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Development of methods for the determination of veterinary drugs in food matrices by Liquid Chromatography – Mass Spectrometry

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DOCTORAL THESIS

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ABSTRACT

The possible presence of veterinary drug residues and other contaminants in edible tissues and even food products is one of the key issues for food safety which arouses great public concern. The main objective of this thesis was the development of sensitive, selective, robust and effective analytical methods for the determination of veterinary drugs in food of animal origin using liquid chromatography hyphenated with mass spectrometric techniques.

Initially, an extended review of the veterinary drug classes and the existing methodologies for their determination is presented. The experimental section of the thesis is constituted of four parts: (1) Multi-residue determination of seventeen sulfonamides and five tetracyclines in fish tissue using a multi-stage LC-ESI-MS/MS approach based on advanced mass spectometric techniques (Chapter 3), (2) Qualitative multi-residue screening methods for 143 veterinary drugs and pharmaceuticals in milk and fish tissue using Liquid Chromatography Quadrupole-Time-Of-Flight Mass Spectrometry (Chapter 4), (3) Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) (Chapter 5) and Multiresidue / Multiclass Determination Of 76 Veterinary Drugs And Pharmaceuticals In Bovine Muscle Tissue By Hydrophilic Interaction Liquid Chromatography-Tandem Mass Spectrometry (HILIC-MS/MS) (Chapter 6).

It is our strong belief that these studies will constitute a step forward in multiresidue veterinary drug analysis, providing rapid and reliable analytical results.

SUBJECT AREA: Analytical Chemistry

KEYWORDS: Veterinary drugs, LC-MS/MS, LC-QTOF MS, multi-residue methods, food matrices

ΠΕΡΙΛΗΨΗ

Η πιθανή παρουσία υπολειμμάτων κτηνιατρικών φαρμάκων και άλλων ρυπαντών σε βρώσιμους ιστούς και στα προϊόντα διατροφής είναι ένα από τα βασικά θέματα για την ασφάλεια των τροφίμων που προκαλεί μεγάλη ανησυχία στην κοινή γνώμη. Ο κύριος στόχος της παρούσας διπλωματικής εργασίας ήταν η ανάπτυξη ευαίσθητων, εκλεκτικών, ανθεκτικών και αποτελεσματικών αναλυτικών μεθόδων για τον προσδιορισμό κτηνιατρικών φαρμάκων σε τρόφιμα ζωικής προέλευσης με τη χρήση υγροχρωματογραφίας συζευγμένης με τεχνικές φασματομετρίας μάζας. Αρχικά παρουσιάζεται μια εκτεταμένη ανασκόπηση των κατηγοριών των κτηνιατρικών φαρμάκων και των υπάρχοντων μεθόδων για τον προσδιορισμό τους. Το πειραματικό μέρος της διατριβής αποτελείται από τέσσερα μέρη: (1) Ανάπτυξη ταχέων μεθόδων διαλογής (screening) για τον προσδιορισμό μη στοχευμένων-ενώσεων σε ζωικούς ιστούς με χρήση προηγμένων τεχνικών σάρωσης διαδοχικής φασματομετρίας μαζών συζευγμένης με υγροχρωματογραφία (Κεφάλαιο 3), (2) Ανάπτυξη πολυ - υπολειμματικής μεθόδου ταυτόχρονου προσδιορισμού 143 κτηνιατρικών φαρμάκων και φαρμακευτικών προϊόντων σε γάλα και ιστό ψαριού με LC-QTOF-MS (Κεφάλαιο 4), (3) Ανάπτυξη πολυ - υπολειμματικής μεθόδου ταυτόχρονου προσδιορισμού 115 κτηνιατρικών φαρμάκων και φαρμακευτικών καταλοίπων σε σκόνη γάλακτος, βούτυρο, ιστό ψαριού και αυγό χρησιμοποιώντας υγροχρωματογραφία υψηλής απόδοσης συζευγμένης με διαδοχική φασματομετρία μαζών (Κεφάλαιο 5) και Ανάπτυξη πολυ - υπολειμματικής μεθόδου προσδιορισμού 76 κτηνιατρικών καταλοίπων και φαρμακευτικών ουσιών σε ζωικούς ιστούς με υγροχρωματογραφία υδρόφιλων αλληλεπιδράσεων συζευγμένης με διαδοχική φασματομετρία μαζών (HILIC-MS/MS, Κεφάλαιο 6).

Πεποίθησή μας είναι ότι οι μελέτες αυτές θα αποτελέσουν ένα βήμα προς τα εμπρός στην πολυ-υπολειμματική ανάλυση κτηνιατρικών φαρμάκων παρέχοντας γρήγορα και αξιόπιστα αναλυτικά αποτελέσματα.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αναλυτική Χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Κτηνιατρικά φάρμακα, LC-MS/MS, LC-QTOF MS, πολύ-υπολειμματικές μέθοδοι, τρόφιμα ζωικής προέλευσης

To my father

CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

This work was conceived and performed at the Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece.

This thesis would not have been possible without the help and support of my supervisor, Dr. Nikolaos Thomaidis. The good advice, encouragement, and immense knowledge of him have been invaluable on both an academic and a personal level and were key motivations throughout my PhD. Dr Thomaidis has been a mentor, from whom I have learnt the vital skill of disciplined critical thinking for which I am extremely grateful.

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CHAPTER 1.

Veterinary drug residues and the role of analytical chemistry

1.1 Introduction

Since always, animal breeding and agriculture have been major human activities, but nowadays they have been evolved into an important economic activity and they have a clear impact on food safety. Over the years, increasing interest has been directed toward maximizing the quantity of food product and at the same time reducing the cost. It is of vital importance to cover the needs for food supplies of an increasing world population, and also comply with legal limits regarding contaminants and veterinary drugs used. Thus, new practices in animal breeding have been designed by controlling various factors such as genetics, nutrition, health, management and the environmental conditions.

During the last decades, a large number of veterinary drugs has been used at therapeutic levels in the systems of livestock breeding in order to improve animal health and prevent stress-induced animal death but also as growth promoters for intensive animal production [1]. Antibacterials (including sulfonamides, tetracyclines, beta-lactams, macrolides etc) are widely used by farmers to fight against bacterial infections [2, 3]. Furthermore, other families of veterinary drugs, such as anthelmintics and coccidiostats, are used for the treatment of parasitic diseases and coccidiosis (an infectious disease caused by a microscopic protozoan parasite), respectively [4, 5].

Their improper use, non-respect of withdrawal periods, and cross-contamination can lead to the presence of residues of veterinary drugs, mainly antimicrobial agents, in food of animal origin. These residues may include the non-altered parent compound as well as metabolites and/or conjugates, and may have direct toxic effects on consumers, e.g. allergic reactions in hypersensitive individuals. Moreover, indirect problems in clinical treatment maybe caused through induction of resistant strains of bacteria (development of bacterial resistance) [6-8].

As a result increasing concern has been expressed for the safeguarding of the public health. In that direction, several associations and international systems of

legal control are working on the quality assurance and control of the animal products entering the food supply.

1.2 Classification of veterinary drugs

1.2.1 Antibacterials

Antibacterial agents can be classified based on their mechanism of action, chemical structure, spectrum of activity or source. Most commonly, the classification is based on the chemical structures, which can provide information on chemical, physical and biological properties. The classes are: aminoglycosides, amphenicols, β-lactams (cephalosporins & penicillins), lincosamides, macrolides, nitrofurans, quinolones, sulfonamides, tetracyclines and miscellaneous.

1.2.1.1 Aminoglycosides

Aminoglycosides are broad-spectrum antibiotics isolated from *Streptomyces* and *Micromonospora* bacteria that exert their antibacterial effect by targeting the bacterial ribosome, thus inhibiting protein synthesis [9]. Their structure contains two or more aminosugars linked by glycosidic bond to an aminocyclitol group, which is 2-deoxystreptamine in most aminoglycosides or streptidine in streptomycin and dehydrostreptomycin. Most aminoglycosides are mixtures of several very similar components differing only in degree of methylation or stereochemistry of the sugar units. Closely related aminocyclitols, such as spectinomycin or apramycin, that also contain an aminocyclitol group but slightly differ in structure, are generally considered part of the aminoglycoside class of antibiotics. They are administered both therapeutically and prophylatically to treat cattle, swine and poultry [10]. Aminoglycosides are not absorbed orally and so are usually administered via intramuscular injection. Residues of these drugs tend to concentrate in the kidney as they are generally excreted through the urinary tract [11].

1.2.1.2 Amphenicols

Amphenicols (chloramphenicol, florfenicol, and thiamphenicol) are broad-spectrum antibiotics with a phenylpropanoid structure, active against a variety of pathogens. They function by blocking the enzyme peptidyl transferase on a ribosome subunit of bacteria [9]. Chloramphenicol was first isolated from cultures of *Streptomyces venezuelae* but is now produced synthetically. It readily forms conjugates with glucuronic acid in the liver of treated animals and therefore appears in kidney mainly as the corresponding glucuronide [12]. However due to the reports of serious side effects (mainly aplastic anemia) in humans, chloramphenicol was banned in the EU, the USA and Canada in the 1990s. Structurally similar thiamphenicol and florfenicol, in which the nitro group of chloramphenicol is replaced by a methyl sulphonyl group (in florfenicol, a hydroxyl group is also replaced by a fluorine), have been permitted as chloramphenicol substitutes.

1.2.1.3 β-Lactams

β-Lactam antibiotics are probably the most widely applied antimicrobial drugs in current veterinary practice. They are divided into two subcategories: penicillins and cephalosporins. These antibacterials have as their basic structure a thiazolidine ring, a β-lactam ring and variable side chains that account for the major differences in their chemical and pharmacological properties [13]. In penicillins, the ring is fused to a five-member thiazolidine ring, while for cephalosporins the ring is fused to a six-member ring. The *β*-lactam ring is responsible for the antimicrobial activity and also for a reduced stability of *β*-lactams. They are thermolabile, unstable in alcohols and acidic conditions [14].

Their mode of action is based on inhibiting bacterial cell wall biosynthesis, which has lethal effect on bacteria. However, bacteria have shown resistant against β-lactam antibiotics [15]. Penicillins are derived from *Penicillium fungi* and are historically significant because they are the first drugs that were effective against many previously serious diseases. They are used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms [16]. Cephalosporins are originally derived from the fungus *Acremonium*, previously known as *Cephalosporium*. First-generation cephalosporins were active predominantly against Gram-positive bacteria but successive generations have increased activity against Gram-negative bacteria, as well.

1.2.1.4 Macrolides and lincosamides

Macrolides are basic macrocyclic antibiotics that have a common 14-, 16-, or 17-membered ring in their structure, which is linked by glycoside bonding to one or more molecules of deoxy sugars, usually cladinose and desosamine. They are widely used in veterinary practice to treat respiratory diseases and to promote growth and are usually used against Gram-positive organisms that are resistant to penicillin treatment. Erythromycin and tylosin are the drugs most commonly given to food-producing animals. Macrolide antibiotics are weak bases readily soluble in common organic solvents [17]. Lincosamides (lincomycin, clindamycin, and pirlimycin) are monoglycosides with an amino acid side chain. The first lincosamide to be discovered was lincomycin, isolated from *Streptomyces lincolnensis*. They are highly effective against a broad spectrum of gram-positive and anaerobic bacteria. Both macrolides and lincosamides target the bacterial ribosome and inhibit protein synthesis [13, 14, 17].

1.2.1.5 Nitrofurans

Nitrofurans are synthetic antibacterial compounds, which contain a characteristic 5-membered nitrofuran ring in their structure. They are used to treat infections caused by protozoa or by certain Gram-positive or Gram-negative bacteria and do not contribute to the development of antimicrobial resistance [9, 13]. The precise mechanism by which nitrofurans exert their antimicrobial effects is not completely clarified, but it is based on inhibition of enzyme systems [18]. They are used in the poultry industry as well as for the treatment of cattle and pigs and residues of them have also been found in farm-raised shrimp and honey [11]. However due to their toxicological effects (carcinogenity and mutagenicity), nitrofurans (nitrofurazone, nitrofurantoin, furaltadone, furazolidone and later also nifursol) were banned in many countries, including the US, the EU, Japan and Australia, starting in mid-1990s to early 2000s.

1.2.1.6 Quinolones

Quinolones are broad spectrum synthetic antibiotics (derived from 3-quinolenecarboxylic acid) that are widely used in aquaculture and poultry farming. They prevent bacterial DNA from unwinding and duplicating.

The first generation of quinolones includes mainly oxolinic acid and nalidixic acid that are effective only against Gram-negative bacteria, while the second-generation quinolones are fluoroquinolones, such as enrofloxacin, danofloxacin and ciprofloxacin. Fluoroquinolones contain a fluorine atom at the C-3 position and a piperazinyl group at the C-7 position, which increases the activity against Gram-positive and Gram-negative bacteria, respectively, and the majority of quinolones in clinical use belong to this subclass [19]. Quinolones are also highly important human drugs, and their widespread use in food-producing animals is of high concern due to the recent evidence of development of bacterial resistance to these antibiotics.

1.2.1.7 Sulfonamides

Sulfonamides are synthetic antibiotics that are used for prophylactic and therapeutic treatment of bacterial and protozoal infections. They share a common chemical nucleus that comes from sulfanilamide and is responsible for the exhibited antimicrobial activity [14]. They have been used clinically for more than 50 years, and during this time over 5000 derivatives have been tested. Sulfonamides show large variations in polarity and exhibit amphoteric properties. In bacteria, antibacterial sulfonamides act as competitive inhibitors of the enzyme dihydropteroate synthetase (DHPS), an enzyme involved in folate synthesis (vitamin B9). As such, the microorganism will be "starved" of folate and die. On the contrary, humans, acquire folate through the diet [20]. Sulfonamides are often administered together with synthetic diaminopyrimidines, such as baquiloprim, ormetoprim or trimethoprim, which act as potentiators of sulfonamides.

1.2.1.8 Tetracyclines

Tetracyclines are broad-spectrum antibiotics that consist of a substituted 2-napthacenecarboxamide molecule. They are widely used in veterinary medicine for cost-effective prophylactic and therapeutic treatment and also as growth-promoting

substances in cattle and poultry but their usefulness has been reduced with the onset of bacterial resistance. Tetracycline antibiotics are protein synthesis inhibitors, inhibiting the binding of aminoacyl-tRNA to the mRNA-ribosome complex [21].

1.2.1.9 Other antibacterials

Unlike the compounds in the preceding groups, several individual antibacterials have heterogenous nature. A tabulated survey of their properties is not possible. However, there are a number of subgroups including diaminopyrimidines quinoxalines, pleuromutilins, peptides or novobiocin and dapsone that merit discussion.

Diaminopyrimidines are a class of organic chemical compounds that include two amine groups on a pyrimidine ring. They include many dihydrofolate reductase inhibitor drugs and the antibiotics iclaprim and trimethoprim. Trimethoprim blocks folic acid synthesis in bacteria at a step later than the sulfonamides [22].

Carbadox and olaquindox are both quinoxaline-1, 4- dioxide antibacterials that are synthetically produced. They are light-sensitive compounds and require special handling precautions during analysis to prevent their decomposition. Metabolism studies have shown that carbadox is rapidly converted into its mono-oxy and desoxy metabolites whereas quinoxaline-2-carbonic acid is considered to be the last remaining major metabolite and may serve as a marker residue. Both carbadox and its desoxy metabolite are carcinogenic compounds [23].

Pleuromutilin and its derivatives are antibacterial drugs that inhibit protein synthesis in bacteria by binding to the peptidyl transferase component of the 50S subunit of ribosomes. This class of antibiotics includes retapamulin, valnemulin and tiamulin [24]. Among the peptides, the main antibacterials are avoparcin, bacitracin, efrotomycin, polymyxin and virginiamycin. Most are complex multicomponent compounds that possess large peptide molecules that often contain D-amino acids in contrast to naturally occurring proteins, which are composed of L-amino acids.These peptides disrupt both Gram positive and Gram negative bacteria by interfering with cell wall and peptidoglycan synthesis [13].

Novobiocin, also known as albamycin or cathomycin, is an aminocoumarin antibiotic that is produced by the actinomycete Streptomyces niveus. Aminocoumarins are very potent inhibitors of bacterial DNA gyrase, with higher potency than fluoroquinolones, but at a different site on the enzyme. Finally, dapsone (diamino-diphenyl sulfone), according to its chemical structure, is not comprehended in any antibacterial class but according to its mechanism of action, it falls onto the sulfonamide group. As an antibacterial, dapsone inhibits bacterial synthesis of dihydrofolic acid, via competition with para-aminobenzoate for the active site of dihydropteroate synthetase. It is used for the treatment of Mycobacterium leprae infections (leprosy) and for a second-line treatment against Pneumocystis jirovecii [13].

1.2.2 Anthelmintics

Anthelmintics (also called parasiticides, endectocides and nematocides) are drugs used to treat parasitic warm infections, including flatworms (tapeworms and flukes) and roundworms (nematodes), which usually infect human, livestock and crops, affecting food production.

They are usually classified into several types on the basis of similar chemical structure and mode of action. Basically, three main families can be distinguished: benzimidazoles, nicotinic receptor agonists and macrocyclic lactones (avermectines and milbemycins) [25]. The benzimidazoles consist of a ring system composed of a benzene ring fused with an imidazole ring. They exert their effect by binding selectively and with high affinity to the beta-subunit of helminth microtubule protein. The target site of the nicotinic agonists (e.g. levamisole, tetrahydropyrimidines) is a pharmacologically distinct nicotinic acetylcholine receptor channel in nematodes. The macrocyclic lactones (e.g. ivermectin, moxidectin) are a group of complex compounds isolated from Streptomyces avermitilis. They act as agonists of a family of invertebrate-specific inhibitory chloride channels that are activated by glutamic acid [26].

The most frequently used anthelmintic compounds are levamisole, several compounds from the benzimidazole group (albendazole, cambendazole, fenbendazole, oxfendazole and thiabendazole) and ivermectin [1]. Other important anthelmintics are dichlorvos and haloxon (organophosphorus cholinesterase

antagonists) and piperazine (gamma-amino-butyric acid agonist at receptors on nematode muscles causing flaccid paralysis). Praziquantel has a selective effect on the tegument of trematodes and increases permeability of calcium while salicylanilides: rafoxanide, oxyclozanide, brotianide and closantel and the substituted phenol, nitroxynil, are proton ionophores [27].

Anthelmintic resistance is wide-spread and a serious threat to effective control of helminth infections and, therefore, new classes of anthelmintics with new new modes of action are being proposed. Thus, a new anthelmintic class named aminoacetonitrile derivative (AAD) has been developed, which is well tolerated and has low toxicity to mammals. The AAD monepantel is effective against some nematodes resistant to other drugs because its mode of action, which is based on a nematode-specific clade of acetylcholine receptor subunits, is different [24].

1.2.3 Beta-agonists

B-agonists are synthetic phenethanolamine compounds and were originally used as therapeutic treatments for asthma and preterm labour in humans [28]. However, these compounds have also been misused as nutrient repartitioning agents in livestock, where they served to divert nutrients from fat deposition in animals to the production of muscle tissues [29]. B-agonists have been banned as growth promoters in many countries including European Union countries and China because of their well-documented adverse effects on human health. Because of diversified analogues and rapid metabolism, highly sensitive analytical methods for quantification and confirmation of trace residues in cattle tissues are necessary for surveillance of feeding processes and food animal origin [30].

1.2.4 Coccidiostats

Coccidiostats are antiprotozoal agents that act upon Coccidia parasites by inhibiting reproduction and retarding the development of the parasite in a host cell [11, 31]. Even minor lesions of the intestinal wall due to coccidiosis can lead to poorer growth of the animal and lower feed conversion, reducing economic viability. They are most commonly used in poultry populations by addition in the feed at the authorized levels and observing the prescribed hygiene

requirements. The disease can also occur in other food producing animals including pigs, calves, and lambs [5].

Coccidiostats can be grouped in two major classes: the polyether ionophore antibiotics (monensin, lasalocid, maduramycin, narasin, salinomycin and semduramycin) and the nonpolyether ionophores (often reffered as synthetic compounds or chemicals). Polyether ionophore antibiotics are produced by fermentation with several strains of *Streptomyces* spp. and *Actinomadura* spp. They have both anticoccidial and antibacterial activity and they are also used as growth-promoting agents and as an active compound against clostridiosis [31].

1.2.5 Hormones

1.2.5.1 Anabolic steroids

Anabolic steroids (ASs) have been extensively used in husbandry practice with beneficial effects such as animal growth promotion and feed efficiency. The use of anabolic steroids for growth promotion purposes in meat producing animals results in an improvement in muscle growth and more lean meat. However, toxicological/epidemiological studies show that there are harmful effects to consumers; as a result the public health is placed in risk. As a consequence, the use of anabolic steroids for fattening purposes has been banned in the European Union since 1986 [32].

1.2.5.2 Corticosteroids

Endogenous corticosteroids are produced by the adrenal cortex (e.g. cortisol) and have important effects on a variety of metabolic events, including glucose and protein metabolism. The overall effect is to increase the blood glucose level by stimulating hepatic synthesis of glucose from amino acids [33]. Nowadays, several exogenous corticosteroids (prednisolone, dexamethasone, betamethasone, methylprednisolone) are authorized for therapy in both human and veterinary practices. They are widely used to combat inflammatory diseases in food-producing animals, but they are also frequently employed as growth promoters.

The European Union banned their administration for fattening purposes in 1996 [34].

1.2.5.3 Thyreostats

Thyreostats are orally active drugs, which upon administration disturb the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine. This goitrogenic activity may be attributed to the presence of athiocarbamidegroup. In livestock,the administration of thyreostats results in a considerable live weight gain, mainly caused by increased water retention in edible tissue and augmented filling of the gastrointestinal tract [35]. Consequently, these growth promoting agents negatively affect the meat quality of treated animals. In addition, xenobiotic thyreostats are listed as compounds with teratogenic and carcinogenic properties and thus pose a possible human health risk. These arguments led in 1981 to a ban on their use for animal production in the European Union [36].

1.2.6 Tranquilizers

Tranquilizers are administered to animals for sedation prior to anesthesia before transport to the market. Stress in animals is known to produce a deterioration of meat quality and pigs, in particular, easily become stressed during transport [1]. Some tranquilizers have analgesic effects (α_2-agonists) but these are the exception since analgesia is not a hall mark of tranquilizers [37].

Tranquilizers are classified into two broad categories in veterinary medicine: major and minor tranquilizers. Major tranquilizers include phenothiazides (acepromazine, promazine, and chlorpromazine), butyrophenones (azaperone, droperidol) and α_2-agonists (xylazine, detomidine, medetomidine, dexmedetomidine etc.) while minor consist of benzodiazepines (diazepam, midazolam, zolazepam) [37]. Most tranquillizers are rapidly metabolized in the animal's body and any residues are concentrated in the liver and/or kidney. These organs should be discarded if tranquillizers have been administered shortly before slaughter [1].

1.3 Residual veterinary drugs in food of animal origin

Organic contaminants that might be present in food, whether from natural or anthropogenic origin, can be divided into four main categories, namely pesticides, persistent environmental chemicals, naturally occurring toxins and veterinary drugs. In the field of food safety, scientists and regulatory agencies need to identify any potential risks to consumers related to the consumption of food [2].

Taking into consideration the inevitable use of veterinary drugs and the assurance of the public health, there are several measures required in order to eliminate the possibility of contamination; extensive analytical control of food, determination of the sources of contamination and strict legislation [38].

The veterinary drug residues in food are a crucial issue in food safety and thus in public health. The concept of zero tolerance, which refers to the total absence of residues, is unrealistic, since the power of analytical chemistry is not limitless. For quite some time, this concept seemed to guarantee the highest degree of food safety as residues could not be found in meat, milk and eggs, due to high detection limits. As the power of analytical chemistry increases, the types of chemicals that can be detected increase, and the limits of concentration at which they can be measured are continually reduced. Analytical Chemistry is the mean to expand and refine our ever-changing perspective of food safety. Since it is impossible to entirely abandon the use of veterinary drugs, a complete risk assessment must be performed in order to evaluate the possible hazards against public health.

1.3.1 Risk evaluation

Although residues from veterinary drugs in food products of animal origin are generally considered safe and well tolerated, they have been associated with a wide range of adverse effects and can represent a risk for consumers. However, the adverse effects from consuming food of animal origin, like meat, milk and eggs, are not very probable since the residues are present at very low concentrations, and thus acute human toxicity is rather unlikely [13].

The main side-effect of the presence of antibacterial residues in food is the development of resistant bacterial strains. Such resistance could be transferred to other bacteria, pathogenic or not, and can be related to the appearance of

antibacterial-resistant microorganisms [8]. Although increased bacterial resistance has several causes, two are the main key factors; the overuse and misuse of antibiotics. Such resistant bacteria may enter the human food supply and cause infectious diseases that can no longer be successfully treated by the antibacterial agent. Furthermore, some substances must receive particular attention due to allergic reactions [39].

Although prophylactic medication with coccidiostats in the feed remains the major way of preventing coccidiosis, the development of resistance by the coccidium to all medications available has been the greatest problem associated with this control [31]. Also, anthelmintic resistance has become entrenched as a perennial programme favourite at any gathering of veterinary parasitologists. Anthelmintic resistance is likely to develop wherever anthelmintics are frequently used and be detected if it is investigated. Worm count or egg count reduction after treatment are useful for the detection of all types of anthelmintic resistances. More economical, faster and more sensitive in vitro assays for the detection of anthelmintic resistance have been developed [40].

Finally, growth promoters (β-agonists, hormones) have been banned in many countries, including European Union countries, because of their well-documented adverse effects on human health, such as food poisoning and cardiovascular and central nervous diseases [16,41], as well as their teratogenic and carcinogenic properties [36].

Risk assessments of veterinary drugs residing in foods are performed by following the integrative steps of hazard identification, hazard characterization, exposure assessment, and risk characterization [42]. At the step of hazard identification, known or potential adverse health effects in humans are identified, which are induced by a veterinary drug or its metabolites that may be present in a particular food.

Toxicological evaluations, toxicokinetic assessments, and cancer/non-cancer evaluations are mainly performed for hazard identification. At the hazard characterization step, the characteristics of the adverse effects associated with a veterinary drug or its metabolites present in food are demonstrated. In addition, the levels that clearly do not cause any adverse effects on human health are evaluated according to dose-response relationships [43].

Maximum acceptable or tolerable levels for chemicals which are neither genotoxic nor carcinogenic, such as acceptable daily intake (ADI), reference dose (RfD), tolerable daily intake (TDI) and provisional tolerable weekly intake (PTWI) for contaminants which may accumulate in the body, are set. Dose–response information is essential for quantifying an adverse health effect. NOAEL (No Observed Adverse Effect Level) is the highest dose of a substance which causes no detectable adverse alteration in line with defined treatment conditions. ADI is generated using conservative statistical extrapolation to humans [44]. The ADI is an estimate of the residue, expressed in term of mg or mg per kg bodyweight, which can be ingested daily over a lifetime with a health risk to the consumer. In calculating an MRL, the ADI, the residue depletion patterns of a compound in the edible tissues of a particular food-producing animal and the theoretical food intakes are taken into account [45].

In case the chemical is evaluated as a complete carcinogen, which means a genotoxic carcinogen, it is recommended to operate a policy of prohibition and control levels “as low as reasonably practicable” [42].

Furthermore, toxicity assays involve the determination of acute toxicity, designated as LD_{50} (the dose that will kill 50% of the animals in a test series), subacute toxicity, determined by animal feeding tests lasting four weeks and chronic toxicity, assessed by animal feeding tests lasting 6 months to 2 years. In chronic toxicity tests attention is especially given to the occurrence of carcinogenic, mutagenic and teratogenic symptoms [38].

1.3.2 Legislation

In order to ensure the safety of the consumers, many agencies worldwide regulate the use of antimicrobials, particularly in food-animal species. The US Department of Agriculture’s (USDA) Food Safety Inspection Service (FSIS) is responsible for the safety of meat, poultry, and egg products in the USA. The European Food Safety Authority (EFSA) is the keystone of the European Union’s (EU) risk assessment regarding food and animal feed safety. The Codex Alimentarius Commission (created by the FAO and WHO) develops food standards, guidelines and related texts such as codes of practice under the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Moreover, VICH, a trilateral (EU-Japan-

USA) program aimed at harmonizing technical requirements for veterinary product registration was officially launched in April 1996.

The European Union (EU) has strictly regulated controls on the use of antibacterial agents, particularly in food–animal species, by publishing different Regulations and Directives. The use of veterinary drugs was regulated through EU Council Regulation 2377/90/EC [46], which has been repealed by Council Regulation 470/2009/EC [47] and describes the procedure for establishing Maximum Residue Limits (MRLs) for veterinary medicinal products in foodstuffs of animal origin.

In 1996, the prohibition of the use of growth promoters is laid down in Council Directives 96/22/EC and 96/23/EC, which also contain guidelines for controlling veterinary drug residues in animals and their products with all the necessary information to set up national monitoring plans [48, 49]. In regulation (EC) No 1831/2003 the European Union (EU) has prohibited the use of antimicrobials as feed additives but allows the usage of anticoccidial drugs to allow for the prevention of coccidiosis, a disease that may cause serious economical consequences [50]. EU-wide surveillance conducted during 2009, reported that coccidiostats continue to be a problem with non-compliance rates of 2.05 and 1.19% in poultry meat and eggs, respectively [5]. In response, the European Food Safety Authority set maximum levels (MLs) for 11 coccidiostats in edible tissues (including eggs) [51].

Most recently, Regulation 37/2010/EC [52] lists pharmacologically active substances and their maximum residue level (MRL) in foodstuffs of animal origin, as well as compounds for which no MRL has been set because no hazard for public health has been observed. As regards the coccidiostat lasalocid, regulation 37/2010/EC was amended in 2012 [53].

The requirements for performance and validation of analytical methods employed in the official residues control for screening and confirmatory purposes are described in European Decision 2002/657/EC [54]. Validation shall demonstrate that the analytical method complies with the criteria applicable for the relevant performance characteristics. Different control purposes require different categories of methods. The following table determines which performance characteristic shall be verified for which type of method [54].

Table 1.1: European Decision's 2002/657/EC requirements

		Detection limit CCß	Decision limit CCα	Trueness /recovery	Precision	Selectivity/ specificity	Applicability/ ruggedness/
Qualitative methods	S	+	-	-	-	+	+
	C	+	+	-	-	+	+
Quantitative methods	S	+	-	-	+	+	+
	C	+	+	+	+	+	+
S = screening methods; C = confirmatory methods; + = determination is mandatory.							

Amending the Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin, a Commission Decision, 2003/181/EC, was regulated [55].

1.4 Analytical methodologies

For all the reasons mentioned above, sensitive and reliable analytical methods for the determination of veterinary drug and pharmaceutical residues in food of animal origin are needed to ensure consumers' safety. An emerging trend in drug residue analysis is the development of generic methods that are capable of monitoring a wide variety of compounds, belonging to different drug classes. This appears as a considerable challenge due to all the pre-mentioned reasons and, as a result, multiclass methods for veterinary drugs are still not so widespread although they are strongly required. An overview of the analytical methodologies developed so far for the multi-residue analysis of veterinary drugs in food matrices using liquid chromatography and mass spectrometric techniques is presented in **Table 1.2.**

Table 1.2: Applications in multi-residue analysis of veterinary drugs in food matrices.

Compounds	Matrix	Sample preparation technique	Stationary phase	Mobile phase	Detection-identification	Recoveries	Reference
SAs (6), TCs (1), Qs (6) and other contaminants (5)	shrimp	extraction with 5% TCA in H_2O (v/v) and cleanup with SPE	Waters YMC Phenyl, (50×4mm, 3 µm)	**A:** 5% (v/v) ACN/H_2O, with 0.1% formic acid, **B:** 85% (v/v) ACN/ H_2O with 0.05% formic acid.	LC–APCI-QIT-MS^n	>40% (Sulfaquinoxaline) - >90% (Sulfamerazine)	Li 2006b [56]
Antibiotics (19), synthetic antibacterials (42), hormonal agents (20), ANTHs (35) and other contaminants (14)	Bovine, porcine, chicken muscle	extraction with ACN-MeOH (95:5, v/v) and delipidation with n-hexane saturated with ACN	TSKgel ODS-100Z (150×2.1mm, 5 µm)	**A:** 10mM ammonium acetate containing 0.3% acetic acid, **B:** ACN / MeOH (2/8, v/v)	LC-ESI-MS/MS (+) and (-)	46.3% (Ciprofloxacin) - 117.7% (Desoxycarbadox)	Yamanda [57]
SAs (15), TCs (5), Qs (4) and β-LACTs (5)	egg	Homogeniztion with sodium succinate buffer and extraction-	phenyl cartridge column,	**A:** 0.1% formic acid in H_2O (v/v), **B:**	LC-ESI-MS/MS (+)	<25% (Amoxicillin) - 80% (SAs)	Heller 2006 [58]

		cleanup with SPE	(50×4mm, 3 µm silica)	ACN			
TCs (7) and Qs (14)	pig tissues	extraction with EDTA-McIlvaine buffer and cleanup with SPE	Acquity UPLC BEH C18 (100×2.1mm, 1.7 µm)	**A:** MeOH/ACN (v/v, 40:60), **B:** 0.2% formic acid in H_2O	LC-ESI-MS/MS (+)	85.6% (Chlortetracycline) - 117.8% (Demeclocycline)	[59] Shao 2007
β-LACTs (3), LINCs (1), MCs (4), Qs (8), Sas (10), TCs (3), ANTHs (1) and other antibiotics (2)	meat	pre-homogeneization of the meat with EDTA –washed sand and PLE with H_2O	XTerra MS C18 (100×2.1 mm, 3.5 µm)	**A:** 10mM formic acid in MeOH **B:** 10mM formic acid in H_2O	LC-ESI-MS/MS (+)	73% (Tetracycline) – 93% (Flumequine)	[60] Carretero 2008
TCs (4), Qs (9), MCs (4), β-LACTs (7), SAs (14) and other antibiotics (1)	chicken, porcine muscle	extraction with MeOH - water (70:30, v/v) with EDTA, dillution	Acquity UPLC BEH C18 (100×2.1mm, 1.7 µm)	**A:** 1 mM oxalic acid with 0.2% formic acid in H_2O **B:** 0.1% formic acid in ACN.	LC-ESI-MS/MS (+)	60.5% (Chlortetracycline) - 96.5% (Spiramycin)	[61] Chico 2008b
TCs (5), MCs	honey	liquid–liquid extraction	Zorbax SB-	**A:** 1mM	LC-ESI-	24% (Streptomycin) -	[62]

(7), AMGs (3), β-LACTs (8), AMPs (2) and SAs (17)		with ACN, 10% TCA in water (v/v) and NFPA - hydrolysis	C18 (50×2.1mm, 1.8 µm)	NFPA in H_2O with 0.5% formic acid, **B:** ACN/MeOH (50/50, v/v) with 0.5% formic acid	MS/MS (+)	226% (Neomycin)	Hammel et al. 2008
Qs (5), TCs (4), MCs (2), SAs (1), AMPs (1), AMGs (1), LINCs (1), COCs (1) and other contaminants (1)	honey	dissolution in water and cleanup with SPE	Phenomenex Polar-RP Synergi (50×2mm, 4 µm)	**ESI (+)**: **A:** 0.1% formic acid in H_2O, **B:** 0.1% formic acid in ACN, **ESI (-): A:** H_2O, **B:** ACN	LC-ESI-MS/MS (+) and (-)	29% (Erythromycin) - 104% (Streptomycin)	[63] Lopez et al. 2008
β-LACTs (16), SAs (10), MCs (10), AMGs (8), TCs (4) and Qs (10)	milk	extraction with ACN and 5% TCA aqueous solution	Symmetry C18, (150×3.9mm, 5 µm)	**A:** 0.1% PFPA in H_2O, **B:** ACN	LC-ESI-MS/MS (+)	-	[64] Gaugain-Juhel. 2009
TCs (4), SAs (4), Qs (4), β-LACTs (3) and MCs (4)	muscle	extraction with MeOH - water (70:30, v/v) with EDTA, dillution	Genesis C18, (50×2.1mm,	**A:** 0.2% formic acid containing	LC-ESI-MS/MS (+)	68% (Ciprofloxacin, Difloxacin) - 95% (Oxytetracycline)	[65] Granelli 2009

			4 µm)	0.1mM oxalic acid in H_2O, **B:** ACN			
TCs (4), SAs (12), Qs (14), MCs (10), LINCs (3) and other antibiotics (1)	milk	extraction with McIlvaine buffer at pH 4.0, TCA, cleanup with SPE	C18 AQUA (150×2.1 mm, 3 µm)	**A:** 0.2% formic acid in H_2O, **B:** 0.2% formic acid in ACN	LC-ESI-MS/MS (+)	94% (Doxycycline) – 112% (Tulathromycin)	[66] Bohm 2009
MCs (4), TCs (4), Qs (5), and SAs (4)	honey	dissolution in Na_2EDTA and cleanup with SPE	Acquity UPLC BEH C18 (100×2.1mm, 1.7 µm)	**A:** MeOH, **B:** 0.05% (v/v) formic acid in H_2O	LC-ESI - MS/MS (+)	53 % (Erythromycin) - 115% (Tylosin)	[67] Martínez Vidal. 2009
TCs (4), MCs (4), Qs (5), SAs (4) and ANTHs (8)	egg	Comparison of SE - QuEChERS - SPE - MSPD	Acquity UPLC BEH C18 (100×2.1mm, 1.7 µm)	**A:** MeOH, **B:** 0.05% (v/v) formic acid in H_2O	LC-ESI - MS/MS (+)	**SEs':** 70.4% (Tetracycline) to 94.4% (Tilmicosin)	[68] Garrido Frenich 2010
AMGs (3), β-LACTs (3), TCs (4), LINCs (2), MCs (4), Qs (4), and SAs (4)	chicken muscle	extraction using (1:1, v/v) of 2% trichloroacetic acid in H_2O – ACN followed by removing fat with hexane	ZIC–HILIC (100×2.1 mm, 3.5 µm)	**A:** 50mM ammonium formate in H_2O at pH 2.5, **B:** ACN	LC-ESI - MS/MS (+)	57% (Erythromycin) - 86% (Danofloxacin)	[69] Chiaochan et. al 2010

Qs (4), TCs (3), MCs (9), β-LACTs (4), SAs (9), AMPs (3), AMGs (6) and NSAIDs (1)	amimal tissue	solid-liquid extraction with ACN / H_2O (86:14, v/v) and defatting with hexane	Atlantis dC18 (20×3.9 mm, 3 µm) and ZIC–HILIC (50×2.1mm, 5 µm) for AMGs	**Reversed Phase**: **A:** 0.1% (v/v) formic acid in H_2O, **B:** ACN **HILIC: A:** 0.4% (v/v) formic acid in H_2O, **B:** ACN	LC - ESI - MS/MS (+) and (-)	-	[70] Martos et al. 2010
SAs (9), Qs (5), MCs (1), β-LACTs (1)	Bovine muscle tissue	Extraction with ACN – d-SPE with PSA (QuEChERS) compared to PLE with water	XTerra MS C18 (100×2.1 mm, 3.5 µm)	**A:**10 mM ammonium formate in H_2O, **B:** 10 mM ammonium formate in MeOH	LC-ESI - MS/MS (+)	25% (Ciproflocaxin) – 93% (Flumequine)	[71] Blasco et al. 2011
TCs (3), SAs (16), β-LACTs (7), Qs (3), MCs (3), LINCs (2)	porcine muscle	solid–liquid extraction with ACN with fast partition at very low temperature	Zorbax Eclipse XDB C-18 (150×4.6 mm, 5 µm)	**A:** H_2O/ACN (95 : 5 v/v) with formic acid 0.1% and **B:** H_2O/ACN (5 : 95 v/v) with formic	LC - ESI - MS/MS (+)	-	[72] Lopes et al. 2011

				acid 0.1%			
SAs (14), TCs (4), Qs (9), β-LACTs (7), MCs (5), LINCs (1) and other antibiotics (1)	egg	PLE with 1:1 (v/v) mixture of ACN and 0.01 mol L−1 succinic acid buffer pH 6.0	Acquity UPLC BEH C18 (100×2.1mm, 1.7 μm)	**A:** oxalic acid 2-hydrate 0.13g/L in H_2O with 0.02% formic acid, **B:** 0.1% (v/v) formic acid in ACN	LC-ESI-MS/MS (+)	47% (Amoxicillin) - 320% (Danofloxacin)	[73] Jiménez et al. 2011
TCs (7), Qs (14), MCs (12), SAs (12), LINCs (3), PLMTs (3) and other antibiotics (3)	cattle and pig muscle	extraction with EDTA-McIlvaine buffer and cleanup with SPE	AQUA C18 (150×2.1 mm, 3 μm)	**A:** 0.2% formic acid in H_2O, B: 0.2% formic acid in ACN	LC - ESI - MS/MS (+)	99% (Nalidixic acid) - 114% (Ofloxacin)	[74] Bohm et al. 2011
β-LACTs (4), SAs (8), TCs (4), Qs (3), MCs (3), NSAIDs (1), ANTHs (1), other antibiotics (1) and other contaminants (1)	milk	extraction with ACN, cleanup with SPE and with an 30 kDa MW cutoff filter	YMC ODS-AQ (100×2 mm, 3 μm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** ACN	LC - ESI - MS/MS (+)	22% (Ampicillin) - 143.3% (Enrofloxacin)	[75] Clark et al. 2011
Qs (11), SAs (20), TCs (4),	bovine kidney	extraction ACN- H_2O (4:1, v/v), cleanup with	Prodigy ODS-3	**A:** 0.1% (v/v) formic acid in	LC - ESI - MS/MS (+)	53 % (Chlortetracycline) - 129% (Semicarbazide)	[76] Schneider

MCs (9), NFs (7), β-LACTs (15), AMPs (1), QUINOXs (1), ANTHs (12), β-AGONs (9), NSAIDs (6), CORTs (7), THYRs (5), other antibiotics (2) and other contaminants (11)		hexane partitioning	(150×3mm, 5 µm)	H_2O, **B:** 0.1% (v/v) formic acid in ACN			et al. 2012
TCs (3), β-LACTs (5), Qs (2), SAs (3) and other contaminants (8)	egg	MeOH:H2O:CH3COOH 80:20:1 (v/v/v), 0.5 g CH3COONa and 2.0 g Na_2SO_4 anhydrous (QuEChERS-like extraction method)	ACE C18 (150×2.1mm, 3 µm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** 0.1% (v/v) formic acid in ACN	LC - ESI - MS/MS (+)	56% (Oxacillin) - 79% (Doxycycline)	[77] Capriotti et al. 2012
SAs (5), Qs (7), TCs (4), MCs (4), ANTHs (8) and other antibiotics (1)	baby food and infant formula	modified QuEChERS approach	Acquity UPLC BEH C18 (100×2.1mm, 1.7 µm)	**A:** MeOH, **B:** 0.05% (v/v) formic acid in H_2O	LC-ESI-MS/MS (+)	baby food: 69.9% (Flumequine) to 122.9% (Sulfaquinoxaline) - infant formula: 70.4% (Flumequine) to 119% (Sulfadimethoxine)	[78] Aguilera-Luiz et al. 2012

Qs (2), SAs (6), MCs (4), ANTHs (7), other antibiotics (1) and other contaminants (1)	chicken	QuEChERS	Acquity UPLC BEH C18 (100×2.1mm, 1.7 µm)	**A:** 0.1% (v/v) formic acid in ACN, B: 0.1% (v/v) formic acid in H_2O	LC-ESI-MS/MS (+)	69% (Sulfadimidine) to 118.3% (Oxolinic acid)	[79] Lopes et al. 2012a
MCs (11), β-LACTs (7), LINCs (2), Qs (1), ANTHs and other antibiotics (1)	milk	Extraction with ACN	HSS T3 column (100×2.1 mm, 1.8 µm)	**A:** 0.05% (v/v) formic acid in H_2O, **B:** ACN	UPLC-ESI-MS/MS (+)	56.9 % (Cefapirin) – 127.6% (Troleandomycin)	[80] Tang et al. 2012
β-LACTs (7), TCs (4), Qs (6), SAs (6), ANTHs (7) and other antibiotics (1)	gilthead sea bream	modified QuEChERS approach	Acquity UPLC BEH C18 (100×2.1mm, 1.7 µm)	**A:** 0.1% (v/v) formic acid in ACN, **B:** 0.1% (v/v) formic acid in H_2O	LC-ESI-MS/MS (+)	74% (Penicillin G) to 117% (Chlortetracycline)	[81] Lopes et al. 2012b
MCs (10), LINCs (3), Qs (13), TCs (8) and other antibiotics (3)	honey	Dillution to McIlvaine buffer, pH 4.0 and cleanup with SPE	AQUA C18 (150×2.1 mm, 3 µm)	**A:** 0.2% (v/v) formic acid in H_2O, **B:** 0.2% (v/v) formic acid in ACN	LC - ESI - MS/MS (+)	92% (Danofloxacin) – 106% Tylosin A	[82] Bohm et al. 2012
β-AGONs (17), β-LACTs (11), THYRs (2),	Raw milk	Extraction with ACN-Ethanol with addition of EDTA	Acquity HSS-T3 column	**ESI (+)**: **A:** 0.1% formic acid in H_2O	LC-ESI-MS/MS (+) and (-)	62% (Amitraz) to 133% (Teridazole)	[83] Zhan et al 2012

QUINOXs (9), LINCs (2), ANTHs (28), Qs (17), SAs (20), TCs (10), AMPs (4), CORTs (5), MCs (10), NSAIDs (25), COCs (12), other antibiotics (2) and other contaminants (81)			(100×2.1 mm, 1.8 µm)	with 0.5 mmol/L ammonium acetate, **B:** 0.1% formic acid in MeOH **ESI (-):** A: 2.5 mmol/L ammonium acetate in H_2O **B:** MeOH			
SAs (4), Qs (2), COCs (7), CORTs (3), other antibiotics (1) and other contaminants (1)	milk	extraction with ACN - cleanup with SPE	Synergi Polar-RP 100 Å (50×2.0 mm, 2.5 µm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** 0.1% (v/v) formic acid in ACN	LC- ESI-MS/MS (+)	65% (Robenidine) – 119% (Sulfamethoxypyridazine)	[84] Nebot 2012
Qs (14), TCs (4), MCs (7), β-LACTs (8), SAs (22), ANTHs (21), other antibiotics (4)	chicken muscle	solid-liquid extraction with EDTA-succinate buffer and acetonitrile	RP-LC column PFP (100×4.6 mm, 3 µm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** 0.1% (v/v) formic acid in ACN	LC - ESI - MS/MS (+)	29% (Ofloxacin) - 98% (Erythromycin)	[85] Biselli et al. 2013

and other contaminants (4)							
β-AGONs (17), THYRs (1), QUINOXs (7) MCs (10), ANTHs (28), Qs (17), NSAIDs (24), SAs (20), β-LACTs (6), AMPs (3), LINCs (1), COCs (13), CORTs (1), other antibiotics (1) and other contaminants (62)	infant formula	extraction with ACN, clean-up by low temperature and water precipitation	Acquity HSS-T3 column (100×2.1 mm, 1.8 µm)	**ESI (+)**: **A:** 0.1% formic acid in H_2O with 0.5 mmol/L ammonium acetate, **B:** 0.1% formic acid in MeOH **ESI (-):** A: 2.5 mmol/L ammonium acetate in H_2O **B:** MeOH	LC-ESI-MS/MS (+) and (-)	59% (Erythromycin) - 133% (Nalidixic acid)	[86] Zhan et al. 2013
AMGs (2), MCs (7), LINC (2), SAs (6), TCs (4), Qs (14) and other antibiotics (1)	chicken meat	extraction with ACN:2% trichloroacetic acid (45:55, v/v) and on-line clean-up using turbulent flow chromatography	Betasil phenyl hexyl (50×2.1 mm, 3 µm)	**A:** 1 mM HFBA with 0.5% formic acid in H_2O, **B:** 0.5% formic acid in ACN/ MeOH (1:1, v/v).	LC-ESI-MS/MS (+)	71% (Neomycin) - 120% (Kanamycin)	[87] Bousova et al. 2013

SAs (13), TCs (4), MCs (4), Qs (11) and AMPs (1)	milk	extraction and protein precipitation with ACN -	Acquity HSS-T3 (100×2.1 mm, 1.8 μm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** 0.1% (v/v) formic acid in ACN	LC- ESI-MS/MS (+) and (-)	-	[88] Freitas et al. 2013
TCs (4), Qs (11), MCs (8), β-LACTs (6), SAs (15), LINCs (1), AMPs (2), ANTHs (19), TRANQs (8), COCs (4), NSAIDs (20) and other antibiotics (3)	milk	Protein precipitation with ACN, dillution with water and cleanup with SPE	Acquity UPLC BEH C18 (50×2.1mm, 1.7 μm)	**A:** 0.1% formic acid in H_2O (v/v), **B:** 0.1% formic acid in H_2O/ACN (1/9, v/v)	UPLC–TOF-MS (+)	76% (Nafcillin) – 186% (Ipronidazole)	[89] Stolker et al. 2008
SAs (23), TCs (6), Qs (14), β-LACTs (12), MCs (10), LINC (4), ANTHs (19), TRANQs (7), COCs (1) and other antibiotics (7)	meat	liquid–liquid–solid extraction (bipolarity extraction) and cleanup with SPE	HSS T3 C18, (100×2.1 mm, 1.8 μm)	**A:** 0.3% (v/v) formic acid and 5% ACN in H_2O, **B:** 0.3% (v/v) formic acid and 5% H_2O in ACN	UPLC - ESI - TOF (+)	13% (Fenbendazole) - 144% (Cefoperazon)	[90] Kaufmann et al. 2008

SAs (15), TCs (4), Qs (11), β-LACTs (7), MCs (10), AMPs (2), ANTHs (19), TRANQs (8), COCs (6), NSAIDs (23) and other antibiotics (2)	egg, fish, meat	extraction with ACN - water and cleanup with SPE	Acquity UPLC BEH C18 (100×2.1mm, 1.7 μm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** ACN/0.1% (v/v) formic acid in H_2O 9/1	HRLC–ESI TOF-MS (+)	32% (Phenylbutazone) – 1274 (Piroxycam)	[91] Peters et al. 2009
SAs (25), TCs (6), Qs (14), MCs (10) β-LACTs (25), QUINOXs (2), LINC (1), ANTHs (34), COCs (2), CORTs (5), β-AGONs (7), NSAIDs (5), TRANQs (2), other antibiotics (3) and other contaminants (8)	milk	extraction and protein precipitation with ACN - ultrafiltration	Acquity UPLC BEH C18 (100×2.1mm, 1.7 μm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** 0.1% (v/v) formic acid in ACN	UPLC - ESI - TOF (+)	1% (Eprinomectin) - 807 % (Danofloxacin)	[92] Ortelli et al. 2009
Sas (8), TCs (4),	milk	extraction with ACN,	YMC ODS-	**A:** 0.1% (v/v)	UHPLC-Q-	42% (Chlortetracycline) –	[93]

β-LACTs (4), MCs (3), Qs (3), ANTHs (1) and other contaminants (2)		cleanup with 3000 Da molecular weight cutoff centrifuge filter	AQ, (100×2.1 mm, 3 μm)	formic acid in H_2O, **B:** ACN	TOF-MS	154% (Sulfachloropyridazine).	Turnipseed et al. 2011
Qs (14), Sas (18), TCs (5), CORTs (7), ANTHs (23), β-AGONs (14), other antibiotics (1) and other contaminants (23)	Meat, milk, egg	extraction with acidified ACN, cleanup with SPE	Zorbax Eclipse XDB C18 (100×3.0 mm, 1.8 μm)	**A:** 5 mmol L^{-1} ammonium formate in H_2O with0.1% formic acid, **B:** 0.1% (v/v) formic acid in ACN	UHPLC-Q-TOF-MS	41.1–120.9% (meat), 52.4–91.9% (milk) and 57.3–118.9% (egg)	[94] Deng et al. 2011
SAs (7), ANTHs (1), other antibiotics (1) and other contaminants (5)	shrimp	QuEChERS	RR Zorbax Eclipse XDB-C18 (50×4.6 mm, 1.8 μm)	**A:** 0.1% (v/v) formic acid in H_2O, B: 0.1% (v/v) formic acid in ACN	LC - ESI - TOF (+)	33% (Sulfamethizole) - 118% (Mebendazole)	[95] Villar-Pulido et al. 2011
β-LACTs (15), Sas (12), MCs (10), TCs (7), AMGs (10) and Qs (10)	Muscle tissues	Two protocols: extraction with ACN and extraction with acidified ACN, cleanup with SPE	RP18 Purospher column (125×3 mm, 5 μm)	A: 1mM HFBA in H_2O with 0.5% formic acid, **B:** 0.5% formic acid in MeOH	LC- orbitrap H-ESI MS (+ and -)	-	[96] Hurtaud-Pessel et al. 2011

				/ ACN (50:50; v/v)			
β-LACTs (5), Sas (8), Qs (1), MCs (2), ANTHs (5), COCs (4) and other contaminants (93)	Meat, milk, liver, corn silage	Extraction with acidified ACN - QuEChERS	Hypersil Gold AQ (50×2.1mm, 1.9 µm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** 0.1% (v/v) formic acid in ACN	LC- orbitrap H-ESI MS (+)	-	[97] Filigenzi et al. 2011
Sas (24), Qs (16), MCs (16), TCs (6), β-LACTs (12), ANTHs (18), COCs (2), TRANQs (7), other antibiotics (1) and other contaminants (3)	muscle, kidney, liver, fish and honey	extraction with ACN and EDTA-succinate buffer, cleanup with SPE	Kinetex Core–Shell, C18 (150×2.1 mm, 2.6 µm)	**A:** 0.3% (v/v) formic acid and 5% ACN in H_2O, **B:** 0.3% (v/v) formic acid and 5% H_2O in ACN	LC- orbitrap H-ESI MS (+ and -)	Kidney: 11.9% (Cephalexin) – 97.5% (Ofloxacin), Honey: 1.2% (Sulfanilamide) -89.6% (Nalidixic acid)	[98] Kaufmann et al. 2011
Qs (5), SAs (1), AMPs (1) and other antibiotics (1)	frog legs, aquacultured Species	extraction with H_2O 1% acetic acid - ACN (1:4, v/v)	YMC ODS-AQ (100×2 mm, 3 µm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** ACN	LC - ESI - Q-TOF (+ and -)	58% (Ciprofloxacin) - 128.8% (Nalidixic acid)	[99] Turnipseed et al. 2012
ANTHs (20) and COCs (14)	feed, milk	Extraction with ACN with NH_4OH -	-	-	DART-orbitrap MS (+	-	[100]

		QuEChERS			and -)		
MCs (6), SAs (4), TCs (4), Qs (12), β-LACTs (5), NFs (2), LINCs (1), ANTHs (1), other antibiotics (1) and other contaminants (46)	feed, fish	Extraction with ACN/H2O (80:20) HCOOH 0.1% & ultrasonic extraction - clean-up by low temperature (freezer for 2h)	Acquity UPLC BEH C18 (100×2.1 mm, 1.7 μm)	**A:** 0.01% (v/v) formic acid and 0.1 nM NH_4Ac in H_2O, **B:** 0.01% (v/v) formic acid and 0.1 nM NH_4Ac in MeOH	UHPLC-Q-TOF-MS (+)	-	[101]
TCs (2), β-LACTs (2) and AMPs (1)	milk	QuEChERS	Gemini C18 (50×2.1 mm, 3 μm)	**A:** H_2O, **B:** MeOH	LC-IT-TOF MS (+ and -)	83% (Ampicillin) – 92% (Tetracycline)	[102]
AMGs (12), β-AGONs (4), MCs (12), β-LACTs (27), Ts (6), Sas (22), Qs (27), AMPs (4), COCs (14), NSAIDs (10), ANTHs (26), TRANQs (3), other antibiotics (6)	milk	extraction with ACN, cleanup with 3000 Da molecular weight cutoff centrifuge filter	YMC ODS-AQ, (100×2.1 mm, 3 μm)	**A:** 0.1% (v/v) formic acid in H_2O, **B:** ACN	UHPLC-Q-TOF-MS (+ and -)	-	[103]

and other contaminants (22)							

Abbreviations: TCA: Trichloroacetic acid, ACN: Acetonitrile, MeOH: Methanol, QuEChERS: Quick Easy Cheap Effective Rugged Safe, SE: Solvent Extraction, SPE: Solid Phase Extraction, PLE: Pressurized Liquid Extraction, MSPD: Matrix Solid Phase Extraction, ANTHs: Anthelmintics, AMGs: Aminoglycisides, AMPs: Amphenicols, β-AGONs: Beta-agonists, β-LACTs: β-Lactams, COCs: Coccidiostats, CORTs: Corticosteroids, LINC: Lincosamides, MCs: Macrolides, NFs: Nitrofurans, NSAIDs: Non Steroidal Anti inflammatory Drugs, PLMTs: Pleuromutullins, Qs: Quinolones, QUINOXs: Quinoxalins, SAs: Sulfonamides, TCs: Tetracyclines, , THYRs: Thyreostats, TRANQs: Tranquilizers

1.4.1 Sample preparation

Sample preparation is the process which includes the isolation and/or preconcentration of compounds of interest from various matrices, the removal of any matrix interferences that may affect the detection system as well as making the analytes more suitable for separation and detection. Even with the advances in the development of highly efficient analytical instrumentation for their final determination, sample preparation is a vital part of the analytical procedure and effective sample preparation is essential for obtaining accurate quantitative results and maintaining instrument performance.

