NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS

SCHOOL OF SCIENCE

DEPARTMENT OF CHEMISTRY

MASTER OF SCIENCE IN “ANALYTICAL CHEMISTRY”

MASTER THESIS

Development of a multi-residue methodology for the determination of veterinary drugs in animal feed by High Performance Liquid Chromatography-tandem Mass Spectrometry (HPLC-MS/MS)

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OCTOBER 2019

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DEFENDING DATE 08/10/2019

ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ ΔΙΠΛΩΜΑΤΟΣ ΕΙΔΙΚΕΥΣΗΣ

Ανάπτυξη πολύ-υπολειμματικής μεθοδολογίας για τον προσδιορισμό κτηνιατρικών φαρμάκων σε ζωοτροφές με Υγροχρωματογραφία Υψηλής Απόδοσης συζευγμένη με διαδοχική Φασματομετρία Μαζών (HPLC-MS/MS)

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ΠΕΡΙΛΗΨΗ

Τα κτηνιατρικά φάρμακα χρησιμοποιούνται ευρέως στην κτηνοτροφία για την αποτροπή ή την αντιμετώπιση ασθενειών, αλλά επίσης και για την επίτευξη καλύτερης απόδοσης. Μεγάλος αριθμός αυτών των φαρμάκων χορηγούνται και για την αντιμετώπιση ανθρώπινων ασθενειών. Η έκθεση του καταναλωτή σε αυτά τα φάρμακα και τα κατάλοιπά τους μπορεί να έχει ανεπιθύμητες επιπτώσεις στην ανθρώπινη υγεία, όπως ανάπτυξη μικροβιακής αντοχής, αλλεργικές αντιδράσεις, τοξικές και πιθανώς καρκινογόνες και τερατογόνες επιδράσεις. Ενώ η Ευρωπαϊκή Ένωση έχει νομοθετήσει τη χρήση των κοκκιδιοστατικών και ιστομονοστατών, όλες οι άλλες κατηγορίες κτηνιατρικών φαρμάκων παραμένουν εκτός επίσημου ελέγχου και νομοθετικού πλαισίου. Εφόσον ο πιο αποτελεσματικός τρόπος χορήγησής τους είναι η άμεση προσθήκη τους στην ζωοτροφή, είναι σημαντικό να μπορούμε να τα ανιχνεύσουμε σε χαμηλές συγκεντρώσεις σε αυτή τη μήτρα. Η μεγαλύτερη πρόκληση έγκειται στην απομόνωση αυτών των φαρμάκων, με τις πολύ διαφορετικές φυσικοχημικές ιδιότητες, από την εξαιρετικά πολύπλοκη μήτρα που είναι οι ζωοτροφές. Ο στόχος αυτής της μελέτης ήταν η ανάπτυξη μιας μεθόδου για τον προσδιορισμό μιας μεγάλης ποικιλίας κτηνιατρικών φαρμάκων, συμπεριλαμβανομένων των, τετρακυκλινών, σουλφοναμιδών, κινολονών, β-λακταμών, μακρολιδών, βενζιμιδαζολών, αμφενικολών, ανθελμινθικών κι αβερμεκτινών, μεταξύ άλλων, χρησιμοποιώντας Αντίστροφης Φάσης Υγροχρωματογραφία Υψηλής Απόδοσης συζευγμένη με φασματομετρία μαζών (RP HPLC-MS/MS). Πραγματοποιήθηκε ενδελεχής μελέτη για την ανάπτυξη και βελτιστοποίηση της προκατεργασίας του δείγματος. Το τελικό πρωτόκολλο περιλάμβανε στερεό-υγρό εκχύλιση χρησιμοποιώντας εκχύλιση με υπέρηχους, ακολουθούμενη από καθαρισμό του δείγματος με καταβύθιση πρωτεϊνών, απομάκρυνση λιπαρών ουσιών με εξάνιο και εκχύλιση στερεάς φάσης (SPE). Η μέθοδος επικυρώθηκε σύμφωνα με τις οδηγίες της απόφασης της Ευρωπαϊκής Επιτροπής 2002/657/EC, με ικανοποιητικά αποτελέσματα όσον αφορά τη γραμμικότητα, την πιστότητα, την ενδιάμεση πιστότητα και την ανιχνευσιμότητα για τις περισσότερες μελετώμενες ενώσεις.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αναλυτική χημεία

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Υγροχρωματογραφία υψηλής πίεσης, υπολειμματική ανάλυση, κτηνιατρικά φάρμακα, ζωοτροφή, Χρωματογραφία αντίστροφης φάσης

ABSTRACT

Veterinary drugs are widely used in animal husbandry not only for the prevention or treatment of diseases but also to achieve greater yield. A large number of veterinary drugs are administered for human diseases treatment. The consumer's overexposure to these drugs and their residues can result in adverse effects on human health, such as antimicrobial resistance development, allergic reactions, toxic and potentially carcinogenic and teratogenic effects. While the EU regulates the use of coccidiostats and histomonostats, all the other categories of veterinary drugs remain out of monitoring and legislation framework. Since the most effective way of administration is their direct addition in the animal feed, it is important to be able to trace them in very low concentrations in that matrix. The most challenging part is the isolation of these drugs with substantially different physicochemical properties from the extremely complex matrix that is animal feed. The aim of this study was the development of a method for the determination of a wide variety of veterinary drugs, including tetracyclines, sulfonamides, quinolones, b-lactams, macrolides, benzimidazoles, amphenicols, anthelmintics and avermectins, among others, using Reversed Phase High Performance Liquid Chromatography-tandem Mass Spectrometry (RP HPLC-MS/MS). A thorough study was performed for the development and optimization of a sample preparation protocol. The final protocol comprised of a solid-liquid extraction using Ultrasonic-Assisted Extraction, followed by a three-step cleanup combining protein precipitation, hexane partitioning and Solid Phase Extraction (SPE). The method was validated in agreement with the guidelines of Commission Decision 2002/657/EC, yielding satisfactory results in terms of linearity, precision, intermediate precision and detectability for the majority of the studied compounds.

SUBJECT AREA: Analytical Chemistry

KEYWORDS: LC-MS/MS, residue analysis, veterinary drugs, animal feed, Reverse-Phase Chromatography

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PREFACE

This work was conceived and carried out at the Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece under the supervision of Professor Nikolaos S. Thomaidis.

I would like to thank my supervisor Dr. Nikolaos Thomaidis for entrusting me with this project. I am extremely honoured and happy to have been part of his research group and grateful for all the unique opportunities presented to me this past year. I would also like to give special thanks to the members of the examination committee, Assistant Professor E. Gikas and Professor A. Oikonomou, for the devotion of their time and their remarks regarding my master thesis.

This journey has proved crucial for further developing and expanding my theoretical knowledge and improving my practical skills in the field of Analytical Chemistry. Post-graduate researcher Marilena Dasenaki and PhD candidate Anastasia Kritikou deserve special mention and thanks for their consistent support and help. Their advice and knowledge have been invaluable throughout my master and I'm deeply grateful to have them by my side.

I would like to acknowledge all the other group members, Maria-Christina Nika, Dimitris Damalas, Giorgos Koulis, Maria Glyniadaki, Anastasia Koupa, Giorgos Gkotsis, Maria Katsa, Elena Panagopoulou, Nikiforos Alygizakis, Reza Aalizadeh, Varvara Nikolopoulou, Sofia Drakopoulou, Stefanos Kokolakis and Panagiota Katsianou, for contributing to the pleasant working environment and seamless cooperation in the lab. At this point, I would like to offer special thanks to PhD candidate Anthi Panara and Dr Marios Kostakis for taking time to train me and transfer their valuable knowledge and my fellow classmates Evagelia Kritikou and Kallirroi Rodi for their daily company, encouragement and sharing of ideas.

Last but not least, I would like to deeply thank my family for their unwavering support and understanding during this effort but also through every step of my life.

CHAPTER 1

VETERINARY DRUGS

1.1 Introduction

Today, in a global marketplace, food quality and safety have gained increasing attention from consumers, governments and food producers. A broad range of chemical contaminants are monitored or controlled in food commodities and products due to their possible adverse effects on human health. Contaminants are either natural compounds, such as mycotoxins, plant toxins or marine toxins or chemical structures developed and manufactured at industrial scale for various applications. For instance, some are used as pesticides, flame retardants or veterinary drugs [1]. Since the 1950s, a large number of veterinary drugs have been used in order to improve animal health, but also as growth promoters for intensive animal production. However, the abuse and overuse of antibiotics in food-producing animals resulted in the presence of these compounds in final products for human consumption, including meat, fish, milk and eggs. The public health hazards related to antimicrobial use in agriculture and aquaculture involve several problems such as the increased risk of developing allergies in individuals with hyper-sensitivity and the development of antibiotic-resistant bacteria [2]. Therefore, animal feedstuffs require sufficient quality from a nutritional point of view and must also comply with legal limits regarding contaminants and antimicrobial agents [3]. Human pharmaceuticals (especially antibiotics) can also be added to animal feed, because of their commercial availability and low cost. In this context, pharmaceutical dosing must be carefully monitored to achieve a compromise between the agronomic results and the negative environmental and sanitary consequences of releasing these drugs to agro-ecosystems [4].

Regarding food industries and authority bodies, the challenge related to the control of veterinary drug residues lies in the management of three factors together. These are: the number of chemical compounds, the range of

matrices and the regulation. Today, there are about 200 veterinary drug residues that need to be taken into account for control in foodstuffs such as meat, fat, milk, egg, fish, seafood and honey. On a chemical analysis standpoint, the scope further extents when matrix derivatives (fresh vs. powder), species (chicken, beef, etc.) and related finished products require attention. The regulation, often different from one region to the other, renders the requirements in terms of limit of detection, usually in the µg/kg levels or even lower in the case of banned compounds. Obviously, the complexity associated with the control of veterinary drugs significantly increases when the impact of the business is global and the portfolio of products broad. The analytical setup required for such monitoring from the farm (raw materials) to the fork (finished products) has to be optimized carefully for ensuring an effective control with regard to the coverage (number of analytes), the throughput (analysis turnaround time) and the analytical cost (cost-effective quality control). In that respect, multiresidue screening methodologies are attractive tools for a reliable consumer protection with regard to the possible presence of veterinary drug residues in food [1].

Although, there is a need for sensitive, specific and rapid analytical methods to monitor the use of veterinary drugs, only a limited number of analytical methods have been published for feed compared to food products. In most cases, the existing methods target compounds belonging to the same or similar chemical groups (e.g. coccidiostats, sulfonamides, macrolides, quinolones, tetracyclines, etc.) based on high performance liquid chromatography (HPLC) combined with ultraviolet (UV) absorbance spectrophotometry, fluorescence (FL) or mass spectrometry (MS) detection [5].

1.2 Feed Composition

Concerning the animal nutritional needs, they require approximately 40 different nutrients to allow them to grow, reproduce and produce milk, eggs or wool [6]. Feed matrices, generally, consists of cereals seeds, legumes and oilseeds, roots, fruits and other plant products and their by-products, animal

and dairy products, oil and fats (vegetable and/ or animal) and many different additives such as enzymes, acidifiers, minerals, vitamins, antimicrobials and antioxidants in varying amounts. Sometimes diets will also contain other additives used in diets for humans and pets such as flavour enhancers, artificial and nutritive sweeteners, colours, lubricants, etc. Within each one of these classes of additives there can be dozens of specific additives manufactured and distributed by a wide variety of companies. In some instances, additives are added to the animal's diet in order to enhance their value for human consumption. Therefore, feed matrices are presented to be extremely complex and variable, due to the components mentioned above, rendering their analysis challenging. Their composition differs not only between the different animal species for which it is intended and their energy requirements, but also between technological groups of the same animal species (piglet, sow, porker or laying hens and broiler). Thus, the development of analytical methods for the determination of low concentrations of a large number of antibacterial substances in feed appears to be laborious and tedious [7,8].

Figure 1. Animal feed [9]

1.3 Veterinary Drugs

One of the most effective ways for farmers to administer medicines to the livestock after veterinary prescription is by medicated feed [10]. Medicated feeds are veterinary medicinal products administered orally after being mixed with animal feed [11]. However, veterinarian and even human pharmaceuticals may be intentionally added to animal feed to enhance animal production. Drugs can reach feeds in three ways: authorised drugs (for therapeutic and prophylactic purposes), unauthorized drugs (as growth promoters to increase yield) and unintentional (as a result of the so-called cross-contamination) [4]. The production and marketing of medicated feed are regulated by the European Commission, and many European countries have implemented residue monitoring plans to control the illegal use of these substances in feed and the misuse of authorized veterinary medicines, and to minimize drug residual occurrence [10].

In this thesis we focused on the analysis of two main veterinary drugs categories, namely, antibacterials and anthelmintics. More specifically the studied compounds, their properties and MS parameters are summarized in Table 1.

1.3.1 Antibiotics

‘Antibiotics’ are organic substances synthesized either naturally by microorganisms through secondary metabolism or artificially from industries, that may kill or inhibit the growth or metabolic activity of other microorganisms. Antibiotics have been utilized in industrial production, agriculture and medicine for over 60 years. Many antibiotics are used worldwide extensively and have been approved for use as drugs for preventing plant, animal and human infections, growth promotion, and as feed additives for animals to prevent or treat diseases. Notably, the largest amounts of antibiotics serve to treat animals rather than treat human diseases. Antibiotics are administered to animals by intravenous, intramuscular or subcutaneous injections, orally in feed or water, locally on the skin and by intramammary and intrauterine infusions. Although the

inclusion of antibiotics in feed for growth promotion in livestock production was banned in EU in 1998, large scale use of antibiotics in animal production is being widely adopted worldwide. These nontherapeutic purposes represent the most often reason of VA use. Sales reports indicate that the USA ranks first in the consumption of VA (over 11,000 tons per year) followed by China (6000 tons per year). Thus, the consumption in both countries is high not only due to the large numbers of livestock but also due to the common practice employing VA as growth feed additives.

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, lincosamides, macrolides, nitrofurans, quinolones, sulfonamides, tetracyclines and miscellaneous [12,13].

1.3.1.1 *β*-Lactams

β-Lactams belong to a group of antibiotics that has been widely used in veterinary medicine for the treatment of diseases that regularly affect livestock animals (e.g., bovine mastitis, pneumonia, bacterial diarrhea and bacterial arthritis) [1]. Even without any signs of disease, these drugs are also administered to animals for preventive and prophylactic purposes. Soon after their spread, b-lactams started to be used as supplements, supplied illegally, in order to promote growth in food-producing animals. Amoxicillin and penicillin V are the most commonly used b-lactam antimicrobials and can also be administered orally [13].

β-Lactam antibiotics are probably the most widely applied antimicrobial drugs in current veterinary practice. They are divided into two subcategories: penicillins and cefalosporins. 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 [14]. In penicillins, the ring is fused to a five-member thiazolidine

ring, while for cefalosporins 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 [15]. Their mode of action is based on inhibiting bacterial cell wall biosynthesis, which has a lethal effect on bacteria. However, bacteria have shown resistance against *β*-lactam antibiotics [16]. 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 [17]. Cefalosporins are originally derived from the fungus *Acremonium*, previously known as *Cephalosporium*. First-generation cefalosporins were active predominantly against Gram-positive bacteria but successive generations have increased activity against Gram-negative bacteria, as well.

1.3.1.2 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 [18]. Chloramphenicol was first isolated from cultures of *Streptomyces venezuelae* but is now produced synthetically [19]. 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.

1.3.1.3 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 [20]. 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 [14,15,20].

1.3.1.4 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 [20]. Quinolones are also highly important human drugs, and their widespread use is of high concern due to the recent evidence of development of bacterial resistance to these antibiotics.

1.3.1.5 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 [15]. 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 [22]. Sulfonamides are often administered together with synthetic diaminopyrimidines, such as baquiloprim, ormetoprim or trimethoprim, which act as potentiators of sulfonamides.

1.3.1.6 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 [23].

1.3.1.7 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 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 [24].

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 [25]. 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 [26].

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* [14].

1.3.2 Anthelmintics

By definition, anthelmintics are drugs that reduce parasite burdens in the animals to a tolerable level, they kill the parasites (vermicide), inhibit their growth or paralyse them (vermifuge). Synthetic and semi-synthetically produced anthelmintics have long been considered the only effective method of controlling helminthosis. The era of modern anthelmintics started in the middle of the 20th century with the introduction of phenothiazine and piperazine, products that are considered to be the first generation of the broad spectrum drugs. The 2nd generation of truly broad spectrum anthelmintics were released in the 1960s and included the benzimidazoles, the

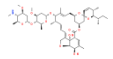
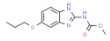
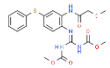
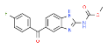
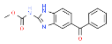
probenzimidazoles, the imidazothiazoles and the tetra-hydro-pyrimidines. Following the early success of the introduction of the benzimidazoles, extensive research programmes were initiated during which successful structural modification resulted in the production of a series of benzimidazoles [27]. Between 1960 and 1980, extraordinary success was achieved in anthelmintic development for animals. In these 20 years, drugs with diverse structure, novel activity and enviable safety were produced for a global livestock industry leading to the productivity gains needed to support a human population that grew by 1.5 billion during the same period. The following 20 years have been spent refining existing molecules with niche activity (parasite and host specificity), improving delivery systems and worrying about the inexorable spread of drug resistance [28]. A 3rd generation of broad spectrum anthelmintics, the macrocyclic lactones, emerged in the early nineteen eighties [27].

