample preparation technique developed in 1989 by Pawliszyn and co-workers at the University of Waterloo.[118] SPME was originally designed as a fused silica fiber coated with a thin polymeric extraction phase for equilibrium based extraction of volatile and semi-volatile organic compounds from air and water for analysis with gas chromatography (GC).[118,119] In the last three decades, the applicability of SPME has substantially expanded to offer numerous geometrical configurations with different substrates and coating chemistries that allow direct extraction of a wide range of analytes from a broad variety of complex liquid and solid matrices, for both GC and LC applications. For volatile and semi-volatile analytes, the extraction process can be carried out either by exposing the SPME extractive phase to the headspace above the sample matrix, or by direct immersion of the extractive phase to the

sample matrix, as shown in Figure 1.14, whereas successful extraction of low volatility compounds is carried out using direct immersion mode only.

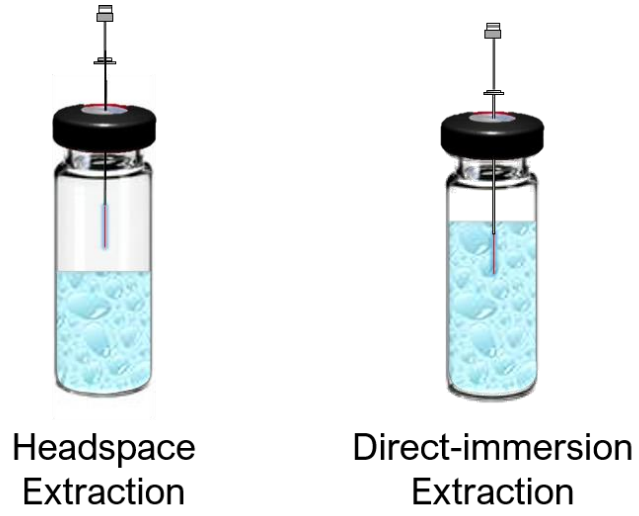


Figure 1.14 SPME modes of extraction

After the extraction process with SPME is complete, the extracted analytes are desorbed from the extractive phase either thermally using high-temperatures, or by using a solvent with strong affinity for the target analytes. The desorption step is generally followed by instrumental analysis in GC or LC platforms.[120,121]

An in-depth understanding of SPME theory is crucial for successful development of SPME methods, as analysts must be able to identify, control, and exploit the various parameters that influence SPME extraction in order to develop and optimize a method for a given application.[122]

First and foremost, unlike SE and SPE, SPME is based on the non-exhaustive extraction of quantities of analytes that are proportional to the concentrations of said analytes in a sample matrix. The extent of extraction is governed by the analyte distribution constant (K_{es}) between the SPME

extractive phase and sample matrix for the analyte of interest, which is expressed by the following equation:

$$K_{es} = \frac{C_e^{\infty}}{C_s^{\infty}} \qquad \textit{Equation 1.1}$$

where C_e^{∞} and C_s^{∞} are the analyte equilibrium concentrations in the extractive phase and the sample, respectively. K_{es} is mainly dependent on the physico-chemical properties of the analyte of interest and the extractive phase used. Extraction conditions that affect K_{es} are temperature, ionic strength, pH, and organic solvent content. At equilibrium, in the case of direct immersion extraction using a liquid-based extractive coating, where the analyte diffuses within the complete volume of the extractive phase during the extraction process, the amount of analyte extracted by the extractive phase (n_e) is given by Equation 1.2:[123,124]

$$n_e = \frac{K_{es}V_e\, V_s\, C_s^0}{K_{es}V_e + V_s} \qquad \textit{Equation 1.2}$$

where C_s^0 is the initial sample concentration, V_s is the sample volume, and V_e is the volume of the extractive phase. When the sample volume, V_s , is much larger than $K_{es}V_e$, Equation 1.2 can be reduced to:

$$n_e = K_{es}V_eC_s^0 \qquad \textit{Equation 1.3}$$

Therefore, according to SPME fundamental principles, as shown in equations 1.2 and 1.3, the amount of analyte extracted by SPME is proportional to the volume of the extractive phase.

In case of solid adsorptive coatings, where the volume of surface-active sites should be taken into consideration, n_e can be calculated using Equation 1.4:

$$n_e = \frac{K_{Afs} V_e V_s C_s^0 (C_{f\,max} - C_f^\infty)}{V_s + K_{Afs} V_e (C_{f\,max} - C_f^\infty)} \qquad \textit{Equation 1.4}$$

where $C_{f\,max}$ is the maximum concentration of active sites in the solid coating, C_f^∞ is the equilibrium concentration of the analyte in the extractive phase, and K_{Afs} represents the analyte's adsorption constant, which is the ratio of the surface concentration of the adsorbed analyte on the porous solid extractive phase (S_{Af}^∞) to the concentration of the analyte in the sample at equilibrium ($K_{Afs} = S_{Af}^\infty / C_s^\infty$).

When extraction is performed from a heterogeneous sample, such as biological fluids or tissue, the amount of analyte extracted by SPME is directly proportional to the unbound concentration of the drug present in the sample system, and can be calculated by modifying Equation 1.2 to the following equation:

$$n_e = \frac{K_{es} V_e V_s C_s^0}{K_{es} V_e + \sum_{i=1}^{i=m} K_{is} V_i + V_s} \qquad \textit{Equation 1.5}$$

Where $K_{is} = C_i^\infty / C_s^\infty$ is the distribution constant of the analyte between the *i*th phase and the matrix of interest with a volume of V_i. All other terms are defined as in the previous equations.

1.6.2 Balanced Analyte Coverage

According to equation 1.5, in case of heterogeneous samples (such as biological fluids or tissue homogenates), the amount of analyte extracted by SPME is directly proportional to the free

(unbound) concentration of the analyte under study.[123] This unique characteristic of SPME enables it to achieve balanced coverage of analytes with a wide range of physico-chemical properties in the presence of matrix components without manifestation of saturation or displacement effects.[125,126] Essentially, due to the presence of binding matrix components, analytes that usually exhibit strong affinities for SPME coatings tend to bind heavily to matrix components, which limits their availability in their free form for extraction by SPME. As a result, adsorptive coatings are unlikely to become saturated by these compounds within a reasonable extraction time. In contrast, compounds with low affinity for SPME extraction phases, such as polar compounds, typically demonstrate low protein binding affinity, resulting in a high proportion of these chemicals becoming freely available in the sample in comparison to hydrophobic compounds. Yet, due to the lower affinity of these compounds to SPME coatings, saturation from these compounds is also unlikely to take place.[127] This phenomenon of balanced analyte coverage, which has been demonstrated by experimental data as well as mathematical models by Alam et al., additionally leads to minimal extraction of phospholipids, resulting in significant reduction of matrix effects when performing quantification using ESI.[126]

1.6.3 Kinetics of solid phase microextraction

The SPME extraction process generally follows the profile shown in Figure 1.15, where the x-axis represents extraction time whereas the y-axis represents the amount of analyte extracted.[128] When the extractive phase is first exposed to the sample, an almost linear mass uptake process ensues until extraction time t_{50}, which is defined as the time needed for 50% of the equilibrium amount of analyte to be extracted from the sample. Afterwards, the rate of extraction slows down until it reaches equilibrium.

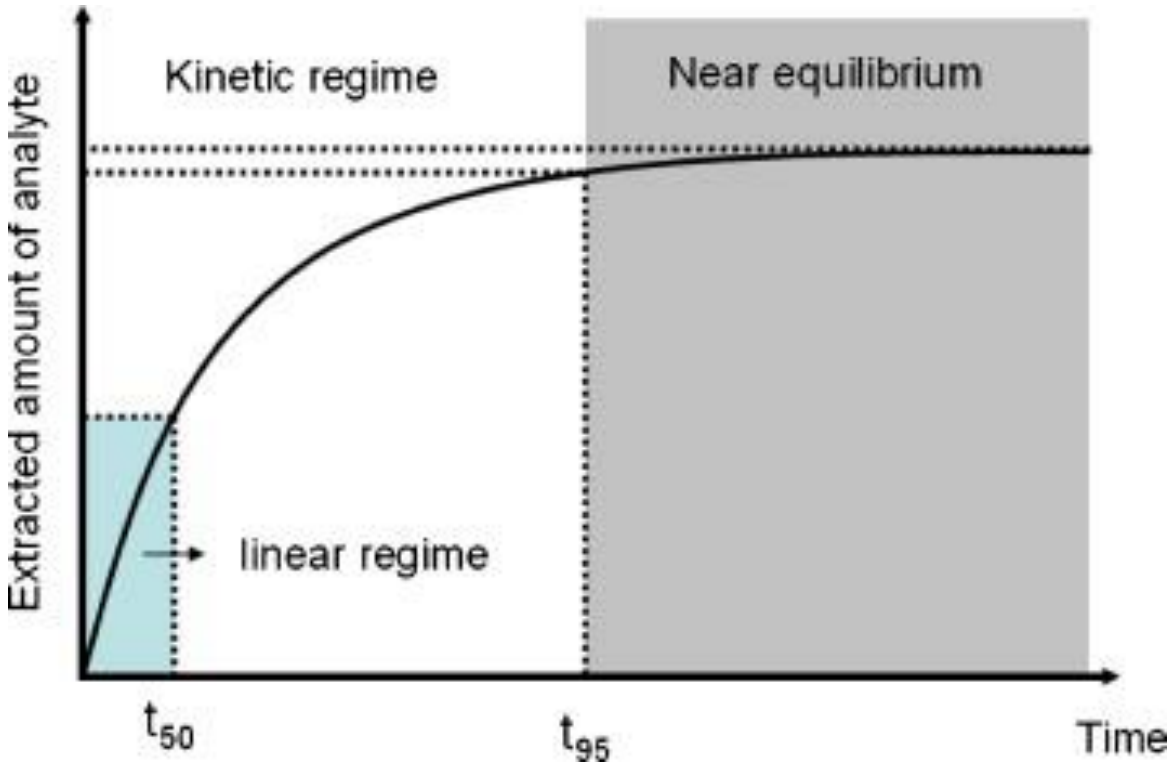


Figure 1.15 Typical extraction time profile for SPME

A kinetic regime is defined by a steady increase in the amount of analyte extracted. Theoretically, the time required to reach equilibrium is infinite.[129] However, in practice, assuming

that the experimental error is typically about 5%, equilibration time is assumed to be achieved when 95% of the equilibrium amount of an analyte is extracted from the sample. Thus, for all intents and purposes, t_{95} is assumed to be the equilibrium time. In a practical agitated system, the main factors that influence the time required to achieve equilibrium can be expressed using the following equation:

$$t_{95} = \frac{3\, K_{es}\, b\, \delta}{D_s} \qquad \textit{Equation 1.6}$$

where b is the fiber coating's thickness, δ is the boundary layer, and D_s is the analyte's diffusion coefficient in the sample fluid. From equation 1.6, we can conclude that the time required to reach equilibrium can be very long for analytes that have either very high affinity for the coating, δ is too large (e.g. no agitation); or the extractive phase is too thick.

When time is of importance, and in cases where it takes a long time for all analytes to reach equilibrium, a kinetic or pre-equilibrium calibration approach can be employed instead. At such conditions, the amount extracted is calculated by using Equation 1.7, where a is a time constant that represents a measure of how fast adsorption equilibrium can be reached in the SPME process, while t is extraction time.[130]

$$n = (1 - e^{-at}) \frac{K_{es} V_e\, V_s\, C_s^0}{K_{es} V_e + V_s} \qquad \textit{Equation 1.7}$$

It is of course obvious from figure 1.15 that maximum detectability and better reproducibility can be achieved through equilibrium extraction. However, for applications where rapid analysis is required, a compromise between extraction efficiency and the overall time of extraction must be

made. Nevertheless, with recent advances in MS capabilities, pre-equilibrium extraction is still capable of achieving low limits of quantitation. In addition, as will be discussed in the following sections, full automation of the extraction step permits implementation of pre-equilibrium extraction without compromising the reproducibility of the method.[131]

1.6.4 Thin Film SPME (TFME)

The low regulatory level requirements that must be met by methods for residue analysis in complex sample matrices are one of the key challenges in the development of said methodologies, as such methods must have superior detectability, often at trace level, for a large number of compounds consisted of a wide range of physico-chemical properties.

According to SPME fundamental principles, as shown in Equation 1.3, the amount of analyte extracted by SPME is proportional to the volume of the extractive phase (V_e); thus, SPME detectability can be improved with the use of larger extractive phase volumes. While this can be accomplished by simply increasing the thickness of the extractive phase, this approach, as specified in equation 1.6, would obviously also lead to longer equilibration times, and thus longer extraction times and lower sample throughput.[132] An alternative approach to increasing the volume of the extractive phase that does not compromise extraction time is to increase the surface area of the coating. This can be achieved either by increasing the diameter of the SPME fiber, or by using a thin-film geometry. It has also been demonstrated that the initial uptake rate of SPME extraction is proportional to the surface area of the extraction phase, as shown in equation 1.8:[133]

$$\frac{dn}{dt} = \frac{D_S A}{\delta} C_S^0 \qquad \textit{Equation 1.8}$$

where n is the mass of analyte extracted over sampling time t, and A is the surface area of the extraction phase. Therefore, the best way to increase the volume of the extraction phase, and thus the detectability of the method, is to use a thin extraction phase with a large surface area, as this configuration would also additionally improve the uptake rate of the method. This theory formed the basis for the development of thin-film SPME (TFME). Another advantage of the TFME geometry as compared to a rod geometry with a larger diameter is the higher surface area-to-volume ratios provided by TFME, which enable configuration of smaller probes that occupy significantly less space than the rod geometry. If made sufficiently large, the rod geometry can cause displacement of the sample solution and limit the maximum sample volume that could be placed in the wells, although smaller sample volumes can also be compensated for by using less desorption solvent volumes.

Although the term TFME may cause some confusion since the extraction phase of traditional SPME fibers is essentially a thin film, TFME in this context refers to a thin rectangular flat surface geometry with relatively larger surface areas and larger volumes of extraction phase compared to traditional fibers.[120,134] The most common format of TFME for LC-MS based analysis consist of a stainless steel blade coated with an extraction phase.[132]

1.6.5 Automated high-throughput SPME

In modern food safety analysis, increased pressure to produce results in the shortest possible turnaround time continues to create high demand for the development of rapid and high-throughput methodologies.[96,134] Within the SPME context, sample throughput can be significantly increased by performing the extraction and desorption of large numbers of samples in parallel with the use

of a multi-well-plate format, resulting in a significant reduction in sample preparation time per sample. This type of high-throughput was successfully introduced in 2008 with the use of the Concept 96 autosampler.[135–137] The Concept 96 autosampler is a fully automated, software-operated, off-line robotic sample-preparation station designed by Professional Analytical Systems (PAS) Technology (Magdala, Germany) to facilitate the simultaneous extraction of up to 96 samples. While the first application of this automated system was aimed at accommodating SPME in its traditional fiber format, the platform has since evolved to accommodate different geometries and coating chemistries.

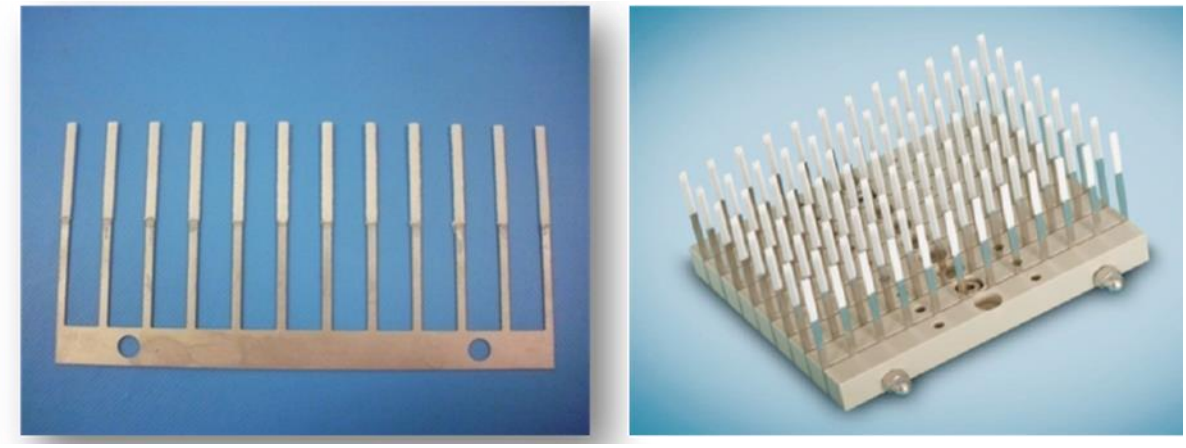


Figure 1.16 Thin Film SPME (TFME)

The mechanical robustness afforded by the rigid metal nature of the TFME blades (shown in figure 1.16) makes it an ideal format for the Concept 96 autosampler.[137]

Combining the advantages of simultaneous extraction of 96 individual samples with larger surface areas and higher recoveries has allowed for successful development of a large assortment

of high-throughput analysis methods that cover a wide range of analytes, including various food, environmental, and bioanalytical applications.[123,131,145,146,132,138–144]

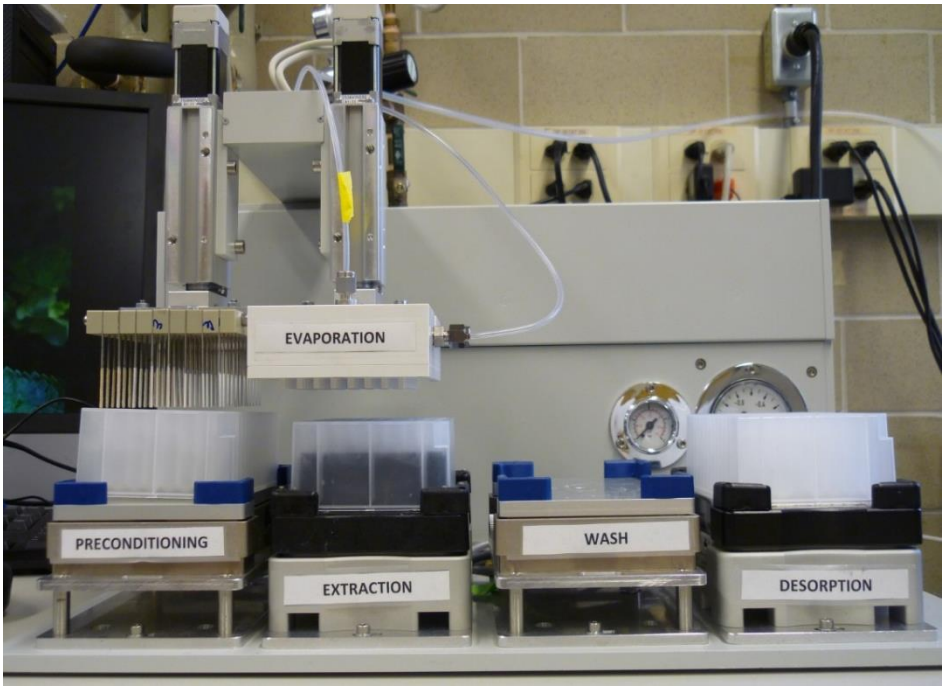


Figure 1.17 Concept 96 autosampler, Professional Analytical Systems (PAS) Technology

Some of the major advantages of this system for analysis of tissue samples are its open-bed configuration and its ability to perform direct extraction from complex matrices such as dense fluids and colloidal suspensions without any need for sample pretreatment or concerns regarding clogging, which is a common concern in SPE systems.[138]

1.6.6 Biocompatible SPME coatings

Certainty, one of the main concerns when selecting an SPME coating for open-bed extraction from tissue homogenate is the potential adhesion of proteins and other macromolecules to the surface of the coating.[147] Adsorbed macromolecules form a diffusion barrier that can affect the

kinetics of analyte uptake and reduce the efficiency of extraction. One of the solutions to minimize the attachment of macromolecules to the coating surface is the use of biocompatible coatings. In the context of SPME, biocompatible coatings serve two purposes: (i) they prevent adverse and/or toxic reactions in living systems and, (ii) they prevent adhesion of macromolecules, such as proteins, to the surface of the coating.[123] The development of biocompatible SPME coatings can be achieved by either direct application of biocompatible extractive phases such as PDMS, or by utilizing biocompatible polymers to immobilize non-biocompatible extractive particles onto SPME substrates. For instance, while Polyacrylonitrile (PAN), considered one of the best polymers in terms of biocompatibility, is not appropriate in itself as an extractive phase for drugs, and whereas good extractive materials are generally not biocompatible, as demonstrated by Musteata and co-workers, PAN can be used as a binder to immobilize these extractive materials onto SPME substrates to form a biocompatible extractive phase.[147] The hydrophilic and negatively charged polyacrylonitrile minimizes the binding of macromolecules such as proteins and allows for selective permeation of small molecules to the extraction phase.[120,126,148] Over the last decade, the emergence of biocompatible coating chemistries for SPME devices, which hinder fouling of the coating by protein adsorption, has enabled employment of direct immersion SPME for analysis of complex samples in many applications.[148–151]

While PAN as a binder offers the desired biocompatibility characteristic, the selection of suitable extractive particles is key to achieving maximum SPME detectability. An ideal coating for multi-class multi-residue analysis would offer extraction efficiency for a large number of compounds with a wide range of physico-chemical properties.[131,134] In this case, the ideal

extractive phase consists of a universal sorbent with affinity for both polar and non-polar compounds. One example of such a sorbent is Hydrophilic-Lipophilic Balance (HLB) particles. HLB particle sorbents are second-generation mesoporous polymers characterized by a high surface area that were specifically designed for extraction of low-molecular-weight polar and non-polar compounds.[152] Due to the respective presence of aromatic rings in divinylbenzene and polar groups in the lactam ring of N-vinylpyrrolidone, as can be seen in Figure 1.18, HLB provides balance between hydrophobic and hydrophilic interactions.

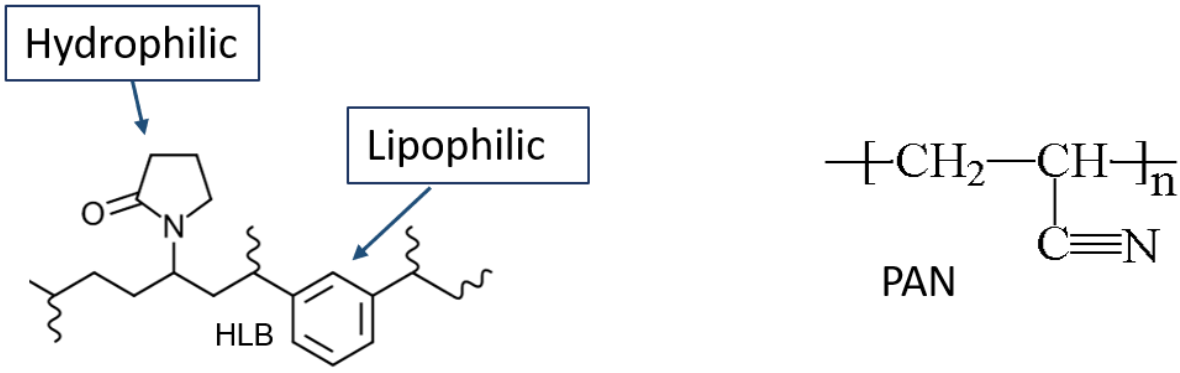


Figure 1.18 HLB copolymer and PAN structures

Another advantage of selecting HLB as an extractive phase is its characteristic wettability: the hydrophilic N-vinylpyrrolidone increases the water wettability of the polymer, which facilitates its interaction with aqueous matrices.

1.6.7 SPME applications in biological tissue analysis

Biocompatible SPME probes have facilitated a growing number of studies for the determination of drug concentrations in targeted tissues and organs.[153] The distinct advantage

offered by SPME in tissue analysis is its tuning capabilities in terms of both the geometry of the device and the coating chemistry. The geometry of the device can be customized and miniaturized to target specific sampling sites with minimal invasiveness, while the coating chemistry can be either selective to target specific analytes or generic to cover a very wide range of analytes. The suitability of DI-SPME methodology has been demonstrated and validated successfully for both ex vivo and in vivo sampling for targeted and untargeted studies for many different types of tissue samples from different matrices, such as fish, as well as muscle, lung, liver, and brain.[154] It has also been applied successfully for monitoring and sampling tumors and other tissues in humans. For instance, SPME fibers were successfully utilized to measure changes in the concentrations of selected neurotransmitters, namely, dopamine, serotonin, gamma amino-butyric acid, and glutamic acid, in rats brains after fluoxetine administration.[155] Another application by Togunde et al. employed in vivo non-lethal SPME sampling coupled with LC-MS/MS to quantify drugs such as fluoxetine, venlafaxine, sertraline, paroxetine, and carbamazepine in rainbow trout and fathead minnow.[156] Recently, Roszkowska and colleagues have developed an in vivo SPME-LC-MS/MS method for the quantitation of the anticancer drug, doxorubicin in pig lung tissues.[157] Preliminary experiments were performed using the ex vivo SPME approach, using lamb's lungs as a surrogate matrix to optimize the extraction conditions and for ex vivo calibration for doxorubicin. As evidenced in the literature, the biocompatibility of SPME together with its flexible tuning capabilities have facilitated numerous applications in biological tissue analysis.

1.7 Research objective

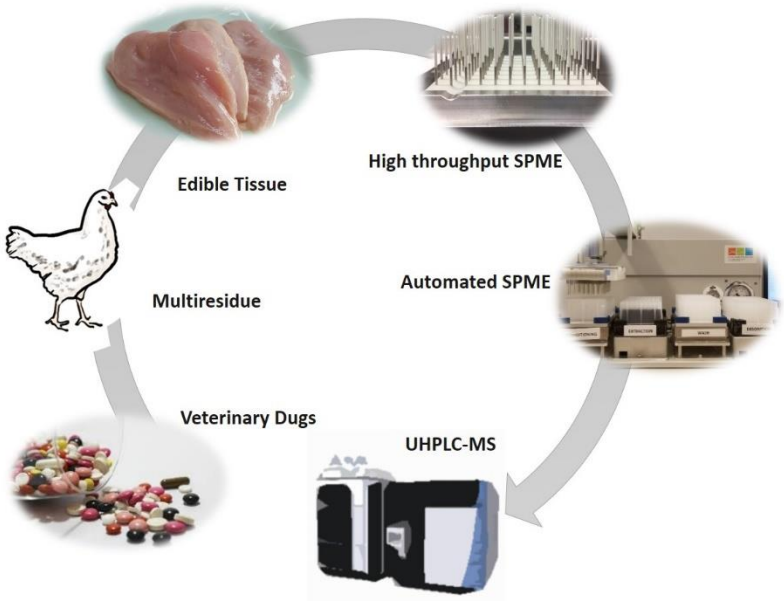
In the field of food safety, the perishable nature of food commodities such as meat demands accurate and fast decisions regarding potential human health risks. Therefore, food safety investigations require the use of fast, efficient, and reliable analytical methods. In addition, due to the large number of samples to be analyzed, high-throughput methods are favored by regulatory agencies and food safety laboratories to save cost and time. However, due to the tedious nature of conventional sample preparation approaches and the large number of steps involved, the possibility of error is very high. Accordingly, automation of the sample preparation step plays a major role in improving efficiency and analytical outcomes.

The main objective of this thesis was to develop a fully automated high-throughput sample preparation method for quantitative analysis of multi-class multi-residue veterinary drugs in animal tissue using the Concept 96-blade SPME robotic system and LC-MS/MS. Chapter 2 is mainly focused on method development, optimization of parameters influencing SPME extraction, and the validation of a fully automated, high-throughput multi-class multi-residue method for quantitative analysis of 77 veterinary drugs using SPME and LC-MS/MS. In chapter 3, the scope of the method developed in chapter 2 was extended to cover more than 100 veterinary drugs in two different matrices: chicken and beef muscle. Moreover, chapter 3 also encompassed a thorough comparison between the developed SPME method and two well-documented generic sample preparation procedures, namely solvent extraction (SE) and QuEChERS. With aims to overcome lengthy chromatographic runs, and reduce sample turnaround time, chapters 4 and 5 focused on rapid screening by direct coupling of SPME to MS. Finally, Chapter 6 summarizes the

main findings and contribution of this work and proposes future directions with regards to the application of SPME in multi-residue analysis of tissue samples.

Chapter 2

Development and Validation of a Fully Automated Solid Phase Microextraction High-throughput Method for Quantitative Analysis of Multi-residue Veterinary Drugs in Chicken Tissue



2.1 Preamble

The materials in this chapter have been published as a research article: Khaled, A.; Gionfriddo, E.; Acquaro, V.; Singh, V.; Pawliszyn, J. Development and Validation of a Fully Automated Solid Phase Microextraction High Throughput Method for Quantitative Analysis of Multi-residue Veterinary Drugs in Chicken Tissue. *Anal. Chim. Acta* **2019**, *1056*, 34–46. Materials for all sections of this current chapter are reprinted from this research article with the permission of Elsevier, (Copyright 2018). Copyright for this work remains the property of Elsevier publications and any further request for re-use of this information should be requested directly from them (DOI: https://doi.org/10.1016/j.aca.2018.12.044).

The contribution of co-author Emanuela Gionfriddo to the work described in this chapter was technical advice at the early stage of method development and assistance in preparing stock solutions. The contribution of Varoon Singh was in preparation of 5 µm HLB particles for the SPME coating. Vinicius Acquaro Jr. assisted in the use of the Statistica software for statistical analysis for the lack of fit and the simplex-lattice mixture design (SLMD). All of the experimental work, experimental planning and design conducted in the laboratory, data processing, analysis, interpretation, and writing were performed by the author of the thesis.

I, Emanuela Gionfriddo, authorize Abir Khaled to use the material for her thesis.

I, Vinicius Acquaro Jr., authorize Abir Khaled to use the material for her thesis.

I, Varoon Singh, authorize Abir Khaled to use the material for her thesis.

2.2 Introduction

As per current agricultural practices, animals raised for food are often housed and transported in high densities, which makes them more prone to increased levels of stress and disease. Aiming to minimize livestock losses and increase production, veterinary drugs (VDs) are thus frequently used to prevent and treat diseases, as well as promote weight gain.[158,159]. In this regard, illegal or improper dosage of VDs as well as failure to comply with stipulated withdrawal dates may lead to the presence of drug residues in the edible tissue of the treated animal. These residues, even at low concentrations, may in turn pose a risk to human health.[159] In this respect, one of the main concerns arising from the overuse of antibiotics in animals is the emergence of resistant bacteria.[91]

Aiming to protect human health, most governments and associated agencies have established monitoring and regulation laws, standards, and procedures with respect to veterinary drug residues in the edible tissues of food-producing animals.[109,160] To this end, maximum residue levels (MRLs), defined as the maximum concentration of residue that can safely remain in the edible tissue of an animal that has been treated with a veterinary drug, are established to set and enforce these regulatory standards.[81] In Canada, VD residues are regulated by MRLs established by the Veterinary Drugs Directorate of Health Canada. Likewise, in the USA, regulatory tolerances of registered veterinary drugs are set by the Food and Drug Administration (FDA) Center for Veterinary Medicine.[84] In the European Union (EU), the use of veterinary drugs is also strictly regulated through EU Council Regulation 2377/90/EC.[161] At the international level, MRLs are established by Codex Alimentarius, a joint initiative between the Food and Agricultural Organization and the World Health Organization.[85] MRLs are also employed in the establishment and monitoring of trading standards so as to ensure the quality of imported and exported meat.[13,86]

Given the growing public interest and concern regarding food safety and taking into account the importance of the meat industry to the global economy, the demand for simple, automated high-throughput analytical procedures for monitoring of drug residues in meat is expected to continue growing. Within this same context, cost-effectiveness plays a large role in analytical method selection, particularly for laboratories that provide regulatory testing for VD residues. In this respect, one approach to increase the efficiency and cost-effectiveness of an assay is to increase the number of analytes that can be determined in a single run or method. Consequently, multi-

residue, multi-class analytical methods have been gaining popularity as cost-effective methods for screening, identification, and quantification of drug residues in food.[32]

Despite the high selectivity and detectability afforded by LC-MS/MS platforms, analysis of VDs at trace levels in complex matrices, such as animal tissues, still requires extensive sample preparation procedures aimed at isolating target analytes from complex matrix constituents, and minimizing interferences and matrix effects.[90] In this respect, multi-residue sample preparation poses a large challenge to analysts due to two main factors: the large quantity and variety of analytes under consideration, and the complexity of the matrix under study. A suitable sample preparation method must enable the detection and quantification of a large quantity of analytes, encompassing a wide range of physico-chemical properties, while offering sufficient sample clean-up so as to minimize interferences and matrix effects - a challenging prospect, given that the studied matrix is characterized by the presence of endogenous compounds as well as macromolecules such as proteins and lipids. One of the first and most commonly employed sample preparation techniques for analysis of tissues is solvent extraction (SE), which involves liquid extraction of analytes from homogenized animal tissue.[85] While this method offers a quick and simple workflow, it also involves the co-extraction of a high number of endogenous sample compounds, which may cause matrix effects in LC-MS/MS analysis. Other disadvantages include the possibility of emulsion formation and the use of large volumes of toxic organic solvents.[162] To minimize interferences and matrix effects, solid phase extraction (SPE) is commonly used for further sample pre-treatment.[98] Another extraction method that offers purification of sample interferences in complex matrices is dispersive SPE (d-SPE), which is widely applied in the

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method.[163–165] However, while SPE and d-SPE techniques are widely employed for sample preparation, such approaches do not always effectively eliminate matrix effects, and may sometimes retain target analytes, thus leading to analyte loss.[90,91] Further, owing to the multiple steps often involved in such methods, employment of such sample preparation strategy may involve long analysis times, the introduction of errors, as well as large costs.[7]

As a well-established and environmentally friendly sample preparation technique, solid SPME enables the attainment of clean sample extracts while minimizing matrix interferences from biological samples.[123,137,166] Matrix-compatible SPME coatings offer adequate robustness for direct immersion in complex matrices and balanced extraction coverage of compounds with a wide range of polarities.[126,167,168] Matrix-compatible coatings, when combined with the open-bed configuration of SPME, facilitate extraction of multi-residue compounds from complex matrices without being burdened by the clogging issues typical of conventional SPE packed bed systems.[120] Boyaci et al. recently developed a fully automated high-throughput thin-film solid phase microextraction (TF-SPME) method where TF-SPME blades coated with C_{18} particles/PAN were utilized to extract 110 doping compounds banned by the World Anti-Doping Agency (WADA) from urine.[131] Among the many configurations offered by SPME, SPME thin films are consisted of a higher surface area as compared to the conventional SPME fiber format, and a comparable or lower thickness.[132] Further, as thin-film SPME offers high-throughput compatibility, use of thin-film SPME offers enhanced throughput of the extraction process due to the simultaneous extraction of 96 individual samples, with minimal use of organic solvents. Another approach to increase

surface area and enhance recovery is the use of round SPME devices with larger outer diameters.[149,169] The purpose of the presented work entailed the development of a simple, green, automated, high throughput sample preparation method for determination of a wide range of veterinary drugs at regulatory levels in chicken muscle. Automated sample preparation improves precision and reproducibility due to the elimination of human error from the procedure. Thus, an automated high throughput DI-SPME LC-MS/MS method is presented in this chapter for analysis of 77 veterinary drug compounds in homogenized tissue from chicken.

2.3 Experimental

2.3.1 Chemicals and materials

The veterinary drugs under study were selected from the list of standards specified in the official method for screening and confirmation of animal drug residues developed by the United States Department of Agriculture (CLG-MRM1.08)[31] and referred to by Schneider et al.[32] Depending on availability of standards, we aimed to include as many analytes from the list which cover a wide range of polarities representing at least 12 classes. Standards were obtained from suppliers listed in Table 2.1. The corresponding class of each target analyte is presented in Table 2.2. LC-MS grade acetonitrile (MeCN), methanol (MeOH), isopropyl alcohol (IPA), water, and formic acid (FA) were purchased from Fisher Scientific (Mississauga, ON, Canada). Polyacrylonitrile (PAN), Dimethyl sulfoxide (DMSO), and N, N-dimethylformamide (DMF), divinylbenzene (DVB), N-vinylpyrrolidone (N-VP), and 2,2'-azobisisobutyronitrile (AIBN) were obtained from Sigma Aldrich (Oakville, ON, Canada). Nunc U96 Deep Well 2 mL and 1 mL plates made of polypropylene were purchased from VWR International (Mississauga, ON, Canada).

Polypropylene inserts (600 µL) for the 1 mL plates were purchased from Analytical Sales and Services (NJ, USA). For preparation of SPME coatings, C_8-benzenesulfonic acid (Mix-mode) was obtained from Supelco (Bellefonte, PA, USA), PS-DVB was purchased from Chromabond, Germany, and HLB particles were synthesized in-house as per the protocol discussed in section 2.3. High-tolerance 304 stainless steel rods (1/16" diameter) were obtained from McMaster-Carr (Aurora, OH, USA) for use as SPME pins.

Table 2.1 Physico-chemical properties and supplier information for all studied compounds.

Compound	**Supplier**	**Formula[1]**	**Molecular Mass (Da)[1]**	**Log P[1]**
2-Aminoflubendazole	Sigma-Aldrich	$C_{14}H_{10}FN_3O$	255.2	1.96
5-Hydroxythiabendazole	Sigma-Aldrich	$C_{10}H_7N_3OS$	217.3	1.73
6-Phenyl-2-thiouracil	Sigma-Aldrich	$C_{10}H_8N_2OS$	204.3	-0.10
Acepromazine Maleate	Sigma-Aldrich	$C_{19}H_{22}N_2OS$	326.5	4.08
Albendazole	Sigma-Aldrich	$C_{12}H_{15}N_3O_2S$	265.3	3.07
Albendazole-2-Aminosulfone	Sigma-Aldrich	$C_{10}H_{13}N_3O_2S$	239.3	0.12
Albendazole Sulfone	Sigma-Aldrich	$C_{12}H_{15}N_3O_4S$	297.1	0.86
Albendazole Sulfoxide	Sigma-Aldrich	$C_{12}H_{15}N_3O_3S$	281.3	0.91
Azaperone	Sigma-Aldrich	$C_{19}H_{22}FN_3O$	327.4	2.50
Betamethasone	Sigma-Aldrich	$C_{22}H_{29}FO_5$	392.2	1.87
Cambendazole	Sigma-Aldrich	$C_{14}H_{14}N_4O_2S$	302.4	2.90
Carbadox	Sigma-Aldrich	$C_{11}H_{10}N_4O_4$	262.2	-1.22
Chlorpromazine HCl	Sigma-Aldrich	$C_{17}H_{20}Cl_2N_2S$	355.3	5.20
Chlortetracycline HCl	Sigma-Aldrich	$C_{22}H_{24}Cl_2N_2O_8$	515.3	1.11
Clenbuterol HCl	Sigma-Aldrich	$C_{12}H_{19}Cl_3N_2O$	313.7	2.61
Clindamycin HCl	Sigma-Aldrich	$C_{18}H_{34}Cl_2N_2O_5S$	461.4	1.83
Cloxacillin Sodium Salt	Sigma-Aldrich	$C_{19}H_{17}ClN_3NaO_5S$	457.9	2.53
Danofloxacin	Sigma-Aldrich	$C_{19}H_{20}FN_3O_3$	357.1	1.20
Desethylene Ciprofloxacin HCl	TRC[2]	$C_{15}H_{17}ClFN_3O_3$	341.8	-0.14
Diclofenac Sodium	Sigma-Aldrich	$C_{14}H_{10}Cl_2NNaO_2$	318.1	4.06

Table 2.1 continued

Compound	Supplier	Formula[1]	Molecular Mass (Da)[1]	Log P[1]
Dicloxacillin Sodium Salt Hydrate	Sigma-Aldrich	$C_{19}H_{16}Cl_2N_3NaO_5S.xH_2O$	492.3	3.02
Difloxacin HCl	Sigma-Aldrich	$C_{21}H_{20}ClF_2N_3O_3$	435.9	2.78
Dimetridazole	Sigma-Aldrich	$C_5H_7N_3O_2$	141.1	0.31
Doxycycline HCl	Sigma-Aldrich	$C_{22}H_{25}ClN_2O_8$	480.9	-0.54
Emamectin Benzoate	Sigma-Aldrich	$C_{56}H_{81}NO_{15}$	1008.2	6.84
Enrofloxacin	Sigma-Aldrich	$C_{19}H_{22}FN_3O_3$	359.4	1.88
Erythromycin	Sigma-Aldrich	$C_{37}H_{67}NO_{13}$	733.9	2.83
Erythromycin	Sigma-Aldrich	$C_{37}H_{67}NO_{13}$	733.9	2.83
Fenbendazole	Sigma-Aldrich	$C_{15}H_{13}N_3O_2S$	299.3	3.75
Fenbendazole Sulfone	Sigma-Aldrich	$C_{15}H_{13}N_3O_4S$	331.3	1.70
Florfenicol amine	Sigma-Aldrich	$C_{10}H_{14}FNO_3S$	247.3	-0.80
Flubendazole	Sigma-Aldrich	$C_{16}H_{12}FN_3O_3$	313.3	3.05
Flunixin	Sigma-Aldrich	$C_{14}H_{11}F_3N_2O_2$	296.2	5.40
Haloperidol	Sigma-Aldrich	$C_{21}H_{23}ClFNO_2$	375.9	3.01
Haloxon	CedarLane	$C_{14}H_{14}Cl_3O_6P$	415.6	2.81
Hydroxy dimetridazole	TRC[2]	$C_5H_7N_3O_3$	157.1	-0.49
Hydroxy ipronidazole	Sigma-Aldrich	$C_7H_{11}N_3O_3$	185.2	0.21
Hydroxy metronidazole	Sigma-Aldrich	$C_6H_9N_3O_4$	187.15	-0.81
Ipronidazole	Sigma-Aldrich	$C_7H_{11}N_3O_2$	169.2	1.18
Ketoprofen	Sigma-Aldrich	$C_{16}H_{14}O_3$	254.3	2.81
Levamisole HCl	Sigma-Aldrich	$C_{11}H_{13}ClN_2S$	240.8	1.85
Lincomycin HCl Monohydrate	Sigma-Aldrich	$C_{18}H_{37}ClN_2O_7S$	461.0	0.91
Mebendazole	Sigma-Aldrich	$C_{16}H_{13}N_3O_3$	295.3	2.83
Mebendazole-amine	Sigma-Aldrich	$C_{14}H_{11}N_3O$	237.3	1.74
Melengestrol Acetate	Sigma-Aldrich	$C_{25}H_{32}O_4$	396.5	4.21
Metronidazole	Sigma-Aldrich	$C_6H_9N_3O_3$	171.2	-0.01
Morantel Tartrate Hydrate	Sigma-Aldrich	$C_{16}H_{22}N_2O_6S\ xH_2O$	370.4	1.97
Norfloxacin	Sigma-Aldrich	$C_{16}H_{18}FN_3O_3$	319.3	0.82
Orbifloxacin	Sigma-Aldrich	$C_{19}H_{20}F_3N_3O_3$	395.4	2.37
Oxacillin Sodium Salt Monohydrate	Sigma-Aldrich	$C_{19}H_{18}N_3O_5SNa\ H_2O$	441.4	2.05
Oxfendazole	Sigma-Aldrich	$C_{15}H_{13}N_3O_3S$	315.3	1.36
Oxyclozanide	Sigma-Aldrich	$C_{13}H_6Cl_5NO_3$	401.5	8.67

Table 2.1 continued

Compound	Supplier	Formula1	Molecular Mass (Da)[1]	Log P[1]
Oxyphenylbutazone	Sigma-Aldrich	$C_{19}H_{20}N_2O_3$	324.4	2.72
Oxytetracycline HCl	Sigma-Aldrich	$C_{22}H_{25}ClN_2O_9$	496.9	-1.50
Phenylbutazone	Sigma-Aldrich	$C_{19}H_{20}N_2O_2$	308.4	3.16
Pirlimycin HCl	TRC[2]	$C_{17}H_{32}Cl_2N_2O_5S$	447.4	1.47
Prednisone	Sigma-Aldrich	$C_{21}H_{26}O_5$	358.4	1.57
Promethazine HCl	Sigma-Aldrich	$C_{17}H_{21}ClN_2S$	320.9	4.78
Propionylpromazine HCl	Sigma-Aldrich	$C_{20}H_{25}ClN_2OS$	376.9	4.61
Ractopamine HCl	Sigma-Aldrich	$C_{18}H_{24}ClNO_3$	337.8	1.65
Sarafloxacin HCl Hydrate	Sigma-Aldrich	$C_{20}H_{17}F_2N_3O_3$ HCl xH_2O	421.8	2.09
Sulfachloropyridazine	Sigma-Aldrich	$C_{10}H_9ClN_4O_2S$	284.7	1.02
Sulfadimethoxine	Sigma-Aldrich	$C_{12}H_{14}N_4O_4S$	310.3	1.48
Sulfadoxine	Sigma-Aldrich	$C_{12}H_{14}N_4O_4S$	310.3	0.34
Sulfaethoxypyridazine	Sigma-Aldrich	$C_{12}H_{14}N_4O_3S$	294.3	0.85
Sulfamerazin	Sigma-Aldrich	$C_{11}H_{12}N_4O_2S$	264.3	0.34
Sulfamethazine	Sigma-Aldrich	$C_{12}H_{14}N_4O_2S$	278.33	0.80
Sulfamethizole	Sigma-Aldrich	$C_9H_{10}N_4O_2S_2$	270.3	0.51
Sulfamethoxazole	Sigma-Aldrich	$C_{10}H_{11}N_3O_3S$	253.3	0.89
Sulfamethoxypyridazine	Sigma-Aldrich	$C_{11}H_{12}N_4O_3S$	280.3	0.32
Sulfapyridine	Sigma-Aldrich	$C_{11}H_{11}N_3O_2S$	249.3	0.03
Sulfaquinoxaline	Sigma-Aldrich	$C_{14}H_{12}N_4O_2S$	300.3	1.30
Sulfathiazole	Sigma-Aldrich	$C_9H_9N_3O_2S_2$	255.3	0.05
Tetracycline HCl	Sigma-Aldrich	$C_{22}H_{25}ClN_2O_8$	480.9	-1.47
Tolfenamic Acid	Sigma-Aldrich	$C_{14}H_{12}ClNO_2$	261.7	5.76
Triclabendazole	Sigma-Aldrich	$C_{14}H_9Cl_3N_2OS$	359.7	5.97
Triclabendazole Sulfoxide	Sigma-Aldrich	$C_{14}H_9Cl_3N_2O_2S$	375.7	4.12
Triflupromazine HCl	Sigma-Aldrich	$C_{18}H_{20}ClF_3N_2S$	388.9	5.70
Tylosin	TRC[2]	$C_{46}H_{77}NO_{17}$	916.1	3.27
Virginiamycin M1	Sigma-Aldrich	$C_{28}H_{35}N_3O_7$	525.6	-0.66
Xylazine HCl	Sigma-Aldrich	$C_{12}H_{17}ClN_2S$	256.8	2.37

[1] Data taken from www.chemspider.com, accessed April 2018, LogP data taken from computational predictions ACD/LogP

[2] Toronto Research Chemicals

Stock solutions of veterinary drugs and deuterated internal standard were prepared by weighing approximately 5 mg of each individual standard, and dissolving individual quantities in 5 mL of either MeCN, MeOH, water, or 10% DMSO in MeCN, in accordance with the solubility of each compound. An internal standard (IS) solution, containing flunixin-d_3 at 1 μg mL^{-1}, was prepared by appropriate dilution of IS stock solution in MeCN. All target analytes were mixed in a composite solution and diluted to 100X, where X represents the MRLs listed in Table 2.6 with MeCN, with the exception of the β-lactams/cephalosporins composite solution, which was prepared and diluted in water at a concentration of 200X. All stock and composite solutions were stored at −30 °C. Spiking solutions and their dilutions were prepared daily for validation experiments. With the exception of β-lactams/cephalosporins solutions, which were stored in plastic, all other standard and composite solutions were stored in amber glass vials closed with fitted PTFE caps.

The MRL values displayed in Table 2.6 were based primarily on Canadian MRL values[81] in poultry, or US tolerance levels[170] in cases where MRL values corresponding to certain analytes were not available in the Canadian database. In cases where MRL values were unavailable for poultry in either database, values in other tissues, such as bovine tissue, were selected instead. In cases where Canadian MRLs were higher than US tolerance levels, such as that established for Ketoprofen, the US regulatory value was selected.

Antibiotic free chicken breast, thighs, and liver from five different sources were purchased from local grocery stores to serve as matrix. Chicken thighs and liver tissue were used only for matrix effects experiments. Each sample was homogenized separately with dry ice, using a

Vitamix blender to obtain a uniform powder. All samples were then combined and ground again with dry ice to produce a pooled matrix. All homogenized samples were first stored in glass jars covered by loose lids overnight at −30 °C to allow for sublimation of dry ice to occur, then subsequently stored at −80 °C until analysis.

2.3.2 LC-MS/MS method

Experiments were performed with the use of a Thermo Accela 1250 pump with an on-line vacuum degasser liquid chromatography system coupled to a triple quadrupole mass spectrometer TSQ Vantage (Thermo Scientific, San Jose, USA). Further instrumental details and optimized LC and MS/MS parameters are provided in Tables 2.2, 2.3, and 2.4. The autosampler, thermostated at 5 °C, was used for high throughput, 10 μL sample injections in full loop mode. A Waters (Mississauga, ON, Canada) Acquity UPLC HSS T3 (100 × 2.1 mm, 1.7 μm) analytical column connected to a guard column (HSS T3, 2.1 × 5 mm, 1.7 μm) was used for separation of the targeted analytes. The column compartment was maintained at 40 °C, and the flow rate was 0.3 mL/min. MeCN/water (70:30, v/v) was used to clean the injection system (flush and wash volumes were 1000 μL and 200 μL, respectively).

Table 2.2 MS/MS optimized parameters, (m/z), and retention times (RT) of compounds, ions used for quantification are bolded.