A typical sample preparation technique consists of an extraction step of the analytes from the matrix and a subsequent purification step of the extract.

1.4.1.1 Sample extraction techniques

A. Liquid extraction (LE)

Liquid extraction is a very popular sample treatment technique. LE entails conventional liquid–liquid extraction (LLE) of target compounds from liquid matrices, such as milk, and the liquid extraction of homogenized tissues such as liver, kidney, and meat, referred to as solvent extraction (SE). To obtain optimal results, the extraction solvent has to be selected in such way that efficient extraction of the target compounds is obtained, whereas the extraction of matrix constituents remains limited in order to prevent excessive matrix effects (ME). The selection of the solvent therefore depends not only on the target compounds, but also on the matrix.

Simple extraction with aqueous buffers (e.g. McIlvaine buffer or succinate buffer) is advantageous for highly polar residues because they reduce non-polar matrix components (e.g. lipids) and extracts can be enriched on reversed phase SPE [56, 59, 66, 74, 82]. A disadvantage is that strongly protein-bound residues are not fully extracted and polar matrix components are co-extracted. Complexing agents are reported to be essential for the extraction of tetracyclines, quinolones and some macrolides, because these compounds have a strong tendency to form chelates with divalent metallic cations present in food samples [56, 85].

In general, the majority of methods employ more efficient organic solvents as extracting agents. Methanol (MeOH) and acetonitrile (ACN) are more adequate as extraction solvents as they can simultaneously precipitate the proteins and extract the target analytes. Many authors, as it is shown by the collected applications presented in **Table 1.2**, prefer ACN over MeOH or ethyl acetate as extraction solvent, because MeOH and ethyl acetate extract too many matrix compounds, complicating the following clean-up steps. However, ACN does not sufficiently extract polar analytes.

A great number of multi-residue analytical methods developed use a combination of water or aqueous buffer and organic solvent as the extraction mixture of the target compounds from the matrix (**Table 1.2**). Kaufmann et al. proposed a bipolar extraction, combining an extraction with ACN and one using a McIlvain buffer-containing complexing agent [90]. With one of the greater challenges in sample preparation being the development of a generic extraction method which should not only cover a vast number of target analytes, but should also be applicable to different types of food and feed matrixes, Mol et al. reported a thorough research comparing the use of ACN, MeOH and acetone (ACE) for the extraction of veterinary drugs, pesticides and toxins from honey, milk, eggs and muscle [104]. However, in the area of multi-residue analysis there is always a compromise between recovery and purity of sample extracts.

Liquid–liquid extraction (LLE) is a widely applied extraction procedure in residue analysis due to its high selectivity compared to simple solvent extraction (SE). LLE applications can also include polar ionisable compounds, which can be extracted by non polar organic solvents using the ion-pair technique: transforming positively charged substances into non-polar neutral compounds in the presence of organic anions, or vice versa [105, 106].

Anastassiades et al. developed a variation of LLE, called QuEChERS sample preparation procedure (standing for Quick, Easy, Cheap, Effective, Rugged and Safe), which has been successfully applied to the analysis of hundreds of pesticide residues [107]. In QuEChERS approach, the high-moisture sample (H_2O is added to dry foods) is extracted with an organic solvent (mainly ACN, but also ethyl acetate or acetone) in the presence of salts (MgSO4, NaCl and/or buffering

agents). The addition of salts induces phase separation of the solvent from the aqueous phase. The residues of interest and matrix co-extractives are separated into the relevant liquid phase based on their polarity with the residues partitioning into the organic phase and matrix co-extractives into the aqueous phase. The extract is subjected to further purification using dispersive-SPE (d-SPE), which entails mixing sorbents with the extract.

Although veterinary drugs present greater diversity in the chemical properties compared to pesticides, making their simultaneous extraction more difficult, many methods have been developed for antibacterial determination using this technique, as shown in **Table 1.2**. The majority of methods based on the QuEChERS approach involve SE with acidic ACN in the presence or absence of EDTA followed by phase separation using anhydrous magnesium sulfate as drying agent. A few methods include a subsequent d-SPE procedure using C18, primary secondary amine (PSA) or a combination of both as sorbent. A thorough optimization of the QuEChERS procedure for the extraction of antibactrerials from animal tissues was performed from Stubbings & Bigwood [108]. QuEChERS flexibility, coupled to low cost and ease of use will undoubtedly result in an increase in its application to residue analysis.

B. Pressurised liquid extraction (PLE)

The use of automated extraction techniques leads to a reduction in uncertainty. Automated methods are generally more reproducible than manual ones and they also decrease the time spent on sample preparation, which is often the bottleneck in analysis. However, their disadvantage is additional extraction cost and instrumental downtime. Pressurised liquid extraction (PLE) is the most widely used instrumental extraction technique in food and feed analysis [109].

Pressurised liquid extraction has received numerous names, such as accelerated solvent extraction (ASE), pressurized fluid extraction (PFE), pressurised hot solvent extraction (PHSE), subcritical solvent extraction (SSE) and hot H_2O extraction (HWE). PLE is carried out at temperatures above the boiling point of the solvent and uses high pressure to maintain the solvent in the liquid phase and achieve fast and efficient extraction of analytes from the solid matrix. HWE has

gained interest during the last years [110]. PHWE is cheaper, cleaner and more environmentally friendly than conventional PLE. Moreover, the dielectric constant (polarity) of water can be significantly reduced with increasing temperature, so under pressure, heated water can behave like an organic solvent, thus making more selective the extraction of moderately polar compounds. Thus, at 100–200 °C, water can act as a medium/non-polar solvent. However, due to the high temperatures involved, the thermal stability of the analytes should always be checked before extraction. For instance, degradation of macrolides has been observed at temperatures above 100 °C [111].

Several authors have demonstrated the feasibility of using PLE for sample preparation in the multi-residue determination of veterinary drug residues in different types of foods (**Table 1.2**). Herranz et al. report a PLE analytical method for the determination of quinolones in table eggs [112]. Berrada et al. determine macrolides in meat and fish using PLE with MeOH [113] while Tao et all determine macrolides and avermectines with PLE with ACN/MeOH [114]. A method for the determination of tetracyclines using trichloracetic acid/acetonitrile as extraction solvents was developed by Yu et al. [115]. PLE was also used for the extraction of sulfonamides from meat samples [116], aminoglycosides from milk [117] and corticosteroids from bovine liver [34].

C. Microwave-assisted extraction

Microwave-assisted extraction (MAE) is a technique which uses microwave energy to heat a solvent in contact with a sample, in order to partition analytes from the sample matrix into the solvent. Using microwave energy allows the solvent to be heated rapidly: an average extraction takes 15–30 min. MAE offers high sample throughput (several samples can be extracted simultaneously) with low solvent consumption (10–30 mL). However, solvent choice is limited, care must be taken not to overheat the sample, additional clean-up of the samples is generally necessary prior to analysis and MAE is not amenable to automation (on-line extraction and detection) [118,119].

Although MAE is established as a routine, well-developed method for sample preparation in environmental analysis (soils, sediments, etc.), only few papers have

been found in the literature for the application of MAE to the extraction of veterinary drugs from solid foodstuffs [120-122]. This is generally due to the limited diffusion of the solvent in samples containing more than 30% of water (as it is the case in food samples), resulting in low analyte recovery. This problem can be circumvented by prior drying of samples by lyophilisation [123].

D. Supercritical fluid extraction

Supercritical fluids (SCFs) include properties of both liquids and gases while their density correlates with temperature and pressure. They offer a considerable promise as a media for selective isolation of target compounds for complex matrices. Carbon dioxide (CO_2) is the most widely used supercritical fluid, because of its inertness, low cost, high purity, low toxicity and low critical parameters (CO_2: Tc = 31.3 °C, Pc = 72.9 atm). Apart from CO_2, other potential SCF solvents are N_2O, xenon, C_2H_6, C_3H_8, n-C_5H_{12}, NH_3, CHF_3, SF_6 and water [124]. Sometimes, the relatively low polarity of CO_2 may be a major problem. By adding a polar modifier (MeOH, EtOH or H_2O), its polarity can be changed to make separation more selective but it also leads to more co-extractants.

Two of the main problems with SFE are the robustness of the method compared to other techniques and that conditions must be consistent for reproducible extractions. This has resulted in reduced interest in the area of residue analysis in the last 10 years [123].

Very few SFE applications have been reported in peer reviewed literature for selective isolation of veterinary drug residues from food samples [125-127].

E. Matrix Solid Phase Dispersion (MSPD)

Matrix solid-phase dispersion (MSPD) is a sample pretreatment procedure that is increasingly used for extracting/purifying analytes from a variety of solid and semi-solid foodstuffs. It is a sample preparation strategy that consists of a manual blending of samples with a bulk dispersing agent, to produce complete disruption of the original matrix structure, thus providing an enhanced surface area for subsequent sample extraction. Usually, the blended material is then transferred

and packed into a column to perform sequential extraction and eventual clean-up with an appropriate solvent or a sequence of solvents. MSPD's biggest advantage is that it can combine the procedures of homogenization, disruption, extraction and clean-up into one simple process.

Since its introduction, it has been widely applied to the isolation of veterinary drugs from samples of animal origin. Milk and its derivates were the most investigated matrices [128-132], but eggs and animal and fish tissues, were also analyzed [133 – 136]. It has been applied in the extraction of coccidiostats [132], tetracyclines [131], cephalosporins [128], macrolides [129], penicillins [130], sulfonamides [134, 136], quinolones [133] and amphenicols [129, 135]. Different bulk materials have been used as matrix dispersing agents, being C18- and C8-bonded silica the most popular by far. Cephalosporins, penicillins and amphenicols were extracted from milk by a polar/non-polar polymer (Abselut Nexus) [128] and Strata X [129] while Kishida used normal-phase MSPD with alumina N–S for the extraction of sulfonamides from meat samples [137]. MSPD with hot water extraction has been employed in some cases for the extraction of antibacterials in food, using a home-made like-PLE apparatus [138-140]. Finally, the use of molecularly imprinted polymers (MIPs), as selective dispersing media for sample clean-up (MI-MSPD) has been reported [133, 135]. The use of MIPs enhances the selectivity and sensitivity of the MSPD procedure, allowing higher recoveries of the target analytes.

1.4.1.2 Sample clean-up/purification techniques

A. Solid-Phase Extraction (SPE)

SPE is the most important sample purification technique in residue analysis and has gradually replaced liquid-liquid extraction and liquid-liquid partitioning. A number of books and review papers have already been written on this topic and can be consulted for more detail [141-144].

A wide choice of sorbents is available which rely on different mechanisms for extraction/retention of analytes. Alumina, amino or strong cation exchangers (SCX) have been proposed for ionic antibacterials, while C18 or polymeric sorbents,

especially Hydrophilic-Liphophilic Balance (HLB) polymeric reversed phases are used for neutral or ionisable compounds working at a pH lower than the pKa of the analytes. HLB sorbent consists of a copolymer of N-vinylpyrrolidone and divinylbenzenes. The hydrophilic N-vinyl pyrrolidone increases the water wettability of the polymer and the lipophilic divinylbenzene provides the reversed-phase retention necessary to retain analytes.

For compounds with varied chemical properties, mixed-mode sorbents are recommended (e.g., Bond Elut SCX cartridges for multiresidue of basic drugs [145]. SPE can be directly used for the extraction of veterinary drugs from liquid food only (e.g., milk, or honey, which can be dissolved in aqueous media). Applications of SPE in multi-residue analysis of veterinary drugs in food matrices are presented in **Table 1.2**.

B. Dispersive SPE (d-SPE)

Dispersive-SPE (d-SPE) is a clean-up technique that involves mixing sorbent with a sample that has been pre-extracted with an appropriate solvent. It is typically part of the QuEChERS method where it follows the extraction step. The appropriate sorbent adsorbs matrix co-extractives onto its surface, leaving analytes of interest in the solvent. C18 sorbents remove highly lipophilic compounds and other sorbents, like amino- or carbon-based phases, are employed mainly for the removal of fatty acids and pigments, respectively. MgSO4 is added to provide additional clean-up by removing residual H2O and some other compounds via chelation. It is an extremely fast, simple and inexpensive process that provides high recovery and reproducibility for many LC- and GC-amenable analytes.

Several analytical methods have used d-SPE as a clean-up step in veterinary residue analysis, mainly using C18 as a sorbent [146-148]. PSA, amine (NH2) and silica have also been reported [71, 95, 108, 149, 150]. d-SPE does not provide the same degree of clean-up as SPE. However, it does provide good recovery and reproducibility, coupled with practical (speed) and cost advantages.

C. Molecularly imprinted polymers (MIPs)

Molecularly imprinted polymers (MIPs) are engineered cross-linked polymers that exhibit high affinity and selectivity towards a target compound or class of structurally related compounds. The use of MIPs as selective sorbents in SPE, so-called molecularly imprinted solid phase extraction (MISPE), is an emerging clean-up procedure for complex matrices such as food. Currently, MIPs are not employed very much for food analysis, in comparison to other methods, but, thanks to their high specificity, reduction in the time of analysis in on-line approaches and reduction in the costs of analysis, rapid increase in the number of applications is foreseeable**.** Different works reported the use of MIPs for extraction and clean-up in β-agonist [151], tetracycline [152], sulfonamide [153], quinolone [154] and chloramphenicol [155] residue analysis.

D. Turbulent flow chromatography (TFC)

A relatively new technique used for sample preparation is the so-called turbulent flow chromatography (TFC) that has shown a great potential for on-line sample pretreatment, in terms of both, high sample throughput and high reproducibility linked to automation. Columns packed with large particle size sorbents (typically 60 μm) allow on-line extraction using high solvent flow rates (typically 4–6 mL min^{-1}), without significant back-pressure. Molecules with low molecular weight diffuse faster than molecules with high molecular weight. Therefore, the small analytes diffuse into the particle pores, whereas the high flow of the mobile phase quickly flushes the large sample compounds (e.g. proteins) into the waste, before they have the opportunity to diffuse into the particle pores. Once trapped the analytes on the turbulent flow column, a back-flushing elution desorbs the analytes and focuses them onto the analytical column for chromatographic separation [2]. The number of TFC applications in food analysis is scarce [87, 156, 157].

E. Molecular weight cut-off devices – Ultra-filtration

The development of multi-residue assays using LC–MS/MS detection has resulted in the alternative purification systems in the field of residue analysis such as ultra-filtration (UF). In residue analysis of food, UF is primarily used to separate analytes of interest from macromolecules, such as proteins, peptides, lipids and sugars,

which may interfere with analysis, particularly affecting ionisation in mass spectrometry. Molecular weight cut-off devices or spin filters coupled to microcentrifuge tubes are the most commonly used formats [123]. Examples of applications include sulfonamides in milk [158], eggs [159] and edible tissues [160], tetracyclines in egg [161] and penicillin G in muscle, kidney and liver [162]. Goto et al. compared several types of membranes for ultra-centrifugation and showed the difference in recoveries of the antibiotics, because some membranes sometimes adsorb tetracycline and penicillins irreversibly [163]. Lately, three multi residue method for the determination of 27, 25 and >150 veterinary drugs in milk, respectively, using extraction with ACN, were reported [75, 93, 103]. Clark et al. [75] used a 30 kDa MW cut-off centrifuge filter while Turnipseed et al. [93, 103] a 3-kD cut-off filter. Moreover, a multi residue method for the analysis of 150 veterinary drugs in milk using a 3-kD cut-off filter has been presented. In this study it was shown that ultrafiltration removes more lipophilic matrix interferences than SPE, but that the removal of hydrophilic interferences is worse [92].

F. Restricted access materials (RAMs)

RAM sorbents are porous chromatographic supports partially based on size-exclusion mechanisms that have been specifically developed for protein removal [164]. RAMs are suitable for on-line SPE, allowing direct injection of complex samples into LC-MS. Macromolecules are excluded from the stationary phase and eluted with the mobile phase and the small molecules are able to permeate through the pores of the RAM sorbent and interact with it by diverse mechanisms. RAMs are frequently used as pre-columns in column switching LC systems, using two pumps and a selection valve with a synchronization unit. These systems allow automate on-line protein removal and analyte preconcentration on the RAM pre-column, and afterwards separation of the target compounds in the analytical column, avoiding or reducing sample pretreatment. However, high fat and protein contents may block the efficient elimination of interferences and decrease column lifetime. Different examples have been published, reporting the application of RAMs in extract purification of food samples e.g. the clean-up of trace levels of tetracycline antibiotics in food [165].

G. Porous monolithic microextraction materials

Thanks to their structure, monoliths have a number of potential advantages like low cost, mechanical robustness and high stability, no void volumes forming at conventional LC flow rates, high hydraulic permeability and dominance of convection over diffusion of mass-exchange under dynamic conditions, which allow the separation of target analytes at extremely high flow rates [166].

They are widely used as sorbents in in-tube solid phase microextraction (SPME). Basically, monolithic materials are divided into two groups: polymer- and silica-based. Up till now, organic polymer and silica-based monoliths have been introduced as extraction media for the microextraction. Generally, the most commonly used organic polymer monoliths can be grouped into one of several broad categories [167]. Poly (methacrylic acid-co-ethylene glycol dimethacrylate) (poly (MAA-co-EGDMA)) monoliths are the most widely reported type to veterinary residue analysis. They have been used for the microextraction of sulfonamides in milk and eggs [168, 169], quinolones in milk, egg, chicken and fish muscles [170, 171], tetracyclines in fish muscle [172] and chlorampenicol in honey, milk, eggs [173]. A hybrid organic-inorganic silica monolith with hydrophobic and strong cation-exchange functional groups was prepared and used for in-tube microextraction of sulfonamides in milk [174]. Also, Stir Bar Sorptive Extraction (SBSE) based on poly (vinylimidazole-co-divinylbenzene) (VIDB) monolithic material as coating was used to directly extract sulfonamide antibacterial residues in milk without de-fats and de-proteins step [175]. SBSE is based on sorptive extraction, whereby the solutes are extracted into a polymer coating on a magnetic stirring rod. The extraction is controlled by the partitioning coefficient of the solutes between the polymer coating and the sample matrix and by the phase ratio between the polymer coating and the sample volume [176].

H. Nanoparticles as sorbent materials

The excellent properties of new nanomaterials were recently exploited for extraction and new clean-up technologies. Carbon nanotubes (CNTs) can be considered excellent materials for SPE because of their large adsorption surface and high affinity for organic compounds. Multi-walled CNTs (MWCNTs) are

preferred over the single-walled CNTs (SWCNTs), as the presence of concentric sheets results in enhanced interaction with the analytes. One of the advantages of carbon-based adsorbent materials is that their sorbent capacity is maintained even in organic media. Recent examples have reported the successful use of CNTs in preparing food samples [166, 177, 178].

1.5 Instrumental analysis

1.5.1 Screening tests

Very popular and quite often used methods for residue screening are methods based on microbial or immunological assay or bioassays [179, 180]. Screening methods usually can provide semi-quantitative or quantitative results, with low rate of false compliant samples. They can also assure high throughput, ease of use, short analysis time, good selectivity, and low cost. It is common practice for routine laboratories to apply screening methods, covering families of antibiotics, and samples found to be non-compliant are then analyzed by confirmatory methods [181]. However, this approach would not be sufficient itself. Positive responses from the rapid tests would need to be correlated with an actual presence of residues in the samples. Thus, very often, screening tests are accompanied by confirmatory methods [182].

1.5.2 Confirmatory methods

Separation techniques, for example gas chromatography (GC), high-performance liquid chromatography (LC), and capillary electrophoresis, have been widely used for the analysis of veterinary drugs residues in food samples. Historically, the control of veterinary drug residues was based on chromatography coupled to non-specific technologies such as fluorimetric detector (FLD), ultra violet detector (UV) and electron capture detector (ECD). However, these techniques suffer some inherent drawbacks: each antibiotic class has to be tested separately, confirmation of the target analytes is based mainly on retention-time comparison to standards and some analytes have to be derivatized to obtain an appropriate limit of detection (LOD).

The first introduction of mass spectrometry (MS) in the 1980s was immediately considered as a revolution in the domain due to its outstanding specificity and sensitivity. Compared with older chromatographic methods based on the use of conventional detectors, the use of separation techniques coupled to very selective MS detector systems, besides supplying precious information about the identity of a specific compound, offers the additional advantage that older laborious and time-consuming sample treatment procedures can be greatly simplified, thereby resulting in faster and low-handling methodologies.

Public Health Agencies in many countries rely on detection by mass spectrometry, which, being a specific detector, affords unambiguous confirmation of contaminants in foodstuff. Commission Decision 2002/657/EC states that “Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods” [54].

1.5.2.1 Capillary electrophoresis

Capillary electrophoresis (CE) is a separative analytical technique which is widely accepted due to its ability to simultaneously determine different analytes with both high efficiency and resolution, low consumption of samples and electrolytes and the possibility of rapid method development [183]. CE is an adequate alternative to chromatographic techniques, mainly when only small sample amounts are available.

The physicochemical properties of some veterinary drugs, their ionizable nature, multiple ionization sites and different water solubilities, make them highly suitable for electrophoretic analysis. Also, CE, as HPLC, allows automation, injection of smaller volumes of samples and multiple modes of detection (ECL, UV, DAD, FLD, MS etc). For these reasons, CE is an analytical tool that has gain importance and today broadens its scope of applications, mainly in the food and drug analysis areas [184,185].

The coupling of CE with MS combines the excellent separation capabilities of CE and the power of MS in analyte identification, with MS/MS being additionally used in structure elucidation or for additional selectivity in order to gain sensitivity by reducing the background noise. CE-MS and CE-MS/MS methods have been

reported for the analysis of quinolones in chicken muscle [186], sulfonamides in pork [116], and sulfonamides and trimethoprim in meat samples [187]. Nevertheless, a small number of applications have been presented describing the simultaneous determination of different groups of veterinary drugs [188,189].

1.5.2.2 Gas chromatography – mass spectrometry

GC is rarely used for the determination of veterinary drugs, due to the polar nature, low volatility and thermal instability of these drugs. Derivatization of polar compounds is advisable to improve peak shape and sensitivity of the method, acetylation being most widely used, since the reaction can be carried directly in aqueous phase. Once again, MS detection offers the major advantage of the qualitative identification of the analytes by their mass spectrum.

In the case of chlorampenicol the high sensitivity of the GC-NCI-MS technique may be advantageous and justify the time necessary for derivatization (silylation) of the antibiotic to enhance its volatility. GC-MS methods based on electron ionization (EI) have historically been used for this purpose [190], but the resulting sensitivity sometimes remains insufficient. Negative chemical ionization (NCI) is more commonly used since it is particularly well adapted for these halogenated substances which exhibit intense electronic capture properties [191]. The same strategy can be successfully applied for measuring other related compounds such as thiamphenicol or florfenicol [192].

A similar approach is also effective for β-agonists for which positive chemical ionization (PCI) is indeed an interesting option providing a wide panel of different mass spectra by varying the nature of the reagent gas in the ionization source. These approaches provide excellent detectability, down to 0.1 µg kg^{-1} (ppb) for chloramphenicol in tissues and at sub-ppb levels for most β-agonists on the basis of one single ion. [191].

In conclusion, there is a downward trend in utilizing GC for the determination of antibacterials in food matrices, due to long analysis time and the additional step of derivatization, as demonstrated by the very small number of recent publications.

1.5.2.3 Liquid Chromatography – mass spectrometry

LC-MS techniques provide a universal approach applicable to the widest number of veterinary drugs and this is the reason why they have today become the technique of choice in the field of the analysis of antibacterial residues in food stuffs.

The combination of atmospheric pressure ionization tandem mass spectrometry (API-MS/MS), with liquid chromatography (LC) and ultra-performance LC (UPLC) is currently the most frequently used technique in antibacterial analysis. The most used atmospheric pressure interfaces are atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). For compounds of moderate to high polarity, ESI constitutes the most important ionization technique in MS coupled to LC for the analysis of organic contaminants, and it dominates the application area of antibacterial analysis (**Table 1.2**).

Among the different mass analyzers usually applied for target analysis, triple quadrupole (QqQ) is the most widely used for measuring and quantifying residues of veterinary drugs. Hybrid quadrupole-linear ion trap (Q-LIT) system combines fully functional quadrupole and linear ion trap-MS within the same instrument and thus, apart from great sensitivity, is capable of producing MS^n spectral information, useful for structure elucidation. Q-LIT has been used in fewer applications than simple triple quadruple formats.

However, a recent trend towards the high-resolution mass spectrometry (HR-MS; i.e. time-of-flight, TOF; Orbitrap; Fourier Transform-Ion Cyclotron Resonance, FT-ICR) is undoubtedly observed. High resolution mass analyzers and hybrid mass analyzers, such as Q-TOF, LIT-Orbitrap, open a new era in food analysis, together with holistic sample preparation and retrospective analysis. Due to their high resolving power, mass accuracy, fragmentation and isotopic pattern elucidation can provide tentative identification of non-target and unknown compounds in food samples. Full scan acquisition mode and MS^n mode are useful tools of these new generation instruments.

The main source of analytical problems encountered by LC-MS users is related to matrix effect problems, particularly when studying complex samples, such as food. It represents certainly one of the main sources of pitfall for the analyst, affecting many aspects of the method performance, such as detection capability,

repeatability and accuracy. Matrix effect mainly appears as ion suppression and it corresponds to the decrease of the evaporation efficiency of the ions of the analyte due to competition effects with co-extracted and co-eluted matrix components. Another proposed mechanism is the competition between analytes and interfering compounds regarding the maximal ionization efficiency of the technique [193-195]. Much less frequently and by a process not yet fully understood, the presence of endogenous compounds in the nanodroplets of the electrosprayed solution can result in an increased ion signals for the analytes compared to those of a reference standard solution.

To overcome matrix effects when quantifying, two practicable approaches can be used. The use of adequate isotope-labeled internal standards and/or analyte quantitation by matrix-matched calibration standards should eliminate the analytical systematic errors (bias) caused by ion suppression or ion enhancement [196].

A. LC-MS/MS Techniques (QqQ and Q-LIT)

Triple quadrupole MS analyzers (QqQ) present the highest sensitivity and selectivity when working in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), by selection of at least two precursor ion-to-product ion transition reactions. The fragmentation of the target compounds in order to detect only specific product ions rather than the entire molecule permits to considerably increase the signal to noise ratio of the target diagnostic signal by decreasing to a major extent the interferences due to other compounds present in the final extract with the same - or very close - molecular weight as the analyte of interest [191]. Under this condition, QqQ MS analyzers are best suited to achieve the strict tolerance levels regulated in various countries for antibacterials in different food matrices.

The large number of veterinary drugs that have to be monitored in order to ensure food safety has caused a steady increase of the number of multi-analyte analytical methods developed in recent years. Applications of multi-analyte methods related to LC coupled to MS/MS are presented in **Table 1.2**.

The application range of MS/MS is today extremely wide, both in terms of target compounds and in terms of possible different acquisition modes. This last

capability authorizes not only very sensitive and specific quantitative target measurements, but also powerful untargeted "fishing" approaches based on advanced scanning techniques like precursor ion scanning or neutral loss scanning, applicable to a class of substances with similar fragmentation patterns [197,198].

A drawback of the QqQ MS arrangement is its relatively long duty cycle (slow scan speed) that limits the number of scans that can be acquired simultaneously. As a result, SRM methods are typically limited to ~100 or 150 target analytes, depending on the chromatographic separation, resulting in a loss of sensitivity. Furthermore, for reliable quantification, two selected reaction monitoring transitions are required and some analytes present only one transition while some transitions are unspecific. In spite these disadvantages, QqQ still remains the analyzer of choice, coupled to liquid chromatography, for the determination of veterinary drugs in food matrices.

B. High-resolution MS Techniques

To overcome the drawbacks associated with QqQ and IT mass analyzers, as well as to meet with the challenge of monitoring drug metabolites that are either more stable of higher toxicological concern than the parent compound [199], high-resolution mass analyzers (Q-TOF, Orbitrap and FT-ICR) are increasingly becoming more popular in analytical laboratories.

The introduction of the reflectron TOF MS which is used to compensate for energy spread from the initial ion velocities has resulted in mass resolving power approaching $\Delta m/m \sim 10{,}000$ [Δm = full-width half-maximum (FWHM)]. Mass accuracy can currently reach values better than 10 ppm in routine conditions with external calibration. The ability of a time-of-flight (TOF) mass spectrometer to assign a mass to compounds with high accuracy (±0.005 Da) enables it to collect full scan data and still detect low levels (ng/g) of contaminants in complex food matrices with sufficient selectivity. A hybrid quadrupole TOF detector (Q-TOF) has the additional ability to obtain MS/MS spectra that can be used to further characterize drug residues. Fourier transform ion cyclotron resonance (FT-ICR) is one of the most sensitive ion-detection methods with mass accuracy of <1 ppm

[200], but the high cost of instrumentation currently restricts their application. An alternative to these techniques is the Orbitrap mass analyzer, launched in 2005. However, it suffers from a slower data acquisition rate than Q-TOF instruments, so it is not fully appropriate for fast chromatography [201]. This system provides outstanding mass accuracy, mass resolution and reliable high-sensitivity MS^n performance, much higher resolving power (R = 100,000) than TOF-MS, which is important when analyzing complex matrices in order to avoid both false positive and false negative results [166].

CHAPTER 2

Scope and Objectives

2.1 The analytical problem

There are three main difficulties that constitute the analytical problem in the residue analysis of veterinary drugs in food. First, there is the large number of compounds with diverse physico-chemical characteristics. In addition, the definition of ‘‘residue’’ of many contaminants includes known metabolites of toxicological interest since many drugs administered to food-producing animals are oxidized, reduced and biotransformed to water-soluble conjugates, primarily by glucuronidation, sulfatation or conjugation with glycine. Such metabolites cannot be ignored, particularly when they are even more hazardous and more persistent than the parent compounds (e.g., nitrofurans are rapidly biochemically transformed into toxic metabolites, which are highly bound to the proteins, so they are stable for longer periods in food-producing animals) [202].

The second problem is the very low concentration levels at which a veterinary drug residue should be analysed, since most of the MRLs and MRPLs established are at the ppb level (parts per billion or μg kg^{-1}). Therefore, analytical methods for the determination of veterinary drug residues in food matrices at trace levels are necessary and the procedures used for selective and quantitative extraction of the analytes, cleanup and enrichment of sample, as well as the sensitive and specific detection should meet the requirements of this challenge.

Finally, the complexity of the matrix should also be taken into consideration. Several edible tissues from food producing animals can be selected for residue surveillance including muscle, liver, kidney, skin and fat, which are normally collected at slaughter houses. In addition, further sample matrix types can be taken on-farm or at production sites, including milk, honey, eggs and fish. All these foods, except honey, are protein rich (from 3% in milk to 20% in meat), which is important for those drugs that bind easily to proteins. They also contain significant amounts of divalent and trivalent cations that form complexes with some antibacterials, increasing their retention in different tissues. In general, many residues are present

in conjugated forms and require liberation through enzymatic or chemical hydrolysis prior to extraction [13].

Due to all the aforementioned reasons and the desire of improving the cost-effectiveness of analytical procedures, the development of multiclass methods which are able to detect, confirm and quantify as many compounds as possible, has become a significant trend in the analysis of residues and contaminants in food samples. Liquid chromatography hyphenated to mass spectrometric techniques dominates in the field of multi-residue determination of veterinary drugs in complex matrices, since it permits excellent sensitivity and selectivity.

2.2 Research Objectives and Scope

The experimental part of this thesis is consisted of 4 individual studies.

In the first study performed a strategy was newly developed to rapidly screen seventeen sulfonamides and five tetracyclines in a single run from the fish tissue using ultra-high performance liquid chromatography (UHPLC) coupled with comprehensive mass spectrometric approaches including precursor ion scan and data dependent scan. The product ions for precursor-ion scanning were selected by studying the MS/MS fragmentation of the analytes. All sulphonamides share the same diagnostic product ion at m/z 156 in positive MS/MS scan, while for tetracycline antibiotics the diagnostic product ion was proved to be at m/z 154. Further characterization of each compound was performed using a data dependent scan.

Separation was performed on a Zorbax Eclipse Plus C18 column with a gradient elution using acetonitrile - 0.1% formic acid mobile phase at a flow rate of 0.1 mL min^{-1}. A comparison of extraction solvents has been performed in order to optimise the extraction procedure for both groups of antibiotics and develop an effective generic extraction procedure.Validation was performed according to Commission Decision 2002/657/EC and satisfactory method performance characteristics were achieved.

In the second study, two wide-scope screening methods have been developed for the identification of veterinary drugs and pharmaceuticals in fish and milk using

liquid chromatography quadrupole time-of-flight (LC-QTOF-MS). The methods were validated using a qualitative approach at two concentration levels. The detection of the residues was accomplished by retention time, accurate mass and the isotopic fit using an in-house database. Product-ion spectra were used for unequivocal identification of the compounds. Generic sample treatments were applied. For fish tissues, a solid-liquid extraction with a mixture of aqueous 0.1% (v/v) formic acid and 0.1 (w/v) EDTA – acetonitrile – methanol (1:1:1, v/v) and cleanup with hexane was performed. Milk was treated with aqueous TCA 5% (w/v) - ACN (3:1, v/v) and then subjected to clean-up with Oasis HLB cartridges. The majority of the compounds were successfully detected and identified at the highest concentration level (more than 80% of the compounds in both matrices). Satisfactory results were obtained at the lowest level as well (more than 60 % of the compounds detected and identified).

In the third study, a simple and sensitive multi-residue method for the determination of 115 veterinary drugs and pharmaceuticals, belonging in more than 20 different classes, in butter, milk powder, egg and fish tissue has been developed. The method involves a simple generic solid-liquid extraction step (solvent extraction, SE) with 0.1% formic acid in aqueous solution of EDTA 0.1% (w/v) – acetonitrile (ACN) – methanol (MeOH) (1:1:1, v/v) with additional ultrasonic-assisted extraction. Precipitation of lipids and proteins was promoted by subjecting the extracts at very low temperature (-23 ºC) for 12 hours. Further cleanup with hexane ensures fat removal from the matrix. Analysis was performed by liquid chromatography coupled with electrospray ionization and tandem mass spectrometry (LC–ESI–MS/MS). Two separate runs were performed for positive and negative ionization in multiple reaction monitoring mode (MRM). Particular attention was devoted to extraction optimization: different sample-to-extracting volume ratios, different concentrations of formic acid in the extraction solvent and different ultrasonic extraction temperatures were tested in butter, egg and milk powder samples. The method was also applied in fish tissue samples. It was validated, on the basis of international guidelines, for all four matrices and quantitative analysis was performed using standard addition method.

Finally, in the fourth study performed, a rapid, sensitive and efficient multiresidue analytical method for the simultaneous determination of 76 veterinary drugs and

pharmaceuticals in bovine muscle tissue by HILIC–MS/MS has been developed. The multi-class method includes aminoglycoside determination which has been rarely reported in multiresidue methods due to aminoglycosides' very different physicochemical properties comparing to other veterinary drugs.The analytical method includes ACN extraction followed by an acidic aqueous buffer extraction, cleanup with HLB cartridges and HILIC-MS/MS determination using bare silica stationary phase (BEH HILIC). The method was thoroughly optimized and validated according to the European Commission Decision 2002/657, demonstrating its good performance and its great potential value in the veterinary drug and pharmaceutical analysis field.

CHAPTER 3

Multi-residue determination of seventeen sulfonamides and five tetracyclines in fish tissue using a multi-stage LC-ESI-MS/MS approach based on advanced mass spectrometric techniques.

3.1 Introduction

In this study, a strategy was newly developed to rapidly screen seventeen sulfonamides and five tetracyclines in a single run from the fish tissue using ultra-high-performance liquid chromatography (UHPLC) coupled with comprehensive mass spectrometric approaches including precursor ion scan and data dependent scan. The product ions for precursor-ion scanning were selected by studying the MS/MS fragmentation of the analytes. All sulphonamides share the same diagnostic product ion at m/z 156 in positive MS/MS scan, while for tetracycline antibiotics the diagnostic product ion was proved to be at m/z 153.8. Further characterization of each compound was performed using a data dependent scan. Separation was performed on a Zorbax Eclipse Plus C18 column with a gradient elution using acetonitrile - 0.1% formic acid mobile phase at a flow rate of 0.1 mL min^{-1}.

This approach has proven to be a powerful, highly selective, and sensitive tool for rapid screening and detection of non targeted components in fish tissue and requires a minimum sample preparation such as one generic extraction step with MeOH:ACN 50:50 v/v acidified with 0.05% formic acid. A single UHPLC run is required for the confirmation of all 22 compounds and validation data is presented for all analytes in fish tissue according to Commission Decision 2002/657/EC. The method has also been applied successfully to porcine and poultry meat. With this method also non-target compounds of these two groups of veterinary drugs can be determined due to their common fragmentation pattern.

3.2 Experimental section

3.2.1 Chemicals and Reagents

Acetonitrile and methanol LC-MS grade were purchased from Fisher Scientific (Fair Lawn, NJ) and formic acid 99% from Fluka (Buchs, Switzerland). Distilled water was provided by a MilliQ purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). Oxytetracycline (OTC), tetracycline (TC), demeclocycline (DMTC), chlortetracycline (CTC), doxycycline (DC), sulfadiazine (SDZ), sulfathiazole (STZ), sulfamerazine (SMR), sulfadimidine (SDD), sulfamethoxypyridazine (SMP), sulfamonomethoxine (SMM), sulfachloropyridazine (SCP), sulfadimethoxine (SDM), sulfamethizole (SMT), sulfamethoxazole (SMTX), sulfisoxazole (SIX), sulfaguanidine (SGN), sulfapyridine (SPD), sulfamoxole (SMX) and sulfaquinoxaline (SQX) were provided from Sigma–Aldrich (Athens, Greece), while sulfadoxine (SDX) and sulfaclozine (SClZ) were donated by the National Laboratory of Residue Analysis of Food of Animal Origin of the Hellenic Ministry of Rural Development and Food. The structures of all the examined compounds are presented in **Figures 3.1** and **3.2**.

Standards stock solutions (500 μg mL^{-1}) of all veterinary drugs were prepared in methanol and were stored at -20 ºC. A multi component solution of the 22 compounds was obtained by diluting the stock solution in methanol to a final concentration of 2 μg mL^{-1}. These solutions were stored at 4 ºC for up to one month. Matrix-matched standards were prepared in the same way as the other samples.

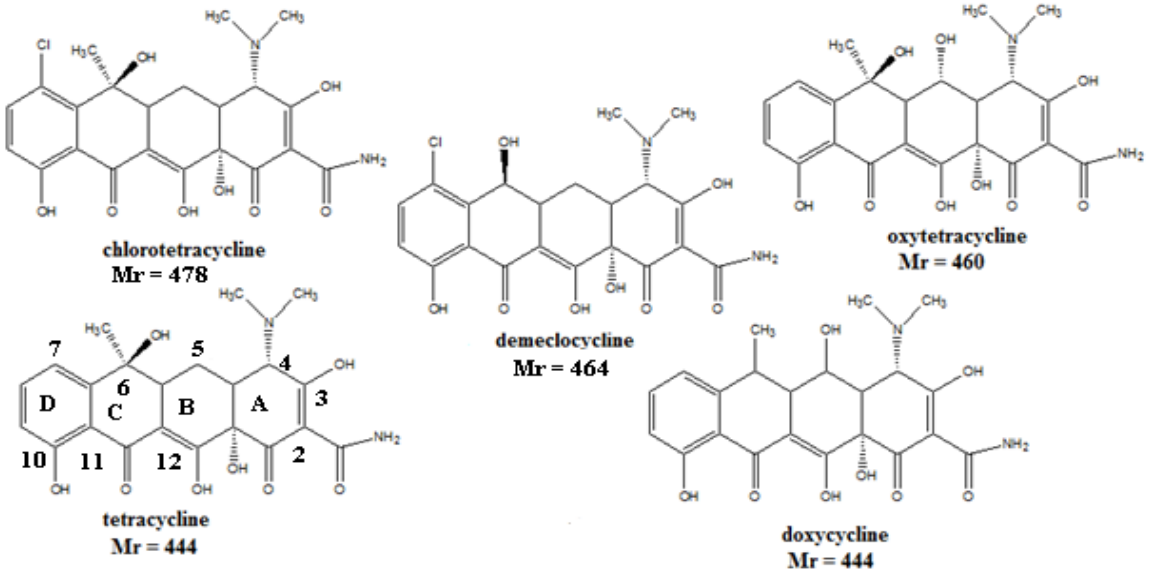


Figure 3.1. Chemical structure of tetracyclines.

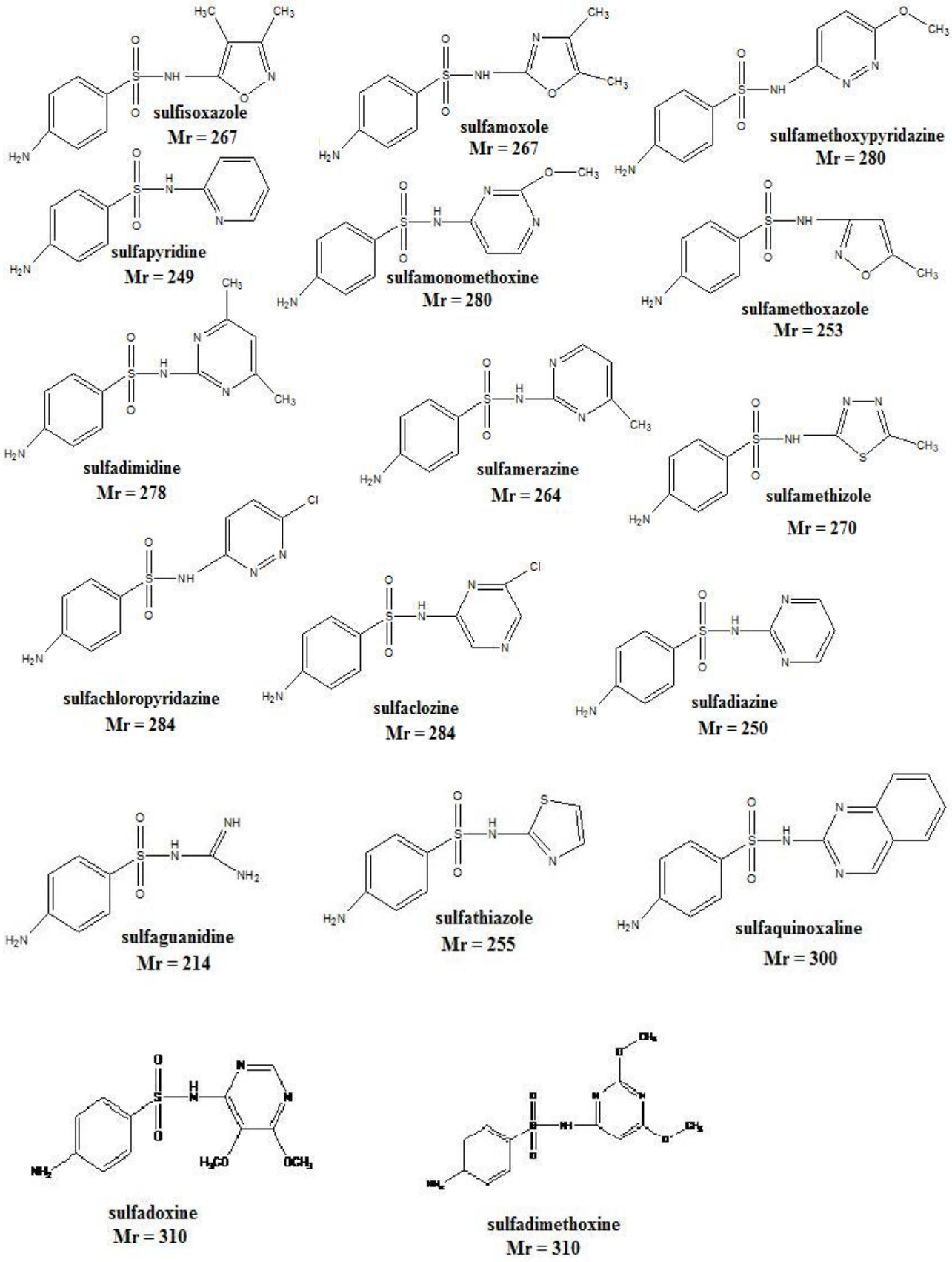


Figure 3.2. Chemical structure of sulfonamides.

3.2.2 Samples

Negative fish (sea bass and sea bream), porcine and poultry tissue samples were used during these experiments. All fish samples were obtained from aquaculture and were confirmed to be free of targeted analyte residues by LC-MS/MS after sample preparation with the procedure developed and optimized. Porcine and poultry tissue samples were obtained by local supermarket and were also evaluated for the survey analysis. All tissue samples were homogenized and stored at −20 ºC until analysis.

3.2.3 UHPLC–MS/MS conditions

A Thermo UHPLC Accela system was connected to a Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument (Thermo, San Jose, CA, USA). A Zorbax Eclipse plus C18 (2.1 mm × 50 mm, 1.8 μm, Agilent, CA, USA) column protected by a guard column was used at a constant flow rate of 100 μL min^{-1}. Mobile phase consisted of water containing 0.1% formic acid (v/v) (solvent A) and acetonitrile (solvent B). The gradient used was: 0–12 min linear gradient from 5 to 50% B; 12–13 min from 50 to 5% B and 13–21 min held at 5% B in order for the column to reequilibrate before the next injection. The full loop injection volume of the tissue extract was set at 10 μL.

The quadrupole instrument was operated in the positive-ion mode under the following conditions: Spray Voltage, 4000 V; Capillary temperature 270 ºC; Sheath gas 60 psi; Auxiliary (drying) gas 20 a.u. Two MS runs were done for each sample: one in the precursor ion scan mode and one in data dependent-scan mode. Different scan events were created for sulfonamides and tetracyclines in both MS modes. For SAs in the precursor-ion scan mode m/z 156 was selected as the product ion, collision energy was set at 22 eV, scan time of 1 s was used in both quadrupoles Q1 and Q3, and the Q1 scan range was set at m/z 210–315. For TCs the product ion was m/z 153.8, collision energy 30 eV, scan time for Q1 and Q3 1 s and the Q1 scan range was set at m/z 440–480. This experiment is used as a survey experiment to trigger a data dependent (DD) scan of the most abundant ion when exceeding a certain threshold, set at 1×10^4 counts for SAs and at 0.1×10^4

counts for TCs. The data dependent scan was performed with a collision energy of 30 and 28 eV for SAs and TCs, respectively.

3.2.4 Sample preparation

Homogenised tissue (1.0 g) was weighed in a 15 mL polypropylene Falcon tube. Samples were fortified as appropriate with the targeted compounds. For method development each experiment consisted of one blank and three fortified blank samples (spikes) in the MRL level. Fortification at a concentration of 100 μg kg^{-1} for each analyte was performed by adding 50 μL of the multi-analyte working solution (concentration 2 μg mL^{-1}). After that the samples were mixed and allowed to stand in the dark for at least 15 min.

Various extraction solvents were tested (acetonitrile, methanol, acetone) as well as two concentrations of formic acid (0.05, 0.1%, v/v) in order to conclude to the most efficient mixture which would provide the highest recovery and the lowest signal suppression for most of the analytes. Signal suppression states the peak ratio of analyte response in matrix extract over the equal concentration of analyte in pure standard solution. Addition of 5mL of acetonitrile, 5mL of methanol and 5 μL of formic acid was followed by vortex shaking for 30 s and a 15-min ultrasonic extraction of the antibiotics from the tissue. Subsequently, the samples were centrifuged (3000 rpm, 10min) and a 4mL aliquot of supernatant sample extract was transferred to a test tube. The extract was evaporated to dryness under a light stream of nitrogen (at 40 ºC). The dry sample was reconstituted in 0.40 mL of aqueous formic acid 0.2%, vortexed and ultrasonicated for 1min. After filtration by a 0.2 μm RC filter into a polypropylene vial (Mini-UniPrep syringeless filters, Whatman, Kent, UK), an aliquot of 10 μL of the extract was analyzed by LC–ESI–MS/MS.

3.2.5 Validation

Validation of the developed procedure was performed according to Commission Decision 2002/657/EC [54] which indicates that for quantitative screening methods the performance characteristics that have to be determined are the detection limit,

CCβ, precision, selectivity/specificity and applicability/ruggedness/stability at the level of interest. SAs and TCs have common Maximum Residue Limit (MRL) in animal tissue, set at 100 μg kg^{-1} and this concentration was selected to be the spiking level for the methods' validation. The validation was performed using fish tissue as the major matrix. Method detection limit (LOD), method quantitation limit (LOQ), precision, selectivity, decision limit (CCα) and detection limit (CCβ), were estimated based on the analysis of spiked blank fish samples. Ruggedness of the method was estimated by analysing spiked poultry and porcine samples. Method precision was expressed as relative standard deviation (%RSD).

3.3 Results and discussion

3.3.1 LC–MS/MS method

Precursor-ion scanning is a powerful approach, since the only knowledge required is the fragmentation pattern of the compound. Searching for common fragment ions can also provide vital information about the putative structure of a metabolite. By fixing the third quadrupole Q3 on selected ion, and scanning the first quadrupole Q1 over an appropriate range, the resulting spectra contain all of the precursor ions that produce the common fragment ion selected at Q3. The pseudo-molecular ions of all the examined compounds are summarized in **Table 3.1**.