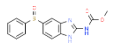
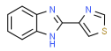
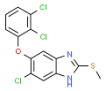
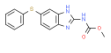
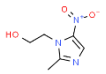
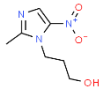
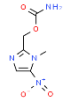
They are usually classified into several types on the basis of similar chemical structure and mode of action. Basically, three main families can be distinguished: benzimidazoles, nicotinic receptor agonists and macrocyclic lactones (avermectines and milbemycins) [29]. The benzimidazoles consist of a ring system composed of a benzene ring fused with an imidazole ring. The determination of this class of residues is problematic because, despite the similarities in their chemical structure and mode of action, their chemical properties (such as lipophilicity and acid–base behaviour) are very different [30]. 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. [31] The most frequently used anthelmintic compounds are levamisole, several compounds from the benzimidazole group (albendazole, cambendazole, fenbendazole, oxfendazole and thiabendazole) and ivermectin [32]. Salicylanilides:

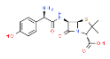
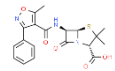
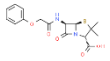
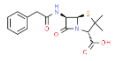
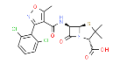
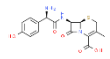
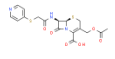
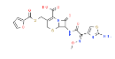
rafoxanide, oxyclozanide, brotianide and closantel and the substituted phenol, nitroxinil, are proton ionophores [33].

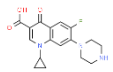
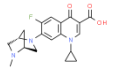
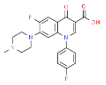
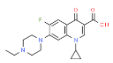
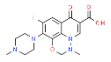
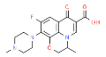
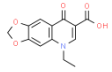
A common concern arising from the use of anthelmintic drugs is the emergence of resistance. Moreover, it is well known that conventional cooking cannot be considered a safeguard against ingestion of residues of anthelmintic veterinary drugs in beef as well as the fermentation process, i.e. levamisole was found in soft and hard (mature) cheeses [30].

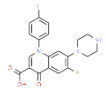
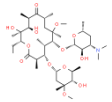
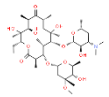
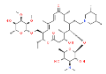
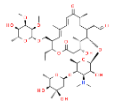
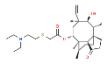
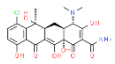
Table 1. Compound properties and MS parameters [34]

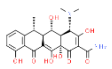
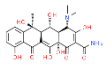
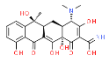
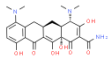
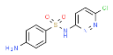
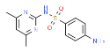
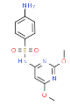
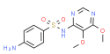
Compound	Monoisotopic Mass (Da)	Molecular Formula	Chemical structure	LogD (pH 5.5)*	LogD (pH 7.4)*	Parent Mass (Da)	Product Ion 1 (Da)	CE (eV)	Product Ion 2 (Da)	CE (eV)	Tube Lens
Avermectins											
Emamectin	885.523865	$C_{49}H_{75}NO_{13}$		3.47	4.99	886.3	157.8	31	301.6	31	123.38
Benzimidazoles											
Albendazole	265.088501	$C_{12}H_{15}N_3O_2S$		2.74	3.1	266	191	31	234	29	85
Febantel	446.126007	$C_{20}H_{22}N_4O_6S$		2.54	2.36	447	383	17	280	31	110
Flubendazole	313.086273	$C_{16}H_{12}FN_3O_3$		2.72	2.79	314	281.9	31	123	35	90
Mebendazole	295.095703	$C_{16}H_{13}N_3O_3$		2.64	2.75	296	264	31	105	35	90

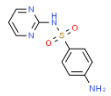
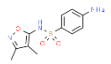
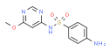
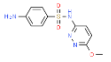
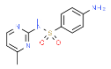
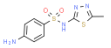
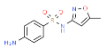
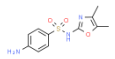
Oxfendazole	315.067749	$C_{15}H_{13}N_3O_3S$		1.55	1.61	316	159	30	191	24	87
Thiabendazole	201.036072	$C_{10}H_7N_3S$		2.38	2.39	202	130.9	35	174.9	35	87
Triclabendazole	357.950104	$C_{14}H_9Cl_3N_2OS$		5.74	5.63	359	273.9	35	171	40	85
Fenbendazole	299.072845	$C_{15}H_{13}N_3O_2S$		3.24	3.54	300	267.8	29	159	33	85
Nitroimidazoles											
Metronidazole	171.064392	$C_6H_9N_3O_3$		0.05	0.05	172	128	13	82.3	25	69
Ternidazole	185.080048	$C_7H_{11}N_3O_3$		0.17	0.17	186	128	15	82.2	28	75
Ronidazole	200.05455	$C_6H_8N_4O_4$		-0.42	-0.42	201	140.1	10	55.5	21	73
Penicillins											

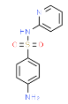
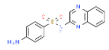
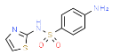
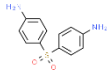
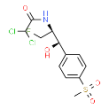
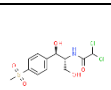
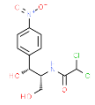
Amoxicillin	365.104553	$C_{16}H_{19}N_3O_5S$		-2.04	-2.72	365.8	348.3	9	133.8	29	77
Oxacillin	401.104553	$C_{19}H_{19}N_3O_5S$		-0.98	-1.66	375.8	143.8	31	173.7	18	97
Penicillin V	350.093628	$C_{16}H_{18}N_2O_5S$		-0.92	-1.68	324.8	127.9	26	173.7	16	86
Penicillin G	334.098724	$C_{16}H_{18}N_2O_4S$		-0.94	-1.7	308.8	173.8	16	127.9	26	93
Dicloxacillin	469.026581	$C_{19}H_{17}Cl_2N_3O_5S$		0.03	-0.65	443.7	127.9	29	211.4	33	115
Cefalosporins											
Cefadroxil	363.088898	$C_{16}H_{17}N_3O_5S$		-3.4	-4.06	364.1	114.1	19	134	29	97
Cefapirin	423.055878	$C_{17}H_{17}N_3O_6S_2$		-2.84	-3.79	423.8	291.5	14	151.8	23	85
Ceftiofur	523.028992	$C_{19}H_{17}N_5O_7S_3$		-1.6	-2.5	523.8	124.9	52	125.9	29	101

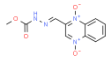
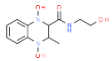
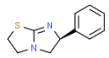
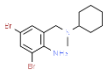
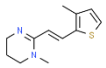
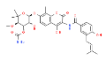
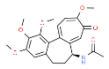
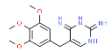
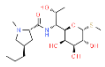
Quinilones											
Ciprofloxacin	331.133209	$C_{17}H_{18}FN_3O_3$		-2.98	-2.23	332.1	288	18	314	22	85
Danofloxacin	357.148865	$C_{19}H_{20}FN_3O_3$		-2.17	-1.4	358	314	20	96	25	85
Difloxacin	399.139435	$C_{21}H_{19}F_2N_3O_3$		-1.71	-1.37	399.9	356	20	299	27	85
Enrofloxacin	359.16452	$C_{19}H_{22}FN_3O_3$		-1.49	-0.86	360.3	316.3	20	342.3	20	85
Flumequine	261.080109	$C_{14}H_{12}FNO_3$		1.08	-0.66	262.1	244	20	201.9	30	85
Marbofloxacin	362.139038	$C_{17}H_{19}FN_4O_4$		-2.02	-2.08	363.1	320	15	71.9	20	85
Ofloxacin	361.143799	$C_{18}H_{20}FN_3O_4$		-1.84	-2.08	362.2	317.9	19	260.9	27	120
Oxolinic acid	261.063721	$C_{13}H_{11}NO_5$		1.2	-0.14	262.1	244	18	158	31	79

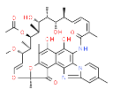
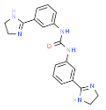
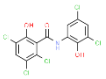
Sarafloxacin	385.12381	$C_{20}H_{17}F_2N_3O_3$		-2.84	-2.22	386	342	18	299	27	85
Macrolides											
Clarithromycin	747.476868	$C_{38}H_{69}NO_{13}$		0.67	2.38	748.9	158	30	590.5	20	123
Erythromycin	733.461243	$C_{37}H_{67}NO_{13}$		-0.02	1.69	734.4	576.3	20	158.1	30	130
Tilmicosin	868.56604	$C_{46}H_{80}N_2O_{13}$		-0.1	1.52	869.4	173.6	42	155.7	44	165
Tylosin	915.519165	$C_{46}H_{77}NO_{17}$		-0.09	1.45	916.8	173.9	36	772.2	28	148
Tiamulin	493.322571	$C_{28}H_{47}NO_4S$		2.53	3.33	494.4	192	21	119	33	101
Tetracyclines											
Chlortetracycline	478.114288	$C_{22}H_{23}ClN_2O_8$		-2.71	-3.13	479	444	20	462	15	90

Doxycycline	444.153259	$C_{22}H_{24}N_2O_8$		-2.98	-3.29	445	427.4	19	267	35	90
Oxytetracycline	460.148193	$C_{22}H_{24}N_2O_9$		-3.85	-4.25	461	425.8	19	442.6	12	90
Tetracycline	444.153259	$C_{22}H_{24}N_2O_8$		-2.77	-3.17	445	410	18	426.4	12	90
Minocycline	457.184906	$C_{23}H_{27}N_3O_7$		-2.65	-2.74	458.3	441.2	19	352.1	29	105
Sulfonamides											
Sulfachloropyridazine	284.013458	$C_{10}H_9ClN_4O_2S$		0.54	-0.82	284.9	155.9	14	92.1	28	87
Sulfadimidine	278.08374	$C_{12}H_{14}N_4O_2S$		0.44	0.3	279	185.9	17	124.1	26	87
Sulfadimethoxine	310.073578	$C_{12}H_{14}N_4O_4S$		0.43	-0.49	311	156	17	108.1	29	87
Sulfadoxine	310.073578	$C_{12}H_{14}N_4O_4S$		0.23	-1.04	311	156	17	108.1	27	87

Sulfadiazine	250.052444	$C_{10}H_{10}N_4O_2S$		-0.09	-0.79	251	156	15	92.2	27	87
Sulfisoxazole	267.067749	$C_{11}H_{13}N_3O_3S$		0.58	-0.77	268	156	13	92.2	27	87
Sulfamonomethoxine	280.063019	$C_{11}H_{12}N_4O_3S$		-0.08	-1.31	281	92.2	29	156	13	87
Sulfamethoxypyridazine	280.063019	$C_{11}H_{12}N_4O_3S$		0.43	-0.29	281	92.2	29	156	13	87
Sulfamerazine	264.068085	$C_{11}H_{12}N_4O_2S$		0.39	0.04	265	156	16	172	16	87
Sulfamethizole	270.024506	$C_9H_{10}N_4O_2S_2$		0.21	-1.14	271	155.9	14	92.2	28	87
Sulfamethoxazole	253.052109	$C_{10}H_{11}N_3O_3S$		0.56	-0.56	253.9	155.8	16	108	25	87
Sulfamoxole	267.067749	$C_{11}H_{13}N_3O_3S$		0.47	0.12	268	156	13	92.2	28	87

Sulfapyridine	249.05719	$C_{11}H_{11}N_3O_2S$		0.47	0.4	250	156	15	184	17	87
Sulfaquinoxaline	300.068085	$C_{14}H_{12}N_4O_2S$		1.5	0.47	301	156	18	92.2	30	87
Sulfathiazole	255.013611	$C_9H_9N_3O_2S_2$		0.41	0.03	255.9	155.9	15	92.2	26	87
Dapsone	248.061951	$C_{12}H_{12}N_2O_2S$		1.08	1.08	249	155.9	14	108	22	79
Amphenicols											
Florfenicol	357.000458	$C_{12}H_{14}Cl_2FNO_4S$		0.4	0.4	356	336	11	185	18	90
Thiamphenicol	355.004791	$C_{12}H_{15}Cl_2NO_5S$		-0.19	-0.19	354	290	11	185	19	90
Chloramphenicol	322.012329	$C_{11}H_{12}Cl_2N_2O_5$		1.02	1.02	321	256.8	13	152.1	19	90
Other											

Carbadox	262.07019	$C_{11}H_{10}N_4O_4$		-0.19	-0.19	262.8	230.7	13	128.9	30	91
Olaquindox	263.090607	$C_{12}H_{13}N_3O_4$		-0.83	-0.83	263.9	220.7	13	142.8	29	105
Levamisol	204.072113	$C_{11}H_{12}N_2S$		0.16	0.25	205	178.1	29	123	31	87
Bromhexine	373.999298	$C_{14}H_{20}Br_2N_2$		1.46	3.09	376.9	114.1	18	263.6	29	78
Morantel	220.103424	$C_{12}H_{16}N_2S$		0.62	0.62	221	123.2	31	111.2	26	96
Novobiocin	612.231934	$C_{31}H_{36}N_2O_{11}$		1.77	-0.02	613	188.8	31	395.6	14	108
Colchicine	399.168182	$C_{22}H_{25}NO_6$		1.1	1.1	400.1	309.9	26	325.9	24	110
Trimethoprim	290.137878	$C_{14}H_{18}N_4O_3$		-1.16	-1.15	290.9	230	25	122.9	30	87
Lincomycin	406.213745	$C_{18}H_{34}N_2O_6S$		-2.08	-0.44	407.3	126.2	30	359.2	17	99

Rifaximin	785.352356	$C_{43}H_{51}N_3O_{11}$		2.06	0.73	786.2	753.7	22	361.5	32	114
Imidocarb	348.169861	$C_{19}H_{20}N_6O$		-2.32	-2.29	349.1	187.8		161.8		
Oxyclozanide	398.879028	$C_{13}H_6Cl_5NO_3$		6.83	4.86	397.8	361.7	25	175.7	38	87

*Predicted values

1.4 Analysis of veterinary drugs

Feedingstuffs are complex matrices that usually contain carbohydrates, proteins, and lipids in high concentrations. Thus, an extraction procedure shall minimize matrix interference and concomitant agents. One of the main challenges faced in the development of a multi-residue method, is to perform a single extraction procedure for all analytes, without compromising performance parameters [11]. When medicated feed is manufactured in the same production lines as non-medicated products, cross-contamination may occur in all stages of feed production, including processing, storage and transport (Commission Directive 2009/8/EC) [3]. Monitoring feed to ensure the absence of an increasing number of undesirable drugs at very low levels requires highly sensitive and selective methods. The main difficulties arise from the complexity and variability of the animal feed matrix and from the frequently low levels of the compounds to be detected. The strategies developed for sample preparation and extraction of drug residues from such matrices usually involve extensive handling and cleanup to improve sensitivity and selectivity, but although extraction, cleanup, and matrix analyte concentration are key steps in determining antimicrobials in complex samples, one should bear in mind that such drugs have very different physicochemical properties [35]. Several extraction approaches have been proposed to the analysis of feedingstuffs and animal products, such as the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach, solid phase extraction (SPE), pressurized liquid extraction (DVPLE), liquid-liquid extraction and matrix solid-phase dispersion, among others [36].

1.4.1 Extraction

The large number of available drugs has caused an increase in the number of analytes to be monitored. Under this situation, it is advisable to perform sample extractions as generic as possible in order to widen the scope of the method and to include as many analytes as possible [4]. In some studies, the extract was simply diluted before analysis, whereas in other studies, a cleanup step was added, such as liquid–liquid extraction (LLE), e.g. by using

hexane to remove lipids, solid-phase extraction (SPE) using various solid phases (Oasis® HLB, ion-exchange, silica, etc.), and tandem-SPE, but also matrix solid-phase dispersion extraction (MSPDE) employing C18 material [2]. Normally, most of these methods are focused on specific groups of residues, not being suitable for wide-scope multi-residue analysis able to cover different classes of residues and contaminants. However, the use of wide-scope methods inherently involves the need for generic extraction procedures, and this strategy shortens the possibilities for cleanup steps, in order to minimize analyte losses. Nevertheless, the lack of selectivity in sample preparation can be compensated by selectivity/sensitivity in instrumental analysis [37].

1.4.2 Detection

During the last decade, analytical instrumentation has improved tremendously and, as a result, the methods used in confirmatory analysis have evolved from single residue methods to multi-class methods including over hundreds of compounds within a single method [38]. Microbiological assays are among the most commonly used techniques for the detection of the majority of antibiotic classes. However, due to the risk of false positive samples, results from microbiological assays typically require confirmation by a confirmatory method, allowing for selective, sensitive, accurate and rapid detection and quantification of antibiotics for an effective surveillance. Commission Decision 2002/657/EC [12] has introduced the concept of identification points (IPs) for confirmatory methods suggesting antibiotic detection should be based on more than a single characteristic [2]. Although several different analytical methods based on liquid chromatography coupled to fluorescence or ultraviolet detection have been developed, the most recent methodology relies on mass spectrometry or, preferably, tandem mass spectrometry detection with triple quadrupole or ion trap analysers, because of the high selectivity and sensitivity provided by this technique. For large screening purposes, an alternative to MS/MS is the application of full scan techniques based on high resolution mass spectrometry (HRMS), using QTOF or Orbitrap analysers [4].

1.4.3 Literature Review

The rising demand for food producing animals has led to the widespread use of veterinary drugs. The ever-growing number of analytes to be monitored combined with the complexity of the feed as a matrix have necessitated the development of sensitive and reliable analytical methodologies for their determination. An overview of the analytical methodologies developed so far for the analysis of veterinary drugs in feed matrices using liquid chromatography coupled to various detection systems is presented in Table 2.