Compound	Class	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	RT (min)	S-Lens value	Polarity
2-Aminoflubendazole	Anthelmintics	256	**123**, 95, 75	27, 38, 56	5.7	131	+
5-Hydroxythiabendazole	Anthelmintics	218	**191**, 147, 81	26, 33, 40	4.2	50	+
6-Phenyl-2-thiouracil	Thyreostats	205	**188**, 103, 146	19, 20, 28	5.7	47	+
Acepromazine Maleate	Tranquilizers	327	**86**, 58, 222	20, 35, 37	6.7	66	+
Albendazole	Anthelmintics	266	**234**, 191, 159	19, 33, 38	6.9	62	+
Albendazole sulfone	Anthelmintics	298	**266**,159, 224	36, 20, 28	6.0	70	+
Albendazole sulfoxide	Anthelmintics	282	**240**, 207, 159	13, 24, 39	5.3	71	+
Albendazole-2-aminosulfone	Anthelmintics	240	**133**,198,106	29,19,42	4.4	63	+
Azaperone	Tranquilizers	328	**165**,123, 121	36, 21, 23	5.2	113	+
Betamethasone	Anti-inflammatories	393	**325**, 373, 347	11, 6, 29	7.2	57	+
Cambendazole	Anthelmintics	303	**217**, 261, 190	28, 18, 190	5.8	110	+
Carbadox	Other	263	**231**, 130, 102	13, 20, 46	5.0	63	+
Chlorpromazine HCl	Tranquilizers	319	**86**, 58, 214	20, 34, 40	7.3	102	+
Chlortetracycline HCl	Tetracyclines	479	**462**,444, 154	20, 16, 28	5.3	121	+
Clenbuterol HCl	β-Agonists	277	**203**, 259, 132	16, 10, 30	5.4	56	+
Clindamycin HCl	Macrolides/lincosamides	425	**126**, 377, 83	30, 19, 53	5.7	114	+
Cloxacillin Sodium Salt	β-Lactams/cephalosporins	436	**277**, 160, 114	14, 12, 31	8.0	61	+
Danofloxacin	Fluoroquinolones	358	**340**, 82,314	23, 41, 17	5	116	+
Desethylene Ciprofloxacin	Fluoroquinolones	306	**288**, 268, 217	19, 26, 38	4.7	61	+
Diclofenac Sodium	Anti-inflammatories	296	**214**, 250, 278	37, 13, 9	9.3	56	+
Dicloxacillin	β-Lactams/cephalosporins	470	**452**, 310, 114	13, 13, 39	8.5	87	+
Difloxacin HCl	Fluoroquinolones	400	**382**, 298, 356,	28, 18, 2	5.4	116	+
Dimetridazole	Coccidiostats	142	**96**, 81, 54	16, 28, 34	4.2	47	+
Doxycycline HCl	Tetracyclines	445	**428**, 267, 321	17, 37, 30	5.8	107	+
Emamectin Benzoate	Anthelmintics	887	**158**, 82, 126	35, 48, 37	9.3	231	+
Enrofloxacin	Fluoroquinolones	360	**342**,316, 245	19, 27, 22	5.1	120	+
Erythromycin	Macrolides/lincosamides	734	**576**,158, 116	30, 17, 36	6.5	94	+
Fenbendazole	Anthelmintics	300	**268**, 159,131	21, 34, 45	7.7	115	+
Fenbendazole Sulfone	Anthelmintics	332	**300**, 159, 131	23, 38, 49	6.8	102	+
Florfenicol amine	Phenicols	248	**230**, 130, 91	12, 25, 46	0.9	48	+
Flubendazole	Anthelmintics	314	**282**, 123, 95	22, 35, 46	7.1	87	+

Table 2.2 Continued

Compound	Class	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	RT (min)	S-Lens value	Polarity
Flunixin	Anti-inflammatories	297	**279**, 264, 236	23, 34, 42	8.5	127	+
Haloperidol	Tranquilizers	376	**165**,123, 95	37, 24, 95	6.7	118	+
Hydroxy dimetridazole	Coccidiostats	158	**80**,140, 55	12, 13, 19	3.5	32	+
Hydroxy ipronidazole	Coccidiostats	186	**168**, 122, 106	13, 20, 35	5.4	46	+
Ipronidazole	Coccidiostats	170	**124**, 109, 96	17, 25, 22	6.4	48	+
Ketoprofen	Anti-inflammatories	255	**209**, 77, 105	39, 22, 13	8.2	51	+
Levamisole HCl	Anthelmintics	205	**178**, 91, 123	22, 39, 29	4.4	51	+
Lincomycin	Macrolides/lincosamides	407	**126**, 359, 389	30, 18,16	4.4	127	+
Mebendazole	Anthelmintics	296	**264**, 105, 77	21, 34, 43	6.9	115	+
Mebendazole-amine	Anthelmintics	238	**105**, 77, 133	25, 26, 37	5.5	58	+
Melengestrol Acetate	Other	397	**279**,337, 221	13, 20, 40	10.2	89	+
Morantel Tartrate Hydrate	Anthelmintics	221	**123**, 111, 164	35, 25, 27	5.6	53	+
Norfloxacin	Fluoroquinolones	320	**302**, 276, 233	16, 24, 21	4.8	53	+
Orbifloxacin	Fluoroquinolones	396	**352**, 295, 226	17, 24, 43	5.2	89	+
Oxacillin Sodium	β-Lactams/cephalosporins	402	**243**, 160, 114	14, 14, 34	7.6	75	+
Oxfendazole	Anthelmintics	316	**159**, 284, 191	32, 18, 22	6.0	122	+
Oxyclozanide	Anthelmintics	402	**186**, 83, 144	22, 22, 44	9.9	62	+
Oxyphenylbutazone	Anti-inflammatories	325	**204**, 148, 232	16, 29, 13	8.2	89	+
Oxytetracycline HCl	Tetracyclines	461	**426**, 443, 201	18, 11, 39	4.9	98	+
Phenylbutazone	Anti-inflammatories	309	**120**, 188, 160	42, 19, 29	9.7	53	+
Pirlimycin HCl	Macrolides/lincosamides	411	**363**,112, 56	29, 17, 49	5.5	111	+
Prednisone	Anti-inflammatories	359	**341**, 313, 147	11, 10, 26	6.7	65	+
Promethazine HCl	Tranquilizers	285	**86**, 198, 71	17, 29, 36	6.7	52	+
Propionylpromazine HCl	Tranquilizers	341	**86**, 58, 236	20, 35, 37	7.1	113	+
Ractopamine HCl	β-Agonists	302	**164**, 284, 107	12, 16, 34	5.0	56	+
Sarafloxacin HCl Hydrate	Fluoroquinolones	386	**342**,299, 368	26, 18, 22	5.4	66	+
Sulfachloropyridazine	Sulfonamides	285	**156**, 92, 108	15, 29, 26	5.9	59	+
Sulfadimethoxine	Sulfonamides	311	**156**, 92, 108	20, 33, 29	6.7	116	+
Sulfadoxine	Sulfonamides	311	**156**, 108, 65	18, 29, 45	6.1	107	+
Sulfaethoxypyridazine	Sulfonamides	295	**156**, 108,92	18, 28, 30	5.4	62	+
Sulfamerazine	Sulfonamides	265	**156**, 172, 108	16, 26, 30	4.9	59	+
Sulfamethazine	Sulfonamides	279	**186**, 156, 124	18, 20, 28	5.3	104	+
Sulfamethizole	Sulfonamides	271	**156**, 92, 108	15, 27, 25	5.3	60	+
Sulfamethoxazole	Sulfonamides	254	**108**, 156, 92	28, 27, 41	6.1	67	+

Table 2.2 Continued

Compound	Class	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	RT (min)	S-Lens value	Polarity
Sulfamethoxazole	Sulfonamides	254	**108**, 156, 92	28, 27, 41	6.1	67	+
Sulfamethoxypyridazine	Sulfonamides	281	**156**, 126, 108	17, 20, 46	6.1	105	+
Sulfapyridine	Sulfonamides	250	**156**, 92, 184	16, 28, 19	4.7	75	+
Sulfaquinoxaline	Sulfonamides	301	**156**, 92, 108	17, 30, 28	6.7	57	+
Sulfathiazole	Sulfonamides	256	**156**, 92, 65	15, 29, 39	4.6	100	+
Tetracycline HCl	Tetracyclines	445	**410**, 154, 427	19, 28, 12	5.1	107	+
Tolfenamic Acid	Anti-inflammatories	262	**214**, 180, 209	16, 41, 27	10.2	51	+
Triclabendazole	Anthelmintics	359	**274**, 344, 171	36, 25, 51	9.8	158	+
Triclabendazole Sulfoxide	Anthelmintics	375	**357**, 360, 242	20, 45, 22	9.0	122	+
Triflupromazine HCl	Tranquilizers	353	**248,** 86, 58	21, 35, 45	7.6	106	+
Tylosin	Macrolides/lincosamides	917	**772**, 174, 101	36, 27, 43	6.7	121	+
Virginiamycin M1	Other	526	**508**, 337, 355	12, 20, 17	7.8	115	+
Xylazine HCl	Tranquilizers	221	**90**, 164, 72	23, 26, 37	5.6	51	+
Flunixin-*d3*	Internal standard	300	**282**	24	8.5	115	+

Table 2.3 Optimized LC conditions

	Optimized LC conditions		
Column	Acquity UPLC HSS T3 C_{18} Column 2.1 x 100 mm, 1.7 µm particle size, Waters, Mississauga, ON, Canada		
Guard cartridge	VanGuard Pre-Column HSS T3 1.7µm, 2.1mm x 5mm		
Mobile phase	A: water with 0.1% formic acid B: acetonitrile with 0.1% formic acid		
Flow rate	300 µL min^{-1}		
Column temperature	40 ˚C		
Autosampler temperature	5 ˚C		
Sample injection volume	10 µL (full loop mode)		
Gradient	Time (min)	%A	%B
	0	97	3
	1	97	3
	11	0	100
	13	0	100
	15	97	3
	18	97	3

The mobile phases were water (solvent A) and MeCN (Solvent B), each containing 0.1% (v/v) formic acid. The gradient was run at 3% B for 1 min, ramped linearly to 100% B until 11 min, and then held at 100% B until 13 min. The column was then returned to 3% B over 2 min, where it was allowed to re-equilibrate for 3 min. MS data was processed using Xcalibur software v.2.1 (Thermo Fisher Scientific, San Jose, USA). Mobile phases were degassed for 30 min in a VWR Scientific, Aquasonic model 75HT (West Chester, PA, USA) ultrasonic bath before use.

Table 2.4 Optimized MS/MS conditions

MS parameters on TSQ Vantage	
Spray voltage	3.5 kV (positive)
Vaporizer temperature	275 °C
Sheet gas and flow	N_2, 30 AU
Auxiliary gas and flow	N_2, 30 AU
Transfer capillary temperature	275 °C
Quadrupole resolution at FWHM	0.7 u
Collision gas and pressure	Argon, 1.5 mTorr
Cycle time	0.4 s

2.3.3 Synthesis of Hydrophilic-Lipophilic Balance (HLB) particles

HLB particles were synthesized via precipitation polymerization by modifying the procedure described elsewhere;[152,171] however it was scaled up and toluene was used as a porogen in order to prepare mesoporous particles. First, 450 mL MeCN and 150 mL toluene were added in a 2 L three-necked round bottom flask equipped with a mechanical stirrer and an inlet for nitrogen gas purging, then purged with nitrogen for 30 min. Following, 42 mL of the monomer/cross linker (DVB) and 18 mL of the functional monomer (N-VP) were added to the solvent mixture. AIBN (500 mg) was used as an initiator for the polymerization reaction. The reaction was thermally controlled at 70 °C for 24 hours. Particles were then rinsed and dried as per previous methods.

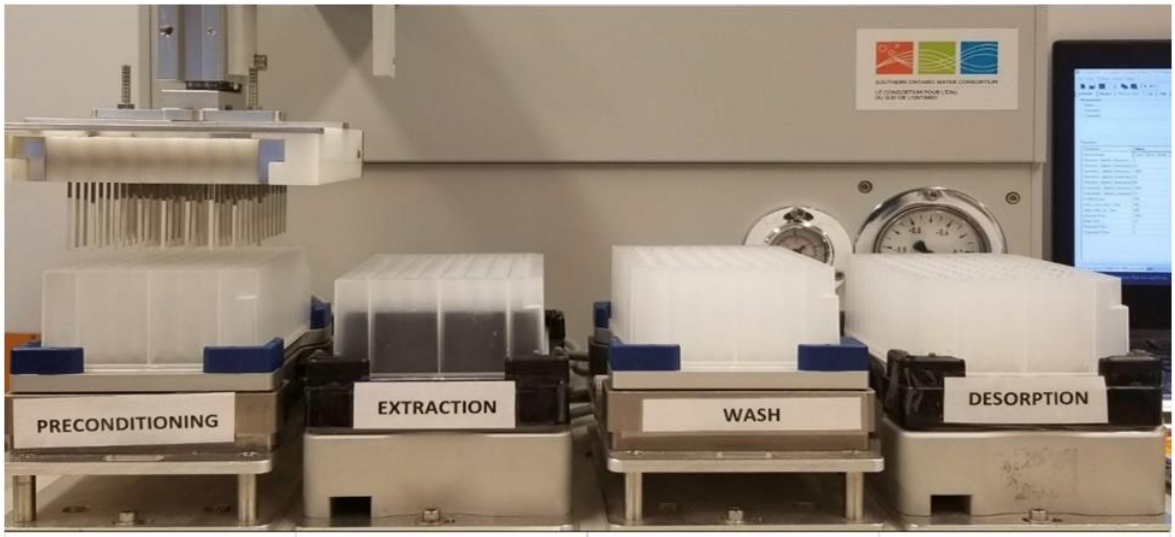
HLB particles were characterized by UltraPlus field emission scanning electron microscopy (FE-SEM, Carl Zeiss, Germany), specific surface area analysis (Autosorb iQ-MP by

Quantachrome (Boyton Beach, Florida)), for determinations of size and shape, and surface area, respectively.

2.3.4 Automated concept 96-pin SPME system

The Concept 96 robotic sample preparation station (Professional Analytical System (PAS) Technology, Magdala, Germany) used in this work for SPME sample preparation is a fully automated, software-operated, off-line bench top robotic station (Figure 2.1). The device executes all steps of the SPME protocol, including preconditioning of the sorbent, SPME extraction, rinsing, and solvent desorption. In addition, the system also contains an evaporation unit that allows for optional drying and reconstitution of extracts and/or preconcentration of analytes. The SPME brush is comprised of 96 SPME pins coated with extraction phase (Figure 2.1). The brush fits standard commercial 96-well-plates, which can be accommodated on the agitators used during subsequent steps of the method, and is compatible with most autosamplers available for standard LC systems.

Stainless steel rods for the SPME brush were cut to a length of 50 mm at the University of Waterloo's Science Technical Services. The SPME coating was comprised of HLB particles suspended in PAN, and immobilized on the stainless steel rod surfaces of the 96-pin SPME brush by dip coating, as per the protocol developed by Gomez-Ríos at al.[151] All prepared coatings had the same length (20 mm) and thickness (60 μm).



Conditioning	Extraction	Rinsing	Desorption
30 min	60 min	10 s	20 min
900 rpm	static	900 rpm	1200 rpm
$MeOH/H_2O$ (50/50 v/v)	Chicken sample	H2O	$MeCN/MeOH/H_2O$ (37.5/37.5/25 v/v/v)
1000 µL	1500 µL	1000 µL	400 µL

Figure 2.1 Concept 96-SPME device and SPME brush with 96 pins. Optimized conditions (time, agitation, solvent, and volume) for conditioning, extraction, rinsing, and desorption steps are shown, respectively, under each agitator-station.

2.3.5 Automated SPME procedure for high-throughput analysis

Spiked chicken samples were prepared by adding 100 µL of the working solution, containing all analytes under study at their respective designated concentrations, and 30 µL of the internal standard working solution to 2.0 g of homogenized chicken tissue. Samples were vortexed manually for 1 min, then placed on a benchtop agitator for 1 h. Samples were then placed in a 4 °C fridge overnight so as to allow for binding equilibria between matrix and analytes to be established.

Following overnight refrigeration, chicken samples were placed on the benchtop agitator for 1 h prior to extraction so as to allow samples to reach room temperature. 6 mL of water were added to each individual spiked chicken sample; samples were then vortexed for 1 min in order to attain homogeneous consistency, and a viscosity suitable for pipetting of samples to wells of the 96 well-plates. Next, 1.5 mL of the diluted chicken samples were transferred to 96 well plates. All final diluted spiked samples had an organic solvent content equivalent to less than 2%.

The Concept 96 system was programmed to consecutively perform the preconditioning of fibers, SPME extraction, fiber rinsing, and solvent desorption. The SPME protocol was executed as follows: prior to extractions, SPME rods were conditioned for 30 min with 1 mL of methanol/water (50:50, v/v) in 96-well-plates with agitation (900 rpm). Next, static extractions were performed from 1.5 mL of diluted chicken tissue samples spiked with the target compounds. For coating and desorption solvent selection, sample matrix was 1 mL of PBS spiked with each target analyte at 50 ng mL^{-1}. Chicken matrix was used for all subsequent steps of the study. The final optimized extraction parameters were static extraction for 60 min at 50 °C. In the fiber rinsing step, SPME pins were rinsed with 1 mL of water for 10 s with agitation (900 rpm). Following, desorption of analytes was carried out in 400 µL of desorption solvent (in the final method) for 20 min with agitation (1200 rpm) in a new 96-well-plate containing desorption solvent. In order to evaluate carryover for each pair of sorbent and desorption solvents, second and third sequential desorption steps were carried out under the same conditions. Lastly, the 96-well-plate containing final extracts was covered with the 96-well-plate lid, and placed in the LC-MS/MS autosampler for further analysis.

The final optimized SPME conditions for preconditioning, extraction, rinsing, and desorption steps are shown in Figure 2.1, while protocols for preparation of the used solutions as well as further details regarding the full analytical procedure can be found in figure 2.2 and 2.3.

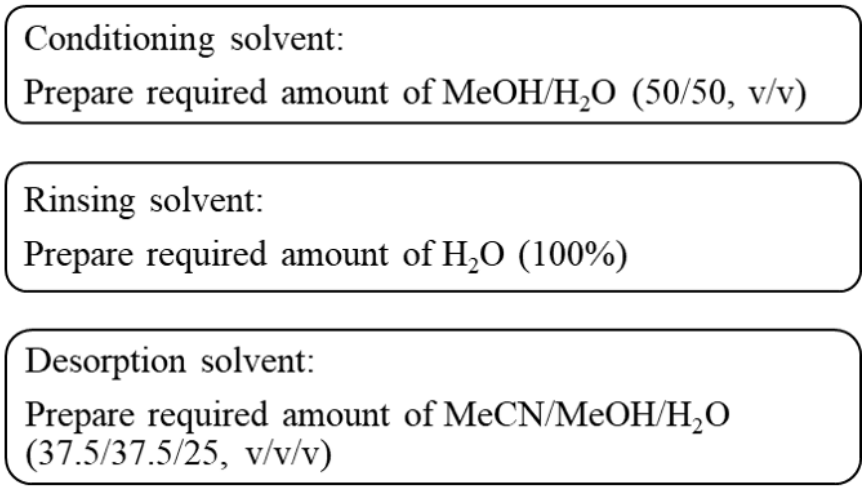


Figure 2.2 Protocol for preparation of conditioning, rinsing and desorption solvents

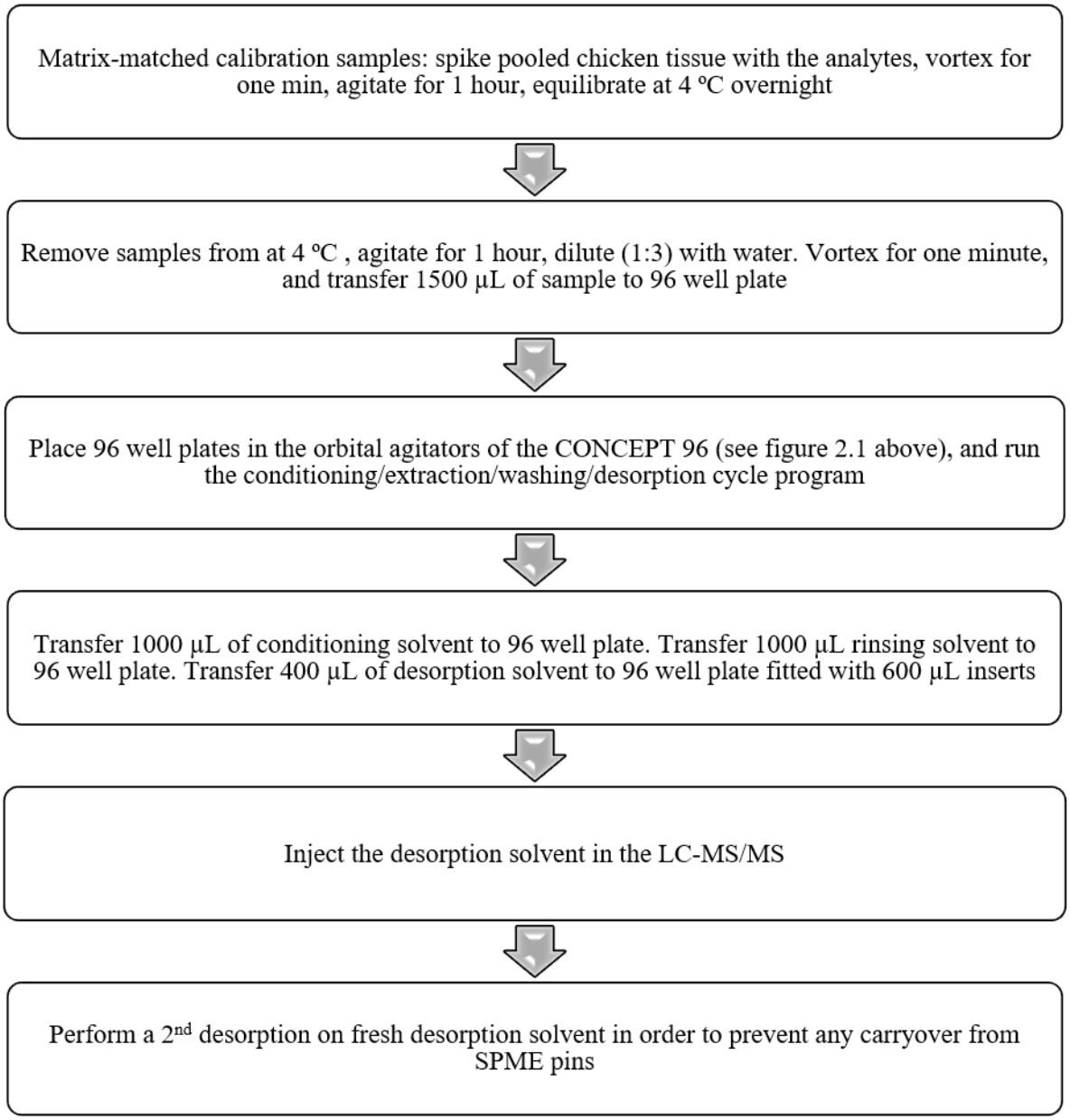


Figure 2.3 Protocol for preparation, extraction, and analysis of veterinary drugs by 96-pin SPME and LC-MS/MS

2.3.6 Optimization of the desorption solvent by experimental design

The composition of the desorption solvent was optimized for effectiveness with respect to ratios of water, methanol, and acetonitrile. Experiments to optimize the desorption solvent were designed based on a simplex-lattice mixture design (SLMD) introduced by Scheffé.[172] The design consists of a symmetrical arrangement of points, referred to as {k, m}-lattice, where k is the number of components, and m is the polynomial model degree. According to Scheffé, in order to better elucidate the shape of the response surface, the best design option comprises the use of a design where points are spread evenly over the whole simplex. The uniformly spaced distribution of points on a simplex is known as a lattice. In addition, the summation of the three portions (factors) in the SLMD must be equal to one. In cases where the optimization process involves multiple responses, it is not feasible to individually optimize each response, as such would necessitate the use of a large number of samples, equal to the dependent variable under study.[173] The Derringer & Suich approach presents an alternative to overcome this drawback, as it allows for the discovery of the best compromised conditions among all investigated responses through the desirability function.[174,175] In total, 14 experiments in triplicate were performed as listed in Table 2.5. Solvent ratios were established through an SLMD with three components, and the polynomial model degree equal to three. Statistical evaluation of data from these experiments was performed with the use of Statistica 13.0 software (TIBCO® Statistica™, CA, USA).

Table 2.5 Proportion of desorption solvent in a simplex lattice mixture design.

Experiment Order	% H_2O	% MeCN	% MeOH
12	16.7	66.7	16.7
6	0.0	33.3	66.7
3	0.0	0.0	100.0
11	66.7	16.7	16.7
10	33.3	33.3	33.3
1	100.0	0.0	0.0
13	16.7	16.7	66.7
2	0.0	100.	0.0
4	33.3	66.7	0.0
8	66.7	0. 0	33.3
7	66.7	33.3	0.0
14	33.3	33.3	33.3
5	33.3	0.0	66.7
9	0.0	66.7	33.3

2.3.7 Validation of the method

The developed method was validated following the guidelines established by the FDA for Method Validation for Drugs and Biologics.[176] The developed method was validated in terms of selectivity, linearity, accuracy, intra- and inter-day precision, stability, and limits of quantification (LOQs). Calibration curves as well as statistical tests were attained with Origin 2018 software (OriginLab Corporation®, MA, USA).

The first step in the validation procedure entailed an evaluation of the selectivity of the method. Method selectivity was assessed through an analysis of 10 blank chicken tissue samples.

Matrix-matched calibration with internal standard (IS) correction was selected as a calibration method for the current work. The matrix-matched calibration curve was prepared by spiking analytes in one lot of blank pooled chicken. Flunixin-d_3 was added to samples as internal standard to compensate for sample variations with respect to matrix, variations from pin to pin, as well as variations in desorption solvent loss due to evaporation during the desorption step.

The linear dynamic range of the LC-MS/MS instrument was determined for each analyte by direct injection of the neat standards prepared in desorption solution. In order to determine the linearity of the method, calibration curve solutions were prepared in a range of 0.1X to 3X in pooled chicken, and analyzed with the proposed SPME-LC-MS/MS method in triplicate. For determinations of accuracy and precision, target analytes were spiked at low-, mid- and high concentration levels (0.3X, 0.9X and 2.5X) in pooled chicken matrix. Internal standard (Flunixin-d_3) was spiked in pooled chicken matrix at 30 ng mL^{-1}. These extractions were performed in six replicates for intra-day precision. Interday precision was evaluated by running three different experiments over three different days.

The stability of the analytes in the desorption solvent for the duration of the analysis period was also assessed. Supposing that one run of the experiment utilizes every one of the 96 SPME pin spaces available in the Concept 96, and taking into account that a total chromatographic time of 18 min is needed per sample, an approximate period of 48 h was calculated as required to complete analysis for each dataset. In addition to the 48 h period, periods of 72 h and one week were also considered in the stability evaluation. Stability samples were prepared in desorption

solvent, stored at 5 °C, and analyzed after 48 h, 72 h, and 1 week; the attained results were then compared with those of freshly prepared standards.

2.4 Results and discussion

2.4.1 LC-MS method

The method was optimized with respect to run time, retention time stability, chromatographic separation, carryover, and sensitivity. Chromatographic separation is especially important in the presence of isobaric compounds such as sulfadoxine and sulfadimethoxine, tetracycline and doxycycline.[177] To this end, different chromatographic columns with different chemistry from various suppliers were tested with respect to their performance for the analytes under study. Based on the results attained in this comparative study, a Waters Acquity T3 C_{18} HSS (100 × 2.1 mm, 1.7 μm) column was selected for further experiments, as it provided the best performance compared to the other evaluated columns in terms of retention of target analytes, as well as peak shapes and resolution. Quality control (QC) samples were run to verify retention time, reproducibility, and instrumental performance. QC samples constituted of extracted blank chicken matrix spiked at 0.5X. The criteria used for verification were: retention time to verify that all analytes fall in retention time windows, peak shape, as well as peak area of the internal standard to make sure it does not deviate by more than 20% from the averages obtained during the initial calibration.

2.4.2 Development of the SPME method

Method optimization was carried out as per the protocol suggested by Risticevic et al.[178] Evaluated parameters included coating chemistry, desorption solvent, extraction and desorption times, agitation rates for extraction and desorption, extraction temperature, and sample and desorption solvent volumes. The first, and most important step in SPME method development entails the selection of a suitable coating in terms of extraction efficiency and carryover. Coating selection is especially important when targeting a large number of analytes with a wide range of polarities such as the ones targeted in this work, which present log P values ranging from −1.50 to 8.67. For this purpose, different polymer chemistries characterized by both polar and nonpolar functional groups to facilitate extraction of compounds with a wide range of polarities were selected for evaluation. Evaluated polymer chemistries included Hydrophilic-Lipophilic Balance (HLB) particles synthesized in-house, commercially available SPE particles, a polar modified polystyrene-divinylbenzene copolymer (PS-DVB), a mix-mode (C_8-SCX), and a 50:50 (w/w) HLB:PS-DVB. At the beginning of the study, preparation of the above listed SPME coatings was first attempted by spraying the particle slurry on the SPME blade's surface, as described in earlier work by Mirnaghi et al.[138] However, due to the sharp edges of the blades and the roughness of the sprayed coating surface, attachment of matrix components, especially fatty residue was observed on the coating surface following extraction. To avoid coating fouling, the geometry of the SPME device was modified to include rounded rods, while smaller HLB particles (1–5 μm) were used to yield a smoother extraction phase surface, which prevented any further attachment of matrix constituents. A rounded SPME geometry has been previously utilized for extraction from complex

biological matrices such as blood by Reyes-Garcés et al.[149] and Vuckovic et al.[135] In addition, static extraction was carried out to totally prevent any mechanical attachment of the sample to the SPME device.

When performing SPME in complex matrices, a rinsing step is usually added after the extraction step and prior to desorption so as to avoid fouling on the sorbent surface. In the current work, water was selected as rinsing solvent. Agitation rate was set up at 900 rpm, and rinsing time was set at 10 s so as to avoid loss of polar analytes.

Desorption-solvent effectiveness was assessed by evaluating desorption solutions with varied composition of the MeCN, MeOH, isopropanol, and water content. The effect of adding formic acid to the desorption solvent was also evaluated. Target analyte extraction amounts by each coating/desorption solvent pair are shown in Appendix A. These results were then compared with the aim of selecting the combination of extraction phase and desorption solvent composition that offers the highest extraction efficiency for most of the analytes, and the least carryover. The attained results, as summarized in Figure 2.4, showed that HLB provided the highest extraction recoveries for most of the target analytes, while mix-mode yielded the poorest recoveries. HLB yielded higher extraction recoveries for hydrophobic analytes, while PS-DVB yielded higher extraction recoveries for more polar compounds.

Although the PS-DVB and 50:50 [w/w] HLB:PS-DVB coatings offered higher extraction efficiencies for polar compounds, they were also shown to be characterized by higher carryover effects for a number of compounds when submitted to second and third desorption cycles. As a result, HLB was selected for further method development as the functional coating polymer.

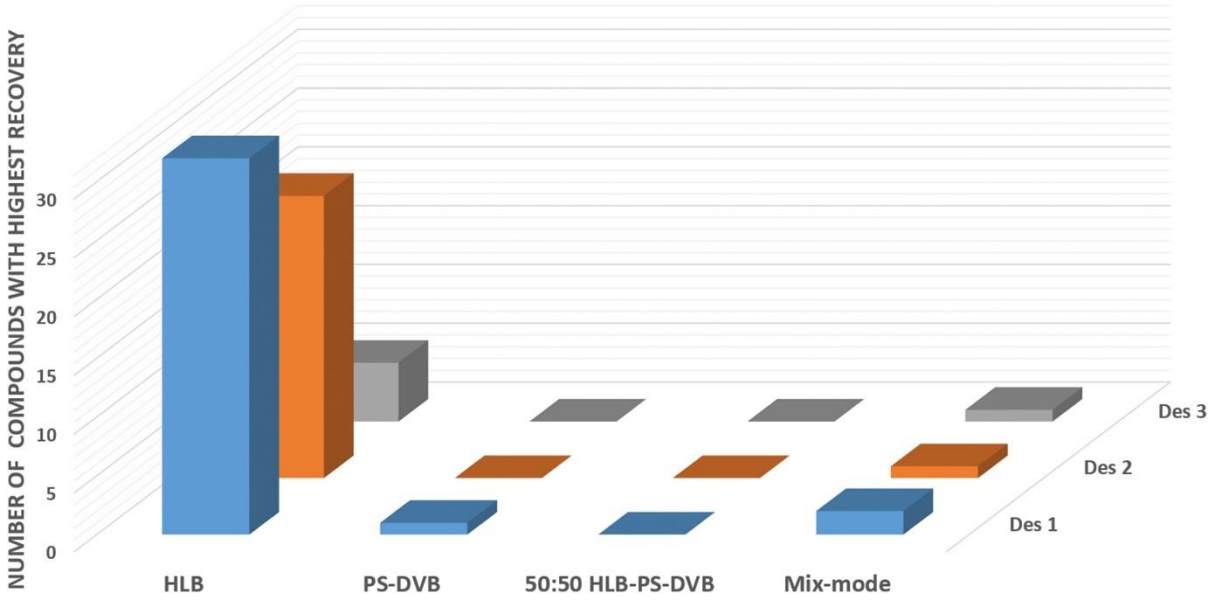


Figure 2.4 Evaluation of 4 SPME coatings (HLB, mix-mode, PS-DVB and 50:50 HLB:PS-DVB) in different desorption solutions (Des 1: MeCN/H_2O 50/50, Des 2: MeCN/MeOH/H_2O/FA 40/40/20/0.1 and Des 3: MeCN/IPA/H_2O/FA 40/40/20/0.1). Extraction volume:1000 µL, extraction time:120 min, concentration of analytes: 50 ng mL^{-1}, extraction matrix: 1X PBS. Desorption time: 120 min, desorption volume: 1000 µL.

2.4.3 Synthesis of Hydrophilic-Lipophilic Balance (HLB) particles

FE-SEM images of the prepared HLB particles show that the attained particles are characterized by a uniform spherical shape and are monodisperse in nature. Surface area analysis of the HLB particles revealed that the particles were microporous and mesoporous in nature, although most of the observed pores were in the mesoporous range (2–8 nm). The specific surface area of the HLB particles, calculated via the Brunauer–Emmett–Teller (BET) method with nitrogen gas used as adsorbate at 77.35 K, was measured at 816.78 m^2g^{-1}. The SEM images in Figure 2.5 (A and B) illustrate how particles initially agglomerated when dried under vacuum. The

SEM images show the obtained particles were spherical in shape and of a size between 1 and 5 μm. Although the HLB particles are embedded in PAN glue (Figure 2.5, C and D), pores present in PAN allow analytes to access the different layers of the HLB coating. These pores enable the diffusion of analytes of interest into the coating but restrict access of macromolecules such as proteins and lipids, resulting in satisfactory extraction of compounds of interest and minimal background interferences. At the same time, the smooth layer of PAN hinders attachment of matrix constituents to the SPME coating.

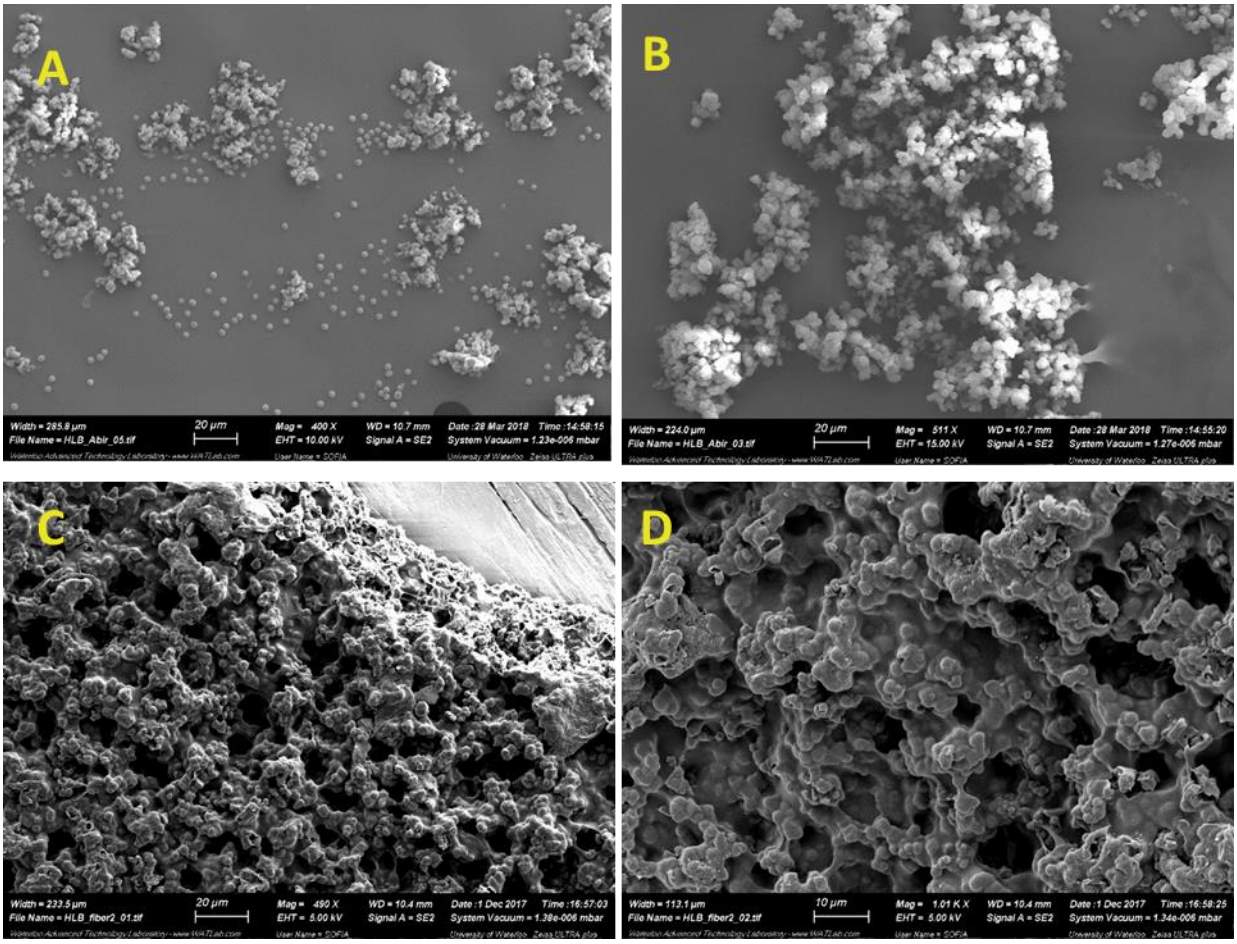


Figure 2.5 FE-SEM images of (A and B) HLB particles and (C and D) HLB particles in PAN glue on stainless steel rod.

2.4.4 Optimization of the desorption solvent by experimental design

In order to optimize the desorption of the analytes under study, which are characterized by a wide range of polarities and physico-chemical properties, after extraction by the HLB coating, optimum desorption solvent composition was investigated via experimental design. In total, 14 experiments in triplicate were performed, and the solvents portions were established through an SLMD with three components and the polynomial model degree equal to three. The use of SLMD

for optimization of desorption solvent composition enables a much more efficient optimization process as compared to sequential testing, particularly when the goal of analysis is detection of a wide range of analytes. Figure 2.6 demonstrates the attained results for the tested compositions with respect to the HLB coating. In this work, the optimum desorption composition was selected as 25:37.5:37.5, v/v/v water: MeCN: MeOH. The optimum desorption solvent composition was in agreement with expected results; considering the wide range of Log P values studied, a considerable amount of water would be required to desorb polar compounds from the fiber coating, while a sufficient amount of organic solvent would be needed to desorb non-polar compounds (Figure 2.6A). The same experimental design was used to assess carryover values; not surprisingly, the attained results converged with the above discussed results. The best conditions to minimize carryover were water (25%), MeCN (75%), and MeOH (0%) (Figure 2.6B). As this test was performed by using the inverse of values obtained for carryover, the maximum point denotes the lowest carryover obtained. Although the attained carryover percentage (less than 3% for all analytes, except for Phenylbutazone and Tolfenamic acid, at 5 and 8%, respectively) in the final desorption solution is considered acceptable in terms of quantitative analysis requirements, potential false positive results should be kept in mind in cases where extractions from samples characterized by high concentrations of target compounds are followed by extractions from low concentration samples or blanks. Therefore, it is recommended that an additional desorption step is performed prior to the next SPME cycle. This additional desorption step, when combined with the conditioning step, allows for effective removal of residual analytes from the SPME coating.

While addition of formic acid to the desorption solvent was found to improve overall desorption efficiency for most compounds, certain compounds, including lactams and fluoroquinolones, failed to present enough stability under such acidic conditions. Erythromycin, in particular, was observed to be very unstable under acidic conditions.[179] Thus, formic acid was not added to the final desorption solution selected in the developed method.

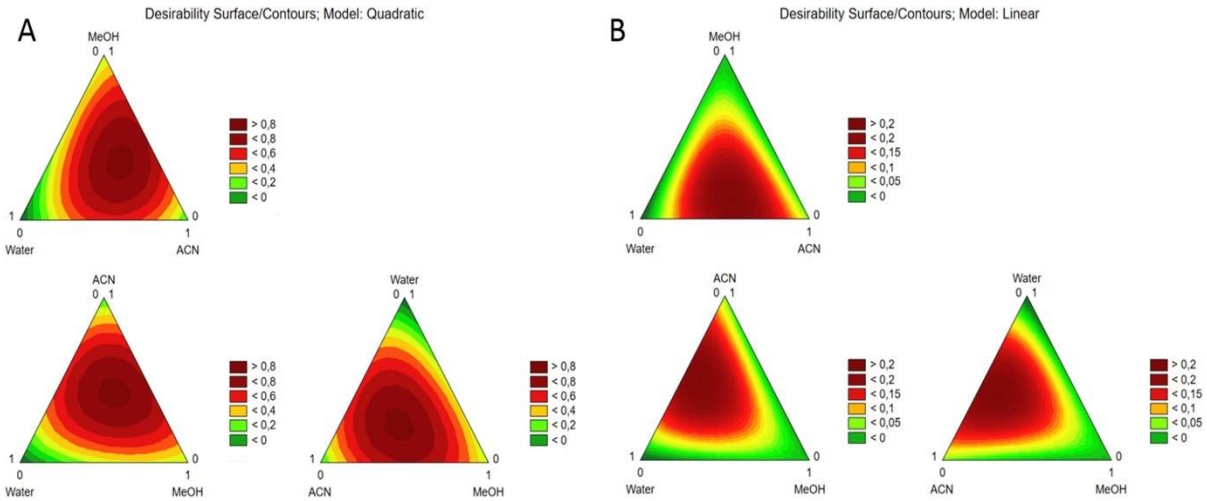


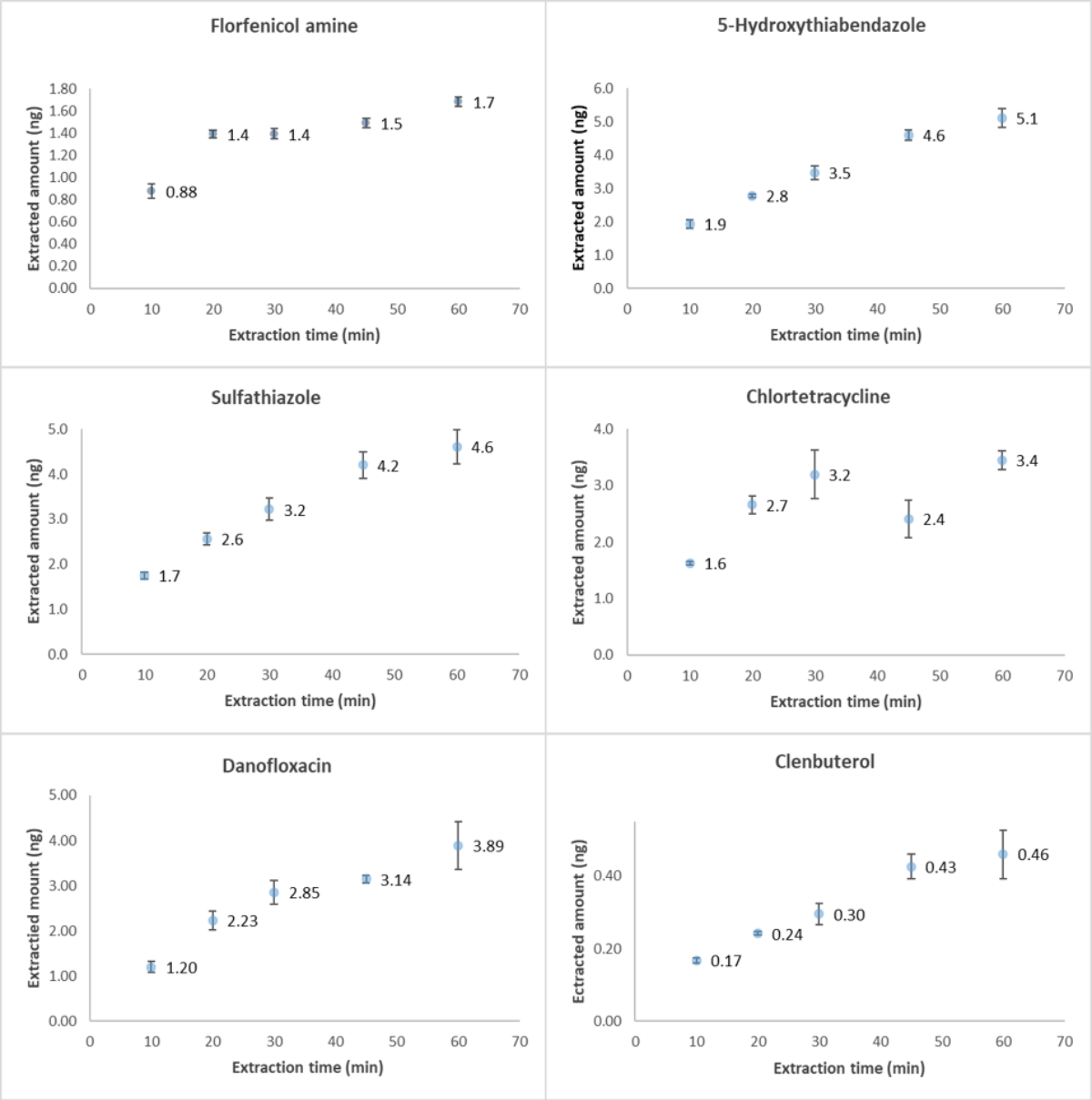
Figure 2.6 Optimum contour plot for special cubic model to fit experimental data for all compounds under study. (A: Desorption, B: Carryover).

2.4.5 Time profiles

Extraction time profiles were determined by extracting spiked chicken samples at 1 MRL level at different time points, within the range of 10–60 min, under optimized conditions. According to the attained results, most polar compounds reached equilibrium within 60 min, while the majority of the hydrophobic compounds under study did not reach equilibrium within this time period. As a compromise between extraction efficiency and overall analysis time, 60 min was selected as the final extraction time. Figure 2.7 presents the extraction time profiles of representative compounds from each class of veterinary drugs. Thus, under the selected extraction

conditions, extraction of most hydrophobic compounds would occur under the pre-equilibrium regime.

However, owing to the automation of the method, which enables precise control over extraction time, carrying out pre-equilibrium extractions will not affect the precision of the method. Desorption time profiles were determined by extracting spiked PBS samples at the 1 MRL level at various times (10–120 min). Although the results showed that most compounds reached quantitative desorption within only 15 min, 20 min were selected as desorption time in order to minimize carryover of the most hydrophobic compounds.


Florfenicol amine
Extracted amount (ng)
Extraction time (min)
0.88
1.4
1.4
1.5
1.7
5-Hydroxythiabendazole
Extracted amount (ng)
Extraction time (min)
1.9
2.8
3.5
4.6
5.1
Sulfathiazole
Extracted amount (ng)
Extraction time (min)
1.7
2.6
3.2
4.2
4.6
Chlortetracycline
Extracted amount (ng)
Extraction time (min)
1.6
2.7
3.2
2.4
3.4
Danofloxacin
Extracted mount (ng)
Extraction time (min)
1.20
2.23
2.85
3.14
3.89
Clenbuterol
Ectracted amount (ng)
Extraction time (min)
0.17
0.24
0.30
0.43
0.46

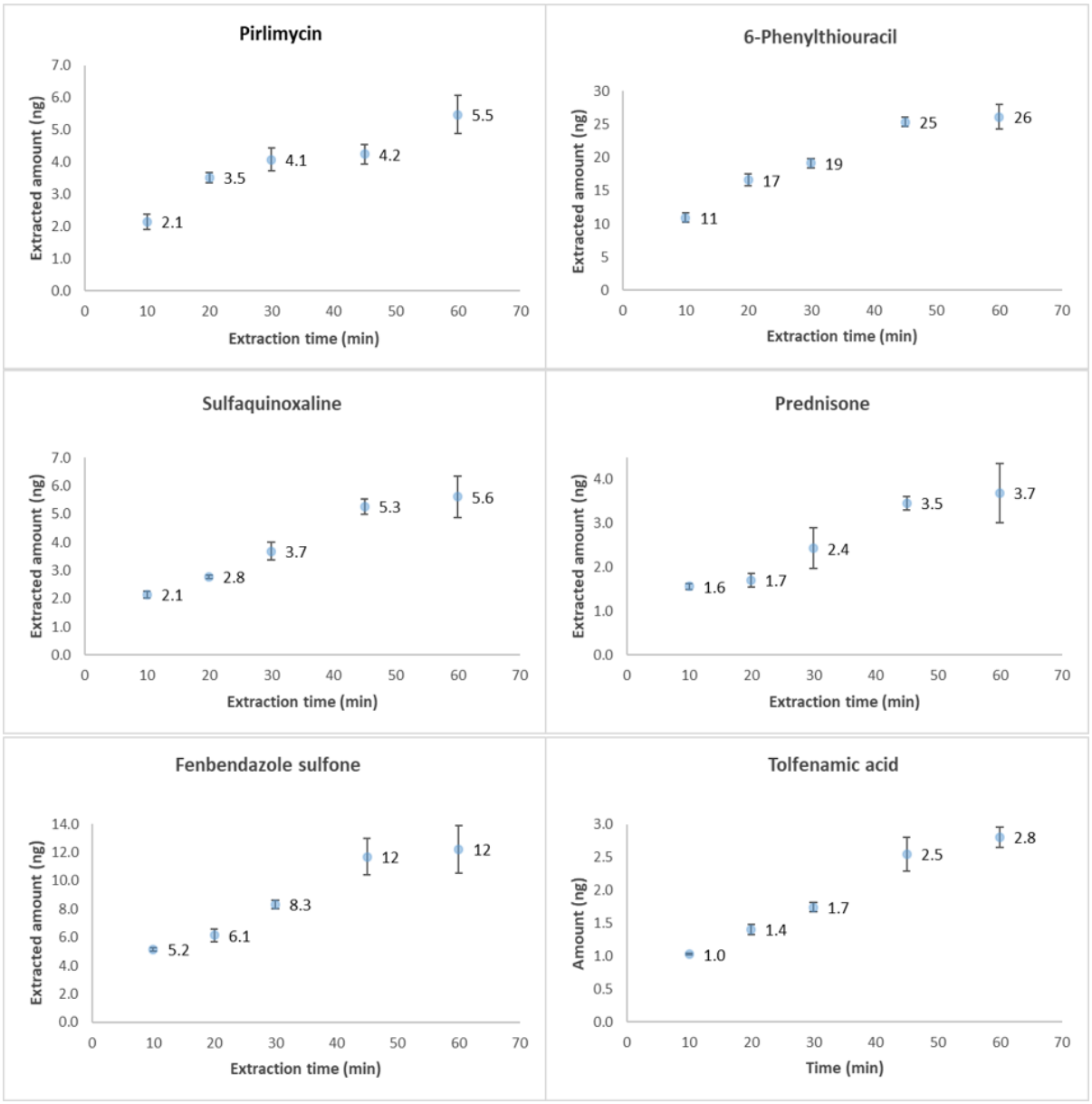


Figure 2.7 Extraction time profiles of selected compounds representing different classes of veterinary drugs.

The final optimized SPME parameters presented in Figure 2.1 yielded sufficient extraction efficiency, minimum carryover, and minimum use of organic solvents, while affording minimum manual handling during the sample preparation steps. A total time of 1 h 21 m is needed to achieve SPME extraction and desorption; supposing the 96-pin system is fully utilized within a run of the proposed workflow (i.e., 96 samples per run), the proposed method thus offers a time per sample of less than 1 min.

2.4.6 Matrix effect

The presence of matrix effects (MEs) is considered one of the main challenges in the multi-residue determination of drugs in tissue by LC-MS due to the complexity of the matrix under study. Matrix effects were calculated by the equation: ME% = (slope of matrix-matched calibration curve − slope of reagent-only calibration curve) × 100%/slope of reagent-only calibration curve.[32] Matrix effects were evaluated for three different types of chicken tissues; breast, liver, and thighs. Figure 2.8 shows the matrix effects for the analytes studied versus their retention time.

Absolute matrix effects were determined by the ratio of the peak areas of analytes spiked at three levels, low (0.3X), medium (0.9X), and high (2.5X), with extracts from the pooled matrix and neat standards at the same concentrations, as described by Matuszewski et al.[180] Absolute matrix effects values are presented in Table 2.6 and Figure 2.9. Absolute matrix effect values for the majority of compounds were within the range of 80–120%. Only florfenicol amine displayed significant signal suppression (31%, 45% and 56% at 0.3X, 0.9X and 2.5X respectively), while six other compounds displayed significant ion enhancement at the 0.3X concentration level.