Table 3.1: Pseudo-molecular ions ($[M+H]^+$), retention times (RT with their SD, n = 18) and characteristic product ions of sulfonamides and tetracyclines.

	$[M+H]^+$	RT ± S (min)	Characteristic ions		$[M+H]^+$	RT ± S (min)	Characteristic ions
SDZ	251	6.42 ± 0.10	156, 92, 108	**SGN**	215	2.55 ± 0.15	156, 92, 108
STZ	256	7.13 ± 0.11	156, 92, 108	**SIX**	268	8.21 ± 0.15	156, 92, 108
SMR	265	7.64 ± 0.07	156, 92, 108	**SMX**	268	11.00 ± 0.09	156, 92, 108
SDD	279	8.43 ± 0.09	156, 92, 108	**SQX**	301	11.94 ± 0.08	156, 92, 108
SMP	281	8.81 ± 0.12	156, 92, 108	**SMTX**	254	10.59 ± 0.12	156, 92, 108
SPD	250	7.24 ± 0.07	156, 92, 108	**SMT**	271	8.74 ± 0.08	156, 92, 108
SMM	281	9.63 ± 0.14	156, 92, 108	**OTC**	461	7.72 ± 0.09	154, 443, 426
SCP	285	9.99 ± 0.10	156, 92, 108	**CTC**	479	9.57 ± 0.08	154, 462, 444
SDX	311	10.33 ± 0.12	156, 92, 108	**DC**	445	9.96 ± 0.07	154, 428, 410
SClZ	285	11.82 ± 0.06	156, 92, 108	**TC**	445	8.15 ± 0.10	154, 427, 410
SDM	311	11.93 ± 0.06	156, 92, 108	**DMTC**	465	8.84 ± 0.07	154, 448, 430

Each sulfonamide and tetracycline standard was diluted in methanol and infused at a flow rate of 10 µL min^{-1} in order to establish the pseudo-molecular ion in a positive ESI mode and check the presence of potential impurity or adduct ions. Only protonated species [M+H]+ were observed for all the compounds and from these experiments, breakdown curves were recorded under different collision energy conditions to select the main characteristic fragments for quantification and analyte confirmation purposes.

Sulfonamides show a very typical fragmentation pattern, which includes ions with m/z 156, 92 and 108, as shown in **Figure 3.3**. As the first ion was found to be the most intense for the majority of sulfonamides, it was selected for our precursor-ion scan experiment to increase the overall sensitivity of such method. Precursor-ion scan survey of m/z 156 was optimized at collision energies of 22 and 30 eV, with the first being the optimum, as the collision energy of 30 eV proved to disassemble the fragment of m/z 156 in the other two characteristic fragments of SAs (m/z 92

and 108), decreasing the sensitivity of the method. This scan was performed in a m/z range of 210–315 within 1 s.

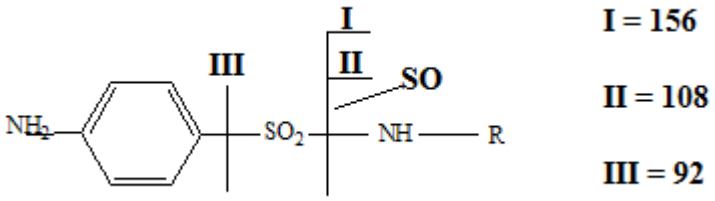


Figure 3.3: Fragmentation pattern of sulfonamides.

The fragmentation patterns of protonated tetracyclines have been reported previously and [203-205] and in general, they show an array of common fragment ions such as ions at m/z 154, 126, 98, and 58 that are identified as characteristic of the whole class of molecules. Possible structures for these common fragment ions are shown in **Figure 3.4** but numerous other resonance structures and other isomers could be proposed [206].

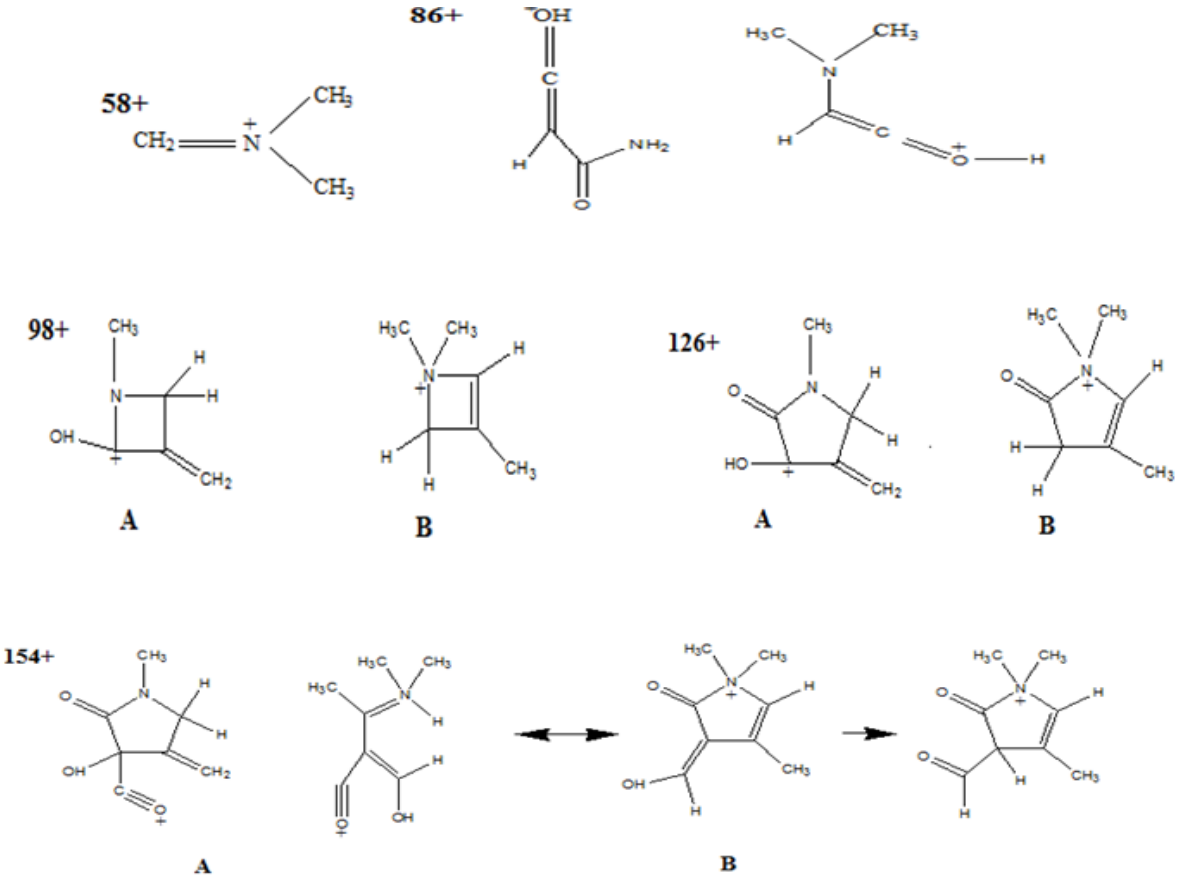
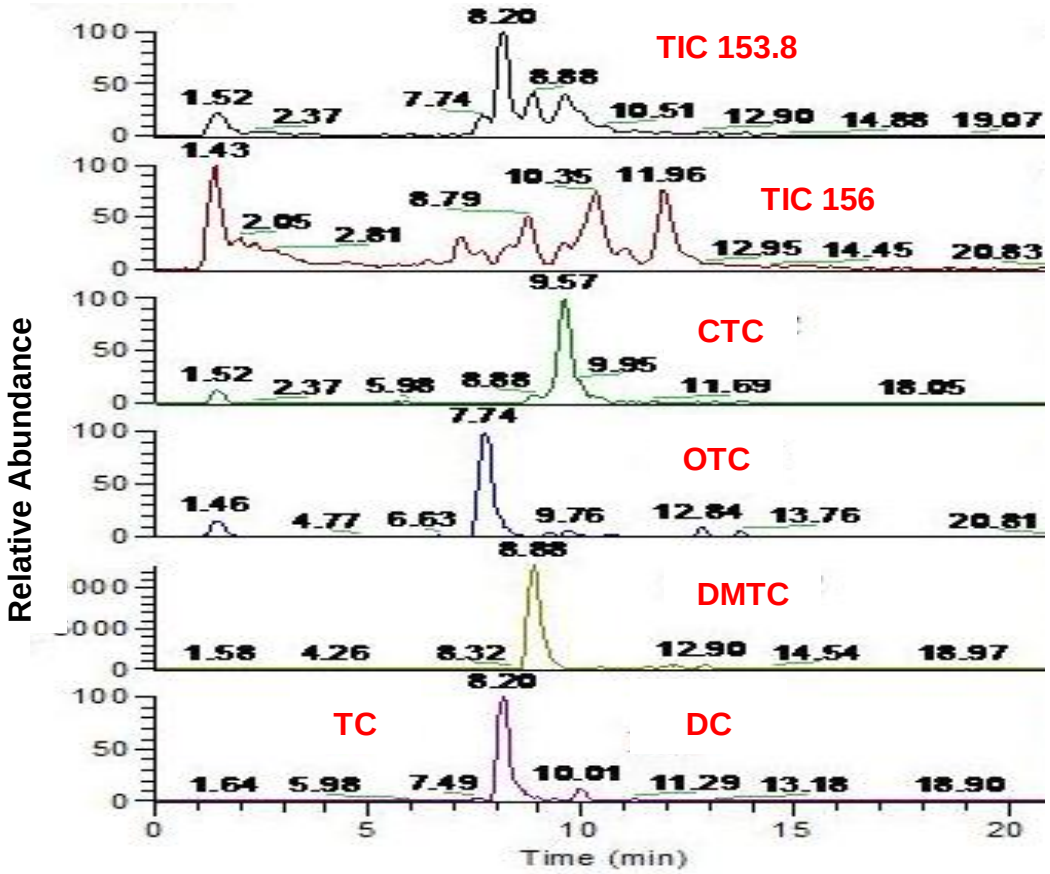


Figure 3.4 Possible structures of the fragment ions with m/z 154, 126, 98, 86, 58 [33].

The fragment ion that was chosen for the precursor-ion scan experiment was the most intense common fragment at m/z 153.8, as it was found from the breakdown curves obtain for each compound. Optimization for the collision energy was performed at 25 and 30 eV and the optimum value was proved to be 30 eV, because the precursor ion was not sufficiently produced at the lower collision energy, resulting in low sensitivity of the detection of tetracyclines. This scan was performed in a different scan event than the precursor-ion scan for sulfonamides and in a m/z range of 440–480, also within 1 s.

The precursor ions chosen for both sulfonamides and tetracyclines not only present the highest intensity, but were also the most selective ones (highest m/z). A chromatogram of all 22 compounds in the precursor-ion scan mode is presented in **Figure 3.5**.



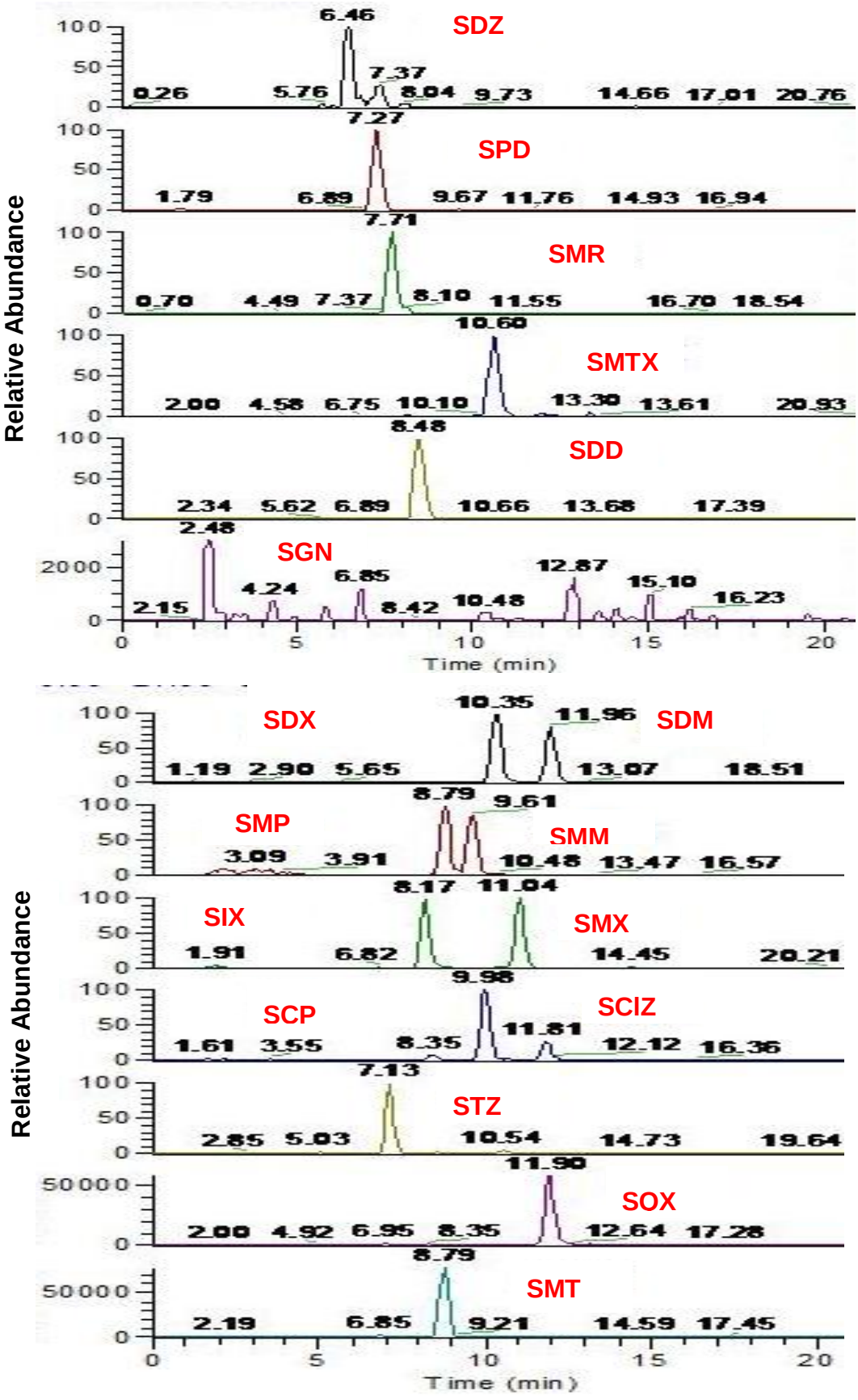


Figure 3.5 Precursor-ion scan chromatograms of a fish tissue spiked with seventeen sulfonamides and five tetracyclines at the MRL level (100 µg kg^{-1}).

Experiments were performed in order to reduce the chromatographic analysis time, but the results showed that when higher flow rates and steeper gradient were used, chromatographic separation was not adequate. There are five pairs of isobaric compounds **(Table 3.1)** that should be separated, namely SMP and SMM, SCP and SClZ, SDZ and SDM, SIX and SMX, and DC and TC and determined independently. In addition, for shorter chromatographic runs, there was a significant loss of sensitivity, especially for the first eluting compounds. These drawbacks lead to choose a chromatographic run that was probably more time-consuming than previously described UHPLC methods, however, it produced the best and most reliable results and permitted the determine all the analytes (22 compounds in total) in one chromatographic run and in concentrations as low as possible.

This experiment was used to trigger a data dependent (DD) scan of the most abundant ion when exceeding a certain threshold. The appearance in this scan of characteristic ions for each group of compounds confirms that the determined compound belongs either to TCs or to SAs. For SAs the characteristic ions are the ions with *m*/*z* 92 and 108, while for protonated tetracyclines, in addition to the characteristic series of fragment ions at *m*/*z* 154, 126, 98 and 58, their pseudo-molecular ions typically lose water, ammonia, or a combination of both, as noted previously [203-205]. DC and DMCTC undergo loss of ammonia instead of dehydration, whereas the other three may undergo dehydration first [203-204]. The three tetracyclines that undergo dehydration (TC, OTC, and CTC) have the common feature that they possess a hydroxyl group at the C6 position, whereas the others do not. The confirmation of tetracycline compounds was performed by monitoring these neutral losses than monitoring the fragment ions cited above since it was proved that it is the fragment ion at *m*/*z* 154 which is dissociated to *m*/*z* 126 and 98 and this cannot occur in a simple MS/MS mode, but it needs a MS^n in order to obtain the small fragments in a full-scan spectra [206].

Two scan events were added in the method, one for sulfonamides and one for tetracyclines, including different signal thresholds and different collision energies. Sulfonamides were more sensitive than tetracyclines in the precursor-ion scan mode and for that the chosen signal threshold was 1×10^4 counts whereas for tetracyclines was 0.1×10^4. The collision energies for the data dependent-scan

mode were also optimized for both groups of veterinary drugs and the optimum values were 30 eV for SAs and 28 eV for TCs. The choice of the optimum collision energy was made based on the appearance in the full-scan spectra of the characteristic fragment ions for each compound, as well as on the repeatability of the spectra obtained. In **Figure 3.6** and **Figure 3.7** two chromatographs are presented, one for SAs and one for TCs, in both precursor-ion scan and data dependent-scan mode, along with the obtained full-scan spectra.

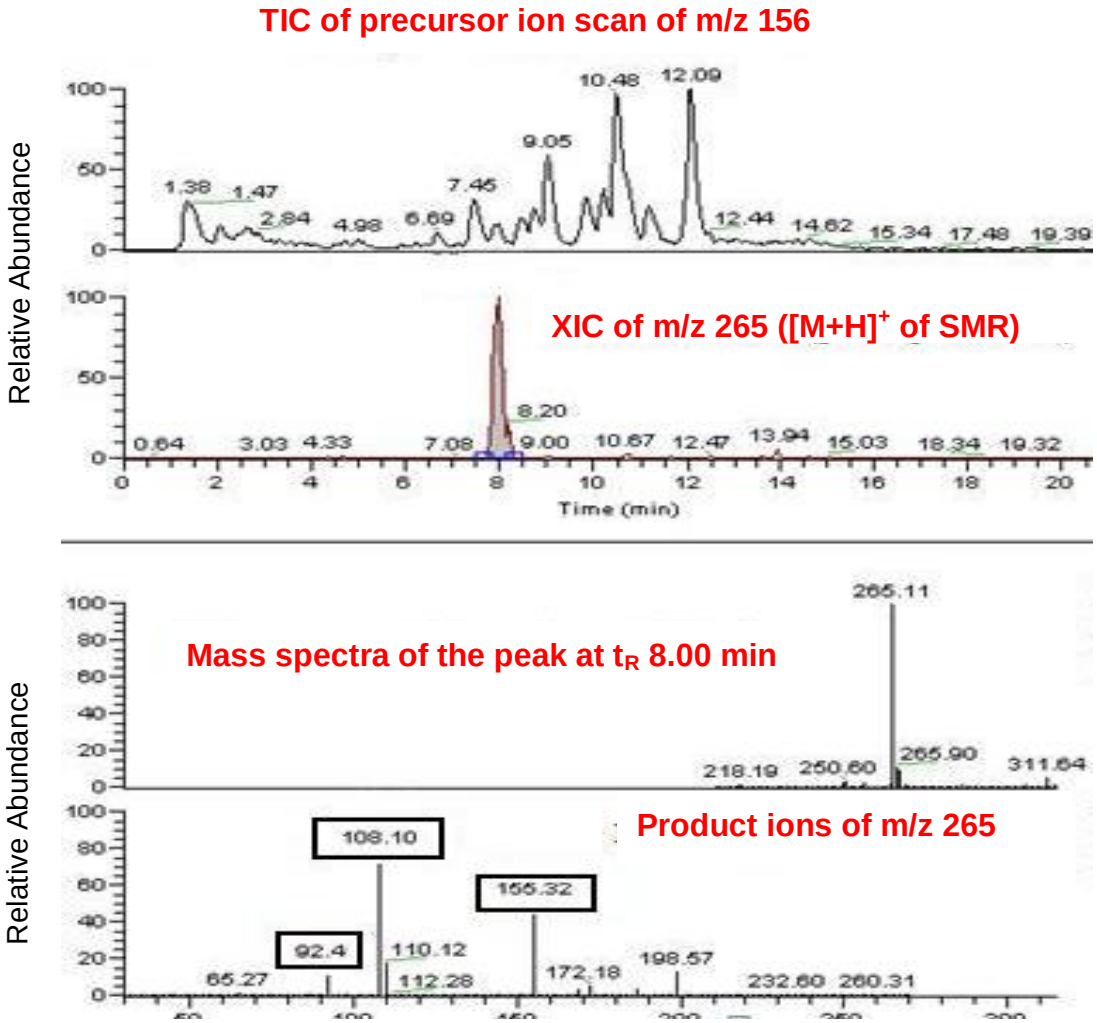


Figure 3.6: Total ion chromatogram and full-scan spectra of sulfamerazine (SMR) spiked in fish tissue at the MRL level.

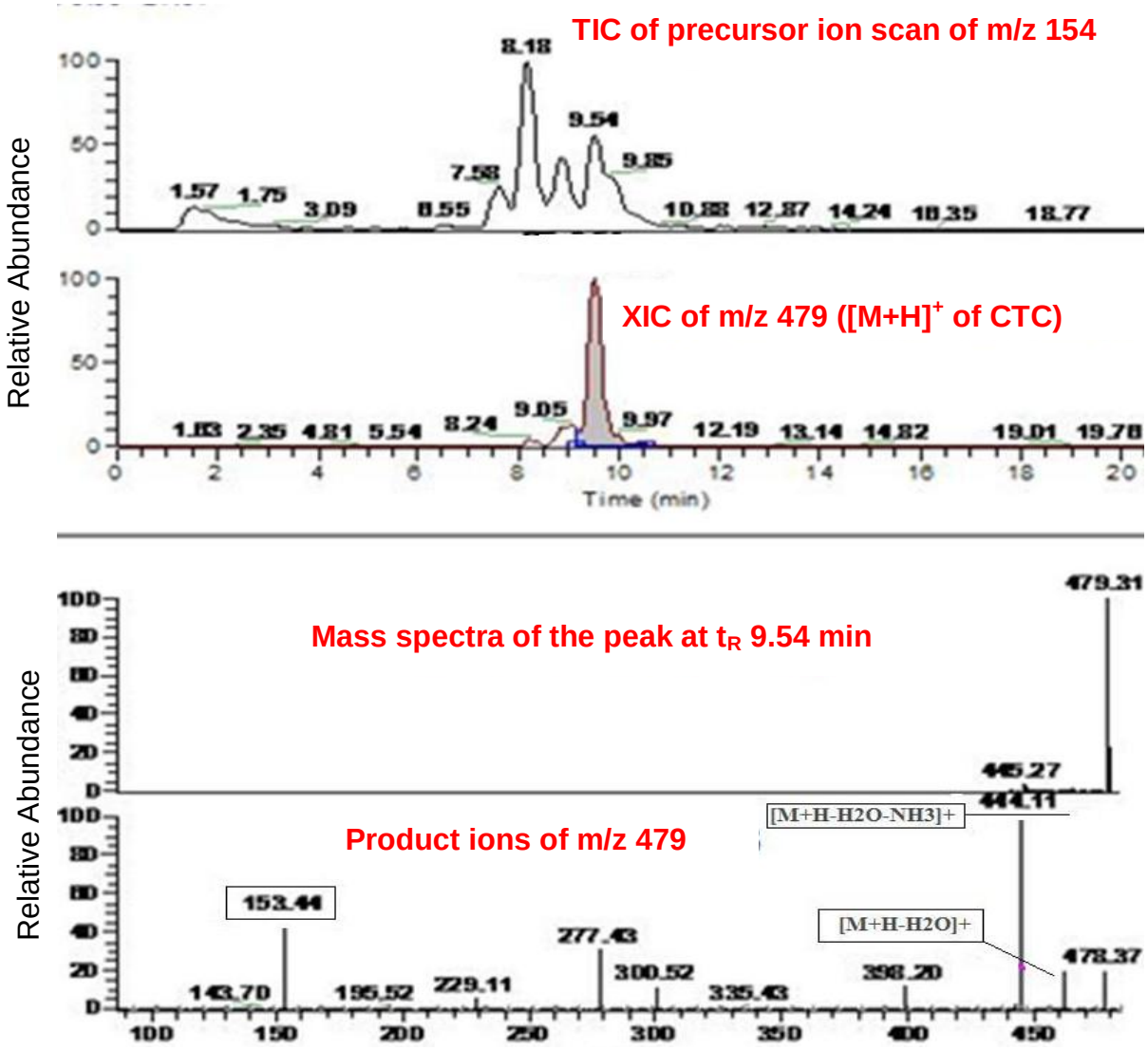


Figure 3.7: Total ion chromatogram and full-scan spectra of chlortetracycline (CTC) spiked in fish tissue at the MRL level.

The identification points of the proposed method consist of one precursor ion (pseudo-molecular ion: 1 point) and three product ions (3 × 1.5 = 4.5 points). Thus, the identification points earned using the precursor-ion scan–data dependent-scan technique are more than the SRM technique with two product ions (in total 5.5 identification points in comparison to 4 points of the SRM method) and therefore it fulfils the required criteria for a reliable identification of the compounds [54].

3.3.2 Sample preparation – extraction procedures

Sulfonamides, which are amphoteric compounds, are readily extracted by organic solvents when they are present in molecular form. Sulfonamides are not very soluble in non-polar solvents, but have good solubility in polar solvents. Therefore, the extraction is generally done with acetonitrile [207,208], chloroform, methylene chloride, acetone, or ethyl acetate [209]. The solubility of tetracyclines is higher in alcohols such as methanol (MeOH) and ethanol, while varied in other organic solvents, as ethyl acetate (EtOAc), acetone, and acetonitrile (ACN). Organic based extractions have been performed with EtOAc, ACN, and methanolic trichloroacetic acid (TCA) [210-212].

The pH value can change the form of analytes in the solvent system and greatly affect the extraction efficiency. In general, the compounds interaction with the matrix could be avoided by extracting at low pH. A possible explanation for this could be that, at neutral conditions, acidic analytes are in their deprotonated state and can interact with (protonated) amino functionalities on matrix material. *Vice versa*, basic analytes can interact with deprotonated acidic functionalities on the matrix material. At low pH the acidic groups are protonated and exist in their neutral state, while basic functionalities are either neutral or protonated (cationic). As a consequence, no or less interactions take place, the analytes do not bind to the matrix and remain dissolved in the extract solution [104].

When most of sulfonamides are extracted in basic conditions, the H^+ dissociation of secondary amino group increases, so that the extraction efficiency of sulfonamides is obviously lower than the extraction process using acidic or neutral conditions. However, there is only a slight difference between using the acidified or non- acidified extraction solution, although there is a great p*K*a value discrepancy for SAs [208].

Acidification of the extraction solvent was mostly beneficial to the extraction of tetracyclines. The pK_{a1} values for OTC, TC, CTC, DMTC and DC vary from 3 to 3.6 [213] and is associated with the deprotonation of C_3 hydroxyl. Loss of protons from O_{12} and dimethylammonium constitutes pK_{a2} (from 7.5 to 8) and pK_{a3} (from 8.9 to 9.8), although the exact assignment of these dissociation constants remains controversial [213, 214]. As indicated by their acid dissociation constants, the TCs

contain localized charges across all pH values and only achieve an overall neutral state as zwitterions. As a result, tetracyclines achieve high molar solubility and best extraction to the organic phase at $pK_{a1} < pH < pK_{a2}$, where they are in equilibrium favouring the zwitterionic state over the fully protonated species [215].

Although a wide range of analytes need to be extracted as efficiently as possible, the co-extraction of bulk matrix constituents like fat, proteins, and carbohydrates is undesirable. They may reduce the lifetime of the UHPLC column and affect the ionization process in LC–MS analysis causing a suppression or enhancement of analyte response [194,195]. Therefore, besides recoveries, such effects were also taken in consideration during evaluation of the extraction procedure.

For method development and optimization of the extraction procedure each experiment consisted of one blank and three fortified blank samples (spikes) in the MRL level. Among all the extraction solvents tested, ACN consistently was the most favourable with respect to matrix effects, but was not suitable for the extraction of tetracyclines. When methanol and acetone were compared, overall matrix effects for sulfonamides were less abundant for acetone, but it caused a sever signal suppression as far as tetracyclines are concerned. However, 50% methanol, giving a relatively high recovery of TCs along with lower signal suppression was chosen along with acidification of the extraction solvent with 0.05% formic acid. The results of the extraction procedure optimization are shown schematically in **Figures 3.8 – 3.11.**

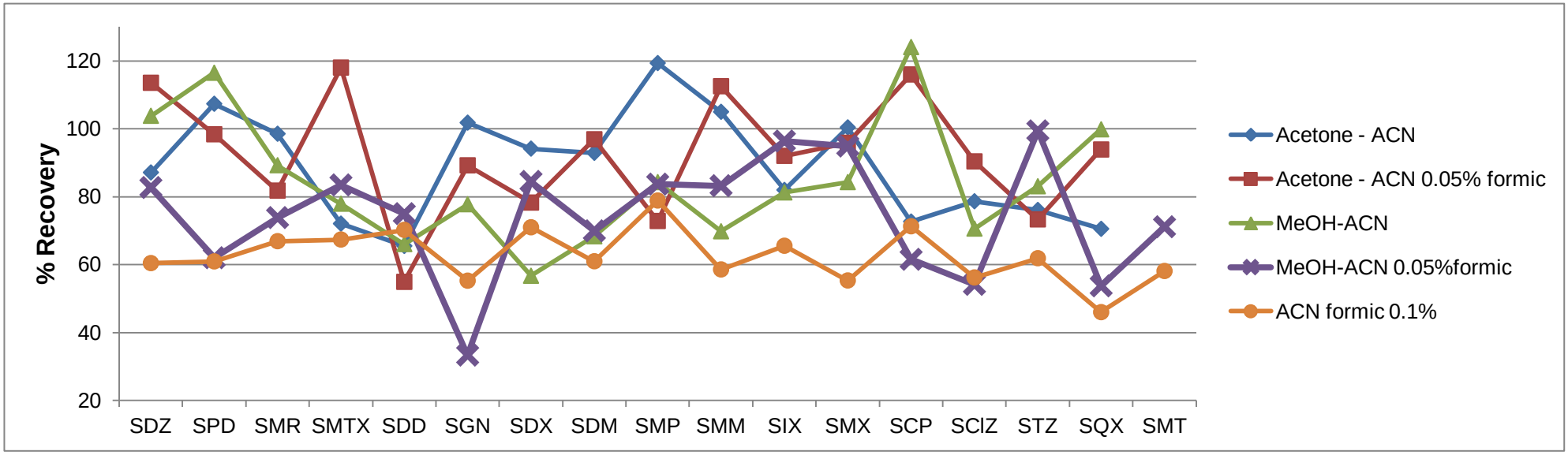


Figure 3.8: Recoveries (%) of 17 sulfonamides from fish tissue in different extraction solvents.

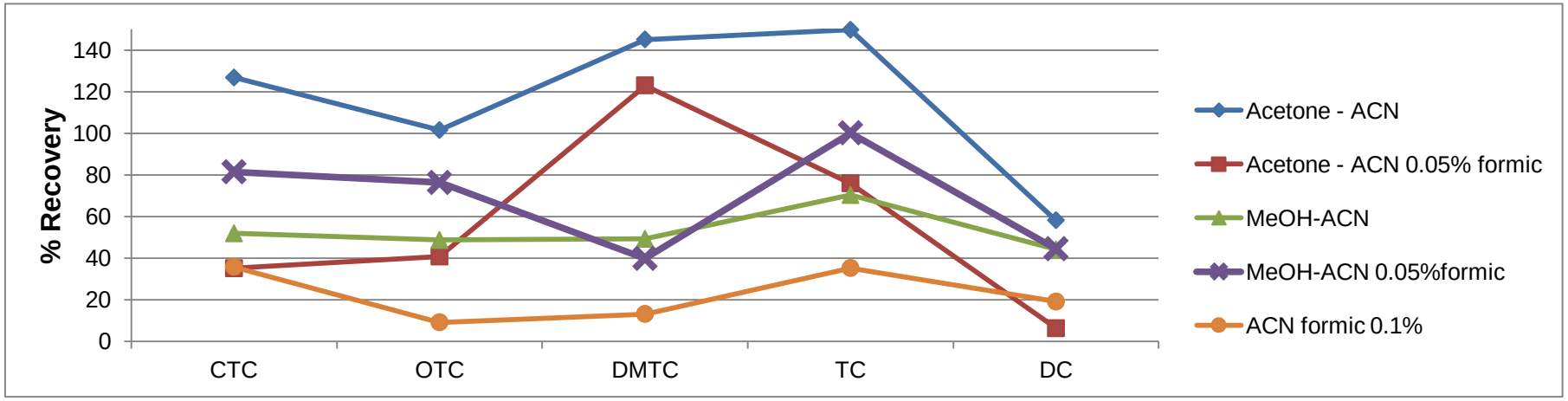


Figure 3.9: Recoveries (%) of 5 tetracyclines from fish tissue in different extraction solvents.

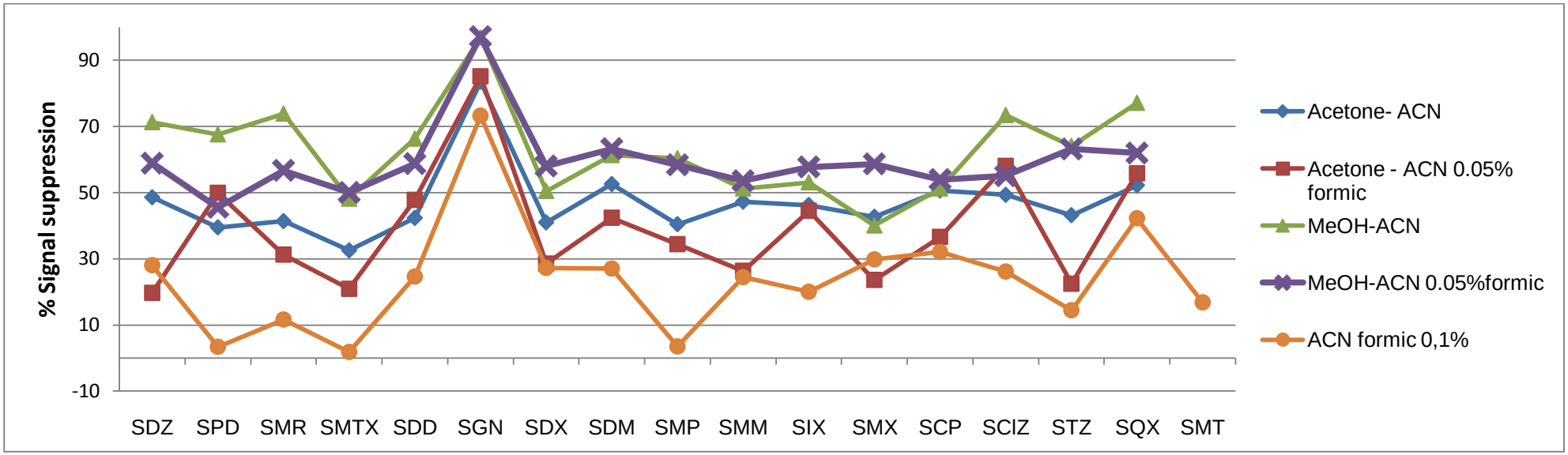


Figure 3.10: Ion suppression after post-extraction spike of sulfonamides in fish tissue extract.

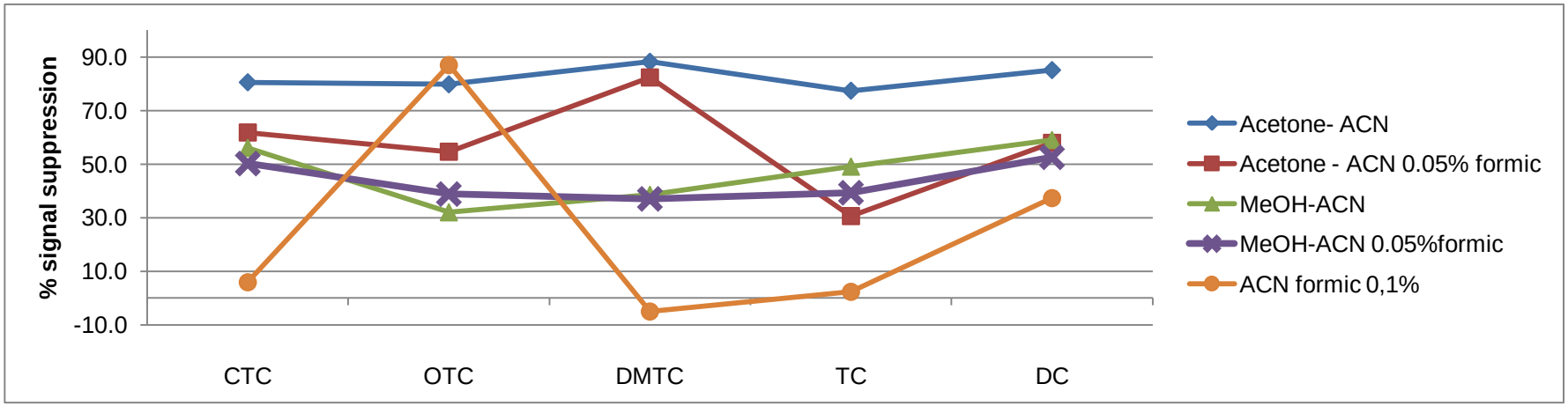


Figure 3.11: Ion suppression after post-extraction spike of tetracyclines in fish tissue extract.

No clean-up was performed in order to make the method fast and simple. This enabled the analysis of a large number of samples (20–30) within a working day. Sensitivity achieved by this simple process was sufficient to determine the analytes at the concentration levels of interest. The use of matrix-matched standards ensured correct quantification of the samples, as ion suppression effects were automatically corrected.

3.3.3 Validation

The applicability of the developed method was tested following the accepted criteria for analytical method validation, as indicated in the Commission Decision 2002/657/EC for quantitative screening methods [54]. Method detection limit (LOD), quantitation limit (LOQ), precision, selectivity, decision limit (CCα) and detection capability (CCβ) were determined for all compounds in fish tissue and precision was also determined for poultry and porcine tissue. All 22 compounds presented very similar behaviour in the three matrices examined, a fact that leads to the conclusion that sensitivity as well as CCα and CCβ are approximately at the same levels as the values that estimated for the fish tissue samples.

3.3.3.1 Limit of detection and limit of quantification

The calibration curves for detection of the analytes were obtained by performing a linear regression analysis on samples spiked with the analytes before the extraction. The linearity obtained for all analytes were good in the investigated interval (20–150 μg kg^{-1}) with correlation coefficients higher than 0.98. The limit of detection of the method was calculated as 3.3 times the standard error of the intercept (S_a) divided to the slope of the calibration curve and the limit of quantification as 10 times the S_a divided to the slope. The results are presented in **Table 3.2**. In every case, LOD was found to be much lower than the MRL.

Table 3.2: LOD, LOQ, CCα and CCβ values for the developed multi-analyte, multi-stage LC–MS/MS method.

Sas	LOD (µg Kg^{-1})	LOQ (µg Kg^{-1})	CCα (µg Kg^{-1})	CCβ (µg Kg^{-1})
SDZ	22	67	16	27
STZ	13	39	9.2	16
SMR	5.7	17	4.0	6.8
SDD	16	49	12	20
SMP	5.9	18	4.1	7.1
SPD	7.4	23	5.0	8.5
SMM	7.0	21	11	19
SCP	16	47	7.0	12
SDX	9.9	30	7.4	13
SClZ	11	32	5.8	9.9
SDM	8.3	25	25	37
SGN	24	73	5.0	8.5
SIX	9.7	29	6.8	12
SMX	7.4	23	5.2	8.9
SQX	16	47	11	19
SMTX	14	44	10	17
SMT	12	36	8.4	14
OTC	26	78	18	31
CTC	15	45	10	18
DC	15	47	11	19
TC	10	31	7.3	12
DMTC	15	46	11	18

3.3.3.2 Decision limit (CCα) and detection capability (CCβ)

For the calculation of CCα and CCβ, all compounds were treated as banned substances since the goal of the developed method is to quantitatively screen for sulfonamides and tetracyclines in the lowest possible level. Decision limit and detection capability of the method where calculated based on the matrix matched calibration curve of samples spiked with the analytes before the extraction [54], according to the following equations: CCα (μg kg^{-1}) = $a/b + 2.33S_a/b$ (α = 1%) and CCβ (μg kg^{-1}) = CCα + $1.64S_a/b$ (β = 5%), where a is the intercept of the matrix-matched curve (20–150 μg kg^{-1}), b is the slope of the matrix-matched curve and S_a is the standard error of the intercept. The results are presented in **Table 3.2**. CCβ values range from 6.8 μg kg^{-1}(SMR) to 37 μg kg^{-1} (SDM). It is concluded that the developed method has a very good applicability for detecting the selected antibiotics with a statistical certainty of 95% in fish tissue since all CCβ values are more than three times below the established MRL (100 μg kg^{-1}).

3.3.3.3 Precision

The repeatability of the analytical method for each matrix was calculated from the analysis of 18 blank fish, poultry and porcine tissue samples (at sets of six) spiked each with the 22 veterinary drugs at the MRL level before the extraction. The analysis was performed by the same operator on three separate occasions in a week period (six experiments per day) and the %RSD of the concentrations determined for each compound were calculated. For the reproducibility of the method three sets of two spiked samples were analysed in three separate occasions and the %RSD of the concentrations determined was calculated. The same methodology was followed for all three matrices. All the results are summarised in **Table 3.3**. It can be observed that the RSDs were always lower than 20% for all the antibiotics, indicating the good performance of the developed method.

Table 3.3: Precision values (as %RSDs) for all analytes in all matrices analysed.

	Fish		**Poultry**		**Porcine**	
	% RSD_r	% RSD_R	% RSD_r	% RSD_R	% RSD_r	% RSD_R
SDZ	8.4	13	5.2	19	8.6	15
STZ	6.8	11	7.5	11	11	11
SMR	12	13	7.3	15	5.4	10
SDD	9.4	11	9.8	12	5.0	11
SMP	8.4	9.6	12	13	6.8	8.0
SPD	4.3	8.8	9.2	15	8.2	11
SMM	8.5	12	14	16	9.3	11
SCP	5.3	11	6.5	8.2	5.3	10
SDX	3.5	8.1	9.0	12	3.9	5.9
SCIZ	13	18	13	18	10	16
SDM	7.1	12	6.5	8.4	4.1	5.6
SGN	16	21	15	16	7.6	13
SIX	8.3	8.7	8.0	10	5.0	8.8
SMX	8.4	11	11	12	6.9	8.6
SQX	13	15	7.1	10	8.8	14
SMTX	8.8	11	4.2	7.8	6.1	11
SMT	6.7	11	10	12	11	12
OTC	15	17	18	18	15	18
CTC	9.2	12	9.2	13	9.1	18
DC	12	14	11	13	11	19
TC	5.7	11	7.3	14	6.9	14
DMTC	11	19	16	17	13	18

3.3.3.4 Specificity/selectivity

The specificity of the method is also good. Analysis of blank samples did not show any peaks in the area of interest. Six sets of compounds, tetracycline/doxycycline, sulfamethoxypyridazine/sulfamonomethoxine, sulfachloropyridazine/sulfaclozine, sulfado-xine/sulfadimethoxine, sulfamoxole/sulfisoxazole and sulfadiazine/sulfapyridine have very similar molecular weights at 445 and 281, 285, 311, 268 and 251–250, respectively. However, these compounds can be easily distinguished on the basis of retention time as it is shown in the chromatogram of the veterinary drugs (**Figure 3.5**).

In addition, 20 fish tissue samples were spiked in the MRL level, randomly with one out of three different standard mixtures, and were analysed. This study showed no false positive or false negative results. The concentration of the antibiotics determined was in every case inside the limits of ± the standard deviation that was calculated in the reproducibility experiments. All these parameters indicate the good performance of the proposed analytical method.

3.3.3.5 Ruggedness

In order to evaluate the ruggedness of the method, three sets of spiked samples at the MRL level were analyzed. Each set consisted of seven samples of fish, poultry and porcine tissue, respectively, spiked randomly with one out of three different standard mixtures. The results showed no false positive or false negative results in every case and they were again consistent with the results of the reproducibility experiments performed for each matrix.

3.3.4 Analysis of real samples

This method has been used to analyze 30 sea bream and sea bass samples available from a local market. Among the 30 samples analyzed, only SIX was found in one of them at a concentration of 45 μg kg^{-1}. Antibiotics were considered as positively identified in fish samples when all the confirmation criteria were met: (i) identical retention time (±2.5% of the one observed for the matrix-matched calibrants) and (ii) actual pseudo-molecular ion ($[M + H]^+$) and characteristic

product ions as shown in **Table 3.1**. **Figure 3.12** shows the chromatogram of the sea bass sample in which SIX was detected and identified.

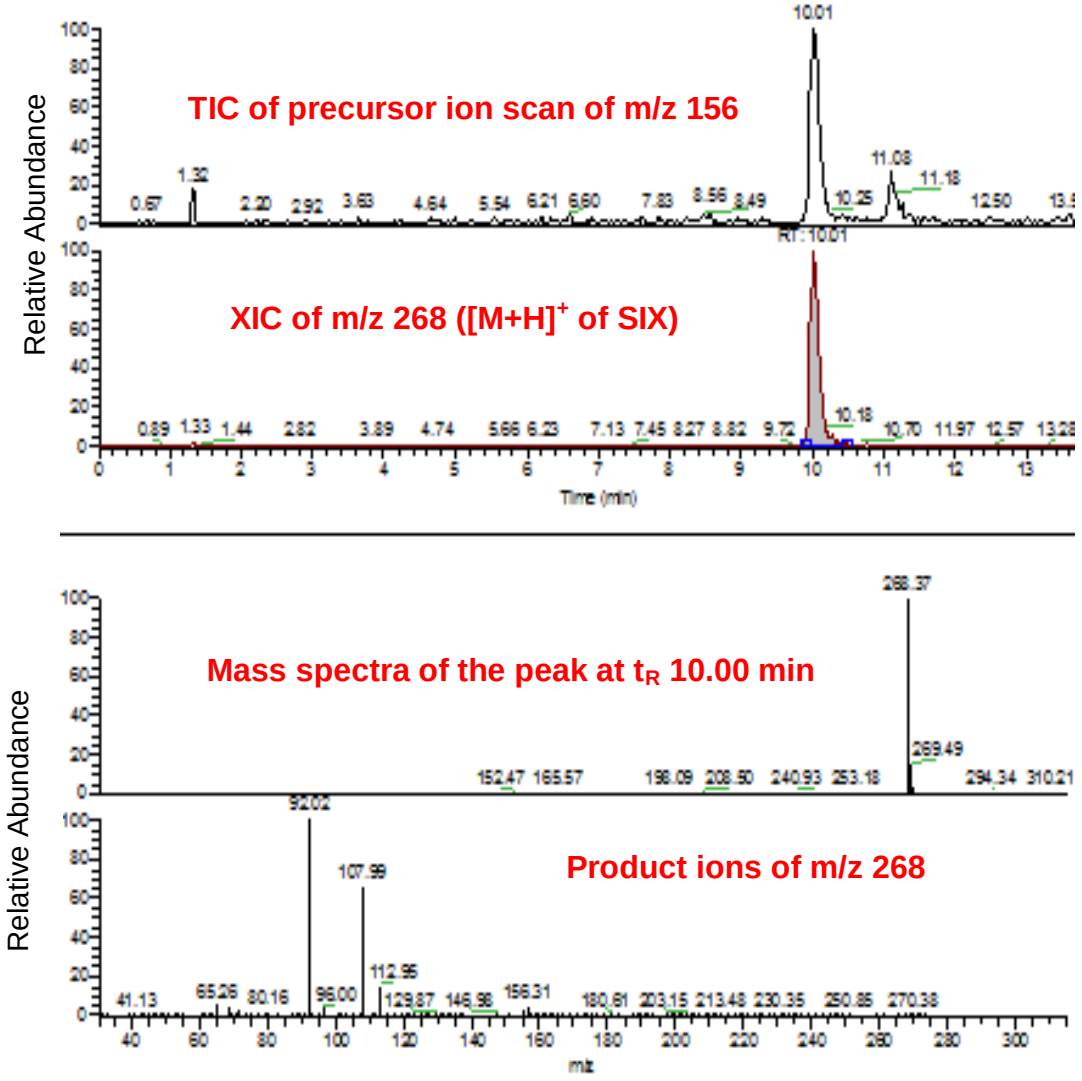


Figure 3.12: Total ion chromatogram and full-scan spectra of sulfisoxazole (SIX) found in a sea bass sample using the developed method.

3.3.5 Conclusions

A multi-stage LC–ESI–MS/MS method for the simultaneous determination of seventeen sulfonamides and five tetracyclines in fish tissue was developed and validated. The determined CCα and CCβ were adequate for SAs and TCs residue analysis, at levels well below the established MRL (100 μg kg^{-1}). This method can

be applied also for the determination of unknown SAs and TCs due to the common fragmentation pattern of all the compounds in each category. Experiments were performed for the optimization of a generic solvent extraction. MeOH–ACN acidified with 0.05% (v/v) of formic acid was finally chosen due to the highest recoveries and the lowest ion suppression, especially for tetracyclines.

The developed method has two main advantages in comparison to current SRM methods. The sample preparation procedure consists of a single generic extraction of the analytes from the matrix, requiring no cleanup step, a fact that makes the method fast and permits the screening analysis of a large number of samples. In addition, it could be applied for the determination of all compounds that belong to the groups of sulfonamides and tetracyclines, which, due to their common fragmentation pattern, provide the same precursor ion. Therefore, also unknown non-targeted compounds belonging to these groups of antibiotics can be determined, something that is not possible with a SRM method, which is used to determine only specific compounds.

CHAPTER 4

Qualitative multi-residue screening methods for 143 veterinary drugs and pharmaceuticals in milk and fish tissue using Liquid Chromatography Quadrupole-Time-Of-Flight Mass Spectrometry

4.1 Introduction

The objective of this work was the development of a reliable, sensitive and modern screening methodology for the rapid detection and identification of 143 veterinary drugs and pharmaceuticals in milk and fish samples based on the use of advanced UHPLC–QTOF–MS technique. Generic sample extraction procedures were used and their efficiency was evaluated. A qualitative method validation of the screening method was performed and subsequently the method was applied to the analysis of different milk and fish samples to test its applicability. Experimental data, including retention times and product ion spectra were obtained for all target compounds and measured exact mass data are presented. The majority of the compounds were successfully detected and identified at the highest concentration level (more than 80% of the compounds in both matrices). Very satisfactory results were obtained in the lowest level as well (more than 60 % of the compounds detected and identified).

4.2 Experimental section

4.2.1 Chemicals and Reagents

All veterinary drug and pharmaceutical standards were of high purity grade (>90%) The vast majority of them were purchased from Sigma-Aldrich (Steinheim, Germany). Sulfadoxine (SDX) and sulfaclozine (SClZ) were donated by the National Laboratory of Residue Analysis of Food of Animal Origin of the Hellenic Ministry of Rural Development and Food. Bacitracin, halofuginone, arprinocid, salinomycin, semduramicin, manduramycin, nigericin, narasin, albendazole sulfone, carprofen, diclofenac, flunixin, mefenamic acid, meloxicam, niflumic acid

and tolfenamic acid were donated by the Veterinary Drug Residues Laboratory of the State General Laboratory of Cyprus.

Acetonitrile (ACN) and methanol (MeOH) LC–MS grade were purchased from Merck (Darmstadt, Germany), while 2-propanol LC-MS grade from Fisher Scientific (Geel, Belgium). Sodium hydroxide monohydrate (NaOH) for trace analysis ≥99.9995% and formic acid 99% were purchased from Fluka (Buchs, Switzerland). Hexane (pesticide analysis grade, 95%) was purchased from Carlo Erba (Milan, Italy) and distilled water was provided by a MilliQ purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). The ethylenediaminetetraacetic acid disodium salt (EDTA) and the trichloroacetic acid (TCA) were of analytical grade and were purchased from Panreac (Barcelona, Spain) and Fisher Scientific (Loughborough, UK), respectively. RC (Regenerated Cellulose) syringe filters (15 mm diameter, 0.22 μm pore size) were provided from Phenomenex (Torrance, CA, USA). Solid phase extraction cartridges were Oasis HLB 3 cc (60 mg) and 6 cc (200 mg) from Waters (Millford, MA).

Stock standard solutions of individual compounds (1000 μg ml^{-1}) were prepared in methanol and stored at −20 ºC in brown glass to prevent the photodegradation. Penicillins, cefalosporines and metformin were dissolved in MilliQ-water and stored at 4 ºC. In quinolone standard solutions, 100 μL of formic acid were added to enhance solubility. Four intermediate standard solutions containing the analytes grouped according to their classification and stability were prepared by dilution of the stock solutions with methanol. The final concentration of these multi-component solutions was 10 μg mL^{-1} and they were also stored at −20 ºC. New ones were prepared every month. All working solutions and calibration standards, containing all the target analytes, were obtained by gradient dilution of the intermediate solutions, in concentrations varying from 1 μg mL^{-1} to 1 ng mL^{-1}. The working solutions were kept at −20 ºC and renewed weekly.