Table 2. Literature review table

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
129 VDs	Chicken, hen, rabbit, horse feed	2.5 g of feed extracted with 2.5 mL of H2O and 7.5 mL of ACN (1 % FA, v/v).	**UHPLC–QqTOF–MS/MS** Chromatographic system Column: Acquity UPLC BEH C18 (2.1 mm×100 mm, 1.7 µm particle size) Mobile phase: A: 0.1 % (v/v) FA and ammonium formate 4 mM in H2O and B: 0.1 % (v/v) FA and ammonium formate 4 mM in MeOH. Total run time: 14 min. Flow rate: 0.3 mL/min. Column temp: 30 °C. Injection volume: 5 µL. Detection system QqTOF. ESI (+) & ESI (-). Resolution: at least 9,000 FWHM. Mass range: m/z 90–1,000, scan time: 0.050 s and interscan time: 0.025 s.	**Recovery**: 60%-125% at 10 µg/kg **LOQ**: 4-200 µg/kg	[37] Aguilera-Luiz M.M et al **(2013)**
116 human and veterinary drugs	Bovine, rabbit, poultry, goat and pork feed	5.0 g of feed extracted with 10 mL ACN 1% HCOOH and sonication.	**UHPLC-QTOF MS** Chromatographic system Column: Acquity UPLC BEH C18 (100 ×2.1 mm, 1.7 µm particle size). Mobile phase: A: H2O and B: MeOH, both with 0.01% (v/v) HCOOH. Gradient. Total run time: 18 min. Flow rate: 0.3 mL/min. Injection volume: 50 µL. Column temp: 40 °C and samples: 5 °C. Detection system QTOF mass spectrometer. ESI (+) & ESI (-). Resolution: 20,000 at FWHM at m/z 556. Mass range: 50–1200, scan time: 0.3 s.	SDL: 0.02- >0.2 mg/kg LOI: 0.02- >0.2 mg/kg	[4] Boix C. et al **(2014)**
33 antibiotics	Piglet, bovine and lamb feed	4g of feed extracted with 15mL of CH3OH/CH3CN/McIlvaine buffer, pH 4.6 (37.5/37.5/25, v/v/v) containing 0.3% of EDTA Na2 (0.5 M) and ultrasonication. Cleanup with d-SPE (250 mg PSA). ***INTERNAL STANDARDS***: (ESI+): $^{13}C_3$-flumequine and d_4-sulfadiazine (ESI-): d_5-chloramphenicol	**LC–ESI-MS/MS** Chromatographic system Column: Zorbax XDB plus (2.1 mm×15 0 mm, particle size 3.5 µm), guard column (2.1mm×12.5mm, particle size 5 µm). ESI (+) mobile phase: A: H2O with 0.1%FA and B: ACN/MeOH (70/30, v/v) with 0.1% FA. Total run time: 28 min. ESI (−) mobile phase: A: H2O containing 5mM ammonium acetate and B: ACN/MeOH (70/30, v/v). Total run time: 21 min. Flow rate: 0.25mL/min. Column temperature: 40 ◦C. Injection volume: 25 µL. Detection system API 3200 triple quadrupole mass spectrometer. ESI (+) & ESI (-). SRM mode.	**Recovery**: 51%-116% **RSD%**: 7.3%-9.0% **LOQ**: 3.8 ng/g-65.0 ng/g	[2] Boscher A. et al **(2010)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
14 prohibited medicinal additives	Pig and poultry compound feed	2g feed extracted with 12mL ACN, addition of 3.5 g anhydrous sodium sulfate and extraction with hexane. ***INTERNAL STANDARDS***: d_3-DMZ, d_3-IPZ, d_3-RN, d_5-CAP, sulfaphenazole	**LC–MS/MS** Chromatographic system Column: Luna C18 (100×2 mm, 3 µm particle size), Security Guard guard cartridge system (20×2mm). Mobile phase: A: H2O acidified with 0.2% acetic acid and B: ACN acidified with 0.2% acetic acid. Gradient. Total run time: 26 min. Flow rate: 0.25 mL/min. Oven temp: 40 ◦C. Detection system TSQ Quantum Ultra EMR**.** ESI (+) & ESI (-).	**Accuracy**: 95.6%-103.3% **%RSD (repeatability)**: 4.3%-23.3%	[37] Cronly M. et al **(2010)**
> 300 VDs	Chicken, rabbit, hen, horse, lamb and pig samples	2.5g of feed extracted with 2.5 mL H2O and 7.5 mL ACN (1% FA v/v).	**UHPLC–Orbitrap–MS** Chromatographic system Column: Hypersil GOLD aQ C18 (100×2.1 mm, 1.7 µm particle size). Mobile phase: A: 0.1% (v/v) FA and ammonium formate 4 mM in H2O and B: 0.1% (v/v) FA and ammonium formate 4 mM in MeOH. Total run time: 14.0 min. Flow rate: 0.3 mL/ min. Column temp: 30ºC. Detection system Single-stage Orbitrap mass spectrometer. HESI-II in ESI (+) & ESI (–). Mass range in full-scan: m/z 70–1000. **HPLC–TOF–MS/MS** Chromatographic system Column: Acquity UPLC BEH C18 (2.1 mm×100 mm, 1.7 µm particle size). Mobile phase and gradient same as above. Flow rate: 0.3 mL/min. Column temp: 30ºC. Detection system QqTOF. ESI (+) & ESI (–). Resolution: at least 9000 FWHM at m/z. Mass range: m/z 90–1000, scan time: 0.050 s and interscan time: 0.025 s.	***ORBITRAP*** **Recovery**: 60%-125% at 10 µg/kg **%RSD**: <25% at 10 µg/kg **LOQ**: 1-12.5 µg/kg ***TOF*** **Recovery**: 60%-125% at 10 µg/kg **%RSD** : <25% at 10 µg/kg **LOQ**: 1-12.5 µg/kg	[3] Gómez-Pérez M. L. et al **(2015)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
48 antimicrobial agents	Pig, poultry, cattle and fish feed	3.0 g of feed extracted with 15 mL ACN/MeOH/H2O (65/25/10) in 1% FA and ultrasonication. ***INTERNAL STANDARDS***: sulfamethoxypyridazine-d_3, sulfadimethoxine-d_6, roxithromycin, demeclocycline hydrochloride, nigericin sodium, decoquinate-d_5, dinitrocarbanilide-d_8, bis-diclazuril, ronidazole-d_3, dimetridazole-d_3, furazolidone-d_4, norfloxacin-d_5	**LC–HRMS** Chromatographic system Column: Altima HP C18 (150 mm×3.2 mm, 5 μm) with guard column (7.5 mm×3.2 mm). Mobile phase: A: 0.5% FA in H2O and B: 0.5% FA in MeOH. Total run time: 23.0 min. Flow rate: 350 μL/min. Injection volume: 15μL. Column temp: 35 ◦C. Detection system H-ESI in positive and negative mode. Mass range: 140-940 m/z with maximum injection time of 500 ms. Resolving power: 50,000, duty cycle: 1.5 s.	**Recovery**: 60.6%-125.4% (pig feed) **%RSDr**: 0.24%-9.65% **%RSDint**: 1.72%-23.5% **LOD**: 10 μg/kg **LOQ**: 25-100 μg/kg	[6] Kaklamanos G. et al **(2013)**
18 antibacterial compounds	Bovine feed samples	3g of feed extracted by PLE (5g diatomaceous earth, H2O–MeOH (95:5). Cleanup with online SPE ***INTERNAL STANDARDS***: sulfathiazole-d_4, penicillin V-d_5	**LC–ESI-MS–MS** Chromatographic system Column: C12 Phenomenex Hydro-RP (50 mm×2 mm i.d., 4 μm, 80 Å) with C12 MAX-RP security guard column (4 mm×3 mm i.d.). Mobile phase: A: HPLC-grade H2O with 0.1% FA and B: MeOH with 0.1% FA. Total run time: 10 min. Flow rate: 0.7 mL/min. Detection system 4000 Q TRAP MS–MS system. Positive-ion mode with TurboIonSpray source.MRM mode. Dwell time: 50 ms.	**Recovery**: 93%-134% **%RSDr**: 0.7%-8.3% **LOD**: 0.09-2.12 μg/kg **LOQ**: 0.25-5.79 μg/kg **CCα**: 10-174 μg/kg **CCβ**: 22-182 μg/kg	[13] Kantiani L. et al **(2010)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
Antiparasitic VDs	Feed samples	10g of feed extracted with 10 mL of deionized water and 10 mL of MeCN followed by 5g of MgSO4/NaCl (4:1, w/w). Cleanup with MgSO4 (150 mg), C18 sorbent (50 mg) and PSA sorbent (50 mg). ***INTERNAL STANDARDS***: MBZ-d_3	**DART-HRMS** Detection system Exactive™ mass spectrometer with DART-SVP ion source. *Positive ion mode*: helium flow: 4.0 L/min, discharge needle voltage: 3.0 kV, grid electrode potential: 250 V. *Negative ion mode*: helium flow: 4 L/min, discharge needle voltage: 3.0 kV, grid electrode potential: –150 V. *Orbitrap MS*: cone voltage:+/- 20 V, monitored m/z range: m/z 50–1000.	**Recovery**: 72%-92% **%RSD**: 3.4%-7.5% **LOQ**: 0.25 mg/kg (chicken feed)	[41] Martínez-Villalba A. et al **(2013)**
35 antibiotics	Fish feed	5 g of feed extracted with 10 mL of ACN/H2O (80:20) 0.1% HCOOH, ultrasonication and storage in freezer (minimum 2 h).	**UHPLC-QTOF MS** Chromatographic system Column: Acquity UHPLC BEH C18 (2.1×100 mm, 1.7 µm particle size). Mobile phase: H2O/MeOH gradient both with 0.01% HCOOH and 0.1 mM NH4Ac. Flow rate: 300 µL/min. Column temp: 60 °C. Detection system Hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer with orthogonal Z-spray-ESI(+). TOF MS resolution: 10,000 FWHM at m/z 556.2771. Mass range: 50−1000. Scan time: 0.2 s, interscan delay: 0.05 s.	SDL: 20-100 µg/kg LOI: 20-100 µg/kg	[42] Nácher-Mestre J. et al **(2013)**
51 antibiotics and 2 benzimidazoles	Pig, cattle, and poultry feed	5.0 g of feed extracted with 25 mL MeOH. ***INTERNAL STANDARDS***: triclabendazole-d_3, trimethoprim-d_9, chloramphenicol-d_5, lomefloxacin	**UHPLC-MS** Chromatographic system Column: Acquity UPLC HSS T3 (150×2.1 mm, 1.7 mm particle size). Mobile phase: *ESI(-)*: A: H2O and B: ACN, post-column delivery of NH4OH, *ESI(+)*: A: 0.05% F.A in H2O (A) and B: 0.05% F.A in ACN (B). Column temp: 50◦C, injection volume: 50 µL. Detection system Waters Acquity TQ mass spectrometer. ESI (+) & ESI (-). 4 MRMs for the ESI (-) and 50 MRMs for the ESI (+).	**Validation Levels**: 0.5-2 µg/kg (macrolides) 1 µg/kg (sulfonamides) 2-100 µg/kg (β-lactams) 1-25 µg/kg (quinolones)	[35] Robert C. et al **(2014)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
6 coccidiostats	Poultry and cattle compound feed	5.0 g of feed extracted with 100 mL of MeOH:H2O mixture (90:10, v:v). Cleanup with silica cartridge (IST Isolute) ***INTERNAL STANDARD***: nigericin	**LC–MS/MS** Chromatographic system Column: RP Nucleosil® C18 (250 mm×4.6 mm, 5 µm particle diameter) with Nucleosil® C18 guard column (7.5 mm×4.6 mm, 5 µm particle diameter). Mobile phase: 94:6 (v:v) mixture of MeOH containing 0.1% FA and H2O containing 0.1% FA. Flow rate: 1.0 mL/min. Column temp: 25 ◦C and sample temperature: 4 ◦C. Injection volume: 40µL. Detection system Quattro LC triple stage quadrupole. ESI (+). MRM mode. Dwell time: 100 ms.	**Recovery**: 86%-120% **%RSD**: 4%-10% (matrix matched stds) **LOD**: 0.001-0.014 mg/kg **LOQ**: 0.007-0.046 mg/kg (poultry) **LOD**: 0.001-0.008 mg/kg **LOQ**: 0.004-0.026 mg/kg (cattle)	[43] Vincent U. et al **(2008)**
77 banned VDs	Feed samples	2 g of feed extracted with 6 mL distilled water, 5 mL ACN containing 1% acetic acid, and the extraction salt packet (4 g MgSO4, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate).	**UHPLC–HRMS** Chromatographic system Column: Hypersil Gold (100×2.1 mm, 1.9 µm). Mobile phase: A: 0.1% acetic acid aqueous solution and B: MeOH:ACN (90:10) 0.1% acetic acid. Total run time: 14.5 min. Flow rate: 400 µL/min, injection volume: 10 µL. Detection system Orbitrap mass spectrometer Exactive™ analyser, H-ESI II. ESI (+) & ESI (-). Resolving power: 50,000 FWHM, maximum inject time: 250 ms. Scan range: 55–1000 m/z.	**Recovery**: 80%-120% **%RSD**: < 20% **LOQ**: < 12.5 µg/kg	[44] León N. et al **(2015)**
9 VDs	Feed samples	2 g of feed extracted with 5 mL of ACN/H2O (80:20, v/v) acidified with 1 % F.A and ultrasonication.	**DESI-HRMS (screening)** **UHPLC-MS/MS (quantification)** Detection system Desorption electrospray ionization (DESI) source coupled to quadrupole-orbitrap mass spectrometer. DESI solvent (ACN/H2O, 80:20 v/v), nebulizer gas: N2 at 9 bar. Electrospray voltage: ±4.8 kV (positive/ negative). Transfer capillary temperature: 250 °C. Scan range: 100–1000 m/z.		[10] Seró R. et al **(2015)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
7 β-agonists	Feed samples	2.5 g of feed extracted with 20 mL of 0.1 N HCl and sonication. Cleanup with SPE. LLE twice with 2 mL of ter-butylmethylether and 1g of sodium chloride. ***INTERNAL STANDARDS***: ractopamine-d_5 (RTP-d_5), clenbuterol-d_9 (CBT-d_9)	**LC-MS** Chromatographic system Column: Luna PFP Phenomenex® (100 mm×2.0 mm, 3µm particle size), guard column (4.0×2.0 mm). Mobile phase: A: H2O containing 10mM acetic acid and B: ACN Flow rate: 0.3mL/min. Injection volume: 25µL. Column support temp: 40 ◦C. Detection system TSQ Quantum Ultra AM triple quadrupole detector. ESI (+).	**Recovery**: 66%-110% **%RSDr**: 7%-12% **CCα**: 0.46-0.87 µg/kg **CCβ**: 0.50-0.92 µg/kg	[45] Juan C. et al **(2010)**
Spectinomycin, halquinol, andzilpaterol	Feed samples	1.00g of feed extracted with 5mL F.A 80%.	**LC-MS** Chromatographic syste *HILIC*: Column: Hypersil Gold HILIC (150×3.0 mm, 5µm Mobile phase: A: H2O and B: ACN with ammonium formate (5 mmol/L) an FA (0.1%) in each phas Total run time: 15min. Injection volume: 5µL in 350µL/min flow. Column tem 40◦C. *RP-LC*: Column: Symmetry C18 (2,1×50 mm, 3.5µm), with Security Guar C18 guard column (4.0×3.0 mm Mobile phase: A: H2O and B: ACN, both containing HFBA (0.12% Total run time: 12min. Injection volume: 5µL in 300µL/min flow. Column ove 40◦C. Detection syste 5500 QTrap hybrid triple quadrupole-linear ion trap mass spectrometer, ES (+) and APCI. MRM aqcuisition mode.	**Recovery**: 80%-92% **%RSDr**: 6.7%-16% **CCα**: 304 µg/kg **CCβ**: 358 µg/kg	[11] Molognoni L. et al **(2018)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
62 VDs	Feed samples	2.0g of feed extarcted with 1mL EDTA 150 mmol/L and 9 mL of ACN:H2O 90:10 acidified with acetic acid (0.1%). Kept at −20◦C for 1h. ***INTERNAL STANDARDS***: DCQ-d_5, DNC-d_8, ROBE-d_8, CAP-d_5, EMA, DEMO, SPY, ENRO-d_5	**LC-MS** Chromatographic system Column: C18 Venusil XBP (50×2.1 mm, 3.0µm particle diameter, 100 Å pore size) with C18 guard column (4.0 mm×3.0 mm). Mobile phase: A: H2O with 0.5 mmol/L ammonium acetate and 0.05% acetic acid and B: MeOH with 0.5 mmol L ammonium acetate and 0.05% acetic acid. Total run time: 13min. Column temp: 30◦C. Flow rate: 0.3 mL/min. Injection volume: 5µL. Detection system Hybrid triple quadrupole-linear ion trap mass spectrometer QTRAP 5500. ESI (+). MRM mode.	**LOD**: 1.25-40 µg/kg **LOQ**: 5-50 µg/kg **CCα**: 6.10-271.40 µg/kg **CCβ**:7.31-342.79 µg/kg	[36] Camargo Valese A. et al **(2017)**
9 β-agonists	Feed samples	5.0 g of feed extracted with 20 mL ACN and ultrasonication. Cleanup with SPE. ***INTERNAL STANDARDS***: salbutamol-d_3, diazepam-d_5, clenbuterol-d_9	**LC–MS-MS** Chromatographic system Column: Agela MP-C18 LC (2.1×150 mm, 3.5 µm particle size). Mobile phase: mixture of 0.1% FA solution and ACN. Total run time: 15 min. Flow rate: 0.2 mL/min. Column temp: 25◦C, injection volume: 20µL. Detection system TSQ Quantum mass spectrometer, ESI (+). MRM acquisition mode.	**Recovery**: 74.9%-110.6% (5 ng/g) **%RSDr**: 3.9%-12.6% **LOD**: 0.2-0.5 ng/g **LOQ**: 0.5-2 ng/g	[46] Suo D. C. et al **(2013)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
Tiamulin, trimethoprim, tylosin, sulfadiazine, sulfamethazine	medicated feed samples	2.00g of feed sample extracted with 10 mL of 0.1% FA in ACN.	**LC-MS/MS** Chromatographic system Column: Kinetex biphenyl (2.1 mm×50 mm, 5µm), guard column (biphenyl 2.0×4.0 mm). Mobile phase: A: 0.1%FA in H2O and B: ACN with 0.1% FA. Total run time: 24.0 min. Flow rate: 400 µL/min. Injection volume: 15 µL. Column temp: 30ºC. Detection system Triple-quadrupole API Qtrap 2000 mass spectrometer. ESI (+). MRM acquisition mode.	**Recovery**: 73.58%-115.21% **%RSDr**: <14% **LOD**: 6.5-34.1 mg/kg **LOQ**: 4.1-16.4 mg/kg	[7] Patyra E. et al **(2018)**
11 antibacterial substances	Feed samples	2 g of feed extracted with 10 mL of 0.1% F.A in ACN.	**HPLC-MS/MS** Chromatographic system Column: Kinetex biphenyl (2.1 mm×50 mm, 5µm), guard column (biphenyl 2.0×4.0 mm). Mobile phase: A: 0.1%FA in Milli-Q H2O and B: 0.1% FA in ACN. Total run time: 24.0 min. Flow rate: 400 µL/min. Injection volume: 15 µL. Column temp: 30ºC. Detection system 2000 Qtrap mass spectrometer. ESI (+). MRM acquisition mode.	**Recovery**: 76.04%-117.39% **%RSDr**: 2.41%-19.76% (250 µg/kg) **LOD**: 79.22-193.60 µg/kg **LOQ**: 133.74-217.69 µg/kg **CCα**: 285.35-401.85 µg/kg **CCβ**: 290.61-602.24 µg/kg	[47] Patyra E. et al **(2018)**
Penicillins	Feed samples	2 g of feed extracted with phosphate buffer (100 mM, pH=8.0, 40 mL). Addition of 300 µL piperidine. Purification with Oasis® HLB.	**LC–MS/MS** Chromatographic system Column: Symmetry C18 (3.0×150 mm, 5 µm). Mobile phase: A: 0.2% F.A in H2O and B: 0.2% FA in MeCN/H2O 9/1 (v/v)). Total run time: 20 min. Injection volume: 50 µL. Flow rate: 0.4 mL/min. Column temp: 40 °C. Detection system Quattro Ultima® tandem mass detector. ESI (-). MRM acquisition mode.	**Recovery**: 88%-116% **%RSDr**: 11%-32% **CCα**: 0.12-0.97 mg/kg **CCβ**: 0.13-1.43 mg/kg	[48] van Holthoon F. et al **(2010)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
Coccidiostats and sulfonamides	Non-target feed and feeds produced after a medicated feed	1 g of feed + 2.5 g of salt mixture ($MgSO_4$:NaCl 60:40 w/w) extracted with 15 mL of ACN:H_2O 60:40 v/v) and 300 µL of hexane. ***INTERNAL STANDARDS***: robenidine-d_8, sulphadimethoxine-d_8, sulphadiazine-d_6	**HPLC-MS/MS** Chromatographic system Column: Synergi Polar-RP (50×2.00 mm, 2.5 µm, 100 Å), Polar-RP security-guard (4.0×2.0 mm). Mobile phase: A: 0.1% F.A in H_2O and B: 0.1% FA inACN. Total run time: 36 min. Injection volume: 15 µL. Flow rate: 0.175 mL/min. Column temp: 24°C Detection system Qtrap 2000™ MS. MRM mode.	**Recovery**: 74%-126% **%RSDr**: 3%-23% **LOD**: 10-50 µg/kg **LOQ**: 40-100 µg/kg **CCα**: 89-2140 µg/kg **CCβ**: 128-2186 µg/kg (coccidiostats)	[49] Gavilán R. E. et al **(2018)**
Amprolium	Chicken feed	10 g of feed extracted with 100 mL MeOH/H_2O 80/20 (v/v).	**LC–MS** Chromatographic system Column: C18 (150 mm×2 mm i.d., 3 µm) Mobile phase: (initial phase pH 2.38 at 25 ◦C) heptafluorobutyric acid (HFBA) 5mM in distilled water and MeOH. Flow rate: 0.2mL/min. Injection volume: 5µL. Detection system Ion trap mass spectrometer. Electrospray capillary: 1.94 kV. Positive ions acquired in SIM mode.	**Recovery**: 96.8% **%RSD**: 13.59% (1mg/kg) **LOD**: 0.061mg/kg **LOQ**: 0.202mg/kg	[50] Squadrone S. et al **(2008)**
20 prohibited VDs	Feed samples	2.0 g of feed extarcted with 15 mL of MeOH:ACN (50:50, v/v) and ultrasonication. Cleanup with 150mg PSA sorbent.	**LC–MS/MS** Chromatographic system Column: Agilent Zorbax SB-Aq C18 (150 mm×2.1 mm, 3.5 µm). Mobile phase: A: ACN and B: 0.1% FA in H_2O. Total run time: 25 min. Flow rate: 0.25 mL/min. Injection volume: 5 µL. Detection system API 4000 triple quadrupole mass spectrometer. ESI (+).	**Recovery**: 56.7%-103% **%RSDr**: <10% **CCα**: 0.42-5.74 µg/kg **CCβ**: 5.70-9.81 µg/kg	[51] Zhang G-J. et al **(2013)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
Monensin, lasalocid, salinomycin, narasin	Feed samples	5.0 g of feed extracted with 100 mL of MeOH:H2O (90:10). ***INTERNAL STANDARD***: nigericin	**LC-MS/MS** Chromatographic system Column: (50 mm×2.1 mm, particle size 5 µm), guard column (10 mm×2.1 mm, particle size 5 µm). Mobile phase: ACN:H2O, containing 20 mM of HFBA. Total run time: 9 min. Flow rate: 300 µL/min. Detection system Triple quadrupole mass spectrometer, ESI (+).	**Recovery**: 69%-122% **LOD**: 0.018-0.056 µg/g **LOQ**: 0.2 µg/g	[52] Huang M. et al **(2011)**
VDs	Feed samples	5 g of feed extracted with 20 mL HCl (0.5 M in aqueous solution). Cleanup with SPE. ***INTERNAL STANDARD***: demeclocycline, sulfadiazine-$^{13}C_6$, trimethoprim-d_9, triclabendazole-d_3	**UHPLC-MS/MS** Chromatographic system Column: Acquity UPLC HSS T3 (150×2.1 mm, 1.7 µm particle size). Mobile phase: A: 0.05% FA in H2O and B: ACN. Flow rate: 0.5 mL/ min with post-column delivery of NH4OH. Column and autosampler temp: 50 and 15°C, respectively. Detection system Waters Acquity TQ mass spectrometer. ESI (+) & ESI (–).	**Recovery**: 89%-107% **%RSDr**: <12.4% **LOD**: 1-100 µg/kg **LOQ**: 3-300 µg/kg **CCα**: 111-5439 µg/kg **CCβ**: 122-5878 µg/kg	[53] Robert C. et al **(2016)**
22 banned or unauthorized VDs	Pig, chicken, cattle, and aquatic feeds	2 g of feed extracted with 24 mL ACN and 1 mL FA and ultrasonication. Cleanup with SPE.	**LC-MS/MS** Chromatographic system Column: RP Agilent ZORBAX SB-Aq (150 mm×2.1 mm i.d., 3.5 µm). Mobile phase: A: 0.1% FA aqueous solution and B: ACN. Column temperature: 35 °C. Injection volume: 5 µL. Flow rate: 0.2mL/min. Detection system API 4000 triple-quadrupole mass spectrometer. ESI (+). SRM mode. **LC-LTQ-Orbitrap MS** Chromatographic system Column: Thermo Scientific Hypersil Gold C18 (100 mm×2.1 mm i.d., 3.5 µm). Same mobile phase composition as for LC-MS/MS analysis. Cartridge temperature: 35 °C. Injection volume: 10 µL. Flow rate: 0.25 mL/min. Detection system LTQ-Orbitrap XL. ESI (+). Resolution:30,000 FWHM (FWHM at m/z 400). Mass range: 65-500 m/z.	*LC-MS/MS* **Recovery**: 52.2%-90.4% **%RSDr**: <15% **CCα**: 2.6-23 µg/kg **CCβ**: 4.2-34 µg/kg *LC-LTQ-Orbitrap MS* **CCα**: 1.0-12 µg/kg **CCβ**: 1.3-19 µg/kg	[54] Wang X. et al **(2013)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
Albendazole	Fish feed	1.0 g of feed extracted with 2 mL of aqueous solution containing 1.0% FA and 8 mL MeOH. ***INTERNAL STANDARDS***: albendazol sulfoxide, phenacetin	**LC-MS/MS** Chromatographic system Column: X-Terra MS C18 column (3.9 mm×100 mm, 3.5 µm). Mobile phase: A: aqueous solution with 0.1% FA and B: ACN. (Isocratic conditions 60:40 v/v, respectively). Flow rate: 0.3 mL/min. Column oven: 25°C. Detection system Triple-quadrupole mass spectrometer. ESI (+). SRM mode.	**Recovery**: 99.4% **%RSDr**: <1.03% **CCα**: 108.4 ng/g **CCβ**: 116.9 ng/g	[55] Busatto Z. et al **(2016)**
11 coccidiostats	Feed samples	5 g of feed extarcted with 10 mL of 10% Na2CO3 solution (w/v) and 15 mL ACN. Re-extraction with CAN. ***INTERNAL STANDARDS***: dinitrocarbanilide-d_8, diclazuril-bis, robenidine-d_8, decoquinate-d_5, nigericin.	**HPLC–ESI–MS/MS** Chromatographic system Column: Purospher C18 (125×3 mm, 5 µm). Mobile phase: A: 0.1% FA in H2O and B: 0.1% FA in ACN. Total run time: 16 min. Column temp: 40°C and samples: 10°C. Injection volume: 20 µL, with a split ratio of 1:5. Flow rate: 1 mL/min. Detection system Micromass Quattro Ultima tandem mass spectrometer. ESI (+) & ESI (-). MRM mode.	**%RSDr**: <23.7% **LOD**: 0.1-10 ppb **LOQ**: 0.33-15 ppb	[56] Delahaut P. et al **(2010)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
β-agonists	Feed samples	2.5 g of feed extracted with 25 mL of 0.2 M phosphoric acid/MeOH (1:1). Addition of 5 mL of 0.25 M sodium acetate buffer pH 4.8. Cleanup with SPE. ***INTERNAL STANDARDS***: clenbuterol-d_6, salbutamol-d_6, ractopamine-d_5 chloride	**LC-MS/MS** Chromatographic system Column: C18 Alltima (150 mm×3.5 mm, i.d. 5µm). Mobile phase: A: 100:0.2 H2O/FA and B: 100:0.2 ACN/FA. Column oven: 30 °C. Flow rate: 0.4 mL/min. Detection system Quattro Ultima triple-quadrupole mass spectrometer. ESI (+): MRM mode.	**Recovery**: 54%-85% **%RSDr**: 5%-24% **CCα**: 0.01-0.28 µg/L **CCβ**: 0.04-0.99 µg/L	[57] Nielen M. W. F. et al **(2008)**
Sulfonamides	Feed samples	1 g of feed extracted with 3 mL of MeOH:H2O (70:30) with FA 0.1%. Kept freezer for 30 min.	**LC-MS/MS** Chromatographic system Column: Zorbax® XDB C18 (150×4.6 mm, 5 µm). Pre-column C18 cartridge (4.0 × 3.0 mm). Mobile phase: A: ammonium acetate 10 mM with 0.1% acetic acid and B: MeOH. Total run time: 9 min. Flow rate: 0.8 mL/min. Injection volume: 20 µL. Detection system API 5000 mass spectrometer, ESI (+). MRM mode. Dwell time: 100ms.	**LOD**: 0.005-0.020 mg/kg **LOQ**: 0.075 mg/kg	[58] Hoff R. B. et al **(2014)**
50 antimicrobials	Pig, poultry and cattle feed	4 g of feed extracted with 15 mL of MeOH/ACN/McIlvaine buffer (37.5/37.5/25, v/v/v). ***INTERNAL STANDARDS***: SDD-^{13}C, NOR-d_5, ROX, DMC, RDZ-d_3, NIG	**LC-MS/MS** Chromatographic system Column: Kinetex XB-C18 (100 mm×2.1 mm, 1.7 µm particle size). Mobile phase: A: 5 mmol/L aqueous FA and B: 50 mmol/L aqueous FA/ACN (10/90, v/v). Total run time: 16 min. Column temp: 35 °C. Injection volume: 8 µL. Flow rate: 0.5 mL/min. Detection system Quattro Premier triple quadrupole mass spectrometer. ESI (+) & ESI (-).	**%RSDr**: 2%-24% (0.05 µg/g) **LOD**: 0.1-71.4 ng/g **LOQ**: 0.2-238.1 ng/g **CCα**: 7.8-1303.0 ng/g (pig feed) **CCβ**: 8.2-1355.9 ng/g (pig feed)	[59] Borràs S. et al **(2013)**