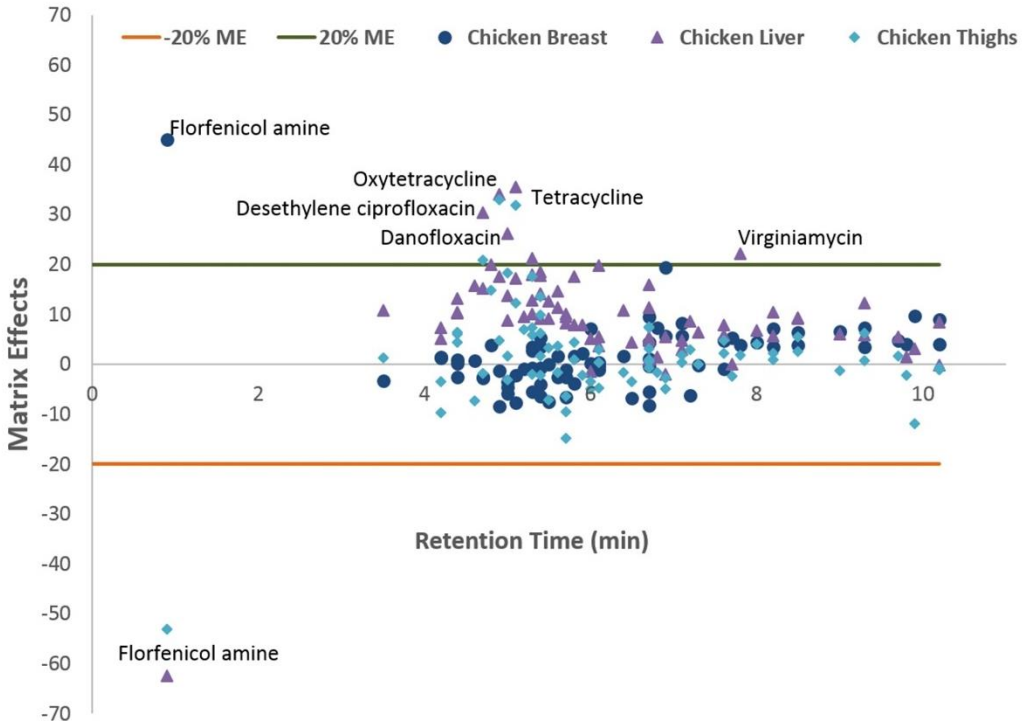


Figure 2.8 Matrix effects (%) of the final method for target analytes in three types of chicken tissue (breast, liver, and thigh) plotted vs. retention time.

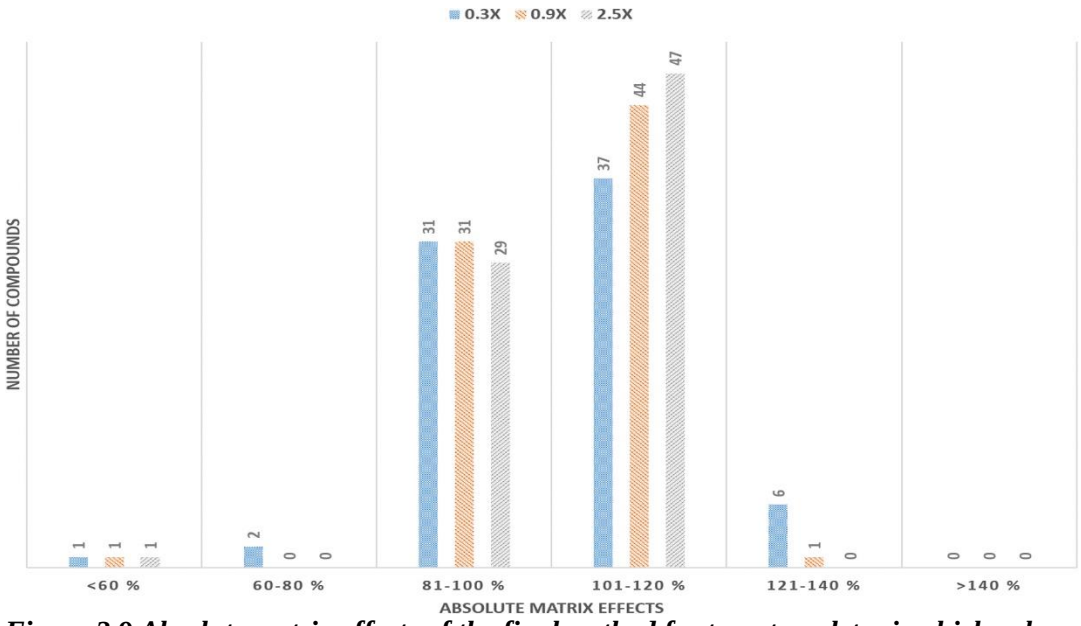


Figure 2.9 Absolute matrix effects of the final method for target analytes in chicken breast tissue.

No significant differences were observed when the results were calculated without normalization with internal standard. This is mainly due to the use of matrix-matched calibration and minimal matrix effects offered by SPME which is capable of isolating and enriching target analytes with effective and efficient sample clean-up. However, we selected to use one internal standard in this method in order to compensate in case of variations from pin to pin, as well as variations in desorption solvent loss due to evaporation during the desorption step. The results confirm that SPME provides clean sample extracts, thus offering reliable results while minimizing analytical instrument maintenance requirements.[120,131]

2.4.7 Validation of the method

The target analytes in this work were selected to represent more than 12 classes of veterinary drugs varying in physiochemical properties. The developed method was validated following FDA guidelines for methods validation for drugs and biologics.[176] The selectivity of the method was evaluated via an analysis of blank chicken samples (n = 10). No background peaks, above a signal-to-noise ratio of 3, were present at the same elution time as the target analytes, showing that the method is free of endogenous interferences. Figure 2.10 displays an example of obtained total ion chromatograms of blank chicken and blank chicken spiked at 0.1X and 1X levels.

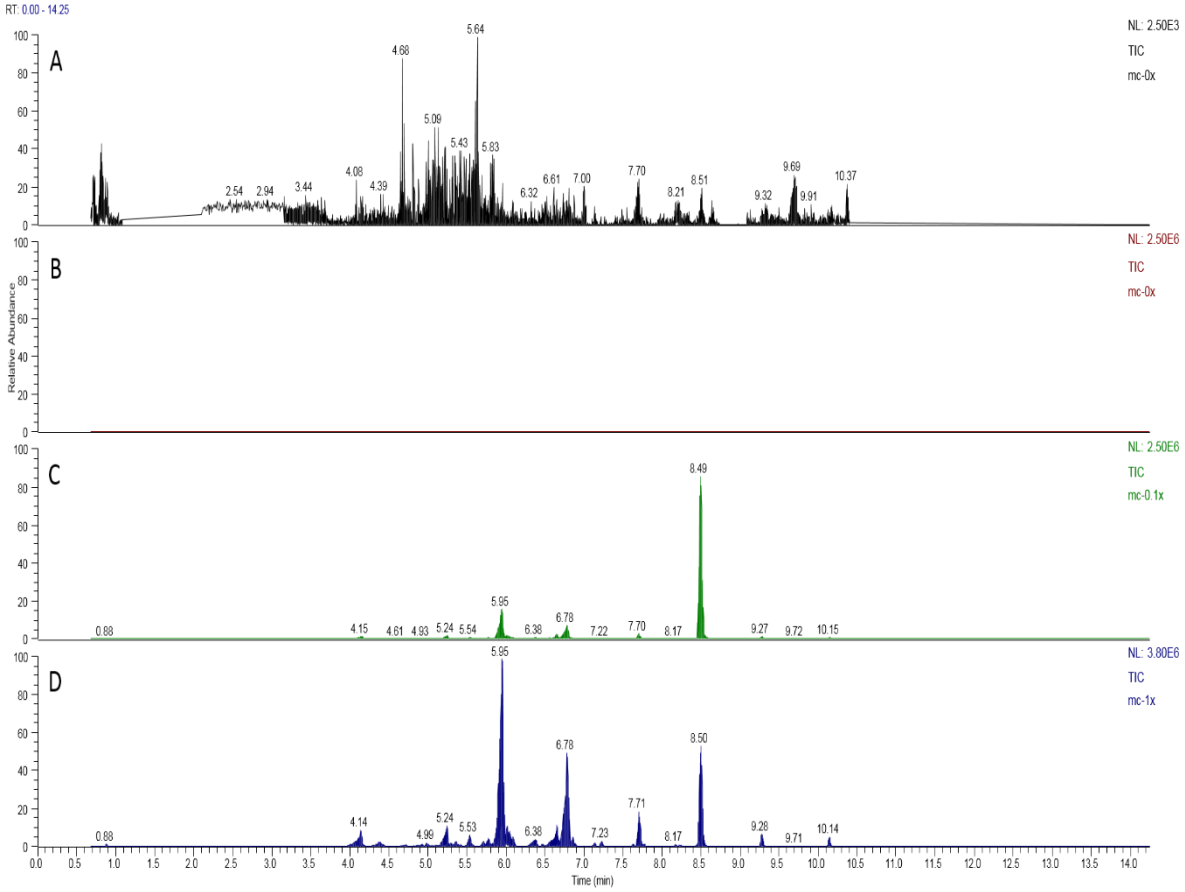


Figure 2.10 Total ion chromatogram.(A and B) Pooled blank chicken sample. (C) Pooled blank chicken spiked with all target analytes at 0.1X level. (D) Pooled blank chicken spiked with all target analytes at 1X level. All chromatograms are normalized to the highest peak except for B which is normalized to the same scale as (C).

Method linearity was evaluated for each compound through the establishment of matrix-matched calibration curves, which were prepared in a range of 0.1–3X in pooled chicken, and analyzed with the proposed SPME-LC-MS/MS method in triplicate. Pearson's coefficient (R) values ranged from 0.9956 to 0.9999 for all analytes under study, indicating good correlation between both axes. Furthermore, the determination coefficient (R^2) was higher than 0.991 for all

compounds, and a lack of fit (LOF) test performed at the 5% level presented no significance difference ($p > 0.05$), indicating that well-adjusted models were obtained for all target compounds.

Linear ranges, limits of quantitation, determination coefficients (R^2), and lack of fit test results are presented in Table 2.6.

The accuracy and precision of the method were calculated using six replicates per concentration ($n = 6$) at three levels; low, mid, and high (0.3X, 0.9X and 2.5X). To evaluate the accuracy of the method, the mean relative recovery of the analyte was calculated by fortifying blank chicken samples at the three concentration levels mentioned above. The spiked samples were quantified using the matrix matched calibration curves. Accuracy of the method as presented in Table 2.6 and Figure 2.11 was within 80–120% for all analytes except for Desethylene Ciprofloxacin (73%) Sulfadimethoxine (74%) and Tetracycline (63%) at low level concentration (0.3X), Oxyclozanide (60%) at mid level concentration (0.9X), 6-phenylthiouracil (134%), Albendazole (121%), and Oxytetracycline (121%) at high concentration level (2.5X). Intra-day and inter-day precision are presented in Figure 2.12.

LOQs were calculated as the lowest point of the matrix matched calibration curves with RSD ≤20%. LOQs equal to 0.1X were achieved for all analytes with the exceptions of 6-Phenylthiouracil, Danofloxacin, Desethylene Ciprofloxacin, Norfloxacin, and Triclabendazole with LOQ of 0.25X and Oxyclozanide 0.9X. This could be attributed to the low affinity of these analytes to the coating or due to poor ionization in electrospray ionization mass spectrometry.

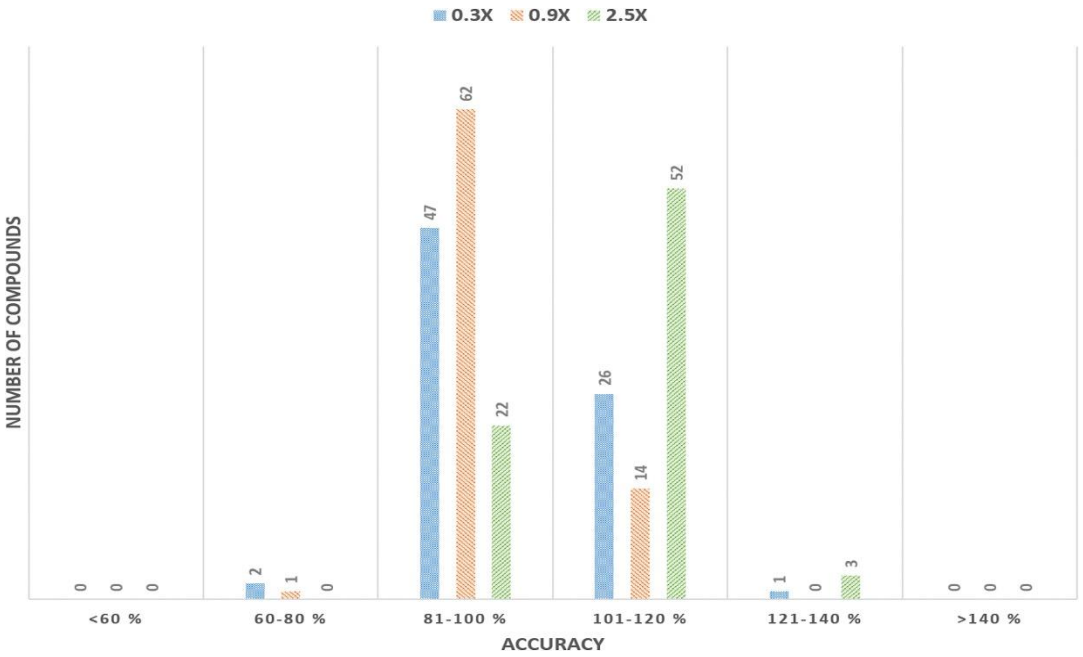


Figure 2.11 Accuracy of the final method for target analytes.

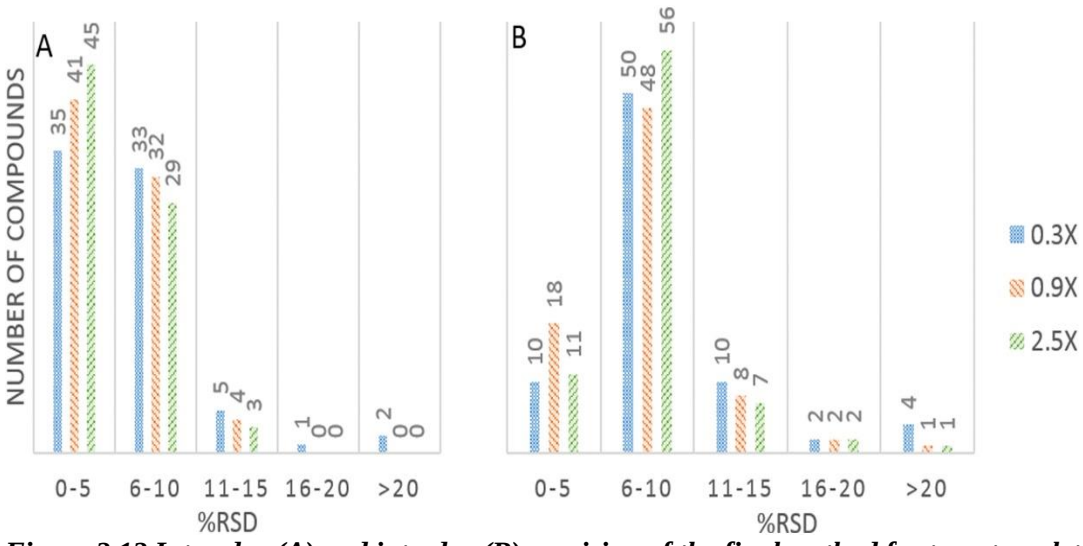


Figure 2.12 Intra-day (A) and interday (B) precision of the final method for target analytes.

Stability results were evaluated by calculating the percentage decrease in calculated concentrations of analytes after 48 h, 72 h, and one week of storage in the desorption solvent relative to the concentration results from the first day of preparation. A statistically significant decrease was only observed after 1 week of storage for tetracyclines and lactams. All other analytes were found to be stable within the studied conditions.

2.4.8 Application of final method towards analysis of chicken samples

The final developed method was used to analyze chicken samples purchased from five local grocery stores. Three different types of chicken tissues were selected: breast, liver, and thighs. Most of the samples analyzed were free from the target analytes, except for chicken samples from one supplier, where the following analytes were present at levels below established MRLs: sulfapyridine, sulfamerazine, enrofloxacin, orbifloxacin, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, doxycycline, sulfadoxine, sulfamethoxazole, sulfaethoxypyridazine, sulfadimethoxine. Although the amounts detected were below limits of quantification, chicken thigh tissue was observed to present consistently higher values of the detected analytes in comparison to the other tested tissues.

Table 2.6 Validation of the developed method under optimized conditions (conditioning: 1.0 mL, 50/50 (MeOH/H_2O, v/v) 30 min; extraction: 1.5mL diluted homogenized chicken, 60 min extraction time; rinsing: 1.0 mL H_2O, 10 sec with agitation; desorption: 0.4 mL 37.

Compound	MRL (X)	Linearity				Accuracy (%)			Intra-day precision (%)			Inter-day precision (%)			Absolute matrix effect (%)		
	ng g^{-1}	Range	LOQ	R^2	LOF	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X
2-Amino flubendazole	10	0.1 – 3X	0.1X	0.9968	0.1668	105	91	92	10	8	7	9	6	9	107	105	100
5-HydroxyThiabendazole	100	0.1 – 3X	0.1X	0.9996	0.6585	90	98	107	3	3	4	5	6	8	102	100	102
6-Phenylthiouracil	400	0.25 – 3X	0.25X	0.9991	0.7475	102	102	134	15	15	6	12	15	9	96	100	102
Acepromazine	10	0.1 – 3X	0.1X	0.9993	0.9666	97	91	93	12	6	6	8	6	10	111	113	107
Albendazole	50	0.1 – 1X	0.1X	0.9963	0.3941	91	94	121	4	6	3	11	16	17	86	93	96
Albendazole 2 aminosulfone	50	0.1 – 3X	0.1X	0.9986	0.313	98	98	110	9	2	2	9	6	10	95	98	100
Albendazole Sulfone	50	0.1 – 3X	0.1X	0.9996	0.9327	96	97	101	4	2	4	5	4	7	100	101	101
Albendazole Sulfoxide	50	0.1 – 3X	0.1X	0.9997	0.9808	99	99	106	5	3	3	8	5	7	97	97	101
Azaperone	10	0.1 – 3X	0.1X	0.9995	0.9611	90	88	96	6	1	5	6	5	8	105	92	99
Betamethasone	100	0.1 – 3X	0.1X	0.9987	0.2617	103	103	98	3	3	4	6	4	10	111	110	109
Cambendazole	10	0.1 – 3X	0.1X	0.9996	0.5943	97	98	102	3	2	3	6	6	4	111	104	101
Carbadox	30	0.1 – 3X	0.1X	0.9994	0.888	90	93	105	3	5	6	6	8	11	93	104	102
Chlorpromazine	10	0.1 – 3X	0.1X	0.9984	0.2482	94	95	114	11	7	2	10	11	8	110	107	104
Chlortetracycline	200	0.1 – 3X	0.1X	0.9989	0.7779	104	94	94	10	9	6	11	7	10	106	108	111
Clenbuterol	10	0.1 – 3X	0.1X	0.9981	0.17	95	101	101	4	8	8	6	9	8	106	103	106
Clindamycin	100	0.1 – 3X	0.1X	0.9993	0.071	102	101	106	6	4	9	7	6	10	103	103	102
Cloxacillin	10	0.1 – 3X	0.1X	0.9995	0.9664	100	96	102	6	4	4	9	7	10	119	98	100
Danofloxacin	70	0.25 – 3X	0.25X	0.9971	0.0611	85	96	115	10	7	6	13	10	7	131	116	109
Desethylene ciproflaxin	100	0.25 – 3X	0.25X	0.9973	0.6129	73	98	114	26	8	3	31	13	10	130	130	106
Diclofenac	200	0.1 – 2X	0.1X	0.9985	0.0504	110	92	100	3	4	2	6	7	5	99	100	99

Table 2.6 Continued

Compound	MRL (X)	Linearity				Accuracy (%)			Intra-day precision (%)			Inter-day precision (%)			Absolute matrix effect (%)		
	ng g^{-1}	Range	LOQ	R^2	LOF	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X
Dicloxacillin	100	0.1 – 3X	0.1X	0.9998	0.9945	102	99	109	9	6	6	7	6	8	131	100	100
Difloxacin	50	0.1 – 3X	0.1X	0.9994	0.8808	103	97	100	8	5	6	7	6	7	104	106	108
Dimetridazole	10	0.1 – 3X	0.1X	0.9982	0.4102	96	97	104	4	6	9	8	7	11	98	97	98
Doxycycline	100	0.1 – 3X	0.1X	0.9993	0.7341	97	96	100	6	9	4	9	8	8	116	107	106
Emamectin	10	0.1 – 3X	0.1X	0.9922	0.3439	110	105	107	4	5	6	13	12	9	101	99	102
Enrofloxacin	20	0.1 – 3X	0.1X	0.9996	0.9574	97	99	114	7	6	4	8	6	9	114	106	105
Erythromycin	125	0.1 – 3X	0.1X	0.9987	0.7575	90	94	98	11	8	7	9	6	10	109	113	109
Fenbendazole	100	0.1 – 3X	0.1X	0.9934	0.0541	100	94	99	8	8	7	11	12	12	82	95	97
Fenbendazole Sulfone	400	0.1 – 3X	0.1X	0.9991	0.8673	102	96	106	8	3	3	14	6	5	77	93	95
Florfenicol amine	100	0.1 – 3X	0.1X	0.9966	0.5284	89	101	100	6	7	9	9	9	10	31	45	56
Flubendazole	10	0.1 – 3X	0.1X	0.9993	0.4049	101	94	104	6	2	2	7	5	4	98	99	98
Flunixin	10	0.1 – 2X	0.1X	0.9993	0.1371	102	94	103	2	1	2	8	8	8	97	97	99
Haloperidol	10	0.1 – 3X	0.1X	0.9974	0.1244	106	92	106	6	8	8	9	6	9	105	105	104
Hydroxy dimetridazole	50	0.1 – 3X	0.1X	0.9991	0.6471	100	95	104	6	3	9	10	5	11	97	101	101
Hydroxy Ipronidazole	10	0.1 – 3X	0.1X	0.9995	0.6841	95	97	92	6	8	5	7	8	8	102	103	101
Ipronidazole	10	0.1 – 3X	0.1X	0.9958	0.8951	102	97	105	3	7	11	9	7	10	99	98	101
Ketoprofen	10	0.1 – 3X	0.1X	0.9996	0.7832	100	98	107	4	1	3	8	7	8	98	99	99
Levamisole	100	0.1 – 3X	0.1X	0.9985	0.1678	92	96	106	3	3	5	4	6	9	99	102	101
Lincomycin	100	0.1 – 2X	0.1X	0.9989	0.885	93	95	112	9	6	3	9	8	4	103	102	102
Mebendazole	10	0.1 – 3X	0.1X	0.9988	0.1994	102	95	107	5	3	3	7	4	5	79	82	85

Table 2.6 Continued

Compound	MRL (X)	Linearity				Accuracy (%)			Intra-day precision (%)			Inter-day precision (%)			Absolute matrix effect (%)		
	ng g^{-1}	Range	LOQ	R^2	LOF	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X
Mebendazole amine	10	0.1 – 3X	0.1X	0.9996	0.7758	92	91	104	5	4	6	8	8	7	107	107	110
Melengestrol Acetate	25	0.1 – 3X	0.1X	0.999	0.4051	108	90	98	3	2	5	11	9	9	94	93	92
Morantel	150	0.1 – 3X	0.1X	0.9988	0.3643	94	94	98	4	7	6	5	6	8	103	105	111
Norfloxacin	50	0.25 – 3X	0.25X	0.9972	0.1145	89	96	114	13	6	5	25	9	8	119	107	100
Orbifloxacin	50	0.1 – 3X	0.1X	0.9997	0.9792	106	101	109	7	9	3	8	8	8	111	105	103
Oxacillin	100	0.1 – 3X	0.1X	0.9969	0.1026	98	100	103	6	4	2	7	5	8	130	101	101
Oxfendazole	800	0.1 – 3X	0.1X	0.9996	0.958	100	98	108	5	2	3	6	4	5	98	99	96
Oxyclozanide	10	0.9 – 3X	0.9X	0.9912	0.267	-	60	86	-	15	13	-	51	30	98	95	96
Oxyphenylbutazone	100	0.1 – 3X	0.1X	0.9981	0.6933	102	101	109	6	7	2	9	8	5	96	97	98
Oxytetracycline	200	0.1 – 3X	0.1X	0.994	0.349	88	104	121	9	12	7	14	12	12	120	118	114
Phenylbutazone	100	0.1 – 3X	0.1X	0.9987	0.5731	92	101	114	4	10	3	8	8	5	97	99	97
Pirlimycin	300	0.1 – 3X	0.1X	0.9992	0.4285	100	97	107	5	2	6	8	5	11	100	101	98
Prednisone	100	0.1 – 3X	0.1X	0.998	0.9244	92	102	101	3	4	3	8	7	8	95	92	97
Promethazine	10	0.1 – 3X	0.1X	0.9988	0.1956	98	97	110	10	9	6	9	8	8	112	112	108
Propionylpromazine	10	0.1 – 3X	0.1X	0.9998	0.9644	94	94	115	7	9	5	6	9	8	107	106	100
Ractopamine	30	0.1 – 3X	0.1X	0.9984	0.0569	94	97	107	4	4	5	6	7	8	98	101	104
Sarafloxacin	50	0.1 – 3X	0.1X	0.9987	0.0651	90	107	100	6	6	7	20	8	13	114	111	104
Sulfachloropyridazine	100	0.1 – 3X	0.1X	0.9986	0.2934	96	99	103	6	3	2	6	5	8	101	100	101
Sulfadimethoxine	100	0.1 – 3X	0.1X	0.9927	0.583	126	96	113	24	15	12	34	16	17	126	114	106
Sulfadoxine	100	0.1 – 3X	0.1X	0.9988	0.3358	99	100	107	3	2	4	5	5	6	104	102	101

Table 2.6 Continued

Compound	MRL (X)	Linearity				Accuracy (%)			Intra-day precision (%)			Inter-day precision (%)			Absolute matrix effect (%)		
	ng g^{-1}	Range	LOQ	R^2	LOF	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X	0.3X	0.9X	2.5X
Sulfaethoxypyridazine	100	0.1 – 3X	0.1X	0.9998	0.7936	92	96	98	3	4	3	4	4	6	101	101	102
Sulfamerazine	100	0.1 – 3X	0.1X	0.9991	0.5314	98	97	108	7	3	4	7	5	9	99	103	103
Sulfamethazine	100	0.1 – 3X	0.1X	0.9986	0.2373	108	99	106	4	6	2	6	4	6	106	102	102
Sulfamethizole	100	0.1 – 3X	0.1X	0.9977	0.1043	96	102	111	7	4	2	7	7	8	99	103	102
Sulfamethoxazole	100	0.1 – 3X	0.1X	0.9987	0.7362	91	99	104	6	3	2	6	7	8	101	102	100
Sulfamethoxypyridazine	100	0.1 – 3X	0.1X	0.9988	0.6857	103	99	100	5	3	1	6	5	5	107	107	104
Sulfapyridine	100	0.1 – 3X	0.1X	0.9993	0.5457	94	99	108	4	4	3	5	6	7	101	100	101
Sulfaquinoxaline	100	0.1 – 3X	0.1X	0.9998	0.9357	96	101	109	4	2	3	5	4	4	98	100	100
Sulfathiazole	100	0.1 – 3X	0.1X	0.9992	0.0588	99	96	105	4	3	8	8	6	10	102	99	103
Tetracycline	200	0.1 – 3X	0.1X	0.9952	0.6236	63	92	107	20	7	8	21	7	7	123	119	118
Tolfenamic Acid	200	0.1 – 2X	0.1X	0.9993	0.2278	103	90	98	3	2	2	5	7	6	99	99	100
Triclabendazole	50	0.25 – 2X	0.25X	0.9972	0.0772	106	84	97	4	4	6	8	12	10	96	96	97
Triclabendazole Sulfoxide	50	0.1 – 2X	0.1X	0.9952	0.0621	103	88	103	9	6	4	18	12	9	97	98	99
Trifluropromazine	10	0.1 – 3X	0.1X	0.9986	0.5501	101	93	110	8	7	7	10	9	8	100	103	100
Tylosin	200	0.1 – 3X	0.1X	0.9962	0.0558	105	94	105	9	5	7	11	7	8	111	107	103
Virginiamycin	100	0.1 – 3X	0.1X	0.9953	0.118	103	98	100	3	2	3	7	5	9	100	103	102
Xylazine	10	0.1 – 3X	0.1X	0.999	0.1677	91	90	93	4	7	7	4	8	9	104	103	112

2.5 Conclusion

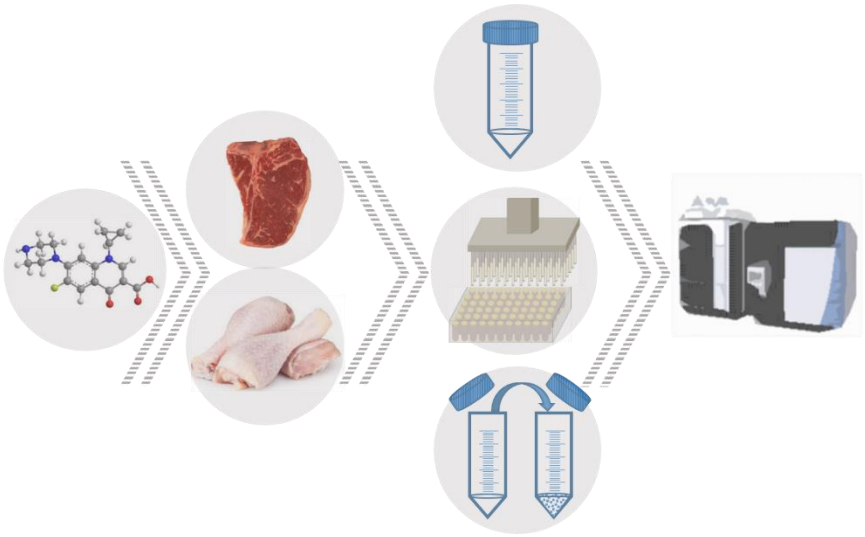
The developed method is the first and most comprehensive SPME approach to date for analysis of multi-residue veterinary drugs in meat in terms of the number of analytes tested as well as with respect to the range of physical and chemical properties covered. The method is fully automated, allowing for simultaneous analysis of up to 96 samples. Therefore, it offers a cost-effective alternative for analysis of veterinary drug residues in meat, additionally offering improved precision and shorter analysis times as compared to traditional sampling procedures.

Given the growing public interest and concern regarding food safety, and taking into account the importance of the meat industry to the global economy, the demand for sophisticated, automated high-throughput analytical procedures for monitoring of drug residues in meat is expected to continue growing. The method is ideal for large-scale monitoring of multi-residue drugs, and is thus proposed as a valuable tool for regulatory monitoring and enforcement of MRLs. In addition to its superior performance and wide coverage, it is environmentally friendly due to the minimum amount of organic solvents needed as compared to traditional methods. Furthermore, it can be potentially adopted for other high-throughput analyses in biological, pharmaceutical, food science, and metabolomics applications. The proposed SPME method for analysis of multi-residue veterinary drugs in meat offers many advantages in comparison to currently adopted approaches. Notably, it offers fully automated and high-throughput monitoring, thus allowing for shorter analysis times per sample as compared to traditional sampling procedures. Further, the range of compounds detectable by the method include analytes from several drug classes, and of varying physical and chemical properties. The validation results and minimal matrix effects demonstrate that the method is suitable, reliable, and offers cleaner extracts as opposed to traditional methods.

It is expected that the developed method will be tested in the near future in different food matrices and for ultra-fast screening of multi-class multi-residue drugs via direct interface to MS technologies. Furthermore, studies to compare results of this method to other established methods for incurred samples will also be considered in the future.

Chapter 3

Comparison of Solid-Phase Microextraction to Solvent Extraction and QuEChERS for Quantitative Analysis of Veterinary Drug Residues in Chicken and Beef Matrices



3.1 Preamble

The materials in this chapter have been published as a research article: Khaled, A.; Singh, V.; Pawliszyn, J. Comparison of Solid-Phase Microextraction to Solvent Extraction and QuEChERS for Quantitative Analysis of Veterinary Drug Residues in Chicken and Beef Matrices. *J. Agric. Food Chem.* **2019**, *67*, 12663–12669. The article is part of the 55th North American Chemical Residue Workshop special issue. Materials for all sections of this current chapter are reprinted from this research article with the permission of Journal of Agricultural and Food Chemistry of the American Chemical Society (ACS), copyright 2019. This work remains the

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The contribution of co-author Varoon Singh was in preparation of HLB particles used to prepare the SPME coating used in this work. All of the experimental design, planning, and work conducted in the laboratory, data processing, analysis, interpretation, and writing were performed by the author of the thesis.

I, Varoon Singh, authorize Abir Khaled to use the material for her thesis.

3.2 Introduction

Veterinary drugs are widely used in food-producing animals to prevent diseases and promote growth.[84,109,181] It is estimated that nearly 80% of the world production of antibiotics is used on animals and that 80% of all antibiotics sales in the U.S. stem from the sale of antibiotics used to protect food-producing animals from infection and promote their growth.[182,183] To ensure that veterinary drugs are used responsibly and that any possible residues left in the edible tissues of animals do not pose health risks to consumers, regulatory agencies worldwide have established maximum residue limits (MRLs) for veterinary drugs in animal-derived foodstuffs.[10,13,82]

MRL compliance assessments demand robust and reliable analytical methods capable of detecting a wide range of potential residues. With recent advancements in liquid chromatography (LC) coupled to mass spectrometry (MS) and tandem mass spectrometry (MS/MS), analytical methods for monitoring veterinary drug residues have expanded from simple procedures only capable of monitoring few analytes of interest to multi-residue methods that include as many

analytes of concern as possible.[96] Unfortunately, MS/MS detection is highly vulnerable to matrix effects as a result of the complexity of animal tissue composition.[184] Co-eluting matrix components can sometimes affect the ionization efficiency of target analytes, leading to either suppression or enhancement of signals. These matrix effects, consequently, may affect the qualitative and quantitative performance of the method.[185] As such, sample extraction procedures capable of isolating analytes of interest while removing interfering matrix compounds are essential in achieving reliable results and maintaining instrument performance.[117] On the other hand, simultaneous extraction and analysis of a wide variety of veterinary drugs with a wide range of physico-chemical properties require employment of non-selective, generic sample preparation procedures.[186]

Among sample preparation procedures used for multi-residue methods, the most frequently reported generic sample preparation procedures are solvent extraction (SE),[13,91] and quick, easy, cheap, effective, rugged, and safe (QuEChERS), a hybrid method combining SE and dispersive solid-phase extraction (d-SPE).[186,187] Both procedures have been widely assessed and validated in the literature and are accepted for routine analysis by many accredited laboratories and governmental organizations.[9,31,32,84,91,185,188]

Recently, a fully automated and high-throughput method using direct immersion solid phase microextraction (DI-SPME) was developed and validated for quantitative analysis of 77 veterinary drugs in chicken tissue.[189] In this work, a SPME phase prepared with a matrix-compatible coating made from a polyacrylonitrile (PAN)/hydrophilic–lipophilic balance (HLB) extraction phase was

demonstrated as capable of extracting a broad range of analytes with a wide range of physiochemical properties from chicken tissue, with minimal matrix effects observed.[189]

The aims of the current study were to expand the scope of the previously developed and validated SPME method[189] by testing its applicability for other matrices of interest (beef tissue) and 25 additional analytes of interest. In addition, this work encompassed a comparison of the developed method to two "gold standard" methods developed and implemented by the United States Department of Agriculture (USDA) for quantitation of multi-residue veterinary drugs in meat, namely, a modified QuEChERS method and a SE method.[9,32,91] This comparison took into consideration factors of impact, such as matrix effects, linearity, limit of quantitation (LOQ), accuracy, repeatability, sample throughput, and environmental footprint.

3.3 Experimental Section

3.3.1 Chemicals and Materials

To expand the applicability of the method, beef muscle tissue was additionally selected as the target matrix and 25 new veterinary drugs not included in the previous study were considered herein for analysis. Standards of these additional veterinary drugs and their suppliers are listed in Table 3.1. The corresponding class of each drug is presented in Table 3.2. The veterinary drugs under study were selected from the list of standards specified in the official method for screening and confirmation of animal drug residues developed by the USDA (CLG-MRM1.08).[31] LC–MS-grade acetonitrile (MeCN), methanol (MeOH), water, and formic acid (FA) were purchased from Fisher Scientific (Mississauga, Ontario, Canada). Polyacrylonitrile (PAN), N,N-

dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Nunc U96 Deep Well 2 and 1 mL plates made of polypropylene (PP) were purchased from VWR International (Mississauga, Ontario, Canada). PP inserts (600 μL) used in the 1 mL 96-well plates used during the desorption step were purchased from Analytical Sales and Services (Flanders, NJ, U.S.A.). The end-capped C_{18} sorbent used for d-SPE was obtained from UCT (Bristol, PA, U.S.A.). High-tolerance 304 stainless-steel rods (1/16 in. diameter) used for SPME pins were purchased from McMaster-Carr (Aurora, OH, U.S.A.). HLB particles were synthesized in-house as previously described.[189]

Stock solutions, deuterated internal standard, and mixed standards were prepared as described previously in chapter 2.[189] Organic beef and chicken muscle tissue samples, five samples of each, were obtained from five different local grocery stores to serve as the pooled matrix. Five distinct samples of non-organic chicken and three non-organic beef were purchased from different local grocery stores for analysis of market samples.

Table 3.1 Physico-chemical properties and supplier information for additional 25 analytes

Compound	**Supplier**	**Formula[1]**	**Molecular Mass (Da)[1]**	**Log P[1]**
6-Propyl-2-thiouracil	Sigma-Aldrich	$C_7H_{10}N$	170.232	1.37
Amoxicillin	Sigma-Aldrich	$C_{16}H_{19}N_3O5_S$	365.404	0.61
Ampicillin	Sigma-Aldrich	$C_{16}H_{19}N_3O_4S$	349.4048	1.35
Bacitracin	Sigma-Aldrich	$C_{66}H_{103}N_{17}O_{16}S$	1422.69	-2.21
Carazolol	Sigma-Aldrich	C18H22N2O2	298.379	3.59
Cefazolin	Sigma-Aldrich	C14H14N8O4S3	454.507	1.13
Ciprofloxacin	Sigma-Aldrich	$C_{17}H_{18}FN_3O_3$	331.341	0.65
Desacetyl cephapirin	TRC[2]	$C_{15}H_{15}N_3O_5S_2$	381.427	0.32
Desethylene ciprofloxacin HCl	TRC[2]	$C_{15}H_{17}ClFN_3O_3$	341.8	-0.14
Dimetridazole	Sigma-Aldrich	$C_5H_7N_3O_2$	141.0	0.31
Florfenicol	Sigma-Aldrich	$C_{12}H_{14}C_{l2}FNO_4S$	357.0	-0.12
Gamithromycin	Sigma-Aldrich	$C_{40}H_{76}N_2O_{12}$	776.5	3.89
Meloxicam	Sigma-Aldrich	$C_{14}H_{13}N_3O_4S_2$	351.0	2.71
Metamizole	Sigma-Aldrich	$C_{13}H_{17}N_3O_4S$	311.0	-0.74
Nafcillin	Sigma-Aldrich	$C_{21}H_{22}N_2O_5S$	414.1	3.52
Norfloxacin	Sigma-Aldrich	$C_{16}H_{18}FN_3O_3$	319.3	0.82
Novobiocin	Sigma-Aldrich	$C_{31}H_{36}N_2O_{11}$	612.2	2.86
Penicillin G	TRC[2]	$C_{16}H_{18}N_2O_4S$	334.0	1.67
Ronidazole	Sigma-Aldrich	$C_6H_8N_4O_4$	200.0	-0.45
Salbutamol	Sigma-Aldrich	$C_{13}H_{21}NO_3$	239.1	0.01
Sulfadiazine	Sigma-Aldrich	$C_{10}H_{10}N_4O_2S$	250.277	-0.12
Thiabendazole	Sigma-Aldrich	$C_{10}H_7N_3S$	201.0	2.47
Tildipirosin	Sigma-Aldrich	$C_{41}H_{71}N_3O_8$	733.5	4.70
Tilmicosin	Sigma-Aldrich	$C_{46}H_{80}N_2O_{13}$	868.5	4.95
Tulathromycin	TRC[2]	$C_{41}H_{79}N_3O_{12}$	805.5	4.07

[1] Data taken from www.chemspider.com, accessed March 2019, LogP data taken from computational predictions ACD/LogP

[2]Toronto Research Chemicals

3.3.2 LC–MS/MS Method

All experiments were carried out using an UltiMate 3000RS HPLC system coupled to a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, U.S.A.). MS/MS analysis was performed in positive ionization mode under selected reaction monitoring (SRM) conditions. Data acquisition and processing were performed using Xcalibur 4.0 and Trace Finder 3.3 software (Thermo Fisher Scientific, San Jose, CA, U.S.A.). Chromatographic conditions and analytical column specifications were set as previously described in chapter 2.[189] A Waters (Mississauga, Ontario, Canada) Acquity UPLC C_{18} HSS T3 (100 × 2.1 mm, 1.7 μm) analytical column connected to a guard column (HSS T3, 2.1 × 5 mm, 1.7 μm) was used for separation of targeted analytes. The column compartment was maintained at 40 °C, and the flow rate was 0.3 mL/min. MeCN/water (70:30, v/v) was used to clean the injection system (flush and wash volumes were 1000 and 200 μL, respectively). The mobile phases were water (solvent A) and MeCN (solvent B), each containing 0.1% (v/v) formic acid. The gradient was run at 3% B for 1 min, ramped linearly to 100% B until 11 min, and then held at 100% B until 13 min. The column was then returned to 3% B over a 2 min period, whereupon it was allowed to re-equilibrate for 3 min. The injection volume was 10 μL, and the autosampler was thermostated at 5 °C. MS data were processed using Xcalibur software version 4.0 (Thermo Fisher Scientific, San Jose, CA, U.S.A.). Mobile phases were degassed for 30 min in a VWR Scientific Aquasonic model 75HT (West Chester, PA, U.S.A.) ultrasonic bath before use. Further instrumental details, LC and MS/MS parameters are provided in Table 3.2 and Tables 2.3 and 2.4 of chapter 2.

Table 3.2 MS/MS optimized parameters, (m/z), and retention times (RT) of compounds, ions used for quantification are bolded.

Compound	Class	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	RT (min)	S-Lens value	Polarity
2-Aminoflubendazole	Anthelmintics	256	**123**, 95	37, 27	6.19	93	+
2-Amino mebendazole	Anthelmintics	238	**105**, 77	25, 34	5.03	88	+
2-Quinoxalinecarboxylic	Others	175	**131**, 104	22, 29	6.15	191	+
5-Hydroxythiabendazole	Anthelmintics	218	**191**, 147	26, 32	4.69	85	+
6-Phenyl-2-thiouracil	Thyreostats	205	**188**, 103	17, 26	6.12	69	+
6-Propyl-2-thiouracil	Thyreostats	171	**154**, 112	17, 19	5.29	57	+
Acepromazine Maleate	Tranquilizers	327	**86**, 254	19, 23	7.21	65	+
Albendazole	Anthelmintics	266	**234**, 191	19, 32	7.35	71	+
Albendazole sulfone	Anthelmintics	298	**266**,159	19, 36	4.94	70	+
Albendazole sulfoxide	Anthelmintics	282	**240**, 208	10, 23	6.50	75	+
Albendazole-2-	Anthelmintics	240	**133**,198	28,19	5.68	63	+
Amoxicillin	β-Lactams/cephalosporins	366	**349**, 114	10, 20	4.18	48	+
Ampicillin	β-Lactams/cephalosporins	350	**106**, 160	18, 10	5.08	54	+
Azaperone	Tranquilizers	328	**165**,123	20, 29	5.68	66	+
Bacitracin	Others	475	**199**, 669	24, 10	6.17	74	+
Betamethasone	Anti-inflammatories	393	**325**, 347	14, 12	6.17	59	+
Cambendazole	Anthelmintics	303	**217**, 261	27, 17	6.27	66	+
Carazolol	Tranquilizers	299	**116, 222**	19, 19	6.38	63	+
Carbadox	Other	263	**231**, 245	10, 16	5.10	67	+
Cephapirin	β-Lactams/cephalosporins	424	**292**, 152	14, 22	4.45	66	+
Cefazolin	β-Lactams/cephalosporins	455	**323**, 333	10, 19	5.71	72	+
Chlortetracycline HCl	Tetracyclines	479	**462**,444	17, 19	5.80	70	+
Chlorpromazine HCl	Tranquilizers	319	**86**, 58	19, 30	7.85	63	+
Ciprofloxacin	Fluoroquinolones	332	**288**, 314	17, 19	5.34	72	+
Clenbuterol HCl	β-Agonists	277	**203**, 259	16, 10	5.89	47	+
Clindamycin HCl	Macrolides/lincosamides	425	**126**, 377	27, 18	6.24	78	+
Cloxacillin Sodium Salt	β-Lactams/cephalosporins	436	**277**, 160	12, 10	8.40	56	+
Danofloxacin	Fluoroquinolones	358	**340**, 255	21, 18	5.40	80	+
Desacetyl cephapirin	β-Lactams/cephalosporins	382	**292**, 226	10, 17	3.50	73	+
Desethylene Ciprofloxacin	Fluoroquinolones	306	**288**, 268	17, 24	4.93	67	+
Diclofenac Sodium	Anti-inflammatories	296	**214**, 215	33, 19	9.66	112	+
Dicloxacillin	β-Lactams/cephalosporins	470	**452**, 212	16, 26	8.90	124	+
Difloxacin HCl	Fluoroquinolones	400	**382**, 356	21, 18	5.89	82	+

Compound	Class	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	RT (min)	S-Lens value	Polarity
Dimetridazole	Coccidiostats	142	**96**, 101	16, 10	4.66	38	+
Doxycycline HCl	Tetracyclines	445	**267**, 321	35, 30	5.54	66	+
Emamectin Benzoate	Anthelmintics	887	**158**, 868	33, 21	9.73	122	+
Enrofloxacin	Fluoroquinolones	360	**342**,316	20, 18	5.56	79	+
Erythromycin	Macrolides/lincosamides	734	**576**,158	17, 27	7.00	85	+
Fenbendazole	Anthelmintics	300	**268**, 159	20, 34	8.20	77	+
Fenbendazole Sulfone	Anthelmintics	332	**300**, 159	21, 37	7.30	81	+
Florfenicol	Phenicols	357	**198**, 182	14, 16	7.40	73	+
Florfenicol amine	Phenicols	248	**230**, 130	10, 24	1.00	50	+
Flubendazole	Anthelmintics	314	**282**, 123	22, 35	7.60	80	+
Flunixin	Anti-inflammatories	297	**279**, 264	22, 33	9.00	75	+
Gamithromycin	Macrolides/lincosamides	778	**619**, 601	28, 31	6.30	121	+
Haloperidol	Tranquilizers	376	**165**,123	22, 36	7.22	78	+
Hydroxy dimetridazole	Coccidiostats	158	**80**,140	10, 10	4.20	30	+
Hydroxy ipronidazole	Coccidiostats	186	**168**, 122	10, 19	5.90	37	+
Hydroxy metronidazole	Coccidiostats	188	**123**, 126	10, 17	3.77	47	+
Ipronidazole	Coccidiostats	170	**124**, 109	17, 24	6.87	54	+
Ketoprofen	Anti-inflammatories	255	**209**, 105	14, 23	8.60	59	+
Levamisole HCl	Anthelmintics	205	**178**, 123	21, 28	4.90	66	+
Lincomycin	Macrolides/lincosamides	407	**126**, 359	26, 18	4.90	76	+
Mebendazole	Anthelmintics	296	**264**, 105	20, 33	7.35	75	+
Melengestrol Acetate	Others	397	**279**,337	20, 13	10.50	88	+
Meloxicam	Anthelmintics	352	**115**, 141	19, 20	8.87	67	+
Metamizole	Anti-inflammatories	218	**56**, 97	17, 12	4.53	47	+
Metronidazole	Anthelmintics	172	**128**, 82	14, 23	4.30	48	+
Morantel Tartrate Hydrate	Anthelmintics	221	**123**, 111	35, 25	6.03	73	+
Nafcillin	β-Lactams/cephalosporins	415	**199**, 171	14, 34	8.60	78	+
Norfloxacin	Fluoroquinolones	320	**302**, 276	20, 17	5.26	76	+
Novobiocin	Others	613	**189**, 133	27, 51	10.15	80	+
Orbifloxacin	Fluoroquinolones	396	**352**, 295	17, 23	5.68	83	+
Oxacillin	β-Lactams/cephalosporins	402	**243**, 160	13, 11	8.10	68	+
Oxfendazole	Anthelmintics	316	**159**, 191	32, 20	6.44	68	+
Oxyclozanide	Anthelmintics	403	**186**, 83	22, 20	6.60	30	+
Oxyphenylbutazone	Anti-inflammatories	325	**160**, 162	20, 19	8.68	69	+
Oxytetracycline HCl	Tetracyclines	461	**426**, 443	18, 10	5.34	67	+
Penicillin G	β-Lactams/cephalosporins	335	**217**, 202	14, 23	7.36	80	+

Compound	Class	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	RT (min)	S-Lens value	Polarity
Phenylbutazone	Anti-inflammatories	309	**120**, 160	42, 19	10.08	71	+
Pirlimycin HCl	Macrolides/lincosamides	411	**363**,112	16, 25	6.04	77	+
Prednisone	Anti-inflammatories	359	**341**, 295	10, 14	7.20	65	+
Promethazine HCl	Tranquilizers	285	**86**, 198	17, 25	7.26	46	+
Propionylpromazine HCl	Tranquilizers	341	**86**, 58	19, 30	7.70	68	+
Ractopamine HCl	β-Agonists	302	**164**, 284	15, 10	5.44	53	+
Ronidazole	Anthelmintics	201	**140**, 55	10, 21	4.75	30	+
Salbutamol	β-Agonists	240	**148**, 222	17, 10	4.22	42	+
Sarafloxacin HCl Hydrate	Fluoroquinolones	386	**299**, 368	26, 21	5.83	85	+
Sulfachloropyridazine	Sulfonamides	285	**156**, 108	16, 24	4.90	57	+
Sulfadiazin	Sulfonamides	251	**156**, 108	14, 22	7.10	48	+
Sulfadimethoxine	Sulfonamides	311	**156**, 108	20, 27	6.53	72	+
Sulfadoxine	Sulfonamides	311	**108**, 154	25, 27	6.60	68	+
Sulfaethoxypyridazine	Sulfonamides	295	**156**, 108	17, 25	5.35	64	+
Sulfamerazine	Sulfonamides	265	**172**, 108	16, 24	5.74	59	+
Sulfamethazine	Sulfonamides	279	**186**, 156	17, 18	5.70	65	+
Sulfamethizole	Sulfonamides	271	**92**, 108	24, 22	6.48	47	+
Sulfamethoxazole	Sulfonamides	254	**108**, 156	23, 15	5.76	154	+
Sulfamethoxypyridazine	Sulfonamides	281	**156**, 108	16, 24	5.20	63	+
Sulfapyridine	Sulfonamides	250	**156**, 108	16, 24	7.08	57	+
Sulfaquinoxaline	Sulfonamides	301	**156**, 108	17, 25	5.05	69	+
Sulfathiazole	Sulfonamides	256	**156**, 108	15, 22	5.54	52	+
Tetracycline HCl	Tetracyclines	445	**410**, 427	18, 10	5.08	67	+
Thiabendazole	Anthelmintics	202	**175**, 131	25, 32	4.74	74	+
Tildipirosin	Macrolides/lincosamides	368	**98**, 637	19, 10	6.45	64	+
Tilmicosin	Macrolides/lincosamides	435	**174**, 99	23, 18	8.10	75	+
Tolfenamic Acid	Anti-inflammatories	262	**244**, 209	15, 27	10.43	47	+
Triclabendazole	Anthelmintics	359	**274**, 344	37, 25, 51	10.20	101	+
Triclabendazole Sulfoxide	Anthelmintics	375	**357**, 360	17, 21	9.39	79	+
Triflupromazine HCl	Tranquilizers	353	**248,** 86	42, 20	8.14	68	+
Tulathromycin	Macrolides/lincosamides	404	**72**, 158	19, 21	5.25	62	+
Tylosin	Macrolides/lincosamides	917	**772**, 174	27, 35	7.22	134	+
Virginiamycin M1	Others	526	**508**, 355	12, 17	8.25	72	+
Xylazine HCl	Tranquilizers	221	**90**, 147	22, 23	5.86	68	+
Flunixin-d_3	Internal standard	300	**282**	22	9.00	74	+

3.3.3 Sample Preparation

Each tissue sample was homogenized under liquid nitrogen using a Freezer/Mill cryogenic grinder (SPEX SamplePrep, LLC, Metuchen, NJ, U.S.A.) to produce a fine uniform powder. All samples were then combined in equal weights and mixed to produce a pooled matrix. All homogenized samples were stored in glass jars at −30 °C until analysis. Spiked tissue samples were prepared by weighing 2.0 g of homogenized tissue into a 50 mL PP tube. Each sample was then spiked with 200 μL of a working solution containing all analytes under study at their respective designated concentrations and 50 μL of an internal standard working solution containing flunixin-d_3 at 1 ng μL^{-1}. Samples were vortexed for 1 min and then placed on a benchtop agitator for 1 h. Subsequently, samples were stored in a 4 °C fridge overnight to allow for proper equilibration and matrix binding of target analytes prior to extraction. Following overnight refrigeration, samples were placed on the benchtop agitator for 1 h prior to extraction to allow samples to reach room temperature.