4.2.2 Samples

Pasteurized whole bovine and ovine milk samples were purchased from local retail food outlets and supermarkets. Samples were stored at 4 ºC before analysis (within 10 days). Cultured fish samples, consisting of gilthead sea bream (Sparus

aurata) and sea bass (Dicentrarchus labrax) samples, were obtained from aquaculture and local fish markets. The fillets (denuded from skin and bone) were homogenized with a high-speed blender and stored at -23 ºC until analysis. One sample of each matrix was tested to ensure that it did not contain any analytes and it was used for negative control and was fortified with target compounds.

4.2.3 Instrumentation

An ultra-performance liquid chromatography (UHPLC) system (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) interfaced to a QTOF mass spectrometer (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used for the screening analysis.

The chromatographic separation was performed on an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm) from Waters (Ireland), thermostated at 30 ºC. The mobile phases were (A) MeOH and (B) aqueous solution with 0.01% formic acid and, with a flow rate of 0.1 mL min^{-1}. The gradient elution program started with 5% MeOH (A), increased to 100% in 7 min, and kept constant for 3 min. Then, the initial conditions were restored and the system was allowed to re-equilibrate for the next injection for 7 min. The injection volume was set up to 5 µL.

The QTOF system was equipped with an electrospray ionization interface (ESI), operating in positive mode, with the following operation parameters: capillary voltage, 2500 V, end plate offset, 500 V, nebulizer pressure, 2 bar (N2), drying gas, 8 L min^{-1} (N_2) and drying temperature, 200 °C.

The QTOF system was operating in bbCID (broadband collision-induced dissociation) acquisition mode and recorded spectra over the range 40–1000 m/z with a scan rate of 2 Hz. Two acquisition functions with different collision energies were performed: the low-energy (LE) function with a collision energy of 4 eV, and the high energy (HE) function with a nominal collision energy of 25 eV.

A QTOF external calibration was daily performed with a sodium formate solution and a segment (0.1-0.25 min) in every chromatogram was used for internal calibration, using a calibrant injection at the beginning of the run. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of

water/isopropanol (1:1). The theoretical exact masses of calibration ions with formulae $Na(NaCOOH)_{1-14}$ in the range of 40-1000 Da were used for calibration. The instrument provided a typical resolving power of over 40,000 at 365 m/z.

4.2.4 Sample preparation

Two different sample preparation protocols were developed and used for the extraction of veterinary drugs and pharmaceuticals from fish and milk samples. A schematic representation of the extraction and clean-up procedures is presented in **Figure 4.1**.

The sample preparation method for the fish tissue samples has been developed and presented in the third study of this thesis which predates this work. Briefly, a 1-g portion of fish tissue sample is weighed and placed into a 15 mL polypropylene centrifuge tube. For spiked samples appropriate volumes of the working standard mix solutions are added and they are allowed to stand for 10-15 minutes. 2 mL of H_2O containing 0.1 % formic acid (v/v) and 0.1 % EDTA (w/v), 2 mL of MeOH and 2 mL of ACN are added in all samples, subsequently. After the addition of each solvent the tube is vortex-mixed for 30 sec. The sample set is placed in an ultrasonic bath at 60 ºC for 20 min, the samples are centrifuged at 4000 rpm for 10 min and the supernatant is decanted into a new polypropylene centrifuge tube. The tubes are placed in -20 ºC for 12 h in order to precipitate the lipids and remaining proteins. After centrifuging and discarding the precipitate, defatting with hexane completes the sample clean-up. The extracts are evaporated to dryness under a nitrogen stream and reconstituted in 0.5 mL of methanol/aqueous solution of formic acid, 0.05% (25:75 v/v), achieving a two-time pre-concentration of the analytes in the samples. Finally the extracts are filtered through a 0.22-μm RC filter to remove matrix interferences.

Milk samples are allowed to thaw and a quick homogenization of the fat is performed by shaking before taking up the test portion of 2 g for the analysis. The samples were extracted with a mixture of aqueous TCA 5% (w/v) - ACN (3:1, v/v) and were then subjected to clean-up with HLB (Hydrophilic-Lipophilic Balance) solid phase extraction cartridges. In particular, 2 mL of whole milk are transferred in a 50 mL polypropylene centrifuge tube. For fortified milk samples, aliquots of 30

and 300 µL of a working solution standard containing all the analytes at a concentration of 1 µg mL^{-1} were added to 2 mL milk samples. Spiked levels obtained were 15 and 150 ng mL^{-1} respectively. When fortified, the samples are vortex-mixed for 30 s and allowed to stand for 10 – 15 min. After addition of 12 ml of aqueous TCA solution 5% (w/v) and 4 ml of ACN the samples are vortexed for 1 min and shaken for 60 minutes using a mechanical shaker in medium speed. Then, the sample tube is centrifuged at 4000 rpm for 5 min and the supernatant is decanted in a new polypropylene tube and diluted to 50 mL with Milli-Q water. The diluted sample extract is adjusted to pH 5.5 by adding ammonia hydroxide 15% (w/v) and afterwards is loaded onto an OASIS HLB (60 mg, 3 mL) cartridge previously conditioned sequentially with 6 mL of MeOH and 6 mL of H2O. The sample is passed through the cartridge at a flow no faster than 1 drop/2 s and, then, it is vacuum-dried for 30 min. No washing step was applied in order to minimize analytes loss. The elution is carried out with 2 × 3 mL of MeOH and the eluent is collected and evaporated to dryness under a stream of nitrogen at 40 ºC.

The residue is redissolved in 0.5 mL of MeOH /aqueous solution of formic acid, 0.05% (25:75 v/v) and filtered through a 0.22-µm RC filter. The pre-concentration factor achieved with this method is four-fold and it is very valuable in improving the sensitivity of the analytical method.

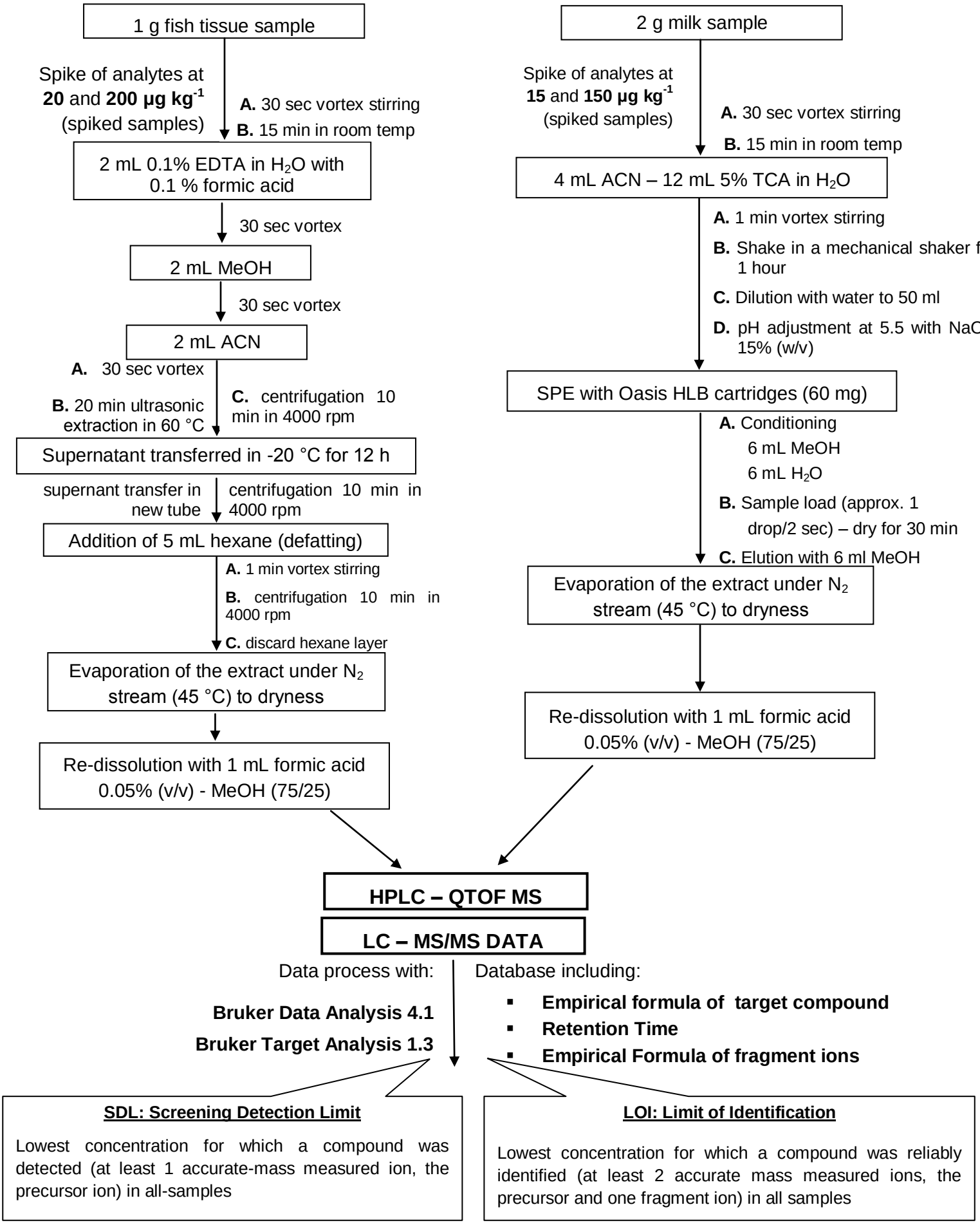


Figure 4. 1: Overall scheme of the screening methods applied

4.2.5 Qualitative Method Validation

Validation of the screening method was performed for qualitative purposes following the strategy described in the literature [101, 216, 217]. A total of twelve fish samples (6 sea bream and 6 sea bass) and twelve milk samples (6 bovine milk and 6 ovine milk samples) were spiked at two concentration levels (20 and 200 μg kg^{-1} for fish samples and 15 and 150 ng ml^{-1} for milk samples). The samples were analyzed together with their respective blanks (non-spiked) and with four reagent blanks to ensure that no laboratory contamination was held during the analysis.

The screening detection limit (SDL) and limit of identification (LOI) were investigated as the main validation parameters to estimate the threshold concentration at which detection and identification, respectively, become reliable (false negative rate: zero). The screening detection limit (SDL) was established as the lowest concentration level tested for which a compound was detected in all the samples, using the most abundant ion (most frequently, the protonated molecule). The limit of identification (LOI) was established as the lowest concentration tested for which a compound was satisfactorily identified in all spiked samples. The identification criterion was the presence of, at least, two m/z ions in either the LE or HE function, at the expected retention time (2.5% Retention Time deviation tolerance) measured at their exact mass (i.e. two peaks in the respective narrow-window extracted ion chromatograms, nw-XIC, ±0.002 mDa) with a maximum mass error of 2 mDa. According to Commission Decision 2002/657/EC, a minimum of three identification points (IPs) is required for authorized substances and four for banned compounds [54].

For HRMS, 2 IPs are earned for the precursor and 2.5 IPs for each product ion. Consequently, when a compound is successfully identified (at LOI) 4.5 IPs are earned, in agreement with the EU requirements. The terms SDL and LOI would be equivalent to the definition of ‘‘screening target concentration’’ and ‘‘detection capability’’, respectively [216], parameters which are being described in the CRL’s 2010 guideline [218].

4.3 Results and Discussion

4.3.1 UPLC-QTOF-MS method development

Data analysis and evaluation was processed with Datanalysis 4.1 and TargetAnalysis 1.3 (Bruker Daltonics, Bremen, Germany). The TOF data were evaluated against an in-house database of 143 pharmaceutical compounds and veterinary drugs. Empirical data were obtained after injection into the UHPLC-QTOF MS of solvent standard solutions for all target analytes in a concentration of 0.5 μg mL^{-1}. The database includes information over the analytes' retention time, molecular formula (in order to extract the accurate mass of the corresponding pseudo-molecular ion and adducts $[M+H]^+$, $[M+Na]^+$, $[M+NH4]^+$), isotopic fit and qualifier ions.

By adding retention time and product ion data for most compounds in the database, the analysis of samples becomes much more efficient as many false detects can be ruled out quickly based initially on retention time matching followed by product ion comparison, if needed. Elemental compositions of the product ions were proposed using the experimental m/z and generating formulas (using Brucker's Data Analysis Smart Formula Tool), restraining the number and type of atoms to those found in the precursor ion. When possible, these structures were compared to fragmentation patterns reported in the literature [219-221]. **Table 4.1** shows the compound name, retention time (min) and exact mass and elemental compositions for precursor and their main fragment ions.

For some compounds, the $[M+H]^+$ did not appear to be the most abundant ion. Ionophores, such as monensin, narasin, nigericin and salinomycin formed ammonium adducts; the same applied for amphenicols and some non-steroidal anti-inflammatory drugs (tolfenamic acid and vedaprofen). Erythromycin presented a very abundant $[M-H_2O+H]^+$ ion, since it is very unstable in acidic conditions and is rapidly subjected to a loss of H_2O. Finally, penicillins were monitored as their $[(M+H_2-CO)+H]^+$ degradation products. The degradation of penicillin G and cloxacillin is presented in **Figure 4. 2** and the same pattern applies to dicloxacillin, oxacillin and penicillin V, as well. Amoxicillin and ampicillin, due to their structure (-NH_2 at the R ring) are not subjected in a β-lactam ring-opening even in very acidic conditions [222].

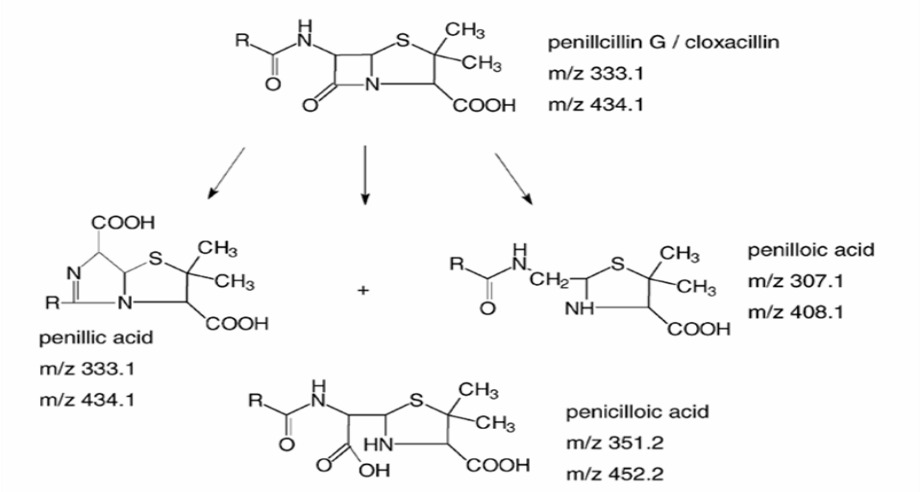


Figure 4.2: Degradation products of penicillin G (R=benzyl) and cloxacillin (R=3-(2-chlorophenyl)-5-methyl-4-isoxazolyl) (from reference 222).

After internal calibration, specific criteria – thresholds were set in order to screen the chromatograms for identified compounds, optimized to prevent false negative results, but also with an acceptable percentage of false positive results. Initially, retention time tolerance should be below 0.25 min, which is lower than 2.5 % RT deviation tolerance, for all the compounds. The mass accuracy of the precursor ion, as well as of the qualifiers, should be below 2 mDa [217]. This threshold is quite strict comparing to ones reported in the literature (e.g. 10 ppm [89, 91, 103], 5 ppm [94]), but it contributes significantly to reduce the number of false positive results. The compliance of ion intensity ratios was not used as a criterion since in large screening methods, the measurement of the ion ratios becomes non-suitable or much more tedious than in target quantitative methods where a limited number of analytes is included in the scope [217]. The isotopic fit is an additional criterion for the identification of a compound [91]. The isotope pattern matching algorithm (SigmaFit) is a feature of Bruker Daltonics software; smaller values show better isotopic fit, however in less abundant peaks, the mSigma values are relatively high, and thus no filtering is carried out for this parameter. Moreover, thresholds for the area and the intensity of the chromatographic peak are set, according to validated experiments, at the level of 2000 and 500, respectively. These thresholds were set, as a compromise of false positive and false negative results, bearing in mind the total number of features of a full-scan chromatogram.

Table 4.1: Retention time, elemental formulas and exact masses for precursor and fragment ions of veterinary drugs and pharmaceuticals.

Compounds	Elemental composition	Monoiso-topic mass	Precursor ion	Precursor ion mass	RT (min)	Fragment ion 1	m/z (fragm 1)	Fragment ion 2	m/z (fragm 2)	Fragment ion 3	m/z (fragm 3)
Quinolones											
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.1332	[M+H]+	332.1405	8.8	$C_{17}H_{17}FN_3O_2^+$	314.1299	$C_{16}H_{19}FN_3O^+$	288.1507		
Danofloxacin	$C_{19}H_{20}FN_3O_3$	357.1489	[M+H]+	358.1561	8.8	$C_{19}H_{19}FN_3O_2^+$	340.1456				
Difloxacin	$C_{21}H_{19}F_2N_3O_3$	399.1394	[M+H]+	400.1467	9.0	$C_{21}H_{18}F_2N_3O_2^+$	382.1362	$C_{20}H_{20}F_2N_3O^+$	356.1569	$C_{17}H_{13}F_2N_2O^+$	299.099
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359.1645	[M+H]+	360.1718	8.8	$C_{18}H_{23}FN_3O^+$	316.182	$C_{19}H_{21}FN_3O_2^+$	342.1612	$C_{17}H_{19}FN_3O_3^+$	332.1405
Flumequine	$C_{14}H_{12}FNO_3$	261.0801	[M+H]+	262.0874	10.6	$C_{14}H_{11}FNO_2^+$	244.0768				
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	362.1390	[M+H]+	363.1463	8.2	$C_{15}H_{15}FN_3O_4^+$	320.1041	$C_{17}H_{18}FN_4O_3^+$	345.1353	$C_4H_{10}N^+$	72.0786
Norfloxacin	$C16_6H_{18}FN_3O_3$	319.1332	[M+H]+	320.1405	8.7	$C_{16}H_{17}FN_3O_2^+$	302.1299	$C_{15}H_{19}FN_3O^+$	276.1507	$C_{13}H_{14}FN_2O^+$	233.1085
Ofloxacin	$C_{18}H_{20}FN_3O_4$	361.1438	[M+H]+	362.1511	8.5	$C_{17}H_{21}FN_3O_2^+$	318.1612	$C_{14}H_{14}FN_2O_2^+$	261.1034		
Oxolinic acid	$C_{13}H_{11}NO_5$	261.0637	[M+H]+	262.0710	10.1	$C_{13}H_{10}NO_4^+$	244.0604				
Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	385.1238	[M+H]+	386.1311	9.0	$C_{20}H_{16}F_2N_3O_2^+$	368.1205	$C_{19}H_{18}F_2N_3O^+$	342.1412		
Tetracyclines											
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.1143	[M+H]+	479.1216	9.8	$C_{22}H_{19}ClNO_7^+$	444.0845	$C_{22}H_{21}ClNO_8^+$	462.095		
Doxycycline	$C_{22}H_{24}N_2O_8$	444.1533	[M+H]+	445.1605	10.3	$C_{22}H_{22}NO_8^+$	428.1350	$C_{16}H_{22}NO_2^+$	260.1648		
Minocycline	$C_{23}H_{27}N_3O_7$	457.1849	[M+H]+	458.1922	9.4	$C_{23}H_{25}N_2O_7^+$	441.1662	$C_{23}H_{23}N_2O_6^+$	423.1551		
Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.1482	[M+H]+	461.1555	9.0	$C_{22}H_{20}NO_8^+$	426.1183	$C_{22}H_{22}NO_9^+$	444.1289		
Tetracycline	$C_{22}H_{24}N_2O_8$	444.1533	[M+H]+	445.1605	9.0	$C_{22}H_{20}NO_7^+$	410.1234	$C_{22}H_{20}NO_8^+$	426.1183	$C_7H_8NO_3^+$	154.0499
Cefalosporines											
Cefaclor	$C_{15}H_{14}C_{l1}N_3O_4S$	367.0394	[M+H]+	368.0466	8.6	$C_7H_8N^+$	106.0651	$C_{10}H_8NO_2^+$	174.0550		
Cefadroxil	$C_{16}H_{17}N_3O_5S$	363.0889	[M+H]+	364.0962	7.0	$C_4H_4NOS^+$	114.0008	$C_{10}H_{11}O_3N_2^+$	208.0842	$C_{10}H_8NO_3^+$	190.0499
Cefalexin	$C_{16}H_{17}N_3O_4S$	347.0940	[M+H]+	348.1013	8.7	$C_7H_8N^+$	106.0651	$C_6H_8NO_2S^+$	158.027	$C_4H_4NOS^+$	114.0008

Cefapirin	$C_{17}H_{17}N_3O_6S_2$	423.0553	[M+H]+	424.0632	1.5	$C_{13}H_{14}N_3OS_2^+$	292.0573	$C_7H_6NOS^+$	152.0165		
Cefazolin	$C_{14}H_{14}N_8O_4S_3$	454.0300	[M+H]+	455.0373	11.3	$C_{11}H_{11}N_6O_4S^+$	353.0557				
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	523.0290	[M+H]+	524.0363	10.4	$C_{16}H_5N_2O^+$	241.0396				
Penicillins											
Amoxicillin	$C_{16}H_{19}N_3O_5S$	365.1045	[M+H]+	366.1118	5.2	$C_9H_{10}N_3^+$	160.0869	$C_4H_4NOS^+$	114.0008	$C_{16}H_{17}N_2O_5S^+$	349.085
Ampicillin	$C_{16}H_{19}N_3O_4S$	349.1096	[M+H]+	350.1169	7.2	$C_7H_8N^+$	106.0651	$C_6H_{10}NO_2S^+$	160.0427	$C_{10}H_8NO_2^+$	174.055
Cloxacillin	$C_{19}H_{18}ClN_3O_5S$	435.0656	[(MH_2-CO)+H]+	410.0936	10.7	$C_7H_{12}NO_2S^+$	174.0583	$C_6H_{10}NS^+$	128.0528		
Dicloxacillin	$C_{19}H_{17}Cl_2N_3O_5S$	469.0266	[(MH_2-CO)+H]+	444.0546	10.8	$C_6H_{10}NO_2S^+$	160.0427	$C_{13}H_9Cl_2N_2O_3^+$	310.9985		
Oxacillin	$C_{19}H_{19}N_3O_5S$	401.1045	[(MH_2-CO)+H]+	376.1326	10.6	$C_6H_{10}NO_2S^+$	160.0427				
Penicillin G	$C_{16}H_{18}N_2O_4S$	334.0987	[(MH_2-CO)+H]+	309.1267	10.3	$C_6H_{10}NS^+$	128.0522	$C_7H_{12}NO_2S^+$	174.0583	$C_7H_7^+$	91.0542
Penicillin V	$C_{16}H_{18}N_2O_5S$	350.0936	[(MH_2-CO)+H]+	325.1217	10.5	$C_{10}H_{13}N_2O_2^+$	193.0972	$C_6H_{10}NS^+$	128.0528	$C_{14}H_{19}N_2O_2S^+$	279.1152
Macrolides											
Azithromycin	$C_{38}H_{72}N_2O_{12}$	748.5085	[M+H]+	749.5158	9.7	$C_8H_{16}NO_2^+$	158.1176	$C_6H_{14}NO^+$	116.107	$C_{30}H_{59}N_2O_9^+$	591.4215
Clarithromycin	$C_{38}H_{69}NO_{13}$	747.4769	[M+H]+	748.4842	10.8	$C_8H_{16}NO_2^+$	158.1176	$C_{30}H_{56}NO_{10}^+$	590.3899		
Erythromycin-H_2O	$C_{37}H_{67}NO_{13}$	733.4612	[M-H2O+H]+	716.4579	10.6	$C_{29}H_{52}NO_9^+$	558.3637	$C_8H_{16}NO_2^+$	158.1174	$C_{29}H_{50}NO_8^+$	540.3538
Tiamulin	$C_{28}H_{47}NO_4S$	493.3226	[M+H]+	494.3299	10.5	$C_8H_{18}NO_2S^+$	192.1053				
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	868.5660	$[M+2H]^{2+}$	435.2894	9.9	$C_{38}H_{67}N_2O_9^+$	695.4841	$C_8H_{16}NO_3^+$	174.1125	$C_5H_7O_2^+$	99.0441
Sulfonamides											
Dapsone	$C_{12}H_{12}N_2O_2S$	248.0619	[M+H]+	249.0692	8.6	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfachloro-pyridazine	$C_{10}H_9ClN_4O_2S$	284.0135	[M+H]+	285.0208	9.0	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfaclozine	$C_{10}H_9ClN_4O_2S$	284.0135	[M+H]+	285.0208	9.8	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfadiazine	$C_{10}H_{10}N4O_2S$	250.0524	[M+H]+	251.0597	7.3	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	310.0736	[M+H]+	311.0809	9.9	$C_4H_4N_4OS^+$	156.0114	$C_6H_6NO^+$	108.0444		

Sulfadimidine	$C_{12}H_{14}N_4O_2S$	278.0837	[M+H]+	279.091	8.8	$C_6H_{10}N_3^+$	124.0869	$C_6H_8N_3O_2S^+$	186.0332	$C_{12}H_4N_4^+$	204.043
Sulfadoxine	$C_{12}H_{14}N_4O_4S$	310.0736	[M+H]+	311.0809	9.3	$C_4H_4N_4OS^+$	156.0114	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495
Sulfaguanidine	$C_7H_{10}N_4O_2S$	214.0524	[M+H]+	215.0597	2.8	$C_6H_6N^+$	92.0495	$C_6H_6NO^+$	108.0444	$C_4H_4N_4OS^+$	156.0114
Sulfamerazine	$C_{11}H_{12}N_4O_2S$	264.0681	[M+H]+	265.0754	8.2	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfameter	$C_{11}H_{12}N_4O_3S$	280.0630	[M+H]+	281.0703	8.6	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfamethizole	$C_9H_{10}N_4O_2S_2$	270.0245	[M+H]+	271.0318	8.7	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.0521	[M+H]+	254.0594	9.1	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfamethoxy-pyridazine	$C_{11}H_{12}N_4O_3S$	280.0630	[M+H]+	281.0703	8.9	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfamono-methoxine	$C_{11}H_{12}N_4O_3S$	280.0630	[M+H]+	281.0703	9.2	$C_6H_6NO^+$	108.0444	$C_4H_4N_4OS^+$	156.0114	$C_6H_6N^+$	92.0495
Sulfamoxole	$C_{11}H_{13}N_3O_3S$	267.0678	[M+H]+	268.075	9.3	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	249.0572	[M+H]+	250.0645	8.0	$C_6H_6NO^+$	108.0444	$C_4H_4N_4OS^+$	156.0114	$C_6H_6N^+$	92.0495
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	300.0681	[M+H]+	301.0754	10.1	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Sulfathiazole	$C_9H_9N_3O_2S_2$	255.0136	[M+H]+	256.0209	7.8	$C_6H_6NO^+$	108.0444	$C_4H_4N_4OS^+$	156.0114	$C_6H_6N^+$	92.0495
Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	267.0678	[M+H]+	268.075	8.5	$C_6H_6NO^+$	108.0444	$C_6H_6N^+$	92.0495	$C_4H_4N_4OS^+$	156.0114
Quinoxalines											
Carbadox	$C_{11}H_{10}N_4O_4$	262.0702	[M+H]+	263.0775	9.2	$C_{10}H_7N_4O_3^+$	231.0513	$C_8H_5N_2^+$	129.0447		
Olaquindox	$C_{12}H_{17}N_3O_4$	267.1219	[M+H]+	268.1292	6.9	$C_{12}H_{11}N_3O_2^+$	229.0846	$C_{10}H_9N_2O_4^+$	221.0553	$C_9H_8N_2O^+$	160.0631
Amphenicols											
Florfenicol	$C_{12}H_{14}Cl_2FNO_4S$	357.0005	$[M+NH_4]+$	375.0343	9.3	$C_{11}H_9Cl_2NO^+$	241.0053				
Thiampenicol	$C_{12}H_{15}Cl_2NO_5S$	355.0048	$[M+NH_4]+$	373.0386	8.5	$C_{11}H_{18}ClN_2O_3^+$	261.1				
Diaminopyrimidines											
Baquiloprin	$C_{17}H_{20}N_6$	308.1749	[M+H]+	309.1822	6.2	$C_{16}H_{18}N_6^+$	294.1587	$C_{11}H_{11}N_2^+$	171.0917		
Trimethoprim	$C_{14}H_{18}N_4O_3$	290.1379	[M+H]+	291.1452	8.3	$C_{12}H_{14}N_4O^+$	230.1176	$C_5H_7N_4^+$	123.0665	$C_{12}H_{12}N_4O_3^+$	261.0957

Other antibiotics											
Lincomycin	$C_{18}H_{34}N_2O_6S$	406.2138	[M+H]+	407.221	8.3	$C_8H_{16}N^+$	126.1277	$C_{17}H_{31}N_2O_6^+$	359.2177		
Novobiocin	$C_{31}H_{36}N_2O_{11}$	612.2319	[M+H]+	613.2392	11.7	$C_{12}H_{13}O_2^+$	189.091	$C_{22}H_{22}NO_6^+$	396.1442	$C_9H_{16}NO_5^+$	218.1023
Rifaximin	$C_{43}H_{51}N_3O_{11}$	785.3524	[M+H]+	786.3596	11.1	$C_{42}H_{48}N_3O_{10}^+$	754.3334				
Althenmintics											
Albendazole	$C_{12}H_{15}N_3O_2S$	265.0885	[M+H]+	266.0958	11.0	$C_{11}H_{12}N_3OS^+$	234.0696	$C_8H_5N_3OS^+$	191.0148	$C_8H_5N_3O^+$	159.0427
Albendazole oxide	$C_{12}H_{15}N_3O_3S$	281.0834	[M+H]+	282.0907	9.9	$C_8H_6N_3O_2S^+$	208.0175	$C_9H_{10}N_3O_3S^+$	240.0437	$C_9H_9N_3O_2^+$	191.0689
Albendazole sulfone	$C_{12}H_{15}N_3O_4S$	297.0783	[M+H]+	298.0856	11.5	$C_{11}H_{12}N_3O_3S^+$	266.0594	$C_8H_6N_3O_3S^+$	224.0124		
Dimetridazole	$C_5H_7N_3O_2$	141.0538	[M+H]+	142.0611	7.9	$C_5H_7N_2^+$	95.0604	$C_4H_5N_2^+$	81.0447		
Febantel	$C_{20}H_{22}N_4O_6S$	446.1260	[M+H]+	447.1333	11.3	No fragments					
Fenbendazole	$C_{15}H_{13}N_3O_2S$	299.0728	[M+H]+	300.0801	11.3	$C_{14}H_{10}N_3OS^+$	268.0539	$C_{10}H_7O_2^+$	159.0441		
Flubendazole	$C_{16}H_{12}FN_3O_3$	313.0863	[M+H]+	314.0935	10.8	$C_{15}H_9FN_3O_2^+$	282.0673	$C_7H_4FO^+$	123.0241		
Levamisol	$C_{11}H_{12}N_2S$	204.0721	[M+H]+	205.0794	7.7	$C_{10}H_{12}NS^+$	178.0685	$C_{10}H_{12}N^+$	146.0964	$C_7H_7S^+$	123.0263
Mebendazole	$C_{16}H_{13}N_3O_3$	295.0957	[M+H]+	296.103	10.7	$C_{15}H_{10}N_3O_2^+$	264.0768				
Metronidazol	$C_6H_9N_3O_3$	171.0644	[M+H]+	172.0717	7.2	$C_4H_6N_3O_2^+$	128.0455	$C_4N_2H_5^+$	82.0525	$C_{11}H_{11}O^+$	
Morantel	$C_{12}H_{16}N_2S$	220.1034	[M+H]+	221.1107	9.0	$C_6H_7S^+$	111.0263	$C_7H_7S^+$	123.0263		
Oxfendazole	$C_{15}H_{13}N_3O_3S$	315.0678	[M+H]+	316.075	10.4	$C_{11}H_{11}O_3^+$	191.0703	$C_{14}H_{10}N_3O_2S^+$	284.0488		
Piperazine	$C_4H_{10}N_2$	86.0844	[M+H]+	87.0917	1.3	$C_4H_7N^+$	70.0651				
Ronidazole	$C_6H_8N_4O_4$	200.0546	[M+H]+	201.0618	7.2	$C_2H_4N_2^+$	56.0369				
Ternidazole	$C_7H_{11}N_3O_3$	185.0800	[M+H]+	186.0873	8.3	$C_4H_6N_3O_2^+$	128.0455	$C_4H_6N_2^+$	82.0525	$C_6HN_2^+$	101.0134
Thiabendazole	$C_{10}H_7N_3S$	201.0361	[M+H]+	202.0433	9.1	$C_9H_7N_2S^+$	175.0324	$C_8H_7N_2^+$	131.0604		
Triclabendazole	$C_{14}H_9C_{l3}N_2OS$	357.9501	[M+H]+	358.9574	11.8	$C_{13}H_7Cl_3N_2OS^+$	343.9334	$C_{13}H_7Cl_3N_2OS^+$	345.931	$C_{12}H_{11}Cl_3N^+$	273.9952
Coccidiostats											

Arprinocid	$C_{12}H_9ClFN_5$	277.0531	[M+H]+	278.0603	10.3	$C_7H_5ClF^+$	143.0058				
Clopidol	$C_7H_7Cl_2NO$	190.9905	[M+H]+	191.9977	8.3	$C_5H_6Cl^+$	101.0153	$C_4H_4Cl^+$	86.9996		
Decoquinate	$C_{24}H_{35}NO_5$	417.2515	[M+H]+	418.2588	12.4	$C_{22}H_{32}NO_5^+$	390.2275	$C_{22}H_{30}NO_4^+$	372.2169		
Diaveridine	$C_{13}H_{16}N_4O_2$	260.1273	[M+H]+	261.1346	8.2	$C_{12}H_{13}N_4O_2^+$	245.1033	$C_5H_7N_4^+$	123.0665	$C_{11}H_{13}N_4O^+$	217.1084
Ethopabate	$C_{12}H_{15}NO_4$	237.1001	[M+H]+	238.1074	10.4	$C_7H_6NO_2^+$	136.0393	$C_9H_{10}NO_2^+$	164.0706	$C_{11}H_{12}NO_3^+$	206.0812
Halofuginone	$C_{16}H_{17}BrClN_3O_3$	413.0142	[M+H]+	414.0215	10.3	$C_5H_{10}NO^+$	100.0757	$C_8H_{10}N^+$	120.0808	$C_6H_{14}NO^+$	116.107
Imidocarb	$C_{19}H_{20}N_6O$	348.1699	[M+H]+	349.1771	7.7	$C_{10}H_{10}N_3O^+$	188.0818	$C_9H_{12}N_3^+$	162.1026		
Lasalocid	$C_{34}H_{54}O_8$	590.3819	[M+H]+	591.3891	10.3	$C_{23}H_{37}O_4^+$	377.2686				
Monensin	$C_{36}H_{62}O_{11}$	670.4291	[M+NH_4]+	688.4630	12.6	$C_{31}H_{43}NO_2^+$	461.3288	$C_{25}H_{41}O_5^+$	421.2949		
Narasin	$C_{43}H_{72}O_{11}$	764.5074	[M+NH_4]+	782.5413	12.4	$C_{23}H_{36}O_6NH_4^+$	426.2850	$[M+Na]^+$	787.4967		
Nigericin	$C_{40}H_{68}O_{11}$	724.4761	[M+NH_4]+	742.5100	12.9	$[M+Na]^+$	747.4654				
Robenidine	$C_{15}H_{13}Cl_2N_5$	333.0548	[M+H]+	334.0621	11.0	$C_7H_5ClN^+$	138.0105	$C_7H_8ClN_2^+$	155.0371	$C_8H_5ClN_3^+$	178.0167
Salinomycin	$C_{42}H_{70}O_{11}$	750.4918	[M+NH_4]+	768.5256	12.3	$C_{23}H_{36}O_6\,NH_4^+$	426.2850				
NSAIDs											
5-Hydroxy-flunixin	$C_{14}H_{11}F_3N_2O_3$	312.0722	[M+H]+	313.0795	11.3	$C_{14}H_{10}F_3N_2O_2^+$	295.0689	$C_5H_8F_3^+$	125.0573		
Aceclofenac	$C_{16}H_{13}Cl_2NO_4$	353.0222	[M+H]+	354.0294	11.7	$C_7H_{15}Cl_2NO_2^+$	215.0474	$C_{13}H_{10}Cl_2N^+$	250.0185		
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	295.0167	[M+H]+	296.024	11.5	$C_{13}H_{10}ClN^+$	215.0496	$C_{13}H_{10}Cl_2N^+$	250.0185		
Flunixin	$C_{14}H_{11}F_3N_2O_2$	296.0773	[M+H]+	297.0845	11.5	$C_{14}H_{10}F_3N_2O^+$	279.0748	$C_{13}H_8F_3N_2O^+$	265.0579		
Ketoprofen	$C_{16}H_{14}O_3$	254.0943	[M+H]+	255.1016	11.0	$C_7H_5O^+$	105.0335	$C_{15}H_{13}O^+$	209.0961	$C_{10}H_9O_3^+$	177.0546
Mefenamic acid	$C_{15}H_{15}NO_2$	241.1103	[M+H]+	242.1176	11.9	$C_{15}H_{14}NO^+$	224.107	$C_{14}H_{11}NO^+$	209.0827	$C_7H_{13}O_2^+$	129.091
Meloxicam	$C_{14}H_{13}N_3O_4S_2$	351.0347	[M+H]+	352.042	11.0	$C_4H_7N_2S^+$	115.0324	$C_5H_5N_2OS^+$	141.0117		
Naproxen	$C_{14}H_{14}O_3$	230.0943	[M+H]+	231.1016	11.1	$C_{13}H_{13}O^+$	185.0961	$C_{10}H_8N_3^+$	170.0713	$C_{12}H_9^+$	153.0699
Niflumic acid	$C_{13}H_9F_3N_2O_2$	282.0616	[M+H]+	283.0689	11.6	$C_{13}H_8F_3N_2O^+$	265.0583	$C_{13}H_7F_2N_2O^+$	245.0521		
Phenylbuntazone	$C_{19}H_{20}N_2O_2$	308.1525	[M+H]+	309.1598	11.3	$C_7H_6NO^+$	120.0444	$C_{11}H_{14}N^+$	160.1121	$C_{13}H_{11}N_2O^+$	211.0866

Tolfenamic acid	$C_{14}H_{12}ClNO_2$	261.0556	$[M+NH_4]+$	279.0895	8.8	No fragments						
Vedaprofen	$C_{19}H_{26}NO_2$	282.1620	$[M+NH_4]+$	300.1958	12.1	$C_{12}H_{11}^+$	155.0855	$C_{18}H_{21}^+$	237.1638			
Beta-agonists												
Cimaterol	$C_{12}H_{17}N_3O$	219.1372	[M+H]+	220.1444	6.8	$C_9H_{10}N_3^+$	160.0869	$C_9H_7N_2^+$	143.0604	$C_{12}H_{16}N_3^+$	202.1339	
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	276.0796	[M+H]+	277.0869	9.1	$C_8H_9Cl_2N_2^+$	203.0137	$C_{12}H_{17}Cl_2N_2^+$	259.0763	$C_{11}H_6NO^+$	168.0444	
Clenpenterol	$C_{13}H_{20}Cl_2N_2O$	290.0953	[M+H]+	291.1025	9.6	$C_8H_9Cl_2N_3^+$	203.0137					
Mabuterol	$C_{13}H_{18}ClF_3N_2O$	310.1060	[M+H]+	311.1133	9.6	$C_9H_9ClF_3N_2^+$	237.0401	$C_9H_8ClF_2N_2^+$	217.0339			
Ractopamine	$C_{18}H_{23}NO_3$	301.1678	[M+H]+	302.1751	8.8	$C_8H_9O^+$	121.0648	$C_7H_7O^+$	107.0491	$C_{10}H_{14}NO^+$	164.1070	
Salbutamol	$C_{13}H_{21}NO_3$	239.1521	[M+H]+	240.1594	7.2	$C_9H_{10}NO^+$	148.0757	$C_8H_9O^+$	121.0648			
Terbutaline	$C_{12}H_{19}NO_3$	225.1365	[M+H]+	226.1438	7.1	$C_8H_{10}NO_2^+$	152.0706	$C_7H_9O_2^+$	125.0597	$C_7H_7O^+$	107.0491	
Steroids												
Betamethasone	$C_{22}H_{29}FO_5$	392.1999	[M+H]+	393.2072	10.8	$C_{22}H_{27}O_4^+$	355.1904	$C_{22}H_{29}O_5^+$	373.201	$C_{22}H_25O_3^+$	337.1798	
Cortisol	$C_{21}H_{30}O_5$	362.2093	[M+H]+	363.2166	10.7	$C_8H_9O^+$	121.0648	$C_{21}H_{27}O3^+$	327.1955	$C_{21}H_{25}O_2^+$	309.1849	
Cortison	$C_{21}H_{28}O_5$	360.1937	[M+H]+	361.201	10.7	$C_{11}H_{15}O^+$	163.1117					
Dexamethazone	$C_{22}H_{29}FO_5$	392.1999	[M+H]+	393.2072	7.4	$C_{22}H_{29}O_5^+$	373.2009					
Methylprednisolone	$C_{22}H_{30}O_5$	374.2093	[M+H]+	375.2166	10.9	$C_{22}H_{29}O_4^+$	357.206	$C_{22}H_{27}O3^+$	339.1955	$C_{22}H_{25}O_2^+$	321.1849	
Progesteron	$C_{21}H_{30}O_2$	314.2246	[M+H]+	315.2319	11.5	$C_7H_9O^+$	109.0648	$C_6H_9O^+$	97.0648	$C_8H_{11}O^+$	123.0804	
Thyreostats												
Methyl-thiouracil	$C_5H_6N_2O_2S$	158.0150	[M+H]+	159.0223	3.9	$C_4H_6NO^+$	84.0444	$C_5H_4NOS^+$	126.0008			
Phenyl-thiouracil	$C_{10}H_8N_2OS$	204.0357	[M+H]+	205.0430	9.8	$C_8H_7^+$	103.0542	$C_9H_8NO^+$	146.06	$C_{10}H_6NOS^+$	188.0165	
Propyl-thiouracil	$C_7H_{10}N_2OS$	170.0514	[M+H]+	171.0587	8.9	$C_6H_{10}NO^+$	112.0757	$C_7H_8NOS^+$	154.0321			
Pharmaceuticals												
Ambroxol	$C_{13}H_{18}Br_2N_2O_2$	391.9735	[M+H]+	392.9808	10.1	$C_6H_6Br_2NO^+$	263.8838	$C_6H_{14}NO^+$	116.1069			
Atenolol	$C_{14}H_{22}N_2O_3$	266.1630	[M+H]+	267.1703	7.1	$C_{11}H_{12}NO_2^+$	190.0863	$C_{11}H_{17}N_2O_3^+$	225.1234	$C_9H_{10}NO_2^+$	164.0706	

Atorvastatin	$C_{33}H_{35}FN_2O_5$	558.2530	[M+H]+	559.2603	11.2	$C_{26}H_{31}FNO_4^+$	440.2232	$C_{27}H_{29}FNO_5^+$	466.2024		
Caffeine	$C_8H_{10}N_4O_2$	194.0804	[M+H]+	195.0877	8.7	$C_5H_7N_4^+$	123.0665	$C_6H_8N_3O^+$	138.0662	$C_5H_8N_3^+$	110.0713
Carbamazepine	$C_{15}H_{12}N_2O$	236.0950	[M+H]+	237.1022	10.6	$C_{14}H_{12}N^+$	194.0964	$C_{14}H_{11}N^+$	193.0886		
Cimetidine	$C_{10}H_{16}N_6S$	252.1157	[M+H]+	253.1230	7.4	$C_5H_7N_2^+$	95.0604	$C_5H_{11}N_4S^+$	159.0699	$C_4H_9N_2S^+$	117.0481
Gemfibrozil	$C_{15}H_{22}O_3$	250.1569	[M+H]+	251.1642	11.9	$C_7H_{13}O_2^+$	129.091				
Haloperidol	$C_{21}H_{23}ClFNO_2$	375.1401	[M+H]+	376.1474	10.4	$C_{10}H_{10}FO^+$	165.071	$C_{21}H_{22}ClFNO^+$	358.1368	$C_7H_4FO^+$	123.0241
Indapamide	$C_{16}H_{16}ClN_3O_3S$	365.0601	[M+H]+	366.0674	10.4	$C_9H_{10}N^+$	132.0808				
Metformin	$C_4H_{11}N_5$	129.1014	[M+H]+	130.1087	3.1	$C_2H_5N_4^+$	85.0509	$C_3H_7N_2^+$	71.0604	$CH_6N_3^+$	60.0556
Metoprolol	$C_{15}H_{25}NO_3$	267.1834	[M+H]+	268.1907	9.3	$C_6H_{12}N^+$	98.0964	$C_6H_{14}NO^+$	116.107	$C_{11}H_{11}O^+$	159.0804
Paracetamol	$C_8H_9NO_2$	151.0633	[M+H]+	152.0706	7.0	$C_6H_8NO^+$	110.0600	$C_6H_5O^+$	93.0335		
Propranolol	$C_{16}H_{21}NO_2$	259.1572	[M+H]+	260.1645	10.3	$C_6H_{14}NO^+$	116.1070	$C_{13}H_{11}O^+$	183.0804	$C_{12}H_{11}^+$	155.0855
Ranitidine	$C_{13}H_{22}N_4O_3S$	314.1413	[M+H]+	315.1485	7.1	$C_5H_{10}N_3O_2S^+$	176.0488	$C_{11}H_{12}NO_2^+$	190.0863		
Simvastatin	$C_{25}H4_2NO_5$	436.3063	[M+NH_4]+	436.3057	12.0	$C_{15}H_{19}^+$	199.1481	$C_{19}H_{25}O_2^+$	285.1850	$C_{17}H_{21}^+$	225.1638
Theophyline	$C_7H_8N_4O_2$	180.0647	[M+H]+	181.0720	8.1	$C_5H_6N_3O^+$	124.0505	$C_4H_6N_3^+$	96.0556	$C_3H_5N_2^+$	69.0447
Tramadol	$C_{16}H_{25}NO_2$	263.1885	[M+H]+	264.1958	9.2	$C_3H_8N^+$	58.0651				
Triamterene	$C_{12}H_{11}N_7$	253.1076	[M+H]+	254.1149	8.9	$C_{12}H_9N_6^+$	237.0883				
Valsartan	$C_{24}H_{29}N_5O_3$	435.2270	[M+H]+	436.2343	11.2	$C_{14}H_{11}N_4^+$	235.0978	$C_{19}H_{19}N_2O^+$	291.1492	$C_{14}H_{11}N_2^+$	207.0917
Others											
Bromhexine	$C_{14}H_{20}Br_2N_2$	373.9993	[M+H]+	375.0066	10.6	$C_7H_6Br_2N^+$	261.8861	$C_7NH_{16}^+$	114.1277		
Chlorpromazine	$C_{17}H_{19}ClN_2S$	318.0957	[M+H]+	319.1030	10.8	$C_5H_{12}N^+$	86.0964	$C_{13}H_9ClNS^+$	246.0139		
Colchicine	$C_{22}H_{25}NO_6$	399.1682	[M+H]+	400.1755	10.3	$C_{20}H_{24}NO_5^+$	358.1649	$C_{20}H_{21}O_5^+$	341.1384	$C_{22}H_{24}NO_5^+$	382.1649
Coumaphos	$C_{14}H_{16}ClO_5PS$	362.0145	[M+H]+	363.0217	11.5	$C_{10}H_9ClO_5PS^+$	306.9591	$C_{12}H_{13}ClO_5PS^+$	334.9904	$C_{10}H_7ClO_2S^+$	226.9923
Melamine	$C_3H_6N_6$	126.0654	[M+H]+	127.0727	2.9	$C_2H_5N_4^+$	85.0509	$C_2H_2N_3^+$	68.0219		

4.3.2 Sample Preparation Optimization

Two different sample preparation procedures were followed for the extraction of veterinary drugs and pharmaceuticals from fish and milk samples. The first method involves a simple generic solid-liquid extraction step of the analytes from fish tissue with 0.1% formic acid in aqueous solution of EDTA 0.1% (w/v) – ACN – MeOH (1:1:1, v/v) and additional ultrasonic-assisted extraction. Precipitation of lipids and proteins was promoted by subjecting the extracts at very low temperature (-23 ºC) for 12 hours and further clean-up with hexane ensures fat removal from the matrix. The developed, optimization and validation of this method is described in the next chapter (Chapter 5). The second protocol consists of a generic extraction of the analytes from milk samples with a combination of ACN/5% aqueous TCA solution 5% (1:3, v/v). Further clean-up with SPE was performed to remove interfering proteins and provide clean and stable extracts. Recoveries were not taken into account in this study as the goal of the screening was to detect and identify the compounds, not their quantification.

Finding suitable extraction conditions for a large range of target analytes displaying different chemical properties (lipophilicity, hydrophilicity, alcaline and acidic characteristics, etc), along with keeping the procedure as short and simple as possible is a great challenge. Having a look at the literature in the field of veterinary drug residues analysis, it becomes apparent that acetonitrile extraction is the most common extraction route used for many veterinary drugs including antibiotics, anthelmintics and coccidiostats [64, 86, 76, 223]. Acidic extraction with trichloroacetic acid was found to be suitable for quinolones, lincomycin, and tetracyclines [56, 64, 87].

The same difficulties apply in the development of a generic solid-phase extraction procedure. If carefully designed, reversed phase SPE can recover most analytes from polar extraction solutions but may lead to losses of some (very polar) analytes [224, 90, 91]. Mixed-mode materials exhibiting both hydrophobic and ion-exchange properties have become a valuable alternative. One of the most widely used sorbent is a copolymer of divinylbenzene and vinylpyrrolidone, which has been commercialized under the trade name Oasis HLB by Waters. It has

become the prime sorbent for multi-residue methods of veterinary drugs and pharmaceuticals due to its broad-applicability spectra [89, 90, 91, 94, 224]. 60 mg and 200 mg cartridges of equal particle size HLB were assayed in order to determine optimum sorbent amount. Slightly better results were obtained with the 60 mg cartridge over the 200 mg cartridge, and the 60 mg size was chosen for further experiments.

The primary extracts had to be diluted in water in order to decrease the % content of ACN (<10%) in the sample that will be loaded in the SPE. This is a practice that many researchers employ in order to avoid breakthrough of analytes during the SPE step due to a moderate amount of ACN in the extracting solution [89, 91, 56].

Proper adjustment of sample pH may be necessary to avoid deprotonation of acidic compounds or protonation of basic compounds and to enhance extraction efficiency of the analytes [224]. As analytes belong to different classes with different pKa values, it is extremely difficult to establish a clean-up method that is effective for all the compounds. The extract pH was adjusted to 5.5 with ammonium hydroxide solution, according to Kaufmann et al. [90] who clearly states that this value constitutes a compromise and might not be the optimum for some very acidic and strongly basic analytes. Neutral pH values of the extract loaded in the SPE have been reported in cases where the extraction was held with water [89, 91] while a pH value of 4 was selected by Bohm et al.,[66, 74] where McIlvaine buffer was used as extraction solvent.

Finally, the effectiveness of MeOH and ACN as elution solvents was evaluated. When eluting with MeOH, higher recoveries of β-lactams and tetracyclines from the milk samples were achieved, since MeOH is a more polar solvent than ACN. On the contrary, less polar compounds, like some macrolides (tiamulin, tylosin) and some ionophore coccidiostats (semduramycin, narasin), presented better results when the elution was performed with ACN. However, MeOH was deemed to be the most suitable elution solvent, because it revealed satisfactory results for the majority of the target analytes.

4.3.3 Qualitative Method Validation

The aim of a qualitative method validation is to ensure the presence of an analyte in a sample at a certain concentration level. As no quantitation is necessary, method recovery, accuracy and precision are not considered [217]. Two different milk matrices (ovine and bovine) and two different fish matrices (sea bream and sea bass) were tested at method validation at two concentration levels: 20 and 200 μg kg^{-1} for fish samples and 15 and 150 ng mL^{-1} for milk samples. The method was considered fully validated for a given compound, at a certain concentration, when it was detected and identified according to the criteria established in all the samples tested (i.e. 6 out of 6 for each matrix). **Table 4.2** summarizes the SDLs and LOIs obtained for each analyte in milk and fish samples.

Table 4.2: Validation Results; Detection and identification limits in spiked milk and fish samples at two concentration levels; SDL and LOI obtained.