COMPOUND	MATRIX	SAMPLE PREPARATION	TECHNIQUE	RESULTS	REFERENCE
13 sulfonamides	Feed samples	5 g of feed extracted with 10 mL ultrapure H_2O and 10 mL acetic acid 0.1% (v/v) in ACN:MeOH (75:25 v/v). Addition of 4.0 g anhydrous magnesium sulfate and 0.5 g sodium acetate. Cleanup with 200 mg of PSA.	**HPLC-MS/MS** Chromatographic system Column: Zorbax Eclipse XDB C-18 (4.6×150 mm, particle size: 5 µm). Mobile phase: A:FA 0.1% in H_2O/ACN (95:5 v/v) and B: FA 0.1% in H_2O/ACN (5:95 v/v). Total run time: 18 min. Flow rate: 0.3 mL/min. Injection volume: 50 µL. Detection system Quattro Premier XE triple quadrupole mass spectrometer. ESI (+).	**Recovery**: 86.0%-106.8% **%RSDr**: 3.6%-19.5% **LOQ**: 0.9-7.1 µg/kg **CCα**: 50.4-55.8 µg/kg **CCβ**: 50.7-55.8 µg/kg	[60] Pereira Lopes R. et al **(2012)**
7 penicillins	Bovine feed	3 g of feed extracted by PLE (with inert diatomaceous earth, hydromatrix). Cleanup with SPE. ***INTERNAL STANDARDS***: amoxicillin-d_4, sulfathiazole-d_4, penicillin G-d_7, penicillin V-d_5	**LC–QLIT-MS/MS** Chromatographic system Column: C12 Phenomenex Hydro-RP (50 mm×2 mm i.d., 4 µm, 80 Å), with a C12 MAXRP security guard column (4 mm×3 mm i.d.). Mobile phase: A: HPLC grade H_2O with 0.1% FA and B: MeOH with 0.1% FA. Total run time: 10min. Loop volume: 100 µL, injection volume: 10 µL. Flow rate: 0.7 mL/min. Detection system 4000 QTrap mass spectrometer. ESI (+). MRM mode. Dwell time: 50ms.	**Recovery**: 71%-115% **%RSDr**: 1%-9% **LOD**: 0.11-4.99 ng/g **LOQ**: 0.25-13.32 ng/g	[61] Kantiani L. et al **(2010)**

1.5 Effects of veterinary drugs

The world population is increasing and there is a growing demand for food, leading to intensification of farming methods and a requirement for more coadjuvants. Potential high profits sometimes lead to fraudulent use of drugs and pesticides. Veterinary drugs in particular can pose a real risk to human health if their residues are allowed to enter the food chain. Parent drugs and their metabolites can occur in foodstuffs individually or as multicomponent mixtures with enhanced adverse effects [30]. Veterinary drugs also represent an important source of environmental pollution due to intensive agri- and aqua-culture production. The drugs can reach the environment through the treatment processes, inappropriate disposal of used containers, unused medicine or livestock feed and manufacturing processes [62].

1.5.1 Effects on human health

Residues of veterinary drugs can be found in foods of animal origin when appropriate withdrawal times are not respected or when prohibited drugs are used by negligence or fraud [63]. The presence of veterinary drug residues in food products constitutes a potential health risk for consumers as they might induce various effects such as allergic reactions, carcinogenic or teratogenic mechanisms, or induce antimicrobial resistance. In particular, antimicrobial resistance is considered as a quickly increasing global threat to the public that now requires appropriate actions across governments and society [1]. Specifically, the misuse of antibiotics can lead to bacteria becoming resistant and 25,000 deaths every year in the EU have been attributed to antimicrobial resistance [63]. Briefly, antimicrobial resistance happens when microorganisms exposed to antimicrobial drugs change and ultimately impair treatment with antibiotics in human medicine. Then, infections persist in the body, with increasing risks of spread to others. Both the World Health Organisation (WHO) and the World Organisation for Animal Health (OIE) have

addressed the threat specifically. In 2009, the WHO created the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) as a response to a worldwide solicitation of experts from human health and veterinary medicine areas. The AGISAR has revised the list of critically important antimicrobials initially published in 2005 with the addition of new substances. The OIE has also issued a list of veterinary drugs of particular concern. Incidentally, veterinary drugs may be used in an incorrect manner with production animals including sometimes disrespect of withdrawal time after treatment; this leads to residues in milk, eggs and edible tissues that can be detected either as the parent compound or metabolites/conjugates. This applies beyond antibiotics as antiparasitics, antiinflammatories, growth promoters or tranquilizers can also be found in food by misuse or incorrect practice at the farm [1]. However, there is a new trend among consumers: greater awareness of the risks and growing demand for quality and safety. This consciousness may be a reason why food analytical methods have experienced a wide range improvement in sensibility, accuracy, rapidness and reliability [29].

1.5.2 Effects on the environment

The majority of VAs causing environmental concern are solely of anthropogenic origin. The primary causes of VAs being released into agro-ecosystems are: firstly, fertilization with animal urine/manure containing antibiotics (direct application from medicated animals or application after composting), sewage sludge, sediment or biosolid; secondly, irrigation with antibiotics-contaminated waste/surface/ groundwater and thirdly, aerial transport. Depending on pharmacokinetics and specific transformation processes in animals, large proportions of the administered antibiotics are usually poorly adsorbed in the animal gut and are excreted with urine and feces in the form of parent compounds or their metabolites, which may also still be active, within a few days of medication.