3.3.3.1 Automated SPME Protocol

The SPME procedure was carried out in accordance with the previously described high-throughput extraction protocol in chapter 2,[189] using the Concept 96 robotic sample preparation station [Professional Analytical System (PAS) Technology, Magdala, Germany].[137] The Concept 96 robotic station uses a 96 SPME pin device shown in Figure 2.1, which is compatible with commercially available 96-well plates. The main steps of automated SPME are summarized in Figures 2.2 and 2.3 of chapter 2. SPME pins for the 96 SPME pin device were prepared using

stainless-steel rods, which were cut to 50 mm length. The HLB/PAN coating was deposited on the stainless-steel rod surfaces by dip coating as previously reported by Gómez-Ríos et al.[190] The coating length and thickness was 20 mm and 60 μm, respectively. To prepare the samples for SPME extraction, 6 mL of water was added to each individual spiked tissue sample and vortexed for 1 min. Next, 1.5 mL of the diluted tissue samples were transferred to the 96-well plate. The 96-well plate was then placed on the extraction plate of the Concept 96 station, which was programmed to consecutively execute the preconditioning of the SPME-coated pins, extraction, rinsing, and solvent desorption. While the SPME protocol normally permits direct transfer of the 96-well plates to the LC autosampler for analysis, for the purpose of maintaining consistency among all methods, in the current work, 300 μL of each sample extract was transferred to PP autosampler vials and 10 μL was injected into the LC system.

3.3.3.2 SE

The SE protocol used in this study was adapted from the USDA multi-class, multi-residue method (MMM) described by Lehotay et al.[91] In brief, each sample was extracted with 10 mL of MeCN/water (4:1, v/v) by vortex shaking (5 min and 1500 rpm in pulse mode) in a multitube vortexer, BenchMixer Multi-Tube Vortexer (Benchmark Scientific, Edison, NJ, U.S.A.). Each sample was then centrifuged for 3 min at 5000 rpm (Jouan B4i centrifuge, Thermo Electron Corporation, Waltham, MA, U.S.A.). A total of 300 μL of the supernatant was transferred to PP autosampler vials, and 10 μL was injected into the LC system. No dilution of the sample was carried out post-extraction.

3.3.3.3 QuEChERS

The QuEChERS method followed in this study is a modified version of the USDA QuEChERS/d-SPE method using C_{18} as the cleanup sorbent as previously described in the literature.[9] In brief, the QuEChERS procedure was carried out using the same described parameters as the SE method above, followed by transfer of full extracts into another 50 mL PP centrifuge tube containing a 500 mg end-capped C_{18} sorbent. The total volume of the extracts is approximately 11.5 mL, taking into account 1.5 mL of water in the 2 g tissue samples. Samples were then shaken in the multitube vortexer for 30 s and centrifuged for 3 min. A total of 300 μL of the final supernatant was transferred to PP autosampler vials. For the purposes of the current work, no dilution was performed post-extraction and 10 μL of the final extract was injected into the LC system.

3.4 Results and Discussion

3.4.1 Matrix Effects

Matrix effects have significant influence on analyte response, being considered one of the main drawbacks of electrospray ionization (ESI)–LC–MS/MS analysis as a result of the co-extraction and co-elution of interfering matrix components.[191,192] Given the complexity of tissue as a matrix, reliable data can thus only be attained by use of sample preparation methods that are designed to minimize matrix effects.[184] In view of this, the degree of matrix effect generated by each method was evaluated by comparing the slope of matrix-matched calibration curves against the slope of calibration standards in the reagent, using the following equation to calculate matrix

effect percentage: ME (%) = (slope of matrix-matched calibration curve – slope of reagent-only calibration curve) × 100/slope of reagent-only calibration curve.[32] No internal standards were used when calculating matrix effects because such results might also be affected by matrix effects, which could, in turn, lead to inaccurate results.[193] Figure 3.1 shows the matrix effect results for the target analytes versus their retention time for the three methods under study in beef and chicken tissue. As demonstrated in Figure 3.1, the SPME protocol yielded the best results among all three methods in both beef and chicken matrices, where only two compounds, namely, florfenicol amine and tildipirosin, displayed signal suppression (−26%) and signal enhancement (55%), respectively, in beef, while only florfenicol amine displayed signal suppression (−31%) in the chicken matrix. The minimal matrix effects achieved by SPME are mainly attributed to the smooth biocompatible PAN layer, which acts as a barrier to prevent interfering matrix components and macromolecules, such as proteins and lipids, from adsorbing to the SPME coating[120,194,195] while still allowing target analytes to adsorb on the HLB coating. In addition, because the extraction rate of analytes by SPME coatings is proportional to the free concentration of analytes present in the sample, only a small amount of phospholipids is extracted, resulting in very low chromatographic backgrounds and minimal suppressive matrix effects, which are usually caused by the highly abundant phospholipids in the ESI source.

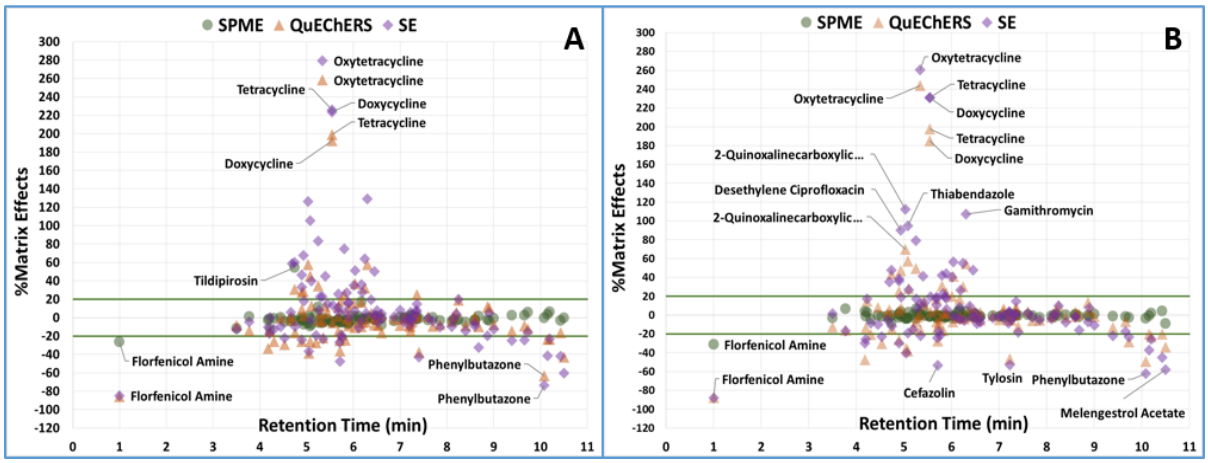


Figure 3.1 MEs (%) of selected drug analytes in (A) beef tissue and (B) chicken tissue versus their retention time for all three sample preparation methods under study. Points above or below the green lines indicate MEs (%) ± 20%.

Measured matrix effects for SE and QuEChERS methods displayed similar trends in both matrices. In the beef matrix, 30 and 42% of analytes extracted by the QuEChERS and SE methods, respectively, displayed either signal enhancement or suppression compared to 30 and 39% in chicken tissue. Full results are listed in Table 3.3 for beef tissue and Table 3.4 for chicken tissue. Tetracyclines, in particular, such as tetracycline, doxycycline, and oxytetracycline, showed significant signal enhancement in both matrices, demonstrating the importance of using a specific tetracycline internal standard and matrix-matched calibration for better quantification of these compounds when using SE or QuEChERS, as previously suggested by Anumol et al.[9] While abundant matrix effects are expected in SE, the obtained results for QuEChERS demonstrate that the d-SPE cleanup step did not yield sufficiently effective sample clean up given the extra time and effort involved in the execution of this extra step. Blasco et al. also observed significant MEs for analytes in beef tissue when using the QuEChERS method compared to pressurized liquid extraction (PLE).[196] A similar observation was also reported by Lehotay et al., wherein the authors

assert that the sample clean up provided by the d-SPE step does not merit the extra time and effort, especially when injecting a small sample equivalent or when a large dilution factor is used.[91] While large dilution factors are commonly used in SE and QuEChERS methods to minimize matrix interferences, high sample dilutions increase limits of detection and reduce overall method sensitivity and, as a result, may require the use of newer and more expensive instrumentation.[32,92,184] In addition, the dilution step introduces an additional step to the already tedious sample preparation process, thus increasing time, cost, and probability of error. While SE and QuEChERS offer a simple sample preparation procedure, the occurrence of abundant matrix effects is a clear drawback that compromises method performance and selectivity as a result of interfering peaks.[90] Implementation of these methods also requires frequent preventive maintenance of expensive equipment, thus leading to significant down time in high-throughput laboratories.[184]

3.4.2 Linearity and LOQs

Linearity and LOQ were assessed using matrix-matched calibration curves at six concentration levels in the range of 0.1–3X, with three replicates at each level. The calibration range was selected to include concentration levels bracketing the MRL. The MRL values shown in Tables 3.3 and 3.4 were based primarily on Canadian MRL[82] values and U.S. tolerances[91,170] in both chicken and beef tissues. In general, Canadian MRLs are either equal or less than U.S. tolerances; however, in cases where Canadian MRLs were higher than U.S. tolerances, the U.S. regulatory value was selected, as is the case for ketoprofen and morantel.

LOQ was established as the lowest point of the matrix-matched calibration curve that produced a response that is both accurate when compared to the expected value (calculated via linear regression) with the range of deviation of ≤30% as well as precise [≤30% relative standard deviation (RSD)].

In terms of linearity, as shown in Figure 3.2, the SPME protocol achieved the best linearity results among all three methods, with determination coefficients (R^2) higher than 0.99 for 99% of the compounds in both matrices compared to 87 and 80% in QuEChERS and SE in beef tissue and 91 and 87% of compounds in chicken, respectively, achieving (R^2) higher than 0.99.

LOQs for all methods were equal or lower than MRL values for all target analytes under study, with the exception of those attained for amoxicillin in the beef matrix with all methods and cephapirin in the chicken matrix with the SPME protocol. It is worth noting here that the MRL value for amoxicillin used in this study is the Canadian value (10 ng g^{-1}), which is 5 times lower than the U.S. value (50 ng g^{-1}). Florfenicol amine could not be quantified adequately with QuEChERS and SE in beef tissue as a result of the high noise attained via these methods at the retention time corresponding to florfenicol amine. In terms of LOQ, as shown in Figure 3.3, and

listed in Tables 3.3 and 3.4, the LOQs obtained with the SPME method were generally lower than those obtained by QuEChERS and SE methods.

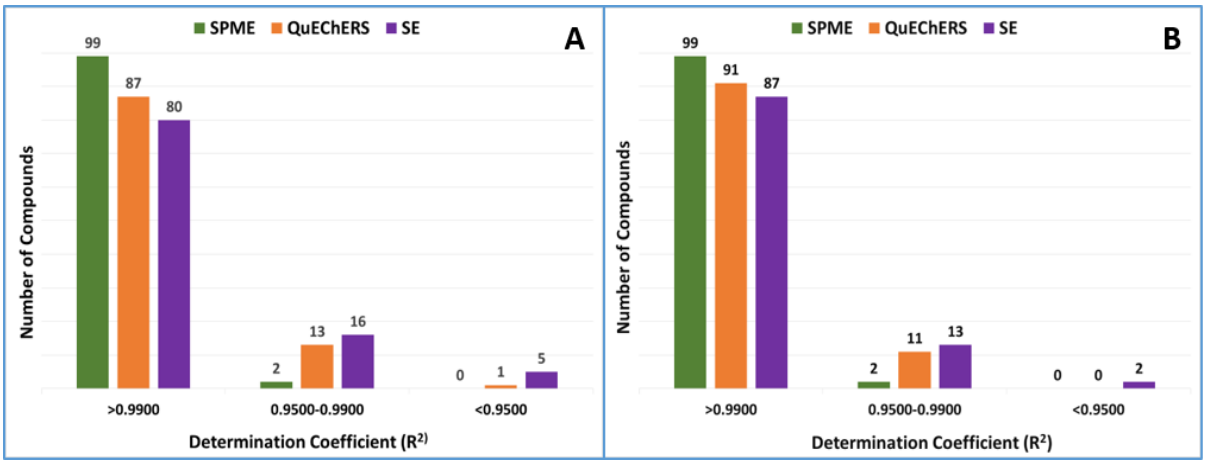


Figure 3.2 Linearity results for selected drug analytes in (A) beef tissue and (B) chicken tissue expressed as the number of compounds corresponding to R^2 value ranges.

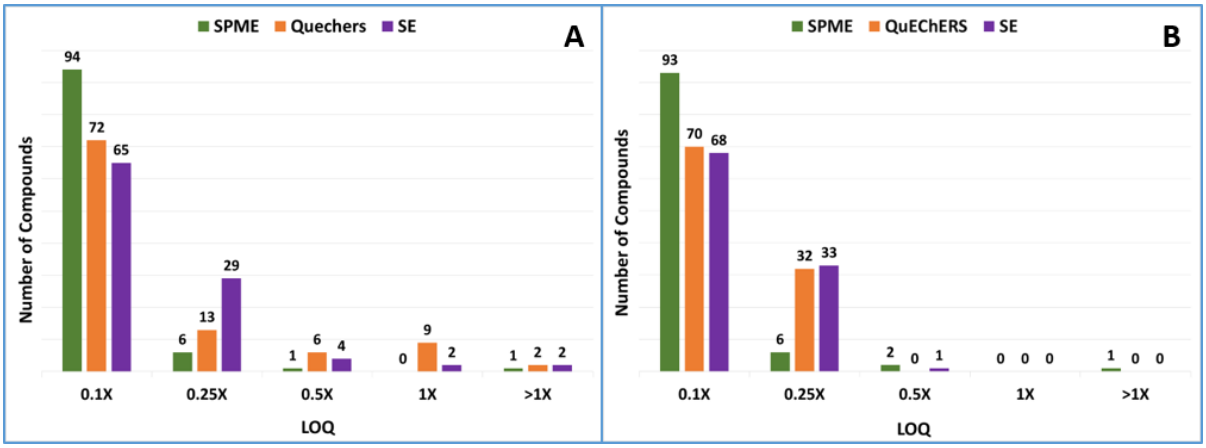


Figure 3.3 Number of compounds corresponding to LOQ values in (A) beef tissue and (B) chicken tissue.

While QuEChERS and SE are exhaustive methods, which allow for higher recoveries, the non-exhaustive extraction provided by SPME enables a lower background noise, resulting in a higher signal-to-noise ratio. Moreover, the use of acetonitrile as an extraction solvent is well-

known to lead to the extraction of sample constituents, such as lipids, to LC–MS, which leads to a noisy background and, thus, compromises high recovery as well as the precision of the analysis.[120] Better LOQs with SPME can also be attributed to lower sample dilution, 1:3 in SPME compared to 1:5 QuEChERS and SE. Moreover, in SPME, the analytes are extracted from a 1500 μL sample volume and desorbed in a 400 μL desorption volume, which leads to the enrichment of the analytes.

Individual LOQ values and determination coefficient values for all compounds under study are listed in Table 3.3 for beef tissue and Table 3.4 for chicken tissue.

3.4.3 Accuracy and Precision

To determine the accuracy and precision of the three methods under study with respect to their intra-day repeatability, pooled matrix blanks were spiked at two concentration levels bracketing the MRL, at concentrations corresponding to 0.75X and 1.5X for each analyte, using six replicates per concentration (n = 6). The accuracy of each method was calculated on the basis of estimated concentration values calculated from the linear regression equation of the matrix-matched calibration curve.

As shown in Figure 3.4, excellent accuracy and repeatability results were achieved with the SPME protocol, with more than 97 and 99% of analytes falling within the 70–120% range of the true concentrations of compounds and RSD of ≤25% at the 0.75X and 1.5X concentration level, respectively, in the beef matrix. In comparison, 86 and 92% of analytes could be quantified by QuEChERS and 90 and 96% by SE, respectively, under the same parameters. However, all methods achieved excellent accuracy and repeatability results in chicken tissue at both

concentration levels, with 97 and 99% in SPME, 96 and 97% in QuEChERS, and 91 and 98% in SE of analytes falling within the 70–120% range of their true concentrations and RSD of ≤25% at both the 0.75X and 1.5X concentration levels, respectively. The better results attained by QuEChERS and SE in the chicken matrix compared to beef could be attributed to the lower fat content of this matrix and less matrix effects.

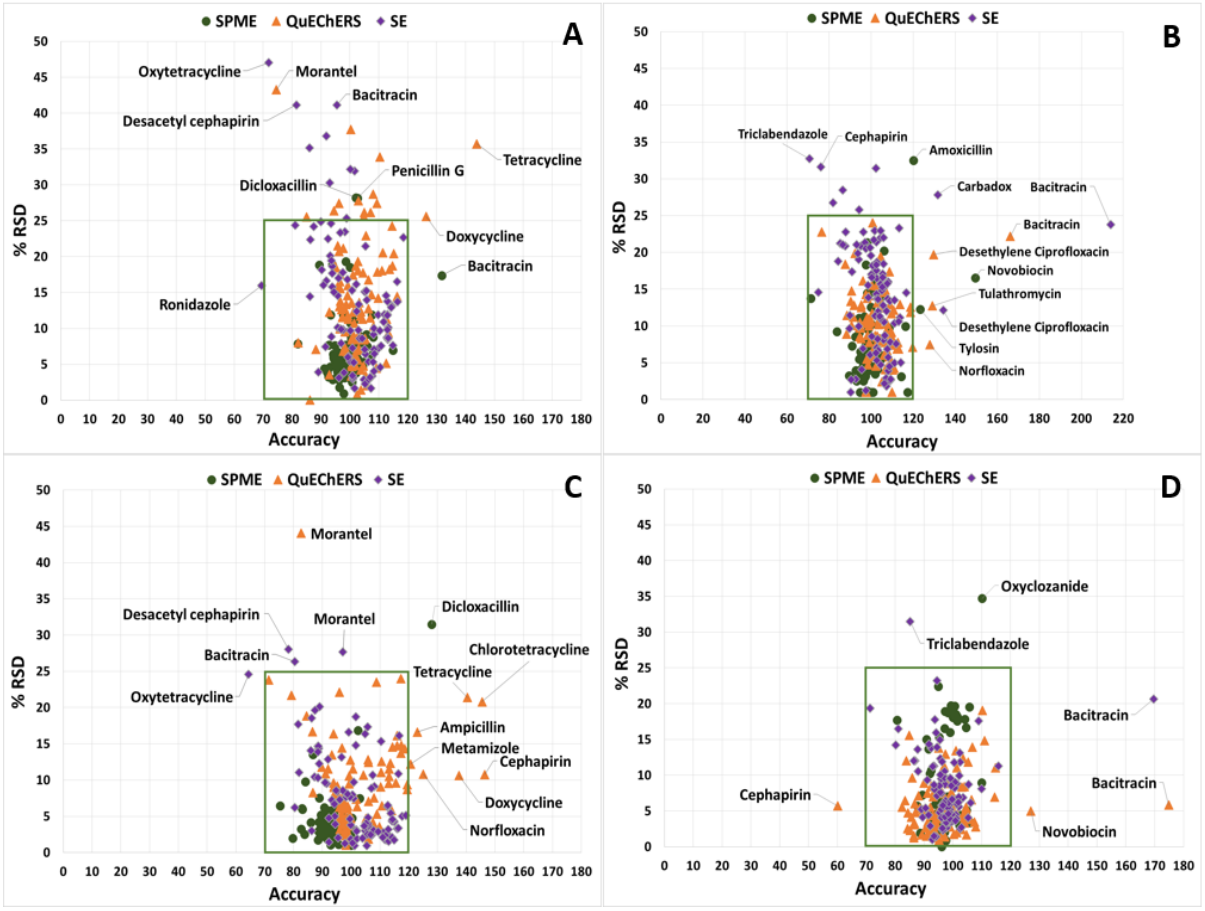


Figure 3.4 Accuracy and repeatability results of the three methods for the target analytes fortified at the 0.75X level in (A) beef tissue and (B) chicken tissue and at the 1.5X level in (C) beef tissue and (D) chicken tissue (n = 6).

The results obtained by the QuEChERS and SE methods in beef tissue further demonstrate the importance of using of matrix-matched calibration with internal standards specific for compounds, such as tetracyclines and lactams. Accuracy and RSD (%) values are listed in Tables 3.3 and 3.4 for each analyte under study in beef and chicken tissues, respectively.

The superior repeatability results, expressed as RSD (%), obtained by SPME can be attributed to lower matrix effects and to the fully automated process of the SPME protocol, which allows for samples to be extracted simultaneously and under the same conditions with minimum random variations, thus reducing the possibility of error during sample preparation. In addition to the superior precision afforded by the SPME protocol, automation of the method allows analysts to perform other activities during the extraction process, thus enabling more efficient use of their time in the laboratory. Conversely, the comparative lower precision of the QuEChERS and SE methods could also be attributed to the pronounced matrix effects compared to SPME.

*Table 3.3 Limits of quantification (LOQ), Linearity (n=3 at each level), and average accuracy (ACC), n = 6 at each level, in beef tissue. * designates compounds which could not be quantified*

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
2-Aminoflubendazole	10	-2	0.1X	0.9981	98 (5)	92 (2)	-5	0.1X	0.9993	104 (4)	105 (4)	**27**	0.1X	0.9999	101 (3)	105 (3)
2-Quinoxalinecarboxylic acid	100	-6	0.1X	1.0000	95 (6)	93 (3)	**58**	0.25X	0.9947	97 (17)	93 (13)	**126**	0.25X	0.9778	**102 (32)**	89 (15)
2-Amino mebendazole	10	-1	0.1X	0.9985	100 (3)	93 (2)	16	0.25X	0.9991	103 (19)	108 (4)	**35**	0.25X	0.9935	107 (3)	105 (16)
5-Hydroxythiabendazole	100	-1	0.1X	0.9955	97 (5)	100 (2)	-3	0.1X	0.9971	102 (9)	99 (4)	**59**	0.1X	0.9952	102 (12)	93 (7)
6-phenyl-thiouracil	400	1	0.1X	0.9990	95 (6)	92 (4)	-5	0.1X	0.9985	93 (4)	98 (4)	-11	0.1X	0.9981	89 (4)	113 (6)
6-propyl-2-thiouracil	50	-2	0.25X	0.9987	99 (7)	100 (5)	5	0.5X	0.9983	96 (16)	102 (6)	23	0.5X	0.9989	97 (8)	101 (10)
Acepromazine	10	-2	0.1X	0.9992	94 (6)	92 (2)	-10	0.1X	0.9994	104 (1)	98 (2)	-1	0.1X	0.9998	105 (3)	101 (1)
Albendazole	50	-2	0.1X	0.9943	99 (4)	93 (1)	2	0.1X	0.9992	99 (11)	94 (8)	6	0.1X	0.9945	113 (8)	113 (3)
Albendazole 2-aminosulfone	50	-7	0.1X	0.9999	97 (5)	94 (3)	2	0.1X	0.9896	97 (10)	111 (13)	18	0.1X	0.9967	110 (12)	113 (7)
Albendazole sulfone	50	-2	0.1X	0.9991	102 (5)	97 (3)	2	0.1X	0.9992	106 (2)	108 (4)	8	0.1X	0.9998	105 (2)	105 (3)
Albendazole sulfoxide	50	-2	0.25X	0.9971	105 (5)	98 (3)	1	0.1X	0.9925	108 (10)	119 (9)	6	0.1X	0.9966	98 (10)	101 (8)
Amoxicillin	10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Ampicillin	10	-1	0.25X	0.9906	89 (19)	84 (10)	**-26**	0.5X	0.9942	**109 (27)**	**123 (17)**	-3	0.5X	0.9923	93 (25)	89 (14)
Azaperone	10	-2	0.1X	0.9997	95 (5)	94 (2)	12	0.1X	0.9991	97 (13)	95 (7)	**26**	0.1X	0.9959	113 (14)	118 (5)
Bacitracin	500	17	0.1X	0.9946	**132 (17)**	89 (8)	17	0.25X	0.9982	**103 (28)**	106 (12)	**36**	0.25X	0.9499	**95 (41)**	**80 (26)**
Betamethasone	100	-2	0.1X	0.9946	108 (9)	98 (5)	-10	1X	0.9554	86 (0)	96 (22)	-3	1X	0.9923	119 (23)	116 (11)
Cambendazole	10	-2	0.1X	0.9988	98 (3)	94 (1)	-5	0.1X	0.9969	99 (13)	95 (9)	5	0.1X	0.9961	112 (12)	116 (3)
Carazolol	10	0	0.1X	0.9989	99 (6)	93 (3)	5	0.1X	0.9955	98 (7)	98 (7)	20	0.1X	0.9986	108 (9)	108 (9)
Carbadox	30	-7	0.1X	0.9988	100 (7)	90 (5)	13	1X	0.9996	105 (18)	113 (12)	**40**	1X	0.9982	93 (20)	92 (13)
Cefazolin	100	-5	0.1X	0.9991	99 (5)	93 (4)	**-36**	0.1X	0.9997	99 (15)	99 (6)	**-48**	0.1X	0.9725	98 (23)	89 (15)
Cephapirin	100	0	0.1X	0.9992	115 (7)	75 (6)	-1	1X	0.9883	95 (19)	**146 (11)**	-10	1X	0.9421	92 (17)	106 (17)

Table 3.3 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Chlortetracycline	200	-4	0.1X	0.9986	99 (7)	92 (4)	25	1X	0.9942	101 (17)	146 (21)	75	1X	0.9965	**100 (32)**	82 (18)
Chlorpromazine	10	-3	0.1X	0.9988	91 (4)	92 (3)	-7	0.1X	0.9993	103 (7)	97 (4)	-10	0.1X	0.9968	111 (5)	106 (4)
Ciprofloxacin	50	-6	0.1X	0.9989	100 (9)	94 (3)	-4	0.5X	0.9887	101 (14)	119 (14)	23	0.5X	0.9970	86 (14)	89 (12)
Clenbuterol	10	-5	0.1X	0.9991	96 (4)	94 (3)	-9	0.1X	0.9998	101 (6)	99 (3)	16	0.1X	0.9980	101 (3)	102 (3)
Clindamycin	100	-2	0.1X	0.9968	106 (7)	89 (3)	32	0.1X	0.9917	115 (20)	117 (14)	64	0.1X	0.9872	94 (19)	92 (10)
Cloxacillin	10	-5	0.1X	0.9993	102 (5)	95 (4)	-9	0.1X	0.9934	110 (14)	119 (9)	-15	0.1X	0.9972	96 (10)	96 (7)
Danofloxacin	70	-4	0.1X	0.9995	93 (8)	89 (3)	12	0.1X	0.9982	103 (6)	99 (6)	45	0.1X	0.9919	97 (23)	87 (10)
Desacetyl cephapirin	100	-12	0.1X	0.9995	93 (12)	91 (0)	-9	0.1X	0.9891	**100 (38)**	109 (23)	-13	0.1X	0.9878	81 (41)	78 (28)
Desethylene Ciprofloxacin	100	-3	0.1X	0.9985	100 (6)	89 (4)	28	0.1X	0.9996	106 (13)	108 (8)	68	0.1X	0.9914	91 (16)	91 (10)
Diclofenac	200	2	0.1X	0.9979	102 (6)	94 (4)	-9	0.1X	0.9997	101 (5)	97 (4)	-24	0.1X	0.9994	101 (3)	100 (2)
Dicloxacillin	100	-3	0.1X	0.9833	102 (28)	**128 (31)**	11	0.25X	0.9959	95 (26)	85 (19)	5	0.25X	0.9919	106 (14)	107 (9)
Difloxacin	50	-7	0.1X	0.9974	100 (8)	95 (2)	10	0.1X	0.9998	96 (21)	106 (4)	17	0.1X	1.0000	100 (5)	106 (4)
Dimetridazole	50	-4	0.1X	0.9995	103 (0)	94 (0)	1	0.1X	0.9933	82 (8)	110 (11)	6	0.1X	0.9980	94 (9)	98 (8)
Doxycycline	100	-5	0.1X	0.9983	101 (11)	91 (6)	193	0.1X	0.9986	126 (26)	137 (11)	223	0.1X	0.9886	90 (18)	80 (6)
Doxycycline	100	-5	0.1X	0.9983	101 (11)	91 (6)	193	0.1X	0.9986	126 (26)	137 (11)	223	0.1X	0.9886	90 (18)	80 (6)
Emamectin	10	6	0.25X	0.9944	100 (11)	94 (6)	-12	0.25X	0.9989	101 (12)	97 (7)	-16	0.25X	0.9958	103 (10)	102 (2)
Enrofloxacin	20	-5	0.1X	0.9994	99 (8)	95 (3)	-6	0.1X	0.9921	103 (18)	106 (13)	14	0.1X	0.9976	94 (19)	99 (17)
Erythromycin	100	1	0.1X	0.9976	103 (6)	89 (2)	-8	0.1X	0.9993	104 (8)	106 (7)	8	0.1X	0.9941	92 (7)	94 (5)
Fenbendazole	100	-3	0.1X	0.9988	99 (5)	88 (2)	6	0.1X	0.9996	99 (16)	94 (9)	4	0.1X	0.9947	113 (12)	112 (2)
Fenbendazole Sulfone	400	0	0.1X	0.9977	100 (3)	96 (1)	1	0.1X	0.9998	100 (10)	98 (6)	1	0.1X	0.9983	110 (7)	112 (2)
Florfenicol	100	-1	0.5X	0.9819	94 (6)	83 (6)	-38	0.1X	0.9861	110 (18)	111 (6)	-43	0.1X	0.9958	105 (6)	101 (8)

Table 3.3 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Florfenicol Amine	100	-26	0.1X	0.9993	96 (3)	97 (3)	-86	*	*	*	*	-85	*	*	*	*
Flubendazole	10	-3	0.1X	0.9969	99 (4)	94 (2)	1	0.1X	0.9987	97 (14)	95 (8)	-1	0.1X	0.9949	108 (10)	113 (2)
Flunixin	20	-1	0.1X	0.9999	98 (1)	100 (1)	-7	0.1X	0.9999	102 (1)	101 (1)	-13	0.1X	0.9999	102 (2)	100 (1)
Gamithromycin	20	0	0.1X	0.9967	104 (9)	91 (6)	**58**	0.1X	0.9949	115 (24)	117 (15)	**129**	0.1X	0.9843	87 (24)	86 (14)
Haloperidol	10	-3	0.1X	0.9962	98 (4)	93 (2)	-6	0.1X	0.9991	99 (12)	97 (7)	-1	0.1X	0.9935	110 (10)	113 (2)
Hydroxy dimetridazole	50	-1	0.25X	0.9996	99 (3)	97 (5)	-14	0.25X	0.9911	114 (18)	104 (11)	-17	0.25X	0.9962	94 (18)	100 (8)
Hydroxy ipronidazole	10	-6	0.1X	0.9996	96 (4)	92 (5)	1	0.1X	0.9962	104 (12)	99 (3)	0	0.1X	0.9977	106 (5)	111 (3)
Hydroxy metronidazole	10	1	0.25X	0.9998	98 (0)	96 (0)	-14	0.25X	0.9984	112 (5)	109 (5)	-4	0.25X	0.9977	112 (11)	98 (8)
Ipronidazole	10	-4	0.1X	0.9993	98 (6)	94 (4)	-2	0.1X	0.9997	99 (7)	98 (5)	1	0.1X	0.9967	108 (5)	112 (2)
Ketoprofen	10	-2	0.1X	0.9999	101 (3)	94 (4)	-2	0.1X	0.9991	105 (5)	106 (2)	-6	0.1X	0.9962	101 (5)	106 (5)
Levamisole	100	0	0.1X	0.9999	95 (6)	92 (4)	9	0.1X	0.9950	96 (22)	100 (13)	**46**	0.1X	0.9976	96 (17)	97 (13)
Lincomycin	100	-2	0.1X	0.9994	97 (6)	91 (5)	**27**	1X	0.9958	108 (29)	116 (15)	**33**	1X	0.9806	86 (35)	86 (19)
Melengestrol Acetate	25	0	0.1X	0.9991	93 (5)	84 (3)	**-43**	1X	0.9972	98 (13)	91 (11)	**-60**	1X	0.9927	99 (17)	92 (5)
Meloxicam	20	-2	0.1X	0.9994	97 (4)	93 (1)	12	0.1X	0.9997	98 (7)	99 (4)	-20	0.1X	0.9987	100 (6)	98 (1)
Metamizole	200	-4	0.1X	0.9934	82 (8)	103 (7)	**-29**	1X	0.9686	106 (23)	121 (12)	**-22**	1X	0.9278	101 (15)	117 (16)
Metronidazole	10	-1	0.1X	0.9999	96 (4)	95 (7)	**-26**	0.5X	0.9957	88 (7)	79 (22)	-13	0.5X	0.9933	96 (15)	110 (15)
Morantel	100	2	0.1X	0.9993	96 (2)	95 (4)	-11	0.1X	0.9883	75 (43)	83 (44)	7	0.1X	0.9828	86 (22)	97 (28)
Nafcillin	100	-3	0.1X	0.9999	102 (4)	95 (3)	1	0.1X	0.9983	107 (11)	110 (9)	-9	0.1X	0.9996	97 (10)	94 (7)
Norfloxacin	50	-6	0.1X	0.9993	100 (7)	92 (4)	-3	0.5X	0.9676	116 (14)	125 (11)	8	0.5X	0.9886	94 (16)	95 (8)
Novobiocin	1000	4	0.1X	0.9946	92 (4)	80 (2)	**-23**	0.25X	0.9934	105 (26)	94 (16)	-41	0.25X	0.9949	96 (3)	86 (3)
Orbifloxacin	50	-3	0.1X	0.9986	101 (8)	95 (3)	-15	0.1X	0.9969	111 (18)	116 (10)	11	0.1X	0.9965	100 (10)	99 (8)

Table 3.3 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Oxacillin	100	-4	0.1X	0.9995	103 (4)	91 (3)	-4	0.1X	0.9955	107 (15)	113 (11)	-10	0.1X	0.9953	94 (15)	97 (9)
Oxfendazole	800	-2	0.1X	0.9984	102 (4)	95 (3)	-1	0.1X	0.9998	105 (3)	99 (2)	2	0.1X	0.9967	108 (3)	104 (2)
Oxyclozanide	10	-8	0.1X	0.9945	100 (19)	102 (17)	-16	0.5X	0.9932	85 (26)	87 (17)	4	0.5X	0.9803	105 (15)	95 (9)
Oxyphenylbutazone	100	1	0.1X	0.9995	100 (6)	98 (3)	-10	0.25X	0.9982	96 (13)	94 (9)	**-32**	0.25X	0.9899	102 (16)	94 (4)
Oxytetracycline	200	-6	0.1X	0.9993	99 (9)	91 (3)	**258**	1X	0.9764	**105 (26)**	117 (24)	**279**	1X	0.9499	**72 (47)**	**64 (25)**
Penicillin G	50	1	0.1X	0.9941	**103 (28)**	87 (14)	25	1X	0.9879	**96 (27)**	71 (24)	15	1X	0.9584	105 (21)	82 (11)
Phenylbutazone	100	0	0.1X	0.9990	96 (7)	96 (3)	**-63**	0.25X	0.9952	113 (11)	110 (4)	**-74**	0.25X	0.9929	101 (7)	92 (1)
Pirlimycin	300	-3	0.1X	0.9961	106 (9)	90 (3)	**37**	0.1X	0.9993	102 (19)	99 (9)	**51**	0.1X	0.9809	90 (25)	88 (14)
Prednisone	100	-2	0.1X	0.9997	104 (8)	96 (4)	-9	0.1X	0.9995	100 (3)	98 (1)	-9	0.1X	0.9995	107 (3)	107 (2)
Promethazine	10	2	0.1X	0.9999	96 (3)	93 (4)	-4	0.1X	0.9992	101 (5)	99 (2)	-3	0.1X	0.9994	108 (4)	108 (3)
Propionylpromazine	10	0	0.1X	0.9999	93 (3)	93 (2)	-9	0.1X	0.9995	102 (5)	96 (3)	-4	0.1X	0.9981	107 (2)	106 (3)
Ractopamine	30	-2	0.1X	0.9990	97 (6)	92 (4)	5	0.1X	0.9961	97 (18)	92 (12)	15	0.1X	0.9953	98 (18)	102 (15)
Ronidazole	10	-3	0.1X	0.9998	99 (5)	93 (6)	-16	0.1X	0.9987	111 (21)	113 (10)	-10	0.1X	0.9926	69 (16)	108 (11)
Salbutamol	10	-13	0.1X	0.9984	98 (12)	94 (5)	-15	0.25X	0.9975	**107 (26)**	114 (15)	0	0.25X	0.9951	**93 (30)**	89 (20)
Oxyphenylbutazone	100	1	0.1X	0.9995	100 (6)	98 (3)	-10	0.25X	0.9982	96 (13)	94 (9)	**-32**	0.25X	0.9899	102 (16)	94 (4)
Sarafloxacin	50	-6	0.1X	0.9961	102 (7)	93 (4)	**-24**	0.1X	0.9919	98 (21)	97 (14)	6	0.1X	0.9984	114 (13)	115 (4)
Sulfadiazin	100	-3	0.1X	0.9996	97 (6)	95 (2)	**-26**	0.1X	0.9968	98 (10)	97 (8)	**-24**	0.1X	0.9985	114 (13)	115 (4)
Sulfadimethoxine	100	0	0.1X	0.9974	100 (4)	95 (2)	0	0.1X	0.9997	101 (11)	97 (6)	0	0.1X	0.9971	113 (9)	112 (2)
Sulfaethoxypyridazine	100	-1	0.1X	0.9977	101 (5)	97 (3)	3	0.1X	0.9988	101 (9)	96 (5)	-1	0.1X	0.9986	113 (10)	114 (2)
Sulfamerazine	100	0	0.1X	0.9985	101 (6)	96 (3)	**-22**	0.1X	1.0000	102 (6)	98 (3)	**-23**	0.1X	0.9995	113 (10)	114 (2)
Sulfamethazine	100	-5	0.1X	0.9960	103 (6)	96 (2)	18	0.1X	0.9996	105 (9)	100 (5)	-19	0.1X	0.9961	115 (8)	115 (2)

Table 3.3 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Sulfamethizole	100	-4	0.1X	0.9997	100 (5)	96 (3)	**-25**	0.1X	0.9988	113 (9)	105 (5)	**-27**	0.1X	0.9999	81 (24)	107 (2)
Sulfamethoxazole	100	-3	0.1X	0.9967	108 (6)	100 (3)	1	0.1X	0.9995	105 (8)	95 (8)	3	0.1X	0.9963	117 (14)	114 (5)
Sulfamethoxypyridazine	100	-1	0.1X	0.9960	102 (5)	100 (3)	-19	0.1X	0.9999	104 (12)	98 (5)	**-21**	0.1X	0.9987	114 (13)	116 (3)
Sulfapyridine	100	4	0.1X	0.9995	99 (6)	97 (4)	**-27**	0.1X	0.9994	101 (9)	97 (5)	-19	0.1X	0.9988	113 (12)	114 (4)
Sulfaquinoxaline	100	-1	0.1X	0.9970	100 (4)	94 (2)	-2	0.1X	0.9999	101 (8)	97 (5)	-4	0.1X	0.9986	111 (8)	111 (2)
Sulfathiazole	100	2	0.1X	0.9995	100 (5)	97 (4)	-39	0.1X	0.9957	93 (12)	87 (8)	-37	0.1X	0.9956	113 (15)	114 (5)
Tetracycline	200	-4	0.1X	0.9979	100 (9)	92 (4)	**199**	0.1X	0.9850	**144 (36)**	**140 (21)**	**226**	0.1X	0.9917	99 (25)	102 (19)
Thiabendazole	100	-9	0.1X	0.9997	96 (4)	96 (3)	**45**	0.1X	0.9990	97 (16)	90 (12)	**105**	0.1X	0.9957	116 (17)	119 (5)
Tildipirosin	400	**55**	0.1X	0.9956	94 (19)	90 (5)	31	0.25X	0.9926	**110 (34)**	116 (16)	**60**	0.25X	0.9826	92 (37)	87 (20)
Tilmicosin	100	-1	0.1X	0.9942	108 (12)	83 (6)	-8	0.1X	0.9978	95 (18)	94 (10)	**50**	0.1X	0.9915	98 (4)	96 (2)
Tolfenamic acid	200	-3	0.1X	0.9957	95 (5)	86 (4)	-17	0.1X	0.9997	103 (9)	100 (5)	**-42**	0.1X	0.9973	103 (9)	102 (3)
Triclabendazole	50	6	0.1X	0.9995	94 (3)	83 (3)	**-24**	0.1X	0.9920	106 (17)	101 (12)	**-23**	0.1X	0.9797	106 (15)	105 (4)
Triclabendazole Sulfoxide	50	2	0.1X	0.9988	98 (7)	90 (3)	-14	0.1X	0.9948	103 (14)	99 (11)	**-25**	0.1X	0.9943	103 (13)	105 (4)
Trifluropromazine	10	-2	0.1X	0.9977	96 (4)	91 (3)	2	0.1X	0.9939	104 (11)	102 (7)	-2	0.1X	0.9912	105 (7)	100 (4)
Tulathromycin	1000	11	0.1X	0.9956	99 (19)	90 (5)	**35**	0.25X	0.9843	115 (19)	114 (12)	**83**	0.25X	0.9804	92 (23)	88 (10)
Tylosin	200	2	0.1X	0.9983	104 (5)	94 (3)	-14	0.1X	0.9994	109 (4)	114 (6)	6	0.1X	0.9961	99 (6)	100 (5)
Virginiamycin	100	-2	0.1X	0.9993	101 (6)	95 (3)	19	0.1X	0.9998	98 (12)	100 (5)	20	0.1X	0.9981	104 (9)	106 (1)
Xylazine	10	-3	0.1X	0.9995	95 (8)	93 (3)	-2	0.1X	0.9991	102 (6)	97 (2)	**27**	0.1X	0.9998	109 (6)	109 (3)

Table 3.4 Limits of quantification (LOQ), Linearity (n=3 at each level), and average accuracy (ACC), n = 6 at each level, in chicken tissue. * designates compounds which could not be quantified

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
2-Aminoflubendazole	10	-3	0.1X	0.9998	97 (5)	95 (3)	-2	0.1X	0.9994	109 (13)	93 (2)	**27**	0.1X	0.9992	103 (7)	96 (2)
2-Quinoxalinecarboxylic acid	100	-4	0.1X	0.9989	96 (7)	95 (7)	**70**	0.1X	0.9986	103 (15)	84 (3)	**112**	0.25X	0.9984	105 (16)	100 (4)
2-Amino mebendazole	10	-1	0.1X	0.9997	95 (6)	95 (3)	20	0.1X	0.9992	112 (7)	95 (5)	**40**	0.1X	0.9953	103 (8)	96 (4)
5-Hydroxythiabendazole	100	-3	0.1X	0.9990	98 (7)	96 (5)	6	0.1X	0.9949	120 (7)	104 (8)	**35**	0.1X	0.9882	90 (11)	101 (9)
6-phenyl-thiouracil	400	3	0.1X	0.9995	91 (7)	88 (6)	-3	0.1X	0.9994	103 (8)	93 (3)	-3	0.1X	0.9997	109 (8)	103 (3)
6-propyl-2-thiouracil	50	1	0.25X	0.9997	94 (9)	95 (22)	-2	0.25X	0.9960	101 (12)	96 (6)	7	0.25X	0.9974	114 (11)	105 (4)
Acepromazine	10	-1	0.1X	0.9986	95 (4)	96 (3)	-2	0.1X	0.9969	107 (8)	105 (2)	3	0.1X	0.9999	100 (6)	98 (4)
Albendazole	50	0	0.1X	0.9976	98 (5)	95 (5)	0	0.1X	0.9992	98 (11)	90 (5)	1	0.1X	0.9917	102 (23)	102 (10)
Albendazole 2-aminosulfone	50	-1	0.1X	0.9997	97 (5)	95 (3)	7	0.1X	0.9985	119 (13)	104 (4)	19	0.1X	0.9995	109 (3)	102 (6)
Albendazole sulfone	50	2	0.1X	0.9978	99 (4)	95 (4)	2	0.1X	0.9999	103 (7)	99 (3)	3	0.1X	0.9993	103 (12)	98 (3)
Albendazole sulfoxide	50	-1	0.1X	0.9983	100 (5)	94 (3)	6	0.1X	0.9975	110 (4)	101 (5)	10	0.1X	0.9989	108 (4)	99 (5)
Amoxicillin	10	4	1X	0.9938	**120 (33)**	97 (19)	**-47**	1X	0.9935	98 (1)	98 (9)	**-30**	0.1X	0.9981	88 (23)	87 (12)
Ampicillin	10	-4	0.25X	0.9995	94 (11)	91 (15)	**-38**	0.25X	0.9973	105 (19)	99 (8)	-19	0.25X	0.9991	104 (22)	94 (16)
Azaperone	10	-2	0.1X	1.0000	97 (6)	95 (4)	17	0.1X	0.9992	103 (10)	91 (3)	19	0.1X	0.9980	103 (18)	96 (6)
Bacitracin	500	5	0.1X	0.9963	116 (10)	104 (5)	17	0.1X	0.9809	**166 (22)**	**175 (6)**	8	0.25X	0.9469	**214 (24)**	**170 (21)**
Betamethasone	100	4	0.1X	0.9973	100 (1)	99 (7)	-2	0.1X	0.9959	88 (9)	84 (12)	-4	0.1X	0.9889	90 (1)	93 (1)
Cambendazole	10	-2	0.1X	0.9993	96 (5)	96 (3)	-4	0.1X	0.9994	100 (11)	87 (1)	-6	0.1X	0.9975	101 (18)	98 (6)
Carazolol	10	-1	0.1X	0.9998	97 (5)	94 (3)	7	0.1X	0.9989	102 (5)	93 (2)	9	0.1X	0.9988	104 (5)	97 (2)
Carbadox	30	3	0.1X	0.9966	101 (4)	94 (6)	28	0.1X	0.9977	97 (14)	85 (16)	**27**	0.1X	0.9116	**132 (28)**	109 (18)

Table 3.4 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Cefazolin	100	-3	0.1X	0.9979	93 (9)	95 (5)	-27	0.1X	0.9978	101 (12)	90 (9)	**-53**	0.25X	0.9951	104 (16)	96 (12)
Cephapirin	100	*	*	*	*	*	8	0.1X	0.9942	76 (23)	**60 (6)**	-10	0.25X	0.9802	**76 (32)**	71 (19)
Chlortetracycline	200	0	0.1X	0.9987	93 (3)	92 (2)	**31**	0.1X	0.9943	104 (11)	97 (11)	**42**	0.25X	0.9996	94 (19)	94 (8)
Chlorpromazine	10	-3	0.1X	0.9978	98 (6)	98 (3)	-5	0.1X	0.9960	103 (9)	97 (5)	-5	0.25X	0.9979	102 (14)	100 (4)
Ciprofloxacin	50	0	0.1X	0.9993	97 (5)	97 (4)	0	0.1X	0.9874	113 (8)	104 (12)	12	0.1X	0.9825	117 (15)	102 (13)
Clenbuterol	10	-2	0.1X	0.9995	99 (7)	97 (6)	2	0.1X	0.9983	108 (7)	92 (8)	18	0.1X	0.9986	107 (3)	97 (4)
Clindamycin	100	0	0.25X	0.9978	100 (4)	98 (3)	30	0.25X	0.9935	112 (7)	99 (9)	**55**	0.25X	0.9923	111 (10)	97 (11)
Cloxacillin	100	0	0.1X	0.9998	97 (4)	94 (4)	-2	0.1X	0.9977	104 (8)	105 (5)	-8	0.1X	0.9966	106 (11)	97 (10)
Danofloxacin	70	6	0.1X	0.9976	90 (3)	89 (2)	15	0.1X	0.9958	111 (4)	95 (14)	**41**	0.1X	0.9958	111 (5)	101 (7)
Desacetyl cephapirin	100	-3	0.1X	0.9994	101 (1)	102 (18)	-13	0.1X	0.9847	107 (10)	107 (14)	2	0.25X	0.9894	113 (23)	94 (23)
Desethylene Ciprofloxacin	100	4	0.1X	0.9965	96 (3)	94 (3)	**47**	0.1X	0.9784	**130 (20**)	110 (19)	**90**	0.25X	0.9886	**134 (12)**	116 (11)
Diclofenac	200	-2	0.1X	0.9995	99 (4)	96 (4)	-7	0.1X	0.9994	95 (13)	103 (3)	-17	0.1X	0.9990	103 (5)	97 (3)
Dicloxacillin	100	-2	0.1X	0.9957	95 (1)	99 (6)	5	0.1X	0.9991	95 (10)	103 (2)	8	0.1X	0.9994	102 (11)	95 (7)
Difloxacin	50	-3	0.1X	0.9995	98 (5)	97 (2)	**24**	0.1X	0.9962	105 (2)	96 (4)	44	0.1X	0.9965	107 (2)	97 (6)
Dimetridazole	50	-1	0.1X	0.9993	97 (18)	92 (14)	2	0.1X	0.9991	100 (11)	103 (8)	-5	0.1X	0.9966	114 (5)	103 (3)
Doxycycline	100	-2	0.1X	0.9960	95 (4)	89 (7)	**185**	0.1X	0.9893	110 (12)	105 (12)	**231**	0.25X	0.9991	95 (21)	96 (9)
Emamectin	10	-3	0.25X	0.9877	99 (6)	91 (5)	**-28**	0.25X	0.9995	98 (10)	99 (5)	**-24**	0.1X	0.9908	90 (10)	96 (9)
Enrofloxacin	20	-1	0.1X	0.9991	97 (3)	96 (3)	3	0.1X	0.9978	107 (4)	100 (6)	20	0.1X	0.9968	108 (6)	96 (7)
Erythromycin	125	-2	0.1X	0.9990	108 (10)	100 (4)	-5	0.1X	0.9947	109 (7)	97 (7)	-1	0.1X	0.9976	106 (8)	101 (8)
Fenbendazole	100	1	0.1X	0.9979	100 (4)	98 (4)	5	0.1X	0.9988	95 (13)	87 (6)	1	0.1X	0.9856	**102 (31)**	103 (12)
Fenbendazole Sulfone	400	-1	0.1X	0.9984	98 (6)	97 (3)	1	0.1X	0.9996	98 (9)	93 (4)	-1	0.1X	0.9965	103 (16)	99 (6)