Positive/Negative results												
	Milk samples (n=12)						Fish Samples (n=12)					
	Detected		Identified				Detected		Identified			
Compounds	15 ng mL^{-1} (n=6)	150 ng mL^{-1} (n=6)	15 ng mL^{-1} (n=6)	150 ng mL^{-1} (n=6)	**SDL** (ng mL^{-1})	**LOI** (ng mL^{-1})	20 µg kg^{-1} (n=6)	200 µg kg^{-1} (n=6)	20 µg kg^{-1} (n=6)	200 µg kg^{-1} (n=6)	**SDL** (µg kg^{-1})	**LOI** (µg kg^{-1})
Quinolones												
Ciprofloxacin	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	6/6	200	200
Danofloxacin	6/6	6/6	6/6	6/6	15	15	3/6	6/6	3/6	6/6	200	200
Difloxacin	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	6/6	200	200
Enrofloxacin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Flumequine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Marbofloxacin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Norfloxacin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Ofloxacin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Oxolinic acid	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sarafloxacin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Tetracyclines												
Chlortetracycline	6/6	6/6	6/6	6/6	15	15	4/6	6/6	4/6	6/6	200	200
Doxycycline	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Minocycline	6/6	6/6	6/6	6/6	15	15	4/6	6/6	4/6	5/6	200	>200
Oxytetracycline	6/6	6/6	5/6	6/6	15	150	4/6	6/6	4/6	6/6	200	200
Tetracycline	6/6	6/6	6/6	6/6	15	15	4/6	6/6	4/6	6/6	200	200
Cefalosporines												

Cefaclor	0/6	0/6	0/6	0/6	>150	>150	0/6	3/6	0/6	2/6	>200	>200
Cefadroxil	3/6	6/6	1/6	6/6	150	150	0/6	6/6	0/6	6/6	200	200
Cefalexin	0/6	6/6	0/6	6/6	150	150	0/6	6/6	0/6	6/6	200	200
Cefapirin	1/6	0/6	0/6	0/6	>150	>150	0/6	0/6	0/6	0/6	>200	>200
Cefazolin	5/6	5/6	0/6	0/6	>150	>150	0/6	0/6	0/6	0/6	>200	>200
Ceftiofur	2/6	6/6	1/6	6/6	150	150	0/6	6/6	0/6	3/6	200	>200
Penicillins												
Amoxicillin	0/6	0/6	0/6	0/6	>150	>150	0/6	6/6	0/6	6/6	200	200
Ampicillin	3/6	0/6	2/6	0/6	>150	>150	0/6	6/6	0/6	5/6	200	>200
Cloxacillin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Dicloxacillin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Oxacillin	6/6	6/6	2/6	6/6	15	150	0/6	2/6	0/6	2/6	>200	>200
Penicillin G	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Penicillin V	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Macrolides												
Azithromycin	0/6	3/6	0/6	3/6	>150	>150	6/6	6/6	6/6	6/6	20	20
Clarithromycin	0/6	0/6	0/6	0/6	>150	>150	6/6	6/6	6/6	6/6	20	20
Erythromycin-OH2	2/6	6/6	2/6	6/6	150	150	6/6	6/6	6/6	6/6	20	20
Tiamulin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Tilmicosin	6/6	6/6	6/6	6/6	15	15	6/6	4/6	6/6	4/6	200	200
Sulfonamides												
Dapsone	5/6	6/6	5/6	6/6	150	150	1/6	6/6	1/6	6/6	200	200
Sulfachloropyridazine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfaclozine	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	6/6	200	200
Sulfadiazine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20

Sulfadimethoxine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfadimidine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfadoxine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfaguanidine	5/6	6/6	5/6	6/6	150	150	6/6	6/6	6/6	6/6	20	20
Sulfamerazine	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	6/6	200	200
Sulfameter	6/6	6/6	6/6	6/6	15	15	4/6	6/6	4/6	6/6	200	200
Sulfamethizole	6/6	6/6	6/6	6/6	15	15	2/6	6/6	2/6	6/6	200	200
Sulfamethoxazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfamethoxypyridazine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfamonomethoxine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfamoxole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfapyridine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfaquinoxaline	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfathiazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Sulfisoxazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Quinoxalines												
Carbadox	6/6	6/6	6/6	6/6	15	15	1/6	6/6	1/6	5/6	200	>200
Olaquindox	6/6	6/6	6/6	6/6	15	15	0/6	3/6	0/6	1/6	>200	>200
Amphenicols												
Florfenicol	6/6	6/6	6/6	6/6	15	15	0/6	6/6	0/6	6/6	200	200
Thiampenicol	6/6	6/6	4/6	2/6	15	>150	0/6	3/6	0/6	2/6	>200	>200
Diaminopyrimidines												
Baquiloprin	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	6/6	200	200
Trimethoprim	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Other antibiotics												

Lincomycin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Novobiocin	0/6	6/6	0/6	6/6	150	150	0/6	6/6	0/6	6/6	200	200
Rifaximin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Althenmintics												
Albendazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Albendazole oxide	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Albendazole sulfone	2/6	2/6	5/6	4/6	>150	>150	6/6	6/6	6/6	6/6	20	20
Dimetridazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Febantel	3/6	6/6	0/6	0/6	150	>150	0/6	0/6	0/6	0/6	>200	>200
Fenbendazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Flubendazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Levamisole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Mebendazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Metronidazol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Morantel	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Oxfendazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Piperazine	0/6	2/6	0/6	2/6	>150	>150	0/6	5/6	0/6	5/6	>200	>200
Ronidazole	6/6	6/6	6/6	6/6	15	15	0/6	4/6	0/6	3/6	>200	>200
Ternidazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Thiabendazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Triclabendazole	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Coccidiostats												
Arprinocid	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Clopidol	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	6/6	200	200
Decoquinate	2/6	6/6	2/6	5/6	150	>150	4/6	6/6	4/6	6/6	200	200

Diaveridine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Ethopabate	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Halofuginone	6/6	6/6	6/6	6/6	15	15	0/6	6/6	0/6	6/6	200	200
Imidocarb	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	4/6	200	>200
Lasalocid	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Monensin	6/6	6/6	6/6	6/6	15	15	0/6	1/6	0/6	1/6	>200	>200
Narasin	0/6	6/6	0/6	3/6	150	>150	0/6	6/6	0/6	6/6	200	200
Nigericin	2/6	6/6	0/6	0/6	150	>150	1/6	6/6	1/6	5/6	200	>200
Robenidine	0/6	6/6	0/6	6/6	150	150	5/6	5/6	5/6	5/6	>200	>200
Salinomycin	2/6	6/6	1/6	1/6	150	>150	1/6	6/6	1/6	3/6	200	>200
NSAIDs												
5-Hydroxy-flunixin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Aceclofenac	6/6	6/6	6/6	6/6	15	15	0/6	6/6	0/6	6/6	200	200
Diclofenac	0/6	6/6	0/6	6/6	150	150	6/6	6/6	6/6	6/6	20	20
Flunixin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Ketoprofen	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Mefenamic acid	5/6	6/6	4/6	6/6	150	150	3/6	6/6	3/6	6/6	200	200
Meloxicam	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Naproxen	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Niflumic acid	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Phenylbuntazone	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Tolfenamic acid	6/6	6/6	0/6	0/6	15	>150	0/6	5/6	0/6	0/6	>200	>200
Vedaprofen	4/6	6/6	3/6	5/6	150	>150	1/6	1/6	1/6	1/6	>200	>200
beta-agonists												
Cimaterol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20

Clenbuterol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Clenpenterol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Mabuterol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Ractopamine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Salbutamol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Terbutaline	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Steroids												
Betamethasone	6/6	6/6	2/6	6/6	15	150	2/6	6/6	2/6	6/6	200	200
Cortisol	6/6	6/6	6/6	6/6	15	15	0/6	6/6	0/6	6/6	200	200
Cortison	6/6	6/6	6/6	6/6	15	15	4/6	6/6	4/6	6/6	200	200
Dexamethazone	5/6	6/6	0/6	0/6	150	>150	3/6	5/6	3/6	0/6	>200	>200
Methylprednisolone	6/6	6/6	5/6	6/6	15	150	5/6	5/6	5/6	4/6	>200	>200
Progesteron	2/6	6/6	2/6	6/6	150	150	5/6	6/6	5/6	6/6	200	200
Thyreostats												
Methyl-thiouracil	0/6	2/6	0/6	2/6	>150	>150	5/6	6/6	5/6	6/6	200	200
Phenyl-thiouracil	3/6	6/6	3/6	6/6	150	150	6/6	6/6	6/6	6/6	20	20
Propyl-thiouracil	1/6	6/6	1/6	6/6	150	150	6/6	6/6	6/6	6/6	20	20
Pharmaceuticals												
Ambroxol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Atenolol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Atorvastatin	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Caffeine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Carbamazepine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Cimetidine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Gemfibrozil	6/6	6/6	6/6	6/6	15	15	4/6	6/6	4/6	6/6	200	200

Haloperidol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Indapamide	6/6	6/6	6/6	6/6	15	15	1/6	6/6	1/6	6/6	200	200
Metformin	6/6	6/6	6/6	6/6	15	15	1/6	6/6	1/6	6/6	200	200
Metoprolol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Paracetamol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Propranolol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Ranitidine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Simvastatin	6/6	6/6	6/6	6/6	15	15	5/6	6/6	5/6	6/6	200	200
Theophyline	6/6	6/6	6/6	6/6	15	15	0/6	6/6	0/6	6/6	200	200
Tramadol	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Triamterene	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Valsartan	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Others												
Bromhexine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Chlorpromazine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Colchicine	6/6	6/6	6/6	6/6	15	15	6/6	6/6	6/6	6/6	20	20
Coumaphos	1/6	6/6	0/6	6/6	150	150	6/6	6/6	6/6	6/6	20	20
Melamine	6/6	6/6	6/6	6/6	15	15	0/6	4/6	0/6	4/6	>200	>200

Milk presented slightly better SDL and LOI values in comparison to fish samples. This could be due to the highest pre-concentration achieved with the sample preparation protocol used for milk which succeeds to a significant increase in sensitivity. The more effective clean-up of milk samples, using SPE, could also lead to reduced matrix effects and increased sensitivity.

The majority of the compounds in both milk and fish samples were detected at the lowest concentration level (78% in milk samples and 60% in fish samples). What is of great significance is that, practically, the same number of compounds were also identified at this level: 74% in milk samples and 60% in fish samples. This comes to contrast with methods reported in literature where the number of identified compounds is drastically decreased at the low concentration level [101, 216, 217] indicating the wide effectiveness and applicability of the proposed methodology.

Overall, for milk samples, the reliable identification using two accurate-mass ions was feasible for 74% of compounds at 15 ng mL^{-1} and for 87 % at 150 ng mL^{-1}. For the remaining 13% of the compounds, LOIs are >150 ng mL^{-1} with this method. However, only 8% of the compounds presented SDLs >150 ng mL^{-1}. For fish samples, as mentioned above, 60% of the compounds were detected and identified at 20 μg kg^{-1}. SDLs for the 29% of the compounds and LOIs for the 24% were calculated at 200 μg kg^{-1} while 11% could not be detected and 16% could not be identified at the concentrations examined. The identification of the anthelmintic levamisole in a spiked milk sample (15 ng mL^{-1}) and in a spiked fish sample (20 μg kg^{-1}) is shown in **Figure 4.2**.

Two compounds (tolfenamic acid and febantel) presented poor or none fragmentation as a result of the collision energy value applied, which was the result of a compromise for all compounds. For these compounds no fragments could be obtained and, therefore, the screening method was limited for detection, since no identification could be performed. Cefaclor, amoxicillin and clarithromycin were not detected neither identified in any of the spiked milk samples while in fish, cefapirin, cefazolin and febantel could be neither detected nor identified at the levels tested. One reason is that some analytes are not ionized very effectively and their levels of detection, even for solvent standards,

are quite high. Many of the cephalosporin compounds fall into this category [103]. Another reason is that these compounds might not be stable under the extraction conditions assayed and that they are metabolized [225, 226] Matrix effects could also highly affect the determination.

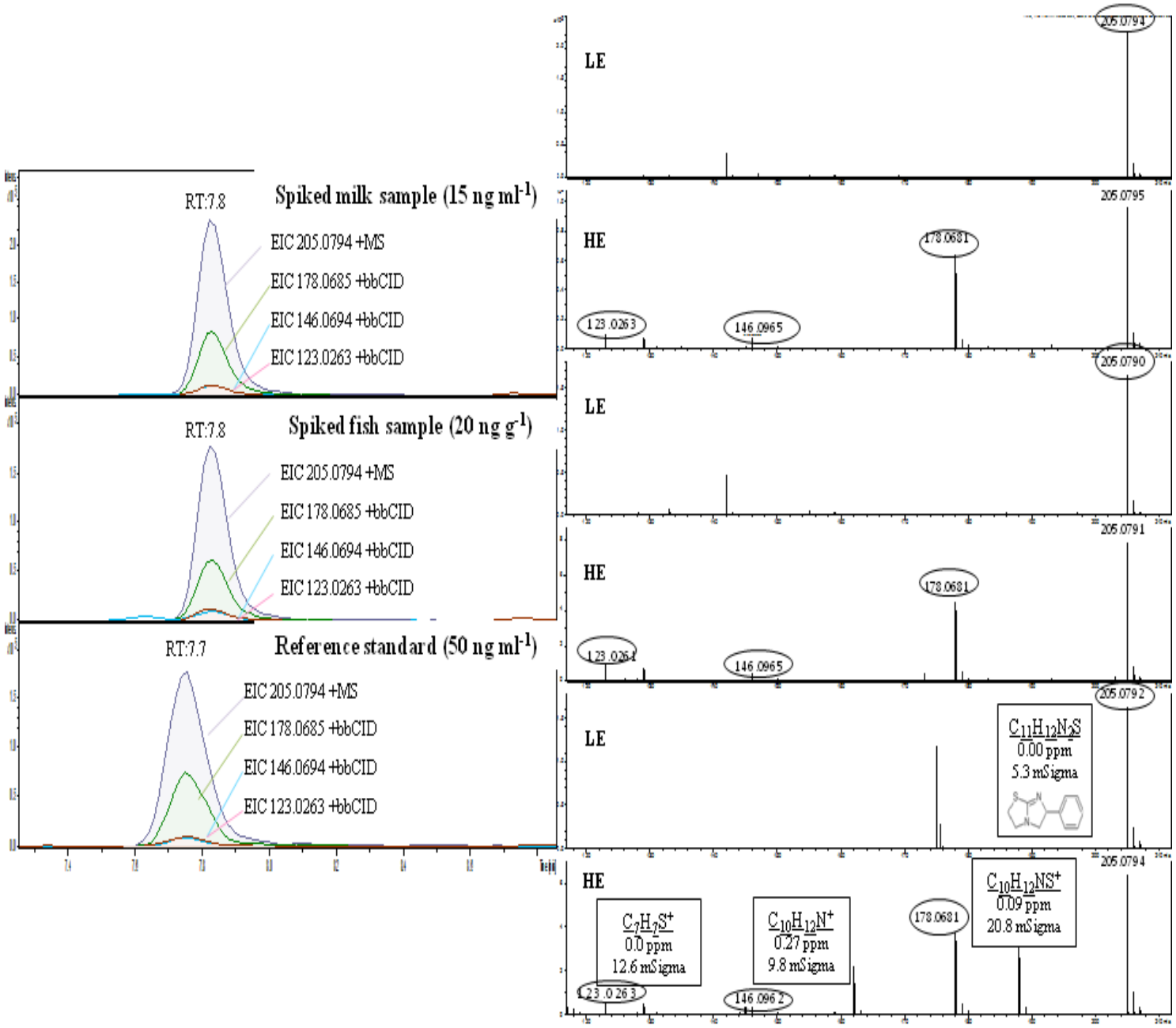


Figure 4.3: nw-XICs of Levamisole corresponding to the protonated molecule at LE and to its fragment ions at HE for 50 ng ml^{-1} reference standard, 15 ng ml^{-1} spiked milk sample and 20 ng g^{-1} spiked fish sample.

4.4 Application to milk and fish samples

To evaluate the applicability of the proposed method in routine analysis, 5 milk samples (4 bovine and 1 ovine) and 5 fish samples (3 sea bream and 2 sea bass) were tested. No positive results were found in any of these samples. The results reported by QTOF MS were confirmed by LC-MS/MS analysis in MRM mode, monitoring two transitions per compound, revealing the absence of any of the target analytes in the samples examined. This indicates the good selectivity of the proposed methodology which presented no false positive results.

4.5 Conclusions

The qualitative validation of a wide-scope screening method including 143 veterinary drugs and pharmaceuticals has been carried out in two types of milk and two types of fish samples. Using this method, data including retention times, as well as accurate mass measurements for precursor and product ions, have been collected for all analytes. Detection, based mainly on the presence of the protonated molecule, as well as identification using a second accurate-mass fragment ion, was feasible in most cases at the lowest level tested (20 μg kg^{-1} for fish samples and 15 ng mL^{-1} for milk samples). Finally, the applicability of the method was examined and no false positive results were revealed in blank samples.

CHAPTER 5

Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using Liquid Chromatography-Tandem Mass Spectrometry

5.1 Introduction

A simple, sensitive and efficient multi-residue and multi-class analytical method for the simultaneous determination of 115 veterinary drugs and pharmaceuticals in milk powder, butter, egg and fish tissue by HPLC–MS/MS has been developed.

The proposed methodology allows the simultaneous extraction of veterinary drugs and pharmaceuticals with very different physicochemical properties from various matrices, employing a simple solvent extraction with 0.1% formic acid in aqueous solution of EDTA 0.1% (w/v) – acetonitrile (ACN) – methanol (MeOH) (1:1:1, v/v) and further ultrasonic-assisted extraction. The extraction procedure was fully optimized in terms of recovery for the three out of four matrices examined. Two separate runs were performed for positive and negative ionization in multiple reaction monitoring mode (MRM) for the determination of all analytes. The method was validated in all four matrices and for over 80% of the analytes, the recoveries were between 50% and 120% in all matrices studied, with RSD values in the range of 1–18%. Limits of detection (LODs) and quantification (LOQs) ranged from 0.008 μg kg-1 (oxfendazole in butter) to 3.15 μg kg-1 (hydrochlorthiazide in egg). The evaluated method provides reliable screening, quantification, and identification of 115 veterinary drug and pharmaceutical residues in foods of animal origin and has been successfully applied in real samples.

This work was presented at the 5th Int. Symp. On Recent Advances in Food Analysis (RAFA 2011, Prague) winning a Poster Award sponsored by Agilent Technologies.

5.2 Experimental

5.2.1 Chemicals and reagents

All the analytes studied are presented in **Table S5.1.** All veterinary drug and pharmaceutical standards were of high purity grade (>90%) The vast majority of them were purchased from Sigma-Aldrich (Steinheim, Germany). Sulfadoxine (SDX) and sulfaclozine (SClZ) were donated by the National Laboratory of Residue Analysis of Food of Animal Origin of the Hellenic Ministry of Rural Development and Food. Halofuginone, bacitracin, arprinocid, salinomycin, semduramicin, carprofen, diclofenac, flunixin, mefenamic acid, meloxicam, niflumic acid as well as the internal standards flunixin-d3 and meloxicam-d3 were donated by the Veterinary Drug Residues Laboratory of the State General Laboratory of Cyprus.

Acetonitrile and methanol LC–MS grade were purchased from Merck (Darmstadt, Germany) while formic acid 99% and ammonium formate from Fluka (Buchs, Switzerland). Hexane (pesticide analysis grade, 95%) was purchased from Carlo Erba (Milan, Italy) and distilled water was provided by a MilliQ purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). The ethylenediaminetetraacetic acid disodium salt (EDTA) was of analytical grade and was purchased from Panreac. RC (Regenerated Cellulose) syringe filters (15 mm diameter, 0.2 μm pore size) were provided from Phenomenex (Torrance, CA, USA)

About 10 mg of each individual standard was accurately weighed and placed in a 10-mL volumetric flask. Penicillins and cefalosporines were dissolved in MilliQ-water while all other analytes in methanol. In quinolone standard solutions, 100 μL of formic acid were added to enhance solubility. Stock solutions of 1000 μg ml-1 of each compound were obtained and stored at −20 °C in brown glass to prevent the photodegradation. Four intermediate standard solutions containing several analytes grouped according to their classification and stability were prepared by dilution of the stock solutions with methanol. The final concentration of these multi-component solutions was 10 μg ml-1 and they were also stored at −20 °C. New ones were prepared every month.

All working solutions and calibration standards were obtained by gradient dilution of the intermediate solutions, in concentrations varying from 1 μg mL^{-1} to 1 ng mL^{-1}. Working standard solution of internal standards in a concentration of 1 μg mL^{-1} came by subsequent dilutions of their stock solutions in methanol. While not in use, the working solutions were kept at −20 °C and renewed weekly.

5.2.2 LC–MS/MS analysis

A Thermo UHPLC Accela system was connected to a Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument (Thermo, San Jose, CA, USA). An Atlantis T3 C18 (100 mm x 2.1 mm, 3 μm, Waters) column protected by a guard column was used at a constant flow rate of 100 μL min^{-1}. Two chromatographic runs were performed in order to determine all analytes in each sample, one in positive ionization mode and one in negative. The mobile phase for the positive mode detection consisted of water containing 0.01% formic acid (v/v) (solvent A) and methanol (solvent B), while for the negative mode detection was modified water (1 mM ammonium formate, A), MeOH (B) and ACN (C)).

The gradient elution programs for both runs are presented in **Tables S5.2** and **S5.3**. The column was thermostated at 30 °C and the full loop injection volume of the extract was set at 10 μL.

As far as the MS parameters are concerned, the mass spectra and the optimum collision energy and tube lens value were obtained for each compound separately by direct infusion of individual standard solutions at concentration of 1 μg mL^{-1} in formic acid : MeOH (75:25, v/v) or ammonium formate : MeOH (75:25, v/v), depending on whether the determination is performed in a positive or a negative ionization mode. The ESI parameters (Spray Voltage, Seath Gas, Auxiliary Gas, Capillary temperature) for each determination are also presented in **Tables S5.2** and **S5.3**.

Multiple reaction monitoring (MRM) was used and detailed parameters for MRM acquisition are presented in **Table 5.1**. Two transitions were selected for identification, but only the most intense one was used for quantification.

Table 5.1: MRM parameters and retention times for all compounds determined.

Compound	ESI	Pseudo Molecular Ion	Product Ion 1	Collision Energy (eV)	Product Ion 2	Collision Energy (eV)	Tube Lens	RT (SD, n=10) (min)
Quinolones								
Ciprofloxacin	+	332	288	18	314	22	85	7.715 (0.029)
Danofloxacin	+	358	96	25	314	20	85	7.776 (0.038)
Difloxacin	+	400	356	20	299	27	85	8.029 (0.022)
Enrofloxacin	+	360	245	25	317	20	85	7.761 (0.032)
Flumequine	+	262	244	20	202	30	85	11.963 (0.024)
Marbofloxacin	+	363	320	15	72	20	85	7.164 (0.025)
Norfloxacin	+	320	276	16	233	23	91	7.613 (0.034)
Ofloxacin	+	362	318	19	261	27	120	7.430 (0.032)
Oxolinic acid	+	262	244	18	158	31	79	10.708 (0.029)
Sarafloxacin	+	386	342	18	299	27	85	8.096 (0.039)
Ciprofloxacin	+	332	288	18	314	22	85	7.715 (0.029)
Tetracyclines								
Chlortetracycline	+	479	444	20	462	15	90	9.407 (0.045)
Doxycycline	+	445	427	19	267	35	90	10.048 (0.034)
Oxytetracycline	+	461	426	19	443	12	90	8.412 (0.018)
Tetracycline	+	445	410	18	426	12	90	8.181 (0.030)
Cefalosporines								
Cefaclor	+	368	174	14	118	32	81	8.070 (0.042)
Cefadroxil	+	364	114	19	134	29	97	5.929 (0.057)
Penicillins								
Amoxicillin	+	366	349	8	114	22	68	5.749 (0.096)

Ampicillin	+	350	106	20	160	12	87	8.170 (0.029)
Macrolides								
Azithromycin	+	750	591	29	158	37	127	8.378 (0.035)
Clarithromycin	+	749	158	30	590	20	123	11.067 (0.039)
Erythromycin	+	734	158	30	576	20	130	10.381 (0.032)
Tiamullin	+	494	192	21	119	33	101	10.152 (0.031)
Tilmicosin	+	869	174	42	156	44	165	8.892 (0.045)
Tylosin	+	917	174	36	772	28	148	10.252 (0.035)
Sulfonamides								
Sulfaclozine (SClZ)	+	285	92	28	156	15	87	10.072 (0.024)
Sulfachloropyridazine (SCP)	+	285	92	28	156	14	87	8.963 (0.029)
Sulfadimidine (SDD)	+	279	186	17	124	26	87	8.751 (0.029)
Sulfadimethoxine (SDM)	+	311	156	17	108	29	87	10.130 (0.017)
Suladoxine (SDX)	+	311	156	17	108	27	87	9.230 (0.020)
Sulfadiazine (SDZ)	+	251	156	15	92	27	87	7.444 (0.026)
Sulfaguanidine (SGN)	+	215	156	14	92	14	87	4.164 (0.087)
Sulfisoxazole (SIX)	+	268	156	13	92	27	87	8.158 (0.017)
Sulfamonomethoxine (SMM)	+	281	156	13	92	29	87	8.615 (0.028)
Sulfamethoxypyridazine (SMP)	+	281	156	13	92	29	87	9.176 (0.018)
Sulfamerazine (SMR)	+	265	156	16	172	16	87	8.132 (0.032)
Sulfamethizole (SMT)	+	271	156	14	92	28	87	8.340 (0.012)
Sulfamethoxazole (SMTX)	+	254	156	16	108	25	87	8.970 (0.018)
Sulfamoxole (SMX)	+	268	156	13	92	28	87	9.162 (0.024)
Sulfapyridine (SPD)	+	250	156	15	184	17	87	7.623 (0.028)
Sulfaquinoxaline (SQX)	+	301	156	18	92	30	87	10.370 (0.030)
Sulfathiazole (STZ)	+	256	156	15	92	26	87	7.347 (0.031)

Dapsone	+	249	156	14	108	22	79	8.242 (0.033)
Other antibiotics								
Bacitracin	+	712	199	42	355.5	29	113	9.968 (0.069)
Lincomycin	+	407	126	30	359	17	99	7.027 (0.041)
Novobiocin	+	613	189	31	396	14	108	16.526 (0.050)
Rifaximin	+	786	754	22	361.5	32	114	13.973 (0.027)
Trimethoprim	+	291	230	25	123	30	87	7.059 (0.034)
Benzimidazoles								
Albendazole	+	266	191	31	234	29	85	13.216 (0.028)
Albendazole sulfone	+	298	159	35	266	19	74	10.485 (0.029)
Febantel	+	447	383	17	280	31	110	14.402 (0.017)
Fenbendazole	+	300	268	29	159	33	85	14.019 (0.017)
Flubendazole	+	314	282	31	123	35	90	12.771 (0.028)
Mebendazole	+	296	264	31	105	35	90	12.493 (0.017)
Oxfendazole	+	316	159	30	191	24	87	11.113 (0.024)
Thiabendazole	+	202	131	35	175	35	87	9.121 (0.021)
Triclabendazole	+	359	274	35	171	40	85	16.100 (0.079)
Nitroimidazoles								
Dimetridazole	+	142	96	18	54	30	80	8.230 (0.033)
Metronidazole	+	172	128	13	82	25	69	7.394 (0.028)
Ternidazole	+	186	128	15	82	28	75	8.371 (0.021)
Ronidazole	+	201	140	10	55.5	21	73	7.273 (0.045)
Coccidiostats								
Aprinocid	+	278	143	28	107	48	95	10.927 (0.018)
Clopidol	+	192	101	27	87	31	99	8.628 (0.015)
Diaveridine	+	261	245	26	123	26	93	6.865 (0.025)

Ethopabate	+	238	206	11	136	27	37	11.291 (0.033)
Halofuginone	+	416	100	26	120	23	73	9.532 (0.045)
Monensin	+	693	461	43	479	51	159	21.119 (0.031)
Salinomycin	+	773	431	48	531	37	138	23.682 (0.073)
Semduramycin	+	895	833	28	851.5	30	116	19.758 (0.053)
Other Anthelmintics								
Levamisol	+	205	178	29	123	30	87	6.501 (0.045)
Morantel	+	221	111	20	177	29	84	7.961 (0.033)
Nitroxinil	-	289	127	34	162	28	87	9.134 (0.032)
Oxyclozanide	-	398	362	25	176	34	79	13.939 (0.065)
NSAIDs								
Carprofen[a]	-	272	226	28	228	23	85	12.856 (0.041)
Diclofenac	+	296	215	19	250	12	72	14.942 (0.031)
Flunixin[a]	-	295	251	15	209	30	85	11.793 (0.032)
Ibuprofen[a]	-	205	161	10	-	-	65	13.253 (0.069)
Ketoprofen	+	255	209	13	105	23	106	13.063 (0.044)
Mefenamic Acid[a]	-	240	196	19	191	26	78	13.491 (0.069)
Meloxicam[b]	-	350	286	16	146	23	67	9.746 (0.040)
Naproxen[b]	-	229	169	10	-	-	76	11.073 (0.047)
Niflumic acid	+	283	265	22	245	28	97	16.485 (0.077)
Salicylic acid	-	137	93	18	65	32	45	5.369 (0.029)
Tolfenamic acid[b]	-	260	216	18	180	24	74	6.226 (0.030)
Amphenicols								
Chloramphenicol	-	321	257	13	152	19	90	9.241 (0.023)
Florfenicol	-	356	336	11	185	18	90	7.684 (0.023)
Thiamphenicol	-	354	290	11	185	19	90	8.176 (0.051)

beta-agonists								
Clenbuterol	+	277	203	26	259	10	82	8.044 (0.032)
Beta-blockers								
Atenolol	+	267	145	26	190	18	94	5.701 (0.032)
Metropolol	+	268	191	17	133	25	96	8.212 (0.034)
Propanolol	+	260	183	19	155	25	99	9.459 (0.031)
Steroids								
Betamethasone Acetate	+	435	415	6	397	10	90	13.365 (0.027)
Cortisol	+	363	309	16	269	21	102	12.267 (0.029)
Cortison	+	361	163	23	145	24	102	11.839 (0.031)
Dexamethasone	+	393	319	17	3369	18	124	13.385 (0.041)
Methylprednisolone	+	375	339	8	357	10	71	12.967 (0.059)
Prednisolone	+	361	343	10	147	24	98	12.210 (0.039)
Progesteron	+	315	109	28	97	24	79	15.361 (0.062)
Analgetics								
Caffeine	+	195	138	18	110	22	87	8.643 (0.018)
Paracetamol	+	152	93	22	110	15	84	7.232 (0.029)
Tramadol	+	264	58	15	246	8	66	8.168 (0.044)
Diuretics								
Furosemide	-	329	285	17	205	25	58	8.176 (0.051)
Hydrochlorthiazide	-	296	269	19	205	22	139	4.977 (0.074)
Indapamine	-	364	189	28	233	21	72	10.426 (0.036)
Triamterene	+	254	237	26	104	36	93	7.945 (0.035)
Statins								
Atorvastatin	+	559	440	22	250	42	123	13.869 (0.031)

Antiepileptic drugs								
Carbamazepine	+	237	194	19	193	32	114	12.027 (0.024)
Antiulcer Drugs								
Cimetidine	+	253	159	13	95	29	73	5.860 (0.055)
Omeprazole	+	330	182	23	149	25	90	11.275 (0.028)
Ranitidine	+	315	176	17	102	31	79	5.724 (0.046)
Fibrates								
Clofibric acid	-	213	127	17	85.4	12	64	10.031 (0.018)
Gemfibrozil	-	249	121	19			83	15.611 (0.092)
Others								
Colchicine	+	400	310	26	326	24	110	10.914 (0.035)
Coumaphos	+	363	227	26	307	17	105	15.134 (0.026)
Theophylline	+	181	124	17	96	22	79	8.023 (0.028)
Valsatran	+	436	207	28	291	16	99	14.420 (0.083)
Internal Standards (IS)								
Flunixin – d3	-	298	254	15			85	11.851 (0.032)
Meloxicam – d3	-	353	149	23			101	9.769 (0.028)

Each chromatographic run was divided into several scan events with a scan time of 20 ms for each transition. LC–MS/MS chromatogram of a spiked egg sample, at a concentration of 100 ng mL^{-1} for 115 target compounds, is presented in **Fig. S5.1**. Instrument control and data acquisition were carried out by using the Xcalibur software, Version 2.3, from Thermo.

5.2.3 Samples and quality control materials

Seventy three (73) milk powder, five (5) butter, twenty two (22) fish (gilthead sea bream and sea bass) and eight (8) egg samples were obtained from several local markets. Upon arrival at the laboratory, milk, butter and egg samples were stored at 4 °C. Fish samples were partially thawed at room temperature and muscle tissue was taken, homogenized and stored at -20 °C until analysis. Whole egg samples (albumen and yolk) were gently homogenized before analysis at room temperature under continuous agitation for 5 min.

One sample of each matrix was repeatedly measured to confirm that no veterinary drugs or pharmaceuticals were present and was used for the preparation of matrix-matched calibration standards and fortified samples for the validation of the method.

Spiked samples were prepared by adding the proper amount (from 10 to 200 μL) of a working solution containing all the analytes at the suitable concentrations, to each 1-g portion of the weighed samples. 50 μL of the Internal Standard working solution were added at each sample to achieve a final concentration of 50 μg kg-1 for each IS. For the evaluation of the different extraction procedures, blank samples were spiked at 100 μg kg^{-1}. Afterwards, there was a waiting period of 15 min for equilibration before starting the extraction step. Blank control samples were extracted and run with each analytical run/batch.

Since there is lack of certified test materials in the examined matrices, and in order to establish method accuracy, two Progetto Trieste (Trieste – Italy) test materials, MI1320 and MI1321, of lyophilized bovine milk containing a certified amount of sulfonamides, tetracyclines and quinolones, were used.

5.2.4 Sample Preparation

A 1-g portion of each properly homogenized sample was weighed and placed into a 15 mL polypropylene centrifuge tube. Afterwards, spiking of the samples with appropriate volumes of the working standard mix solutions (target compounds and IS) was performed. As mentioned above (paragraph 2.3), the blank samples fortified with the target compounds were used during the optimization and validation of the developed procedure. All spiked samples were allowed to stand for 10‑15 minutes before proceeding.

To extract the drug residues and precipitate the proteins, 2 mL of H_2O containing 0.1 % formic acid (v/v) and 0.1 % EDTA (w/v) were added to the samples and subsequently 2 mL of MeOH and 2 mL of ACN. The addition of this chelating agent improves the extraction recovery of some antibiotics, especially of tetracyclines, as it prevents their rapid chelation with metal ions [85]. After the addition of each solvent the tube was vortex-mixed for 30 sec. The sample set was placed in an ultrasonic bath at 60 ºC for 20 min in order an ultrasonic-assisted extraction of the veterinary drugs and pharmaceuticals from the matrix to take place.

Thereafter, the samples were centrifuged at 4000 rpm for 10 min and the supernatant was decanted into a new polypropylene centrifuge tube. The tubes were placed in -20 °C for 12 h in order to precipitate the lipids and remaining proteins. The samples were again centrifuged, the precipitate was thrown away and the supernant was transferred in another tube were the extracts were defatted using 5 mL of hexane, vortexed for 1 min, and then centrifuged for 10 min 4000 rpm. The hexane layer was aspirated to waste and the final extracts were evaporated to dryness under a nitrogen stream at a temperature not exceeding 45°C. The resulting residues were reconstituted in 1 mL of methanol/aqueous solution of formic acid, 0.05% (25:75 v/v) and then filtered through a 0.22-μm RC filter. Appropriate volumes of working multi-analyte solutions were added to blank aliquots at this step, to prepare the range of matrix-matched standards required. After vortex-mixing for 10 s, each extract was then transferred into a vial, and 10 μL was injected into the LC-MS/MS system.

5.2.5 Method validation

An in-house validation protocol was carried out, taking into consideration the requirements outlined in Commission Decision 2002/657/EC, in order to establish the performance characteristics of the method, ensuring the adequate identification, confirmation and quantification of the target compounds.

Identification and confirmation of the analytes were carried out by retention times, identification points of each analyte as required by the EU validation criteria, and relative ion ratio of selected MRM transitions. For each compound, the MRM transition with the highest intensity was used for quantification (quantifier), while the other transition was used for confirmation (qualifier).

The selected solvent extraction procedure was validated in terms of selectivity, linearity, trueness, repeatability, inter-day precision, limits of detection (LODs) and limits of quantification (LOQs). These validation parameters were evaluated in all four matrices examined in this study (milk powder, butter, fish tissue and egg). The use of internal standards was only feasible for some NSAIDs (carprofen, flunixin, ibuprofen, mefenamic acid, meloxicam, naproxen and tolfenamic acid) since it was the only group of analytes for which isotopic labeled internal standards were available (flunixin-d3 and meloxicam-d3). The choice of the adequate IS was made based on the retention time of the analytes. Flunixin-d3 was used in the quantification of carprofen, flunixin, ibuprofen and mefenamic acid while meloxicam-d3 meloxicam, naproxen and tolfenamic acid.

Because the aim of the study was the simultaneous quantification of target compounds at the lowest achievable level, no focus was put on the decision limit, CCα, and the detection capability, CCβ, which are parameters for compliance analysis by authorities [40].

5.2.5.1 Instrument performance

Calibration curves in pure solvent were constructed for all compounds by plotting the peak area against the concentration of the eight corresponding calibration standards (1 to 200 ng mL^{-1}). The calibration curves for NSAIDs were constructed by calculating the ratio of each peak area relative to the

corresponding IS. The linearity of the LC-MS/MS method was evaluated assessing the regression coefficient measured for each analyte. Concentrations were measured five times for each level (n=5). Instrumental LODs were calculated as 3.3 times the standard deviation (SD) of the peak area (or ratio of peak areas with the IS) of the analyte in the five replicates of the lowest concentration standard solution for each compound (0.1, 0.5, 2 or 10 ng mL^{-1}) divided by the slope of the calibration curve. LOQs were calculated as 10 times the SD divided by the slope.

5.2.5.2 Method performance

The selectivity and specificity was assessed by analyzing 10 blank samples from each matrix. The absence of background peaks, above a signal-to-noise ratio of 3, at the retention times of the target compounds showed that the method is free of endogenous interferences.

Standard solution calibration curves in matrix extracts were obtained by addition of the target compounds in blank milk powder, butter, fish and egg extracts in different concentrations (1 to 200 ng mL^{-1}, eight concentration levels). The matrix-matched calibration curve (samples spiked before the extraction) in milk powder was developed by fortifying eight 1 g fractions of blank material with the appropriate volumes from two multi-analyte working solutions of 0.1 µg mL^{-1} and 1 µg mL^{-1} (10 µL to 200 µL, 1 to 200 µg kg^{-1}). The exact same fortification was performed in eight 1 g fractions of blank butter, fish tissue and egg.

In order to evaluate the trueness of the method, recovery studies were carried out. Overall recoveries for all compounds in each matrix were calculated by dividing the matrix-matched calibration curve slope (samples spiked before the extraction) to the slope of the standard solution calibration curve in matrix extracts. Absolute recoveries were determined by comparing samples spiked at the VL (Validation Level, 100 µg kg^{-1}) before and after the extraction. In addition to recovery studies, trueness of the method was assessed by analyzing proficiency test materials of lyophilized bovine milk for 19 target compounds belonging in 3 different groups and having different physicochemical properties. The z-score assigned to each laboratory was calculated from the equation:

$$Z\text{ - score} = (x - X) / \sigma \quad (1)$$

x is the analyte concentration value reported by the laboratory, X is the assigned value and σ is the target value for standard deviation calculated from bX.

$$b = \%RSD / 100 \quad (2)$$

RSD is the relative standard deviation value coming from Horwitz equation [227]

$$\%RSD = 2^{(1-0.5 \log X)} \quad (3)$$

and X is expressed as a dimensionless concentration.

The within-day and between-day precision (repeatability and reproducibility, respectively) are expressed as RSD% and were evaluated by spiking six blank samples (n=6) at the VL for all four matrices. The determination of reproducibility was carried out on three different days.

For the calculation of the method's LODs and LOQs fortification of six blank samples of each matrix was performed in very low concentration of analytes. The SD of the peak area of the six replicates (or the peak area ratio for NSAIDs) is calculated in the lowest concentration that every analyte is determined in each matrix (0.1, 0.5, 2 or 10 μg kg^{-1}). 3.3 times the SD divided to the slope of the matrix-matched calibration curve provides method's LOD for each analyte in each matrix while 10 times this ratio provides the method's LOQs.

Finally, the matrix effect was studied by evaluating the ionic suppression and enhancement effects, comparing standard solution calibration curves for all analytes prepared in solvent and in each matrix, separately.

5.3 Results and Discussion

5.3.1 LC-ESI-MS/MS determination

The aim of the development of this method was to determine a high number of substances with sufficient separation on the column and high sensitivity for the mass spectrometric measurement.

At first, the selection and tuning of the precursor and product ions were carried out. Direct infusion of individual veterinary drugs and pharmaceutical solutions at

concentration of 1 µg mL^{-1} in formic acid 0.1%: MeOH (75:25, v/v) or ammonium formate 1 mM: MeOH (75:25, v/v) was performed, depending on if the determination of the analyte is achieved in positive or negative ionization mode, respectively. The mass spectra for all analytes were obtained along with analyte dependent parameters, such as collision energy and tube lens, which were optimized and calculated automatically. For each compound, the MRM transition with the highest intensity was used for quantification (quantifier), while the other transition was used for confirmation (qualifier). A quantitative data processing method was established using the most abundant SRM transition for each residue. **Table 5.1** gives the specific MS/MS parameters and the retention time of all target drugs in the study.

The protonated ($[M+H]^+$) or deprotonated ($M-H]^-$) molecular ions were selected as the precursor ions for the majority of the compounds. The exceptions were the antibiotic bacitracin, where the doubly charged molecule was used as the precursor ion, the anti-ulcer drug omeprazole which subjects to a loss of $-CH_3$ and the coccidiostats monensin, salinomycin and semduramycin for which the sodium adducts were chosen for analysis since they are thought to give the most reproducible results [228].

Electrospray parameters, such as seath gas, auxiliary gas, spray voltage and capillary temperature, were studied. The optimization was performed using flow injection analysis (FIA) with the carrier solution being the analysis' mobile phase in different proportions of aqueous/organic solvent. MS parameters were optimized in both positive and negative ionization modes with variation of a single setting at a time and evaluation of the target compounds' sensitivity. The optimum ESI parameters that were chosen for positive and negative ionization determination are shown in **Tables S5.2** and **S5.3.**

The chromatographic analysis was based on a LC–MS/MS methodology. Separation was performed in a reversed phase Atlantis T3 C18 (100 mm x 2.1 mm, 3 µm, Waters) column and the mobile phases chosen were aqueous formic acid 0.01% (v/v) and methanol (ESI^+) and 1 mM ammonium formate in water, MeOH and ACN (ESI^-).

Due to the very different nature of the analytes, a gradient program from 90% of aqueous phase to 100% methanol was performed in ESI+ chromatographic analysis in order to elute the analytes in a reasonably short time. In ESI- the gradient program starts from 70% aqueous phase to 100 % MeOH and the retention times range from 5.4 min (salicylic acid) to 15.6 (gemfibrozil). In positive mode determination all analytes were separated and eluted from 4.2 min (sulfaguanidine) to 23.7 min (salinomycin) with the ionophore coccidiostats monensin, semduramycin and salinomycin being the most strongly retained compounds in the analytical column and having, consequently, the highest retention times.

Besides eluting the strongly retained compounds, it is essential to raise the content of organic solvent during the gradient profile to avoid an increase of the contamination of the column due to poorly eluting matrix constituents such as lipids at low concentrations of organic solvent [67]. Furthermore, in the first 3 min of the analysis major interferences are presented due to polar matrix constituents that are eluted and co-elution with the analytes would lead to large signal suppression. Therefore, it was essential that the target analytes began to elute after the third minute of the analysis.

Despite the large number of targeted analytes, complete separation of compounds with mass transitions in common was achieved. Seven sets of compounds, azithromycin/clarithromycin, oxolinic acid/flumequine, tetracycline/doxycycline, sulfamethoxypyridazine/sulfamonomethoxine, sulfachloropyridazine/sulfaclozine, sulfadoxine/sulfadimethoxine and sulfamoxole/sulfisoxazole have similar pseudomolecular ions at 749, 262, 445, 281, 285, 311 and 268, respectively. However, these compounds can be easily distinguished on the basis of retention time indicating the excellent specificity of the developed method. Chromatograms of the veterinary drugs and pharmaceuticals obtained for a spiked egg sample with all compounds at the validation levels (VL) are shown in **Figure S5.1**. The long, broad tailing of chlortetracycline's and doxycycline's chromatographic peaks has been attributed to both tautomerization and epimerization conversion processes occurring in the LC column, very likely catalyzed by residual silanol groups [77].

Finally, another parameter that affects the chromatographic analysis and needs to be taken into consideration is the composition of the re-constitution solvent of the extract after the evaporation step. The high content of water (about 90%) in the final extract is preferred for the hydrophilic analytes while the high content of organic solvent (about 50%) is more suitable for the hydrophobic analytes [86]. In the present study the re-constitution solvent consisted of methanol/aqueous solution of formic acid, 0.05% (25:75 v/v) and this choice was made after an extensive optimization performed for most of the analytes.

5.3.2 Sample Preparation Optimization

Sample preparation is the process which includes the isolation and/or preconcentration of compounds of interest from various matrices, the removal of any matrix interferences that may affect the detection system as well as making the analytes more suitable for separation and detection. Even with the advances in the development of highly efficient analytical instrumentation for their final determination, sample preparation is a vital part of the analytical procedure and effective sample preparation is essential for obtaining accurate quantitative results and maintaining instrument performance. A typical sample preparation technique consists of an extraction step of the antibacterials from the matrix and a subsequent purification step of the extract [13].

To obtain optimal results, the extraction solvent has to be selected in such way that efficient extraction of the target compounds is obtained, whereas the extraction of matrix constituents remains limited in order to prevent excessive matrix effects (ME). The selection of the solvent therefore depends not only on the target compounds, but also on the matrix.

Simple extraction with aqueous buffers (e.g. McIlvaine buffer or succinate buffer) is advantageous for highly polar residues because, except from the extraction efficiency they also reduce co-extraction of non-polar matrix components (e.g. lipids). However, strongly protein-bound residues are not fully extracted and polar matrix components are co-extracted. Complexing agents are reported to be essential for the extraction of tetracyclines and some macrolides, because these

compounds have a strong tendency to form chelates with divalent metallic cations present in food samples [85].

In general, the majority of methods employ more efficient organic solvents as extracting agents. Methanol (MeOH) and acetonitrile (ACN) are more adequate as extraction solvents as they can simultaneously precipitate the proteins, denature enzymes and extract the target analytes. Many authors, as it is shown by the collected applications presented in **Table 1.2**, prefer ACN over MeOH as extraction solvent. Morever, MeOH extracts too many matrix compounds, complicating the following clean-up steps and ACN does not sufficiently extract polar analytes [13].

A number of multi-residue analytical methods developed use a combination of water or aqueous buffer and organic solvent as the extraction mixture of the target compounds from the matrix (see Table 1). In this work a combination of 0.1% formic acid in aqueous solution of EDTA 0.1% (w/v), acetonitrile (ACN) and methanol (MeOH) was used as the extraction solvent and various sample-to-extracting volume ratios were tested. An organic solvent content of 50% and 67% was tested in the first experiment with the aqueous solvent being 1% formic acid (v/v) in 0.1% EDTA solution (w/v). As it was expected, the higher content of aqueous solvent promoted the extraction of rather polar compounds like b-lactams, quinolones and tetracyclines (log Kow <2) but more hydrophobic compounds like NSAIDs, benzimidazoles, coccidiostats and some anthelmintcs (morantel, nitroxinil, oxyclozanide) were not satisfactorily recovered. In addition, the higher content of water would make the evaporation step more time-consuming. Consequently, the 67% of organic solvent content was selected, which corresponds to a proportion 1:1:1 of the three solvents of choice. All extraction optimization results for butter, egg and milk powder are schematically presented in **Figures S5.2**, **S5.3** and **S5.4**.

The overall volume of the extraction solvent was then optimized, evaluating the difference between 6 mL (2 mL of aqueous solution of EDTA 0.1% (w/v), 2 mL of ACN and 2 mL of MeOH) and 9 mL (3 mL of each). Although a slight improvement of the recoveries was observed when increasing the volume of the extraction solvent, it was not significant enough to compensate for the higher

amount of solvents used and the raise of the time needed in the evaporation step. These factors would increase the cost and decrease the rapidity of the developed method and so a final volume of 6 mL of the extraction solvent was chosen.

Afterwards, the % content of the EDTA solution in formic acid was studied. Since some of the target analytes have amphoteric characteristics, we expected the extraction yield to be significantly affected by the pH value, and indeed it turned out to be a significant factor. Aqueous EDTA solutions (0.1% w/v) with 1%, 0.5% and 0.1% were tested and the results, in terms of recovery, were evaluated. Coming in accordance to previous reported studies, quinolones and tetracyclines presented higher recoveries when extraction was performed in most acidic conditions (1% formic acid) [64, 76, 87, 229]. Conversely, other veterinary drugs like sulfonamides, macrolides and NSAIDs provided the best recovery results when extracted with 0.1% formic acid [64, 76, 87, 230].

After the solid-liquid extraction with the final combination of extraction solvents, an additional ultrasonic-assisted extraction was performed in order to obtain higher recovery yields. Ultrasound can be considered as a useful alternative for solid sample pretreatment because the energy imparted facilitates and accelerates some steps, such as dissolution, fusion, and leaching, among others [231]. In milk powder samples, the ultrasonic extraction was held in three different temperatures, 30 ºC, 50 ºC and 60 ºC in order to investigate the relation between extraction temperature and recovery of the analytes. For 102 out of 115 compounds the recovery value was increased when the extraction temperature was set > 30 ºC, with the 72 of them presenting best results when the temperature was set at 60 ºC (**Figure S5.4**). Therefore, the temperature of choice for the extraction was 60 ºC.

After ultrasonic – assisted extraction the samples were subjected in low-temprature clean up. Low temperature clean up is a rather recently reported technique that amplifies the precipitation of proteins and fat from the sample [86, 232]. In an effort to obtain even more clear extracts further defatting with hexane followed.

Finally, a preconcentration step was evaluated, in order to improve the sensitivity of the analytical method. To carry out this step, the extract was evaporated under a stream of nitrogen and the final residue was dissolved in 0.5 mL of a mixture of methanol/aqueous solution of formic acid, 0.05% (25:75 v/v). However, the result was more turbid samples and lower recoveries of the analytes due to the higher amount of matrix components which provoke severe matrix effects and interfere in the detection of analytes in the ESI source.

Recovery values for the majority of the target compounds were satisfactory (>50%). The NSAIDs carprofen, diclofenac, mefenamic acid and tolfenamic acid presented low extraction recoveries in milk, with the two latter compounds having recovery values <50% also in butter. This comes as a verification of the fact that not always satisfactory correction occurs when analogue ILIS are used. Flunixin-d3, used as the ILIS for carprofen and mefenamic acid, and meloxicam-d3, used for tolfenamic acid, did not manage to compensate for the recovery losses. Since NSAIDs are compounds that bind in proteins, low recoveries in some cases could come as a result for the lack of a hydrolysis step in the sample preparation [233]. Low recovery values for acidic NSAIDs in milk have been previously reported [92].

The anthelmintic oxyclozanide, the antiparasitic drug coumaphos and the fibrate gemfibrozil have shown limited extraction from all three matrices tested. Previous reports have already pointed out the difficulty in oxyclozanide's determination in milk reporting unacceptable recovery and precision values [223]. According to Caldow's study, acetic acid 1% in acetone seems to be the most efficient extraction solvent for oxyclozanide [234].To the best of our knowledge, there are no previous references for the determination of gemfibrozil and coumaphos in butter, milk and egg.

Triclabendazole's poor recovery in butter is due to butters high content of fat in which the compound and its metabolites are strongly bound [235]. The same applies for ionophore coccidiostats (monensin, salinomycin and semduramycin) that also presented low recoveries in butter samples due to their hydrophobicity, which makes their separation from fatty samples nearly impossible [86].

5.3.3 Method Validation

5.3.3.1 Identification

An analyte was considered as positively identified and confirmed in a sample when the criteria established in the EU Commission Decision 2002/657/EC were met:

- the ratio of the relative (to the IS) retention time of the analyte to that of the same analyte in standard solution was within ± 2.5 % tolerance
- the presence of a signal at each of the two SRMs for the analyte was achieved (the use of two selected precursor-product ion transition per compound counts for four identification points, which fulfill the EU identification points requirement)
- the signal intensity ratios of the two MS/MS transitions (quantifier and qualifier) with those obtained using fortified blank samples was within the tolerance defined [54].

In the present work, when no Internal Standard (IS) is used (determination of analytes in positive ionization mode), the identification of the target compounds was carried out by searching in the appropriate Retention Time Windows (RTW), which were given by the mean retention time ± three standard deviations of the retention time of ten blank samples spiked at 100 μg kg^{-1} (VL) for each compound [68, 78]. The retention times of all target compounds is presented in **Table 5.1**.

5.3.3.2 Selectivity

The selectivity of the method was evaluated by the analysis of 10 control blank samples from all four matrices. The absence of any signal at the same elution time as the target veterinary drugs and pharmaceuticals indicated the absence of chemical or matrix interferences that may give a false positive signal.

5.3.3.3 Linearity

The linearity of calibration curves was assessed by using an eight-point calibration curve of standards in pure solvent as well as in blank milk powder, fish tissue, egg and butter extracts at different concentrations (1 to 200 ng mL^{-1}). This number of levels was chosen in order to achieve the optimal concentration range for each target analyte, considering the large differences in sensitivity between the single substances. Each calibration standard was injected in each batch in duplicate. Peak area was used as the analytical response versus concentration in all cases except from some NSAIDs (carprofen, flunixin, ibuprofen, mefenamic acid, meloxicam, naproxen and tolfenamic acid) for which the peak area ratio of the analyte/IS was used. Calibration curves were obtained by least-squares linear regression analysis and acceptable linear regression R^2 values were obtained for all compounds over the concentration ranges. Determination coefficient values for standard solution curves were >0.997 (Table S3), and >0.990 for all standard solution calibration curves in extracts of all four matrices examined (4×115 curves). Based on these results, a good linearity was proven and allowed coverage of all drugs within the working range.