In manure sampled within 2 days after oral application of tetracyclines, more than 72% of the drug was excreted unaltered. Similar findings were reported

for fluoroquinolones wherein more than 90% was excreted by pigs after oral administration, predominantly as the parent compound. The amounts of antibiotics excreted may also vary with the dosage level, type and age of the animal. In excreta, concentrations of antibiotics can even increase due to retransformation of metabolites back to the parent compound. Many investigations have been conducted to examine the residual levels of antibiotics in feces and manures from animal farms. Apart from animal excreta, dust from animal farming zones is also one of the notable routes of entry of VAs into the environment. Antibiotic residues (<1.5 μg L^{-1}) have been detected in the final effluents of wastewater treatment plants (WWTPs) in Canada, Europe and the USA, meaning that they are now an increasingly recognized environmental risk. Zhang and Li (2011) summarized the frequent occurrence of *β*-lactams, sulfonamides, quinolones, tetracyclines, macrolides and other VAs in sewage, activated sludge, digested sludge and urban biosolids from WWTPs which can enter the agro-ecosystems.

VAs may enter water bodies through urban sewage systems, wastewater from animal breeding, run-off or leaching from terrestrial ecosystems. A survey by Kolpin et al. (2002) revealed that 27% of 139 rivers in the USA contained 0.7 μg L^{-1} of VAs.

VAs that end up on land are available for plant uptake. This type of exposure leads to bioaccumulation and phytotoxicity in crop plants. Absorption, transport and accumulation of VAs were noted in the edible parts of important vegetable crops like carrot, green onion and cabbage, potato, and leek. Hu et al. (2010) found that VAs are mainly taken up through water transport and passive absorption in crop plants, especially vegetables. Their distribution in vegetables was in the order: leaf>steam>root because of biological accumulation. Consuming these VA accumulated plant parts leads to greater health risks.

Human contact with these resistant bacteria in the agricultural environment, or ingestion of resistant bacteria through the consumption of raw vegetables and/or fruits might increase the chance of exchanging resistance

determinants between the human and environmental microbiome. These may subsequently contribute to the threat of incurable infections in humans.

Monitoring and controlling the spread of VA contaminants in agricultural fields and to their crops is critical for preventing damage to agroecosystems and ultimately, consumers who purchase foodstuffs derived from them [12].

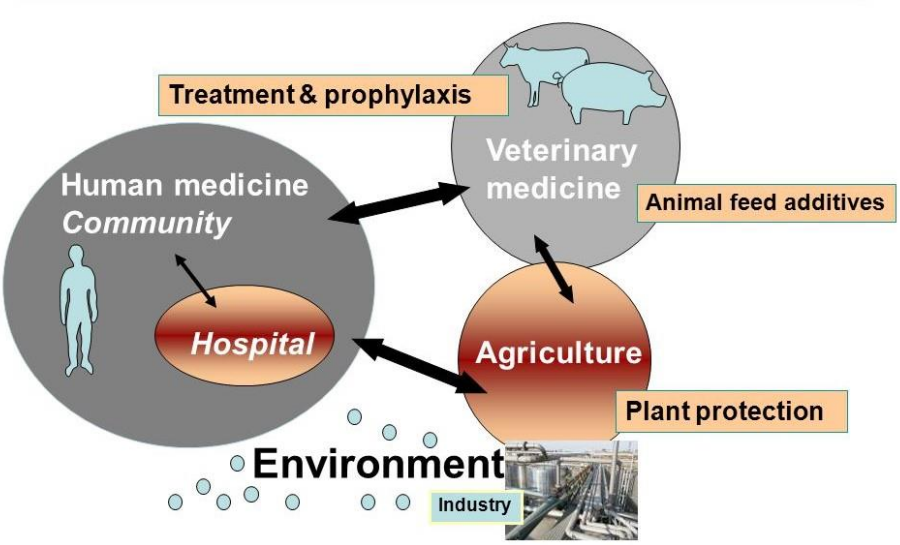


Figure 2. The antibiotics cycle. [64]

1.6 Legislation

In order to ensure the safety of the consumers, many agencies worldwide regulate the use of antimicrobials 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 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. [65,66]

Several European nations (Sweden, Germany, Denmark, etc.) have now banned the use of antibiotics in animal husbandry, that are important in the treatment of human diseases. In 2013, the US Food and Drug Administration (FDA) issued voluntary guidelines for the producers of veterinary drugs that are added to water or animal feed, with the aim of eliminating the use of medically important antibiotics as growth promoters by the end of 2016. Also, the Canadian government in 2014 implemented a voluntary strategy similar to the effort made by the FDA. Some nations, for example Mexico, South Korea and New Zealand have all banned the use of antibiotic growth promoters, but the drugs are still authorized in Japan. Antibiotic growth promoters are not banned in most nations such as Argentina, Brazil, China, India, Indonesia, Philippines, Russia and South Africa. The World Health Organization (WHO), World Organisation for Animal Health (OIE) and FDA listed a few antibiotic classes as 'critically important antibiotics (CIAs)' which include cefalosporins, fluoroquinolones, macrolides, sulfonamides, tetracyclines, phenicols, pleuromutilins, glycopeptides, aminoglycosides, cyclic esters, carbapenems, lipopeptides, oxazolidinones and mycobacterial drugs (FAI, 2016). Some argue that CIAs should be banned from use in food-producing animals, while others are concerned that a complete ban would significantly limit options for some disease treatments and potential consequences could include treatment failures with associated welfare and productivity costs. A compromise may be needed to make use of CIAs the last resort treatment option with the inclusion of additional steps to ensure their responsible use [12].

The European Union 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 [64], which has been repealed by Council Regulation 470/2009/EC [65] and describes the procedure for establishing Maximum Residue Limits (MRLs) for veterinary medicinal products in foodstuffs of animal origin

The general guidelines for animal feed sampling and analysis methods are laid out in Commission Regulation 152/2009, and its most recent amendment

by Commission Regulation 691/2013, where Olaquindox and Carbadox are classified as undesirable additives in feed. The proposed methodology for their determination is based on HPLC with UV detection and the LOQ is 5 mg/kg for both compounds. Commission Directive 2009/8/EC was implemented due to unavoidable carry-over problems of coccidiostats during feed manufacture. This Directive is amended by Regulation 574/2011, which sets up MLs for these drugs in non-target feed (non-medicated) following carry-over [49].

Although a variety of food matrices is officially regulated with MRLs available, animal feed pertaining to veterinary drugs (apart from coccidiostats and histomonostats) remains outside of a precise legislative framework. The only regulation currently available is EC/1831/2003 which prohibits 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 [70].

European Decision 2002/657/EC describes the requirements for performance and validation of analytical methods employed in the official residues control for screening and confirmatory purposes [68]. A confirmatory method used for residue analysis should be able to confirm the identity of a compound beyond reasonable doubt. To provide an adequate instrumental set-up, Commission Decision 2002/657/EC introduced the concept of “identification points”. A second aspect to assure unequivocal confirmation is the establishment of ion ratio and retention time criteria. Currently, the gold standard for confirmatory analysis of most veterinary drug residues is liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) in selected reaction monitoring (SRM) acquisition mode, isolating one precursor ion and monitoring two a priori selected product ions, yielding 4 identification points.

In residue analysis, MS, either in combination with LC or gas chromatography (GC), was assigned as the main technique for confirmation of the identity of banned and regulated substances. Among different working fields, some differences in the identification criteria exist, but they all rely on the detection of fragment or product ions [38].

CHAPTER 2
ANALYTICAL METHODOLOGIES

2.1 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.

2.1.1 Sample extraction techniques

2.1.1.1 Liquid extraction (LE)

Liquid extraction is a very popular sample treatment technique. 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 [72-76]. 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 [72,77].

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.

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. 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 [78]. 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 [79,80].

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 [81]. 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. 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 [82]. QuEChERS flexibility, coupled to low cost and ease of use will undoubtedly result in an increase in its application to residue analysis.

2.1.1.2 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE) is an interesting process to obtain highly valuable compounds and could contribute to the increase in the value of some food by-products when used as sources of natural compounds. The main benefits will be a more effective extraction, thus saving energy, and also the use of moderate temperatures, which is beneficial for heat-sensitive compounds. For a successful application of the UAE, it is necessary to consider the influence of several process variables, the main ones being the applied ultrasonic power, the frequency, the extraction temperature, the reactor characteristics, and the solvent–sample interaction. The highest extraction rate is usually achieved in the first few minutes, which is the most profitable period [83].

Figure 3. Ultrasound Assisted Extraction device with heating option. [84]

2.1.1.3 Microwave Assisted Extraction (MAE)

In the recent years, use of microwaves for extraction of pollutants from mainly environmental matrices has gained great interest. Specifically, the extraction of organic compounds by microwave irradiation appeared with the work of Ganzler et al. in 1986. Since then, the technique has attracted growing interest, and it has been widely used in analytical chemistry. MAE has successfully been adopted for various classes of micro-pollutants (e.g. flame retardants, surfactants, pharmaceutical and personal care products) due to the small number of conditions affecting extraction, speed, reduction of organic solvent consumption, relatively low cost and increased sample throughput. In MAE, microwave energy is used to heat solvents in contact with solid samples or liquid samples and to promote partition of the analytes from sample matrix into the solvent (the extractant). Microwave energy is a non-ionizing radiation (frequency 300–300,000 MHz) that causes molecular motion by migration of ions and rotation of dipoles. Thus, the principle of MAE is based on the direct effect of microwaves on molecules of the extraction system caused by two mechanisms, ionic conduction and, dipole rotation. It should be noted that, unlike usual conventional forms of heating (convection and conduction), microwaves heat the extracted system directly, leading to very short extraction times. Heat generation in the sample in the microwave field requires the presence of a dielectric compound. The greater the dielectric constant, the more thermal energy is released and the more rapid is the heating for a given frequency. Consequently, the effect of microwave energy is strongly dependent on the nature of both the solvent and the matrix. Most of the time, the solvent chosen has a high dielectric constant, so that it strongly absorbs the microwave energy. However, in some cases, only the sample matrix may be heated, so that the solutes are released in a cold solvent (this is particularly useful for thermolabile components, to prevent their degradation). The technical application of microwave energy to the samples may be performed using either closed vessels (under controlled pressure and temperature), or open vessels (at atmospheric pressure). These two technologies are commonly named pressurized MAE (PMAE) or focused MAE

(FMAE), respectively. Whereas in open vessels the temperature is limited by the boiling point of the solvent at atmospheric pressure, in closed vessels the temperature may be elevated by simply applying the adequate pressure. The main parameters influencing MAE performance include: nature of the solvent and the matrix; solvent volume; microwave power; exposure time; and, temperature [85,86].

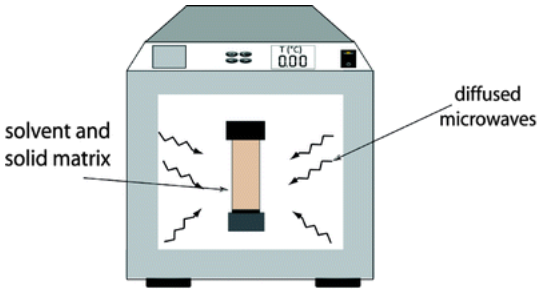


Figure 4. Microwave Assisted Extraction device. [87]

2.1.2 Sample cleanup/purification techniques

2.1.2.1 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 [88-91].

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) [92].

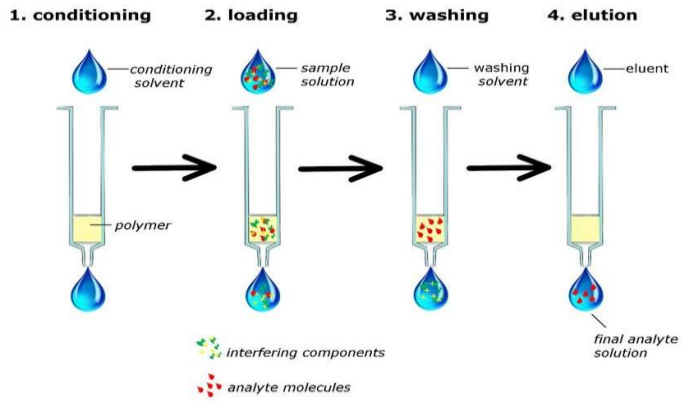


Figure 5. SPE steps. [93]

2.1.2.2 Dispersive SPE (d-SPE)

Dispersive-SPE (d-SPE) is a cleanup 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. $MgSO_4$ is added to provide additional cleanup by removing residual H_2O 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 cleanup step in veterinary residue analysis, mainly using C18 as a sorbent [94-96]. PSA, amine (NH2) and silica have also been reported [82,97-100]. d-SPE does not provide the same degree of cleanup as SPE. However, it does provide good recovery and reproducibility, coupled with practical (speed) and cost advantages.

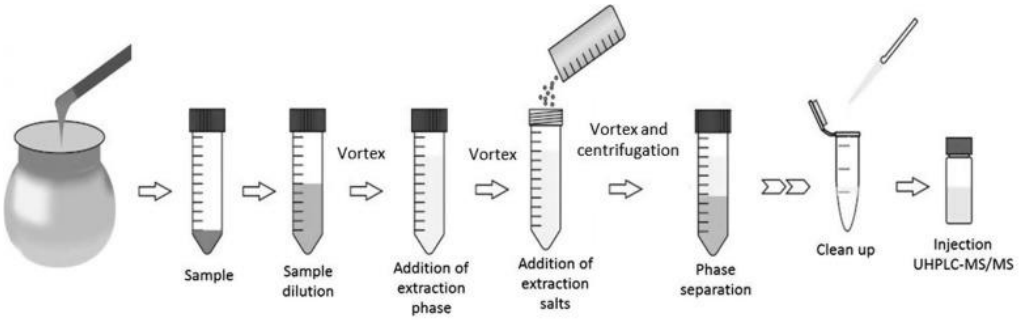


Figure 6. QuEChERS steps. [101]

CHAPTER 3

INTRUMENTAL ANALYSIS

3.1 High Pressure Liquid Chromatography

The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time “on-column”. Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved. A detection unit (e.g. UV detector) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system (computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or to the waste. In general, an HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit. The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve [102].

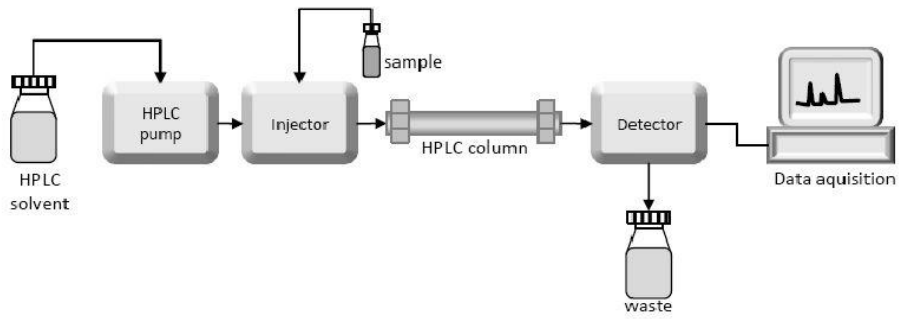


Figure 7. HPLC system components. [103]

3.2 Mass spectrometry

Mass spectrometry is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances.

This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed during the formation of ionic and neutral species.

3.2.1 Basic Principle

A mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

Components

The instrument consists of three major components:

- Ion Source: For producing gaseous ions from the substance being studied.

- Analyzer: For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.
- Detector System: For detecting the ions and recording the relative abundance of each of the resolved ionic species.

In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10-6 to 10-8 mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries [104].

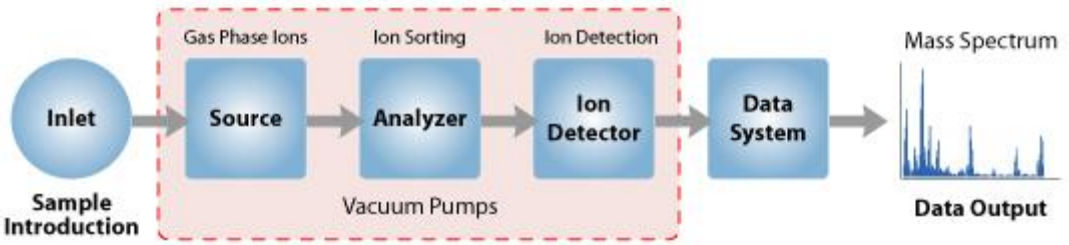


Figure 8. Mass spectrometer components. [104]

3.2.1.1 Ion Source

API-ES is a process of ionization followed by evaporation. It occurs in three basic steps: (1) nebulization and charging, (2) desolvation and (3) ion evaporation.

Nebulization

The HPLC effluent is pumped through a nebulizing needle which is at ground potential. The spray goes through a semi-cylindrical electrode which is at a high potential. The potential difference between the needle and the electrode produces a strong electrical field. This field charges the surface of the liquid and forms a spray of charged droplets. There is a concentric flow of gas which assists in the nebulization process.

Desolvation

The charged droplets are attracted toward the capillary sampling orifice. There is a counterflow of heated nitrogen drying gas which shrinks the droplets and carries away the uncharged material.

Ionization

As the droplets shrink, they approach a point where the electrostatic (coulombic) forces exceed the cohesive forces. This process continues until the analyte ions are ultimately desorbed into the gas phase. These gas-phase ions pass through the capillary sampling orifice into the low pressure region of the ion source and the mass analyzer [105].