Table 3.4 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Florfenicol	300	0	0.1X	0.9976	106 (8)	105 (5)	-13	0.1X	0.9978	89 (11)	87 (3)	-18	0.1X	0.9911	106 (13)	88 (14)
Florfenicol Amine	100	-31	0.1X	0.9976	103 (8)	92 (10)	**-88**	0.1X	0.9953	108 (6)	105 (6)	**-88**	0.25X	0.9884	113 (11)	106 (6)
Flubendazole	10	0	0.1X	0.9994	96 (6)	96 (3)	4	0.1X	0.9992	91 (10)	88 (7)	2	0.1X	0.9943	96 (23)	96 (8)
Flunixin	20	1	0.1X	0.9992	98 (1)	98 (1)	-2	0.1X	0.9998	97 (1)	93 (1)	-11	0.1X	0.9998	97 (1)	94 (1)
Gamithromycin	20	3	0.1X	0.9987	98 (4)	95 (7)	**55**	0.1X	0.9869	111 (12)	114 (7)	**107**	0.1X	0.9868	106 (17)	92 (14)
Haloperidol	10	1	0.1X	0.9996	97 (4)	96 (2)	2	0.1X	0.9984	95 (10)	90 (5)	3	0.1X	0.9939	100 (20)	101 (9)
Hydroxy dimetridazole	50	-1	0.25X	0.9993	100 (6)	96 (4)	-14	0.25X	0.9992	113 (7)	100 (5)	**-26**	0.1X	0.9979	88 (21)	89 (5)
Hydroxy ipronidazole	10	-1	0.1X	0.9997	97 (6)	92 (4)	1	0.1X	0.9979	108 (6)	94 (4)	6	0.1X	0.9997	109 (3)	99 (3)
Hydroxy metronidazole	10	7	0.25X	0.9967	106 (20)	106 (20)	-16	0.25X	0.9988	110 (1)	91 (7)	-17	0.25X	0.9994	75 (15)	93 (11)
Ipronidazole	10	-1	0.1X	0.9992	96 (5)	95 (5)	0	0.1X	0.9997	108 (10)	94 (2)	-3	0.1X	0.9961	99 (14)	95 (5)
Ketoprofen	10	1	0.1X	0.9997	98 (7)	98 (4)	-2	0.1X	0.9950	96 (16)	104 (5)	-2	0.25X	0.9937	107 (9)	97 (9)
Levamisole	100	-3	0.1X	0.9994	100 (8)	96 (4)	4	0.1X	0.9990	110 (8)	96 (5)	**36**	0.1X	0.9984	107 (3)	98 (3)
Lincomycin	100	1	0.1X	0.9992	97 (5)	97 (3)	25	0.1X	0.9944	99 (14)	99 (7)	**38**	0.25X	0.9984	106 (15)	96 (15)
Mebendazole	10	1	0.1X	0.9996	97 (4)	97 (4)	7	0.1X	0.9997	95 (8)	93 (8)	1	0.1X	0.9968	100 (17)	96 (5)
Melengestrol Acetate	25	-9	0.1X	0.9995	114 (3)	97 (5)	**-34**	0.1X	0.9984	91 (15)	83 (6)	**-58**	0.25X	0.9823	85 (21)	95 (14)
Meloxicam	20	0	0.1X	0.9990	95 (4)	96 (4)	13	0.1X	0.9992	98 (5)	96 (2)	-7	0.1X	0.9989	93 (3)	92 (3)
Metamizole	200	-2	0.1X	0.9999	98 (8)	95 (8)	**-30**	0.1X	0.9936	119 (12)	101 (11)	-23	0.1X	0.9997	108 (14)	88 (11)
Metronidazole	10	-5	0.1X	0.9989	101 (7)	92 (11)	**-21**	0.1X	0.9966	107 (8)	101 (6)	-9	0.25X	0.9997	108 (7)	101 (5)
Morantel	150	-4	0.1X	0.9993	97 (3)	96 (3)	-7	0.1X	0.9999	101 (6)	86 (4)	-1	0.1X	0.9989	96 (7)	90 (4)
Nafcillin	100	0	0.1X	0.9997	103 (4)	102 (3)	3	0.1X	0.9991	104 (8)	106 (4)	-2	0.1X	0.9965	104 (11)	96 (10)
Norfloxacin	50	6	0.1X	0.9990	97 (6)	96 (5)	-7	0.1X	0.9847	128 (7)	111 (15)	17	0.25X	0.9900	105 (23)	110 (8)

Table 3.4 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Novobiocin	1000	-1	0.25X	0.9952	149 (17)	99 (4)	-20	0.25X	0.9976	114 (13)	127 (5)	**-37**	0.25X	0.9941	86 (21)	93 (11)
Orbifloxacin	50	-3	0.1X	0.9994	97 (4)	96 (2)	-7	0.1X	0.9962	107 (4)	94 (8)	17	0.1X	0.9946	109 (4)	95 (9)
Oxacillin	100	-1	0.1X	0.9996	97 (4)	94 (4)	-2	0.1X	0.9953	107 (8)	106 (7)	-4	0.25X	0.9979	93 (21)	97 (10)
Oxfendazole	800	-1	0.1X	0.9961	102 (7)	99 (4)	1	0.1X	0.9996	110 (5)	100 (2)	-2	0.1X	0.9940	94 (20)	96 (12)
Oxyclozanide	10	-2	0.5X	0.9982	100 (10)	**110 (35)**	-5	0.5X	0.9960	95 (11)	87 (5)	-3	0.25X	0.9834	**87 (28)**	99 (10)
Oxyphenylbutazone	100	-2	0.1X	0.9992	105 (5)	106 (3)	1	0.1X	0.9972	91 (11)	91 (2)	-16	0.1X	0.9991	95 (4)	93 (2)
Oxytetracycline	200	1	0.1X	0.9987	93 (3)	93 (4)	**244**	0.1X	0.9971	105 (15)	108 (3)	**261**	0.1X	0.9957	106 (22)	105 (9)
Penicillin G	50	-1	0.5X	0.9945	98 (21)	105 (17)	8	0.5X	0.9913	92 (20)	104 (3)	14	0.1X	0.9946	98 (16)	87 (12)
Phenylbutazone	100	-3	0.1X	0.9999	102 (3)	101 (4)	**-49**	0.1X	0.9983	98 (11)	104 (4)	-62	0.1X	0.9981	90 (3)	92 (4)
Pirlimycin	300	-1	0.1X	0.9984	96 (3)	95 (3)	**41**	0.1X	0.9924	110 (9)	102 (9)	57	0.25X	0.9990	97 (21)	96 (10)
Prednisone	100	0	0.1X	0.9987	96 (6)	96 (3)	-5	0.1X	0.9998	99 (10)	91 (3)	-9	0.1X	0.9997	103 (7)	98 (3)
Promethazine	10	-2	0.1X	0.9990	95 (6)	96 (3)	-5	0.1X	0.9973	109 (9)	101 (2)	-5	0.25X	0.9995	102 (6)	99 (5)
Propionylpromazine	10	-1	0.1X	0.9990	97 (5)	97 (2)	-6	0.1X	0.9970	103 (8)	98 (5)	-2	0.25X	0.9990	104 (11)	99 (4)
Ractopamine	30	-1	0.1X	0.9994	97 (7)	97 (4)	11	0.1X	0.9997	108 (6)	92 (6)	13	0.1X	0.9987	103 (14)	98 (3)
Ronidazole	10	0	0.1X	0.9997	98 (10)	97 (10)	-15	0.1X	0.9997	101 (24)	94 (8)	-12	0.1X	0.9956	84 (19)	100 (12)
Salbutamol	10	3	0.25X	0.9993	95 (4)	96 (4)	15	0.25X	0.9836	105 (12)	97 (13)	17	0.25X	0.9992	94 (26)	94 (18)
Sarafloxacin	50	-2	0.1X	0.9970	97 (11)	95 (15)	-16	0.1X	0.9968	106 (6)	99 (7)	**23**	0.1X	0.9916	112 (8)	99 (10)
Sulfadiazin	100	1	0.1X	0.9958	98 (14)	99 (19)	**-26**	0.1X	0.9988	103 (13)	89 (2)	**-30**	0.1X	0.9994	105 (15)	96 (3)
Sulfadimethoxine	100	-2	0.1X	0.9980	104 (16)	101 (20)	7	0.1X	0.9998	102 (9)	94 (2)	0	0.1X	0.9951	106 (18)	104 (7)
Sulfadoxine	100	-3	0.1X	0.9978	105 (15)	101 (19)	-2	0.1X	0.9999	106 (8)	95 (1)	-9	0.1X	0.9991	103 (19)	102 (7)
Sulfaethoxypyridazine	100	-1	0.1X	0.9984	103 (15)	100 (18)	3	0.1X	0.9995	107 (9)	97 (1)	-8	0.1X	0.9976	103 (18)	102 (6)

Table 3.4 continued

Compound	MRL	SPME					QuEChERS					SE				
		%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X	%	LOQ	R^2	0.75X	1.5X
	(ng g-1)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)	ME			ACC (RSD)	ACC (RSD)
Sulfamerazine	100	0	0.1X	0.9976	101 (15)	99 (19)	-16	0.1X	0.9971	95 (10)	88 (5)	**-22**	0.1X	0.9989	108 (16)	98 (5)
Sulfamethazine	100	-1	0.1X	0.9978	106 (14)	102 (18)	-10	0.1X	0.9999	110 (8)	97 (3)	-17	0.1X	0.9998	109 (13)	100 (4)
Sulfamethizole	100	-1	0.1X	0.9969	100 (13)	99 (16)	-13	0.1X	0.9982	90 (13)	90 (5)	**-24**	0.1X	0.9963	109 (3)	99 (6)
Sulfamethoxazole	100	1	0.1X	0.9971	105 (15)	104 (18)	4	0.1X	0.9998	101 (7)	95 (5)	-2	0.1X	0.9923	103 (21)	102 (6)
Sulfamethoxypyridazine	100	-1	0.1X	0.9981	104 (14)	101 (18)	-4	0.1X	0.9999	107 (10)	92 (2)	**-22**	0.1X	0.9996	108 (16)	101 (4)
Sulfapyridine	100	0	0.1X	0.9966	100 (15)	98 (19)	-13	0.1X	0.9995	105 (11)	91 (3)	-15	0.1X	0.9998	107 (12)	99 (4)
Sulfaquinoxaline	100	0	0.1X	0.9984	102 (16)	99 (20)	5	0.1X	0.9997	98 (6)	95 (3)	-3	0.1X	0.9986	103 (14)	101 (4)
Sulfathiazole	100	-4	0.1X	0.9986	101 (8)	100 (6)	**-35**	0.1X	0.9992	104 (14)	87 (2)	**-40**	0.1X	0.9991	105 (15)	97 (4)
Tetracycline	200	-2	0.1X	0.9982	93 (4)	96 (4)	**198**	0.1X	0.9994	100 (16)	104 (3)	**231**	0.1X	0.9990	97 (20)	93 (9)
Thiabendazole	100	-3	0.1X	0.9984	100 (8)	98 (7)	**58**	0.1X	0.9995	105 (15)	85 (2)	**95**	0.1X	0.9987	106 (17)	98 (5)
Tildipirosin	400	-8	0.1X	0.9945	71 (14)	81 (18)	**44**	0.1X	0.9887	107 (15)	101 (13)	**48**	0.25X	0.9934	82 (27)	80 (14)
Tilmicosin	100	-1	0.1X	0.9983	96 (4)	96 (6)	-2	0.1X	0.9997	95 (9)	86 (3)	**48**	0.1X	0.9918	111 (13)	92 (14)
Tolfenamic acid	200	4	0.1X	0.9979	117 (1)	96 (0)	-20	0.1X	0.9992	98 (14)	108 (3)	**-45**	0.1X	0.9970	94 (10)	91 (5)
Triclabendazole	50	2	0.1X	0.9968	101 (8)	99 (4)	**-23**	0.1X	0.9968	88 (18)	82 (6)	**-26**	0.25X	0.9596	71 (33)	85 (31)
Triclabendazole Sulfoxide	50	-1	0.1X	0.9988	105 (6)	97 (4)	-13	0.1X	0.9980	92 (13)	84 (5)	**-22**	0.25X	0.9896	91 (17)	91 (9)
Trifluropromazine	10	-2	0.1X	0.9979	99 (6)	103 (3)	1	0.1X	0.9970	99 (10)	93 (7)	1	0.25X	0.9942	101 (22)	99 (9)
Tulathromycin	150	-4	0.1X	0.9845	84 (9)	97 (17)	**49**	0.1X	0.9889	**129 (13)**	115 (11)	**79**	0.25X	0.9937	99 (21)	81 (17)
Tylosin	200	0	0.1X	0.9992	123 (12)	110 (9)	**-46**	0.1X	0.9711	109 (17)	86 (10)	**-53**	0.25X	0.9996	100 (9)	97 (6)
Virginiamycin	100	-1	0.1X	0.9999	103 (5)	103 (4)	10	0.1X	0.9987	99 (8)	93 (3)	10	0.1X	0.9919	100 (17)	100 (7)
Xylazine	10	-2	0.1X	0.9996	94 (5)	95 (4)	3	0.1X	0.9975	109 (8)	96 (8)	20	0.1X	0.9966	106 (2)	97 (6)

3.4.4 Sample Throughput and Environmental Footprint

In terms of time required to perform extraction, the SE method necessitated a total time of 9 min, including 5 min for extraction and 4 min for centrifugation, while the QuEChERS required a total of 14 min as a result of the additional time needed to weigh the 500 mg of C_{18}, followed by the 30 s shake on the vortexer, and another round of centrifugation. In our lab setup, while 15 samples could be simultaneously processed on the multivortexer, the centrifuge was a limiting factor, because only four tubes could be processed at a time as a result of the centrifuge capacity. As a result, it took approximately 9 min to extract four samples using the SE protocol and 14 min using the QuEChERS protocol. Therefore, total sample extraction times per sample corresponded to over 2 min/sample for SE and over 3 min/sample for QuEChERS of active analyst time. While the QuEChERS and SE extraction methods offer a simple method for extraction, the manual nature of the extraction process, which has not been automated to date, renders the method as tedious and time-consuming while additionally introducing a significant source of variation. While the SPME protocol necessitates 80 min for extraction and desorption, up to 96 samples can be extracted simultaneously, thus affording total sample preparation times of less than 1 min per sample. Moreover, the automated nature of the protocol enables performance of other lab duties throughout the sample preparation procedure, given that the analyst is not actively engaged with the sample preparation process throughout the 80 min.

In addition to high-throughput extraction, another main advantage of SPME is its minimal use of organic solvents compared to SE and QuEChERS. The SPME protocol used in this work requires only 0.3 mL of organic solvent per sample (0.15 mL MeOH + 0.15 mL MeCN) for the desorption step, an amount that can be further reduced if smaller inserts are available

commercially. In contrast, SE and QuEChERS require a minimum of 8 mL of MeCN per sample. This clear advantage of SPME not only leads to obvious savings on reagent costs but also to a significant reduction in the environmental footprint as well as a decreased degree of exposure of analysts to organic solvents.

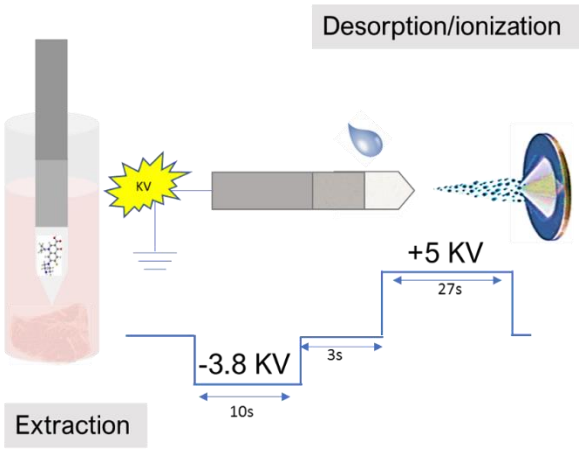
Nonetheless, while SPME offers several advantages over SE and QuEChERS in terms of significantly cleaner sample extracts, excellent accuracy and precision, high-throughput, automation, and ease of use, applicability for onsite and in vivo extraction, and a much lower environmental footprint, SPME method development requires careful considerations, such as optimization of the extraction phase and extraction and desorption conditions, which require a fundamental understanding of the theory and principles of SPME.

3.4.5 Analysis of Market Samples

Five chicken tissue samples and three beef tissue samples obtained from different local grocery stores were submitted to analysis for determination of veterinary drugs, using the three methods described above. None of the veterinary drugs under study were found in any of the samples at levels near the calculated LOQs.

Chapter 4

Optimization of Coated Blade Spray for Rapid Screening and Quantitation of 105 Veterinary Drugs in Biological Tissue Samples



4.1 Preamble

The materials in this chapter have been published as a research article in Analytical Chemistry: Khaled, A.; Gómez-Ríos, G. A.; Pawliszyn, J. Optimization of Coated Blade Spray for Rapid Screening and Quantitation of 105 Veterinary Drugs in Biological Tissue Samples. *Anal. Chem.* 2020, *92* (8), 5937-5943. Materials for all sections of this current chapter are reprinted from this research article with the permission of American Chemical Society (ACS), Copyright, 2020. Copyright for this work remains the property of ACS publications and any further request for re-use of this information should be requested directly from them. (https://doi.org/10.1021/acs.analchem.0c00093).

The contribution of co-author Germán Augusto Gómez-Ríos to the work described in this chapter was technical advice at the early stage of method development. All of the experimental planning, design, and experimental work conducted in the laboratory, data processing, analysis, interpretation, and writing were performed by the author of the thesis.

I, Germán Augusto Gómez-Ríos, authorize Abir Khaled to use the material for her thesis.

4.2 Introduction

A chief concern for human health nowadays is the presence of drug and chemical residues in the edible tissues of food animals.[7,197,198] As the number of residues and contaminants found to present a risk to human health continues to expand, the demand for highly efficient analytical methods capable of monitoring a wide variety of analytes rapidly and simultaneously in one single run continues to grow around the globe.[15,92] One approach to increase the efficiency and cost-effectiveness of an analytical method is to increase the number of analytes that can be determined in a single run. Regulatory bodies and testing laboratories strive to increase the scope of their analytical methods as a means to analyze more compounds per sample, reduce the overall amount of required samples, and increase the probability of detecting residue violations. As a result, multi-residue multi-class methods that are capable of analyzing as many analytes as possible in a single run are becoming very popular.[32,38,84,87,88] Moreover, developing methods that are capable of screening in both negative and positive ionization modes in the same run can also help to increase the scope of analytical methods. Thanks to its selective and fast scanning capabilities, tandem Mass spectrometry (MS/MS) is arguably considered one of the most effective analytical techniques for fast screening and quantitation of a large number of target analytes within one short run.[199]

However, matrices such as biological tissues can be extremely complex to analyze via MS due to their high fat and protein content, as such compounds are very likely to cause interferences in the MS.[200] As such, the introduction of such matrices to the MS often requires multiple sample preparation steps and chromatographic separation to prevent interferences from matrix co-extractives. Conventional sample preparation methods often include solvent extraction (SE) or protein precipitation (PP) followed by additional extract clean-up with either solid phase extraction (SPE) or dispersive SPE (d-SPE).[92,96,201,202] These steps can be very time consuming and may not be suitable for the direct and high-throughput screening requirements of modern analysis of real samples.[139,200] An alternative approach to this lengthy workflow is to simplify sample preparation and eliminate chromatographic separation by directly introducing the sample to the MS.[200,203–205] Direct-to-MS analysis techniques offer the advantages of obtaining analytical results in the order of seconds and screening hundreds of samples in a short period of time.[96] However, in case of tissue samples, minimal sample preparation can introduce an abundance of matrix interferences to the MS. Solid phase microextraction (SPME) has consistently demonstrated its ability to isolate and enrich a wide variety of analytes from biological samples with minimal coextraction of proteins, salts, and other matrix macromolecules.[147,189,206] SPME-based devices have also been successfully used to integrate sample preparation and direct sample introduction into the MS system in a single device.[120,141,204,207,208] One of these devices is the coated blade spray (CBS), which has been widely and successfully applied for rapid qualitative and quantitative analysis of biological fluids such as blood, plasma, and urine.[151,208–210] In addition to its direct-to-MS capability, CBS also offers high throughput capabilities and can be easily automated.[139,151,204]

Several other direct-to-MS methods have been studied and demonstrated suitable for analysis of tissue samples, but none so far for multi-residue analysis of more than 100 analytes in both negative and positive mode in a single run.[14,84,200,202,211] CBS has not been investigated yet for analysis of tissue samples as, unlike mere direct-sample-to-MS technologies, there is need to remove any tissue debris potentially adhered to the device prior to instrumental analysis. In previous CBS studies on biological fluids which are less challenging than homogenized tissue, loosely attached macromolecules and cellular debris were removed by rinsing with water, followed by a light wipe with a Kimwipe in the rare cases that the attachments were not dislodged by the quick rinse.[146] However, in automated high-throughput workflows, using Kimwipe is not a practical approach. Thus, another coating design of the CBS device was needed to minimize/prevent any potential attachments to the device. In this study, we present the optimization of the CBS design to improve and maximize its performance for analysis of complex matrices such as biological tissue samples. In order to prevent adhesion of tissue debris and macromolecules to the uncoated part of the blade, we first applied a thin layer, approximately 5 µm in thickness, of polyacrylonitrile (PAN) to the blade prior to the application of the HLB/PAN extraction phase. The length of the undercoat PAN layer, 20 mm, was 10 mm longer than that of the HLB coating so as to ensure no contact between the bare stainless-steel and the sample during the extraction step. PAN was selected in this study as the protective undercoating layer because it is one of the most commonly used polymers in the biomedical field in terms of biocompatibility.[147] In addition, it is compatible with the extractive phase as it is used in this study as the binder to immobilize the extractive particles (HLB) on the CBS substrate. The biocompatible PAN

undercoat layer served to seal the surface of the stainless steel that comes into contact with the sample during the extraction process, thus eliminating the adhesion of matrix debris and macromolecules onto the bare stainless-steel surface. It is worth noting that this protective layer was applied under the extraction phase and not on top of it so as to prevent changes in the thickness of the coating and the slowing of the extraction or elution kinetics. While the main purpose of the undercoat is to eliminate adhesion of matrix macromolecules onto the bare stainless-steel surface, it also serves the following purposes: 1) the sample is in contact with a biocompatible surface during extraction and 2) the undercoating serves as a primer binder layer where the extractive phase can better adhere to the stainless-steel support. This new device feature enabled the use of CBS-MS/MS for the analysis of 105 veterinary drugs in homogenized bovine tissue. Furthermore, the proposed analytical method allows for the first time screening in both negative and positive modes in a single run using a single sampling device. In addition, when 96 samples are submitted to extraction simultaneously, total analysis time drops to 1 min per sample. As part of the validation procedure, the method was evaluated with respect to matrix effects, selectivity, linearity, accuracy, intra-day repeatability, and limit of quantitation (LOQ).

4.3 Experimental Section

4.3.1 Target Analytes

A total of 114 target analytes, comprising drugs with a wide range of physico-chemical properties (log P −1.85–9.36), were initially selected for this investigation. These analytes include 103 veterinary drugs that were successfully quantified below the regulatory residue limits in a previous report using SPME and liquid chromatography-electrospray ionization - tandem mass

spectrometry (UHPLC-ESI-MS/MS) in positive ionization mode.[206] An additional 11 new target analytes that ionize in negative mode were added to this study to evaluate the performance of CBS in negative mode. Further details pertaining to these compounds, including their physico-chemical properties, drug classes, supplier information, and ionization mode, can be found in Table 4.1 and 4.2. Sulfadoxine was also not further investigated in this study as it is an isobar of sulfadimethoxine. All analytes were selected from the list of standards specified in the official method developed by the United States Department of Agriculture for screening and confirmation of animal drug residues (CLG-MRM1.08).[31] A total of 9 analytes (bolded in Table 4.1) were excluded from validation studies following final optimization of the multi-residue method due to poor ionization efficiencies at the maximum residue limit (MRL). While the detection limit for these compounds could certainly be improved were they to be analyzed separately, optimized method conditions, including desorption solvent, were mainly targeted at optimizing negative mode compounds due to the complexity of negative ionization, leading to compromised performance of certain compounds with low MRL values. The MRL values, listed in Table 4.3, were based primarily on Canadian MRL values and US tolerances in beef tissues.[82,91] In general, Canadian MRLs are either equal or lower than US tolerances; however, in cases where Canadian MRLs were higher than US tolerances, the US regulatory value was selected, as is the case for ketoprofen and morantel. Two isotopically labelled internal standards (IS), namely *d5*-Enrofloxacin for positive mode analytes and *d3*-Thiamphenicol for negative mode analytes, were procured from Sigma Aldrich (Oakville, ON, Canada).

4.3.2 Materials and supplies

LC/MS-grade methanol (MeOH), acetonitrile (MeCN), and water were purchased from Fisher Scientific (Mississauga, ON, Canada). Ammonium fluoride, polyacrylonitrile (PAN), HPLC grade Acetone, and Dimethyl Sulfoxide (DMSO) were purchased from Sigma Aldrich (Oakville, ON, Canada). Stainless steel blades were purchased from Shimifrez Incorporated (Concord, ON, CAN). The blades were first coated with a 20 mm layer of biocompatible PAN and then coated with a slurry of PAN and Hydrophilic-Lipophilic Balanced (HLB) particles. The HLB particles (~5 μm diameter) were synthesized in house and characterized as previously described.[189] The procedure used to coat the CBS devices was reported elsewhere.[209] The extraction phase coating length used was 10 mm, and the thickness was 10 μm. Figure 4.1 shows a schematic of the optimized CBS design with the undercoat layer, as well as the positioning of the blade during extraction. Nunc U96 600 μL, 1 mL Deep, and 2mL well-plates made of polypropylene were purchased from VWR International (Mississauga, ON, Canada). Individual stock standard solutions were prepared in either MeCN, MeOH, water, or 10% DMSO in MeCN, in accordance with the solubility of each compound, and stored at −80 °C.

Organic beef muscle tissue samples were acquired from three different local grocery stores to serve as a pooled matrix.

Table 4.1 Physico-chemical properties, class, and supplier information for analytes under study.

	Compound	**Supplier**	**Class**	**Formula[1]**	**Molecular Mass (Da)[a]**	**Log P[a]**
1	2-Aminoflubendazole	Sigma-Aldrich	Anthelmintics	$C_{14}H_{10}FN_3O$	255.2	1.96
2	2-Amino mebendazole	Sigma-Aldrich	Anthelmintics	$C_{14}H_{11}N_3O$	237.3	1.74
3	2-Mercaptobenzimidazole	Sigma-Aldrich	Anthelmintics	$C_7H_6N_2$	150.2	-0.38
4	**2-Quinoxalinecarboxylic acid**	**Sigma-Aldrich**	**Others**	**$C_9H_6N_2$**	**174.2**	**1.80**
5	2-Thiouracil	Sigma-Aldrich	Thyreostats	$C_4H_4N_2$	128.2	-1.85
6	5-Hydroxythiabendazole	Sigma-Aldrich	Anthelmintics	$C_{10}H_7N_3OS$	217.3	1.73
7	**6-Methyl-2-thiouracil**	**Sigma-Aldrich**	**Thyreostats**	**$C_5H_6N_2$**	**142.2**	**0.31**
8	6-Phenyl-2-thiouracil	Sigma-Aldrich	Thyreostats	$C_{10}H_8N_2OS$	204.3	-0.10
9	6-Propyl-2-thiouracil	Sigma-Aldrich	Thyreostats	$C_7H_{10}N$	170.2	1.37
10	Acepromazine Maleate	Sigma-Aldrich	Tranquilizers	$C_{19}H_{22}N_2OS$	326.5	4.08
11	Albendazole	Sigma-Aldrich	Anthelmintics	$C_{12}H_{15}N_3O_2S$	265.3	3.07
12	Albendazole-2-aminosulfone	Sigma-Aldrich	Anthelmintics	$C_{10}H_{13}N_3O_2S$	239.3	0.12
13	Albendazole Sulfone	Sigma-Aldrich	Anthelmintics	$C_{12}H_{15}N_3O_4S$	297.1	0.86
14	Albendazole Sulfoxide	Sigma-Aldrich	Anthelmintics	$C_{12}H_{15}N_3O_3S$	281.3	0.91
15	**Ampicillin**	**Sigma-Aldrich**	**β-Lactams/cephalosporins**	**$C_{16}H_{19}N_3O_4S$**	**349.4**	**1.35**
16	Azaperone	Sigma-Aldrich	Tranquilizers	$C_{19}H_{22}FN_3O$	327.4	2.50
17	**Bacitracin**	**Sigma-Aldrich**	**Others**	**$C_{66}H_{103}N_{17}O_{16}S$**	**1422.7**	**-2.21**
18	Betamethasone	Sigma-Aldrich	Anti-inflammatories	$C_{22}H_{29}FO_5$	392.2	1.87
19	Bithionol	Sigma-Aldrich	Anthelmintics	$C_{12}H_6Cl$	356.1	5.51
20	Cambendazole	Sigma-Aldrich	Anthelmintics	$C_{14}H_{14}N_4O_2S$	302.4	2.90
21	Carazolol	Sigma-Aldrich	Tranquilizers	$C_{18}H_{22}N_2O_2$	298.4	3.59

Table 4.1 continued

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[a]	Log P[a]
22	Carbadox	Sigma-Aldrich	Others	$C_{11}H_{10}N_4O_4$	262.2	-1.22
22	Carbadox	Sigma-Aldrich	Others	$C_{11}H_{10}N_4O_4$	262.2	-1.22
23	Cefazolin	Sigma-Aldrich	β-Lactams/cephalosporins	C14H14N8O4S3	454.507	1.13
24	Cephapirin	Sigma-Aldrich	β-Lactams/cephalosporins	$C1_7H_{17}N_3O_6S_2$	423.5	0.79
25	Chloramphenicol	Sigma-Aldrich	Phenicols	$C_{11}H_{12}C_{l2}N_2O_5$	323.1	1.02
26	Chlorpromazine HCl	Sigma-Aldrich	Tranquilizers	$C_{17}H_{20}Cl_2N_2S$	355.3	5.20
27	Chlortetracycline HCl	Sigma-Aldrich	Tetracyclines	$C_{22}H_{24}Cl_2N_2O_8$	515.3	1.11
28	Ciprofloxacin	Sigma-Aldrich	Fluoroquinolones	$C_{17}H_{18}FN_3O_3$	331.341	0.65
29	Clenbuterol HCl	Sigma-Aldrich	β-Agonists	$C_{12}H_{19}Cl_3N_2O$	313.7	2.61
30	Clindamycin HCl	Sigma-Aldrich	Macrolides/lincosamides	$C_{18}H_{34}Cl_2N_2O_5S$	461.4	1.83
31	Clorsulon	Sigma-Aldrich	Anthelmintics	$C_8H_8C_{l3}N_3O_4S_2$	380.7	1.04
32	Closantel	Sigma-Aldrich	Anthelmintics	$C_{22}H_{14}C_{l2}I_2N_2O_2$	663.1	9.08
33	Cloxacillin Sodium Salt	Sigma-Aldrich	β-Lactams/cephalosporins	$C_{19}H_{17}ClN_3NaO_5S$	457.9	2.53
34	Danofloxacin	Sigma-Aldrich	Fluoroquinolones	$C_{19}H_{20}FN_3O_3$	357.1	1.20
35	Desacetyl cephapirin	Toronto Research Chemicals	β-Lactams/cephalosporins	$C_{15}H_{15}N_3O_5S_2$	381.427	0.32
36	Desethylene ciprofloxacin HCl	Toronto Research Chemicals	Fluoroquinolones	$C_{15}H_{17}ClFN_3O_3$	341.8	-0.14
37	Diclofenac Sodium	Sigma-Aldrich	Anti-inflammatories	$C_{14}H_{10}Cl_2NNaO_2$	318.1	4.06
38	Dicloxacillin Sodium Salt Hydrate	Sigma-Aldrich	β-Lactams/cephalosporins	$C_{19}H_{16}Cl_2N_3NaO_5S.$ xH_2O	492.3	3.02
39	Difloxacin HCl	Sigma-Aldrich	Fluoroquinolones	$C_{21}H_{20}ClF_2N_3O_3$	435.9	2.78
40	Dimetridazole	Sigma-Aldrich	Coccidiostats	$C_5H_7N_3O_2$	141.0	0.31

Table 4.1 continued

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[a]	Log P[a]
41	Doxycycline HCl	Sigma-Aldrich	Tetracyclines	$C_{22}H_{25}ClN_2O_8$	480.9	-0.54
42	Emamectin Benzoate	Sigma-Aldrich	Anthelmintics	$C_{56}H_{81}NO_{15}$	1008.2	6.84
43	Enrofloxacin	Sigma-Aldrich	Fluoroquinolones	$C_{19}H_{22}FN_3O_3$	359.4	1.88
44	Eprinomectin	Sigma-Aldrich	Anthelmintics	$C_{50}H_{75}NO_{14}$	914.1	6.22
45	Erythromycin	Sigma-Aldrich	Macrolides/lincosamides	$C_{37}H_{67}NO_{13}$	733.9	2.83
46	Fenbendazole Sulfone	Sigma-Aldrich	Anthelmintics	$C_{15}H_{13}N_3O_4S$	331.3	1.70
47	**Florfenicol**	**Sigma-Aldrich**	**Phenicols**	**$C_{12}H_{14}Cl_2FNO_4S$**	**357.0**	**-0.12**
48	**Florfenicol amine**	**Sigma-Aldrich**	**Phenicols**	**$C_{10}H_{14}FNO_3S$**	**247.3**	**-0.80**
49	Flubendazole	Sigma-Aldrich	Anthelmintics	$C_{16}H_{12}FN_3O_3$	313.3	3.05
50	Flunixin	Sigma-Aldrich	Anti-inflammatories	$C_{14}H_{11}F_3N_2O_2$	296.2	5.40
51	Gamithromycin	Sigma-Aldrich	Macrolides/lincosamides	$C_{40}H_{76}N_2O_{12}$	776.5	3.89
52	Haloperidol	Sigma-Aldrich	Tranquilizers	$C_{21}H_{23}ClFNO_2$	375.9	3.01
53	**Hydroxy dimetridazole**	**Toronto Research Chemicals**	**Coccidiostats**	**$C_5H_7N_3O_3$**	**157.1**	**-0.49**
54	Hydroxy ipronidazole	Sigma-Aldrich	Coccidiostats	$C_7H_{11}N_3O_3$	185.2	0.21
55	**Hydroxy metronidazole**	**Sigma-Aldrich**	**Coccidiostats**	**$C_6H_9N_3O_4$**	**187.15**	**-0.81**
56	Ipronidazole	Sigma-Aldrich	Coccidiostats	$C_7H_{11}N_3O_2$	169.2	1.18
57	Ketoprofen	Sigma-Aldrich	Anti-inflammatories	$C_{16}H_{14}O_3$	254.3	2.81
58	Lasalocid A	Sigma-Aldrich	Coccidiostats	$C_{34}H_{54}O_8$	590.8	6.55
59	Levamisole HCl	Sigma-Aldrich	Anthelmintics	$C_{11}H_{13}ClN_2S$	240.8	1.85
60	Lincomycin HCl Monohydrate	Sigma-Aldrich	Macrolides/lincosamides	$C_{18}H_{37}ClN_2O_7S$	461.0	0.91
61	Mebendazole	Sigma-Aldrich	Anthelmintics	$C_{16}H_{13}N_3O_3$	295.3	2.83

Table 4.1 continued

	Compound	**Supplier**	**Class**	**Formula[1]**	**Molecular Mass (Da)[a]**	**Log P[a]**
62	Melengestrol Acetate	Sigma-Aldrich	Others	$C_{25}H_{32}O_4$	396.5	4.21
63	Meloxicam	Sigma-Aldrich	Anti-inflammatories	$C_{14}H_{13}N_3O_4S_2$	351.0	2.71
64	Metamizole	Sigma-Aldrich	Anti-inflammatories	$C_{13}H_{17}N_3O_4S$	311.0	-0.74
65	Metronidazole	Sigma-Aldrich	Coccidiostats	$C_6H_9N_3O_3$	171.2	-0.01
66	Morantel Tartrate Hydrate	Sigma-Aldrich	Anthelmintics	$C_{16}H_{22}N_2O_6S\ xH_2O$	370.4	1.97
67	Nafcillin	Sigma-Aldrich	β-Lactams/cephalosporins	$C_{21}H_{22}N_2O_5S$	414.1	3.52
68	Nitroxynil	Sigma-Aldrich	Anthelmintics	$C_7H_{3I}N_2O_3$	290.0	2.80
69	Norfloxacin	Sigma-Aldrich	Fluoroquinolones	$C_{16}H_{18}FN_3O_3$	319.3	0.82
70	Novobiocin	Sigma-Aldrich	Novobiocin	$C_{31}H_{36}N_2O_{11}$	612.2	2.86
71	Orbifloxacin	Sigma-Aldrich	Fluoroquinolones	$C_{19}H_{20}F_3N_3O_3$	395.4	2.37
72	Oxacillin Sodium Salt Monohydrate	Sigma-Aldrich	β-Lactams/cephalosporins	$C_{19}H_{18}N_3O_5SNa\ H_2O$	441.4	2.05
73	Oxfendazole	Sigma-Aldrich	Anthelmintics	$C_{15}H_{13}N_3O_3S$	315.3	1.36
74	Oxyclozanide	Sigma-Aldrich	Anthelmintics	$C_{13}H_6Cl_5NO_3$	401.5	8.67
75	Oxyphenylbutazone	Sigma-Aldrich	Anti-inflammatories	$C_{19}H_{20}N_2O_3$	324.4	2.72
76	Oxytetracycline HCl	Sigma-Aldrich	Tetracyclines	$C_{22}H_{25}ClN_2O_9$	496.9	-1.50
77	**Penicillin G**	**Toronto Research Chemicals**	**β-Lactams/cephalosporins**	**$C_{16}H_{18}N_2O_4S$**	**334.0**	**1.67**
78	Phenylbutazone	Sigma-Aldrich	Anti-inflammatories	$C_{19}H_{20}N_2O_2$	308.4	3.16
79	Pirlimycin HCl	Toronto Research Chemicals	Macrolides/lincosamides	$C_{17}H_{32}Cl_2N_2O_5S$	447.4	1.47
80	Prednisone	Sigma-Aldrich	Anti-inflammatories	$C_{21}H_{26}O_5$	358.4	1.57
81	Promethazine HCl	Sigma-Aldrich	Tranquilizers	$C_{17}H_{21}ClN_2S$	320.9	4.78

Table 4.1 continued

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[a]	Log P[a]
82	Propionylpromazine HCl	Sigma-Aldrich	Tranquilizers	$C_{20}H_{25}ClN_2OS$	376.9	4.61
83	Ractopamine HCl	Sigma-Aldrich	β-Agonists	$C_{18}H_{24}ClNO_3$	337.8	1.65
84	Rafoxanide	Sigma-Aldrich	Anthelmintics	$C_{19}H_{11}C_{l2}I_2NO_3$	626.0	9.36
85	Ronidazole	Sigma-Aldrich	Coccidiostats	$C_6H_8N_4O_4$	200.0	-0.45
86	Salbutamol	Sigma-Aldrich	β-Agonists	$C_{13}H_{21}NO_3$	239.1	0.01
87	Sarafloxacin HCl Hydrate	Sigma-Aldrich	Fluoroquinolones	$C_{20}H_{17}F_2N_3O_3$ HCl xH_2O	421.8	2.09
88	Sulfachloropyridazine	Sigma-Aldrich	Sulfonamides	$C_{10}H_9ClN_4O_2S$	284.7	1.02
89	Sulfadiazine	Sigma-Aldrich	Sulfonamides	$C_{10}H_{10}N_4O_2S$	250.277	-0.12
90	Sulfadimethoxine	Sigma-Aldrich	Sulfonamides	$C_{12}H_{14}N_4O_4S$	310.3	1.48
91	**Sulfadoxine**	**Sigma-Aldrich**	**Sulfonamides**	**$C_{12}H_{14}N_4O_4S$**	**310.3**	**0.34**
92	Sulfaethoxypyridazine	Sigma-Aldrich	Sulfonamides	$C_{12}H_{14}N_4O_3S$	294.3	0.85
93	Sulfamerazine	Sigma-Aldrich	Sulfonamides	$C_{11}H_{12}N_4O_2S$	264.0	0.34
94	Sulfamethazine	Sigma-Aldrich	Sulfonamides	$C_{12}H_{14}N_4O_2S$	278.0	0.80
95	Sulfamethizole	Sigma-Aldrich	Sulfonamides	$C_9H_{10}N_4O_2S_2$	270.0	0.51
96	Sulfamethoxazole	Sigma-Aldrich	Sulfonamides	$C_{10}H_{11}N_3O_3S$	253.0	0.89
97	Sulfamethoxypyridazine	Sigma-Aldrich	Sulfonamides	$C_{11}H_{12}N_4O_3S$	280.3	0.32
98	Sulfanitran	Sigma-Aldrich	Sulfonamides	$C_{14}H_{13}N_3O_5S$	335.3	2.98
99	Sulfapyridine	Sigma-Aldrich	Sulfonamides	$C_{11}H_{11}N_3O_2S$	249.3	0.03
100	Sulfaquinoxaline	Sigma-Aldrich	Sulfonamides	$C_{14}H_{12}N_4O_2S$	300.0	1.30
101	Sulfathiazole	Sigma-Aldrich	Sulfonamides	$C_9H_9N_3O_2S_2$	255.3	0.05

Table 4.1 continued

	Compound	Supplier	Class	Formula1	Molecular Mass (Da)[a]	Log P[a]
102	Tetracycline HCl	Sigma-Aldrich	Tetracyclines	$C_{22}H_{25}ClN_2O_8$	480.9	-1.47
103	Thiabendazole	Sigma-Aldrich	Anthelmintics	$C_{10}H_7N_3S$	201.0	2.47
104	Thiamphenicol	Sigma-Aldrich	Phenicols	$C_{12}H_{15}C_{l2}NO_5S$	356.2	-0.27
105	Tildipirosin	Sigma-Aldrich	Macrolides/lincosamides	$C_{41}H_{71}N_3O_8$	733.5	4.70
106	Tilmicosin	Sigma-Aldrich	Macrolides/lincosamides	$C_{46}H_{80}N_2O_{13}$	868.5	4.95
107	Tolfenamic acid	Sigma-Aldrich	Anti-inflammatories	$C_{14}H_{12}ClNO_2$	261.7	5.76
108	Triclabendazole	Sigma-Aldrich	Anthelmintics	$C_{14}H_9Cl_3N_2OS$	359.7	5.97
109	Triclabendazole Sulfoxide	Sigma-Aldrich	Anthelmintics	$C_{14}H_9Cl_3N_2O_2S$	375.7	4.12
110	Triflupromazine HCl	Sigma-Aldrich	Tranquilizers	$C_{18}H_{20}ClF_3N_2S$	388.9	5.70
111	Tulathromycin	Toronto Research Chemicals	Macrolides/lincosamides	$C_{41}H_{79}N_3O_{12}$	805.5	4.07
112	Tylosin	Toronto Research Chemicals	Macrolides/lincosamides	$C_{46}H_{77}NO_{17}$	916.1	3.27
113	Virginiamycin M1	Sigma-Aldrich	Others	$C_{28}H_{35}N_3O_7$	525.6	-0.66
114	Xylazine HCl	Sigma-Aldrich	Tranquilizers	$C_{12}H_{17}ClN_2S$	256.8	2.37

[a] Data taken from www.chemspider.com. LogP data taken from computational predictions ACD/LogP.

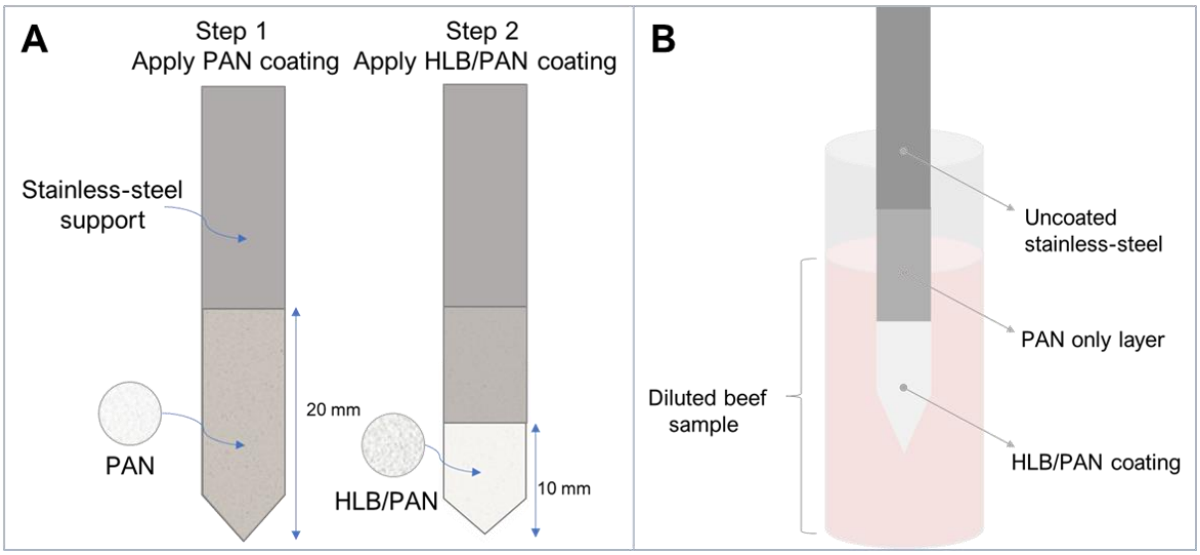


Figure 4.1 Schematics of the blade with the undercoat layer (A) and the blade position in the sample during extraction (B).

4.3.3 Sample Preparation

Utilizing a Freezer/Mill® Cryogenic Grinder (SPEX SamplePrep, LLC, Metuchen, NJ, USA), beef tissue samples were homogenized and mixed in equal portions under liquid nitrogen to produce a uniform fine powder. Homogenized samples were stored in glass jars at −80 °C until analysis. Spiked tissue samples were prepared by weighing 2.0 g of homogenized tissue that had been brought to room temperature into a 50 mL polypropylene (PP) tube. Each sample was then spiked with 100 µL of a working solution containing all analytes under study at their respective designated concentrations, and 60 µL of the IS working solution containing 1 ng μL^{-1} of each IS. Samples were vortexed for 1 min, then placed on a benchtop agitator for 1h. Subsequently, samples were stored in a 4ºC fridge overnight so as to allow for proper equilibration and matrix binding of target analytes prior to extraction. Prior to extraction, samples were placed on the benchtop agitator for 1h so as to allow samples to reach room temperature. Next, 6mL of water were added to each

individual spiked tissue sample and vortexed for 1 min in order to attain homogeneous consistency suitable for pipetting of samples to the wells of the 96 well-plates. 1500 µL of diluted beef sample was then transferred to each well of the 96 well-plate. A detailed schematic of the sample preparation steps is illustrated in Figure 4.2.

4.3.4 Analytical protocols.

The CBS analytical workflow consisted of four main steps: (1) Conditioning of the blades with MeOH:H_2O (50:50, v/v/), (2) Extraction of target analytes from the matrix prepared as described above; (3) rinsing of coating with 15:85 acetone:H_2O to remove any fatty residue, salts, or loose debris from the blade; (4) analyte desorption and ionization, whereby 12 µL of MeOH:MeCN:H_2O (80:15:5, v/v/v) with 5 mM ammonium fluoride was pipetted onto the coating of individual blades as they were placed in front of the MS inlet. We observed that negative mode does not drive the elution/ionization solvent to the tip of the blade. Therefore, in order to achieve a stable spray in negative ionization mode and ensure reproducible results, it is crucial to make sure that the tip of the blade is properly wetted with the elution/ionization solvent prior to applying the voltage. Another factor that possibly helped us achieve successful negative ionization was the addition of a small concentration of ammonium fluoride (5mM) to the elution/ionization solvent as a means to increase ionization efficiency in negative ionization mode.[212] The enhanced ionization may be explained by the strong electronegativity of the fluoride ion, which enhances deprotonation in negative mode. Steps 1-3 were automated with the use of a Concept-96 system (Professional Analytical Systems (PAS) Technology, Magdala, Germany), as described elsewhere.[137] Analytical parameters such as extraction time, desorption solvent and time, and

rinsing solvent and time were investigated for optimum S/N ratio rather than signal only.[208] The CBS extraction and desorption/ionization workflow with the final optimized parameters as well as the developed analytical workflow are illustrated in further details in Figure 4.2. All CBS devices were single use.

4.3.5 Instruments and Conditions

Analysis was carried out on a TSQ Quantiva from Thermo Scientific (San Jose, CA, USA) with data analysis completed using TraceFinder 4.1, also from Thermo Scientific. Desorption and ionization experiments were performed using a custom CBS interface that was built at the University of Waterloo.[210]

After 10s of analyte desorption/elution with the desorption solvent mentioned above, analyses were performed in negative ionization mode at minus 3.8 kV voltage for the first 10s of the analytical run, followed by 27s in positive mode at a plus 5 kV voltage, whereupon an electrospray event was produced at the tip of the blade, directly introducing the eluted analytes into the MS, as illustrated in Figure 4.2. Two single reaction monitoring (SRM) transitions were used for each analyte, one for quantitation and one for confirmation, whereas only one transition was needed for each IS. MS/MS analyte transitions and conditions were optimized via direct infusion from methanolic and acetonitrile standards. The transitions (shown in Table 4.2) were selected based on the optimal S/N at a 1 MRL concentration level in beef matrix. Detailed information regarding optimized instrumental parameters (collision energy and RF-lens values)

and monitored SRM transitions can be found in Table 4.2. The SRM dwell time was set at 5 ms per transition. Transfer line temperature was set to 300 °C.

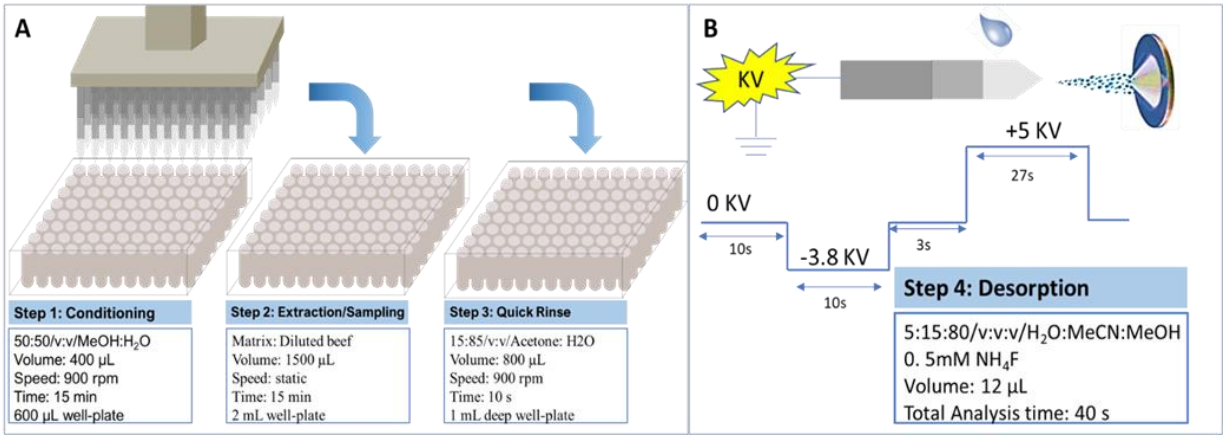


Figure 4.2 Proposed experimental setup for the conditioning, extraction, rinsing (A), and elution/ionization (B) of analytes from complex matrices via optimized CBS method.