5.3.3.4 Precision

The precision of this method was demonstrated in term of repeatability (intra-day precision) and within-laboratory reproducibility (inter-day precision), which were expressed as the %RSD values of set of 6 replicate analysis at the VL in every matrix. Reproducibility experiments lasted three consecutive days. Repeatability results for milk powder, fish tissue, butter and egg spiked samples (n=6 for each matrix) are presented in **Table 5.2** In butter the %RSDs range from 2.4% (morantel) to 15% (bacitracin), in fish tissue from 1.7% (meloxicam) to 17% (semduramycin), in milk powder from 2.2% (clenbuterol) to 18% (chlortetracycline) and in egg samples from 3.1% (triamterene) to 16% (colchicine).

It can be observed that relative standard deviations were always lower than 20 for all the veterinary drugs and pharmaceuticals and for all matrices assayed. Moreover, the obtained RSD values of the within-laboratory reproducibility did not

exceed in any case the acceptable values calculated from the Horwitz equation. These results indicate the good precision and reliability of the developed method.

Table 5.2. %Recovery and repeatability (expressed as %RSD) results in the VL for all matrices tested.

	Butter (n=6)		Fish tissue (n=6)		Egg (n=6)		Milk powder (n=6)	
Compound	**% Recovery**	**% RSD**	**% Recovery**	**% RSD**	**% Recovery**	**% RSD**	**% Recovery**	**% RSD**
Quinolones								
Ciprofloxacin	87.2	4.7	82.5	13	93.0	9.7	72.0	4.4
Danofloxacin	93.8	5.5	84.9	13	107	9.7	101	6.5
Difloxacin	109	5.0	86.8	9.6	63.5	13	84.9	7.4
Enrofloxacin	103	6.2	91.1	12	107	6.3	87.2	7.6
Flumequine	103	5.5	85.3	8.0	47.2	5.5	79.6	6.6
Marbofloxacin	94.4	2.7	101	3.2	78.4	4.7	70.3	6.1
Norfloxacin	98.3	4.9	96.4	5.5	84.8	5.7	71.1	6.1
Ofloxacin	99.8	2.9	109	8.1	64.8	8.0	75.7	7.2
Oxolinic acid	95.5	5.5	92	4.5	101	4.0	74.0	6.6
Sarafloxacin	95.9	4.9	83.7	9.6	58.1	8.4	80.2	6.4
Tetracyclines								
Chlortetracycline	86.5	5.6	59.6	8.9	74.2	4.3	70.4	18
Doxycycline	78.7	11	74.8	11	52.9	10	64.2	13
Oxytetracycline	83.7	6.3	84.0	6.2	80.7	10	59.5	9.5
Tetracycline	66.3	5.5	80.4	7.1	28.3	8.8	53.1	7.1
Cefalosporines								
Cefaclor	106	11	72.3	11	66.7	11	59.7	12
Cefadroxil	94.2	10	73.7	13	41.5	5.0	41.8	8.6
Penicillins								
Amoxicillin	69.8	12	95.6	12	59.4	11	74.6	11
Ampicillin	90.9	7.8	68.3	11	62.2	6.7	113	15
Macrolides								
Azithromycin	88.2	5.3	95.0	10	69.8	12	104	6.7
Clarithromycin	84.8	4.8	88.2	10	85.7	7.5	109	4.5
Erythromycin	71.1	6.7	94.5	5.2	56.9	12	88.3	6.4
Tiamullin	93.7	3.1	95.4	5.9	58.8	5.5	99.1	9.7
Tylosin	83.6	6.8	85.9	8.6	72.6	8.2	95.7	9.6

Tilmicosin	99.1	5.5	93.5	5.2	66.4	5.5	87.4	11
Sulfonamides								
SClZ	104	3.2	99.1	9.5	50.3	6.7	69.2	14
SCP	101	8.6	92.2	3.9	57.0	7.7	55.7	8.1
SDD	92.2	5.8	85.9	7.5	71.3	10	50.7	8.8
SDM	83.2	6.5	77.0	6.2	59.4	6.0	60.3	5.9
SDX	85.1	6.2	76.4	3.2	64.5	8.3	51.6	6.9
SDZ	86.7	7.7	99.6	6.0	63.3	3.6	44.0	7.3
SGN	71.2	11	91.3	11	66.6	13	50.3	12
SIX	80.0	7.2	93.1	3.0	36.9	11	55.2	11
SMM	85.2	7.7	85.2	8.1	60.4	5.7	56.1	6.4
SMP	88.9	5.0	88.8	6.5	80.3	7.5	80.3	8.5
SMR	86.4	6.7	82.9	10	67.2	7.3	65.6	10
SMT	74.7	12	82.2	9.8	58.1	12	60.6	11
SMTX	99.4	5.6	89.7	7.5	64.8	12	65.4	7.2
SMX	96.3	6.7	98.7	3.5	45.8	7.3	62.2	8.1
SPD	98.4	4.3	86.0	12	72.5	8.8	62.2	5.9
SQX	84.2	6.6	81.1	4.6	52.5	4.1	58.1	12
STZ	93.8	6.3	78.2	11	57.5	9.6	69.7	9.1
Dapsone	81.0	11	87.1	8.8	54.2	9.2	82.1	9.2
Other antibiotics								
Bacitracin	56.3	15	73.8	11	72.3	7.2	77.2	12
Lincomycin	89.1	7.3	94.2	4.1	103	7.4	79.0	9.3
Novobiocin	42.3	12	72.2	9.9	63.1	10	63.6	10
Rifaximin	83.7	5.6	92.5	5.1	68.1	8.4	91.1	7.8
Trimethroprim	98.1	8.1	95.2	5.4	63.6	7.1	66.8	6.4
Benzimidazoles								
Albendazole	53.3	9.3	64.8	9.1	88.6	11	87.2	8.5
Albendazole sulfone	89.7	3.6	90.3	6.1	61.2	10	115	9.5
Febantel	70.4	11	81.2	11	55.0	12	61.9	12
Fenbendazole	63.6	13	32.4	15	41.8	8.8	18.0	10
Flubendazole	79.2	8.8	90.8	8.1	57.7	13	61.8	4.7
Mebendazole	95.9	7.4	91.5	6.2	53.8	6.7	70.1	4.3
Oxfendazole	102	2.6	95.3	3.4	65.9	8.2	83.2	7.4
Thiabendazole	101	4.9	94.8	5.9	56.1	6.8	74.8	4.0
Triclabendazole	12.2	14	62.3	6.4	51.0	7.7	94.8	13
Nitroimidazoles								
Dimetridazole	46.2	11	68.8	6.5	74.7	6.1	69.6	5.4

Metronidazole	110	7.0	75.9	13	60.1	7.1	68.6	14
Ronidazole	109	5.6	91.7	8.0	63.9	13	77.2	8.5
Ternidazole	115	6.6	96.2	11	74.4	6.0	82.7	4.0
Coccidiostats								
Aprinocid	90.8	4.6	70.2	4.8	69.4	8.6	65.0	6.5
Clopidol	97.1	6.8	91.0	9.0	57.2	11	78.0	13
Diaveridine	77.7	11	105	4.9	60.4	6.4	77.2	7.6
Ethopabate	89.8	4.0	90.0	5.3	72.4	6.5	83.3	5.0
Halofuginone	82.4	9.0	78.7	10	37.6	9.4	76.1	11
Monensin	41.4	5.6	72.9	12	72.0	6.5	50.1	5.9
Salinomycin	29.0	14	87.2	9.8	85.2	5.9	62.0	11
Semduramycin	44.2	13	78.6	17	94.7	13	101	8.7
Other Anthelmintics								
Levamisol	88.4	13	90.8	2.7	48.2	3.9	75.5	5.5
Morantel	99.3	2.4	82.9	8.5	61.6	6.6	78.3	8.2
Nitroxinil	79.7	8.5	55.5	11	66.0	5.4	63.6	14
Oxyclozanide	4.3	4.3	18.3	9.6	31.6	12	4.7	9.4
NSAIDs								
Carprofen	66.0	12	62.6	9.1	44.9	13	37.8	6.9
Diclofenac	63.4	11	59.5	10	51.0	10	36.8	13
Flunixin	87.7	7.7	95.2	7.4	79.8	8.5	57.4	10
Ibuprofen	60.7	10	54.9	11	62.2	13	98.7	11
Ketoprofen	84.2	10	96.6	6.2	65.5	9.2	75.2	10
Mefenamic acid	6.6	11	58.1	7.8	44.5	11	63.7	13
Meloxicam	102	4.9	67.5	1.7	77.3	4.2	86.5	12
Naproxen	106	8.6	86.9	13	76.8	12	92.6	12
Niflumic acid	87.6	11	63.4	5.8	50.6	8.8	64.6	6.6
Salicylic acid	82.1	7.7	80.9	5.9	83.8	12	60.2	9.6
Tolfenamic acid	9.2	11	31.8	9.3	58.5	11	10.9	11
Amphenicols								
Chloramphenicol	84.9	8.5	64.1	7.5	86.6	11	63.0	11
Florfenicol	84.8	11	65.2	13	78.7	9.1	67.8	10
Thiamphenicol	81.5	8.4	59.7	8.6	82.3	11	57.1	11
beta-agonists								
Clenbuterol	92.7	7.4	88.8	10	58.5	3.7	96.3	2.2
Beta-blockers								
Atenolol	84.8	6.4	100	4.2	68.2	9.8	100	2.3
Metoprolol	109	12	88.5	9.6	67.0	9.1	73.1	6.2

Propanolol	85.8	5.5	84.3	11	47.2	9.2	68.2	13
Steroids								
Betamethasone acetate	75.3	9.5	71.2	12	83.6	12	84.7	11
Cortisol	82.7	6.7	73.3	11	63.5	7.6	78.6	8.0
Cortisone	98.8	6.9	72.6	8.8	63.0	7.2	94.9	10
Dexamethasone	70.3	11	97.0	14	76.8	7.3	53.0	11
Methylprednisolone	105	4.8	88.9	7.5	62.6	9.8	70.4	14
Prednisolone	92.4	9.2	76.6	13	74.7	3.9	86.9	6.8
Progesterone	115	12	75.7	10	58.2	7.9	66.3	4.2
Analgetics								
Caffeine	98.1	11	55.8	8.3	90.0	4.7	110	3.4
Paracetamol	109	8.6	78.9	10	110	10	114	12
Tramadol	107	7.2	83.2	11	60.1	10	94.6	8.7
Diuretics								
Furosemide	64.8	3.8	77.8	5.3	71.3	7.4	44.6	10
Hydrochlorthiazide	90.7	12	72.3	11	88.8	11	40.4	12
Indapamine	91.7	2.9	58.2	4.7	74.8	6.5	68.9	9.9
Triamterene	101	8.5	89.1	8.7	51.6	3.1	73.0	6.2
Statins								
Atorvastatin	59.2	14	63.7	11	40.4	12	33.7	14
Antiepileptic drugs								
Carbamazepine	104	6.2	114	9.9	63.9	9.6	74.2	8.6
Antiulcer Drugs								
Cimetidine	80.2	9.2	92.6	7.9	114	9.2	77.8	7.6
Omeprazole	68.6	13	95.9	3.1	98.6	7.0	100	4.9
Ranitidine	63.6	13	84.5	7.3	67.2	7.1	84.0	9.2
Fibrates								
Clofibric acid	51.5	5.84	65.2	6.8	59.7	9.6	85.2	5.9
Gemfibrozil	10.3	11	22.3	10	62.2	7.3	22.4	13
Others								
Colchicine	84.0	7.2	91.1	9.9	84.6	16	86.0	7.4
Coumaphos	26.6	13	21.9	10	22.3	12	18.8	8.5
Theophylline	96.5	8.2	95.2	11	57.7	14	95.8	9.3
Valsartan	59.3	11	84.1	13	49.1	12	63.2	8.2

5.3.3.5 Trueness

The trueness of the method was estimated through recovery studies. Average recoveries of each analyte at the Validation Level (100 μg kg^{-1}) were calculated performing the analysis in 6 replicates for each matrix (**Table 5.2**). Overall recoveries for all target compounds in butter, fish tissue, egg and milk powder were also calculated. Overall recovery for each compound was assessed by comparing the slope of the the matrix-matched calibration curve (samples spiked before the extraction) (A) to the slope of the standard solution calibration curve in matrix extracts (B).

OVERALL REC (%) = A/B × 100 (4)

This parameter is of great value since it indicates the recovery of each analyte within the whole working range of concentrations for each matrix, separately. Overall recoveries for butter are presented in **Table 5.3**. For the other three matrices the results are presented in Electronic Supplementary Material 1 (ESM 1) in **Tables S5.4, S5.5, S5.6.**

Table 5.3: Performance and validation data of the analytical method in butter.

Compound	Slope matrix matched curve	Slope standard solution curve in matrix extracts	Overall recovery (%)	Matrix effect (%)	LOD (μg Kg^{-1})	LOQ (μg Kg^{-1})
Quinolones						
Ciprofloxacin	463480	460364	101	68	0.059	0.18
Danofloxacin	65906	66527	99.1	83	0.30	0.91
Difloxacin	989318	1092360	90.6	11	0.018	0.053
Enrofloxacin	447855	445422	101	90	0.019	0.056
Flumequine	1372960	1177780	117	-25	0.017	0.051
Marbofloxacin	547875	512314	107	64	0.040	0.12
Norfloxacin	425116	472336	90.0	73	0.064	0.19
Ofloxacin	1083970	994331	109	73	0.034	0.10
Oxolinic acid	1382460	1712640	80.7	162	0.017	0.051
Sarafloxacin	522881	468447	112	-23	0.013	0.039
Tetracyclines						
Chlortetracycline	210289	220660	95.3	105	0.094	0.28
Doxycycline	866383	1185820	73.1	105	0.041	0.12

Oxytetracycline	562185	707888	79.4	63	0.028	0.084
Tetracycline	287902	565617	50.9	91	0.040	0.12
Cefalosporines						
Cefaclor	17010	13846	123	-55	0.48	1.4
Cefadroxil	14708	12581	117	-61	0.29	0.86
Penicillins						
Amoxicillin	8507	7507	113	-62	0.51	1.5
Ampicillin	9444	8358	113	-52	0.38	1.2
Macrolides						
Azithromycin	55968	88538	63.2	209	0.10	0.31
Clarithromycin	162759	220562	73.8	14	0.074	0.22
Erythromycin	4037	6887	58.6	11	0.49	1.46
Tiamullin	910082	1085370	83.8	-9	0.010	0.030
Tylosin	24300	38346	63.4	20	0.41	1.23
Tilmicosin	44534	55723	79.9	94	0.30	0.91
Sulfonamides						
SClZ	39715	42509	93.4	-48	0.20	0.61
SCP	78270	86571	90.4	-46	0.12	0.36
SDD	355019	455522	77.9	-15	0.086	0.26
SDM	338416	532608	63.5	-3	0.10	0.30
SDX	438945	617109	71.1	-2	0.094	0.28
SDZ	71746	73728	97.3	-31	0.12	0.35
SGN	48461	64305	75.4	-54	0.13	0.40
SIX	199534	188372	106	-47	0.11	0.33
SMM	119712	161653	74.1	-9	0.10	0.31
SMP	78289	109657	71.4	-9	0.073	0.22
SMR	109450	122133	89.6	-26	0.092	0.28
SMT	243914	288233	84.6	-44	0.13	0.38
SMTX	207163	195599	106	-41	0.078	0.23
SMX	201324	228083	88.3	-37	0.10	0.31
SPD	175623	200245	87.7	-33	0.070	0.21
SQX	89071	149645	59.5	-10	0.10	0.30
STZ	214072	186111	115	-51	0.073	0.22
Dapsone	257154	342391	75.1	-49	0.19	0.58
Other antibiotics						
Bacitracin	10827	24013	45.1	33	0.15	0.45
Lincomycin	720193	726292	99.2	-5	0.023	0.070
Novobiocin	16210	44412	36.5	48	0.19	0.58

Rifaximin	574044	835486	68.7	20	0.015	0.046
Trimethroprim	501502	453749	111	-26	0.025	0.076
Benzimidazoles						
Albendazole	1122920	2364790	47.5	-8	0.017	0.052
Albendazole sulfone	1283570	1462660	87.8	-11	0.011	0.033
Febantel	388798	480591	80.9	82	0.075	0.22
Fenbendazole	1529243	3623800	42.2	-8	0.020	0.059
Flubendazole	2349500	3596740	65.3	0	0.024	0.072
Mebendazole	2002800	1831910	109	-40	0.021	0.062
Oxfendazole	1901900	2261780	84.1	-6	0.0083	0.025
Thiabendazole	500017	557204	89.7	-13	0.074	0.22
Triclabendazole	22223	964455	2.3	8	0.12	0.35
Nitroimidazoles						
Dimetridazole	152643	284246	53.7	-39	0.11	0.34
Metronidazole	562869	461290	122	-38	0.018	0.053
Ronidazole	120646	86063	140	-47	0.017	0.050
Ternidazole	696286	567108	123	-37	0.068	0.20
Coccidiostats						
Aprinocid	1190690	1414240	84.2	-4	0.013	0.040
Clopidol	27474	22565	122	-35	0.38	1.15
Diaveridine	1467440	1445080	102	-25	0.037	0.11
Ethopabate	791863	876555	90.3	-12	0.012	0.036
Halofuginone	20750	26562	78.1	8	0.46	1.37
Monensin	115342	465089	24.8	104	0.037	0.11
Salinomycin	20775	262979	7.9	-11	0.26	0.77
Semduramycin	137645	384548	35.8	-2	0.059	0.18
Other Anthelmintics						
Levamisol	230640	207226	111	-30	0.17	0.52
Morantel	107340	109004	98.5	-33	0.070	0.21
Nitroxinil	25987	42101	61.7	108	0.060	0.18
Oxyclozanide	695	18537	3.7	38	0.87	2.6
NSAIDs						
Carprofen	0.0001294	0.0002640	49.0	22	0.30	0.90
Diclofenac	76475	139046	55.0	-19	0.079	0.24
Flunixin	0.0218276	0.0220700	98.9	-34	0.0064	0.019
Ibuprofen	0.0000982	0.0001400	70.1	-49	0.33	1.00
Ketoprofen	188048	233190	80.6	-25	0.14	0.42
Mefenamic acid	0.0003020	0.0038700	7.8	6	0.26	0.78

Meloxicam	0.0553025	0.0557000	99.3	-5	0.018	0.053
Naproxen	0.0023887	0.0021500	111.1	-12	0.11	0.32
Niflumic acid	4303695	4342780	99.1	1	0.017	0.050
Salicylic acid	28763	49774	57.8	167	0.071	0.21
Tolfenamic acid	0.0005384	0.0191200	2.8	22	0.41	1.23
Amphenicols						
Chloramphenicol	16348	20574	79.5	54	0.21	0.64
Florfenicol	21134	27819	76.0	1	0.14	0.43
Thiamphenicol	5494	8043	68.3	27	0.16	0.49
beta-agonists						
Clenbuterol	503442	457563	110	-23	0.020	0.060
Beta-blockers						
Atenolol	124444	121700	102	-17	0.10	0.29
Metoprolol	75853	63606	119	-35	0.16	0.49
Propanolol	134941	168315	80.2	0	0.075	0.23
Steroids						
Betamethasone acetate	16659	23123	72.0	6	0.54	1.6
Cortisol	28676	34779	82.5	-20	0.45	1.3
Cortisone	64570	57972	111	-40	0.46	1.4
Dexamethasone	6284	7107	88.4	-16	0.46	1.4
Methylprednisolone	25865	28129	92.0	-15	0.33	0.98
Prednisolone	84454	87067	97.0	-25	0.13	0.38
Progesterone	67647	66977	101	-22	0.40	1.19
Analgetics						
Caffeine	94859	83209	114	-46	0.14	0.41
Paracetamol	19532	16795	116	-35	0.52	1.6
Tramadol	208993	165325	126	-37	0.10	0.31
Diuretics						
Furosemide	6586	11048	59.6	32	0.26	0.79
Hydrochlorthiazide	2517	2438	103	237	0.21	0.62
Indapamine	11787	15043	78.4	-11	0.15	0.46
Triamterene	1204680	1089870	111	-8	0.025	0.074
Statins						
Atorvastatin	86594	200448	43.2	15	0.084	0.25
Benzodiazepams						
Carbamazepine	210165	190884	110	-39	0.084	0.25
Antiulcer Drugs						
Cimetidine	260820	262854	99.2	-1	0.13	0.39

Omeprazole	75595	182795	41.4	70	0.15	0.46
Ranitidine	109095	211702	51.5	38	0.075	0.23
Fibrates						
Clofibric acid	23073	34616	66.7	66	0.21	0.63
Gemfibrozil	1872	13308	14.1	52	0.21	0.63
Others						
Colchicine	99097	134873	73.5	20	0.068	0.20
Coumaphos	107482	721359	14.9	-13	0.094	0.28
Theophylline	60366	51157	118	-63	0.18	0.55
Valsartan	25569	68128	37.5	0	0.090	0.27

In butter, for over 90% of the analytes, recoveries were between 50% and 120%. Only 5 compounds presented recoveries lower than 50% in fish tissue (oxyclozanide, coumaphos, gemfibrozil, tolfenamic acid and fenbendazole) with the 61% of the veterinary drugs and pharmaceuticals giving recoveries >80%. In milk and egg the recoveries for the majority of the analytes lie between 50-80% (66 and 78 compounds, respectively).This is due to the complexity of these matrices which have a high protein and lipid content. The compounds may bind to the lipoproteins and not be sufficiently extracted from the matrix which in some cases also forms emulsions and foams with the extraction solvents [88, 68]. However, recovery values were > 50% for more than 85% of the analytes in both egg and milk powder. In conclusion, although for several compounds the recovery values were not close to 100%, they are considered acceptable since they were reproducible (**Table 5.2**). A schematic presentation of these results is demonstrated in **Figure 5.1**.

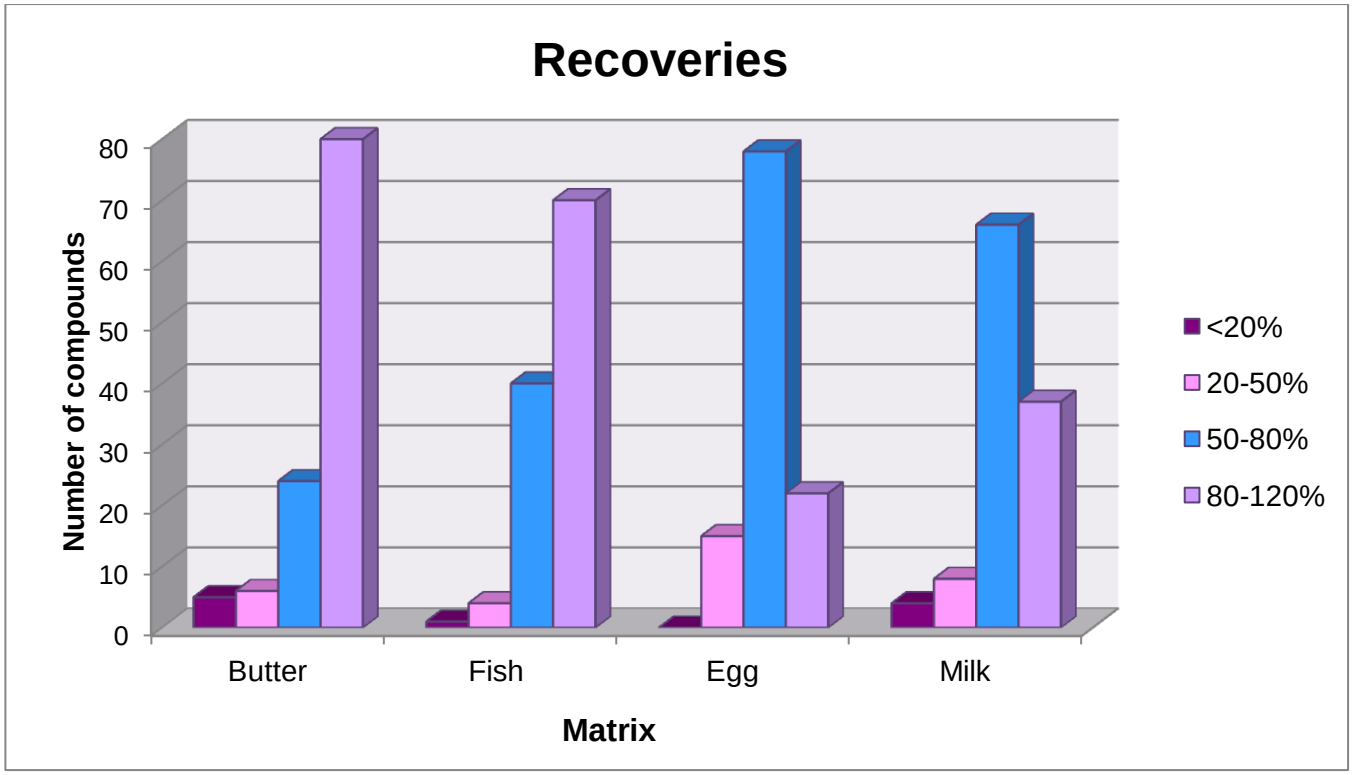


Figure 5.1: Schematic presentation of the recovery results of all target analytes in all studied matrices.

Additionally, trueness was verified by analyzing test materials provided from proficiency testing. As it is not possible to obtain certified reference or proficiency test material containing all analytes simultaneously, the developed method was applied to proficiency testing material for several target compounds only. Sulfonamides, tetracyclines and quinolones were the compounds of choice.

MI1320A and MI1321A test materials were obtained by Progetto Trieste, a proficiency testing service by Test Veritas S.r.l. They consisted of lyophilized bovine milk samples, blank, incurred and spiked, provided for performance evaluation of confirmatory techniques. 54 laboratories from 22 countries participated in this proficiency testing scheme and the compounds detected and quantified were three sulfonamides (sulfadimidine, sulfamerazine and sulfadiazine), one tetracycline (oxytetracycline) and one quinolone (enrofloxacin). In all cases, the z scores achieved were less than 2, fulfilling the proficiency test criteria for successful participation.

Results are presented in **Table 5.4**. A diagram reflecting the dispersion of the calculated concentrations of all laboratories for sulfamerazine in test material

MI1320A-2 is shown in **Figure S5.5**. **Figure S5.6** shows the chromatogram of a proficiency test lyophilized bovine milk sample containing three sulfonamides (MI1320A-2). For comparison, the chromatogram of the blank sample (MI1320A-1) spiked with 35 µg kg^{-1} of all sulfonamide analytes is also presented (**Figure S5.7**).

Table 5.4: Results of the assessment of proficiency testing materials.

Test material code	Sample	Analytes tested	Analytes detected	Assigned value (µg Kg^{-1})	Calculated concentration (µg Kg^{-1})	z-score
MI1320A-1	blank	sulfadimidine, sulfadimethoxine, sulfaquinoxaline, sulfamerazine, sulfadiazine, sulfamethoxypyridazine, sulfamonomethoxine, sulfathiazole	none	< 10 for all analytes except from sulfamonomethoxine < 12.1	< LOQ for all analytes	–
MI1320A-2	incurred	sulfadimidine, sulfadimethoxine, sulfaquinoxaline, sulfamerazine, sulfadiazine, sulfamethoxypyridazine, sulfamonomethoxine, sulfathiazole	sulfadimidine	40.29	33.3	**- 0.67**
			sulfamerazine	34.39	36.0	**0.18**
			sulfadiazine	38.53	34.0	**- 0.45**
MI1321A-1	incurred	oxytetracycline, chlortetracycline , doxycycline, tetracycline, enrofloxacin, ciprofloxacin , flumequine, marbofloxacin, danofloxacin, sarafloxacin, oxolinic acid	oxytetracycline	109.97	144.4	**1.40**
MI1321A-2	spiked	oxytetracycline, chlortetracycline , doxycycline, tetracycline, enrofloxacin, ciprofloxacin , flumequine, marbofloxacin, danofloxacin, sarafloxacin, oxolinic acid	enrofloxacin	87.23	104.6	**0.86**

5.3.3.6 LODs & LOQs

LODs and LOQs were calculated by analyzing blank samples spiked at 0.1, 0.5, 2 or 10 μg kg^{-1}, according to each analyte's sensitivity, as described in the Experimental section. For instrumental LODs and LOQs standard solutions in the same concentrations were analyzed in quintuplicate. Instrumental LODs ranged from 0.0037 ng ml^{-1} (mebendazole) to 2.3 (hydrochlorthiazide). Results are shown in Table **S5.7**.

In butter the lowest LODs and LOQs were achieved, ranging from 0.0064 μg kg−1 for flunixin (LOQ 0.019 μg kg^{-1}) to 0.87 μg kg^{-1} for the anthelmintic oxyclozanide (LOQ 2.6 μg kg^{-1}). All results are presented in **Table 5.3**.

Similarly low values of LOD and LOQ were obtained also for egg samples, although eggs constitute a very complex matrix with high lipid and protein content. Results are shown in **Table S5.4** and, as it can be seen, LOD values vary from 0.0028 μg kg^{-1} (tetracycline) to 3.2 μg kg^{-1} (hydrochlorthiazide) and LOQs from 0.0083 μg kg^{-1} (tetracycline) to 9.5 μg kg^{-1} (hydrochlorthiazide).To the best of our knowledge these are the lowest LODs and LOQs reported in literature for multi-residue/multi-analyte determination in egg.

In fish tissue and in milk powder the LODs and LOQs were slightly elevated. In fish tissue LOQs were in all cases below 5 μg kg^{-1} except for hydrochlorthiazide and danofloxacin who's LOQs were 6.7 and 5.6 μg kg^{-1}, respectively. For these compounds there is no MRL established in fish tissue. Finally, LODs in milk powder ranged from 0.0033 μg kg^{-1} (flunixin) to 4.4 μg kg^{-1} (hydrochlorthiazide). All LODs and LOQs in fish tissue and milk powder are presented in **Tables S5.5** and **S5.6**.

For some compounds, like azithromycin, lowest method LODs than instrumental LODs were obtained due to the severe matrix enhancement of these compound to the matrices examined (+208% in butter, +808% in egg, +462% in milk powder). The same applies for oxolinic acid and salicylic acid (+162% and +167% in butter, respectively, +181% and + 227% in egg, +116% & 107% in fish tissue and 103% & 82% in milk powder), for hydrochlorthiazide in butter (+ 237%) and carprofen in milk powder (+589%).

5.3.3.7 Matrix Effect

When complex samples, such as milk, muscle or egg are analyzed, LC–MS/MS measurements, especially in the ESI mode, might significantly be influenced by matrix effects. Matrix effects derive from various physical and chemical processes and may be difficult or impossible to eliminate. They relate to the concentrations and protonation levels of co-extracted components and can be variable and unpredictable in occurrence. Matrix effects are co-dependent and can affect the ionization efficiency of the analytes, leading to suppression or enhancement of the signal depending on the analyte/matrix combination. Obviously, this affects the quantification, unless matrix effects are minimized or compensated [68, 236]. The best way to compensate the matrix effect is the use of isotope labeled internal standards (ILIS). However, these compounds are not available for many veterinary drugs, they increase severely the cost of the analysis and it is well known that an adequate correction is assured only when the own ILIS is used [237]. The use of analogue ILIS is not always satisfactory [236, 237]. Therefore, other approaches such as matrix-matched calibration or standard addition method can be used for proper quantification of the samples [68, 78, 87, 236].

To evaluate matrix effect, the slopes obtained in the matrix-matched calibration curves were compared with those obtained with solvent standards. Matrix effects (ME%) were calculated by subtracting 1 from the ratio between the standard solution calibration curve slope in matrix extracts (B) and in pure solvent (C) for each compound, and then multiplying by 100:

$$ME\ (\%) = ((B/C) - 1) \times 100 \qquad (5)$$

The signal is enhanced if the value is positive, whereas it is suppressed if the value is negative. A signal enhancement or suppression effect is considered as acceptable if the matrix effect values range from -20% to +20% (36).

% Matrix effects higher than 20% or lower than -20% indicate a strong matrix effect. It can be observed that a significant matrix effect was noticed for 75 compounds in butter, 100 compounds in fish tissue, 89 compounds in milk powder and 81 compounds in egg whereas tolerable matrix effect was observed

for the rest of compounds. The majority of the compounds in butter, fish tissue and milk powder were subjected to a signal suppression (72 in butter, 95 in fish tissue, 85 in milk powder) while a signal enhancement was observed for most of the compounds in egg samples (73 compounds). Matrix effect results for all compounds in each matrix are presented in **Table 5.3** and **5.S4, 5.S5** and **5.S6**.

Briefly, azithromycin and erythromycin were subjected to significant signal enhancement in all matrices examined, a fact that comes in agreement with other studies which indicate signal enhancement for macrolides [80, 87]. Tetracyclines presented severe signal enhancement in egg samples while for sulfonamides, especially in fish tissue, calibration curves in matrix were found to have a slope lower than the calibration curves in solvent, indicating signal suppression [81]. Amphenicols showed an increase of their signal when measured in standards prepared in fish tissue extracts and conversely, nitroimidazoles were subjected in extended signal suppression in all matrices studied in this work. Nitroimidazoles' signal suppression is not an unprecedented phenomenon [238]. Steroids also reveal a decrease in their signal in butter, milk and fish matrix-matched standards compared to standards in solvent.

5.4 Application to Real Samples

To evaluate the applicability of the proposed method, seventy three (73) milk powder, five (5) butter, twenty two (22) fish tissue and eight (8) egg samples were analysed. A calibration standard (solvent standard), a matrix blank, a spiked blank sample at 50 μg kg^{-1} and a matrix-matched standard at the same concentration were included in the analysis of each batch of samples in order to check the reliability of the proposed method.

The retention time, quantification and confirmation transitions and relative ion intensities of the detected ions in unknown samples were compared to those of corresponding spiked samples and matrix-matched calibration standards in the same batch to confirm the identity of the detected analytes using the criteria established by Decision Commission 657/2002/EC. The standard addition method was used for quantification. The application of the standard addition method with spiking standards to the samples pre-extraction is recommended to

get highly reliable quantitative results independently from correction factors both for recovery and matrix effects.

Analysis of the samples showed that progesterone was repeatedly present in butter and milk powder samples since it has an endogenous origin [39]. Only few other compounds were detected in the samples. Oxfendazole and albendazone sulfone were detected in two milk powder samples at 1 μg kg^{-1} and 0.54 μg kg^{-1}, respectively. These concentrations, although they are higher than LOQ, are very low and far below the MRL established for these benzimidazoles in milk (10 μg kg^{-1} and 100 μg kg^{-1}). Several quinolones were detected in milk (ciprofloxacin at 7.3 μg kg^{-1} and norfloxacin at 2.2 μg kg^{-1}) and fish tissue samples (flumequine at 4.6 μg kg^{-1} and enrofloxacin at 4.8 μg kg^{-1}). Thiabendazole was detected and quantified at 9.7 μg kg^{-1} in one butter sample and, finally, caffeine was present in 6 milk powder samples, at concentrations ranging from 2.4 to 32 μg kg^{-1}. There was no compound detected and quantitated that would exceed the MRL established in Regulation 37/2010/EC. The obtained results are in agreement with other studies [78, 81, 83, 86, 92].

5.5 Conclusions

A simple, sensitive and efficient multi-residue and multi-class analytical method for the simultaneous determination of 115 veterinary drugs and pharmaceuticals in milk powder, butter, egg and fish tissue by HPLC–MS/MS has been developed. Among the different classes of veterinary drugs, the method allows also the determination of TCs, polar penicillins, cefalosporins and ionophores, whose simultaneous analysis in multiclass methods often presents a problem.

The proposed methodology allows the simultaneous extraction of veterinary drugs and pharmaceuticals with very different physicochemical properties from various matrices, employing a simple solvent extraction with 0.1% formic acid in aqueous solution of EDTA 0.1% (w/v) – acetonitrile (ACN) – methanol (MeOH) (1:1:1, v/v) and further ultrasonic-assisted extraction. The extraction procedure was fully optimized in terms of recovery for the three out of four matrices examined.

Good validation parameters such as linearity, recovery, precision and LOQs were obtained indicating the suitability of the proposed solvent extraction method for the analysis of veterinary drugs and pharmaceuticals.

Only a few veterinary drugs and pharmaceuticals were detected in egg, milk powder and butter samples taken from different markets, and their concentrations were below the MRL established for each compound detected. Furthermore, the method was successfully applied in two proficiency test samples of lyophilized bovine milk containing several target analytes (sulfonamides, tetracyclines and quinolones). In all cases a z-score of <2 was achieved, indicating the excellent accuracy of the proposed method.

CHAPTER 6

Multiresidue / Multiclass Determination Of 76 Veterinary Drugs And Pharmaceuticals In Bovine Muscle Tissue By Hydrophilic Interaction Liquid Chromatography-Tandem Mass Spectrometry

6.1 Introduction

Multi-residue methods reported in literature are mainly based on reversed phase (RP) separation due to its efficient separation of analytes within broad range of polarity. However, highly polar compounds undergo early elution on traditional RP stationary phases, leading to lower sensitivity of the mass spectrometric (MS) detection due to (1) high matrix effects and (2) high water percentage in the mobile phase at the beginning of the run resulting in lower ionization efficiency in the MS interface. The analysis of highly hydrophilic and polar compounds by hydrophilic interaction chromatography (HILIC) coupled to MS has been demonstrated as a valuable complementary approach to RPLC [239].

Several applications of HILIC in the analysis of veterinary drugs and pharmaceuticals in food products have been reported the last decade [69, 70, 240-244]. Great attention has been paid in the determination of aminoglycoside antibiotics (AGs) with HILIC [70, 241, 243, 244] due to their extremely polar character which makes them practically unretainable in RP coloums. The simultaneous determination of AGs and other veterinary drugs in one method and in a single chromatographic run, has only been reported once so far [69]. Chiaonchan et al. reported the development of a multi-residue method for the determination of 24 veterinary drugs, mainly antibiotics, in chicken muscle, without however including the most strongly retained AGs.

In this study, a simple, rapid and sensitive multiresidue method for the simultaneous determination of 74 veterinary drugs and pharmaceuticals in bovine muscle tissue has been developed and validated according to the requirements of European Commission Decision 2002/657/EC. The analytes belong in 13

different classes, including strongly retained AGs like apramycin and neomycin which have never been previously included in a multi-residue method. The method combines a two-step extraction procedure (extraction with acetonitrile (ACN) followed by an acidic aqueous buffer extraction) with Hydrophilic Interaction Liquid Chromatography - Tandem Mass Spectrometry (HILIC-MS/MS) determination, allowing confirmation and quantification in a single chromatographic run. Further cleanup with solid phase extraction (SPE) using HLB cartridges was performed.

A thorough ionization study of aminoglycosides was performed in order to increase their sensitivity and significant differences in the abundance of the precursor ions of the analytes were revealed, depending on the composition of the mobile phase tested. To the best of our knowledge, any similar study concerning aminoglycosides has not been reported previously. Further gradient elution optimization and injection solvent optimization were performed for all target analytes. The chromatographic column used was an Acquity UPLC BEH HILIC and the mobile phase consisted of ACN, MeOH and ammonium formate 1mM with 0.1% formic acid, using a ternary gradient.

The method was validated according to the European Commission Decision 2002/657. Quantitative analysis was performed using the standard addition method. Recoveries varied from 37.4% (bromhexine) to 106% (kanamycin) in the lowest validation level and only the 18% of the compounds showed recovery < 70%. CCβ varied from 2.4 μg kg^{-1} (salinomycin) to 1302 (apramycin)

6.2 Experimental

6.2.1 Chemicals and reagents

All the analytes studied are presented in **Table 6.1**. All veterinary drug and pharmaceutical standards were of high purity grade (>90%) and the majority of them was purchased from Sigma-Aldrich (Steinheim, Germany) along with six internal standards (IS, amikacin, decoquinate d^5, fenbendazole d_3, flubendazole, phenylbutazone - (diphenyl-$^{13}C_{12}$) and triclabendazole d_3). Amikacin and

flubendazole are not regulated in bovine muscle tissue and thus were used as internal standards. Arprinocid, salinomycin, semduramicin, manduramycin, narasin, albendazole sulfone, flunixin and meloxicam and the IS nigericin, flunixin d_3 and meloxicam d_3 were donated by the Veterinary Drug Residues Laboratory of the State General Laboratory of Cyprus. Sulfadiazine d_4, sulfadimidine d_4 and sulfadimethoxine d_4 were obtained from Toronto Research Chemicals (Toronto, Canada).

Acetonitrile (ACN) and methanol (MeOH) LC–MS grade were purchased from Merck (Darmstadt, Germany). Formic acid 99% and ammonium formate (>90% purity) were purchased from Fluka (Buchs, Switzerland). All other solid reagents used were of analytical grade. Ethylenediaminetetraacetic acid disodium salt (EDTA), sodium hydroxide monohydrate (NaOH) and sodium chloride (NaCl) were purchased from Panreac (Barcelona, Spain) while trichloroacetic acid (TCA) and ammonium acetate from Fisher Scientific (Loughborough, UK). Distilled water was provided by a MilliQ purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). RC (Regenerated Cellulose) syringe filters (15 mm diameter, 0.22 μm pore size) were provided from Phenomenex (Torrance, CA, USA). Solid phase extraction cartridges were Oasis HLB 6 cc (200 mg) from Waters (Millford, MA).

Stock standard solution of individual veterinary drugs were prepared at a concentration of 1000 μg mL^{-1} by diluting the proper amount of each standard in the suitable dilution solvent. For apramycin a stock standard solution of 5000 μg mL^{-1} was constructed. Aminoglycosides and penicillins were dissolved in MilliQ-water while all other analytes in methanol. In benzimidazole standard solutions, 100 μL of NaOH were added to enhance solubility. Stock solutions were stored at −20 °C in brown glass in order to avoid photodegradation and new ones were prepared every six months, except for aminoglycosides which were prepared every 3 months.

Four intermediate standard solutions were prepared, all in ACN. The first one contained only aminoglycosides and the second one consisted of the compounds prohibited in bovine tissue (dapsone, phenylbutazone, chlorpromazine and bromhexine). For dapsone and phenylbutazone a Recommended Concentration

(RC) in animal tissue is set [218]. All other veterinary drugs examined were included in the last two intermediate solutions. The final concentration of each analyte in these intermediate standards corresponded to 200-fold the MRL or ML established for each compound in bovine tissue or the Validation Concentration (VC) of choice for the compounds where no MRL is established. The MRLs, MLs, RCs and VCs for all the determined analytes are summarized in **Table S6.1**. Validation Levels (VLs), which represent the concentration that the validation was performed for each compound, are also presented in this Table. The intermediate standard solutions were also stored at −20 °C and were conserved for one month except for the one containing aminoglycoside antibiotics which was newly prepared every week. Another intermediate standard solution was prepared for the twelve internal standards at 10 μg mL^{-1} by dilution of their stock solutions in ACN.

Working solutions were constructed by mixing the appropriate amounts of the intermediate standard solutions and diluting with ACN. One working solution of all analytes was obtained with variable concentrations equivalent with 50 times the MRL, ML or VC of each compound. For prohibited veterinary drugs a separate working solution was constructed at analogous concentration (50×VC). The working solution of internal standards was prepared at a concentration of 2.5 μg mL^{-1}. New working solutions were prepared every day of analysis.

6.2.2 Instrumentation.

A Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument was connected to a Thermo UHPLC Accela system (Thermo, San Jose, CA, USA). An Acquity UPLC BEH HILIC (100 mm x 2.1 mm, 1.7 μm, Waters) column was used at a constant flow rate of 100 μL min^{-1}. The determination was performed in positive ionization mode and the mobile phase consisted of ACN (solvent A), aqueous ammonium formate 1mM with 0.1 formic acid (v/v, solvent B) and MeOH (solvent C). The gradient profile started at 80% of solvent A and 20% B (0% C), and decreased linearly to 0% A and 95% B (5%C) in 10 min. This composition was held for additional 4 min before being returned to the initial conditions, in order for all strongly retained aminoglycosides to elute. A 6 min re-

equilibration step gave a total run time of 20 min. This re-equilibration step is rather long for a UPLC column but the equilibration of the column is very significant in HILIC in order to achieve retention time reproducibility [245]. The full loop injection volume of the extract was set at 10 µL. Spray Voltage was set at 4000 V, Seath Gas and Auxiliary Gas were set at 25 psi and 10 a.u respectively and Capillary temperature was set at 300 °C.

Single Reaction Monitoring (SRM) was used and the selected transitions, collision energies and tube lenses are presented in Table 2. Two SRM transitions were monitored for identification and the most intense one was used for quantification.

Table 6.1: MS/MS parameters and retention times for all analytes and internal standards.

Compounds	Pseudo Molecular Ion (m/z)	Quantifier Ion, Q (m/z)	Collision Energy (eV)	Qualifier ion, q, (m/z)	Collision Energy (eV)	Tube Lens (V)	Internal Standard
Aminoglycosides							
Apramycin (Q)	540	378	19			123	Amikacin
Apramycin (2+) (q)	271	217	14			74	
Dihydrostreptomycin	584	262.5	31	246	35	132	Amikacin
Gentamycin	478	322	15	167	22	119	-
Kanamycin	485	163	27	324	16	120	Amikacin
Neomycin	615	163	34	161	28	119	-
Streptomycin	582	245.5	36	263	31	131	Amikacin
Penicillins							
Ampicillin	350	106	25	174	17	89	-
Cloxacillin	410	178	32	174	19	80	-
Dicloxacillin	444	211	33	128	29	115	-
Oxacillin	376	174	18	144	31	97	-
Penicillin G	309	174	16	128	26	93	-
Penicillin V	325	128	26	174	16	86	-
Macrolides							
Azithromycin	750	158	37	591	29	127	-
Clarithromycin	749	158	30	591	20	123	-

Erythromycin	734	158	30	576	20	130	-
Tiamullin	494	192	21	119	33	101	-
Tilmicosin	869	174	42	156	44	165	-
Tylosin	917	174	36	772	28	148	-
Sulfonamides							
Dapsone	249	156	14	108	22	79	sulfadimidine d_4
Sulfachloropyridazine	285	156	14	92	28	87	sulfadimethoxine d_4
Sulfadiazine	251	156	15	92	27	87	sulfadiazine d_4
Sulfadimethoxine	311	156	17	108	29	87	sulfadimethoxine d_4
Sulfadimidine	279	186	17	124	26	87	sulfadimidine d_4
Sulfaguanidine	215	156	14	92	14	87	sulfadiazine d_4
Sulfamerazine	265	172	16	156	16	87	sulfadimethoxine d_4
Sulfamethizole	271	156	14	92	28	87	sulfadimethoxine d_4
Sulfamethoxazole	254	156	16	108	25	87	sulfadimidine d_4
Sulfamonomethoxine	281	92	29	156	13	87	sulfadimethoxine d_4
Sulfapyridine	250	156	15	184	17	87	sulfadimethoxine d_4
Sulfaquinoxaline	301	156	18	92	30	87	sulfadimethoxine d_4
Sulfathiazole	256	156	15	92	26	87	sulfadimidine d_4
Sulfisoxazole	268	156	13	92	27	87	sulfadimethoxine d_4
Diaminopyrimidines							
Baquiloprim	309	171	29	123	28	113	-
Trimethoprim	291	230	25	123	30	87	-
Other antibiotics							

Rifaximin	786	754	22	362	32	114	-
Anthelmintics							
Albendazole	266	234	29	191	31	85	Flubendazole
Albendazole sulfo(oxide)	282	240	13	208	24	99	Fenbendazole d_3
Albendazole sulfone	298	266	19	159	35	74	Fenbendazole d_3
Febantel	447	383	17	280	31	110	Flubendazole
Fenbendazole	300	268	29	159	33	85	Fenbendazole d_3
Levamisole	205	178	29	123	31	87	-
Mebendazole	296	264	31	105	35	90	Fenbendazole d_3
Morantel	221	123	31	111	26	96	-
Oxfendazole	316	159	30	191	24	87	Flubendazole
Thiabendazole	202	131	35	175	35	87	Flubendazole
Triclabendazole	359	274	35	171	40	85	Triclabendazole d_3
Coccidiostats							
Amprolium	243	150	12	122	24	69	-
Aprinocid	278	143	28	107	48	95	-
Clopidol	192	101	27	87	31	99	-
Decoquinate	418	204	41	232	35	119	Decoquinate d_5
Diaveridine	261	245	26	123	26	93	-
Ethopabate	238	206	11	136	27	37	-
Maduramycin (NH_4^+)	935	629	30	647	17	129	Nigericin
Monensin (Na^+)	693	461	51	501	52	159	Nigericin
Narasin (NH_4^+)	782	747	19	373	30	99	Nigericin

Robenidine	334	155	20	138	27	81	Nigericin
Salinomycin (NH_4^+)	768	733	18	373	32	109	Nigericin
Semduramycin (Na^+) (Q)	895	833	28			116	Nigericin
Semduramycin (NH_4^+) (q)	890	629	22			117	
NSAIDs							
5-Hydroxyflunixin	313	295	23	227	30	96	Flunixin d_3
Flunixin	298	280	23	109	44	92	Flunixin d_3
Meloxicam	352	115	22	141	23	85	Meloxicam d_3
Phenylbutazone	309	160	20	211	16	94	Phenylbutazone $^{13}C_{12}$
Thyreostats							
6-phenyl-2-thiouracil	205	146	19	103	26	68	-
Tranquilizers							
Chlorpromazine	319	86	20	246	23	69	-
Pharmaceuticals							
Ambroxol	379	264	20	104	51	95	-
Atenolol	267	145	26	190	18	94	-
atorvastatin	559	440	22	250	42	123	-
caffeine	195	138	18	110	22	87	-
Carbamazepin	237	194	19	193	32	114	-
Metropolol	268	191	17	133	25	96	-
Propanolol	260	183	19	155	25	99	-
Simvastatin	419	225	22	199	7	103	-

Tramadol	264	58	15	246	8	66	-
Triamterene	254	237	26	104	36	93	-
Valsartan	436	291	16	207	28	99	-
Others							
Bromhexine	377	114	18	264	29	78	-
Internal Standards							
Amikacin	586	424	20			87	
Decoquinate d_5	423	377	25			87	
Fenbendazole d_3	303	268	31			87	
Flubendazole	314	282	31			90	
Flunixin d_3	300	282	25			91	
Meloxicam d_3	355	115	25			132	
Nigericin (NH_4^+)	742	461	28			101	
Phenylbutazone - (diphenyl-$^{13}C_{12}$)	321	166	21			90	
Sulfadiazine d4	255	160	16			96	
Sulfadimethoxine d4	315	156	22			90	
Sulfadimidine d4	283	186	18			101	
Triclabendazole d3	364	201	29			130	

Each chromatographic run comprised several scan events with a scan time of 20 ms for each transition. Instrument control and data acquisition were carried out by using the Xcalibur software, Version 2.3, from Thermo.

6.2.3 Samples

Bovine tissue samples were obtained from local supermarkets. Upon arrival at the laboratory the samples were homogenized and refrigerated at -20 °C until analysis.

6.2.4 Sample preparation

A 5.0-g portion of bovine muscle was weighed into a 50-mL polypropylene centrifuge tube and 100 µL of the IS working solution were added to achieve a final concentration of 50 µg kg^{-1} of each internal standard. For fortified samples, aliquots of 50, 100 and 150 µL of the working solution standard containing all the analytes at a concentration of 50×VL were added to 5 g of sample. Spiked levels obtained were 0.5×VL, 1×VL and 1.5×VL, respectively. For prohibited compounds 100, 150 and 200 µL were added in order to aquire 1×VL, 1.5×VL and 2×VL concentration levels as indicated in European Commission 2002/657/EC.

When fortified, either with all analytes (fortified samples) or just with the IS mix, the samples are vortex-mixed for 30 s and allowed to stand for 10 – 15 min. After addition of 10 mL of ACN the samples are vortexed for 1 min and shaken for 30 minutes using a mechanical shaker. Then, the sample tube is centrifuged at 4000 rpm for 5 min and the supernatant is decanted in a glass tube. The acetonitrile extract is evaporated to final volume 1.0 mL under a stream of nitrogen at 30 °C. A volume of 20 mL of an aqueous extraction solvent is subsequently added to the sample. This extraction solvent, consisting of 10 mM ammonium acetate, 0.4 mM EDTA, 1% NaCl (w/v) and 2% TCA (w/v) in H_2O, has been previously reported in literature to be adequate for aminoglycosides' extraction [246]. The samples are vortexed for 1 min and shaken for 60 minutes using a mechanical shaker. Afterwards, the sample tube is centrifuged at 4000 rpm for 5 min and the

supernatant is decanted in a new polypropylene tube. The sample extract is adjusted to pH 6.5 by adding ammonia hydroxide 30% (w/v) and afterwards is loaded onto an OASIS HLB (200 mg, 6 mL) cartridge previously conditioned sequentially with 6 mL of MeOH and 6 mL of H_2O. The sample is passed through the cartridge at a flow no faster than 1 drop/s and, then, it is vacuum-dried for approximately 15 min. The elution of the analytes was carried out with 2×0.5 mL of aqueous formic acid 10% (v/v) and 3×1 mL of ACN. The eluate is collected and combined with the 1-mL ACN extract. At this step proper volumes of working solutions were added to blank aliquots, to prepare the range of matrix-matched standards required. Finally, 500 μL of the combined extract were transferred in a vial and 10 μL was injected into the HILIC-MS/MS system.