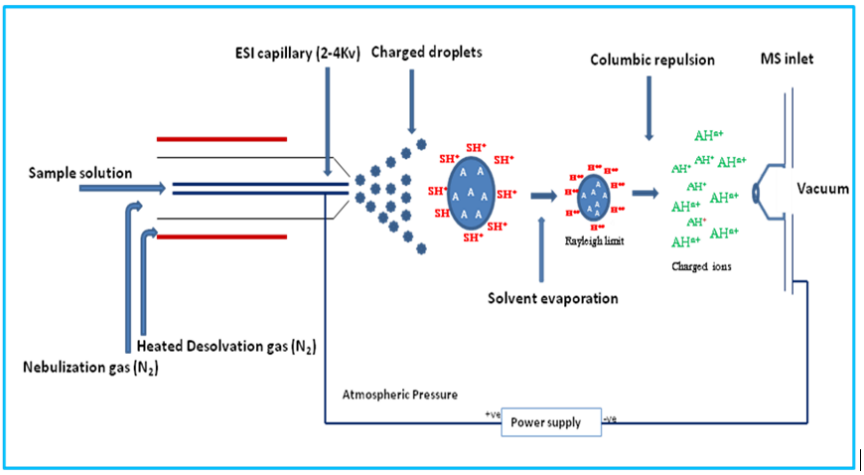


Figure 9. Sample ionization by Electrospray Ionization. [106]

3.3 Liquid Chromatography tandem Mass Spectrometry

LC-MS is a hyphenated technique, which combines the separating power of HPLC with the detection power/capability of mass spectrometry [107]. 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.

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 (HRMS; 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 [108-110]. 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 [111].

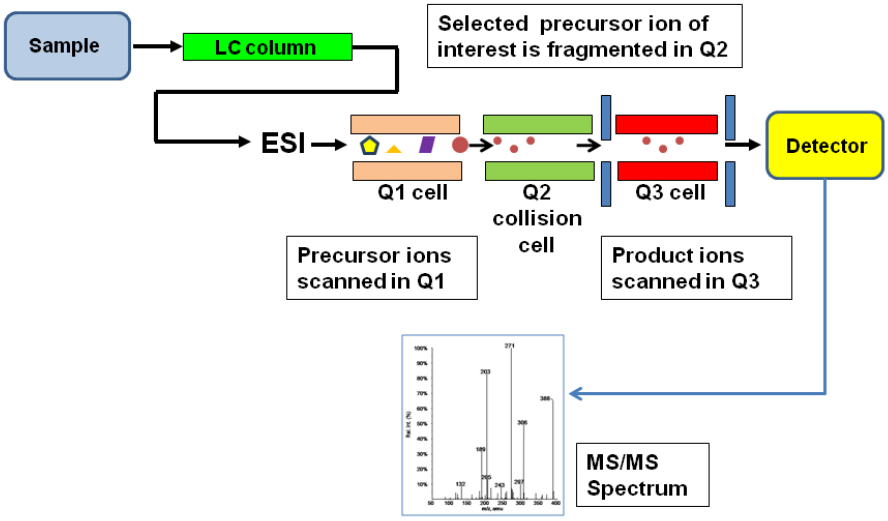


Figure 10. HPLC system coupled to triple quadrupole system. [112]

3.4 LC-MS/MS Techniques: Advantages

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 [113]. 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. The application range of MS/MS is extremely wide today, 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 [114,115].

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 of these disadvantages, QqQ, coupled to liquid chromatography, still remains the analyzer of choice for the determination of veterinary drugs in food matrices.

CHAPTER 4

RESEARCH OBJECTIVE AND SCOPE

4.1 The analytical problem

Regarding the residue analysis of veterinary drugs in feed, there are several difficulties that constitute the analytical problem. Firstly, there is a 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) [116].

Another main analytical problem is the low concentration levels at which a veterinary drug residue should be analysed, since most of the MRLs and MRPLs established for food, to be taken as a guide, are at the ppb level (parts per billion or μg kg^{-1}). Therefore, analytical methods for the determination of veterinary drug residues in feed 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 be the main aspect to be taken into consideration. Animal feed is generally extremely diverse in its components, depending on its intended use, and contains a lot of aminoacids, which is important for those drugs that bind easily to proteins and fatty ingredients [117].

Due to all the aforementioned reasons and the desire to improve the cost-effectiveness of analytical procedures, the development of multiclass methods which are able to detect, confirm and quantify as many analytes as possible, has become a significant trend in the analysis of residues and contaminants in feed 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.

4.2 Scope

A broad range of veterinary drugs are administrated in animal husbandry in order to improve animal health but also as growth promoters for intensive animal production. Quality animal nutrition, free of contaminants and carry-over residues is of paramount importance for the food safety of humans, since through the food chain, these residues can be detected in animal tissues, food of animal origin and even plants and vegetables irrigated with contaminated water and eventually reach the human organism.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is an alternative technical approach that is now popular for the screening of more than 100 veterinary drugs in a single run. By the end of the 20th century, LC-MS/MS had evolved dramatically as a major analytical tool, providing both sufficient sensitivity to reach the regulatory limits and adequate certainty in the identification of the compounds detected. Furthermore, LC-MS/MS is versatile enough to be used either as a screening tool or a quantitative method (or both), depending on the application [1].

The aim of this study was the development of robust and reliable UHPLC-MS/MS methodology for the simultaneous and reliable identification and quantitative determination of 70 VDs from different groups. A thorough investigation of the sample preparation parameters and variables was carried out. The final sample preparation protocol comprised of an Ultrasonic-assisted solid-liquid extraction of the analytes from the matrix

combined with a three-phased cleanup procedure. The cleanup included overnight freezing of the sample to achieve protein precipitation, hexane partitioning for the removal of lipids and SPE. The developed method was validated in agreement with the guidelines of Commission Decision 2002/657/EC and satisfactory method performance characteristics were achieved.

CHAPTER 5

INSTRUMENTATION, LAB EQUIPMENT AND REAGENTS

5.1 Instrumentation

A Thermo UHPLC Accela system was connected to a Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument (Thermo, San Jose, CA, USA). (Figure 11)

Instrument control and data acquisition were carried out by using the Xcalibur software, Version 2.3, from Thermo.

Figure 11. Thermo Scientific TSQ Quantum Access Triple Quadrupole Instrument. [118]

5.2 Laboratory Equipment

In the laboratory equipment used were included mobile phase solvent filtration apparatus (Millipore, XX15.04705), calibrated analytical balance with four decimal digits (Sartorius-Basic), ultra-pure water apparatus 18.2 MΩ / cm (Millipore Direct-Q UV), ultrasonic bath (Metason 60 Stuers), a Vortex spinner apparatus (Velp Scientifica), a centrifugation apparatus (Rotofix 32 Hettich) and a pH meter (HQ30d, HACH).

They were also used, 5, 10, 50, 100 and 250 mL volumetric flasks, 10 mL siphon 100 mL beakers, 15 mL and 50 mL plastic centrifuge tubes, 10 and 50 and 100 mL volumetric cylinders, calibrated 10, 100 and 1000 mL pipettes, plastic pasteur pipettes, and 10 mL test tubes.

5.3 Chemicals and Reagents

All veterinary drug and pharmaceutical standards were of high purity grade (>90%) They were purchased from Fluka Analytical, Alfa Aesar or Riedel-de-Haen with the vast majority of them being purchased from Sigma–Aldrich (Steinheim, Germany). 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 Fluka (Buchs, Switzerland) 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 Macherey-Nagel (Duren, Germany).

5.3.1 Preparation of standard solutions

About 10 mg of each individual standard was accurately weighed and placed in a 10-mL volumetric flask and were dissolved in the appropriate solvent (methanol, water, acetonitrile or DMSO). For those compounds that no bulk was available at the time, standards previously prepared were used. Stock solutions of 1000 mg mL^{-1} of each compound were obtained and stored at -20 ^{o}C in brown glass to prevent photo degradation. Four intermediate standard solutions at a concentration of 10 mg mL^{-1} for all compounds were prepared by mixing and dilution of the stock solutions with methanol. Three final working solutions were prepared by diluting all the compounds in methanol at final concentration 1 mg mL^{-1} and were also stored at -20 ^{o}C. New standard solutions of 1000 mg mL^{-1} were prepared every month for the group of *β*-lactams. All working solutions and calibration standards were obtained by

gradient dilution of the intermediate solutions, in concentrations varying from 1 mg mL^{-1} to 200 ng mL^{-1}. The working standard solution of internal standards in a concentration of 5 mg mL^{-1} came by subsequent dilutions of their stock solutions in methanol. While not in use, the working solutions were kept at -20 ^{o}C and renewed weekly. Matrix-matched standards were prepared in the same way as the other samples.

CHAPTER 6

METHOD DEVELOPMENT AND VALIDATION

6.1 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 mm, Waters) column protected by a guard column was used at a constant flow rate of 100 mL 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 B) and methanol (solvent C), while for the negative mode detection was modified water (1 mM ammonium formate (B)), methanol (C) and acetonitrile (D). The gradient elution programmes for both runs are presented in Tables 3 and 4. The necessary time for the re-equilibration of the analytical column was in both cases 10 min, the column was thermostated at 30 ℃ 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 values used were provided by a previous study, where they were obtained for each separate compound by direct infusion of individual standard solutions at concentration of 1 mg 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 presented in Tables 5. Multiple reaction monitoring (MRM) was used and detailed parameters for MRM acquisition are presented in Table 2. Two transitions were selected for identification, but only the most intense one was used for quantification. Each chromatographic run was divided into 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.

Table 3. Gradient elution programme for positive ESI.

TIME	A%	B%	C%	D%	FLOW (µL/MIN)
0.00	0.00	90.00	10.00	0.00	100.00
10.00	0.00	0.00	100.00	0.00	100.00
27.00	0.00	0.00	100.00	0.00	100.00
27.50	0.00	90.00	10.00	0.00	100.00
28.00	0.00	90.00	10.00	0.00	200.00
33.00	0.00	90.00	10.00	0.00	200.00
33.50	0.00	90.00	10.00	0.00	100.00
37.00	0.00	90.00	10.00	0.00	100.00

Table 4. Gradient programm for negative ESI.

TIME	A%	B%	C%	D%	FLOW (µL/MIN
0.00	0.00	70.00	25.00	5.00	100.00
10.00	0.00	0.00	95.00	5.00	100.00
19.00	0.00	0.00	95.00	5.00	100.00
19.50	0.00	70.00	25.00	5.00	200.00
23.50	0.00	70.00	25.00	5.00	200.00
23.60	0.00	70.00	25.00	5.00	100.00
25.00	0.00	70.00	25.00	5.00	100.00

Table 5. ESI parameters for positive and negative ionization mode.

ESI MODE	**+**		**ESI MODE**	**-**
Spray voltage	4000		Spray voltage	4000
Sheath gas pressure	25		Sheath gas pressure	25
Auxiliary gas pressure	10		Auxiliary gas pressure	10
Capillary temperature	300		Capillary temperature	300
Collision pressure	1.5		Collision pressure	1.5
Gradient	90:10		Gradient	70:25:5
Mobile phase B	F.A 0.01%		Mobile phase B	1mM A.F

6.2 Samples and quality control materials

A poultry feed sample was used during these experiments. It was obtained from NIREUS and was confirmed to be free of the targeted analyte residues by LC-MS/MS. It was homogenized and stored at room temperature until analysis. The blank feed was repeatedly measured to confirm that no VDs 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 of the working solutions at the suitable concentrations, to each 2-g portion of the weighed samples. Suitable volume of the internal standard working solution was added at each sample to achieve a final concentration of 150 μg kg^{-1} for each IS. For the evaluation of the different extraction procedures, blank samples were spiked at 300 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.

6.3 Sample preparation

A schematic diagram of the sample preparation (spiking, extraction and cleanup process) is presented in Fig. 12. 2 g of properly homogenized sample were weighed and placed into a 50 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, 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 min before proceeding. To extract the drug residues and precipitate the proteins, 10 mL of extraction solvent containing ACN, MeOH, EDTA 0.1% (w/v) in water and formic acid 0.1% (v/v) 1:1:1 were added to the samples. The addition of EDTA which is a chelating agent improves the extraction recovery of some antibiotics, especially of tetracyclines, as it prevents their rapid chelation with metal ions. After the addition of the solvent the tube was vortex-mixed for 30 s. The samples were placed on a mechanical shaking device for 30 min and afterwards in an ultrasonic bath at 65 ℃ for 25 min in order for 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 15 mL polypropylene centrifuge tube. The tubes were placed in the freezer overnight (12-16 h) in order to precipitate the lipids and remaining proteins. The next day, the samples were again centrifuged, the precipitate was thrown away and the supernatant was transferred in another 50 mL tube were the extracts were defatted using 5 mL of hexane, vortexed for 1 min, and then centrifuged at 4000 rpm for 5 min. The hexane layer was aspirated to waste and 5 mL of the final extract were diluted to a final volume of 70 mL with water (in order to achieve a final organic solvent content of less than 5% for the SPE cartridges to work). Following that, the SPE cleanup procedure takes place, where the HLB (Oasis HLB, 6cc, 200mg) cartridges are initially conditioned with 5 mL MeOH and 5 mL water to activate the bed and remove any impurities, then the sample is loaded and set at a dropwise flow rate. After the sample had

passed through the cartridge, the bed was left to dry for 15 min and then the analytes were eluted with 5 mL MeOH containing 0.1% (v/v) formic acid. Finally, the samples were evaporated to dryness under a nitrogen stream at a temperature not exceeding 45 ℃. 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-mm RC filter into vials. Appropriate volumes of working multi-analyte solutions were added to blank aliquots at this step, to prepare the range of matrix-matched standards required.

6.4 Method Development

Since the chromatographic and detection parameters for the targeted compounds were already optimized in a previous study, this study's focus was the sample extraction and cleanup optimization.

The first step was the determination of the most suitable solvent for the sufficient extraction of the majority, if not all, of the targeted analytes. For that purpose four different extraction solvents were tested: (A) ACN:MeOH (1:1), (B) ACN:MeOH (1:1) -F.A 0.1% (v/v), (C) ACN:MeOH:aqueous EDTA 0.1% (w/v) (1:1:1) -FA 0.1% (v/v) and (D) ACN:MeOH:McIlvaine buffer pH 4.6 (1:1:1). Generally, mixtures of organic solvents with water are used for the extraction of veterinary drugs. [37,3,5,41,42,43,10,47,49] Furthermore, as tetracyclines form a chelate complex with divalent metal cations and bind with proteins [2], the use of a stronger chelating agent of cations such as EDTA, or/and a strong acid, such as formic acid, or an acidic deproteinizing agent to eliminate proteins may greatly improve tetracycline extraction. The McIlvaine buffer has also been successfully applied for the extraction of veterinary drugs from feed matrices.

In a second step, purification of the samples was implemented in the form of Solid Phase Extraction (SPE), which seems to be a favorable sample cleanup procedure, as it can be used both to purify the extracts and possibly pre-concentrate them. Different types of cartridges have been used for feed matrices (HLB, WCX, SCX, MCX, C18) depending on the targeted group of substances. In this study HLB (Oasis HLB,Waters) and C18 (Strata-X C18,

Phenomenex) were the cartridges chosen, bearing in mind the wide range of physicochemical properties of the studied compounds. The conditioning (5 mL MeOH and 5 mL H_2O), wash (5 mL H_2O) and elution (5 mL MeOH) steps were the same for both procedures. A 5-mL portion of the sample needed to be first diluted in a final volume of 70 mL with H_2O, for optimum cartridge performance.

Upon deciding on the most efficient extraction solvent and SPE cartridge type, we experimented with two assisted extraction methodologies: Ultrasound Assisted Extraction (UAE) and Microwave Assisted Extraction (MAE). Ultrasonic and microwave radiation are known to accelerate the extraction process. These techniques provide shorter extraction times, low solvent consumption and better recoveries than classical extraction techniques. MAE is a technique that has the ability to reduce extraction times and organic solvent consumption, as well as perform multiple extractions and improve recovery. To be comparable with the UAE, the same extraction temperature (65 ℃) and overall time (25 min) were used, with a ramp time to the final temperature of 5 minutes, at 400 W.

Next, these two techniques were also compared coupled with a different cleanup procedure based on the QuEChERS approach, which is widely used in residue analysis, due to its low cost, high throughput, ease of use and acceptable recoveries. The supernatant of the sample was added to a tube with 1 g of Na_2SO_4 and a mix of 0.150 g PSA and 0.150 g C18 sorbents in order to remove water and undesirable co-extractives. While $MgSO_4$ is the most frequently used salt in the place of Na_2SO_4, the latter was chosen, as it is known that Fluoroquinolones form stable complexes with Mg (II).

Early on, it became evident that a high degree of sample purification would be required, due to the complexity of the matrix and the multitude of interfering agents. SPE, yielding the best results and “cleanest” extracts, was an unavoidable step and therefore was optimized in regards to the elution solvent and the wash step. This optimization was performed on microwave-mediated extracts with the additional steps of protein precipitation, by overnight freezing, and hexane partitioning to remove lipids. Placing the samples in the

freezer, at -20 ℃, overnight stretches the analysis time to two days, but it is a necessary compromise for better results. Methanol is the most commonly used elution solvent and was compared with an elution solvent consisting of MeOH-F.A 0.1% (v/v). Another comparison was between extracts obtained from cartridges washed with 5 mL of H_2O and eluted with MeOH and extracts where the wash step was foregone. The wash eluate of the first extracts was checked for possible leaching of the analytes, especially of the more water-soluble ones.

Finally, after concluding the appropriate extraction solvent, purification technique and its consequent optimization, MAE and UAE, coupled to SPE were compared to decide on a final sample preparation procedure. In this test freezing and hexane partitioning were also used.