Table 4.2 MS/MS optimized parameters, (m/z); ions used for quantification are bolded.

	Compound	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	S-Lens value	Polarity
1	2-Aminoflubendazole	256	**95**, 123	27, 37	93	+
2	2-Amino mebendazole	238	77, **105**	34, 25	88	+
3	2-Mercaptobenzimidazole	151	**93,** 118	23, 25	70	+
4	2-Quinoxalinecarboxylic acid	175	104, **131**	29, 22	191	+
5	5-Hydroxythiabendazole	218	147, **191**	32, 26	85	+
6	2- Thiouracil	127	42, **58**	33, 16	34	-
7	6-Phenyl-2-thiouracil	205	**103**, 188	26, 17	69	+
8	6-Propyl-2-thiouracil	171	112, **154**	19, 17	57	+
9	Acepromazine Maleate	327	86, **254**	19, 23	65	+
10	Albendazole	266	191, **234**	32, 19	71	+
11	Albendazole sulfone	298	**159**, 266	36, 19	79	+
12	Albendazole sulfoxide	282	240, **208**	23, 10	75	+
13	Albendazole-2-aminosulfone	240	133,**198**	28,19	75	+
14	Amoxicillin	366	114, **349**	20, 10	48	+
15	Ampicillin	350	**106**, 160	18, 10	54	+
16	Azaperone	328	123,**165**	29, 20	66	+
17	Bacitracin	475	**199**, 669	24, 10	74	+

	Compound	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	S-Lens value	Polarity
18	Betamethasone	393	325, **373**	12, 14	59	+
19	Bithionol	355	161, **192**	22, 24	72	-
20	Cambendazole	303	**217**, 261	27, 17	66	+
21	Carazolol	299	116, **222**	19, 19	63	+
22	Carbadox	263	**231**, 245	10, 16	67	+
23	Cephapirin	424	152, **292**	22, 14	66	+
24	Cefazolin	455	**323**, 333	10, 19	72	+
25	Chloramphenicol	321	**152,** 257	16, 10	53	-
26	Chlortetracycline HCl	479	444, **462**	19, 17	70	+
27	Chlorpromazine HCl	319	**58**, 86	30, 19	63	+
28	Ciprofloxacin	332	**288**, 314	17, 19	72	+
29	Clenbuterol HCl	277	**168**, 203	29, 16	47	+
30	Clindamycin HCl	425	**126**, 377	27, 18	78	+
31	Clorsulon	380	**342,** 344	10, 12	46	-
32	Closantel	661	315, **345**	32, 35	207	-
33	Cloxacillin Sodium Salt	436	**160**, 277	10, 12	56	+
34	Danofloxacin	358	**314**, 340	17, 21	80	+
35	Desacetyl cephapirin	382	226, **292**	17, 10	73	+
36	Desethylene Ciprofloxacin	306	268, **288**	24, 17	67	+
37	Diclofenac Sodium	296	**214**, 277	33, 11	112	+
38	Dicloxacillin Sodium Salt	470	**212**, 355	26, 16	124	+
39	Difloxacin HCl	400	356, **382**	18, 21	82	+
40	Dimetridazole	142	**96**, 101	16, 10	38	+
41	Doxycycline HCl	445	**321**, 428	30, 17	66	+
42	Emamectin Benzoate	887	**158**, 868	33, 21	120	+
43	Enrofloxacin	360	**316**, 342	18, 20	79	+
44	Eprinomectin	937	**490,** 504	79, 50	164	+
45	Erythromycin	735	522, **558**	17, 34	85	+
46	Fenbendazole Sulfone	332	159, **300**	37, 21	81	+
47	Florfenicol	357	182, **198**	16, 14	73	+
48	Florfenicol amine	248	151, **230**	25, 10	50	+
49	Flubendazole	314	123, **282**	35, 22	80	+
50	Flunixin	297	**264**, 279	33, 22	75	+
51	Gamithromycin	778	601, **619**	31, 28	121	+
52	Haloperidol	376	**123**, 165	36, 22	78	+
53	Hydroxy dimetridazole	158	**80**,140	10, 10	30	+
54	Hydroxy ipronidazole	186	122, **168**	19, 10	37	+

	Compound	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	S-Lens value	Polarity
55	Hydroxy metronidazole	188	**123**, 126	10, 17	47	+
56	Ipronidazole	170	**109**, 124	24, 17	54	+
57	Ketoprofen	255	**105**, 209	23, 14	59	+
58	Lasalocid A	589	**235,** 571	30, 28	113	-
59	Levamisole HCl	205	123, **178**	28, 21	66	+
60	Lincomycin HCl	407	126, **359**	26, 18	76	+
61	Mebendazole	296	105, **264**,	33, 20	75	+
62	Melengestrol Acetate	397	279, **337**	20, 13	88	+
63	Meloxicam	352	**115**, 141	19, 20	67	+
64	Metamizole	218	**56**, 97	17, 12	47	+
65	Metronidazole	172	82, **128**	23, 14	48	+
66	Morantel Tartrate Hydrate	221	**123**, 111	35, 25	73	+
67	Nafcillin	415	171, **199**	34, 14	78	+
68	Nitroxynil	289	**127,** 192	27, 20	99	-
69	Norfloxacin	320	**276**, 302	17, 20	76	+
70	Novobiocin	613	**218**, 396	13, 15	80	+
71	Orbifloxacin	396	**295**, 352	23, 17	83	+
72	Oxacillin Sodium Salt	402	**160**, 243	11, 13	68	+
73	Oxfendazole	316	**191**, 284	20, 18	68	+
74	Oxyclozanide	400	**364**, 382	17, 22	84	-
75	Oxyphenylbutazone	325	**160**, 162	20, 19	69	+
76	Oxytetracycline HCl	461	**426**, 443	18, 10	67	+
71	Orbifloxacin	396	**295**, 352	23, 17	83	+
72	Oxacillin Sodium Salt	402	**160**, 243	11, 13	68	+
73	Oxfendazole	316	**191**, 284	20, 18	68	+
74	Oxyclozanide	400	**364**, 382	17, 22	84	-
75	Oxyphenylbutazone	325	**160**, 162	20, 19	69	+
76	Oxytetracycline HCl	461	**426**, 443	18, 10	67	+
77	Penicillin G	335	202, **217**	23, 14	80	+
78	Phenylbutazone	309	120, **160**	42, 19	71	+
79	Pirlimycin HCl	411	112, **363**	25, 16	77	+
81	Prednisone	359	**237**, 341	19, 10	65	+
82	Promethazine HCl	285	86, **198**	17, 25	46	+
83	Propionylpromazine HCl	341	58, **268**	30, 23	68	+
84	Ractopamine HCl	302	**164**, 284	15, 10	53	+
85	Rafoxanide	624	**345,** 513	32, 36	188	-
86	Ronidazole	201	**140**, 55	10, 21	30	+

	Compound	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	S-Lens value	Polarity
87	Salbutamol	240	**148**, 222	17, 10	42	+
88	Sarafloxacin HCl Hydrate	386	**342**, 368	18, 21	85	+
89	Sulfachloropyridazine	285	108, **156**	24, 16	57	+
90	Sulfadiazin	251	**92**, 108	24, 22	48	+
91	Sulfadimethoxine	311	108, **156**	27, 20	72	+
92	Sulfaethoxypyridazine	295	108, **140**	25, 18	64	+
93	Sulfamerazine	265	108, **172**	24, 16	59	+
94	Sulfamethazine	279	156, **186**	18, 17	65	+
95	Sulfamethizole	271	**92**, 108	24, 22	47	+
96	Sulfamethoxazole	254	**108**, 156	23, 15	154	+
97	Sulfamethoxypyridazine	281	108, **156**	24, 16	63	+
98	Sulfanitran	334	198, **270**	28, 24	94	-
99	Sulfapyridine	250	108, **184**	24, 18	57	+
100	Sulfaquinoxaline	301	108, **156**	25, 17	69	+
101	Sulfathiazole	256	108, **156**	22, 15	52	+
102	Tetracycline HCl	445	**410**, 154	18, 28	67	+
103	Thiabendazole	202	131, **175**	32, 25	74	+
104	Thiamphenicol	354	185, **290**	19, 13	77	-
105	Tildipirosin	368	88, **98**	19, 28	64	+
106	Tilmicosin	435	174, **695**	23, 16	75	+
107	Tolfenamic Acid	262	**209**, 244	27, 15	47	+
108	Triclabendazole	359	**274**, 344	37, 25	101	+
109	Triclabendazole Sulfoxide	375	357, **360**	17, 21	79	+
110	Triflupromazine HCl	353	86, **248**	20, 42	68	+
111	Tulathromycin	404	**72**, 230	19, 10	62	+
112	Tylosin	917	**174**, 772	35, 27	134	+
113	Virginiamycin	526	**355**, 508	17, 12	72	+
114	Xylazine HCl	221	**90**, 147	22, 23	68	+
115	Enrofloxacin-*d5*	365	**321**	19	149	+
116	Thiamphenicol-*d3*	357	**188**	19	68	-

4.4 Results and discussion

4.4.1 Matrix effects

The occurrence of matrix effects is undoubtedly a major drawback of ESI due to co-eluting matrix interferences and ionization competition.[213] In the absence of chromatographic separation, matrix effects become even more pronounced, especially when more than 100 extracted analytes are ionized simultaneously. To the best of our knowledge, an evaluation of matrix effects for such a large number of analytes in a complex matrix such as tissue has yet to be reported in the literature for any ambient MS technique.

In this study, matrix effects were examined by comparing the slopes of matrix-matched calibration curves and reagent-only calibration curves via the following equation: ME% = (slope of matrix-matched calibration curve − slope of reagent-only calibration curve) × 100% / slope of reagent-only calibration curve. For matrix effects evaluation, matrix matched samples in the range of 0.5-3X were prepared by blotting the proper amount of calibration solutions on blades that were exposed to the blank matrix as per the analytical procedure described above, while reagent-only samples were prepared using the same procedure but using water as a matrix instead of beef. For the matrix effects calibration curves, the amount of analyte spotted on the blade was determined by comparing and approximating to the signal produced at its respective MRL value.

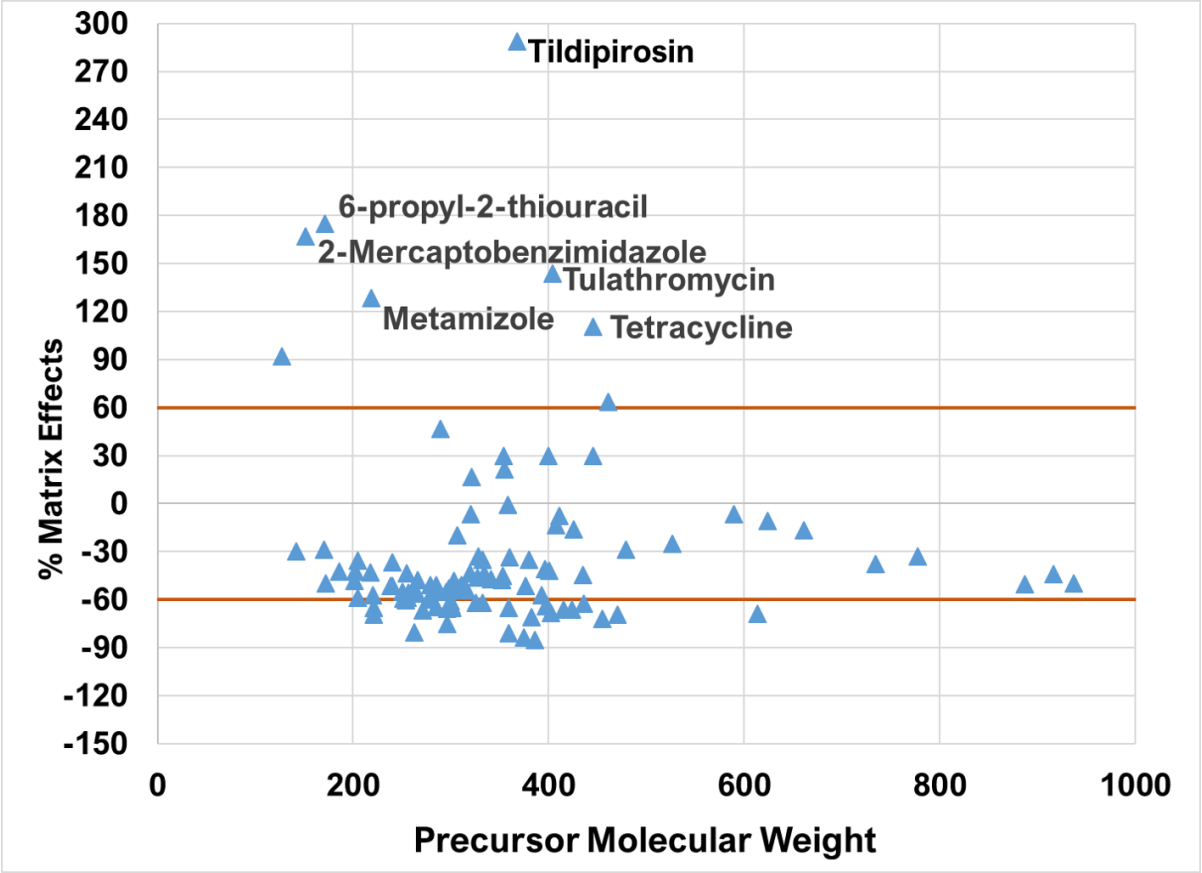


Figure 4.3 %MEs of selected drug analytes in beef tissue vs. their precursor molecular weight. Points above or below the red lines indicate %MEs ±60%. Compounds that underwent suppression or enhancement by more than 100% are labeled.

Figure 4.3 shows the matrix effects for the analytes studied versus their precursor molecular weight. Internal standard correction was not used when evaluating matrix effects. While there is no reference or golden standard to compare these results to, they are somewhat comparable to what is usually attained with LC-MS/MS methods using conventional sample preparation techniques.[91]

As shown in Figure 4.3, and Table 4.3,while most of the analytes underwent signal suppression, signal suppression or enhancement for the majority of analytes (69%) was less than 60%, which we consider remarkable in the absence of chromatographic separation given that more

than 100 analytes are being ionized simultaneously. This promising outcome is not surprising due to the biocompatible nature of the SPME coating used in the CBS devices and its ability to simultaneously isolate and enrich a wide variety of analytes with minimal co-extraction of proteins, lipids, salts, and other matrix macromolecules present in the matrix.[147,151] In addition, optimizing the extraction time based on S/N has proven again that shorter extraction times minimize the unnecessary coextraction of undesired molecules that may cause higher noise or ionization suppression, without compromising detection limits.[204] With that said, matrix effects in direct-to-MS methods cannot be eliminated entirely. However, they can be minimized and/or compensated by using matrix matched calibration curves and labeled isotopic internal standards with structural analogues that mimic the analytes of interest. The ideal internal standard for any analyte is a stable isotope labeled form of said analyte. However, due to the large number of analytes included in this study, this approach is not practical. A more practical approach is to use one class specific internal standard for each class of drugs, especially for analytes that exhibited significant matrix effects.

4.4.2 Selectivity

One potential drawback of direct-to-MS techniques is the lack of retention time, a factor that might lead to selectivity issues. Selectivity, which is the ability of a method to discriminate between the analyte of interest and closely related matrix components is typically the main consideration for identification of analytes in regulatory methods.[93,214] In the absence of retention time, another criterion that can be used for identification of target analytes is the ion ratio between the quantifying and qualifying ion transition signals. However, there is a concern that ion ratios

might be affected by interfering matrix components.[95,215] Matrix components in challenging matrices might interfere with either the qualifying ion or the quantifying ion, resulting in a significant deviation in ion ratios which can negatively impact qualitative outcome. Criteria for acceptable ion ratio deviations from the expected ion ratio vary depending on the country, however, a recent study by Berendsen et al. recommended a fixed ion ratio deviation tolerance of ±50% for veterinary drugs in challenging matrices such as animal tissue to minimize false negative findings.[214,215]

To study the influence of matrix components on ion ratio behavior, the data obtained from the matrix-matched calibration and reagent-only calibration, as described in the matrix effects section above, was used to calculate ion ratios in both matrix and reagent-only samples. Ion ratios were calculated by dividing the area of the less intense ion by the area of the higher intensity ion to ensure that the ion ratio was less than 1, although for certain analytes, the less intense ion was used for quantitative purposes, due to better S/N in the sample matrix. The reference ion ratio for each analyte was calculated as the average ratio obtained from all concentrations of the reagent-only calibration standards in the same sequence as the matrix calibration standards. The average matrix ion ratio for each analyte was the average ratio obtained from all concentrations of the matrix calibration standards. The relative deviation of matrix ion ratios from the corresponding reference ion ratios, in percent, was calculated by subtracting the reference ion ratio from each of the average matrix ion ratio and then dividing by the reference ion ratio.

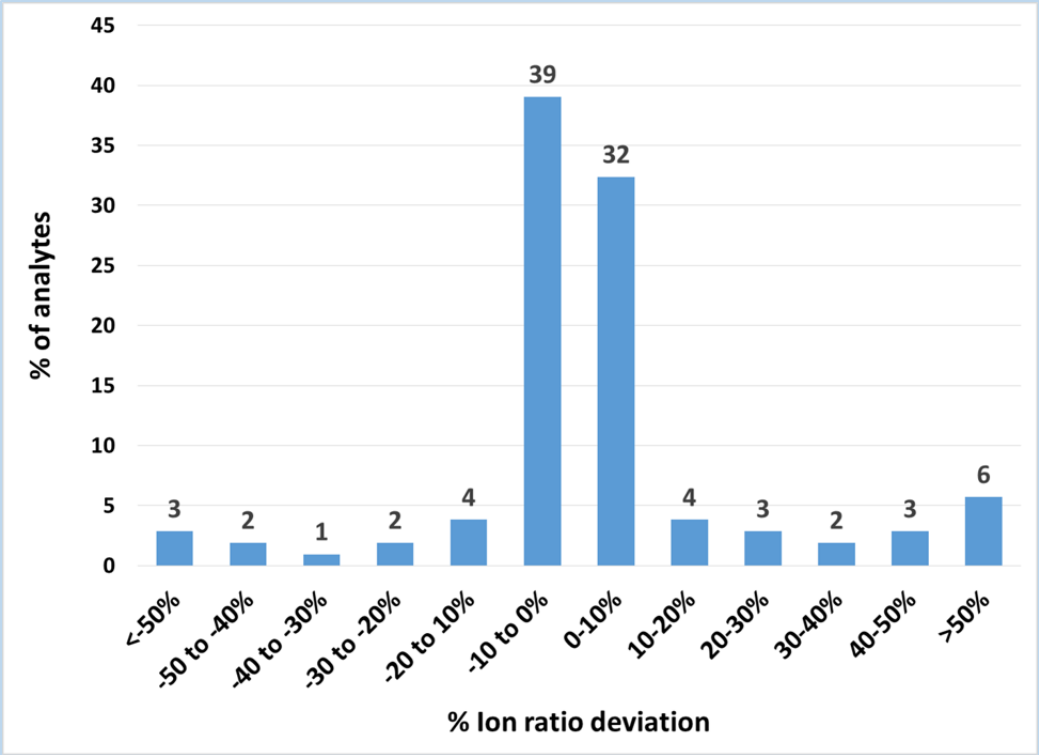


Figure 4.4 Distribution of relative ion ratio deviation value for target analytes in beef tissue from the average reference ratio.

As presented in figure 4.4, the majority of analytes (71%) exhibited relative ion ratio deviation values that were within ±10% of the reference ion ratio value. Only 9% of the analytes exhibited relative ion ratio deviation values that fell outside the ±50% level proposed by Berendsen at al.[214]

The extent of ion ratio variability in the matrix was also assessed at three different concentrations (0.75X, 1X, and 1.5X). As shown in Figure 4.5, the trend of variation was mostly independent on the concentrations except for four analytes, namely 6-phenyl thiouracil, cefazolin, diclofenac, and emamectin. Individual ion ratio deviation values for each analytes at each concentration are shown in Table 4.3. We found that higher deviations for these compounds were

mainly due to the presence of matrix components that interfere with qualifier ions that have low S/N range (<10) as also observed by Mol et al.[216] One particular compound, 6-phenyl thiouracil, displayed a significantly high relative ion ratio deviation (average 403%) due to a significant matrix interference with the qualifier ion transition (205→188). It is also worth noting that this transition is a neutral loss which is not favored for selectivity purposes. This observation further validates recommendations by Berendsen et al. to avoid neutral losses of 17 and 18 Da for proper identification of target analytes.[214] In future studies, we suggest the re-evaluation of the most intense ion transitions for these analytes and the selection of qualifier ions based on S/N levels in the matrix similar to the procedure followed with quantifier ion transitions.

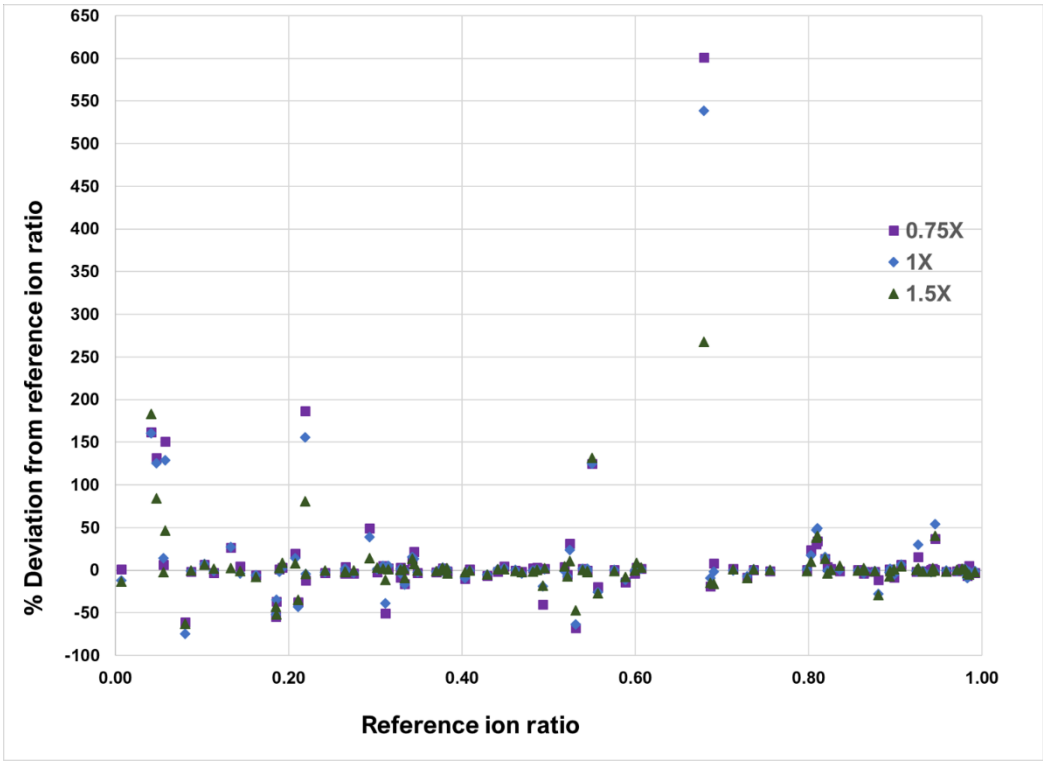


Figure 4.5 Relative ion ratio deviation (%) at three concentration levels (0.75X, 1X, and 1.5X) versus reference ion ratio value.

4.4.3 Linearity and limits of quantitation

Matrix-matched calibration curves were used to assess linearity and limits of quantitation. Calibration functions were constructed on the basis of the signal ratio of the analyte and IS (A/Is) for 7 concentration levels (from 0.25–2.5X) in three independent replicates. The calibration range was selected to include concentration levels bracketing the MRL. Despite the use of only one internal standard per ionization mode for correction for all the analytes, as illustrated in Figure 4.6, the majority of the target analytes (55%) achieved R^2 values higher than 0.99, while the other 45% achieved R^2 values between 0.95 and 0.99. While most analytes exhibited excellent linearity results, the suboptimal linearity results for the remaining analytes could be attributed to inappropriate internal standard correction or low detectability due to suboptimal coating chemistry or inappropriate desorption solvent.

LOQ was established as the lowest point of the matrix-matched calibration curve that produced a response that is both accurate when compared to the expected value (calculated via linear regression), within a 70−120% range of the true concentrations, as well as precise (≤25% RSD). As shown in Table 4.3, all analytes under study achieved low enough LOQs to ensure proper determination at the maximum residue levels set in Canada and the US. Individual determination coefficients and LOQ values for all compounds under study are listed in Table 4.3.

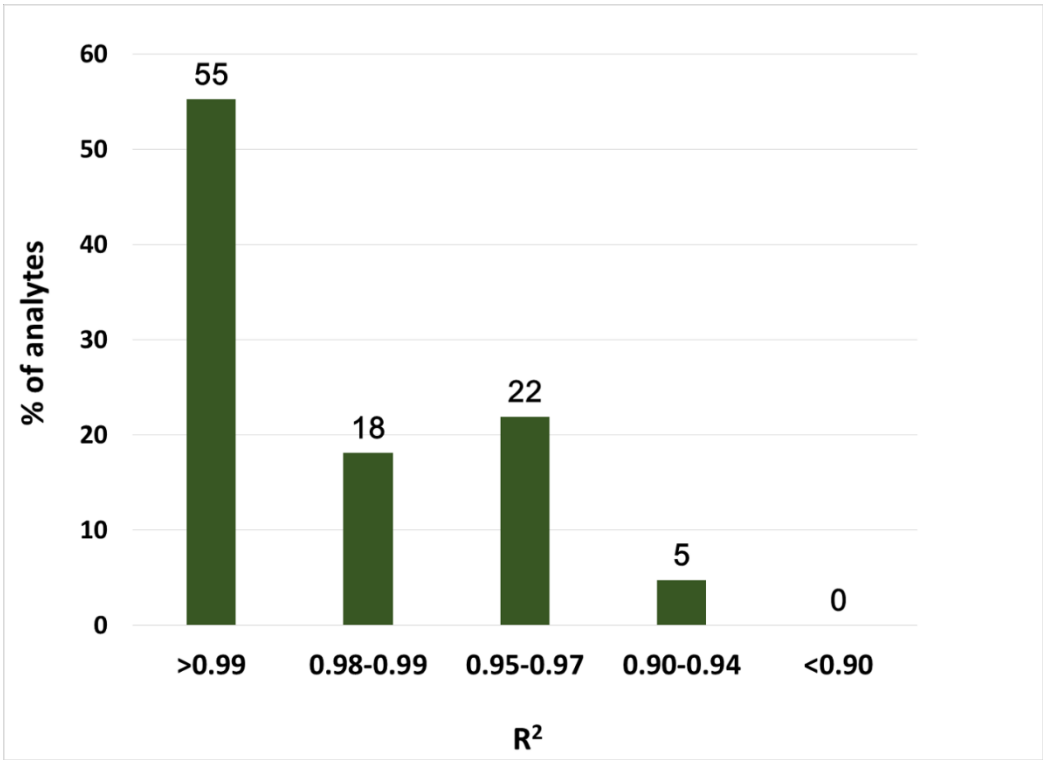


Figure 4.6 Linearity results of the optimized CBS method for the selected drug analytes in beef tissue expressed in percentage of analytes corresponding to R^2 value ranges.

4.4.4 Accuracy and precision

To evaluate the accuracy and precision of the method with respect to intra-day repeatability, pooled matrix blanks were spiked at three validation levels; low, mid, and high (0.4X, 0.75X and 1.5X) for each analyte, using six replicates per concentration (n=6). Accuracy was calculated based on estimated concentration values calculated from the linear regression equation of the matrix-matched calibration curve.

As presented in Figure 4.7 and Table 4.3, the majority of the analytes fell within a 70−120% range of the true concentrations of compounds, and yielded RSDs of ≤25% at the 0.4, 0.75 and

1.5X concentration levels, thus confirming that they can be quantitatively determined. Individual accuracy and %RSD values are listed on Table 4.3 for each analyte under study.

While the majority of analytes exhibited excellent results in terms of linearity, accuracy, and repeatability, the suboptimal analytical figures of merit for other compounds could be attributed to many reasons, such as inappropriate internal standard correction, unsuitable coating chemistry, or inappropriate elution/ionization solvent. These results were expected, considering that only one internal standard was used for correction per ionization mode and that the elution/ionization solvent was mainly optimized for negative ionization mode. The main purpose of this work was not SPME method optimization or validation, rather it was designed mainly to evaluate and report the ability of CBS to perform direct extraction from homogenized tissue samples and perform screening and quantitation in both negative and positive ionization modes with a single device in a single analytical run. Therefore, compromises were made, as it is usually the practice in multi-class multi-residue methods.

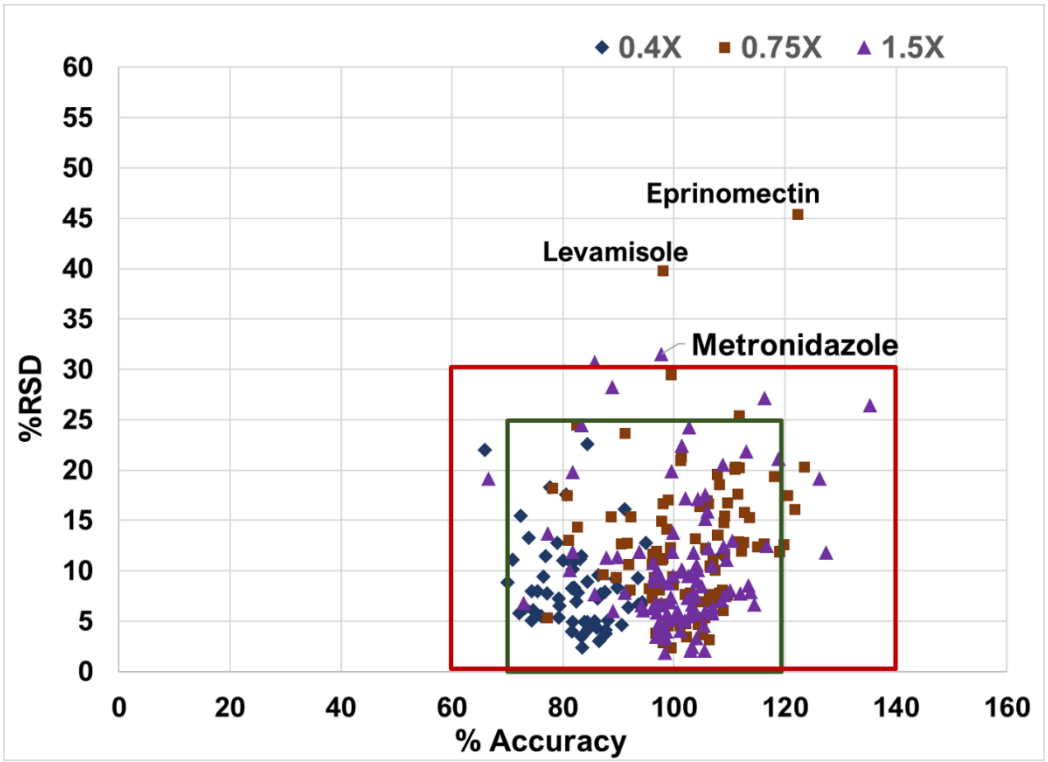


Figure 4.7 Accuracy and repeatability results of the optimized CBS method for the target analytes fortified at the 0.4, 0.75 and 1.5X levels in beef tissue (n = 6).

4.5 Conclusion and future perspectives

The present study demonstrates that the optimized CBS technique with the undercoating is a suitable approach to facilitate both rapid and high-throughput screening and quantitative determination of multi-residue drugs in complex matrices such as beef tissues in both negative and positive ionization modes, even when using only one IS per each ionization mode for such a wide range of analytes. Pairing the current method with automated CBS desorption/ionization will further improve the efficiency and robustness of the CBS technique for complex matrices such as biological tissues. Quantitative results can be further improved by using more analyte-specific internal standards, or at least one IS per each class of drugs. That said, it is important to be aware

that without the use of ion mobility or chromatography for separation prior to MS, it is difficult to distinguish between isomeric and isobaric analytes when employing direct-to-MS technology. In addition, while matrix effects can be minimized, they cannot be completely eliminated in direct to MS methods; thus, the use of matrix-matched calibration curves and the incorporation of analyte-specific internal standards remain the optimal procedure to attain reliable quantitative results in direct-to-MS analysis.

Table 4.3 Figures of merit for the quantitation of multi-residue veterinary drugs in beef tissue via undercoated CBS-MS/MS

Compound	MRL			%Accuracy (n=6)			%Repeatability (n=6)			% Matrix Effects	Ion Ratio Average (all concentrations)			% Ion Ratio Deviation Matrix		
	(ng g^{-1})	R^2	LOQ	0.4X	0.75X	1.5X	0.4X	0.75X	1.5X		Reagent	Matrix	% Deviation	0.75X	1X	1.5X
2-amino mebendazole	10	0.9931	0.5X		108	98		19	7	-52	0.98	0.91	-7	-7	-9	-3
2-Aminoflubendazole	10	0.9932	0.5X		110	97		17	7	-55	0.19	0.12	-37	-36	-35	-52
2-Mercaptobenzimidazole	25	0.9768	1X			67			19	167	0.04	0.11	171	162	161	153
2-Thiouracil	400	0.9448	1X			116			27	92	0.08	0.03	-66	-61	-74	-63
5-Hydroxythiabendazole	100	0.9809	0.5X		107	107		7	6	-43	0.80	0.80	0	1	1	-1
6-phenyl-thiouracil	400	0.9824	0.75X		105	102		16	17	-36	0.68	3.42	403	601	538	268
6-propyl-2-thiouracil	50	0.9934	0.75X			109			12	175	0.60	0.62	3	3	2	8
Acepromazine	10	0.9884	0.5X		108	91		7	8	-46	0.14	0.14	0	5	-4	-1
Albendazole	50	0.9714	0.5X		119	101		12	5	-48	0.44	0.44	0	-2	1	1
Albendazole 2-aminosulfone	50	0.9961	0.25X	79	92	97	7	11	10	-36	0.40	0.37	-9	-10	-9	-3
Albendazole sulfone	50	0.9967	0.25X	83	107	114	3	10	8	-53	0.93	0.92	-1	-1	-1	-1
Albendazole sulfoxide	50	0.9965	0.25X	76	102	110	6	5	8	-55	0.98	0.98	1	1	-1	2
Azaperone	10	0.9932	0.25X	77	104	98	8	5	9	-33	0.83	0.83	0	2	-1	0
Betamethasone	100	0.9980	0.25X	94	111	113	7	18	9	-57	0.06	0.06	5	7	14	-3
Bithionol	10	0.9827	0.5X		91	113		24	22	21	0.89	0.86	-4	1	-3	-7
Cambendazole	10	0.9946	0.5X		108	101		8	4	-48	0.74	0.75	2	0	1	1
Carazolol	10	0.9924	0.25X	86	103	98	4	8	7	-61	0.45	0.46	3	4	3	1
Carbadox	30	0.9984	0.25X	79	96	99	7	7	6	-55	0.69	0.72	4	8	-2	-16
Cefazolin	100	0.9904	0.5X		109	100		8	9	-66	0.05	0.11	130	132	126	84
Cephapirin	100	0.9250	0.25X	97	98	73	11	17	7	-72	0.49	0.49	1	3	1	0

Compound	MRL			%Accuracy (n=6)			%Repeatability (n=6)			% Matrix Effects	Ion Ratio Average (all concentrations)			% Ion Ratio Deviation Matrix		
	(ng g^{-1})	R^2	LOQ	0.4X	0.75X	1.5X	0.4X	0.75X	1.5X		Reagent	Matrix	% Deviation	0.75X	1X	1.5X
Chloramphenicol	10	0.9950	0.5X		104	104		5	7	17	0.93	1.18	27	16	30	3
Chlortetracycline	200	0.9986	0.25X	82	98	104	8	15	10	-29	0.01	0.01	-6	1	-12	-14
Chlorpromazine	10	0.9605	0.5X	90	99	86	10	12	8	-43	0.47	0.44	-5	-2	-4	-2
Clorsulon	100	0.9577	1X			100			12	-35	0.43	0.40	-8	-7	-5	-5
Cimaterol	10	0.9755	0.5X		98	81		11	10	-57	0.59	0.51	-13	-14	-11	-8
Ciprofloxacin	50	0.9983	0.25X	79	90	97	5	9	6	-35	0.81	1.15	42	34	49	40
Clenbuterol	10	0.9909	0.25X	79	96	96	13	9	6	-60	0.33	0.31	-6	-9	-4	0
Clindamycin	100	0.9860	0.25X	83	97	97	8	9	9	-16	0.09	0.09	-1	-2	-1	-1
Closantel	50	0.9899	1X			**135**			26	-17	0.84	0.85	1	-1	1	5
Cloxacillin	10	0.9792	0.25X	91	118	106	16	19	16	-62	0.69	0.57	-18	-19	-10	-15
Danofloxacin	70	0.9982	0.25X	73	92	99	6	8	5	-1	0.22	0.21	-5	-12	-5	-5
Desacetyl Cephapirin	100	0.9988	0.25X	88	91	90	8	13	11	-71	0.82	0.83	1	4	0	-4
Desethylene Ciprofloxacin	100	0.9987	0.25X	77	89	96	11	15	11	-20	0.28	0.27	-3	-3	-3	0
Diclofenac	200	0.9800	0.25X	90	108	103	8	20	10	-75	0.22	0.62	182	187	156	81
Dicloxacillin	100	0.9713	0.5X	94	111	97	12	20	10	-69	0.31	0.18	-42	-50	-39	-11
Difloxacin	100	0.9988	0.25X	88	104	97	5	5	4	-42	0.90	0.85	-5	-8	-5	1
Dimetridazole	50	0.9948	0.25X	83	81	82	11	17	20	-30	0.49	0.35	-30	-41	-19	-18
Doxycycline	100	0.9971	0.25X	86	104	104	7	13	11	30	0.10	0.11	7	7	8	7
Emamectin	10	0.9798	0.5X	84	120	94	18	13	12	-50	0.06	0.14	140	151	129	46
Enrofloxacin	20	0.9997	0.25X	83	99	98	2	2	2	-33	0.55	1.25	127	125	125	132
Eprinomectin	100	0.9812	0.75X			83			24	-50	0.13	0.18	34	27	27	3

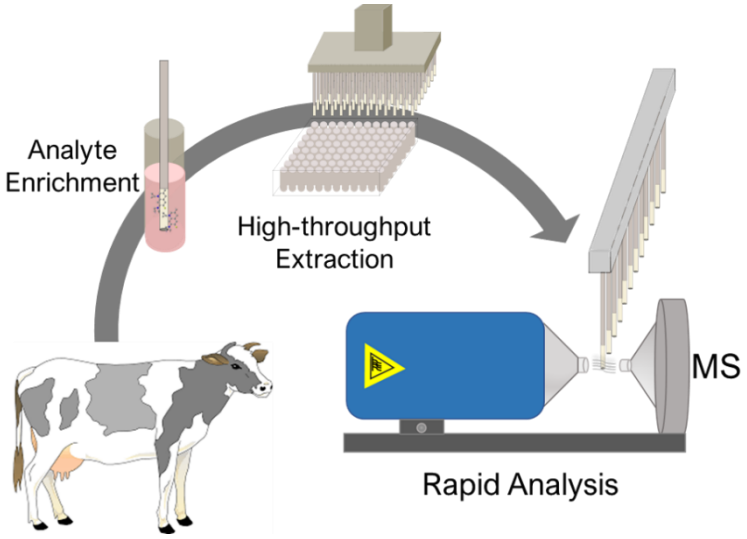
Compound	MRL			%Accuracy (n=6)			%Repeatability (n=6)			% Matrix Effects	Ion Ratio Average (all concentrations)			% Ion Ratio Deviation Matrix		
	(ng g^{-1})	R^2	LOQ	0.4X	0.75X	1.5X	0.4X	0.75X	1.5X		Reagent	Matrix	% Deviation	0.75X	1X	1.5X
Erythromycin	100	0.9929	0.25X	80	106	99	11	17	7	-38	0.73	0.67	-8	-9	-7	-9
Fenbendazole Sulfone	400	0.9956	0.25X	93	112	107	9	13	6	-62	0.54	0.54	-1	0	-1	-1
Flubendazole	10	0.9869	0.25X	95	109	107	13	15	6	-64	0.31	0.33	4	2	5	1
Flunixin	20	0.9900	0.5X		112	97		20	5	-55	0.35	0.34	-3	-3	-2	0
Gamithromycin	20	0.9814	0.25X	81	111	107	18	20	10	-33	0.19	0.19	-2	1	-2	2
Haloperidol	10	0.9850	0.5X		105	94		5	7	-51	0.98	0.99	1	1	2	1
Hydroxy ipronidazole	10	0.9445	0.5X		98	88		11	11	-42	0.34	0.39	14	11	15	13
Ipronidazole	10	0.9762	0.25X	82	101	104	10	21	17	-29	0.91	0.98	8	6	6	5
Ketoprofen	10	0.9760	0.5X		112	101		12	10	-44	0.52	0.49	-5	-5	-7	-7
Lasalocid A	1000	0.9791	0.5X		112	104		25	12	-7	0.19	0.20	5	3	2	9
Levamisole	100	0.9361	0.5X		98	89		**40**	28	-59	0.37	0.36	-2	-3	-2	-1
Lincomycin	100	0.9977	0.25X	74	87	97	13	10	10	-13	0.11	0.11	-2	-3	-1	2
Mebendazole	10	0.9892	0.25X	91	108	106	8	14	6	-66	0.21	0.24	17	20	14	8
Melengestrol Acetate	25	0.9508	0.5X		119	105		12	8	-64	0.82	0.95	16	13	16	13
Meloxicam	20	0.9924	0.5X		106	106		11	2	-48	0.41	0.41	1	1	0	0
Metamizole	200	0.9675	0.25X	66	78	77	22	18	14	129	0.56	0.43	-23	-20	-26	-27
Metronidazole	10	0.9641	0.5X		81	98		13	**32**	-50	0.48	0.48	0	3	0	-2
Morantel	100	0.9757	0.25X	78	106	102	18	12	7	-69	0.86	0.86	-1	-2	0	0
Nafcillin	100	0.9922	0.25X	92	115	112	6	12	8	-66	0.54	0.55	0	-1	2	-2
Nitroxynil	50	0.9787	0.5X	107	99	101		14	22	47	0.38	0.38	0	0	-1	1
Norfloxacin	50	0.9997	0.25X	82	92	97	8	13	10	-7	0.81	1.11	37	30	47	38

Compound	MRL			%Accuracy (n=6)			%Repeatability (n=6)			% Matrix Effects	Ion Ratio Average (all concentrations)			% Ion Ratio Deviation Matrix		
	(ng g^{-1})	R^2	LOQ	0.4X	0.75X	1.5X	0.4X	0.75X	1.5X		Reagent	Matrix	% Deviation	0.75X	1X	1.5X
Novobiocin	1000	0.9727	0.5X		109	97		15	9	-69	0.89	0.90	0	1	2	-1
Orbifloxacin	50	0.9969	0.25X	86	97	97	5	4	3	-41	0.88	0.86	-1	-2	-1	-1
Oxacillin	100	0.9819	0.25X	88	120	127	4	18	12	-68	0.94	0.94	-1	2	-1	2
Oxfendazole	800	0.9923	0.25X	94	107	101	7	11	6	-54	0.97	0.97	0	-1	-1	0
Oxyclozanide	10	0.9455	1X			119			21	30	0.52	0.54	4	4	-1	5
Oxyphenylbutazone	100	0.9820	0.5X	95	113	109	9	16	11	-62	0.38	0.38	-2	-2	-3	-3
Oxytetracycline	200	0.9995	0.25X	82	96	104	11	11	9	64	0.38	0.39	3	2	3	2
Phenylbutazone	100	0.9903	0.5X		116	101		13	10	-53	0.31	0.33	6	5	5	2
Pirlimycin	300	0.9902	0.25X	72	92	106	15	15	18	-8	0.38	0.38	1	-1	2	2
Prednisone	100	0.9940	0.25X	94	114	117	7	15	13	-65	0.19	0.09	-52	-55	-52	-43
Promethazine	10	0.9905	0.5X		110	89		8	6	-51	0.58	0.57	0	1	0	-1
Propionylpromazine	10	0.9812	0.5X	65	101	111		21	13	-47	0.88	0.72	-18	-12	-28	-29
Ractopamine	30	0.9961	0.25X	83	95	99	11	8	9	-53	0.80	0.98	22	24	17	10
Rafoxanide	10	0.9589	1X			126			19	-11	0.27	0.27	1	4	-1	-2
Ronidazole	10	0.9581	0.75X		82	86		24	**31**	-48	0.29	0.43	47	49	39	14
Salbutamol	10	0.9874	0.25X	72	83	82	6	14	12	-51	0.52	0.68	29	32	24	11
Sarafloxacin	50	0.9978	0.25X	86	96	94	3	12	6	-85	0.95	1.37	45	37	54	41
Sulfachloropyridazine	100	0.9948	0.25X	84	106	109	5	8	8	-64	0.54	0.54	1	2	1	1
Sulfadiazin	100	0.9913	0.25X	76	97	100	9	12	5	-59	0.96	0.95	-1	-2	-1	-2
Sulfadimethoxine	100	0.9951	0.25X	84	106	103	4	3	2	-49	0.46	0.46	0	0	0	-1
Sulfaethoxypyridazine	100	0.9908	0.25X	88	106	104	4	5	6	-55	0.86	0.86	0	0	0	0

Compound	MRL			%Accuracy (n=6)			%Repeatability (n=6)			% Matrix Effects	Ion Ratio Average (all concentrations)			% Ion Ratio Deviation Matrix		
	(ng g^{-1})	R^2	LOQ	0.4X	0.75X	1.5X	0.4X	0.75X	1.5X		Reagent	Matrix	% Deviation	0.75X	1X	1.5X
Sulfamerazine	100	0.9910	0.25X	74	100	104	5	9	3	-56	0.95	0.94	0	1	-1	0
Sulfamethazine	100	0.9902	0.25X	75	102	105	6	8	5	-51	0.24	0.24	-2	-3	-3	0
Sulfamethizole	100	0.9923	0.25X	84	105	103	5	4	6	-67	0.92	0.92	-1	-1	-1	0
Sulfamethoxazole	100	0.9966	0.25X	82	105	108	4	7	7	-61	0.94	0.93	-1	0	-2	-1
Sulfamethoxypyridazine	100	0.9923	0.25X	82	102	104	5	3	6	-55	0.76	0.75	0	-1	0	0
Sulfanitran	10	0.9773	0.25X	109	109	114	12	12	7	-44	0.33	0.34	1	1	0	2
Sulfapyridine	100	0.9901	0.25X	74	98	103	8	7	2	-55	0.99	0.97	-3	-3	-1	-2
Sulfaquinoxaline	100	0.9941	0.25X	91	108	107	5	14	6	-65	0.60	0.59	-2	-4	-1	0
Sulfathiazole	100	0.9902	0.25X	82	99	99	7	5	3	-59	0.61	0.63	3	2	2	3
Tetracycline	200	0.9903	0.25X	86	97	99	10	8	7	111	0.38	0.38	1	0	0	2
Thiabendazole	100	0.9963	0.25X	87	109	101	8	6	6	-42	0.71	0.72	1	2	0	1
Thiamphenicol	10	0.9986	0.25X	83	101	102	8	5	5	30	0.99	0.98	-1	6	-2	-5
Tildipirosin	400	0.9855	0.25X	70	99	106	9	17	15	289	0.16	0.15	-6	-6	-6	-8
Tilmicosin	100	0.9974	0.25X	84	100	103	9	9	8	-45	0.33	0.34	2	3	2	0
Tolfenamic acid	200	0.9653	1X			103			24	-80	0.21	0.13	-40	-37	-43	-35
Triclabendazole	50	0.9707	0.75X		123	109		20	21	-81	0.86	0.84	-2	-3	-4	2
Triclabendazole Sulfoxide	50	0.9934	1X			106			12	-83	0.53	0.19	-64	-67	-63	-47
Trifluropromazine	10	0.9881	0.25X	84	122	100	23	16	14	-45	0.27	0.26	-3	-4	2	-2
Tulathromycin	1000	0.9878	0.25X	71	99	100	11	30	20	144	0.30	0.30	0	-2	1	3
Tylosin	200	0.9935	0.25X	75	106	103	8	17	9	-44	0.50	0.50	1	1	1	2
Virginiamycin	100	0.9958	0.25X	89	113	103	9	13	7	-25	0.33	0.28	-17	-16	-17	-9
Xylazine	10	0.9986	0.5X		98	99		3	4	-65	0.34	0.41	18	22	13	7

Chapter 5

Rapid and High-throughput Screening of Multi-residue Pharmaceutical Drugs in Bovine Tissue using Solid Phase Microextraction and Direct Analysis in Real Time-Tandem Mass Spectrometry (SPME-DART-MS/MS)



5.1 Preamble

The materials in this chapter have been accepted as a research article in Talanta: Khaled, A.; Belinato, J. R.; Pawliszyn, J. Rapid and High-throughput Screening of Multi-Residue Pharmaceutical Drugs in Bovine Tissue Using Solid Phase Microextraction and Direct Analysis in Real Time-Tandem Mass Spectrometry (SPME-DART-MS/MS). *Talanta* 2020, 121095. Materials for all sections of this current chapter are reprinted from this research article with the permission of Elsevier, Copyright, 2020. Copyright for this work remains the property of Elsevier

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The contribution of co-author Joao Raul Belinato to the work described in this chapter include assistance in the execution of selected experiments and in processing data. All of the experimental planning, design, and experimental work conducted in the laboratory, data analysis, interpretation, and writing were performed by the author of the thesis.

I, Joao Raul Belinato, authorize Abir Khaled to use the material for her thesis.