6.2.5 Method validation

The method was validated in bovine tissue according to European Commission Decision 2002/657 at three concentration levels. Validation was performed at 0.5×MRL – 1×MRL – 1.5×MRL and 0.5×ML – 1×ML – 1.5×ML where one exists. For prohibited compounds the VLs corresponded to 1×VC, 1.5×VC and 2×VC. For dapsone and phenylbutazone the VCs match the RC that has been set for them in animal tissues (5 μg kg^{-1}). Finally, for compounds that no MRL or MRPL has been established the VCs chosen are presented in Table S1. When no MRL was specified for a particular compound in bovine tissue but there was an MRL established in another matrix (e.g. milk) this concentration was used as the VC. The VLs (Validation Levels) for these compounds corresponded to 0.5×VC, 1×VC and 1.5×VC. Overall, the VL for each compound is the final concentration of choice in which the validation was performed.

Identification and confirmation of the analytes were carried out by retention times, selected SRM transitions and the relative ion ratio of them as required by the EU validation criteria. The developed procedure was validated in terms of selectivity/specificity, linearity, accuracy through recovery studies, intra and interday precision, limits of detection (LODs) and quantification (LOQs), decision limit (CCα) and detection capability (CCβ). Matrix effects were also evaluated.

Since no certified reference materials (CRMs) were available, fortified blank bovine tissue samples were employed for the validation.

The verification of the selectivity/specificity of the method was performed by analyzing 20 blank bovine tissue samples.

Linearity was assessed both in standard solution calibration curves in pure solvent and in matrix extracts. For building up the calibration curves, pure solvent aliquots and blank bovine tissue extracts were fortified with the analytes in 6 different levels, from 0.25×VL to 4×VL. Calibration curves were constructed by plotting the peak area against the concentration of the calibration standards except for analytes for which an internal standard is used for quantification (e.g sulfonamides etc). In this case the calibration curves were constructed by calculating the ratio of each peak area relative to the corresponding IS. Matrix matched calibration curves by spiking the analytes in the matrix before the extraction were also obtained in the same levels by fortifying blank muscle tissue samples with the target compounds and analyzing them with the sample preparation defined in Section 6.2.4. Overall matrix effects were calculated by comparing standard solution curves prepared in solvent and in blank bovine tissue extracts for all analytes.

For accuracy estimation, three batches of 6 blank bovine tissue samples (n=18) were enriched with veterinary drugs and pharmaceuticals at 0.5, 1 and 1.5 times the VL (1, 1.5 and 2 times the VL for prohibited veterinary drugs). These samples were analyzed during three different laboratory days and recoveries in each concentration were determined by comparing samples spiked before and after the extraction. Intra-day precision (repeatability) was evaluated by analyzing six blank samples per validation level (n=6) in the same day and under the same conditions. Inter-day precision (reproducibility) was evaluated by analyzing six blank samples per validation level (n=6) during three different days. Precision in both cases is expressed as % Relative Standard Deviation (%RSD).

Although not indicated in Commission Decision 2002/657/EC, limits of detection (LODs) and limits of quantification (LOQs) were also investigated. Blank bovine tissue samples enriched with all analytes before the extraction at descending

order were analysed and LODs and LOQs were determined based on Signal to Noise ratios (LOD S/N ≥ 3 and LOQ S/N ≥ 10).

Finally, the method's decision limit (CCα) and detection capability (CCβ) were calculated for all compounds examined as stated in Commission Decision 2002/657/EC [54]. CCα is defined as the lowest concentration level of a certain compound at which it can be concluded that a sample is non compliant with an error probability α. For compounds with established MRLs and MLs CCα was calculated as the MRL (or ML) plus 1.64 times the standard deviation of the inter-day precision at the MRL (or ML) level (1×VL). For compounds with no set MRL (or ML) the calibration curve approach was followed. CCα was calculated as the concentration at the y-intercept plus 2.33 times the standard deviation of the reproducibility at the lowest concentration level (1×VL for non-authorized compounds and 0.5×VL for other analytes).

CCβ is the lowest concentration of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. It is calculated as the decision limit plus 1.64 times the standard deviation of the reproducibility at the corresponding concentrations.

6.3 Results and discussion

6.3.1 LC-ESI-MS/MS determination

6.3.1.1 ESI-MS/MS optimization – Ionization study of Aminoglycosides

The ultimate goal of this study was the development of a multi-residue method for the determination of polar and non-polar veterinary drugs and pharmaceuticals, including aminoglycosides which have rarely been included in multi-residue/multi-class analytical methods. Since aminoglycosides' different physicochemical properties render their simultaneous determination with other veterinary drugs quite problematic, an extended investigation of the chromatographic behavior of

these antibiotics was performed in order to increase their sensitivity and make their simultaneous chromatographic detection with other drugs efficient.

Initially, experiments for the determination and tuning of the precursor and product ions for the 6 aminoglycosides under HILIC conditions were carried out. Direct infusion of individual standards of each aminoglycoside at concentration of 10 μg mL^{-1} in three different solvents – mobile phases was performed in positive ionization mode. The solvents tested were

(A) ACN/aqueous ammonium formate 1 mM with 0.1 % formic acid (60/40, v/v),

(B) (ACN /aqueous ammonium formate 1 mM) with 0.1 % formic acid (60/40, v/v),

(C) ACN/MeOH/aqueous ammonium formate 1 mM with 0.1 % formic acid (50/10/40, v/v),

The mass spectra for all aminoglycosides were obtained in full-scan MS mode and the abundance of precursor ions was compared at the different mobile phases. The mass spectra of streptomycin and dihydrostreptomycin revealed monoprotonated ions $[M+H]^+$ as base peaks. For streptomycin also the ion with m/z 600 was observed, which corresponds to the $[M+H_2O+H]^+$ ion. For kanamycin, the sodiated ion $[M+Na]^+$ appeared as the base peak with the monoprotonated ion at lower abundance. For apramycin and neomycin both single-charged $[M+H]^+$ and double-charged $[M+2H]^{2+}$ pseudomolecular ions were revealed in the mass spectra along with the formation of a strong sodium adduct of neomycin.

Gentamicin, as it is well known, is not a single molecule but a complex of three major and several minor components [247]. Gentamicin's forms C1 ($C_{21}H_{43}N_5O_7$, Mr: 477), C1a ($C_{19}H_{39}N_5O_7$, Mr: 449) and C2 ($C_{20}H_{41}N_5O_7$, Mr: 463) are the three major components of the drug complex. C2 form consists of two stereoisomers (C2 and C2a). In gentamicin's mass spectra the ions $[C2/C2a+Na]^+$ (m/z 486), $[C1a+Na]^+$ (m/z 472), $[C1+ Na]^+$ (m/z 500) and $[C1+ H]^+$ (m/z 478) were obtained.

The solvent composition that presented the highest signal/noise ratio of the precursor ions of AGs was ACN/aqueous ammonium formate 1 mM with 0.1 % formic acid (60/40, v/v). Neither the addition of formic acid in the organic phase nor the addition of MeOH resulted in increasing the ionization efficiency. For

apramycin, gentamicin and neomycin the sensitivity acquired (abundance of precursor ion) was rather insufficient, especially bearing in mind the low MRL of gentamicin in muscle (50 μg kg^{-1}). The low water content solvents are not suitable for these aminoglycosides which are not even well soluble in such solvents [248]. These compounds are the most strongly retained aminoglycosides in HILIC columns and they often need a very significant increase to the aqueous phase content in the mobile phase to be eluted (up to 95% aqueous phase) [243, 248].

Taking these facts into consideration a further optimization of the ionization efficiency of apramycin, gentamicin and neomycin was held. Direct infusion of AGs' standards prepared in various solvents was performed and the abundance of precursor ions was compared. The aqueous/organic ratio of all the tested solvents was 95/5 (v/v) to match the eluting conditions of the analytes. Organic phase consisted of methanol or acetonitrile and the aqueous phase of water with different mobile phase additives, such as formic acid, acetic acid, ammonium acetate and ammonium formate at various concentrations. All solvents – mobile phases examined are summarized in **Table S6.2** and the schematic results of the optimization are presented in **Figures S6.1 – S6.3**.

Apramycin and gentamicin showed maximum abundance when ammonium formate 1mM with 0.1% formic acid – MeOH (95/5, v/v) was used as the mobile phase for the infusion experiments (standard solution solvent). Neomycin also gave satisfactory results in this mobile phase, mainly for the $[M+H]^+$ ion. The optimum mobile phase for neomycin proved to be ammonium formate 1mM with 0.0.5 % formic acid – ACN (95/5, v/v) (for $[M+H]^+$ ion) but this was not suitable for the other aminoglycosides which did not present satisfactory ionization. In most cases MeOH as the organic solvent induced signal enhancement for the aminoglycosides, while the increase of the ionic strength (buffer concentration) caused signal suppression, as expected in ESI [243].

Different percentages of formic acid (0.01–0.1 %) were also tested. A significant increase in the $[M+H]^+$ abundance was observed when increasing the formic acid, favouring the formation of the charged species. Thus, ammonium formate 1 mM with 0.1% formic acid – MeOH (95/5, v/v) was chosen as the optimum mobile

phase to elute these three compounds from HILIC columns achieving maximum ionization.

Subsequently, collision energies for all analytes were optimized in order to find the most abundant product ions, selecting the most sensitive transition for quantification purposes and a second one for confirmation. For most of the compounds the $[M+H]^+$ appeared to be the most abundant ion. Penicillins were monitored as their $[(M+H_2\text{-}CO)+H]^+$ degradation products since they are subjected in a β-lactam ring-opening.

Ionophores, such as monensin and semduramycin formed strong sodium adducts while for narasin, nigericin, manduramycin and salinomycin ammonium adducts where the most abundant ones. Sodium adducts of aminoglycosides showed very poor fragmentation with low abundance of product ions, which would decrease the sensitivity of the method. As a result, monoprotonated ions were selected as precursor ions for all aminoglycosides except for apramycin for which a product ion of the $[M+2H]^+$ ion was selected as the qualifier. The precursor and product ions selected for all other target analytes, are presented in **Table 6.1**.

6.3.1.2 LC-MS/MS optimization

The lack of retention of highly hydrophilic compounds on reversed phase chromatography (RP) had at first been supplemented with ion exchange chromatography or ion paring on RP. However, these techniques were not suitable for the determination of strongly hydrophilic compounds which could not receive charge in solution and which had not been able to receive retention on any stationary phase [249]. The problem has been overcome in LC developing hydrophilic interaction chromatography (HILIC) [250].

In the present study, HILIC was the technique of choice for the rapid and reliable determination of veterinary drugs and pharmaceuticals for two reasons: firstly because very polar compounds like aminoglycosides, sulfaguanidine and amprolium present lack of retention on reversed phase chromatographic columns. The second reason is that an increased MS sensitivity is generally observed in HILIC due to mobile phase's high organic content which assures

high efficiency of spraying and desolvation techniques and, since for many of the target analytes MRLs on MLs are established at particularly low concentration levels (< 10 μg kg^{-1}), low CCα and CCβ values must be achieved in order to meet EU requirements.

Two chromatographic columns with different stationary phases were tested: bare silica (Acquity BEH HILIC, 2.1 × 100 mm, 1.7 μm, Waters) and aminopropyl (APS-2 Hypersil, 2.1 × 50 mm, 3 μm, Thermo). In aminopropyl stationary phase the functional group is an aminopropyl ligand with a primary amino group that is positively charged while bare silica the underivatised silanol groups act as the functional group and are themselves both acidic and hydrophilic in nature. Bare silica also belongs in the charged stationary phases (negatively charged) [249]. Strong electrostatic interactions can take place between the analytes and the charged stationary phases. The hydrophilic partition of the analytes and hydrogen bonding also contribute to the retention.

The mobile phase that was used was a mixture of ACN (solvent A), ammonium formate 1mM with 0.1% formic acid (solvent B) and MeOH (solvent C), as chosen from the ionization optimization experiments. A gradient program of 80% A – 20% B (t_0) increasing linearly to 95% B – 5% C in 8 min and being held at this percentage for additional 5 min, at a flow rate of 100 μL min^{-1}, was tested in both HILIC columns and the elution of all analytes was compared, in terms of peak area and peak shape. Most of the non polar or medium polarity analytes were adequately separated and determined in both stationary phases, showing low retention times, good peak shape and satisfactory sensitivity. However, the chromatographic behaviour of aminoglycosides was significantly different in these two HILIC columns.

APS-2 Hypersil revealed very strong retention of AGs leading to bad peak shape with considerable peak tailing (symmetry > 1.4). Although AGs are positively charged compounds and the predominance of repulsion effects with the positevely charged aminopropyl phase would be expected, partitioning and hydrogen bonding seemed to be the preponderant retention mechanism leading to strong retention of the compounds in the column. The increase of the flow rate

to 300 μL min^{-1} resulted in a noticeable improvement of the peak shape but it could not compensate for the significant decrease in sensitivity.

On the other hand, when BEH HILIC bare silica column was tested, aminoglycosides were less retained and the peak shapes were improved. The use of mobile phase with pH <3 with silica stationary phase led to sharper and more symmetrical peaks due to minimised silanol interactions, as it has already been noted in previous studies [244]. Thus, BEH HILIC column was used for the simultaneous determination of 76 veterinary drugs and pharmaceuticals.

Subsequently, different elution programs were tested in BEH HILIC and the sensitivity of each compound (peak area) along with the peak shape and signal to noise were compared for all target analytes. Due to the very different physicochemical properties of the target compounds (from polar aminoglycosides to hydrophobic ionophore coccidiostats), no isocratic elution program was feasible and so a gradient program was used in order to elute all compounds in one chromatographic run. Seven different gradient elution programs were tested and they are presented in **Table S6.2**. They all start with 80% ACN – 20% ammonium formate 1mM with 0.1% formic acid (t_0) except for gradient 5 that starts with 75% ACN – 20% ammonium formate 1mM with 0.1% formic acid – 5% MeOH.

The elution of the analytes is held either in different isocratic steps in the same chromatogram (Gradients 1, 4, 5, 6) or in linear gradients (Gradients 2, 3, 7). In order to test the efficiency of the isocratic elution for all analytes, two different isocratic elution steps needed to be set in each chromatographic program. The first one was set in 80% organic - 20% aqueous phase for 8 min to elute all analytes except for aminoglycosides and then a sharp increase of the aqueous phase (in 0.1min) led to the second isocratic step (90% or 95% of the aqueous phase for 5 min). Aminoglycosides were eluted during the second isocratic step. Three different linear gradients were also tested, starting from 80% organic phase and coming up to a very high portion of aqueous mobile phase (90% or 95%) in 10 min, a portion which is maintained constant for additional 4 min to complete the elution of even the most strongly retained analytes (neomycin and apramycin).

Aminoglycosides showed very interesting variations in their peak shape and sensitivity during these experiments. A mixture of 95% ammonium formate 1mM with 0.1% formic acid with 5% MeOH proved to be the most suitable mobile phase for the elution of apramycin, gentamycin and neomycin coming in agreement with the ionization optimization experiments. A significant increase in the sensitivity of those compounds is observed when linear gradient elution is performed, rather than isocratic elution; peak shape was also improved. A linear gradient starting from 80% ACN – 20% ammonium formate 1mM with 0.1% formic acid to 95% ammonium formate 1mM with 0.1% formic acid – 5% MeOH gave the most satisfactory results, with the sensitivity of apramycin, kanamycin and neomycin being almost two times higher than when isocratic elution is performed. Taking into consideration that these are the most strongly retained compounds in HILIC columns it is safe to conclude that a linear increase of the aqueous mobile phase reduces effectively the retention of the analytes, promoting their elution with maximum sensitivity. However, this does not seem to be the case for the other three aminoglycosides (streptomycin, dihydrostreptomycin and gentamycin) which presented insignificant differences in their sensitivity and chromatographic behaviour. Chromatograms of apramycin, kanamycin and neomycin obtained (A) with the linear gradient of choice and (B) with isocratic elution with 95% ammonium formate 1mM with 0.1% formic acid – 5% MeOH are presented in **Figure 6.1.** As it can be seen, significant increase in the peak area as well as in the signal to noise ratio is achieved when the analytes are eluted using a linear gradient program. In addition, the retention times of the compounds when linear gradient is used are considerably reduced resulting in a smaller overall run time, ensuring the analysis of a larger number of samples in one laboratory day.

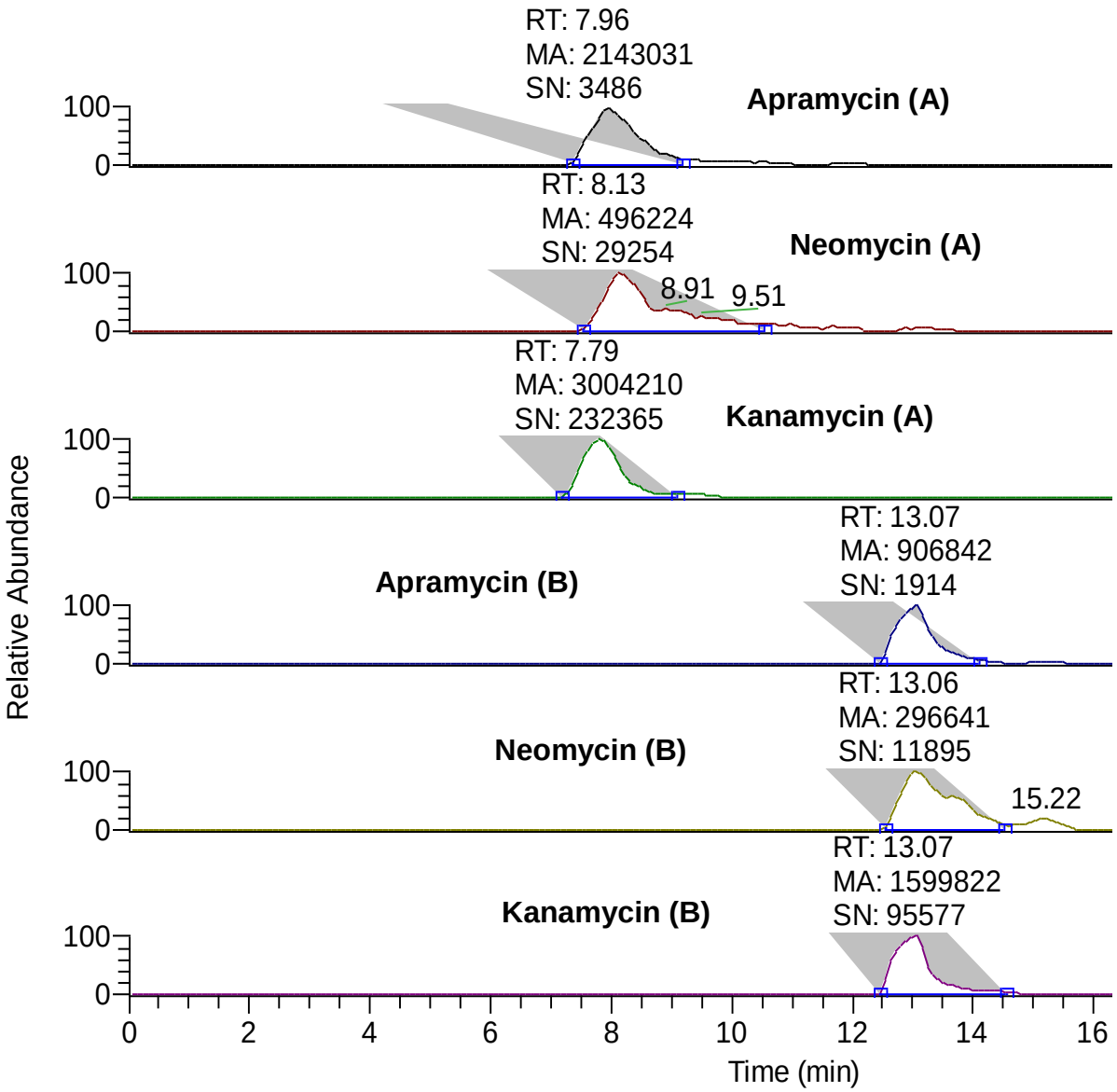


Figure 6.1: Chromatograms of apramycin, neomycin and kanamycin obtained (A) with a linear gradient starting from 80% ACN – 20% ammonium formate 1mM with 0.1% formic acid to 95% ammonium formate 1mM with 0.1% formic acid – 5% MeOH in 10 min and (B) with isocratic elution with 95% ammonium formate 1mM with 0.1% formic acid – 5% MeOH

All results for aminoglycosides in the optimization of the elution program experiments are presented in **Figure S6.4**. Each gradient program was tested by measuring three times a standard solution of all analytes at a concentration of 2×VL. Results are presented as %Relative Peak Area where the first injection of gradient 1 represents 100%. Standard deviations are also calculated and shown in the chart. In addition, %RSDs of the retention times of AGs in each gradient were calculated. %RSDs were in all cases <1.5%, indicating the excellent stability and repeatability of the chromatographic detection.

Unlike aminoglycoside compounds, for most of the other analytes the differences in sensitivity were negligible when changing the gradient elution program. **Figure S6.5** illustrates the compounds with the most noticeable variations in their sensitivity during elution program optimization experiments.

Thus, the ternary linear gradient starting from 80% ACN – 20% ammonium formate 1mM with 0.1% formic acid, increasing to 95% ammonium formate 1mM with 0.1% formic acid – 5% MeOH in 10 min and remaining stable in this composition for additional 4 min was chosen as the final elution program for the determination of veterinary drugs and pharmaceuticals. Along with the re-equilibration time needed, the overall run time was 20 min. This total run time is quite small for a multi-residue method including AGs, especially the strongly retained neomycin and apramycin. It should be noted that even specific HILIC methods for the determination of AGs which contain the strongly retained AGs report overall run times > 15 min [241, 243].

Finally, the injection solvent composition was investigated and optimized in terms of sensitivity (peak area) and peak shape. Five different standard solutions of all analytes at a concentration of 2×VL were constructed in five different injection solvents and each one was measured in triplicate with the final gradient program (gradient 2). The injection solvents tested were (I) 50 MeOH:50 FA 0.1%, (II) 20 MeOH:80 FA 0.1%, (III) 80 MeOH:20 FA 0.1%, (IV) 60 ACN:20 MeOH: 20 FA 0.1% and (V) 80ACN: 20 FA 0.1%. The results of the optimization for all compounds are summarized in **Figure S6.6**. Results are presented as %Relative peak area with the first injection of the standard solution in solvent (I) representing 100%. Standard deviations of the measurements are displayed in

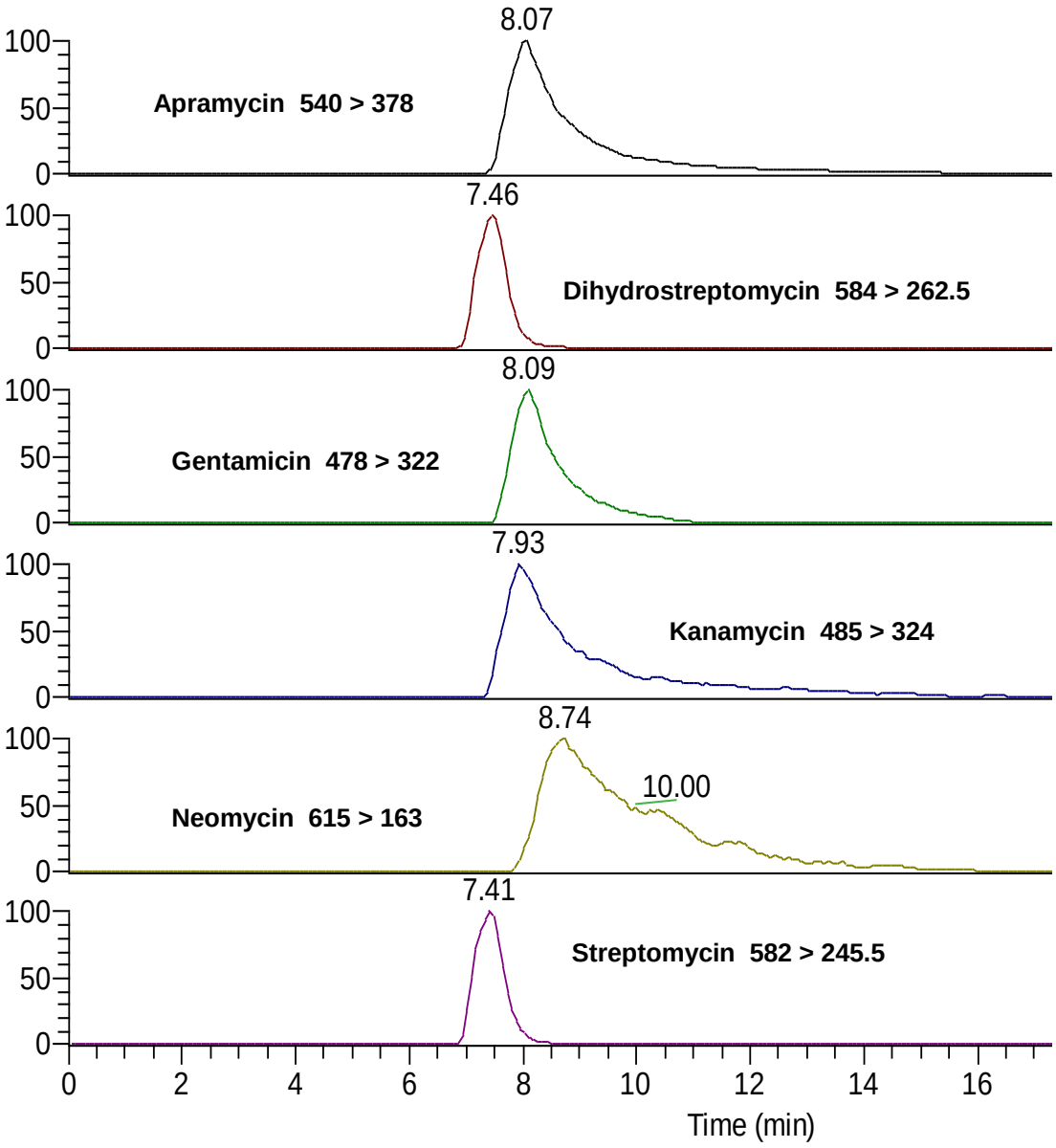
the graphs. %RSDs of the retention times were for all the target compounds <1.5%.

The increase in the portion of ACN in the injection solvent resulted in a significant improvement in the peak shape of most of the compounds. The sensitivity was also increased in most cases, with the exception of streptomycin, dihydrostreptomycin and gentamycin which were favored by the presence of MeOH in the final injection solvent.

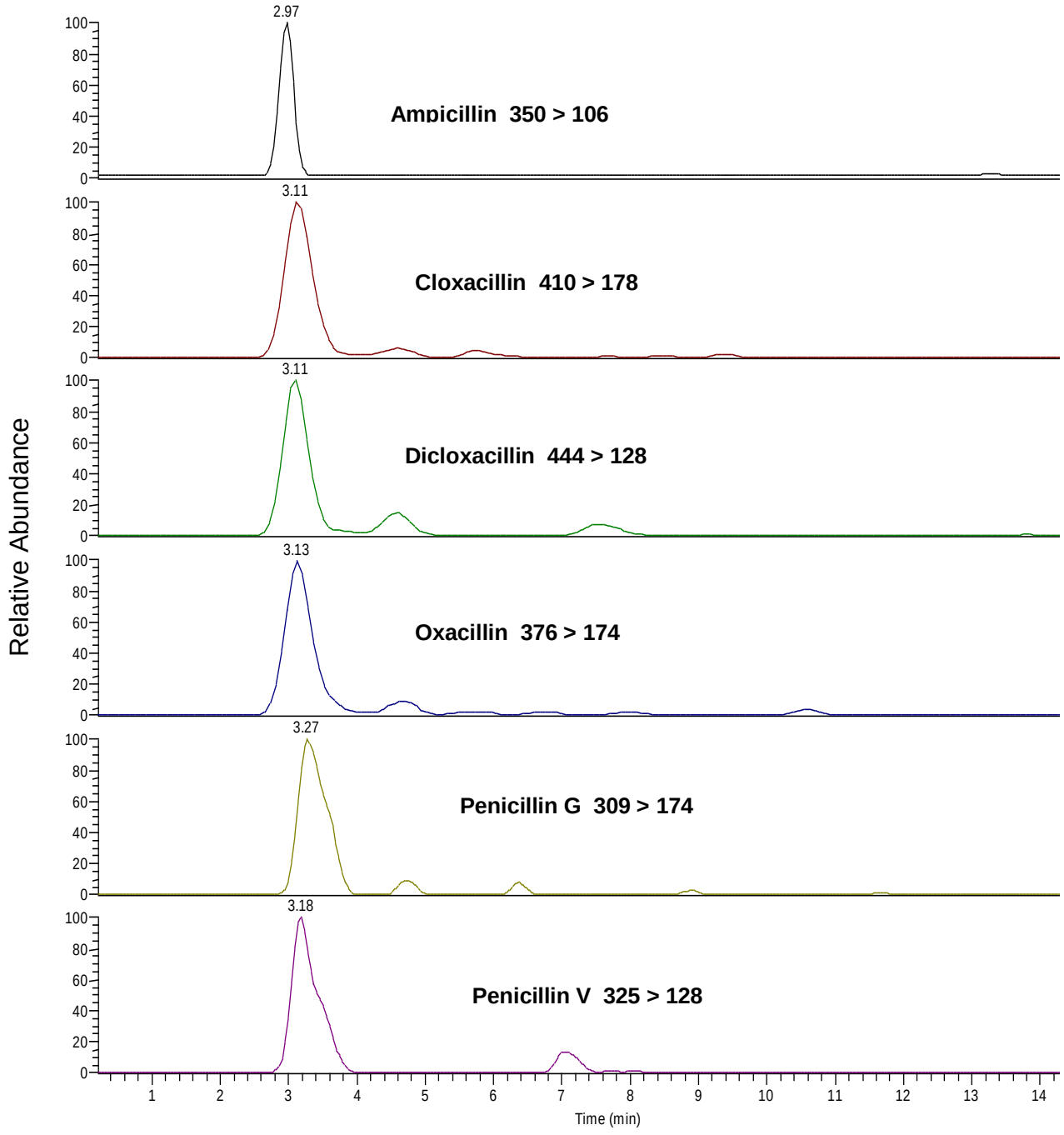
Conclusively, in the developed HILIC-MS/MS method significantly low sensitivity is achieved for many compounds in a quite short total run time, comparing to other HILIC methods reported in the literature but also to reversed-phase chromatographic methods. Apart from aminoglycosides which were thoroughly investigated and optimized, many non-polar and hydrophobic compounds (ionophore coccidiostats, macrolides, statins) could be detected at very low concentration levels due to their affinity with the high organic portion of the mobile phase and the high ionization efficiency they presented in HILIC conditions. This has been very important in order to meet the EU Regulation requirements which in some cases (eg. for coccidiostats) has set MRLs and MLs at low-ppb levels. Further discussion about the sensitivity of the developed method is held in Method Validation Section (Section 6.3.3).

A HILIC–MS/MS chromatogram of a spiked bovine tissue sample at the 1×VL concentration (1.5×VL for prohibited substances) is presented in **Figure 6.1**

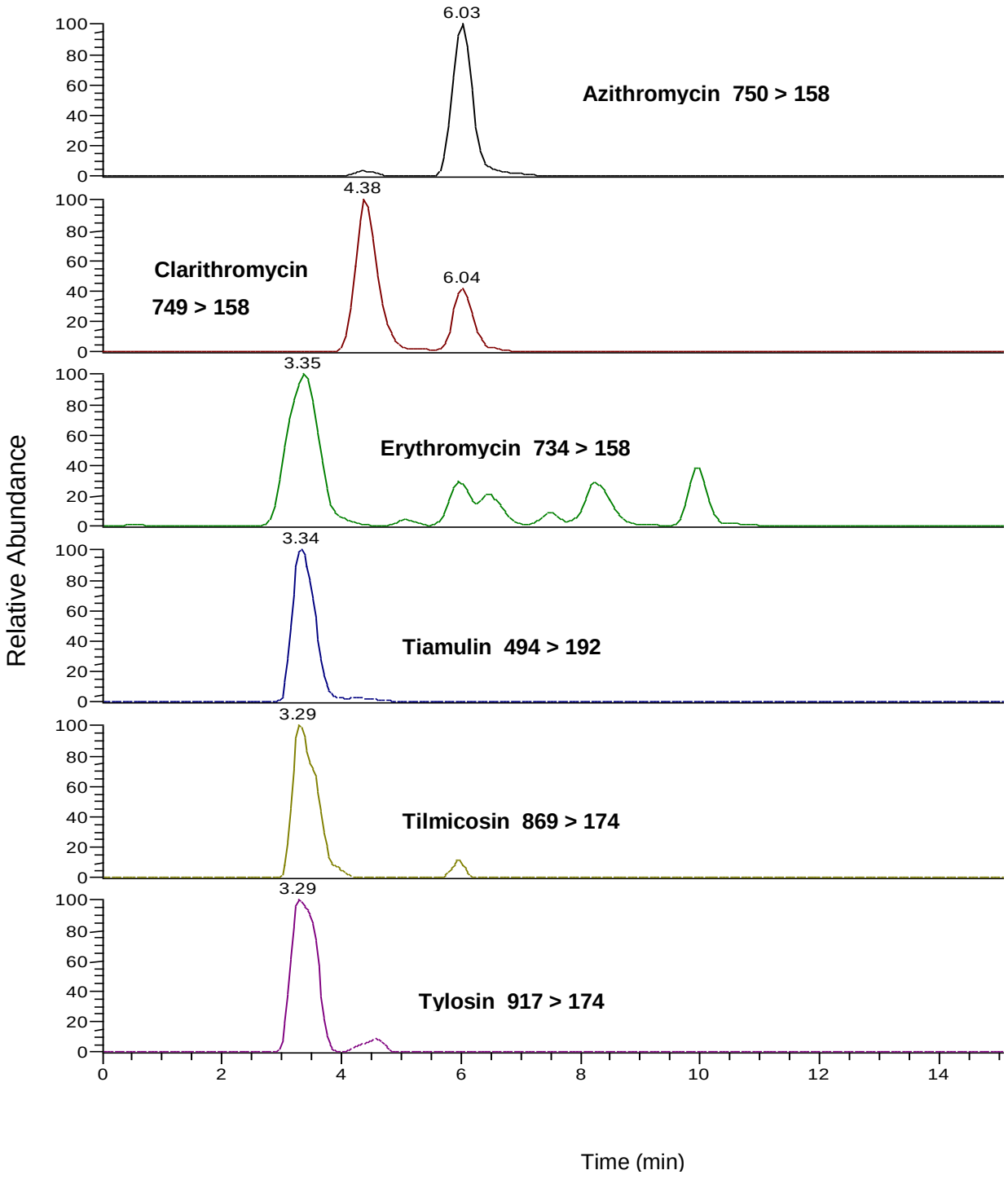
Aminoglycosides



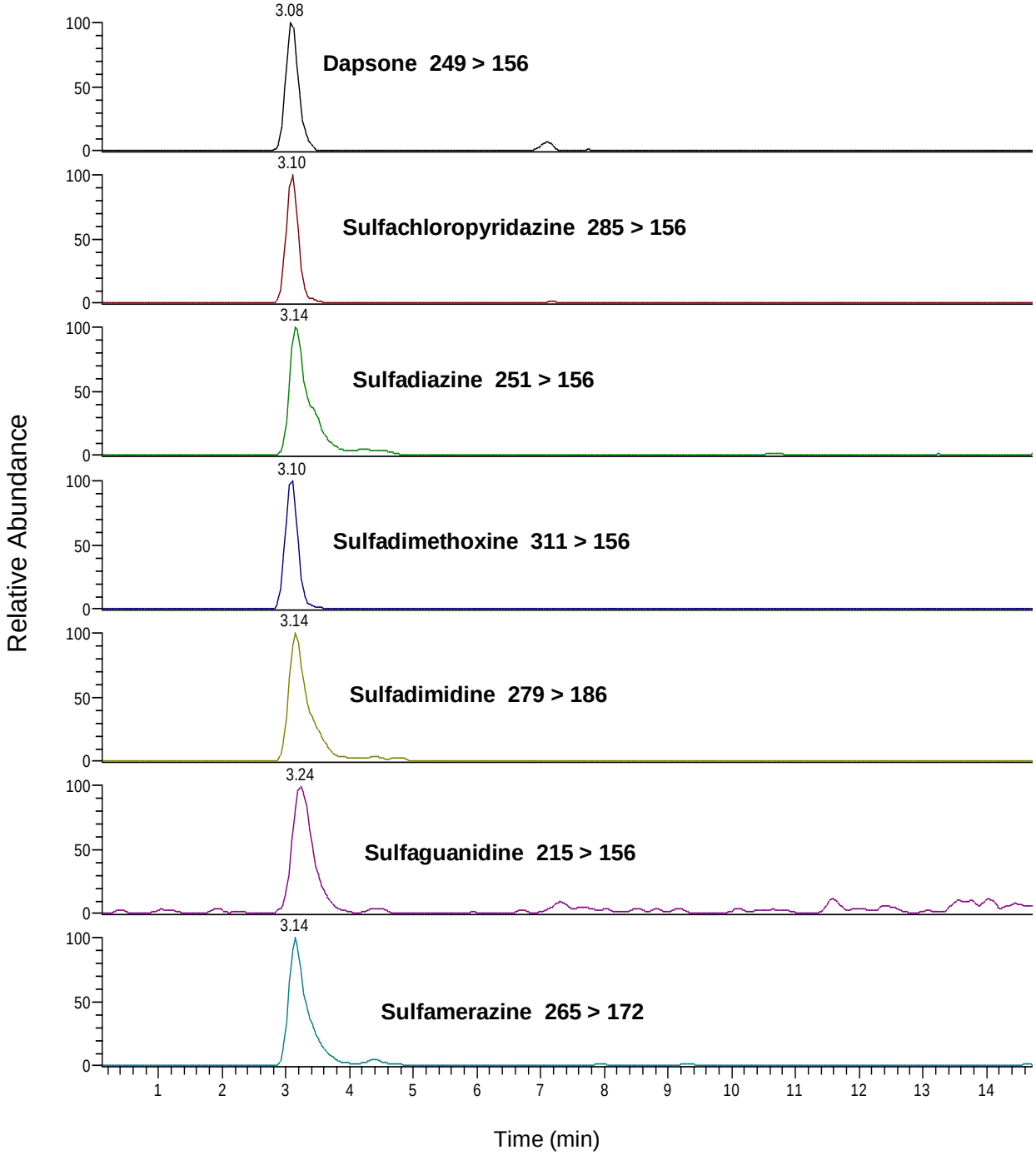
Penicilins


Relative Abundance
2.97
Ampicillin 350 > 106
3.11
Cloxacillin 410 > 178
3.11
Dicloxacillin 444 > 128
3.13
Oxacillin 376 > 174
3.27
Penicillin G 309 > 174
3.18
Penicillin V 325 > 128
Time (min)

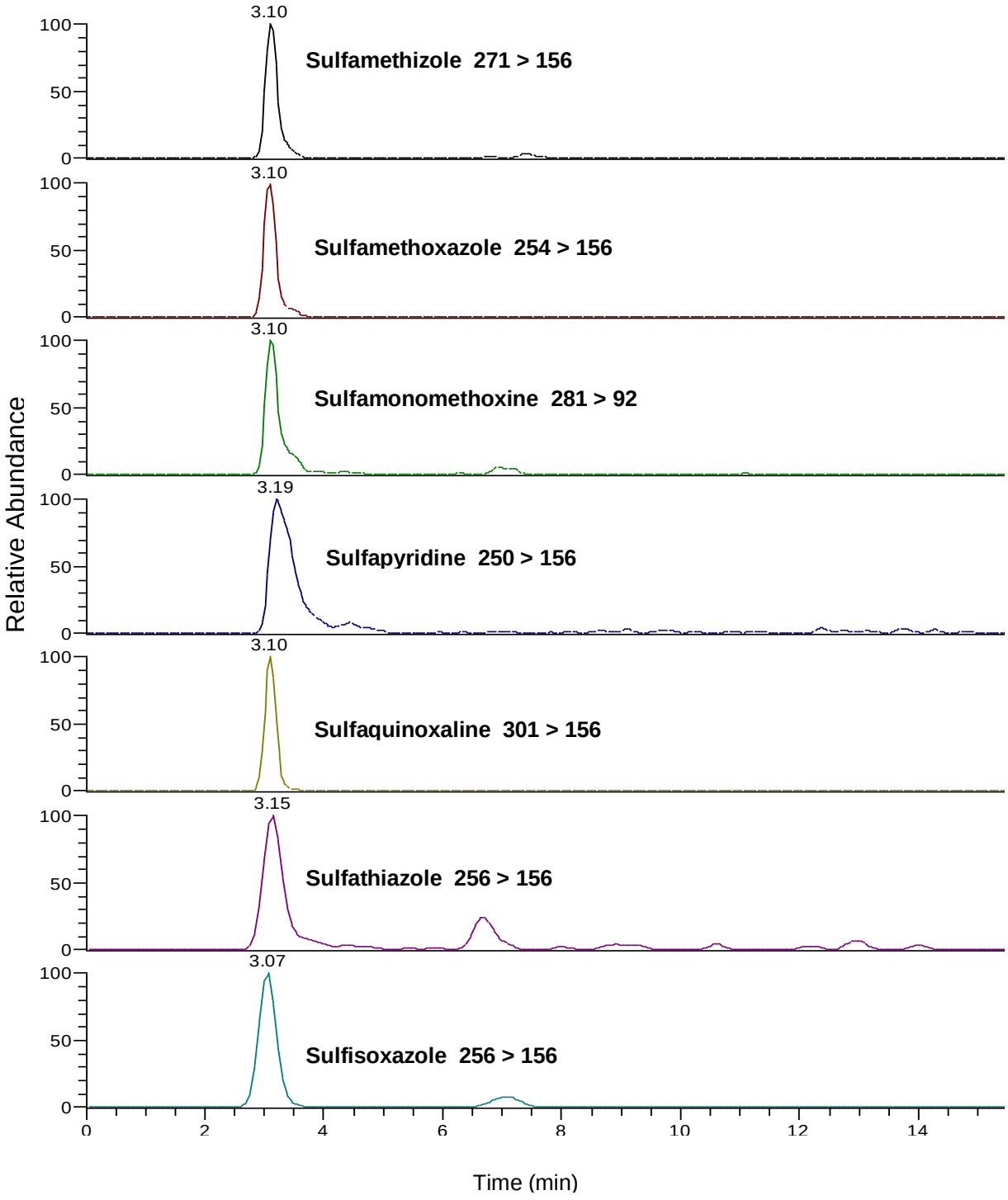
Macrolides

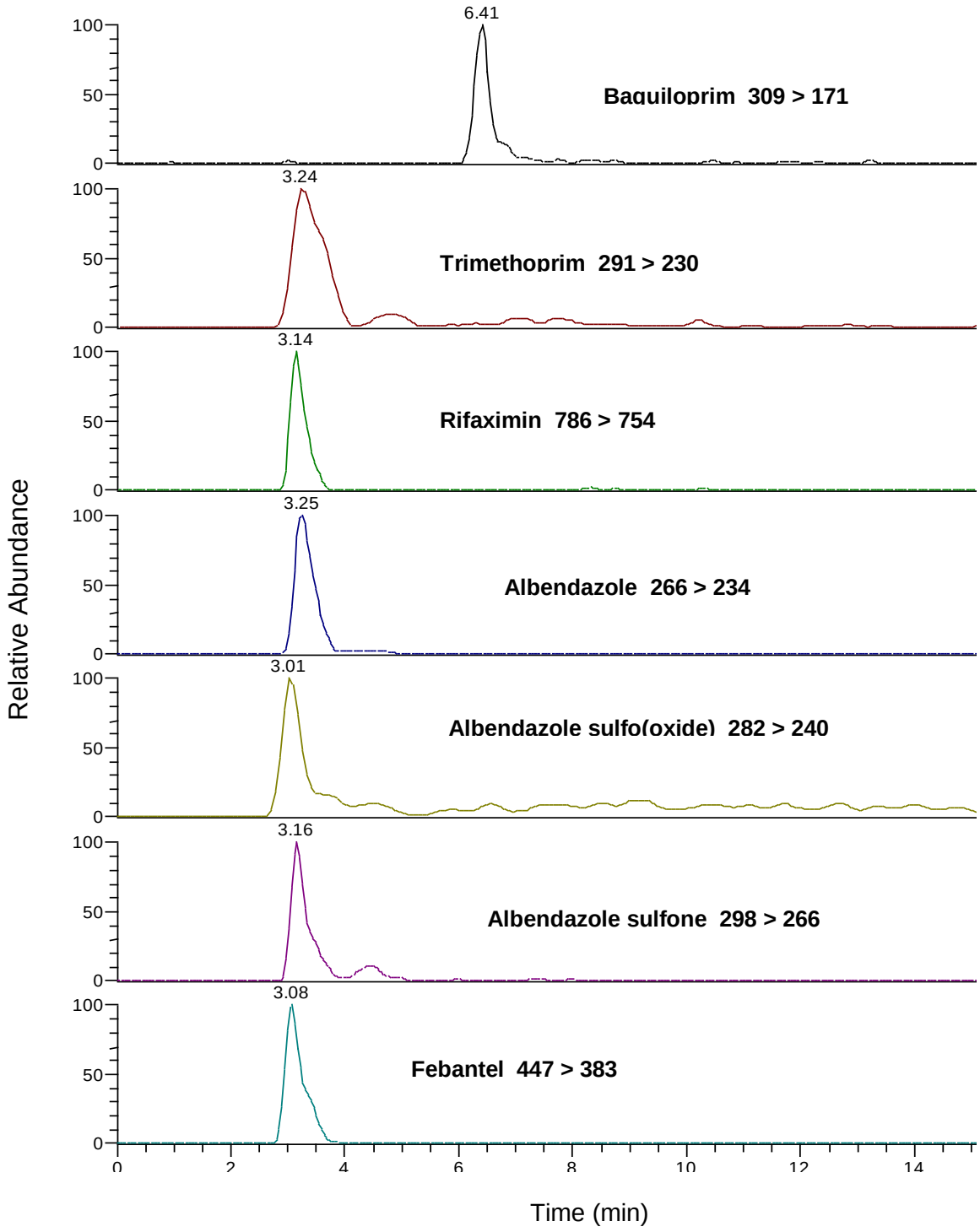

6.03
Azithromycin 750 > 158
4.38
Clarithromycin
749 > 158
6.04
3.35
Erythromycin 734 > 158
3.34
Tiamulin 494 > 192
3.29
Tilmicosin 869 > 174
3.29
Tylosin 917 > 174
Relative Abundance
Time (min)

Sulfonamides

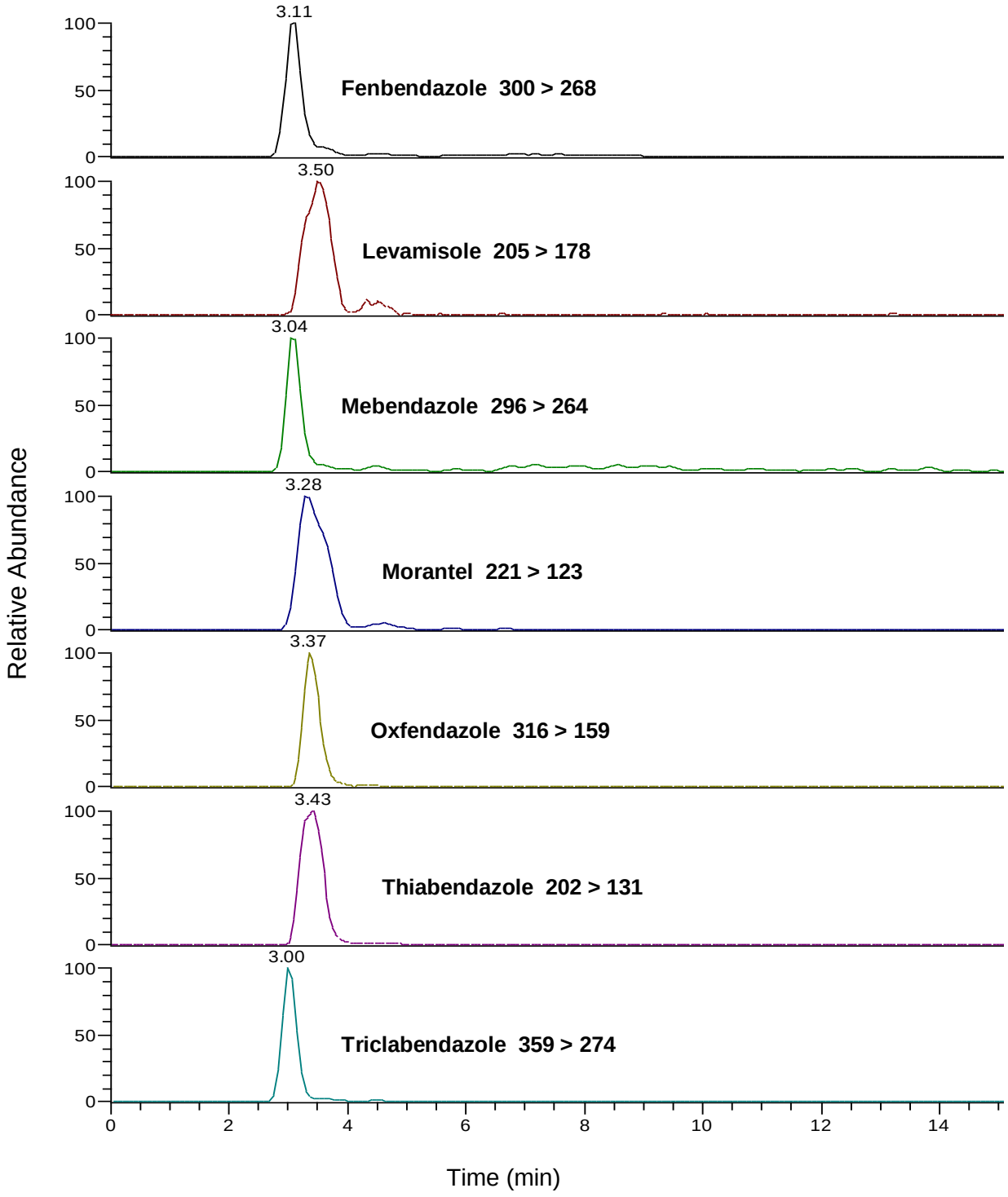


Sulfonamides (B)

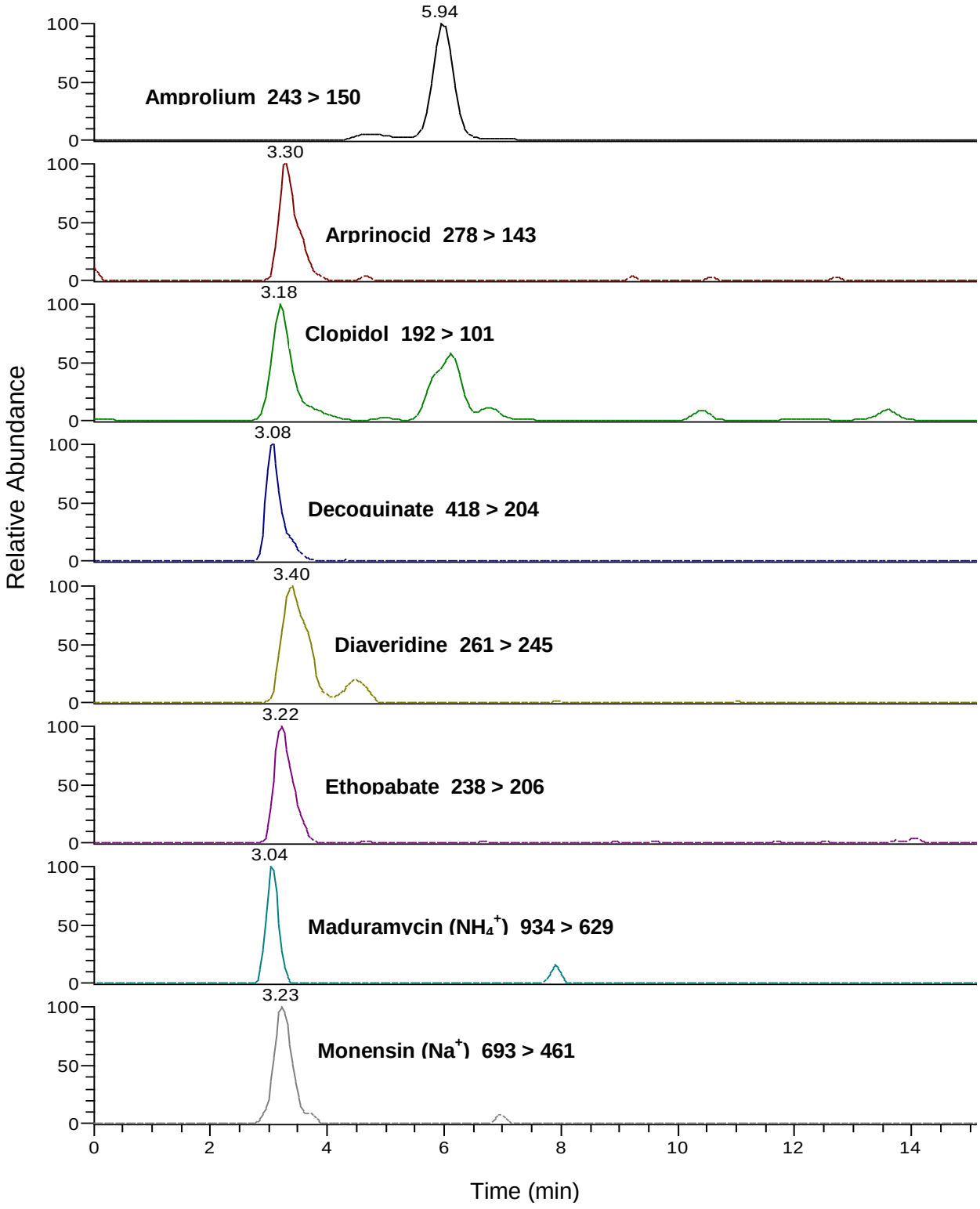



6.41
Baquiloprim 309 > 171
3.24
Trimethoprim 291 > 230
3.14
Rifaximin 786 > 754
3.25
Albendazole 266 > 234
3.01
Albendazole sulfo(oxide) 282 > 240
3.16
Albendazole sulfone 298 > 266
3.08
Febantel 447 > 383
Relative Abundance
Time (min)