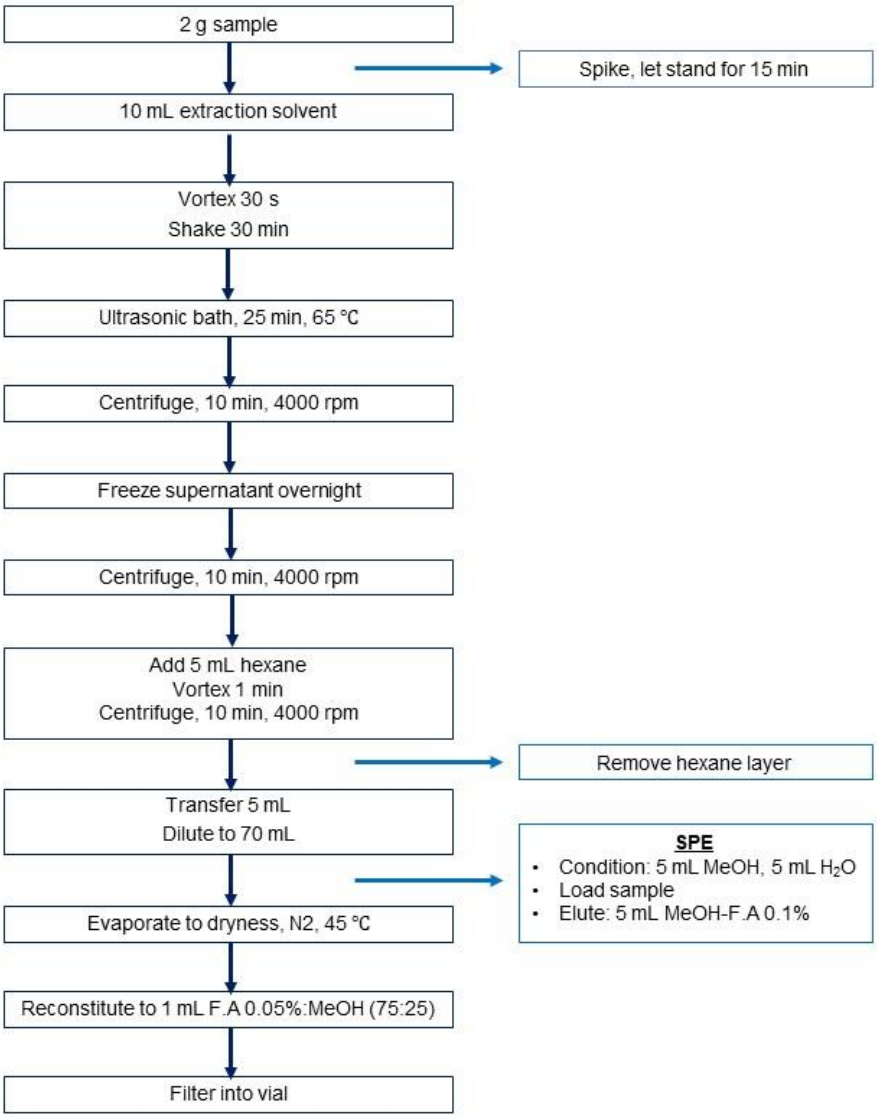


Figure 12. Developed methodology step by step.

6.5 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 extraction procedure was validated in terms of selectivity, linearity, trueness, repeatability, inter-day precision, limits of detection (LODs) and limits of quantification (LOQs). The use of internal standards was only feasible for some antibiotics since there were limited isotopically labeled internal standards available (sulfadiazine-d4, sulfadimidine-d4, sulfadimethoxine-d4, olaquindox-d3, ivermectin-d2, triclabendazole-d3). The choice of the adequate IS was made based on the retention time of the analytes. 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, CCa, and the detection capability, CCb, which are parameters for compliance analysis by authorities [25].

6.5.1 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 were within the tolerance defined [119].

6.5.2 Selectivity/Specificity

The selectivity and specificity were assessed by analyzing blank samples of the 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.

6.5.3 Linearity

The linearity of calibration curves was assessed by using a seven-point calibration curve of standards in pure solvent as well as in blank poultry feed at seven different concentrations and three levels (1, 2, 5 ,10, 20, 50, 75 ng mL^{-1} for the low level, 10, 30, 50, 75, 100, 150, 200 ng mL^{-1} for the medium level and 100, 200, 300, 500, 600, 800, 1000 ng mL^{-1} for the high level). This number of concentration 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. Peak area and peak area ratio of the analyte/IS were used as the analytical response versus concentration in all cases. 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.

6.5.4 Precision

The precision of this method was demonstrated in terms of repeatability (intra-day precision) and within-laboratory reproducibility (inter-day precision). Repeatability and reproducibility were expressed as the %RSD values of a set of 6 replicate analysis for repeatability and 6 replicate for reproducibility at the 3 concentration levels examined (0.5, 1 and 2 times the VL).

6.5.5 Trueness

The trueness of the method was estimated through recovery studies. Average recoveries of each analyte at the Validation Level (20 μg kg^{-1} for the low level, 100 μg kg^{-1} for the medium level and 400 μg kg^{-1} for the high level) were calculated performing the analysis in 6 replicates of the matrix and are portrayed in Table 6.

6.5.6 LODs and LOQs

LODs and LOQs were calculated by analyzing blank samples spiked at 10, 50, or 400 μg kg^{-1}, according to each analyte's sensitivity, as described in the Experimental section. Results are shown in Table 7.

6.5.7 Matrix Effect

When complex samples, such as animal feed, 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 [71]. 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 each substance's own ILIS is used. The use of analogue ILIS is not always satisfactory [119].

To evaluate the 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:

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

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%.

6.6 Instrument performance

Calibration curves in pure solvent were constructed for all compounds by plotting the peak area against the concentration of the seven corresponding calibration standards. The calibration curves for the compounds corrected by the IS 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. 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 six replicates of the lowest concentration of the spiked sample for each compound divided by the slope of the calibration curve. LOQs were calculated as 10 times the SD divided by the slope.

6.7 Method performance

Matrix-matched calibration curves were obtained by addition of the target compounds in blank feed extracts at different concentrations (1–75, 10-200 and 100-1000 ng $mL^{-}1$, seven concentration levels). The standard addition curve was developed by fortifying seven 2 g fractions of blank material with the appropriate volumes from three multi-analyte working solutions of 200 ng mL^{-1}, 1 mg mL^{-1} and 5 mg mL^{-1} (1–75, 10–200 and 100-1000 ng kg^{-1}).

In order to evaluate the trueness of the method, recovery studies were carried out. 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 three different levels (0.5VL, VL and 2VL). The determination of reproducibility was carried out on a different day. For the calculation of the method's LODs and LOQs fortification of six blank samples was performed in very low concentration of analytes (0.5VL). The SD of the peak area of the six replicates (or the peak area ratio) is calculated in the lowest concentration that every analyte is determined in the matrix (10, 50 or 400 ng kg^{-1}). 3.3 times the SD divided to the slope of the standard addition curve provides method's LOD for each analyte 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 calibration curves for all analytes prepared in solvent and in matrix, separately.

CHAPTER 7

RESULTS AND DISCUSSION

7.1 Sample preparation optimization

The ultimate goal of this study was the development of a multi-residue methodology for the determination of a wide range of veterinary drugs belonging in different family groups. The chromatographic behavior of the compounds has been explored in detail and their separation and detection parameters have been optimized in a previous study. Therefore, the current study focused on the successful and reliable extraction of the analytes from animal feed, which is one of the most complex and difficult to handle matrices.

Animal feed inherently contains a lot of ingredients in order to meet the nutritional needs of animals, such as aminoacids and fatty acids, which renders the analysis of this matrix challenging and laborious. From an instrumental//technical point of view the samples need to be cleaned, or they can prove troublesome for the HPLC column and the mass analyzer, while also providing acceptable recoveries of the analytes.

As it is described in 6.4 the first step of the method development was the identification of the most effective extraction solvent. Solvents (C) and (D) provided the best recoveries and lowest matrix effect values for the targeted compounds, with comparable results between the two. The reason solvent (C) was ultimately preferred over solvent (D) is the ease and speed of its preparation, since the McIlvaine buffer used in solvent (D) consists of two separate solvents (citric acid 0.1 M and disodium hydrogen phosphate 0.2 M) and needs to be pH-adjusted first.

In the SPE purification step that followed, out of the two cartridge types, HLB and Strata-X C18, the HLB proved slightly more beneficial for the majority of analytes. That is to be expected though, as an HLB cartridge, with its versatile packing material, appeals to a wider range of compounds, both non-polar and more polar.

To accelerate and facilitate the extraction of the analytes from the matrix, UAE and MAE were tested and both turned out comparable results. Thus, they were next combined with a d-SPE cleanup procedure. While the d-SPE approach offered good results, the physical extract of the sample demanded further cleanup in order for a proper, reliable analysis to be carried out instrumentally. The QuEChERS approach was abandoned in favor of the much “cleaner” extract-yielding SPE.

The initial SPE procedure, with the chosen HLB cartridge, was optimized during the wash and elution step. The addition of the overnight freezing of the samples, in order to precipitate proteins and the hexane partitioning step for the removal of fats, while significantly increasing the time of analysis, serve to further relieve the matrix of its undesirable interfering components. Between the two elution solvents tested, MeOH-F.A 0.1% (v/v) yielded overall better recoveries and physically clearer eluates. On the topic of washing the cartridge bed before the elution of the analytes, no washing is preferable, as some of the more polar compounds were found to be washed away too, resulting in lower recoveries.

The final decision, following the solvent extraction and the optimization of the cleanup procedure, lain in the choice of the most suitable and effective assisted extraction technique (UAE or MAE). While both techniques show promising and comparable results in terms of recovery, again the factors of ease of use, time and cost-effectiveness were evaluated and UAE dominated over MAE. A comparison of the obtained recoveries of all analytes for all the tested parameters is schematically presented in Figure 13.

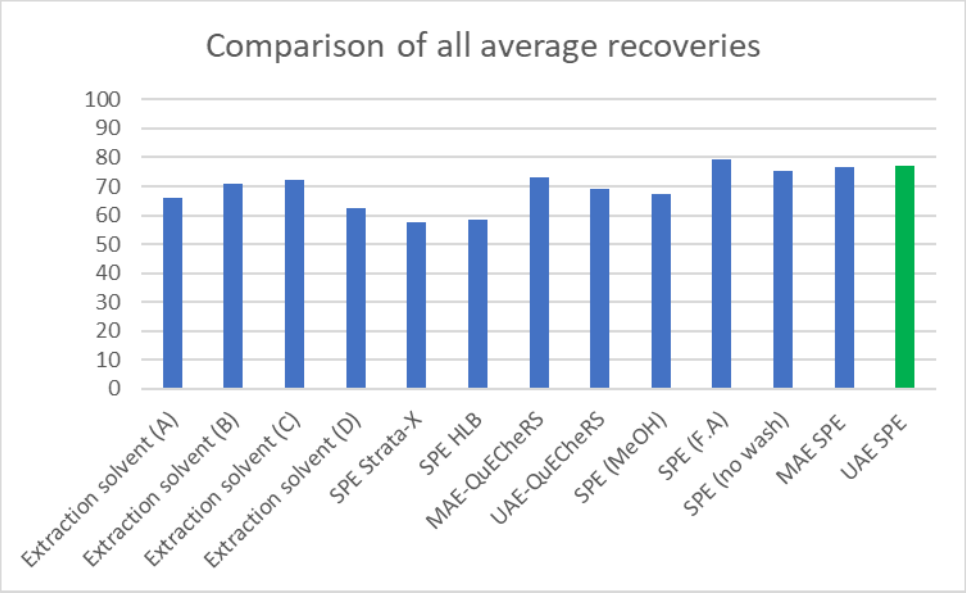


Figure 13. Comparison of recoveries of all tested parameters.

7.2 Method Validation

7.2.1 Linearity

The linearity of calibration curves was assessed using a seven-point standard solution calibration curve in pure solvents as well as in blank sample extracts at different concentrations. 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 IS versus the analyte concentrations when an IS correction was used. The regression line of the form y = bx + a and the correlation coefficients R^2, for spiked and matrix-matched samples were determined. Figure 14 shows indicatively the calibration curves for the spiked sample of sulfadimidine, sulfadiazine, flumequine, penicillin G, clarithromysin, febantel, emamectin, carbadox and oxytetracycline and Figure 15 shows the calibration curves of the same compounds in matrix extract.

For instrument linearity, the calibration parameters showed good linearity since correlation coefficients were >0.99 for all analytes except amphenicols

(0.9670 for chloramphenicol). R^2 ranged from 0.9907 (novobiocin) to 0.9997 (sulfamethizole) for standard solution curves.

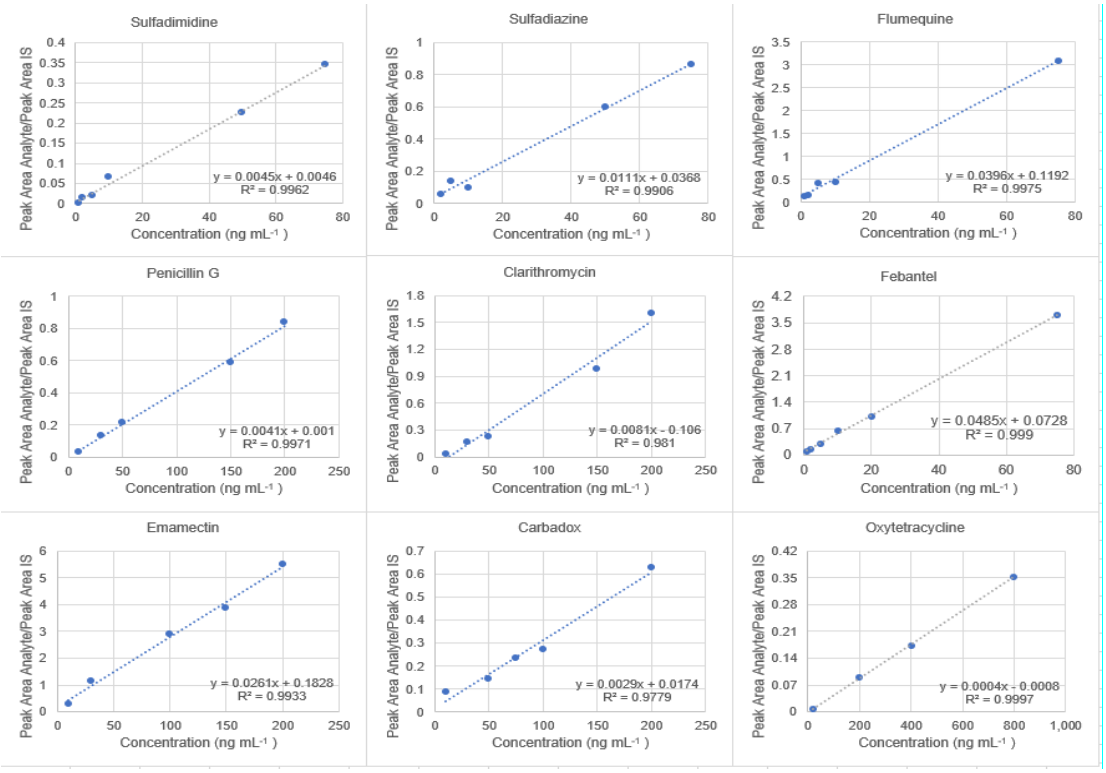


Figure 14. Linearity curves of spiked samples of indicative compounds.

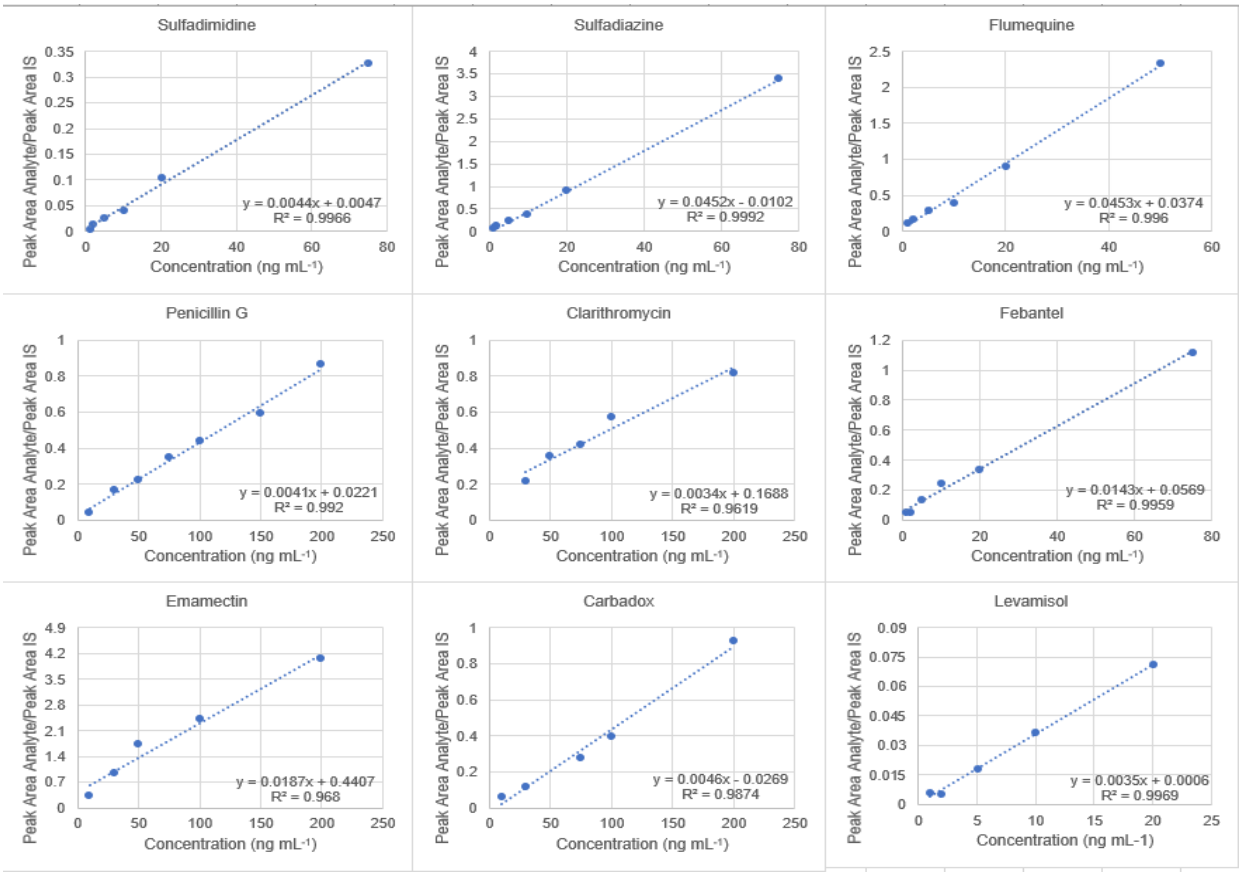


Figure 15. Linearity curves of matrix-matched standards of indicative compounds.