5.2 Introduction

Many pharmaceutical compounds are considered as emerging contaminants in both environment and food.[217] One source of these contaminants in animal-derived food is the widespread use of veterinary drugs as therapeutic drugs and growth promoters in animal husbandry.[218,219] The monitoring of veterinary drug residues in food-producing animal tissues has gained significance for several reasons, including their potential risk to human health as well as their potential role on microbial antibiotic resistance.[7,14–17,197,198] These concerns have led to stringent food safety regulations, prompting increased demand for rapid and high-throughput analysis of real samples. Mass spectrometry (MS) is presently recognized as one of the most powerful techniques available for qualitative and quantitative analysis of a wide range of analytes and matrices due its speed, selectivity, and high detectability.[5,199,220–222] However, conventional MS instrumental analysis is typically preceded by a classical analytical workflow that often includes sampling, sample preparation, extraction, clean-up, and chromatographic separation, with

sample clean-up and separation considered as the two main bottlenecks of the process.[92,96,98,202,223–225] While every step of the analytical workflow is crucial for method performance, the slowest steps determine the overall speed of the process.[223–226]

In the food analysis field, where fast decisions need to be made regarding food safety, use of conventional sample preparation and chromatographic methods may not always be suitable.[22] Given the perishable nature of food, as well as the potential catastrophic risks to human health associated with the distribution of contaminated food, food safety investigations demand the use of extremely fast, efficient, and reliable analytical methods. An alternative to overcome lengthy workflows and improve efficiency is to minimize sample preparation steps and skip chromatographic separation altogether.[96,225,227,228]

This solution to the constraints of conventional analytical techniques has laid the foundation for the development of ambient ionization mass spectrometry (AIMS), which encompasses a group of direct-to-MS techniques where analyte desorption/ionization take place in the atmospheric environment.[139,208,221,222,225,229,230] The direct analysis in real time (DART) approach, introduced by Cody and Laramee in 2005, is one of the pioneers in this increasingly popular field.[231] Since its first introduction, DART has become one of the most commonly used analytical techniques in the food analysis field due to its commercial availability and its versatile sample-introduction approach.[225,232] However, despite the abundance of applications of DART-MS in food analysis, there are no studies on the analysis of multi-residue veterinary drugs in food-animal tissue.[233,234] To the best of our knowledge, there is only one reported use of DART-MS in this field to date: a report by Martínez-Villalba et al. on the analysis of 34 antiparasitic veterinary drugs, namely

benzimidazoles in bovine milk and coccidiostats in chicken feed.[235] The reported method entailed extensive QuEChERS sample preparation and clean-up steps. We believe the evidenced lack of research in this field could be attributed to many factors, such as the complex nature of animal tissue and the tendency of DART to break down labile compounds, such as some pharmaceutical drugs, compared to other soft ionization techniques such as electrospray ionization (ESI) techniques.[54,96,221,222,226,236–239]

To maximize the performance of DART, an ideal sample introduction method should be able to enrich analytes and reduce matrix interferences to minimal levels while maintaining accuracy and reproducibility. As already demonstrated by its successful coupling with many other AIMS techniques, SPME stands as an ideal candidate for this application due to its intrinsic features, which allow integration of sampling, extraction, clean-up, and instrumental introduction into a single step.[120,141,204,207,208,221,222,240] Another distinctive feature of SPME in ambient MS analysis is that SPME devices can be used as the desorption/ionization probe.[225] Moreover, biocompatible coatings used in SPME have proven to minimize co-extraction of interferences commonly found in biological samples such as macromolecules, phospholipids, and salts [120,147,189,206]. Furthermore, SPME can be successfully automated and used in high-throughput format for rapid qualitative and quantitative analyses of complex matrices.[139,189,204,206,240]

SPME–DART-MS/MS has been successfully applied to different food matrices and biofluids using a variety of geometrical SPME configurations; of these, meshes have been shown to offer the best detectability and successful geometry for most biological matrices.[139–141,230] However, the use of meshes for extraction from diluted homogenized animal tissue samples is not

applicable due to potential attachment of matrix debris and macromolecules to the mesh. Recently, an alternative geometry of SPME, namely the SPME pin, was developed specifically for direct immersion extraction of diluted homogenized biological tissue samples. Comprised of stainless-steel rods coated with biocompatible hydrophilic–lipophilic balance HLB/ polyacrylonitrile (PAN), these SPME pins were successfully used for high-throughput extraction and quantitative analysis of more than 100 multi-residue veterinary drugs in chicken and beef tissue via LC-MS/MS.[189,206] In this study, we investigate the feasibility of using the said SPME pins with DART-MS/MS for rapid monitoring and quantitation of multi-residue drugs in animal-food tissue.

To the best of our knowledge, this is the first study entailing the use of DART-MS/MS for screening of multi-residue drugs in beef tissue. In addition, the developed method was evaluated with respect to selectivity, linearity, accuracy, intra-day repeatability, and limit of quantitation (LOQ).

5.3 Materials and Methods

5.3.1 Target Analytes

A total of 98 veterinary drugs encompassing a wide range of physico-chemical properties (log P −1.85–9.36) were initially selected for analysis as part of this study. These compounds were successfully quantified below their MRLs in our previous reports using SPME-LC-ESI-MS/MS,[206] and coated blade spray (CBS)-MS/MS,[240] with the exception of florfenicol and florfenicol amine in case of CBS-MS/MS. Analytes were selected from the list of standards specified in the official method developed by the United States Department of Agriculture for screening and confirmation

of animal drug residues (CLG-MRM1.08).[31] Further details pertaining to these compounds, including their physico-chemical properties, drug classes, and supplier information, are provided in Table 5.1.

A total of 46 analytes (bolded in Table 5.1) were excluded from further evaluation studies following final method optimization due to a lack of quantifiable signals at their respective MRLs. The MRL of a given compound is defined by Health Canada as the maximum concentration of residue that could safely remain in the tissue or food product derived from a food-producing animal that has been treated with a veterinary drug.[82] MRL values utilized in this work (listed in Table 5.1) were based primarily on Canadian MRL values and US tolerances in beef tissues.[82,91] In general, Canadian MRLs are either equal or lower than US tolerances; however, in cases where Canadian MRLs were higher than US tolerances, the US regulatory value was selected, as is the case for morantel and ketoprofen. Sulfadoxine was also not evaluated in this study as it is an isobar of sulfadimethoxine. Two isotopically labelled internal standards (IS), namely sulfamethazine-(phenyl-$^{13}C_6$) and xylazine-d_6 were procured from Sigma Aldrich (Oakville, ON, Canada).

Table 5.1 Physico-chemical properties, class, and supplier information for analytes under study.

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[1]	Log P[1]	MRL (ng g-1)
1	2-Aminoflubendazole	Sigma-Aldrich	Anthelmintics	$C_{14}H_{10}FN_3O$	255.2	1.96	10
2	**2-Amino mebendazole**	**Sigma-Aldrich**	**Anthelmintics**	**$C_{14}H_{11}N_3O$**	**237.3**	**1.74**	**10**
3	**2-Quinoxalinecarboxylic acid**	**Sigma-Aldrich**	**Others**	**$C_9H_6N_2$**	**174.2**	**1.80**	**100**
4	5-Hydroxythiabendazole	Sigma-Aldrich	Anthelmintics	$C_{10}H_7N_3OS$	217.3	1.73	100
5	6-Phenyl-2-thiouracil	Sigma-Aldrich	Thyreostats	$C_{10}H_8N_2OS$	204.3	-0.10	400
6	**6-Propyl-2-thiouracil**	**Sigma-Aldrich**	**Thyreostats**	**$C_7H_{10}N$**	**170.2**	**1.37**	**50**
7	Acepromazine Maleate	Sigma-Aldrich	Tranquilizers	$C_{19}H_{22}N_2OS$	326.5	4.08	10
8	Albendazole	Sigma-Aldrich	Anthelmintics	$C_{12}H_{15}N_3O_2S$	265.3	3.07	50
9	Albendazole-2-aminosulfone	Sigma-Aldrich	Anthelmintics	$C_{10}H_{13}N_3O_2S$	239.3	0.12	50
10	**Albendazole Sulfone**	**Sigma-Aldrich**	**Anthelmintics**	**$C_{12}H_{15}N_3O_4S$**	**297.1**	**0.86**	**50**
11	Albendazole Sulfoxide	Sigma-Aldrich	Anthelmintics	$C_{12}H_{15}N_3O_3S$	281.3	0.91	50
12	**Ampicillin**	**Sigma-Aldrich**	**β-Lactams/cephalosporins**	**$C_{16}H_{19}N_3O_4S$**	**349.4**	**1.35**	**10**
13	Azaperone	Sigma-Aldrich	Tranquilizers	$C_{19}H_{22}FN_3O$	327.4	2.50	10
14	**Betamethasone**	**Sigma-Aldrich**	**Anti-inflammatories**	**$C_{22}H_{29}FO_5$**	**392.2**	**1.87**	**100**
15	Cambendazole	Sigma-Aldrich	Anthelmintics	$C_{14}H_{14}N_4O_2S$	302.4	2.90	10
16	Carazolol	Sigma-Aldrich	Tranquilizers	$C_{18}H_{22}N_2O_2$	298.4	3.59	10
17	Carbadox	Sigma-Aldrich	Others	$C_{11}H_{10}N_4O_4$	262.2	-1.22	30
18	**Cefazolin**	**Sigma-Aldrich**	**β-Lactams/cephalosporins**	**$C_{14}H_{14}N_8O_4S_3$**	**454.507**	**1.13**	**100**

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[1]	Log P[1]	MRL (ng g-1)
19	Chlorpromazine HCl	Sigma-Aldrich	Tranquilizers	$C_{17}H_{20}Cl_2N_2S$	355.3	5.20	10
20	**Chlortetracycline HCl**	**Sigma-Aldrich**	**Tetracyclines**	**$C_{22}H_{24}Cl_2N_2O_8$**	**515.3**	**1.11**	**200**
21	**Ciprofloxacin**	**Sigma-Aldrich**	**Fluoroquinolones**	**$C_{17}H_{18}FN_3O_3$**	**331.341**	**0.65**	**50**
22	**Clenbuterol HCl**	**Sigma-Aldrich**	**β-Agonists**	**$C_{12}H_{19}Cl_3N_2O$**	**313.7**	**2.61**	**10**
23	Clindamycin HCl	Sigma-Aldrich	Macrolides/lincosamides	$C_{18}H_{34}Cl_2N_2O_5S$	461.4	1.83	100
24	**Cloxacillin Sodium Salt**	**Sigma-Aldrich**	**β-Lactams/cephalosporins**	**$C_{19}H_{17}ClN_3NaO_5S$**	**457.9**	**2.53**	**100**
25	Danofloxacin	Sigma-Aldrich	Fluoroquinolones	$C_{19}H_{20}FN_3O_3$	357.1	1.20	70
26	**Desacetyl cephapirin**	**TRC[2]**	**β-Lactams/cephalosporins**	**$C_{15}H_{15}N_3O_5S_2$**	**381.427**	**0.32**	**100**
27	**Desethylene ciprofloxacin**	**TRC[2]**	**Fluoroquinolones**	**$C_{15}H_{17}ClFN_3O_3$**	**341.8**	**-0.14**	**100**
28	Diclofenac Sodium	Sigma-Aldrich	Anti-inflammatories	$C_{14}H_{10}Cl_2NNaO_2$	318.1	4.06	200
29	**Dicloxacillin**	**Sigma-Aldrich**	**β-Lactams/cephalosporins**	**$C_{19}H_{16}Cl_2N_3NaO_5S.xH_2O$**	**492.3**	**3.02**	**100**
30	**Difloxacin HCl**	**Sigma-Aldrich**	**Fluoroquinolones**	**$C_{21}H_{20}ClF_2N_3O_3$**	**435.9**	**2.78**	**50**
31	Dimetridazole	Sigma-Aldrich	Coccidiostats	$C_5H_7N_3O_2$	141.0	0.31	50
32	**Doxycycline HCl**	**Sigma-Aldrich**	**Tetracyclines**	**$C_{22}H_{25}ClN_2O_8$**	**480.9**	**-0.54**	**100**
33	**Emamectin Benzoate**	**Sigma-Aldrich**	**Anthelmintics**	**$C_{56}H_{81}NO_{15}$**	**1008.2**	**6.84**	**10**
34	**Enrofloxacin**	**Sigma-Aldrich**	**Fluoroquinolones**	**$C_{19}H_{22}FN_3O_3$**	**359.4**	**1.88**	**20**
35	**Erythromycin**	**Sigma-Aldrich**	**Macrolides/lincosamides**	**$C_{37}H_{67}NO_{13}$**	**733.9**	**2.83**	**125**
36	Fenbendazole Sulfone	Sigma-Aldrich	Anthelmintics	$C_{15}H_{13}N_3O_4S$	331.3	1.70	400
37	**Florfenicol**	**Sigma-Aldrich**	**Phenicols**	**$C_{12}H_{14}Cl_2FNO_4S$**	**357.0**	**-0.12**	**300**
38	**Florfenicol amine**	**Sigma-Aldrich**	**Phenicols**	**$C_{10}H_{14}FNO_3S$**	**247.3**	**-0.80**	**100**
39	**Flubendazole**	**Sigma-Aldrich**	**Anthelmintics**	**$C_{16}H_{12}FN_3O_3$**	**313.3**	**3.05**	**10**
40	**Flunixin**	**Sigma-Aldrich**	**Anti-inflammatories**	**$C_{14}H_{11}F_3N_2O_2$**	**296.2**	**5.40**	**20**
41	**Gamithromycin**	**Sigma-Aldrich**	**Macrolides/lincosamides**	**$C_{40}H_{76}N_2O_{12}$**	**776.5**	**3.89**	**20**

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[1]	Log P[1]	MRL (ng g-1)
42	Haloperidol	Sigma-Aldrich	Tranquilizers	$C_{21}H_{23}ClFNO_2$	375.9	3.01	10
43	Hydroxy dimetridazole	TRC[2]	Coccidiostats	$C_5H_7N_3O_3$	157.1	-0.49	50
44	**Hydroxy ipronidazole**	**Sigma-Aldrich**	**Coccidiostats**	**$C_7H_{11}N_3O_3$**	**185.2**	**0.21**	**10**
45	Hydroxy metronidazole	Sigma-Aldrich	Coccidiostats	$C_6H_9N_3O_4$	187.15	-0.81	10
46	Ipronidazole	Sigma-Aldrich	Coccidiostats	$C_7H_{11}N_3O_2$	169.2	1.18	10
47	**Ketoprofen**	**Sigma-Aldrich**	**Anti-inflammatories**	**$C_{16}H_{14}O_3$**	**254.3**	**2.81**	**10**
48	Levamisole HCl	Sigma-Aldrich	Anthelmintics	$C_{11}H_{13}ClN_2S$	240.8	1.85	100
49	Lincomycin HCl Monohydrate	Sigma-Aldrich	Macrolides/lincosamides	$C_{18}H_{37}ClN_2O_7S$	461.0	0.91	100
50	Mebendazole	Sigma-Aldrich	Anthelmintics	$C_{16}H_{13}N_3O_3$	295.3	2.83	10
51	**Melengestrol Acetate**	**Sigma-Aldrich**	**Others**	**$C_{25}H_{32}O_4$**	**396.5**	**4.21**	**25**
52	Meloxicam	Sigma-Aldrich	Anti-inflammatories	$C_{14}H_{13}N_3O_4S_2$	351.0	2.71	20
53	Metamizole	Sigma-Aldrich	Anti-inflammatories	$C_{13}H_{17}N_3O_4S$	311.0	-0.74	200
54	Metronidazole	Sigma-Aldrich	Coccidiostats	$C_6H_9N_3O_3$	171.2	-0.01	10
55	Morantel Tartrate Hydrate	Sigma-Aldrich	Anthelmintics	$C_{16}H_{22}N_2O_6S\ xH_2O$	370.4	1.97	150
56	**Nafcillin**	**Sigma-Aldrich**	**β-Lactams/cephalosporins**	**$C_{21}H_{22}N_2O_5S$**	**414.1**	**3.52**	**100**
57	**Norfloxacin**	**Sigma-Aldrich**	**Fluoroquinolones**	**$C_{16}H_{18}FN_3O_3$**	**319.3**	**0.82**	**50**
58	**Novobiocin**	**Sigma-Aldrich**	**Novobiocin**	**$C_{31}H_{36}N_2O_{11}$**	**612.2**	**2.86**	**1000**
59	Orbifloxacin	Sigma-Aldrich	Fluoroquinolones	$C_{19}H_{20}F_3N_3O_3$	395.4	2.37	50
60	**Oxacillin Sodium Salt**	**Sigma-Aldrich**	**β-Lactams/cephalosporins**	**$C_{19}H_{18}N_3O_5SNa\ H_2O$**	**441.4**	**2.05**	**100**
61	Oxfendazole	Sigma-Aldrich	Anthelmintics	$C_{15}H_{13}N_3O_3S$	315.3	1.36	800
62	**Oxyclozanide**	**Sigma-Aldrich**	**Anthelmintics**	**$C_{13}H_6Cl_5NO_3$**	**401.5**	**8.67**	**10**

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[1]	Log P[1]	MRL (ng g-1)
63	**Oxyphenylbutazone**	**Sigma-Aldrich**	**Anti-inflammatories**	**$C_{19}H_{20}N_2O_3$**	**324.4**	**2.72**	**100**
64	**Oxytetracycline HCl**	**Sigma-Aldrich**	**Tetracyclines**	**$C_{22}H_{25}ClN_2O_9$**	**496.9**	**-1.50**	**200**
65	**Penicillin G**	**TRC**	**β-Lactams/cephalosporins**	**$C_{16}H_{18}N_2O_4S$**	**334.0**	**1.67**	**50**
66	Phenylbutazone	Sigma-Aldrich	Anti-inflammatories	$C_{19}H_{20}N_2O_2$	308.4	3.16	100
67	Pirlimycin HCl	TRC[2]	Macrolides/lincosamides	$C_{17}H_{32}Cl_2N_2O_5S$	447.4	1.47	300
68	**Prednisone**	**Sigma-Aldrich**	**Anti-inflammatories**	**$C_{21}H_{26}O_5$**	**358.4**	**1.57**	**100**
69	Promethazine HCl	Sigma-Aldrich	Tranquilizers	$C_{17}H_{21}ClN_2S$	320.9	4.78	10
70	Propionylpromazine HCl	Sigma-Aldrich	Tranquilizers	$C_{20}H_{25}ClN_2OS$	376.9	4.61	10
71	**Ractopamine HCl**	**Sigma-Aldrich**	**β-Agonists**	**$C_{18}H_{24}ClNO_3$**	**337.8**	**1.65**	**30**
72	Ronidazole	Sigma-Aldrich	Coccidiostats	$C_6H_8N_4O_4$	200.0	-0.45	10
73	**Salbutamol**	**Sigma-Aldrich**	**β-Agonists**	**$C_{13}H_{21}NO_3$**	**239.1**	**0.01**	**10**
74	**Sarafloxacin HCl Hydrate**	**Sigma-Aldrich**	**Fluoroquinolones**	**$C_{20}H_{17}F_2N_3O_3$ HCl xH_2O**	**421.8**	**2.09**	**50**
75	Sulfachloropyridazine	Sigma-Aldrich	Sulfonamides	$C_{10}H_9ClN_4O_2S$	284.7	1.02	100
76	Sulfadiazine	Sigma-Aldrich	Sulfonamides	$C_{10}H_{10}N_4O_2S$	250.277	-0.12	100
77	Sulfadimethoxine	Sigma-Aldrich	Sulfonamides	$C_{12}H_{14}N_4O_4S$	310.3	1.48	100
78	Sulfaethoxypyridazine	Sigma-Aldrich	Sulfonamides	$C_{12}H_{14}N_4O_3S$	294.3	0.85	100
79	Sulfamerazine	Sigma-Aldrich	Sulfonamides	$C_{11}H_{12}N_4O_2S$	264.0	0.34	100
80	Sulfamethazine	Sigma-Aldrich	Sulfonamides	$C_{12}H_{14}N_4O_2S$	278.0	0.80	100
81	Sulfamethizole	Sigma-Aldrich	Sulfonamides	$C_9H_{10}N_4O_2S_2$	270.0	0.51	100
82	Sulfamethoxazole	Sigma-Aldrich	Sulfonamides	$C_{10}H_{11}N_3O_3S$	253.0	0.89	100
83	Sulfamethoxypyridazine	Sigma-Aldrich	Sulfonamides	$C_{11}H_{12}N_4O_3S$	280.3	0.32	100
84	Sulfapyridine	Sigma-Aldrich	Sulfonamides	$C_{11}H_{11}N_3O_2S$	249.3	0.03	100

	Compound	Supplier	Class	Formula[1]	Molecular Mass (Da)[1]	Log P[1]	MRL (ng g-1)
85	Sulfaquinoxaline	Sigma-Aldrich	Sulfonamides	$C_{14}H_{12}N_4O_2S$	300.0	1.30	100
86	Sulfathiazole	Sigma-Aldrich	Sulfonamides	$C_9H_9N_3O_2S_2$	255.3	0.05	100
87	**Tetracycline HCl**	**Sigma-Aldrich**	**Tetracyclines**	**$C_{22}H_{25}ClN_2O_8$**	**480.9**	**-1.47**	**200**
85	Sulfaquinoxaline	Sigma-Aldrich	Sulfonamides	$C_{14}H_{12}N_4O_2S$	300.0	1.30	100
86	Sulfathiazole	Sigma-Aldrich	Sulfonamides	$C_9H_9N_3O_2S_2$	255.3	0.05	100
87	**Tetracycline HCl**	**Sigma-Aldrich**	**Tetracyclines**	**$C_{22}H_{25}ClN_2O_8$**	**480.9**	**-1.47**	**200**
88	Thiabendazole	Sigma-Aldrich	Anthelmintics	$C_{10}H_7N_3S$	201.0	2.47	100
89	**Tildipirosin**	**Sigma-Aldrich**	**Macrolides/lincosamides**	**$C_{41}H_{71}N_3O_8$**	**733.5**	**4.70**	**400**
90	**Tilmicosin**	**Sigma-Aldrich**	**Macrolides/lincosamides**	**$C_{46}H_{80}N_2O_{13}$**	**868.5**	**4.95**	**100**
91	Tolfenamic acid	Sigma-Aldrich	Anti-inflammatories	$C_{14}H_{12}ClNO_2$	261.7	5.76	200
92	Triclabendazole	Sigma-Aldrich	Anthelmintics	$C_{14}H_9Cl_3N_2OS$	359.7	5.97	50
93	**Triclabendazole Sulfoxide**	**Sigma-Aldrich**	**Anthelmintics**	**$C_{14}H_9Cl_3N_2O_2S$**	**375.7**	**4.12**	**50**
94	Triflupromazine HCl	Sigma-Aldrich	Tranquilizers	$C_{18}H_{20}ClF_3N_2S$	388.9	5.70	10
95	**Tulathromycin**	**TRC[2]**	**Macrolides/lincosamides**	**$C_{41}H_{79}N_3O_{12}$**	**805.5**	**4.07**	**150**
96	**Tylosin**	**TRC[2]**	**Macrolides/lincosamides**	**$C_{46}H_{77}NO_{17}$**	**916.1**	**3.27**	**200**
97	**Virginiamycin M1**	**Sigma-Aldrich**	**Others**	**$C_{28}H_{35}N_3O_7$**	**525.6**	**-0.66**	**100**
98	Xylazine HCl	Sigma-Aldrich	Tranquilizers	$C_{12}H_{17}ClN_2S$	256.8	2.37	10

[1] Data taken from www.chemspider.com, accessed Nov, 2019, LogP data taken from computational predictions ACD/LogP

[2] Toronto Research Chemicals

5.3.2 Reagents and materials

LC-MS-grade methanol (MeOH), acetonitrile (MeCN), and water were purchased from Fisher Scientific (Mississauga, ON, Canada). Polyacrylonitrile (PAN), and Dimethyl Sulfoxide (DMSO) were purchased from Sigma Aldrich (Oakville, ON, Canada). SPME pins, shown in Figure 5.1, were manufactured in-house using high-tolerance 304 stainless steel rods (1/16" diameter) purchased from McMaster-Carr (Aurora, OH, USA). The pins were cut to a length of 55 mm at the University of Waterloo's Science Technical Services (UW-STC) and coated with a slurry of HLB particles suspended in PAN as a binder at a thickness of 30 μm and a length of 1 cm. HLB particles were synthesized in house and characterized as previously described.[189] The SPME coating was immobilized on the stainless-steel rod surface by dip coating, as per the protocol developed by Gomez-Ríos at al.[209] Utilization of this dip-coating procedure, along with the use of small HLB particles (5 μm), provided a smooth coating surface that prevented attachment of matrix debris to the SPME coating. To ensure good reproducibility and a high-throughput desorption process, a custom-made SPME holder compatible with the commercial DART rail was designed at UW-STC to accommodate up to 12 SPME pins, as can be seen in Figure 5.1. Nunc U96 600 μL, 1 mL Deep, and 2mL well-plates made of polypropylene were purchased from VWR International (Mississauga, ON, Canada). Individual stock standard solutions were prepared in either MeCN, MeOH, water, or 10% DMSO in MeCN as described previously, in accordance with the solubility of each compound, and stored at −80 °C.[189]

Assorted organic beef muscle samples were used to serve as a pooled blank matrix. These samples were obtained from three different local grocery stores and carefully selected to ensure that they came from different sources and that the cows have not been treated with any chemicals

or pharmaceutical drugs. Three distinct samples of non-organic beef were purchased from different local grocery stores for analysis of market samples.

5.3.3 Sample Preparation

Utilizing a Freezer/Mill® Cryogenic Grinder (SPEX SamplePrep, LLC, Metuchen, NJ, USA), beef tissue samples were homogenized under liquid nitrogen to produce a uniform fine powder. Organic beef samples were mixed in equal portions and homogenized to obtain a pooled blank matrix. Homogenized samples were stored in glass jars at −80 °C until analysis. Sample preparation procedure was carried out in accordance with previously optimized and described protocol.[189,206] A detailed schematic of the sample preparation steps is illustrated in Figures 2.2 and 2.3 in chapter 2.

Spiked tissue samples were prepared by weighing 2.0 g of room-temperature homogenized tissue into a 50 mL polypropylene (PP) tube. Each sample was then spiked with 100 µL of a working solution containing all analytes under study at their respective designated concentrations, and 60 µL of the IS working solution containing 1 ng μL^{-1} of each IS. Samples were vortexed for 1 min, and then placed on a benchtop agitator for 1h. Subsequently, samples were stored in a 4ºC fridge overnight to allow for proper equilibration and matrix binding of target analytes prior to extraction. Prior to extraction, samples were placed on the benchtop agitator for 1h to allow samples to reach room temperature. Next, 6mL of water were added to each individual spiked tissue sample, which was then vortexed for 1 min in order to attain a homogeneous consistency suitable for volumetric dispensing of 1500 µL of diluted beef sample to each well of the 96 well-

plate. A schematic of the detailed sample preparation steps is illustrated in Figures 2.2 and 2.3 in chapter 2.

5.3.4 Extraction and Analytical Procedure

As shown in Figure 5.1, the SPME-DART analytical workflow consisted of the following steps: (1) conditioning of the SPME coating for 15 minutes with MeOH:H_2O (50:50, v/v); (2) static extraction of target analytes from matrix (prepared as described above) for 45 minutes; (3) rinsing of coating in water for 10 seconds so as to remove any loosely attached matrix constituents that could induce ion enhancement or suppression; and (4) placement of SPME pins in the 12-pin SPME holder, with subsequent positioning of holder on the DART rail for thermal desorption. Steps 1-3 were performed automatically using a Concept-96 system (Professional Analytical Systems (PAS) Technology, Magdala, Germany) as described elsewhere [137,189]. Sample preparation details and extraction parameters are illustrated in Figures 2.2 and 2.3 in chapter 2, and Figure 5.1. While we determined that the pins can be reused by implementing a thorough cleaning step with a mixture of 50:25:25 v/v/v, MeOH:Isopropanol:MeCN for 30 min following desorption, pins were not re-used in this study.

Extraction parameters were evaluated and optimized in a previous study.[189] However, extraction time was re-optimized for this study and a pre-equilibrium extraction time of 45 min was chosen as a compromise between time and signal intensity. Extraction temperature was set at

30 °C so as to avoid temperature fluctuations originating from ambient temperature changes during extraction.

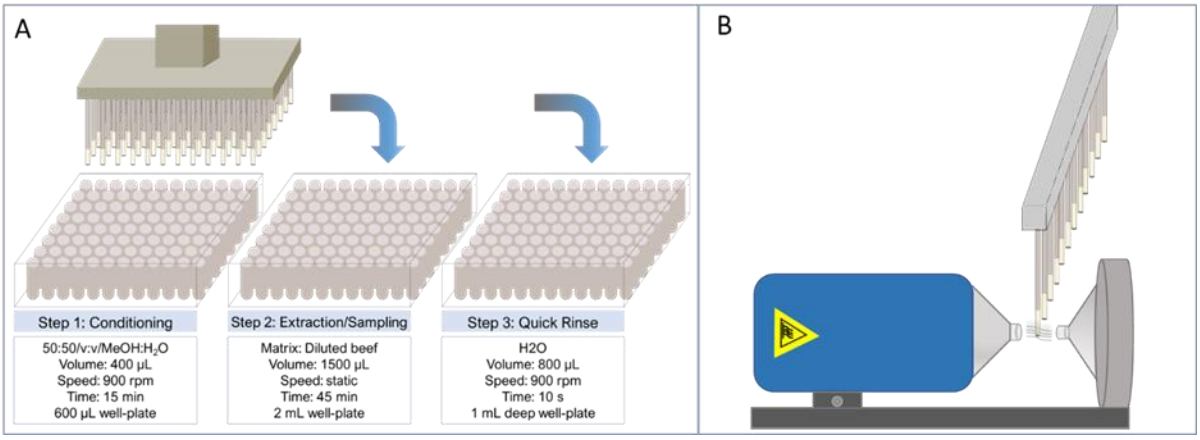

Figure 5.1 SPME-DART-MS/MS analytical workflow, A: Automated high-throughput extraction, B: Automated high-throughput desorption/ionization

5.3.5 SPME-DART-MS Setup

All experiments were performed using a DART-Standardized Voltage and Pressure (DART-SVP) model ion source (IonSense, Inc., Saugus, MA, USA) coupled to a TSQ Quantiva from Thermo Scientific (San Jose, CA, USA) via a Vapur interface (IonSense, Inc.). Data analysis was completed using TraceFinder 4.1, also from Thermo Scientific. Two single reaction monitoring (SRM) transitions were used for each analyte, one for quantitation and one for confirmation, whereas only one transition was used for each IS. MS/MS analyte transitions and conditions were optimized via direct infusion from methanolic and acetonitrile standards. Detailed information regarding optimized instrumental parameters (collision energy and RF-lens values) and monitored SRM transitions can be found in Tables 5.2. The SRM dwell time was set at 10 ms per transition. Transfer line temperature was set to 300 °C.

The DART-SVP was fitted with a single dimensional motorized linear rail, which was controlled through the DART-SVP web-based software to automatically position the SPME pins in front of the DART source by manipulating the 12-pin SPME holder. Each SPME pin was directly positioned between the DART source and MS inlet, where the analytes enriched on the SPME coating were thermally desorbed by the heated gas stream and then ionized by the plasma excited-state species for MS analysis.

The DART ion source was operated in positive ion mode with helium as the ionizing medium, using the following conditions: high voltage (HV) electrode, −3000 V; discharge electrode, +350 V; grid voltage, +350 V. Since DART-SVP utilizes N_2 and He gas during active runs, the amount of gas entering the mass spectrometer was reduced using a membrane pump (adjusted to blue indicator, position 4 to avoid compromising the vacuum. Prior to analyses, the level of background noise was assessed by obtaining background signals for both ambient air and preconditioned pins at the MS/MS transitions of the monitored analytes.

Table 5.2 MS/MS optimized parameters, (m/z), ions used for quantification are bolded.

	Compound	**Precursor ion m/z**	**Product ions m/z**	**Collision Energy (eV)**	**S-Lens value**	**Polarity**
1	2-Aminoflubendazole	256	**123**, 95	37, 27	93	+
2	2-Amino mebendazole	238	**105**, 77	25, 34	88	+
4	2-Quinoxalinecarboxylic acid	175	**131**, 104	22, 29	191	+
5	5-Hydroxythiabendazole	218	**191**, 147	26, 32	85	+
6	6-Phenyl-2-thiouracil	205	**103**, 188	17, 26	69	+
7	6-Propyl-2-thiouracil	171	**154**, 112	17, 19	57	+
8	Acepromazine Maleate	327	**86**, 254	19, 23	65	+
9	Albendazole	266	**234**, 191	19, 32	71	+
10	Albendazole sulfone	298	**266**,159	19, 36	79	+
11	Albendazole sulfoxide	282	**240**, 208	10, 23	75	+

	Compound	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	S-Lens value	Polarity
12	Albendazole-2-aminosulfone	240	**133**,198	28,19	75	+
13	Ampicillin	350	**106**, 160	18, 10	54	+
14	Azaperone	328	**165**,123	20, 29	66	+
15	Betamethasone	393	**325**, 347	14, 12	59	+
16	Cambendazole	303	**217**, 261	27, 17	66	+
17	Carazolol	299	**116, 222**	19, 19	63	+
18	Carbadox	263	**231**, 245	10, 16	67	+
19	Cefazolin	455	**323**, 333	10, 19	72	+
20	Chlortetracycline HCl	479	**462**,444	17, 19	70	+
21	Chlorpromazine HCl	319	**86**, 58	19, 30	63	+
22	Ciprofloxacin	332	**288**, 314	17, 19	72	+
23	Clenbuterol HCl	277	**203**, 168	16, 29	47	+
24	Clindamycin HCl	425	**126**, 377	27, 18	78	+
25	Cloxacillin Sodium Salt	436	**277**, 160	12, 10	56	+
26	Danofloxacin	358	**340**, 314	21, 17	80	+
27	Desacetyl cephapirin	382	**292**, 226	10, 17	73	+
28	Desethylene Ciprofloxacin	306	**288**, 268	17, 24	67	+
29	Diclofenac Sodium	296	**214**, 277	33, 11	112	+
30	Dicloxacillin Sodium Salt	470	**452**, 212	16, 26	124	+
31	Difloxacin HCl	400	**382**, 356	21, 18	82	+
32	Dimetridazole	142	**96**, 101	16, 10	38	+
33	Doxycycline HCl	445	321, **428**	30, 17	66	+
34	Emamectin Benzoate	887	**158**, 868	33, 21	120	+
35	Enrofloxacin	360	**342**,316	20, 18	79	+
36	Erythromycin	735	**522**, 558	17, 34	85	+
37	Fenbendazole Sulfone	332	**300**, 159	21, 37	81	+
38	Florfenicol	357	**198**, 182	14, 16	73	+
39	Florfenicol amine	248	**230**, 130	10, 25	50	+
40	Flubendazole	314	**282**, 123	22, 35	80	+
41	Flunixin	297	**279**, 264	22, 33	75	+
42	Gamithromycin	778	**619**, 601	28, 31	121	+
43	Haloperidol	376	**165**,123	22, 36	78	+
44	Hydroxy dimetridazole	158	**80**,140	10, 10	30	+
45	Hydroxy ipronidazole	186	**168**, 122	10, 19	37	+
46	Hydroxy metronidazole	188	**123**, 126	10, 17	47	+
47	Ipronidazole	170	**124**, 109	17, 24	54	+
48	Ketoprofen	255	**209**, 105	14, 23	59	+

	Compound	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	S-Lens value	Polarity
49	Levamisole HCl	205	**178**, 123	21, 28	66	+
50	Lincomycin HCl	407	**126**, 359	26, 18	76	+
51	Mebendazole	296	**264**, 105	20, 33	75	+
52	Melengestrol Acetate	397	**279**, 337	20, 13	88	+
53	Meloxicam	352	**115**, 141	19, 20	67	+
54	Metamizole	218	**56**, 97	17, 12	47	+
55	Metronidazole	172	**128**, 82	14, 23	48	+
56	Morantel Tartrate Hydrate	221	**123**, 111	35, 25	73	+
57	Nafcillin	415	**199**, 171	14, 34	78	+
58	Norfloxacin	320	**302**, 276	20, 17	76	+
59	Novobiocin	613	**218**, 396	13, 15	80	+
60	Orbifloxacin	396	**352**, 295	17, 23	83	+
61	Oxacillin Sodium Salt	402	**243**, 160	13, 11	68	+
62	Oxyclozanide	402	**186**, 83	22, 22,	62	+
63	Oxfendazole	316	**191**, 284	20, 18	68	+
64	Oxytetracycline HCl	461	**426**, 443	18, 10	67	+
65	Penicillin G	335	**217**, 202	14, 23	80	+
66	Phenylbutazone	309	**120**, 160	42, 19	71	+
67	Pirlimycin HCl	411	**363**,112	16, 25	77	+
68	Prednisone	359	**341**, 237	10, 19	65	+
69	Promethazine HCl	285	**86**, 198	17, 25	46	+
70	Propionylpromazine HCl	341	**58,** 268	30, 23	68	+
71	Ractopamine HCl	302	**164**, 284	15, 10	53	+
72	Ronidazole	201	**140**, 55	10, 21	30	+
73	Salbutamol	240	**148**, 222	17, 10	42	+
74	Sarafloxacin HCl Hydrate	386	**342**, 368	18, 21	85	+
75	Sulfachloropyridazine	285	**156**, 108	16, 24	57	+
76	Sulfadiazin	251	**92**, 108	24, 22	48	+
77	Sulfadimethoxine	311	**156**, 108	20, 27	72	+
78	Sulfaethoxypyridazine	295	**108**, 140	25, 18	64	+
79	Sulfamerazine	265	**172**, 108	16, 24	59	+
80	Sulfamethazine	279	**186**, 156	17, 18	65	+
81	Sulfamethizole	271	**92**, 108	24, 22	47	+
82	Sulfamethoxazole	254	**108**, 156	23, 15	154	+
83	Sulfamethoxypyridazine	281	**156**, 108	16, 24	63	+
84	Sulfapyridine	250	**108**, 184	24, 18	57	+
85	Sulfaquinoxaline	301	**156**, 108	17, 25	69	+

	Compound	Precursor ion m/z	Product ions m/z	Collision Energy (eV)	S-Lens value	Polarity
86	Sulfathiazole	256	**156**, 108	15, 22	52	+
87	Tetracycline HCl	445	**410**, 427	18, 10	67	+
88	Thiabendazole	202	**175**, 131	25, 32	74	+
89	Tildipirosin	368	**98**, 88	28, 19	64	+
90	Tilmicosin	435	**174**, 695	23, 16	75	+
91	Tolfenamic Acid	262	**244**, 209	15, 27	47	+
92	Triclabendazole	359	**274**, 344	37, 25	101	+
93	Triclabendazole Sulfoxide	375	**357**, 360	17, 21	79	+
94	Triflupromazine HCl	353	**248,** 86	42, 20	68	+
95	Tulathromycin	404	**72**, 230	19, 10	62	+
96	Tylosin	917	**772**, 174	27, 35	134	+
97	Virginiamycin	526	**508**, 355	12, 17	72	+
98	Xylazine HCl	221	**90**, 147	22, 23	68	+
99	*Sulfamethazine-(phenyl-$^{13}C_6$)*	285	**186**	19	149	+
100	*Xylazine-d_6*	227	**90**	22	71	+

5.4 Results and discussion

5.4.1 Optimization of DART parameters

Ionization gas temperature is considered one of the most critical parameters influencing the outcome of DART–MS analysis.[225,241,242] For this reason, the ionization gas temperature was investigated in the range of 200–500 °C in 50 °C increments. The optimum temperature was determined by plotting the intensity of the quantifier ion signal onto a graph against the temperature as shown in Figure 5.2. While 500 °C yielded higher intensities for all analytes, we observed discoloration of the coating at this temperature. The observed discoloration could be due to thermal degradation of PAN which is not stable at such high temperatures. No visible discoloration of the coating was observed at 450 °C. In addition, the coating was inspected under

the microscope and no visible damage was observed. Moreover, as shown by the results in Figure 5.2, the efficiency of extraction was improved at high temperatures. As a result, 450 °C was selected as the optimum temperature. Figure 5.2 shows the effect of temperatures on signal intensities of quantifier ions for representative target analytes. The same trend was observed for all detected analytes. None of the analytes was detected at 200 °C. It is important to note that the software's set temperature is usually higher than the real temperature of the ionization region due to the mixing of ionization gas with cooler atmospheric gas.[242] A study by Rothenbacher et al. indicated that a temperature of 450°C set by the DART software corresponded to an actual temperature of 310°C in the ionization region.[243]

Other key parameters influencing DART performance are the distance between the DART gun and the sample, and the distance between the sample and the MS inlet. Higher signal intensities were observed for all detected analytes when the DART gun was closer to the SPME pin. This could be caused by an increase in thermal desorption due to the increase in the sample temperature as the distance from the DART source is decreased. Another explanation for this is that the rapidly moving ionization gas exiting from the DART source disperse and dilute as it moves farther from the DART source. To ensure reproducibility and avoid contact between the DART gun and the SPME pin, we selected a distance of 2mm between the DART gun and the sample as optimum. The placement of the tip of the SPME pin with respect to the DART gun was also optimized with regards to intensity of the analyte signal. Optimum positioning of the pin tip was found to be at slightly below the center line of the ionization-gas beam so as to avoid blockage of the flow of

desorbed analyte ions into MS. The distance between the SPME pin and the MS inlet was also optimized at 4 mm.

Another important factor that affects the intensity of the signal and number of scans per peak is the DART rail speed.[242] Rail speed was thus investigated to ensure adequate interaction between the sample and the ionizing gas. Optimum speed was found to be the lowest setting possible on the DART software, which is 0.2 mm s–1. The lowest speed ensured maximum interaction between the analytes and the ionization gas. At this speed, we estimated the desorption time of analytes from the surface of the SPME coating to be 15 s. This demonstrates that prolonged exposure of analytes to the ionizing gas increases the probability of analyte molecules colliding with ionization gas molecules, resulting in an increased production of ions, and thus higher signals.[244]

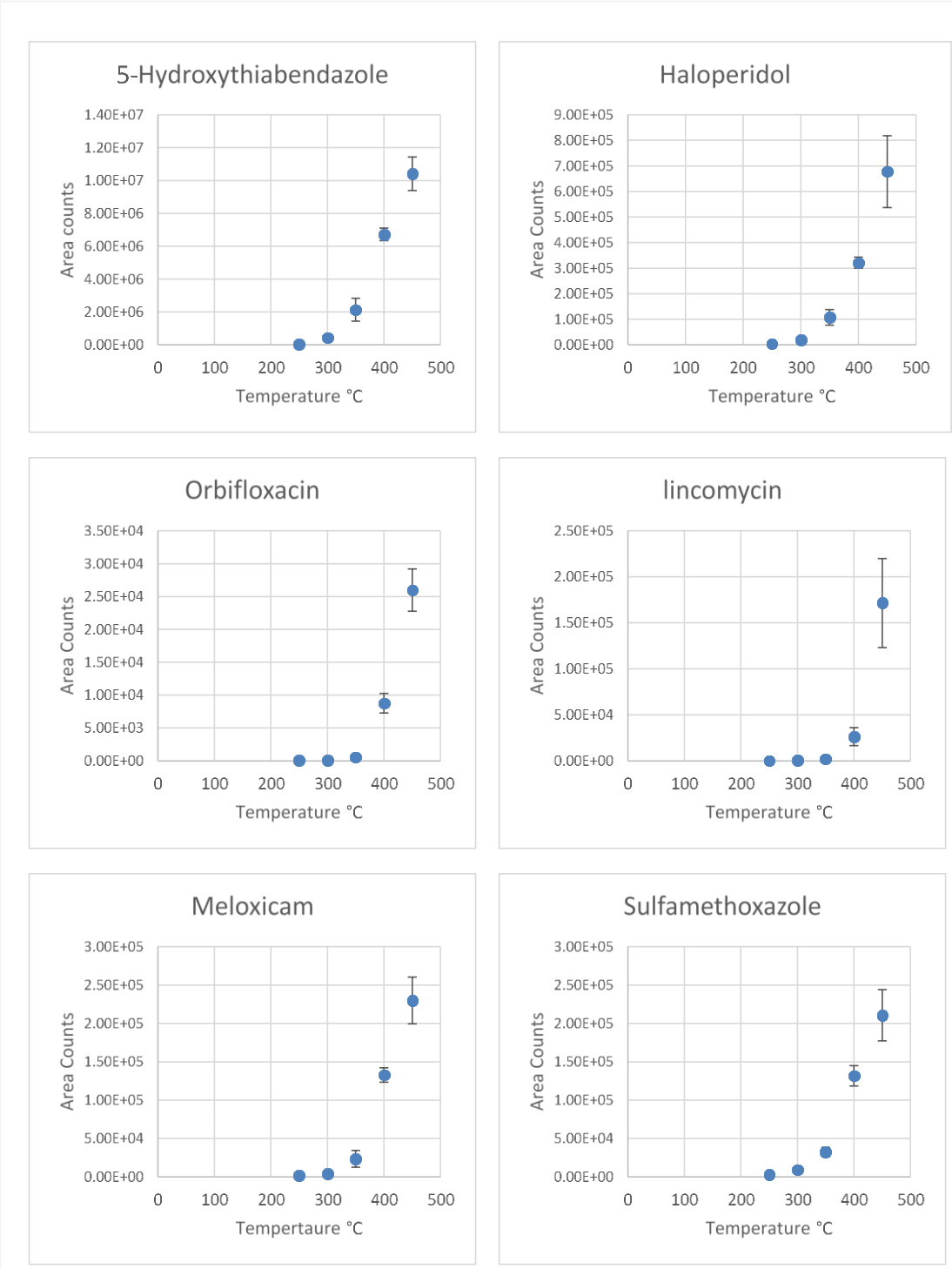

Figure 5.2 Effect of DART temperature on analyte detection

5.4.2 DART ionization of veterinary drugs

Under the above tested parameters, no quantifiable signals were detected for 46 analytes at their respective MRL levels. These analytes are listed in bold font in Table 5.1. However, all of these compounds were successfully quantified well below their MRL levels using SPME-LC- ESI-MS/MS[206] and CBS-MS/MS with the exception of florfenicol and florfenicol amine in case of CBS-MS/MS. The lack of quantifiable signals in DART for these target analytes compared to ESI could be due to many factors. One of these factors could be the fact that in DART, desorption takes place from a smaller surface area of the coating in comparison to solvent desorption typical of LC applications. Other factors include a combination of volatility versus thermal stability of analytes, since DART has a tendency to break down labile bonds in comparison to the soft ionization in ESI.[226,238] For example, none of the 8 β-Lactams/cephalosporins and four tetracyclines, which are considered thermally labile and prone to heat degradation,[54,245] were detected in DART at the MRL. Conversely, all 12 sulfonamides, which are considered more thermotolerant, were detected below their MRLs. This disadvantage limits the scope of DART applicability to somewhat volatile and thermally stable analytes.[232,242,246,247]

While Cody et al. have suggested that desorption in DART may include both thermal and non-thermal processes, according to the results presented by Harris et al., thermal desorption can be considered as the predominant pathway for energy deposition.[238] In our investigation, we found that lower temperatures did not provide higher signals for any of the compounds under investigation. The strong dependence of DART on thermal desorption might pose an even bigger challenge for multi-residue analysis involving groups of compounds characterized by a wide

variety of physico-chemical characteristics, since certain less volatile analytes need very high temperatures to be desorbed, while others might undergo thermal degradation or pyrolysis at such high temperatures.[242,247] On the other hand, very volatile compounds could desorb too quickly, thus resulting in insufficient scans per peak. One possible solution for this is to couple DART to a temperature-programmable sample heater as suggested in previous studies, a technique which could also facilitate thermal separation [248–250]. However, this solution might significantly extend analysis run time, as an additional cooling period for DART would be necessary before the introduction of each new sample. Another useful solution would be to lower the current rail speed limit and introduce a programmable rail speed option that would allow the rail to reduce speed or even pause when the pin is positioned directly in front of the MS inlet. This option would extend the interaction time between the sample and the ionization gas, and thus increase the probability of analyte molecules colliding with the gas, resulting in higher signals.

We also observed that the highest molecular weight precursor detected in this study was 425 (clindamycin). While factors such as molecular weight, volatility, and thermal stability of the analyte might influence DART ionization, unfortunately, our limited knowledge of the underlying mechanisms of the DART ionization and desorption processes, which are beyond the scope of this study, does not allow us to fully explain the reasons why several target analytes were not detected.

5.4.3 Selectivity

In the absence of chromatography, one of the main drawbacks of AIMS techniques is the lack of retention time, which hinders such techniques in terms of selectivity. In the absence of

retention time, another criterion that can be used for identification of target analytes is the ion ratio between the quantifying and qualifying ion transition signals.

In this study, no significant interferences were observed in blank samples near LOQ levels for any of the 52 detected analytes (listed in Table 5.3).

Another main drawback of AIMS techniques is the lack of means for separating isomers and isobars prior to MS analysis. Selectivity issues in AIMS techniques can be addressed without compromising analysis time by coupling said techniques with a complementary rapid ion separation/filter device such as an ion mobility spectrometer (IMS). Likewise, high resolution mass analyzers combined with MS^n capabilities may also provide solutions to obtain higher levels of confidence in the process of analyte identification and confirmation.

5.4.4 Linearity and limits of quantitation

Matrix-matched calibration curves were used to assess linearity and limits of quantitation of the detected analytes. Calibration curves were constructed using analyte/IS peak area ratios (A/Is) for 7 concentration levels (0.25–3X), using three independent replicates for each level. The calibration range was selected to include concentration levels bracketing the MRLs of the selected analytes. Despite the use of only two internal standards for all analytes, 32 target analytes (62% of the total number of analytes detected) achieved determination coefficients (R^2) higher than 0.99. These results can be further improved by using more analyte-specific internal standards. Moreover, a lack-of-fit test was performed with the use of Statistica 13.0 software (TIBCO® Statistica™, CA, USA) at the 5% level to confirm linearity of the calibration curves. None of the detected analytes presented a lack-of-fit ($p > 0.05$), indicating that the lack-of-fit was not significant at 95%

confidence level relative to pure error and thus confirming a good fit for all compounds, except for 5-hydroxythiabendazole and oxfendazole.

LOQ was established as the lowest point of the matrix-matched calibration curve that produced a response that is both accurate when compared to the expected value (calculated via linear regression) with a range of deviation ≤25%, as well as precise (≤25% RSD). As shown in Table 5.3, all analytes under study achieved low enough LOQs to ensure proper determination at the maximum residue levels set for Canada and the US. Individual determination coefficients (R^2), LOQ values, and lack-of-fit test results are presented in Table 5.3.

In terms of detectability and in comparison to other ESI based AIMS techniques such as CBS-MS/MS[240], it is important to recognize that DART has limited analyte scope which limits its application to compounds that are somewhat volatile and thermally stable.[232] Unfortunately, this limitation of DART excludes its application to many pharmaceutical compounds which are usually thermally labile.

5.4.5 Accuracy and precision

To evaluate the accuracy and precision of the method with respect to intra-day repeatability, pooled matrix blanks were spiked at three levels; low, mid, and high (0.5X, 1X and 2X), for each analyte using six replicates per concentration (n=6). Accuracy was calculated based on estimated concentration values obtained from the linear regression equation of the matrix-matched calibration curve.

As presented in Figure 5.3 and Table 5.3, the majority of analytes fell within the 70−120% range of the true concentrations of compounds, with RSD values ≤25% at the 0.5, 1, and 2X concentration levels, thus indicating that these compounds can be quantitatively determined by the optimized DART method.

Individual accuracy and %RSD values are listed Tables 5.3 for each analyte under study. The results showed that two internal standards were sufficient to ensure reliable, precise, and accurate analytical data. For quantitative performance criteria such as linearity, repeatability, and accuracy DART performance was found to be comparable to CBS-MS/MS for compounds that could be detected with DART. These results demonstrate that DART has the potential to be used

for rapid screening and semi-quantitative multi-residue analysis of certain classes of veterinary drugs in animal tissue.