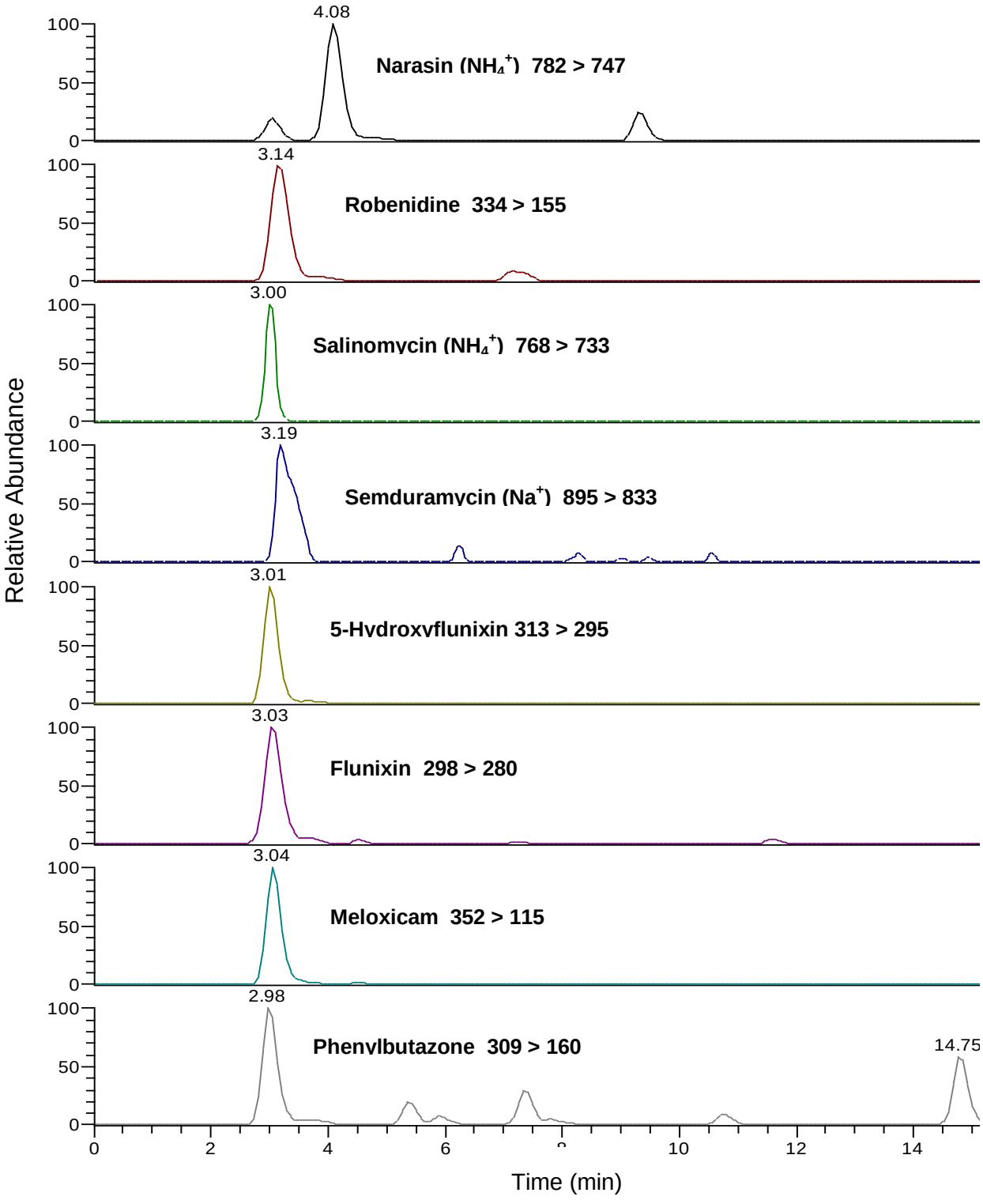
Anthelmintics (B)

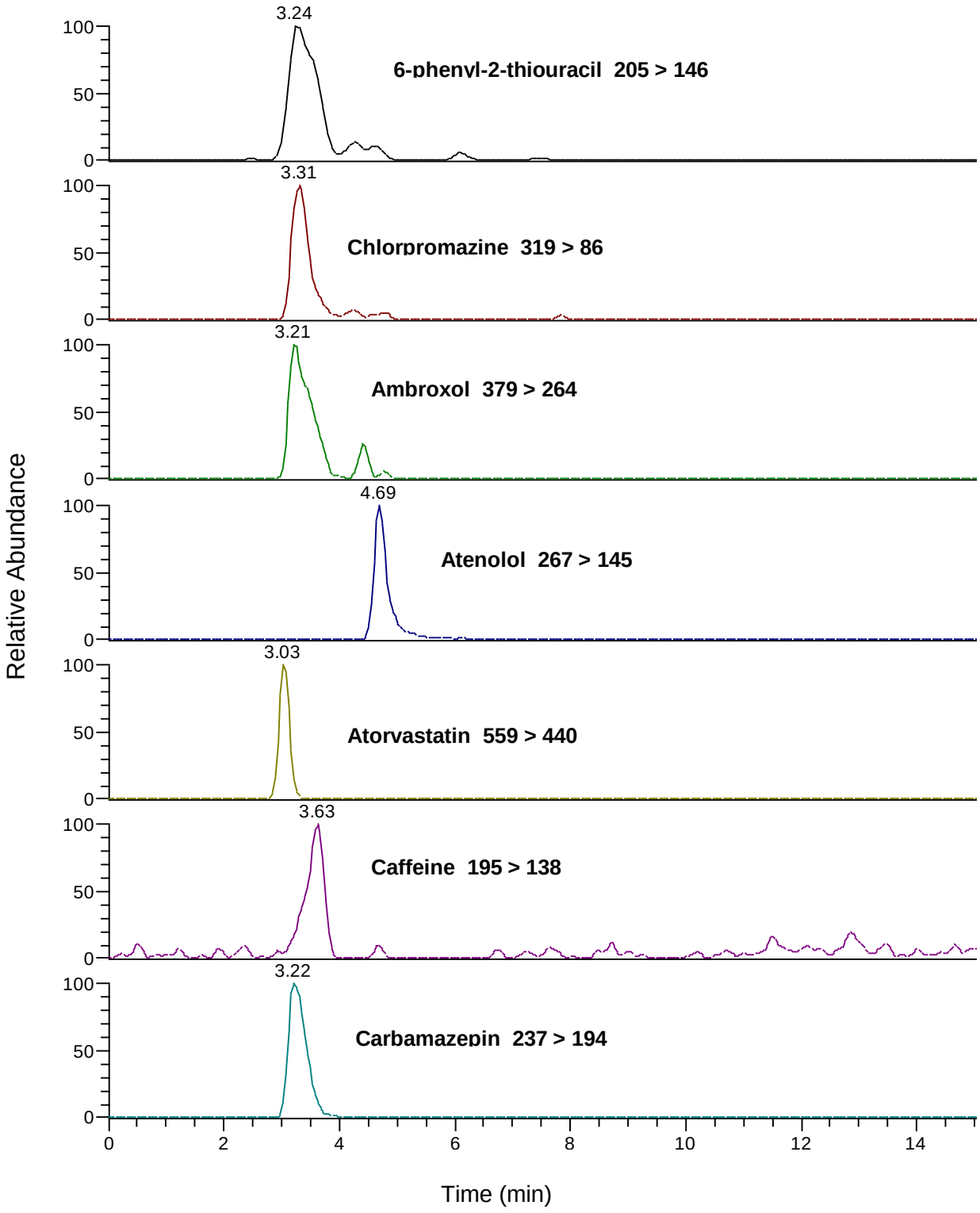


Coccidiostats

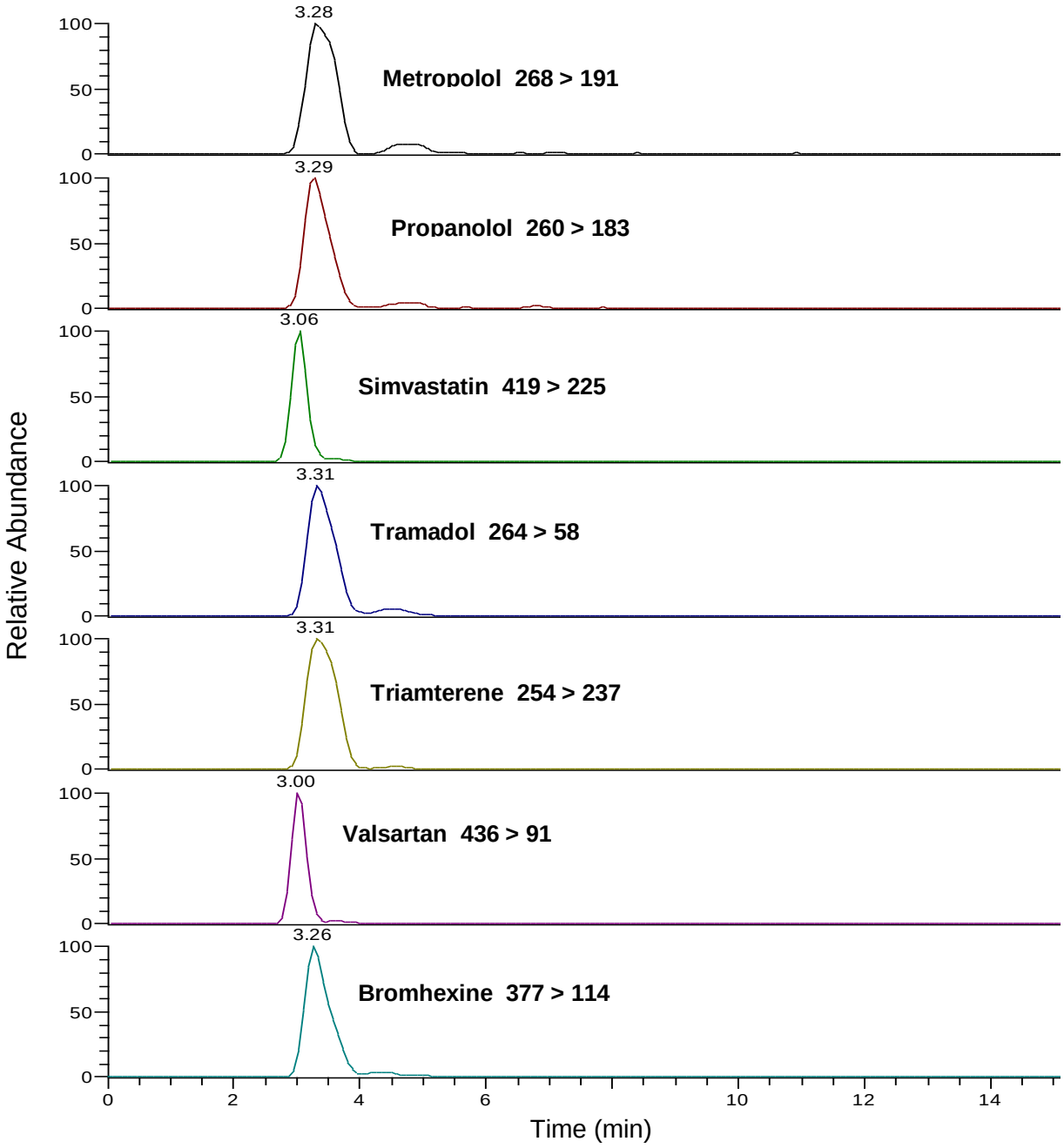


Coccidiostats (B) – NSAIDs

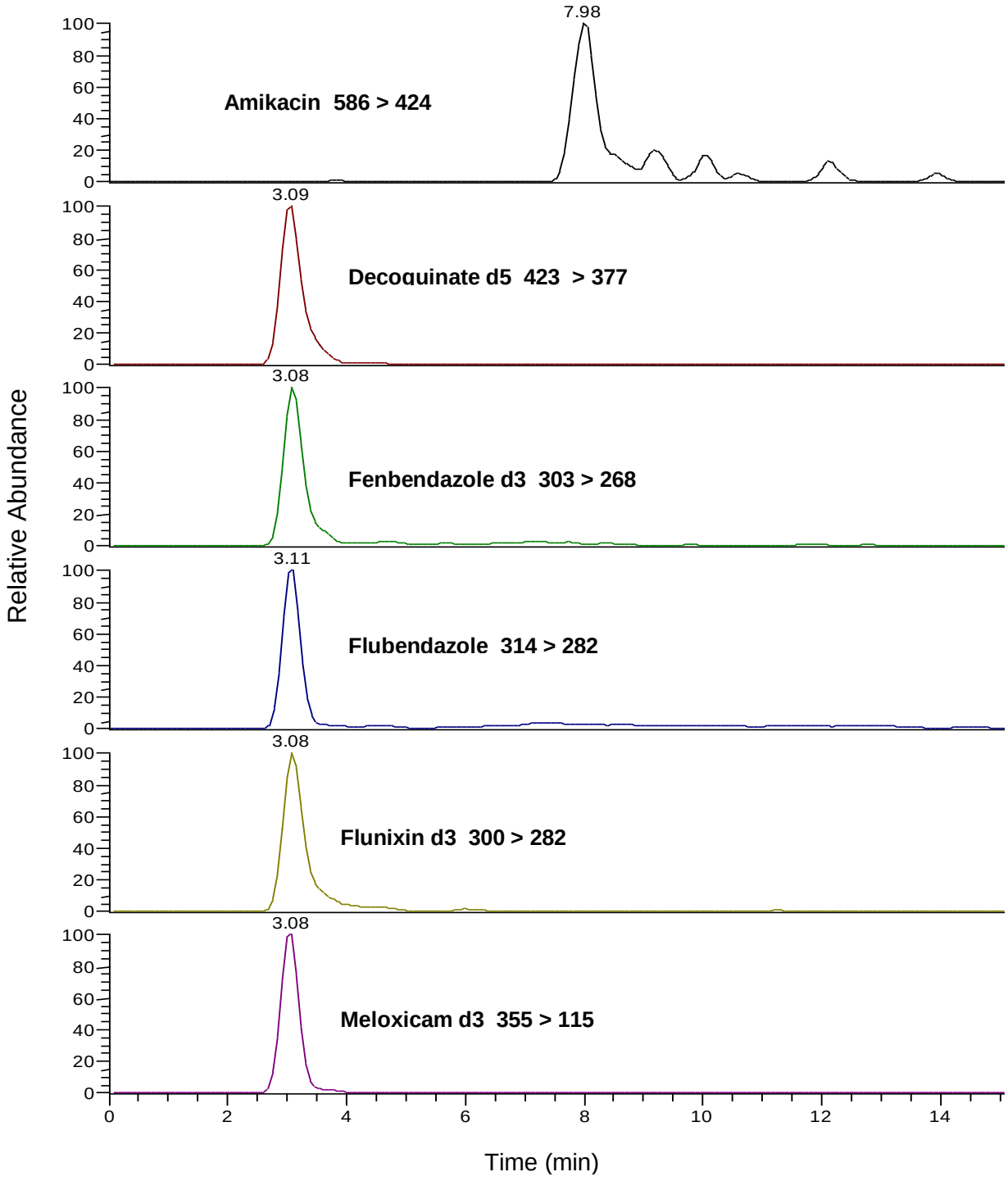



3.24
6-phenyl-2-thiouracil 205 > 146
3.31
Chlorpromazine 319 > 86
3.21
Ambroxol 379 > 264
4.69
Atenolol 267 > 145
3.03
Atorvastatin 559 > 440
3.63
Caffeine 195 > 138
3.22
Carbamazepin 237 > 194
Relative Abundance
Time (min)

Pharmaceuticals - Others



Internal Standards



Internal Standards (B)

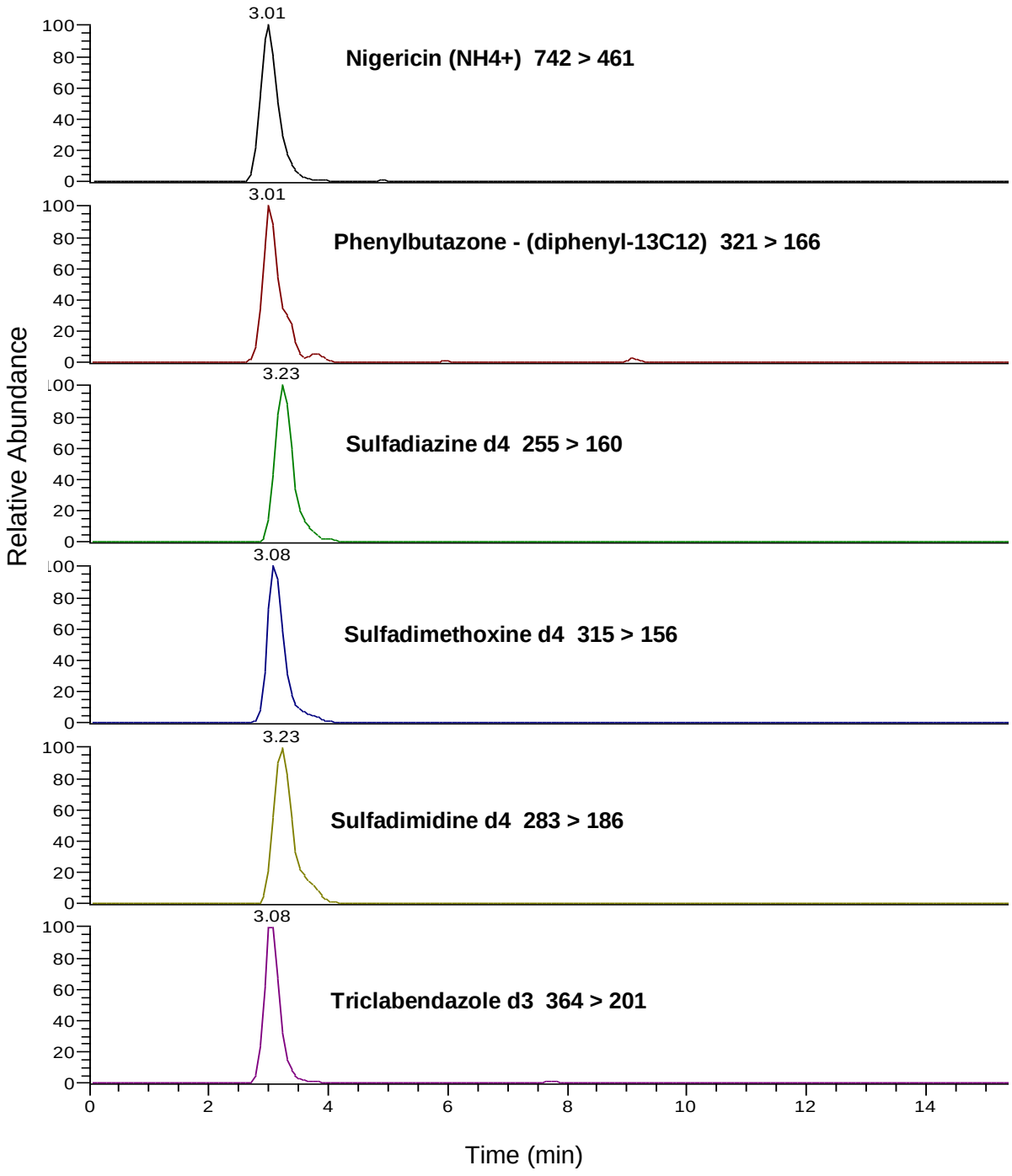


Figure 6.2: SRM chromatogram of spiked bovine muscle tissue sample at a fortification level of 1×VL for all target compounds (1.5×VL for prohibited veterinary drugs).

6.3.2 Sample Preparation

Finding suitable extraction conditions for the simultaneous extraction of the extremely polar aminoglycoside antibiotics along with a large number of other compounds with different physicochemical properties (lipophilicity, hydrophilicity, alcaline and acidic characteristics, etc) composes a great challenge in multiresidue analysis and has rarely been reported previously. An additional difficulty is presented in obtaining a final extract suitable for AGs' determination and HILIC-compatible (>60% ACN) at the same time since AGs are not even well soluble at low water content solvents [248].

In this study, a two-step sample preparation protocol was used for the simultaneous extraction of the 76 veterinary drugs and pharmaceuticals, along with 12 internal standards, from bovine muscle tissue samples. The first step consists of a solvent extraction with ACN, employed in order to extract the medium polarity and non-polar compounds with high lipophilicity. ACN has been reported to effectively extract antibiotics, anthelmintics and coccidiostats from different matrices [75, 223, 228]. The ACN extract is evaporated to a final volume of 1.0 mL after the extraction.

The second step consists of a solvent extraction with an aqueous buffer, in strong acidic conditions (2% TCA). The second extraction solvent consists of ammonium acetate, sodium chloride, EDTA and TCA and is mainly targeted in the extraction of aminoglycosides and other polar compounds like penicillins, thyreostats and sulfaguanidine. Aminoglycosides tend to bind to proteins and so strong acidic conditions are necessary for their extraction from tissue. EDTA is used as a chelating agent to improve the extraction recovery of aminoglycosides as it prevents their rapid chelation with metal ions [243]. After the extraction further clean up and preconcentration of the samples is performed using SPE with HLB cartridges. This copolymer of divinylbenzene and vinylpyrrolidone sorbent has broad applicability spectra due to its hydrophilic and lipophilic properties and has been widely used in the field of veterinary drug and pharmaceutical multiresidue analysis [75, 89, 90, 91, 224].

After the extraction the pH of the extract was adjusted to 6.5 with NaOH 30%, w/v. Approximately 6 drops of NaOH solvent were needed to obtain the desired

pH value and the value was verified using a pH-meter device. Different pH values were tested (5.5, 6.5 and 7.5) with pH 6.5 proving to be optimum for aminoglycosides' determination. The effect of pH value on other compounds' recoveries was insignificant and thus this value was chosen and used in the final sample preparation protocol. Finally, for the elution of the analytes a mixture of aqueous formic acid 10 % (v/v) and ACN (1:4) was used. Strong acidic conditions are required in order to elute AGs [246, 248, 251] and 2×0.5 mL 10% of aqueous formic acid were used as indicated by Lehotay et al [246]. An organic solvent was also used since in the field of multiresidue analysis the vast majority of studies report the use of organic solvents for the elution of veterinary drugs and pharmaceuticals [75, 89, 90, 91, 224]. An organic solvent is also required for gentamicin's elution [248].

The eluate (4.0 mL) was mixed with the remaining 1.0 mL of the ACN extract from the previous extraction step; the final extract consists of a 5-mL solution of 80% ACN and 20% aqueous formic acid, a constitution that matches the optimum ACN/formic acid portion that has resulted from the injection solvent optimization (Section 6.3.1.2).

6.3.3 Method Validation

The method was validated according to European Commission Decision 2002/657/EC. This Decision establishes criteria and validation procedures to ensure the quality and comparability of analytical results obtained. Some analytes are authorized, having corresponding MRL values, some are prohibited and some are not at all regulated, making the validation procedure for multi-residue methods rather difficult to design.

To the best of our knowledge, it is the first method reported in literature, including AGs, in which EU Regulation requirements are fulfilled for so many veterinary drugs with large variations in the established MRLs and MLs (from 1000 μg kg^{-1} for apramycin to 2 μg kg^{-1} for some coccidiostats).

6.3.3.1 Identification, confirmation and quantification

According to the criteria established in the EU Commission Decision 2002/657/EC an analyte is considered as positively identified and confirmed in a sample when the relative retention time ratio of the analyte in the sample and in standard solution is within ± 2.5 % tolerance, both SRMs for the analyte are present in the sample and the ratio of the intensities of the two transitions (quantifier and qualifier) matches the one obtained using fortified blank samples within the defined tolerance [54]. The use of two selected precursor-product ion transition per compound counts for four identification points, which fulfill the EU identification point's requirement. Ion Ratios for all compounds at the 1×VL level are presented in **Table 6.2**, along with the corresponding tolerances.

The quantification procedure of a target analyte that is detected in an unknown sample involves the use of the standard addition method with calibration samples that were spiked with the analytes before extraction, in different concentrations. This quantification method is recommended to get reliable quantitative results without the need for correction for recovery losses and for matrix effects. The use of isotopic analogue internal standards, where available, also ensures the reliability of the quantitation since their use can efficiently compensate for matrix suppression or enhancement as well as for recovery losses.

Table 6.2: Ion Ratios for all compounds in spiked samples at the 1×VL level.

Compound (n=10)	Ion Ratio (q/Q) × 100%	RSD %	Tolerance levels (2002/657/EE)	Compound (n=10)	Ion Ratio (q/Q) × 100%	RSD %	Tolerance levels (2002/657/EE)
Aminoglycosides				Febantel	33.2	6.7	± 25 %
Apramycin	67.1	8.4	± 20 %	Fenbendazole	84.0	6.0	± 20 %
Dihydrostreptomycin	57.0	7.6	± 20 %	Levamisole	36.5	14.4	± 25 %
Gentamycin	4.3	25.8	± 50 %	Mebendazole	29.7	9.9	± 25 %
Kanamycin	89.5	7.3	± 20 %	Morantel	85.7	4.9	± 20 %
Neomycin	70.6	12.8	± 20 %	Oxfendazole	29.3	17.0	± 25 %
Streptomycin	37.6	14.8	± 25 %	Thiabendazole	92.0	5.7	± 20 %
Penicillins				Triclabendazole	6.1	3.3	± 50 %
Ampicillin	78.2	21.1	± 20 %	***Coccidiostats***			
Cloxacillin	92.2	5.3	± 20 %	Amprolium	16.7	10.3	± 30 %
Dicloxacillin	74.5	10.0	± 20 %	Aprinocid	13.4	16.3	± 30 %
Oxacillin	72.8	12.7	± 20 %	Clopidol	18.9	19.9	± 30 %
Penicillin G	88.5	4.5	± 20 %	Decoquinate	60.3	8.8	± 20 %
Penicillin V	90.5	15.7	± 20 %	Diaveridine	38.2	15.2	± 25 %
Macrolides				Ethopabate	44.7	21.2	± 25 %
Azithromycin	24.2	17.7	± 25 %	Maduramycin (NH_4^+)	32.4	18.7	± 25 %
Clarithromycin	29.4	18.7	± 25 %	Monensin (Na^+)	24.8	20.5	± 25 %
Erythromycin	77.2	4.5	± 20 %	Narasin (NH_4^+)	13.8	23.8	± 30 %
Tiamullin	13.5	3.8	± 30 %	Robenidine	76.7	14.7	± 20 %
Tilmicosin	21.7	20.2	± 25 %	Salinomycin (NH_4^+)	39.8	18.0	± 25 %
Tylosin	57.9	16.8	± 20 %	Semduramycin (Na^+)	26.1	6.8	± 25 %

Sulfonamides				***NSAIDs***			
Dapsone	45.4	21.3	± 25 %	5-Hydroxyflunixin	2.3	12.1	± 50 %
Sulfachloropyridazine	34.9	12.1	± 25 %	Flunixin	4.3	13.1	± 50 %
Sulfadiazine	46.6	16.1	± 25 %	Meloxicam	59.0	10.6	± 20 %
Sulfadimethoxine	32.4	3.6	± 25 %	Phenylbutazone	44.6	22.5	± 25 %
Sulfadimidine	28.1	6.0	± 25 %	***Thyreostats***			
Sulfaguanidine	1.4	18.8	± 50 %	6-phenyl-2-thiouracil	0.5	17.6	± 50 %
Sulfamerazine	92.0	9.3	± 20 %	***Tranquilizers***			
Sulfamethizole	32.9	12.0	± 25 %	Chlorpromazine	23.1	20.0	± 25 %
Sulfamethoxazole	50.5	6.2	± 20 %	***Pharmaceuticals***			
Sulfamonomethoxine	94.8	6.3	± 20 %	Ambroxol	5.3	29.2	± 50 %
Sulfapyridine	72.7	12.1	± 20 %	Atenolol	62.3	11.9	± 50 %
Sulfaquinoxaline	32.9	12.7	± 25 %	Atorvastatin	48.5	7.2	± 25 %
Sulfathiazole	40.5	15.5	± 25 %	Caffeine	46.8	12.3	± 25 %
Sulfisoxazole	31.8	11.2	± 25 %	Carbamazepin	24.8	10.5	± 25 %
Diaminopyrimidines				Metropolol	64.4	8.4	± 20 %
Baquiloprim	33.4	15.7	± 25 %	Propranolol	34.5	8.2	± 25 %
Trimethoprim	25.1	6.8	± 25 %	Simvastatin	63.2	16.1	± 20 %
Other antibiotics				Tramadol	11.0	3.7	± 30 %
Rifaximin	14.6	15.9	± 30 %	Triamterene	19.2	4.9	± 30 %
Anthelmintics				Valsartan	81.3	5.1	± 20 %
Albendazole	98.0	3.4	± 20 %	**Others**			
Albendazole sulfo(oxide)	59.0	7.4	± 20 %	Bromhexine	67.8	17.3	± 20 %
Albendazole sulfone	65.5	9.2	± 20 %				

6.3.3.2 Selectivity/specificity

The selectivity of the method was evaluated extracting and analyzing 20 control blank bovine tissue samples. No background peaks, above a signal-to-noise ratio of 3, were present at the same elution time as the target veterinary drugs and pharmaceuticals. This shows that the method is free of endogeneous interferences.

6.3.3.3 Linearity

The linearity of calibration curves was assessed by using a six-point standard solution calibration curve in pure solvents as well as in blank bovine mucle tissue extracts at different concentrations (0.25 to 4×VL for each target compound). The linear regression analysis was carried out by plotting the peak area versus the analyte concentrations for compounds with no corresponding IS and the peak area ratio of the analyte and I.S. versus the analyte concentrations, when an IS correction was used. The calibration parameters showed good linearity since correlation coefficients were >0.99 for all analytes. R^2 ranged from 0.998 (triamterene) to 0.9998 (erythromycin) for standard solution curves in pure solvent and from 0.998 (sulfamethizole) to 0.9999 (erythromycin) in matrix extracts.

6.3.3.4 Precision

The precision of this method was calculated as intra-day precision (repeatability) and inter-day precision (within-laboratory reproducibility), Repeatability and reproducibility were expressed as the %RSD values of set of 6 replicate analysis at the 3 concentration levels examined (0.5, 1 and 1.5 times the VL except for prohibited veterinary drugs for which the concentrations corresponded at 1, 1.5 and 2 times the VL). Reproducibility experiments lasted three consecutive days. Precision results for all compounds in all concentration levels are presented in

Table 6.3.

Table 6.3: Precision, LOD, LOQ, CCα and CCβ values for target veterinary drugs and pharmaceuticals.

	Repeatability % RSD			Reproducibility % RSD			LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	MRL ($\mu g\ kg^{-1}$)	CCα ($\mu g\ kg^{-1}$)	CCβ ($\mu g\ kg^{-1}$)
Compound	**0.5 × VL**	**1× VL**	**1.5×VL**	**0.5 × VL**	**1× VL**	**1.5×VL**					
Aminoglycosides											
Apramycin	8.8	10	6.7	13	10	8.9	22	74	1000	1151	1302
Dihydrostreptomycin	15	12	10	16	15	9	9.8	32	500	594	688
Gentamycin	16.1	12.0	5.9	14	9.3	6.8	7.0	23	50	58	66
Kanamycin	9.4	11.0	4.3	12	10	6.1	17	56	100	118	135
Neomycin	7.5	7.9	5.5	14	11	6.8	49	163	500	539	577
Streptomycin	17	14	13	17	15	15	54	178	500	660	820
Penicillins											
Ampicillin	6.2	5.1	10	14	8.6	8.3	3.8	13	50	56	60
Cloxacillin	12	10	9.0	18	15	9.8	1.1	3.5	300	355	389
Dicloxacillin	12	11	4.3	11	14	15	2.4	8.0	300	340	364
Oxacillin	8.8	6.9	6.6	12	7.7	13	1.5	4.9	300	325	340
Penicillin G	10	14	11	13	7.0	15	2.1	7.0	50	55	58
Penicillin V	5.2	9.3	9.1	12	16	12	3.5	12	-	5.4	8.5
Macrolides											
Azithromycin	9.7	5.9	3.4	14	12	12	0.48	1.6	-	13	21
Clarithromycin	14	13	11	17	12	14	0.90	3.0	-	20	32
Erythromycin	13	13	6.2	14	11	11	51	167	200	225	240
Tiamullin	6.1	8.2	1.5	11	8.1	8.3	0.066	0.22	50	110	116
Tilmicosin	11	6.5	5.4	12	6.7	13	0.56	1.9	100	54	56

Tylosin	12	4.2	9.2	13	13	11	2.2	7.3	100	115	125
Sulfonamides											
Dapsone*	8.9	8.9	8.2	18	17	6.0	0.11	0.37	prohibited	2.7	3.7
Sulfachloropyridazine	7.1	9.6	12	17	12	14	0.16	0.52	100	116	126
Sulfadiazine	11	9.0	5.1	11	14	12	1.9	6.3	100	122	135
Sulfadimethoxine	7.9	4.2	2.1	12	14	4.3	0.053	0.18	100	119	130
Sulfadimidine	7.7	5.5	6.5	7.5	5.4	12	0.022	0.072	100	109	114
Sulfaguanidine	5.7	8.5	11.0	14	7.4	11	1.8	5.9	100	111	118
Sulfamerazine	12	6.2	6.5	14	13	14	0.42	1.4	100	121	135
Sulfamethizole	13	8.6	10.3	15	13	12	0.47	1.5	100	116	125
Sulfamethoxazole	8.8	9.1	1.9	13	16	6.3	0.12	0.40	100	118	129
Sulfamonomethoxine	8.9	7.7	2.3	15	4.5	8.8	0.49	1.6	100	107	112
Sulfapyridine	12	5.0	6.6	9.7	14	14	0.74	2.4	100	124	138
Sulfaquinoxaline	11	7.6	2.9	16	14	8.4	0.15	0.48	100	122	135
Sulfathiazole	10	6.1	2.1	13	12	10	2.0	6.5	100	118	128
Sulfisoxazole	6.9	8.6	5.0	11	13	6.1	0.26	0.86	100	119	131
Diaminopyrimidines											
Baquiloprim	9.5	14	7.6	12	9.5	7.0	0.30	1.0	-	8.5	12
Trimethoprim	9.5	10	6.4	15	11	13	0.072	0.24	50	57	61
Other antibiotics											
Rifaximin	6.1	7.0	4.1	13	12	15	0.18	0.59	-	8.4	13.0
Anthelmintics											
Albendazole	6.1	5.7	5.6	6.7	15	14	0.058	0.19	100	121	134
Albendazole sulfo(oxide)	13	11	7.6	12	11	6.1	0.14	0.46	100	120	132

Albendazole sulfone	12	7.0	4.3	14	15	11	0.14	0.45	100	124	138
Febantel	8.5	8.0	4.4	10	13	11	0.017	0.056	50	60	66
Fenbendazole	4.7	10	4.5	9.8	11	11	0.0092	0.030	50	57	62
Levamisole	10	11	6.8	11	11	14	0.047	0.16	10	12	13
Mebendazole	13	13	7.3	16	10	10	0.078	0.26	-	6.5	11
Morantel	5.8	1.7	3.9	13	16	7.8	0.10	0.33	100	122	135
Oxfendazole	13	14	9.4	17	12	15	0.078	0.26	50	59	65
Thiabendazole	8.3	9.0	7.0	10	7.9	7.0	0.076	0.25	100	112	120
Triclabendazole	7.2	6.6	11.8	17	8.7	8.6	0.027	0.088	225	252	269
Coccidiostats											
Amprolium	17	16	6.6	16	14	9.7	0.091	0.30	-	3.2	5.1
Aprinocid	12	7.3	2.1	16	13	8.0	0.27	0.88	-	7.0	9.5
Clopidol	11	8.1	8.5	10	10	15	0.97	3.2	-	4.7	7.2
Decoquinate	6.5	7.4	6.9	9.0	10	13	0.044	0.15	20	3.8	4.9
Diaveridine	5.1	5.5	3.7	9.6	9.9	6.3	0.028	0.093	-	2.4	3.4
Ethopabate	16	13	5.7	14	11	6.5	0.11	0.36	-	4.2	6.1
Maduramycin (NH_4^+)	18	10	9.2	18	13	15	0.13	0.42	2	2.3	2.5
Monensin (Na^+)	14	15	15	13	19	9.7	0.095	0.31	2	2.4	2.6
Narasin (NH_4^+)	13	7.7	10	17	18	14	0.036	0.12	5	6.2	6.9
Robenidine	10	12	5.6	15	15	10	0.081	0.27	5	6.1	6.7
Salinomycin (NH_4^+)	11	5.7	10	15	11	11	0.012	0.038	2	2.3	2.4
Semduramycin (Na^+)	6.7	8.0	8.3	9.4	12	10	0.025	0.082	2	2.3	2.5
NSAIDs											
5-Hydroxyflunixin	6.4	4.1	3.8	6.6	12	9.9	0.015	0.049	-	2.3	3.0

Flunixin	7.2	7.4	1.7	11	12	8.4	0.034	0.11	20	23	25
Meloxicam	5.0	5.5	6.8	13	9.0	10	0.047	0.16	20	22	23
Phenylbutazone*	10	4.7	3.1	17	13	6.9	0.33	1.1	-	2.2	3.0
Thyreostats											
6-phenyl-2-thiouracil	7.8	6.2	2.1	9.5	5.6	7.2	1.2	3.9	-	2.5	3.2
Tranquilizers											
Chlorpromazine*	9.3	6.8	7.0	17	12	6.8	0.054	0.18	prohibited	4.1	6.6
Pharmaceuticals											
Ambroxol	6.7	8.0	1.8	18	6.4	12	0.36	1.2	-	8.6	13
Atenolol	13	11	11	17	11	9.8	0.65	2.1	-	7.7	13
Atorvastatin	5.2	5.7	4.1	15	15	9.8	0.21	0.68	-	13	17
Caffeine	14	12	8.4	14	12	15	0.72	2.4	-	32	37
Carbamazepin	8.5	6.8	6.5	16	13	8.1	0.32	1.1	-	14	19
Metropolol	8.3	5.7	5.0	12	7.8	11	0.39	1.3	-	20	26
Propranolol	8.4	6.8	2.3	10	6.3	6.6	0.086	0.28	-	27	30
Simvastatin	13	5.5	8.2	16	11	14	0.43	1.4	-	18	25
Tramadol	7.9	10	5.3	11	15	5.9	0.049	0.16	-	11	15
Triamterene	4.9	4.3	2.7	16	9.7	17	0.024	0.081	-	11	16
Valsartan	10	7.9	3.0	16	17	12	0.061	0.20	-	15	20
Others											
Bromhexine*	14	14	4.5	14	14	8.6	0.068	0.22	prohibited	5.1	6.0
* prohibited compounds for which the concentrations examined correspond in 1×VL, 1.5×VL and 2×VL											

%Relative standard deviations were always < 20% for all the veterinary drugs and pharmaceuticals tested, indicating the good precision of the preposed methodology. Repeatability in the lowest validation level for all compounds varied from 4.7% (fenbendazole) to 18% (manduramycin).The obtained %RSDs under reproducibility conditions in the lowest validation level ranged from 6.6% (5-hydroxyflunixin) to 19% (manduramycin).

6.3.3.5 Accuracy

The accuracy of the method was estimated through recovery studies, since there is a lack of certified reference material for all the target analytes in the examined matrix. Average recoveries of each analyte were calculated performing the analysis in 18 replicates at each validation level in three different days (6 samples per day per validation level). These results of the recovery study are given in **Table 6.4.** Recoveries at the 0.5×VL varied from 37.4% (bromhexine) to 106% (kanamycin). In spite that some compounds present recovery values not close to 100%, they are considered acceptable since they were reproducible (**Table 6.3**)

6.3.3.6 LODs & LOQs

LODs and LOQs were evaluated as described in the Experimental Section, showing the obtained results in **Table 6.3**. LOQs ranged from 0.030 μg kg^{-1} (fenbendazole) to 178 μg kg^{-1} (streptomycin) and were in all cases lower than the corresponding MRL or ML where one established.

6.3.3.7 Decision limit (CCα) and Detection capability (CCβ)

CCα and CCβ calculation was performed following two different different procedures, based on whether there is an established MRL or ML for the target analyte, or not. All the compounds that do not have established MRLs were treated as banned compounds and the CCα and CCβ were calculated through the calibration curve procedure. Decision limits ranged from 2.3 μg kg^{-1}

(salinomycin, ML: 2 µg kg^{-1}) to 1151 µg kg^{-1} (apramycin, MRL: 1000 µg kg^{-1}) and detection capability from 2.4 µg kg^{-1} (salinomycin) to 1302 (apramycin).

Table 6.4: % Recoveries and % Matrix effect values for all target veterinary drugs and pharmaceuticals.

	0.5 × VL			1× VL			1.5×VL			% ME
Compound	Recoveries	SD	% RSD	Recoveries	SD	% RSD	Recoveries	SD	% RSD	
Aminoglycosides										
Apramycin	101.2	10.0	9.9	97.4	10.4	10.7	99.1	9.0	9.1	-61
Dihydrostreptomycin	80.3	11.5	14.3	77.6	10.9	14.1	79.2	9.8	12.4	-74
Gentamycin	64.3	11.3	17.6	64.3	10.1	15.7	65.5	9.4	14.4	-75
Kanamycin	105.9	13.0	12.2	106.9	12.5	11.7	110.4	11.2	10.2	-34
Neomycin	62.8	7.3	11.9	60.0	5.6	9.4	59.8	5.3	8.9	-53
Streptomycin	97.4	12.8	13.1	98.0	11.4	11.7	96.9	8.2	8.5	-81
Penicillins										
Ampicillin	94.4	12	13	88.0	7.2	8.2	109	10	9.4	-95
Cloxacillin	76.4	14	19	69.6	11	16	78.4	12	15	-99
Dicloxacillin	74.3	12	16	61.1	9.0	15	73.4	13	17	-97
Oxacillin	76.2	8.4	11	65.2	5.0	7.6	71.1	11	15	-99
Penicillin G	90.6	14	16	93.0	7.6	8.2	63.7	9.8	15	-99
Penicillin V	70.7	7.5	11	73.9	10	14	84.0	9.7	12	-99
Macrolides										
Azithromycin	69.8	13	18	78.5	8.9	11	92.5	10	11	-92
Clarithromycin	75.2	12	16	73.7	8.8	12	77.9	12	15	-95
Erythromycin	99.3	16	16	69.0	7.8	11	84.9	9.4	11	-97
Tiamullin	76.1	7.6	10.0	71.9	6.5	9.0	100	8.0	8.0	-73
Tilmicosin	70.1	8.5	12.1	72.4	4.4	6.1	90.2	12	13	-87
Tylosin	76.6	12.4	16.2	70.5	8.3	12	85.0	8.5	10	-91

Sulfonamides										
Dapsone*	70.8	12	17	58.9	9.2	16	55.0	4.6	8.5	27
Sulfachloropyridazine	78.7	14	18	81.2	9.6	12	80.4	10	13	-40
Sulfadiazine	79.3	9.6	12	94.7	12	13	94.7	12	13	-28
Sulfadimethoxine	75.5	11	15	84.8	12	14	101	5.7	5.6	6
Sulfadimidine	86.3	7.6	8.8	96.8	6.8	7.0	86.7	9.4	11	-89
Sulfaguanidine	88.7	11	13	94.7	7.7	8.1	94.6	16	17	-58
Sulfamerazine	96.3	12	13	103.8	12	12	85.0	10	12	-85
Sulfamethizole	82.6	12	15	76.4	9.2	12	75.0	9.9	13	-78
Sulfamethoxazole	83.0	13	16	68.6	9.7	14	78.6	7.0	8.8	5
Sulfamonomethoxine	78.3	13	16	94.2	7.9	8.4	88.2	7.5	8.5	-77
Sulfapyridine	102.7	8.5	8.2	102.6	11	11	78.7	11	14	-87
Sulfaquinoxaline	80.8	15	19	99.9	15	15	100	11	11	-15
Sulfathiazole	76.2	11	14	83.8	13	16	91.0	8.4	9.2	-95
Sulfisoxazole	87.1	13.2	15.1	90.7	14	15	104	12	11	13
Diaminopyrimidines										
Baquiloprim	71.3	6.9	9.6	74.5	9.1	12	81.6	7.7	9.4	-95
Trimethoprim	85.8	14.9	17.3	77.9	13	17	80.2	10	12	-76
Other antibiotics										
Rifaximin	68.0	8.1	12	65.7	7.5	11	86.4	15	17	-95
Anthelmintics										
Albendazole	98.9	10.0	10	82.5	13	16	90.0	11	12	11
Albendazole sulfo(oxide)	84.8	11.4	13	109	14	13	75.7	7.9	10	-94
Albendazole sulfone	78.5	11.0	14	95.8	12	12	91.9	10	11	-88

Febantel	86.7	9.2	10.6	87.9	12	14	91.4	13	15	48
Fenbendazole	74.2	7.0	9.4	74.2	8.1	11	85.5	8.4	9.8	22
Levamisole	92.2	11	12	87.4	12	14	86.2	12	14	-50
Mebendazole	69.9	8.8	13	78.0	9.5	12	83.4	7.6	9.1	-70
Morantel	89.0	12	13	86.0	14	16	91.1	6.6	7.3	-69
Oxfendazole	95.7	15	16	90.7	12	14	85.0	12	14	-83
Thiabendazole	101.8	8.4	8.3	93.0	7.9	8.5	89.1	6.8	7.6	42
Triclabendazole	70.3	8.1	12	80.8	11	13	89.2	7.9	8.9	-22
Coccidiostats										
Amprolium	71.9	10	14	85.0	12	14	74.5	8.0	11	-79
Aprinocid	77.3	8.8	11	82.4	11	13	106	14	13	-89
Clopidol	73.9	10	14	76.7	9.5	12	88.7	12	14	-97
Decoquinate	70.6	6.4	9.0	83.8	13	16	104	14	14	15
Diaveridine	72.1	6.1	8.4	99.5	9.4	9.5	82.0	5.1	6.2	-55
Ethopabate	88.2	12	14	89.7	9.1	10	95.4	5.7	5.9	-92
Maduramycin (NH_4^+)	79.1	15	19	77.0	8.8	11	77.3	12	15	-38
Monensin (Na^+)	54.7	7.9	14	56.7	10	18	69.0	9.7	14	100
Narasin (NH_4^+)	61.9	9.6	15	88.0	14	16	71.7	12	16	-55
Robenidine	66.3	8.4	13	84.9	16	18	70.7	7.3	10	-26
Salinomycin (NH_4^+)	70.1	9.8	14	75.4	8.7	12	76.0	7.5	9.9	-39
Semduramycin (Na^+)	73.1	5.7	7.8	83.1	10	12	88.0	11	12	59
NSAIDs										
5-Hydroxyflunixin	63.0	5.4	8.5	74.7	9.8	13	102	11	10	117
Flunixin	86.9	9.7	11	81.3	9.1	11	100	11	11	15

Meloxicam	67.9	8.7	13	69.4	9.8	14	97.8	9.4	9.6	1
Phenylbutazone*	73.5	9.7	13	58.9	7.2	12	83.7	6.4	7.7	-97
Thyreostats										
6-phenyl-2-thiouracil	89.6	8.0	8.9	80.9	4.8	5.9	102	8.8	8.6	-77
Tranquilizers										
Chlorpromazine*	66.4	10	15	97.4	13	13	88.2	6.2	7.1	-65
Pharmaceuticals										
Ambroxol	60.5	11	18	52.4	9.4	18	82.9	9.4	11	-93
Atenolol	71.2	9.0	13	90.0	11	12	90.7	9.3	10	-96
Atorvastatin	57.2	8.7	15	68.7	12	17	69.0	6.1	8.8	-36
Caffeine	79.9	12	16	90.1	11	12	80.8	12	14	-97
Carbamazepin	79.3	11	14	87.6	14	16	91.8	7.1	7.8	-93
Metropolol	86.1	14	16	94.8	6.9	7.3	94.0	10	11	-21
Propranolol	75.5	8.4	11	75.6	10	14	85.8	5.4	6.3	-36
Simvastatin	84.2	15	18	74.4	10	14	79.4	10	13	-42
Tramadol	79.8	12.7	16.0	88.2	11	13	93.1	5.4	5.8	-45
Triamterene	67.1	12.0	17.9	86.4	9.0	10	95.4	14	15	53
Valsartan	62.8	11.3	18.0	57.7	9.8	17	66.2	7.6	11	-57
Others										
Bromhexine*	37.4	6.6	18	36.0	6.2	17	40.7	4.7	12	-61
* prohibited compounds for which the concentrations examined correspond in 1×VL, 1.5×VL and 2×VL										

6.3.3.8 Matrix Effect

When complex samples, such as muscle are analyzed with LC–ESI-MS/MS significant matrix effects might influence the analysis. the ionization efficiency of the analytes is affect by matrix effects, leading to suppression or enhancement of the signal depending on the analyte/matrix combination. The use of isotope labeled internal standards (ILIS) and the standard addition quantification method can be used to compensate for matrix effect interferences.

To evaluate matrix effect, the slopes obtained from the standard solution curves in solvent were compared with those obtained in matrix extracts. Matrix effects (ME%) were calculated by subtracting 1 from the ratio between the slope of the standard solution curve constructed in matrix extracts (A) and in pure solvent (B), and then multiplying by 100:

$$ME\ (\%) = ((A/B) - 1) \times 100 \qquad (1)$$

The signal is enhanced if the value is positive, whereas it is suppressed if the value is negative. Matrix effect values are presented in **Table 6.4.** Strong signal suppression was revealed for the vast majority of the analytes (63 out of 76 compounds).The quantification of the samples was performed using a standard addition approach.

6.4 Application to Real Samples

To evaluate the applicability of the proposed method in routine analysis, 10 bovine muscle tissue samples, obtained by local supermarkets, were tested. No positive results were found in any of these samples.

6.5 Conclusions

A rapid, sensitive and efficient multiresidue analytical method for the simultaneous determination of 6 AGs and 70 other veterinary drugs and pharmaceuticals in bovine muscle tissue by HILIC–MS/MS has been developed. The simultaneous determination of AGs with other veterinary drugs has only been reported once previously (69) and in much less extend (3 AGs and 21 other

compounds). Aminoglycosides present very different physicochemical properties compared to other drugs making their simultaneous chromatographic detection and matrix extraction quite challenging. The method includes ACN extraction followed by an acidic aqueous buffer extraction, cleanup with HLB cartridges and HILIC-MS/MS determination in bare silica stationary phase (BEH HILIC).

The strict EU legislation that sets MRLs and MLs for some veterinary drugs at the low ppb level (<10 μg kg^{-1}) poses another significant obstacle in multi-class/multiresidue analysis. The developed method was thoroughly optimized and validated fulfilling all European Commission Decision 2002/657 requirements, revealing good validation parameters and thus indicating its great value in the veterinary drug and pharmaceutical analysis field.

ACN	Acetonitrile
AGs	Aminoglycosides
APCI	Atmospheric Pressure Chemical Ionization
ASE	Accelerated solvent extraction
d-SPE	Dispersive SPE
ESI	Electrospray Ionization
GC	Gas chromatography
H_2O	Water
HCl	Hydrochloric acid
HCOOH	Formic acid
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IS	Internal standard
IT	Ion Trap
LC	Liquid chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LIT	Linear Ion Trap
LE	Liquid Extraction
LLE	Liquid-Liquid Extraction
LOD	Limit of detection
LOQ	Limit of Quantification
m/z	Mass to charge ratio
MeOH	Methanol
MIPs	Molecularly imprinted polymers
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix Solid Phase Dispersion
PLE	Pressurised liquid extraction
QC	Quality Control
QqQ	Triple Quadrupole

RAMs	Restricted access materials
RP-LC	Reversed phase-liquid chromatography
RSD	Relative standard deviation
RT	Retention time
SAs	Sulfonamides
SE	Solvent extraction
SNR	Signal to noise ratio
SPE	Solid Phase Extraction
SRM	Single reaction monitoring
TFC	Turbulent flow chromatography
TOF	Time-of-Flight
UHPLC	Ultra High Performance Liquid Chromatography

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