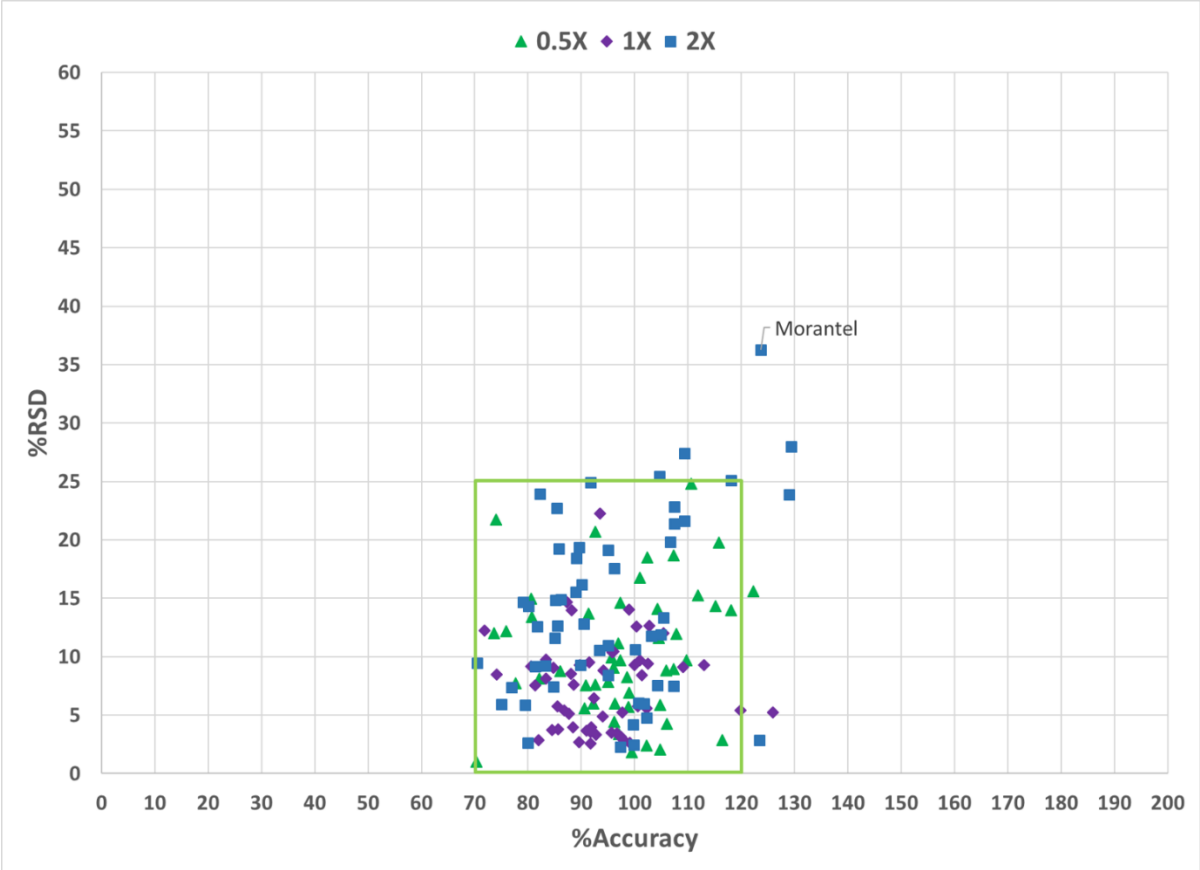

Figure 5.3 Accuracy and repeatability results of the optimized DART method for target analytes fortified at the 0.5, 1, and 2X levels in beef tissue (n = 6).

Individual accuracy and %RSD values are listed Tables 5.3 for each analyte under study. The results showed that two internal standards were sufficient to ensure reliable, precise, and accurate analytical data.

Table 5.3 Figures of merit for the quantitation of multi-residue veterinary drugs in beef tissue via DART-MS/MS.

				Accuracy (%)			Repeatability (%)		
Compound	**R^2**	**p-value**	**LOQ**	**0.5X**	**1X**	**2X**	**0.5X**	**1X**	**2X**
2-Aminoflubendazole	0.9958[a]	0.8714	0.25X	91	85	107	8	6	8
5-Hydroxythiabendazole	0.9614[a]	**0.0000**	0.25X	99	82	85	7	3	7
6-phenyl-thiouracil	0.9895[b]	0.2779	0.25X	87	87	97	8	3	13
Acepromazine	0.9919[b]	0.3575	0.25X	92	100	109	6	13	27
Albendazole	0.9919[b]	0.0619	0.75X		100	80		9	3
Albendazole 2-aminosulfone	0.9958[b]	0.4335	0.25X	105	101	129	6	10	28
Albendazole sulfoxide	0.9789[b]	0.0538	0.75X		109	77		9	7
Azaperone	0.9848[c]	0.1165	0.25X	119	107	107	6	4	20
Cambendazole	0.9990[a]	0.9038	0.25X	99	98	95	8	3	8
Carazolol	0.9965 [b]	0.3685	0.25X	97	99	100	12	4	19
Carbadox	0.992 [a]	0.7490	0.25X	97	98	105	10	5	12
Chlorpromazine	0.9951 [a]	0.8568	0.25X	102	99	85	19	14	23
Clindamycin	0.9902 [b]	0.0891	0.25X	110	99	106	12	11	18
Danofloxacin	0.9740 [b]	0.1183	0.25X	111	113	105	25	9	25
Diclofenac	0.9928 [a]	0.6758	0.25X	105	126	123	12	5	3
Dimetridazole	0.9916 [a]	0.9095	0.25X	78	81	80	8	8	14
Fenbendazole Sulfone	0.9784 [b]	0.0642	0.25X	86	90	95	25	16	9
Haloperidol	0.9919 [a]	0.0503	0.25X	108	101	107	12	6	21
Hydroxy dimetridazole	0.9919 [a]	0.8280	0.75X		74	82		8	13
Hydroxy metronidazole	0.9961 [a]	0.0583	0.25X	118	120	95	14	5	16
Ipronidazole	0.9951 [a]	0.9482	0.25X	74	88	85	22	14	19
Levamisole	0.9962 [a]	0.0994	0.25X	96	88	85	4	5	12
Lincomycin	0.9755 [a]	0.2257	0.25X	116	96	92	20	10	15
Mebendazole	0.9731 [a]	0.2323	0.25X	107	83	105	9	10	25
Meloxicam	0.9863 [a]	0.1598	0.25X	91	86	79	14	4	13
Metamizole	0.9812 [a]	0.1723	0.25X	104	83	79	14	8	15
Metronidazole	0.9809 [a]	0.5908	0.25X	81	81	86	13	9	6

Table 5.3 continued

				Accuracy (%)			Repeatability (%)		
Compound	**R^2**	**p-value**	**LOQ**	**0.5X**	**1X**	**2X**	**0.5X**	**1X**	**2X**
Morantel	0.9912 [b]	0.5398	0.25X	115	103	124	14	13	36
Orbifloxacin	0.9896 [a]	0.6392	0.25X	107	101	118	19	8	25
Oxfendazole	0.9887 [b]	**0.0012**	0.75X		102	75		9	6
Phenylbutazone	0.9889 [c]	0.2747	0.75X		117	97		10	9
Pirlimycin	0.9811 [a]	0.1656	0.25X	111	91	86	12	9	19
Promethazine	0.9899 [a]	0.1832	0.25X	97	92	81	15	10	9
Propionylpromazine	0.9795 [a]	0.7307	0.25X	74	88	90	12	9	13
Ronidazole	0.9861 [a]	0.8379	0.25X	76	72	82	12	12	24
Sulfachloropyridazine	0.9967 [a]	0.0755	0.25X	97	85	95	11	9	11
Sulfadiazin	0.9996 [a]	0.8323	0.25X	96	88	102	9	4	5
Sulfadimethoxine	0.9997 [a]	0.9629	0.25X	97	99	101	3	3	6
Sulfaethoxypyridazine	0.9971 [c]	0.4047	0.25X	107	101	109	8	4	11
Sulfamerazine	0.9998 [a]	0.9474	0.25X	99	96	102	6	4	6
Sulfamethazine	0.9996 [a]	0.9512	0.25X	99	97	97	2	3	2
Sulfamethizole	0.9929 [a]	0.2125	0.25X	105	92	103	2	4	12
Sulfamethoxazole	0.9954 [a]	0.5247	0.25X	106	93	100	9	3	11
Sulfamethoxypyridazine	0.9970 [b]	0.4465	0.25X	91	91	105	15	8	9
Sulfapyridine	0.9992 [a]	0.5705	0.25X	96	92	100	10	4	4
Sulfaquinoxaline	0.9969 [a]	0.6650	0.25X	86	89	96	9	8	18
Sulfathiazole	0.9992 [a]	0.9042	0.25X	91	92	104	6	3	8
Thiabendazole	0.9973 [a]	0.9148	0.25X	82	87	89	8	5	16
Tolfenamic acid	0.9750 [a]	0.7471	0.50X	116	90	109	3	3	22
Triclabendazole	0.9826 [a]	0.6715	0.25X	70	94	129	1	22	24
Trifluropromazine	0.9950 [a]	0.7250	0.25X	81	90	83	15	9	9
Xylazine	0.9986 [b]	0.1293	0.25X	102	97	100	2	3	2

5.5 Analysis of market beef samples

The proposed method was used to analyze beef samples purchased from three local grocery stores. All the samples analyzed were free from the target analytes near or above their LOQ levels, however traces of phenyl thiouracil were detected below quantitation limits in two market samples. 6-phenyl-thiouracil was tentatively identified based on the presence of detectable signals for both SRM transition ions corresponding to 6-phenyl-thiouracil (205→188) and (205→103) as shown Figure 5.4.

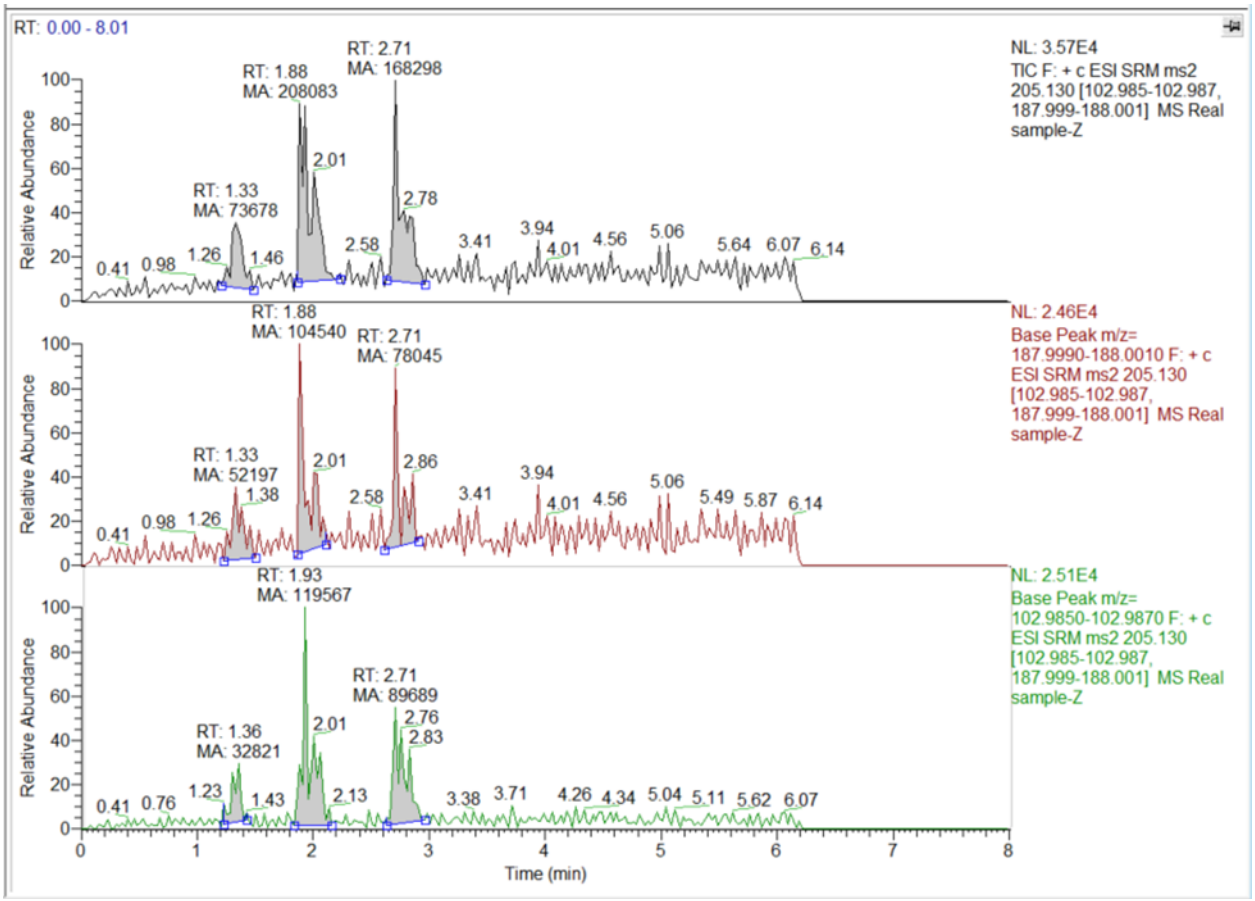

Figure 5.4 Ion chronograms obtained for desorption of 3x extraction from one market beef sample and 3x extraction from pooled blank matrix for 6-phenyl-thiouracil (precursor ion 205), TIC (black), transition ions 205→188 (red) and 205→103 (green).

5.6 Conclusion

Despite the limited analyte scope and lower detectability levels of SPME-DART-MS/MS compared to ESI-based methods for veterinary drugs, this study has demonstrated that the method can be successfully used for rapid screening and semi-quantitative multi-residue analysis of certain classes of veterinary drugs in complex matrices such as beef tissues. Its main attractive features are high-throughput, simplicity, operational cost, and real-time analysis. It is important however to recognize the limitations of this method for certain analytes which lack enough volatility and thermal stability. Unlike soft ionization methods such as ESI, DART tends to break down some labile bonds. DART-MS/MS is still a relatively new technology and more in-depth studies are needed to understand the underlying ionization mechanisms and limitations of this technique in order to increase its detectability for a wider range of analytes.

Similar to all AIMS techniques, another limitation of this method is the compromise on selectivity due to the absence of chromatographic separation; however, this can be compensated for by using ion mobility spectrometry and high resolution mass analyzers with MS^n capabilities. It is envisioned that the described approach can be implemented on site for rapid and real-time screening of food products or living animals by performing in-vivo chemical biopsy sampling using appropriate SPME device.[120]

Chapter 6
Summary and Future Perspectives

6.1 Summary

Rigorous monitoring of veterinary drug residues in food-producing animal tissues plays a crucial role in the protection of human health. Given the perishable nature of food, residue investigations demand the use of extremely fast, efficient, and reliable analytical methods. Recent advances in liquid chromatography and mass spectrometry in terms of speed and detectability have put the pressure on analytical scientists to explore and develop new sampling technologies that meet the demand for fast and accurate chemical analysis methods. Solid phase microextraction (SPME) techniques have been well explored in terms of their applicability in food and biological analysis for determination of a wide range of analytes with different physical and chemical properties. Accordingly, this thesis presented novel methodologies based on SPME technology as an alternative tool for efficient high-throughput sample preparation and reliable analysis of multi-class multi-residue pharmaceutical drugs in animal-derived tissue samples.

Aiming to take advantage of their mechanical robustness and high recovery extraction, the suitability of thin film SPME blades (TFME) for the abovementioned application was initially assessed as the starting point of this thesis. However, the sharp edges of the blades and the rough surface of the extractive phase were shown to cause adhesion of matrix components to the coating that proved difficult to dislodge by just rinsing the coating. The extent of adhesion was exacerbated when agitating the sample during extraction due to fat destabilization and its agglomeration on the coating. In order to prevent adhesion of matrix components to the SPME coating, four measures

were taken: i) employment of a geometry with no edges such as round pins with a large outer diameter to maintain the advantage of a large surface area, ii) preparation of a smoother coating, which was accomplished by synthesizing HLB particles characterized by a smaller size (~5 µm) in comparison to commercial particles (30-60 µm), iii) exploration of new coating techniques such as dip coating to achieve a smoother coating surface, and iv) performance of static extraction to prevent destabilization of fat. The combination of the first three approaches succeeded in creating a smooth coating, while static extraction prevented the destabilization of fat and its agglomeration on the coating. Consequently, the developed round SPME pins, with their large surface area and smooth coating surface, allowed successful direct immersion extraction in homogenized animal tissue.

After establishing a successful direct immersion extraction protocol capable of avoiding fouling of the coating, a fully automated high-throughput multi-class, multi-residue method was successfully developed and validated for quantitative analysis of 77 veterinary drugs in homogenized chicken muscle via SPME and UHPLC-ESI-MS/MS. The developed method is the first and most comprehensive SPME approach in terms of the number of analytes tested as well as with respect to the range of physical and chemical properties covered. Further, in an evaluative comparison against SE and QuEChERS, the SPME method was demonstrated to produce extracts with significantly less matrix effects compared to SE and QuEChERS, while also offering better accuracy, precision, and improved limits of quantification, in addition to significant improvement in sample throughput and reduction in environmental footprint, as demonstrated in chapter 3.

The clean extracts provided by SPME, evidenced by the minimal to almost non-existent matrix effects in comparison to conventional methods, motivated us to push the limits of SPME even further so as to achieve methods as short as 1 minute per sample, a feat accomplished by skipping liquid chromatography altogether by direct coupling SPME to mass spectrometry. In addition, we aimed to develop short methods that allow rapid screening in both positive and negative modes. As a result, we added 11 veterinary drugs that ionize in negative mode to investigate the suitability of coated blade spray for rapid screening of multi-residue drugs in both negative and positive mode. Since CBS employs sword-like, stainless steel blades that are characterized by sharp edges, minor attachment of matrix components was initially observed, although this drawback was mainly observed to occur on the uncoated part of the blade, most specifically at the junction between the bare stainless pin and the coating. In order to prevent adhesion of macromolecules to the uncoated part of the blade, we applied a thin layer of PAN to the blade prior to application of the HLB/PAN extraction phase. The length of the undercoat PAN layer was 10 mm longer than that of the HLB coating so as to ensure no contact between the bare stainless-steel and sample during the extraction step. This improvement to the CBS blades successfully ensured that matrix components did not adhere to any part of the sampling device. In addition, we shortened the extraction time to 15 minutes as shorter extraction times were shown to lead to better signal to noise ratios (S/N) as well as minimize the unnecessary co-extraction of undesired molecules that may cause higher noise or ionization suppression. The optimized CBS device allowed for rapid and high-throughput screening and quantitation of 105 veterinary drugs in beef muscle in both negative and positive ionization modes in one single run with the use of a

single CBS device, achieving analysis times as short as 1 min per sample. In chapter 5, we explored another well-known ambient ionization technique, DART. While meshes have been shown to offer the best detectability and successful geometry for biological matrices, they are not applicable for extraction from diluted homogenized animal tissue samples due to potential attachment of matrix debris and macromolecules to the mesh. As a result, we investigated the applicability of the SPME pins designed and used in chapter 2 and 3 for coupling with DART. While CBS-MS/MS demonstrated a much wider analytical scope than SPME-DART-MS/MS, both methods demonstrated suitability for rapid screening and quantitation of multi-residue residue veterinary drugs in beef muscle.

6.2 Future directions

The advancements and applications introduced in this thesis bespeak a promising future for the applicability of the SPME as an alternative sample preparation tool for direct-immersion extraction from highly complex samples such as diluted homogenized beef and chicken tissue. The wide range of analytes and the challenging matrices covered by these methods demonstrate SPME as a universal sample preparation tool suitable for a wide range of analytes and complex solid sample matrices.

This work opens new paths for new applications, such as untargeted analyses and metabolomics studies aimed at profiling animal tissue samples for a variety of purposes; for instance, to study the effect of animal feed on the quality of meat, although the possibilities are virtually limitless. One such application we are currently working on is the detection of meat adulteration. Promising results have been achieved so far by using the method developed in

Chapter 2 with HRMS for untargeted metabolomics of beef and pork muscle so as to detect adulteration or contamination of ground beef samples with pork meat. The results obtained so far allowed us to detect ground beef samples containing over 5% pork meat.

In terms of residue analysis, the use of SPME with HRMS can be very useful to monitor fraudulent practices such as using mixtures of several unknown pharmaceutical drugs at very low amounts to obtain a synergistic effect for growth promotion.

While this work has demonstrated the suitability of SPME for extraction of a wide range of analytes characterized by a wide range of polarities, it is worth pointing out that the HLB coating still has limitations in terms of extracting very polar compounds (i.e. log $p < -2$). Accordingly, future work should be aimed towards the discovery of novel coating chemistries that can continue to drive possibilities for a more universal extractive phase that further expands the SPME analytical scope to include very non-polar compounds.

Certainly, the direct coupling of SPME to MS has great potential for rapid qualitative and semi-quantitative analysis in food safety applications, especially when used with portable mass spectrometers for in-vivo and on-site analysis. However, similar to all AIMS techniques, the direct coupling of SPME to MS has a number of challenges due to the absence of chromatographic retention and lack of selectivity and reproducibility. While the collection of MS^3 spectra and accurate mass capabilities of the latest HRMS instruments can enhance the selectivity of AIMS techniques, future work needs to be directed towards coupling SPME to non-chromatographic separations, such as ion mobility or differential mobility, prior to MS detection so as to reduce isobaric interference and to introduce additional identification features for qualitative analysis. In

the case of DART for example, additional compound identification information can be provided by introducing a thermal desorption gradient to selectively desorb analytes at different temperatures.

In contrast to clinical applications, where the in-vivo DI-SPME approach was successfully utilized to analyze intact tissue samples that were incurred with target drugs,[120,153–157] in case of food producing animals, it was extremely challenging to obtain intact animal tissue samples that were naturally incurred with measurable concentrations of veterinary drugs. This would have enabled us to assess the detection capability of the developed SPME methods to extract from intact tissue. However, given the proven success of DI-SPME in clinical studies for measuring the concentrations of targeted drugs in intact tissue samples, we have no doubt that the techniques developed in this thesis can be implemented successfully in the future on site for rapid and real-time screening of living animals by performing in-vivo chemical biopsy sampling using appropriate SPME devices.

Finally, this thesis has effectively demonstrated the development and validation of novel SPME-based technologies for reliable automated and high-throughput multi-class multi-residue analysis in animal tissue samples. Further work involving these methods will likely expand upon their applications to different food matrices such as animal feed and other foods derived from animals such as organs and eggs. Ultimately, one of the main future directions of this work is the possible implementation of these methods as fully validated official methods in regulatory monitoring of veterinary drugs.

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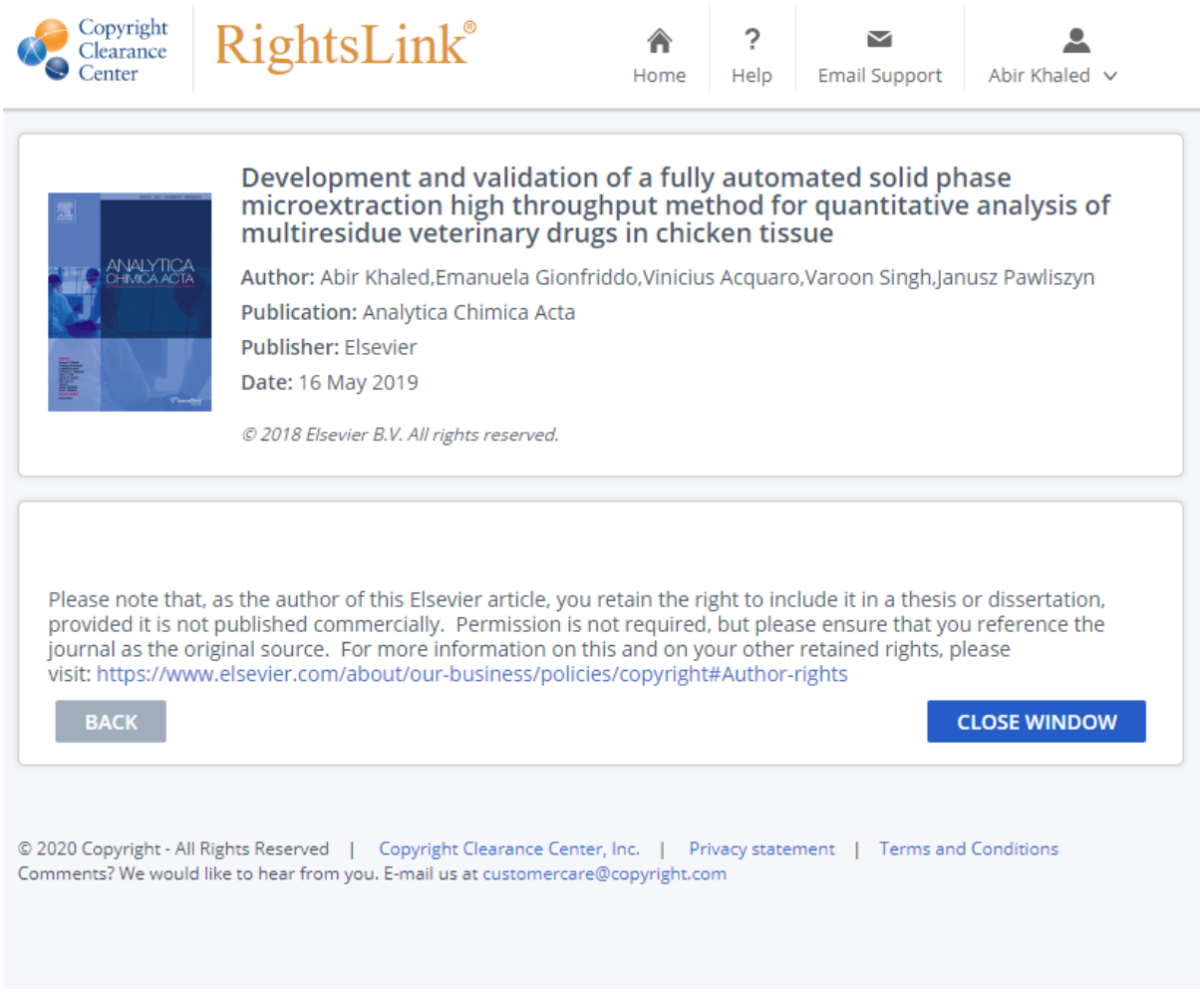

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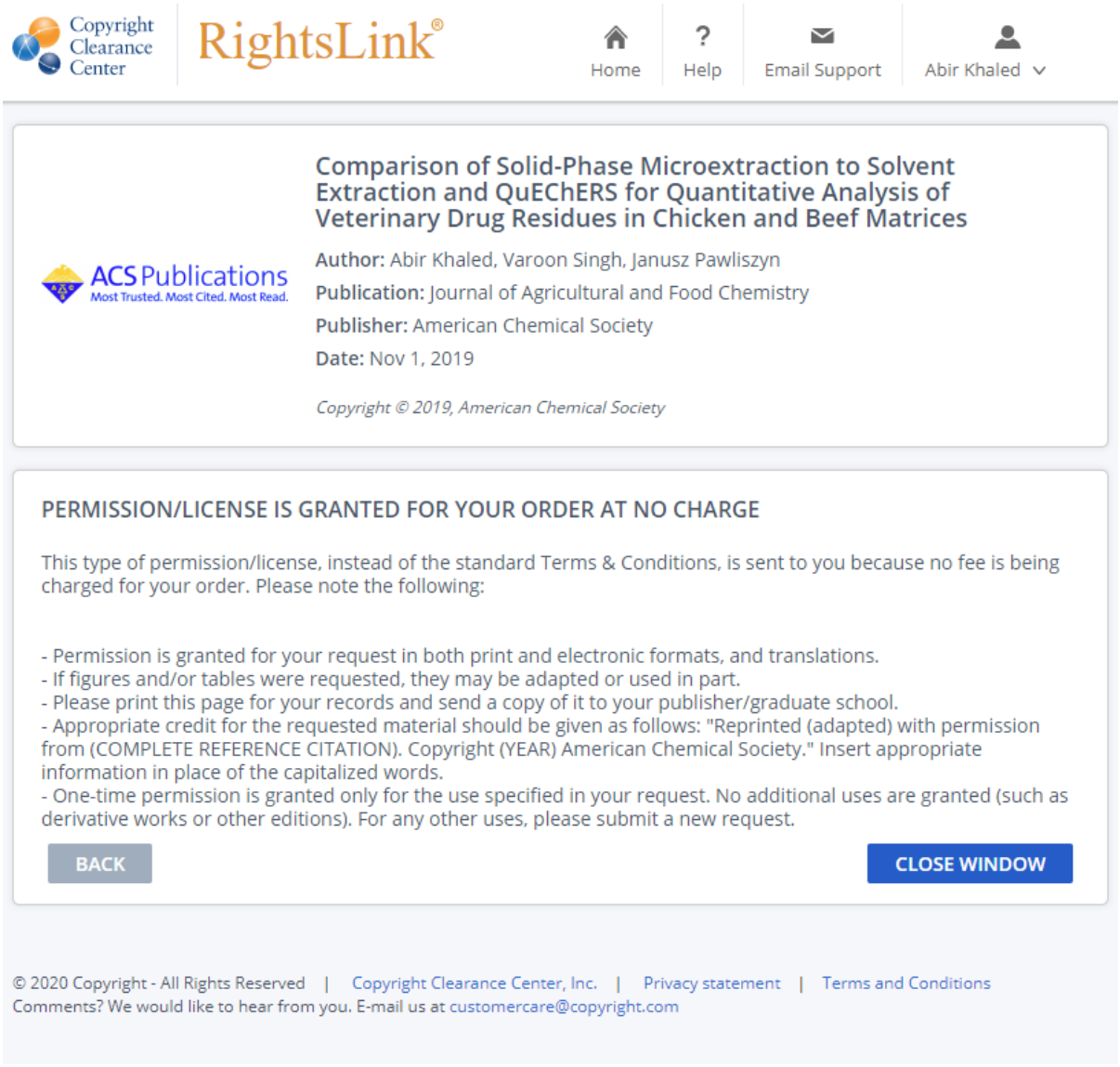

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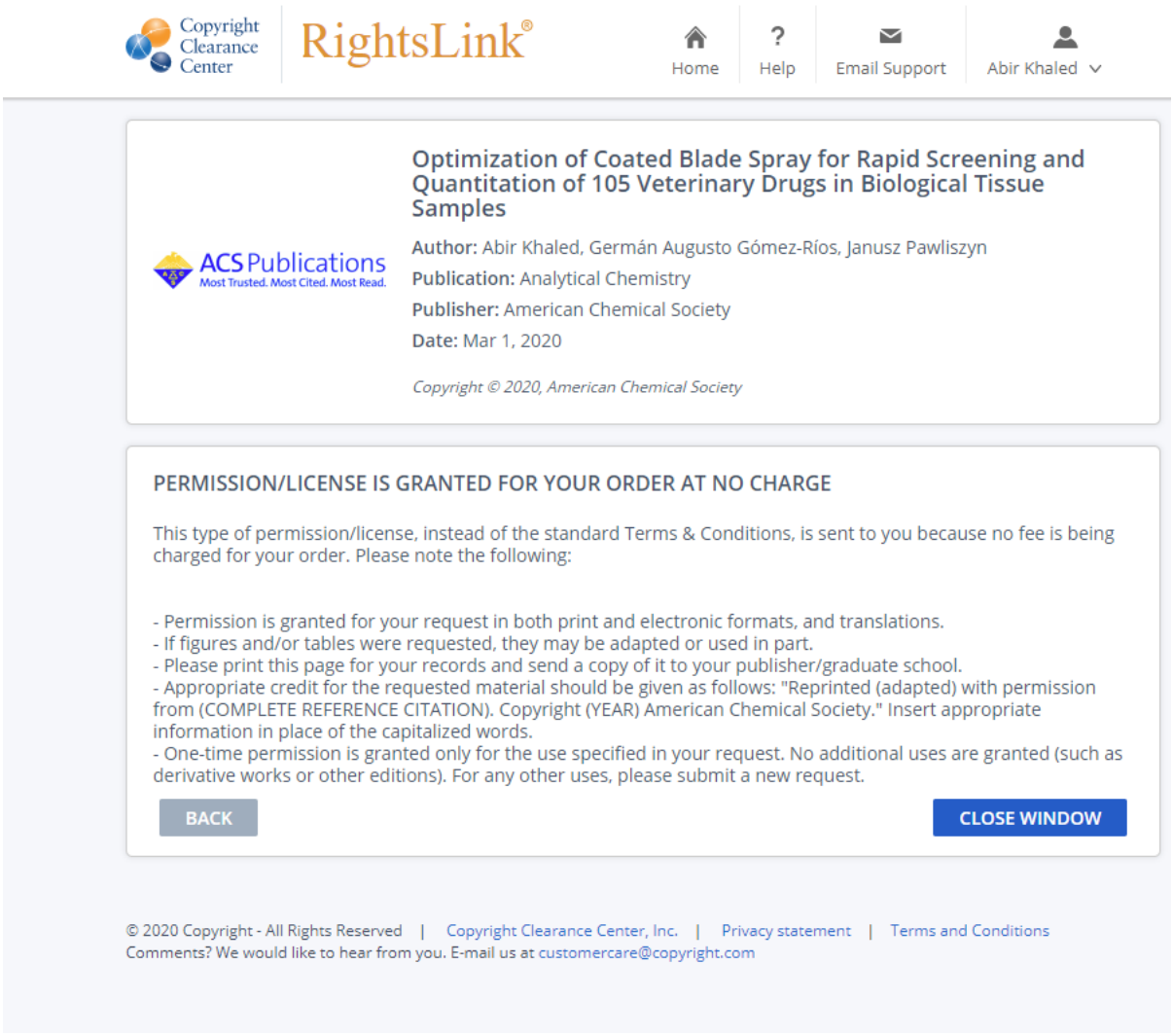

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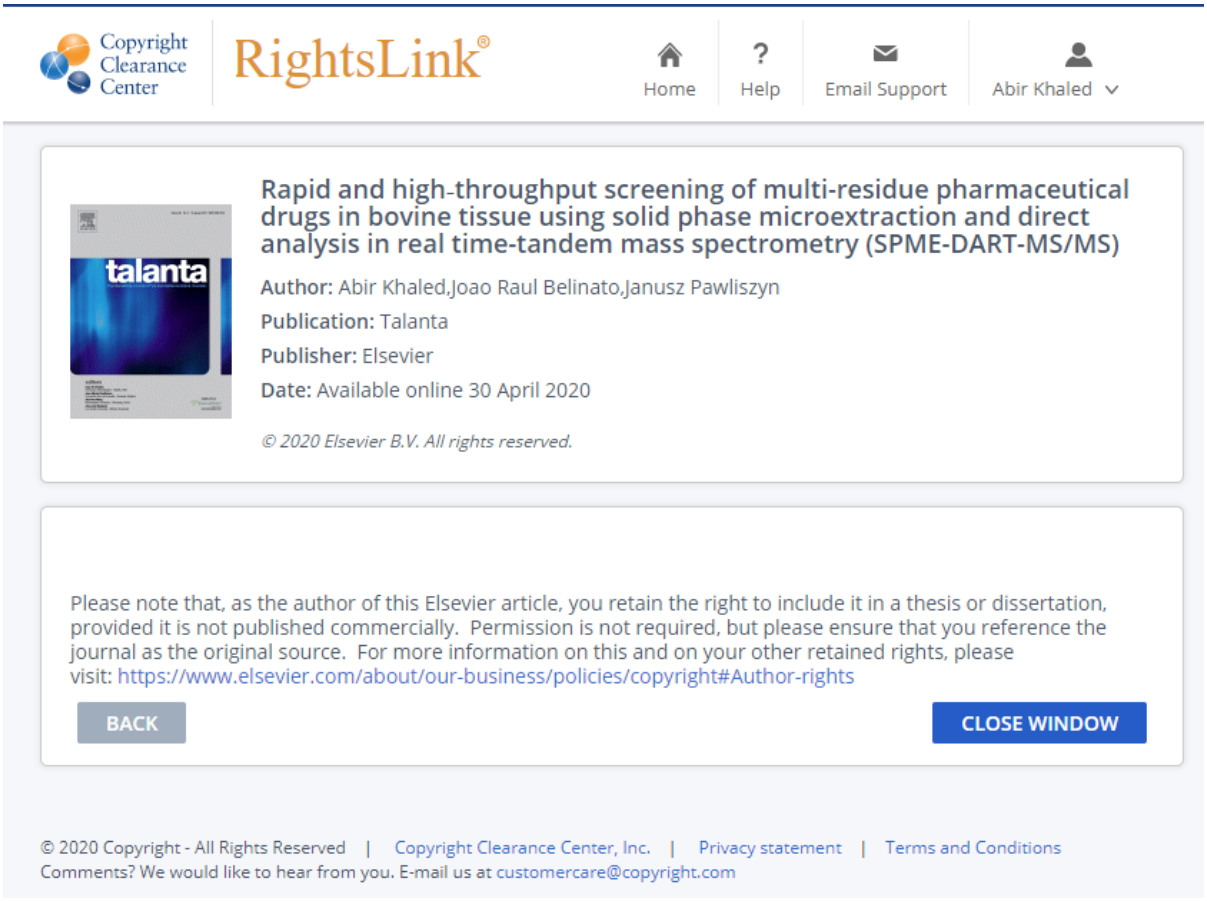

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Appendix A

Evaluation of four SPME coatings

Table 6.1 Evaluation of 4 SPME coatings (HLB, mix-mode, PS-DVB-WAX and 50:50 [w/w] HLB:PS-DVB-WAX in different desorption solutions (MeCN/H2O 50/50, MeCN/MeOH/H2O/FA 40/40/20/0.1 and MeCN/IPA/H2O/FA 40/40/20/0.1). Results are expressed as amount (ng) of compound extracted. The %RSD of three replicates are also shown. Extraction volume: 1000 µL, extraction time: 120 min, concentration of analytes: 50 ng mL-1, extraction matrix: PBS. Desorption time: 120 min, desorption volume: 1000 µL. All results are based on 3 replicates.

Compound	HLB						PS-DVB-WAX						50:50 [w/w] HLB:PS-DVB-WAX						Mix-mode					
	MeCN/H_2O (50/50)		MeCN/MeOH/H_2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H_2O/ FA (40/40/20/0.1)		MeCN/IPA/H_2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H_2O/ FA (40/40/20/0.1)		MeCN/IPA/H_2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H_2O/ FA (40/40/20/0.1)		MeCN/IPA/H_2O/ FA (40/40/20/0.1)	
	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%
2-Amino flubendazole	38.4	23	38.7	3	37.5	27	47.9	7	40.2	7	36.3	26	41.6	14	40.8	5	43.0	7	25.8	10	15.7	6	19.0	4
5-HydroxyThiabendazole	49.8	2	44.9	5	47.7	8	64.3	4	49.9	5	48.4	13	56.9	5	49.7	3	49.6	6	14.9	15	10.1	6	9.7	26
Acepromazine	16.3	16	41.7	4	35.9	4	1.2	44	46.1	6	38.5	4	2.7	3	47.3	4	39.5	4	12.6	16	28.5	5	25.0	6
Albendazole	34.9	6	36.2	3	35.0	3	47.1	2	45.0	2	40.4	2	41.8	2	44.6	2	41.3	1	38.9	11	34.9	5	36.4	1
Albendazole Sulfone	47.0	2	48.7	2	51.3	7	52.0	3	47.2	5	46.4	6	48.3	4	50.4	4	49.7	8	26.4	11	28.2	6	27.0	9
Albendazole Sulfoxide	60.5	4	58.7	4	57.4	4	60.7	6	49.4	7	44.9	8	57.3	5	52.3	5	49.7	4	25.8	13	24.4	6	22.4	8
Albendazole-2-aminosulfone	48.8	3	43.7	4	40.6	6	60.3	4	44.8	8	40.3	7	52.7	7	46.7	3	42.5	5	11.4	11	6.6	8	5.7	20
Azaperone	41.5	13	20.2	7	37.4	7	27.1	10	45.2	3	47.5	8	32.9	3	32.9	7	40.6	4	47.8	41	4.3	49	10.5	14
Betamethasone	48.2	2	46.6	2	47.0	3	45.9	6	39.8	10	36.8	11	48.0	5	48.9	3	46.4	3	41.1	11	40.9	4	40.6	5
Cambendazole	46.5	3	48.1	3	47.2	4	55.3	4	49.0	4	45.1	5	50.4	3	50.6	2	47.9	2	37.0	13	30.8	5	31.7	2
Carbadox	38.4	5	38.3	5	35.8	7	47.3	6	39.2	9	33.3	8	42.5	6	40.5	5	37.3	8	8.6	19	9.1	13	7.2	20
Chlorpromazine	7.7	26	32.9	9	31.4	5	0.5	54	37.5	7	32.4	9	1.3	5	38.8	5	33.1	8	9.1	18	23.4	5	22.2	8
Chlortetracycline	30.1	6	21.5	33	10.2	48	14.7	19	16.7	8	6.1	63	26.9	7	23.4	11	10.4	69	20.3	14	4.8	51	2.6	13
Clenbuterol	34.1	8	48.8	4	45.5	3	2.1	58	47.3	14	41.7	8	5.3	3	50.5	3	45.3	4	9.1	20	15.4	13	12.6	12
Clindamycin	41.5	5	41.0	1	41.0	4	42.1	4	39.3	8	35.6	9	46.0	4	48.0	2	45.5	2	37.3	13	30.3	5	30.7	6
Cloxacillin	47.7	1	46.6	5	42.7	7	35.4	11	32.1	9	25.1	15	47.9	6	47.6	5	41.3	8	35.4	12	37.8	8	33.4	5
Danofloxacin	38.8	21	61.2	15	9.3	12	22.2	29	43.9	23	8.7	30	26.7	21	57.2	10	14.8	17	4.4	8	6.2	8	2.5	41
Desethylene Ciprofloxacin	25.7	27	19.1	23	47.7	34	15.1	32	13.3	37	36.9	37	15.8	33	17.7	22	53.1	22	4.2	16	2.6	41	4.4	42
Diclofenac	51.5	7	47.2	1	43.7	5	33.9	11	40.8	10	34.4	11	45.0	7	46.8	2	41.6	6	31.1	12	30.5	6	26.4	8
Dicloxacillin	51.4	2	46.9	3	44.6	12	39.1	2	31.9	9	27.0	19	51.2	4	45.4	7	42.8	14	43.9	11	42.4	9	41.3	6
Difloxacin	36.3	5	51.8	7	36.1	2	30.7	10	35.0	11	23.1	12	39.6	8	51.0	4	29.7	37	23.5	13	12.8	28	10.0	39
Dimetridazole	25.3	5	26.5	5	17.3	10	39.2	13	34.9	8	20.2	15	27.1	7	30.3	10	20.6	29	2.0	11	2.2	8	1.2	44
Emamectin	1.0	32	4.0	17	4.2	8	0.5	19	2.0	26	2.2	18	0.9	13	4.0	8	4.2	6	0.9	26	3.0	5	3.9	12
Erythromycin	39.0	12	12.7	22	32.7	18	7.8	21	11.2	19	19.9	27	20.4	3	18.5	20	36.2	14	26.0	13	3.5	12	17.4	8

Table A.1 continued

Compound	HLB						PS-DVB-WAX						50:50 [w/w] HLB:PS-DVB-WAX						Mix-mode					
	MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)	
	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%
Fenbendazole	45.3	3	45.6	1	42.7	1	49.5	4	48.6	3	42.9	2	43.7	5	48.3	1	43.3	3	41.3	10	38.9	4	39.1	5
Fenbendazole Sulfone	48.2	1	48.1	1	45.6	5	57.0	3	47.3	4	43.1	5	51.9	8	50.3	3	45.7	1	39.1	9	38.2	5	35.6	4
Florfenicol amine	31.8	10	28.1	21	18.7	19	41.3	8	29.3	21	21.6	11	33.8	13	24.8	9	18.5	17	3.1	17	1.9	8	1.1	23
Flubendazole	47.6	3	49.1	1	47.5	2	55.5	4	51.5	5	45.5	4	49.9	2	52.6	2	47.7	1	41.0	15	41.4	3	40.3	3
Flunixin	50.3	4	46.7	1	44.8	1	34.3	7	39.0	11	34.9	9	44.7	6	45.6	5	43.5	4	27.3	13	26.6	7	24.5	9
Haloperidol	17.9	19	49.9	5	43.7	3	1.2	39	55.0	17	46.3	9	2.7	5	54.1	12	47.2	6	20.0	11	39.8	14	34.8	5
Hydroxy dimetridazole	12.2	12	13.9	8	9.6	16	28.9	9	23.9	13	18.3	12	21.9	10	20.5	14	16.8	8	0.5	10	0.6	18	0.2	58
Hydroxy Ipronidazole	35.4	5	35.6	4	31.2	4	43.3	5	35.7	9	30.5	10	39.1	7	36.8	6	33.7	5	3.1	14	3.4	9	2.5	23
Ipronidazole	44.8	2	46.0	2	43.5	3	52.0	4	45.1	6	41.3	5	46.3	5	45.3	3	44.1	4	6.7	14	7.1	7	5.9	20
Ketoprofen	44.7	5	45.7	2	42.7	4	39.8	5	39.1	9	33.4	7	42.4	4	44.0	4	40.9	5	18.0	18	19.6	8	15.8	12
Levamisole	25.4	12	44.9	9	38.8	5	28.7	13	48.5	6	42.0	5	31.2	8	48.1	7	42.0	4	9.4	12	11.2	4	6.9	12
Lincomycin	46.2	5	36.8	4	36.3	7	34.6	15	23.8	14	20.9	15	46.7	10	36.1	7	35.9	6	23.3	6	14	10	13.9	2
Mebendazole	46.2	4	46.1	1	42.8	2	54.7	2	46.2	5	40.9	3	47.7	6	48.1	1	43.0	3	38.9	9	37.0	5	35.4	5
Mebendazole amine	54.3	2	48.2	3	44.8	2	63.5	3	51.4	4	45.4	6	55.8	7	50.2	3	46.3	2	33.0	8	20.2	5	20.6	4
Melengestrol Acetate	36.9	7	41.0	6	39.7	4	32.6	2	33.0	7	30.5	8	35.9	6	42.0	6	39.1	8	32.6	39	41.0	10	37.8	8
Orbifloxacin	43.1	14	42.1	11	37.0	4	31.0	28	25.7	23	20.4	16	40.4	15	37.1	7	33.5	5	13.8	20	12.0	12	7.7	16
Oxacillin	48.6	5	48.4	3	44.4	9	37.1	10	32.2	11	26.0	13	48.3	6	45.2	3	41.6	6	30.2	18	31.4	8	28.5	10
Oxfendazole	46.1	4	43.0	6	44.7	5	49.9	4	39.1	8	37.6	7	47.5	10	41.7	5	41.0	5	31.4	7	26.1	2	28.0	6
Oxyphenylbutazone	22.6	4	21.3	6	24.3	8	16.1	8	20.1	12	20.2	10	24.2	5	26.7	7	28.9	4	4.8	28	8.0	7	7.6	16
Oxytetracycline	43.6	5	310	27	24.6	18	14.8	40	17.2	14	9.6	25	32.5	3	29.7	12	20.4	29	11.8	26	2.5	36	2.4	27
Phenylbutazone	22.4	4	17.0	4	21.4	6	16.8	6	17.0	11	18.7	13	23.5	5	22.0	9	25.0	5	10.4	25	13.0	13	13.8	11
Pirlimycin	36.0	10	37.7	1	37.6	4	14.3	11	28.1	12	24.8	14	23.4	5	39.7	4	39.0	5	16.2	6	18.5	7	19.1	5
Prednisone	48.3	1	48.9	2	48.1	6	47.0	9	40.7	9	37.7	10	49.3	3	48.8	3	47.3	4	39.2	12	40.8	8	38.8	1
Promethazine	12.5	19	41.2	6	34.8	7	1.0	43	46.1	6	38.7	2	2.3	3	47.4	2	39.1	3	12.0	14	28.8	4	23.7	24
Propionylpromazine	11.4	21	38.6	5	21.2	4	0.7	48	41.4	8	21.8	5	1.8	5	43.5	5	23.2	4	10.2	12	28.0	5	15.4	4
Ractopamine	44.7	2	50.8	2	47.9	3	7.5	27	52.3	9	45.3	5	13.6	2	54.9	3	48.7	1	13.1	11	17	6	14.3	12
Sarafloxacin	29.6	12	42.8	12	36.6	8	22.5	11	28.7	14	20.9	14	30.1	13	40.7	5	30.8	12	7.7	6	6.8	20	6.2	11
Sulfachloropyridazine	29.5	7	27.3	6	24.6	9	31.3	13	26.0	12	22.0	16	32.7	11	29.1	10	27.1	10	1.4	20	1.5	14	1.1	23
Sulfaethoxypyridazine	47.8	5	46.1	1	41.7	6	51.4	8	42.4	9	36.0	11	48.6	8	46.4	6	40.5	6	8.6	17	8.8	9	6.9	20

Table A.1 continued

Compound	HLB						PS-DVB-WAX						50:50 [w/w] HLB:PS-DVB-WAX						Mix-mode					
	MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)		MeCN/H_2O (50/50)		MeCN/MeOH/H2O/ FA (40/40/20/0.1)		MeCN/IPA/H2O/ FA (40/40/20/0.1)	
	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%	ng	RSD%
Sulfamerazine	34.1	5	34.7	4	32.4	8	36.4	7	29.6	15	26.1	10	37.2	8	34.7	10	33.6	11	1.6	13	1.9	7	1.2	40
Sulfamethazine	42.4	6	39.2	3	34.5	12	40.5	6	32.3	10	28.4	12	42.5	9	38.4	7	34.7	5	4.9	21	4.7	9	3.7	24
Sulfamethizole	21.3	8	21.2	3	19.2	12	26.8	10	23.9	11	22.0	18	25.3	11	24.7	11	23.7	8	1.5	15	1.3	35	1.4	29
Sulfamethoxazole	26.1	7	25.1	2	23.1	7	28.4	9	23.9	12	21.0	15	30.8	7	28.1	12	28.0	10	0.7	12	0.8	10	0.4	51
Sulfapyridine	43.0	5	41.3	2	42.6	11	50.9	6	40.3	8	40.6	12	46.8	7	43	6	44.5	4	3.4	17	3.5	8	2.5	19
Sulfaquinoxaline	48.2	9	43.9	3	38.5	7	45.1	9	38.1	10	31.0	12	48.7	10	45.1	4	37.1	8	6.5	19	6.4	12	4.6	23
Sulfathiazole	34.6	7	30.2	12	34.7	12	43.7	7	35.5	185	37.8	10	39.9	6	35	7	36.6	8	2.1	9	2.4	7	1.5	32
Tetracycline	46.0	5	34.4	29	26.0	18	18.6	15	20.6	11	10.6	30	35.2	9	32.3	11	21.3	29	18.2	7	4.0	32	3.9	21
Tolfenamic Acid	51.5	4	48.0	2	42.9	4	32.0	10	47.0	10	39.5	9	42.1	6	49.0	5	41.7	9	37.9	10	40.0	4	35.2	9
Trifluropromazine	8.8	22	39.5	4	39.1	5	0.6	51	41.8	5	39.0	5	1.4	3	44.0	3	40.1	4	8.6	14	31.3	5	29.6	5
Tylosin	8.0	12	8.0	5	7.7	12	4.1	19	3.6	10	3.4	9	6.9	9	7.0	5	6.9	5	8.4	42	7.3	12	8.8	13
Virginiamycin	42.4	8	36.2	3	37.1	7	32.9	12	26.1	12	24.1	15	41.5	6	37.8	2	36.3	3	35.9	24	34.8	6	34.3	6
Xylazine	19.4	17	50.7	6	46.6	2	1.5	53	52.3	10	45.5	10	3.5	4	53.8	5	47.5	3	11.5	20	21.5	13	17.1	13
Tetracycline	46.0	5	34.4	29	26.0	18	18.6	15	20.6	11	10.6	30	35.2	9	32.3	11	21.3	29	18.2	7	4.0	32	3.9	21
Tolfenamic Acid	51.5	4	48.0	2	42.9	4	32.0	10	47.0	10	39.5	9	42.1	6	49.0	5	41.7	9	37.9	10	40.0	4	35.2	9
Trifluropromazine	8.8	22	39.5	4	39.1	5	0.6	51	41.8	5	39.0	5	1.4	3	44.0	3	40.1	4	8.6	14	31.3	5	29.6	5
Tylosin	8.0	12	8.0	5	7.7	12	4.1	19	3.6	10	3.4	9	6.9	9	7.0	5	6.9	5	8.4	42	7.3	12	8.8	13
Virginiamycin	42.4	8	36.2	3	37.1	7	32.9	12	26.1	12	24.1	15	41.5	6	37.8	2	36.3	3	35.9	24	34.8	6	34.3	6
Xylazine	19.4	17	50.7	6	46.6	2	1.5	53	52.3	10	45.5	10	3.5	4	53.8	5	47.5	3	11.5	20	21.5	13	17.1	13