7.2.2 Precision

The precision of this method was calculated as intra-day precision (repeatability) and inter-day precision (within-laboratory reproducibility).

It can be observed that relative standard deviations were in most cases lower than 20%. Moreover, the obtained %RSD values of the within-laboratory reproducibility did not exceed 25%. These results indicate the good precision and reliability of the developed methodology for the majority of the compounds but also highlights the need for a separate, more specific sample handling procedure for the extraction and detection of some particularly challenging groups of analytes, namely avermectins (abamectin, ivermectin, moxidectin, doramectin, erpinomectin) and β-lactams (penicillins and cefalosporins). Precision results for all compounds in all concentration levels are presented in Table 6.

Table 6. Within-day and intra-day precision results.

		Repeatability									Reproducibility								
		0.5VL (n=6)			1VL (n=6)			2VL (n=6)			0.5VL (n=12)			1VL (n=12)			2VL (n=12)		
	COMPOUND	%Rec	SD	%RSDr	%Rec	SD	%RSDr	%Rec	SD	%RSDr	%Rec	SD	%RSD	%Rec	SD	%RSD	%Rec	SD	%RSD
VL 20 µg/kg	Sulfachloropyridazine	40.1	2.4	5.9	76.0	8.1	10.6	36.7	4.9	13.4	42.9	5.2	12.0	55.5	20.7	37.2	41.7	7.5	17.9
	Sulfadimidine	109	10.1	9.3	108	14.4	13.3	112	10.1	9.0	117	16.3	13.9	99.4	17.3	17.4	105	12.0	11.4
	Sulfadimethoxine	128	13.4	10.5	113	9.2	8.1	106	13.2	12.4	120	15.0	12.5	113	7.9	7.0	105	9.8	9.3
	Sulfadoxine	96.3	14.0	14.5	114	12.5	11.0	114	9.1	8.0	99.6	14.5	12.0	109	12.8	11.7	112	9.0	8.1
	Sulfadiazine	94.0	16.1	17.1	58.6	9.9	16.9	104	7.9	7.6	90.3	15.7	17.4	70.1	15.8	22.5	109	12.3	11.2
	Sulfisoxazole	95.1	15.3	16.1	100	11.9	11.9	90.3	7.8	8.6	94.8	16.6	17.5	87.8	17.4	19.8	83.2	12.0	14.5
	Sulfamonomethoxine	46.1	7.1	15.4	70.0	6.4	9.1	80.4	5.4	6.8	44.5	8.1	18.3	58.1	14.5	24.9	76.5	14.9	19.5
	Sulfamethoxypyridazine	91.9	10.8	11.8	69.8	12.0	17.2	73.6	9.4	12.7	80.2	16.3	20.3	63.8	12.1	19.0	73.7	9.3	12.6
	Sulfamerazine	89.2	6.7	7.5	92.4	16.6	18.0	70.1	6.0	8.6	93.6	8.7	9.3	90.7	19.0	21.0	66.7	8.4	12.6
	Sulfamethizole	93.4	11.5	12.3	60.3	13.5	22.4	79.9	4.4	5.5	86.2	17.8	20.7	57.8	11.6	20.1	67.6	13.5	19.9
	Sulfamethoxazole	26.7	2.5	9.5	29.1	1.9	6.5	60.5	9.2	15.2	32.6	6.8	20.7	34.8	7.2	20.7	58.3	9.6	16.5
	Sulfamoxole	47.4	8.8	18.5	56.4	9.9	17.5	80.2	10.4	13.0	47.2	8.3	17.6	58.3	9.3	16.0	81.3	11.0	13.6
	Sulfapyridine	105	2.7	2.5	79.7	10.5	6.6	64.7	7.4	11.5	99.5	10.9	11.0	77.8	16.0	10.3	64.7	5.3	8.2
	Sulfaquinoxaline	103	17.2	16.7	98.6	16.9	17.1	61.6	5.1	8.3	105	17.2	16.3	102	16.3	16.0	66.1	7.2	10.9
	Sulfathiazole	88.3	12.4	14.1	81.9	16.4	20.0	95.3	4.6	4.8	92.6	15.8	17.1	91.0	16.8	18.4	93.9	8.7	9.3
	Dapsone	76.0	6.5	8.6	58.7	10.7	18.2	93.9	20.5	21.8	71.7	12.0	16.7	67.5	15.0	22.3	93.9	15.9	17.0
	Ciprofloxacin	71.2	15.8	11.1	85.8	16.1	18.8	67.7	10.9	8.1	68.8	16.7	12.1	81.6	17.4	21.3	72.7	17.6	12.1
	Danofloxacin	127	12.7	5.0	82.0	16.2	19.8	99.6	15.6	15.7	108	41.2	19.1	78.4	12.1	15.4	110	18.9	17.3
	Difloxacin	93.5	15.1	16.1	75.5	13.4	17.8	92.0	14.8	16.1	87.0	16.5	19.0	75.1	13.1	17.4	102	17.4	17.0
	Enrofloxacin	106	11.7	11.1	117	18.2	15.5	118	32.6	12.8	99.0	19.5	19.7	106	17.2	16.2	114	50.3	18.1
	Flumequine	86.1	13.6	15.8	78.6	11.4	14.5	91.5	3.1	3.4	89.0	16.3	18.3	86.5	12.8	14.8	107	17.2	16.1
	Marbofloxacin	95.4	14.0	14.7	112	15.6	13.9	89.2	10.4	11.7	87.9	17.6	20.0	93.8	22.6	24.1	94.3	10.0	10.6

	Ofloxacin	101	17.2	17.1	93.5	18.8	20.1	102	8.4	8.2	93.0	20.7	22.3	100	19.3	19.2	110	11.1	10.0
	Oxolinic acid	122	14.6	12.0	79.6	14.2	17.8	125	4.1	3.3	104	21.9	21.0	77.5	12.1	15.6	120	7.8	6.4
	Sarafloxacin	67.5	12.1	17.9	91.0	11.1	12.2	84.2	9.0	10.7	59.7	13.6	22.8	90.2	11.1	12.3	95.6	16.9	17.7
	Tiamulin	89.5	6.5	7.3	79.9	14.4	18.1	79.8	3.7	4.7	83.2	15.0	18.1	95.3	20.1	21.1	72.9	10.4	14.2
	Albendazole	91.2	19.3	21.2	65.9	9.1	13.9	88.6	16.0	18.0	90.2	19.5	21.7	72.4	12.8	17.7	82.5	14.9	18.1
	Febantel	112	8.6	7.7	109	17.8	16.3	108	15.2	14.1	101	16.3	16.2	107.0	21.3	19.9	99.0	21.1	21.3
	Flubendazole	36.4	5.8	15.8	51.6	5.0	9.7	85.6	10.7	12.4	43.0	8.8	20.4	49.8	6.2	12.4	87.5	15.2	17.4
	Mebendazole	42.0	4.3	10.2	57.4	11.4	19.9	77.2	7.0	9.1	36.2	7.6	21.0	52.6	11.4	21.7	80.0	14.9	18.7
	Oxfendazole	90.7	8.5	9.4	95.1	13.9	14.6	91.9	2.5	2.7	100	15.7	15.7	89.1	12.8	14.4	88.9	7.0	7.8
	Thiabendazole	87.1	13.9	15.9	90.9	8.7	9.5	106	6.2	5.8	84.4	16.4	19.4	78.8	19.2	24.4	100.1	12.5	12.4
	Triclabendazole	19.9	4.1	20.6	30.0	5.1	17.1	59.8	6.4	10.6	20.5	3.8	18.6	32.2	8.9	27.7	67.8	11.7	17.3
	Fenbendazole	23.8	8.9	37.4	40.5	11.9	29.4	79.3	11.1	14.0	30.3	13.7	45.4	42.8	11.9	27.9	80.8	13.0	16.1
	Levamisol	22.9	0.9	3.9	27.7	4.6	16.4	23.5	1.0	4.3	24.9	3.4	13.6	28.6	4.8	17.0	25.3	3.8	15.0
	Colchicine	81.8	12.6	15.4	66.2	17.1	12.9	72.2	5.5	7.7	73.8	15.2	20.5	115	23.2	20.2	81.1	15.2	18.7
	Trimethoprim	101	14.2	14.1	111	23.6	10.6	76	6.4	8.4	112	17.9	16.0	107	31.9	14.8	74.2	7.1	9.6
	Rifaximin	118	22.4	19.1	71	11.8	16.7	70	10.0	14.4	118	21.1	17.9	69.2	12.8	18.5	69.6	12.4	17.8
VL 100 µg/kg	Amoxicillin	104	19.8	19.0	99.2	14.2	14.3	93.9	17.2	18.4	110	21.0	19.1	111	28.1	25.2	87.3	15.7	18.0
	Oxacillin	n.d	n.d	n.d	80.1	20.9	26.1	122	12.0	9.8	n.d	n.d	n.d	85.3	19.0	22.3	112.1	18.6	16.6
	Penicillin V	88.1	17.7	20.1	45.4	7.6	16.8	104	14.0	13.5	91.0	18.9	20.7	40.6	8.2	20.1	100.4	19.8	19.7
	Penicillin G	89.1	18.0	20.2	84.2	27.5	16.4	113	22.1	19.5	88.8	18.5	20.8	81.2	24.6	15.1	102.9	20.5	19.9
	Dicloxacillin	84.9	14.0	16.5	110	39.5	36.1	61.2	14.0	22.9	95.0	19.8	20.8	72.4	49.6	68.5	62.6	13.1	20.9
	Clarithromycin	83.7	28.3	16.9	103	20.4	19.8	87.0	15.3	17.6	83.3	29.5	17.7	119	29.4	24.7	82.2	14.4	17.5
	Erythromycin	83.2	10.6	12.7	75.1	14.2	18.9	23.6	3.4	14.4	80.6	14.0	17.4	77.0	11.8	15.4	23.3	3.7	16.0
	Tilmicosin	98.7	11.1	11.3	77.9	17.7	22.7	93.3	16.5	17.7	104.4	15.5	14.9	84.4	17.5	20.7	93.6	17.7	19.0
	Tylosin	33.6	5.2	15.5	75.3	14.1	18.8	79.1	9.7	12.2	30.8	6.5	21.2	53.2	30.7	57.7	79.2	9.5	11.9
	Carbadox	76.2	8.0	10.5	79.5	13.2	16.6	93.1	4.3	4.6	78.5	12.3	15.6	81.9	12.3	15.0	99.0	10.2	10.3

	Olaquindox	100	8.9	8.9	18.7	3.5	18.8	14.3	1.7	11.8	94.5	18.8	19.8	17.4	3.1	17.7	14.5	1.8	12.5
	Bromohexine	8.71	1.6	18.6	7.70	1.5	19.1	10.3	0.7	7.2	9.70	2.1	21.7	7.10	1.4	19.7	9.39	1.4	14.9
	Morantel	55.5	2.2	3.9	68.9	10.3	15.0	66.8	3.2	4.8	50.5	7.8	15.4	60.6	12.9	21.3	65.1	5.6	8.6
	Novobiocin	65.03	12.4	19.1	18.03	3.5	19.6	30.01	6.1	20.2	62.23	11.7	18.8	16.87	3.2	18.8	32.46	5.4	16.6
	Lincomycin	7.86	1.2	15.3	7.18	1.2	17.1	5.66	0.7	11.6	7.67	1.3	17.2	7.73	1.3	16.5	5.79	0.6	10.1
	Emamectin	38.6	3.0	7.7	63.9	11.0	17.2	88.6	19.1	21.6	37.7	3.7	9.9	69.9	12.9	18.5	92.4	16.1	17.4
	Metronidazole	17.1	1.9	11.1	26.5	4.4	16.5	21.1	2.5	12.0	18.8	3.8	20.4	23.5	4.6	19.5	19.9	2.5	12.8
	Ternidazole	26.3	3.3	12.6	26.6	5.0	18.7	36.9	4.7	12.8	24.1	3.7	15.4	28.1	5.0	17.8	40.7	7.3	18.0
	Ronidazole	28.5	3.8	13.5	42.3	5.9	13.9	48.4	6.2	12.8	31.8	6.4	20.2	38.2	6.1	15.9	50.9	8.2	16.0
VL 400 µg/kg	Cefadroxil	n.d	n.d	n.d	4.35	0.7	16.6	5.93	1.4	23.9	5.14	3.3	63.3	4.24	0.8	18.6	5.65	1.0	18.6
	Cefapirin	67.4	11.0	16.2	32.5	7.3	22.3	41.7	4.0	9.7	61.5	12.0	19.6	32.1	4.8	15.1	37.3	6.6	17.8
	Ceftiofur	22.8	4.6	20.1	16.6	3.7	22.0	18.5	3.3	17.7	23.4	4.8	20.5	16.6	3.9	23.5	18.0	3.7	20.4
	Imidocarb	n.d	n.d	n.d	60.8	5.5	9.0	82.8	5.5	6.7	n.d	n.d	n.d	59.0	8.3	14.1	72.7	12.8	17.6
	Chlortetracycline	32.4	3.8	11.8	32.6	3.7	11.2	43.7	3.3	7.6	30.4	4.5	14.6	42.0	9.9	23.6	51.9	9.3	17.9
	Doxycycline	104	15.4	14.8	81.1	8.0	9.9	124	7.0	5.7	118	24.9	21.1	78.2	7.2	9.2	122	10.1	8.3
	Oxytetracycline	39.2	5.9	15.0	38.2	2.1	5.5	50.6	3.6	7.1	46.6	9.9	21.3	42.7	5.6	13.0	55.0	6.0	10.9
	Tetracycline	33.0	5.1	15.5	33.6	3.1	9.3	44.2	4.5	10.2	35.4	6.8	19.1	35.6	3.9	10.9	47.6	5.7	12.1
	Minocycline	82.3	6.4	7.8	78.6	3.9	5.0	60.0	1.9	3.2	75.5	9.1	12.0	74.3	6.5	8.7	71.9	13.5	18.7
	Florfenicol	50.8	1.2	2.4	51.2	2.2	4.3	44.9	1.3	2.9	51.7	3.5	6.7	49.6	2.5	5.1	48.3	4.3	8.9
	Thiamphenicol	62.2	4.0	6.4	58.1	2.2	3.7	48.1	1.1	2.3	64.5	3.4	5.3	58.1	3.5	6.0	55.5	10.2	18.3
	Chloramphenicol	49.1	3.6	7.4	47.3	1.9	4.0	42.5	0.9	2.2	50.0	3.2	6.5	47.4	1.4	2.9	46.8	5.3	11.4
	Oxyclozanide	26.1	3.8	14.6	40.7	7.2	17.7	40.0	1.4	3.4	25.0	10.4	12.6	43.6	8.0	18.3	39.7	4.1	10.2

7.2.3 Accuracy

The accuracy of the method was estimated through recovery studies. Average recoveries of each analyte were calculated performing the analysis in 6 replicates at each validation level at two different days. The results of the recovery study are given in Table 6. Recoveries at the 0.5×VL varied from 7.86% (lincomycin) to 128% (sulfadimethoxine). Despite the fact that some compounds present recovery values not close to 100%, they are considered acceptable since they were reproducible.

7.2.4 LODs & LOQs

LODs and LOQs were calculated as described in section 8.1.6. The obtained results are presented in Table 7.

Table 7. Method recoveries, linearity, detectability and matrix effect results.

	%Recovery	R^2	Matrix Factor	% Matrix Effect	LOD (µg/kg)	LOQ (µg/kg)
COMPOUND						
Sulfachloropyridazine	108	0.995	1.12	11.5	3.20	9.60
Sulfadimidine	90.7	0.996	1.10	10.0	0.94	2.81
Sulfadimethoxine	96.4	0.995	1.16	15.6	0.32	0.96
Sulfadoxine	91.1	0.999	1.50	50.0	0.50	1.51
Sulfadiazine	106	0.991	6.11	510.8	1.61	4.84
Sulfisoxazole	90.0	0.995	1.00	0.0	3.03	9.09
Sulfamonomethoxine	87.7	0.995	1.52	52.4	2.97	8.92
Sulfamethoxypyridazine	76.4	0.998	1.00	0.0	2.68	8.04
Sulfamerazine	99.1	0.998	0.89	-11.1	3.09	9.28
Sulfamethizole	72.1	0.989	0.59	-40.9	6.29	18.88
Sulfamethoxazole	77.0	0.989	0.35	-65.2	9.68	29.04
Sulfamoxole	82.3	0.993	0.82	-17.7	3.72	11.17
Sulfapyridine	90.5	0.996	1.00	0.0	1.66	4.97
Sulfaquinoxaline	86.0	0.997	1.19	18.8	0.83	2.49
Sulfathiazole	89.5	0.998	1.61	60.9	3.80	11.41
Dapsone	73.3	0.978	0.57	-42.9	5.61	16.83
Ciprofloxacin	92.8	0.993	2.45	145.0	1.00	3.00
Danofloxacin	106	0.994	2.30	130.0	2.21	6.64
Difloxacin	107	0.993	1.56	56.4	1.11	3.34
Enrofloxacin	118	0.990	1.87	86.9	0.08	0.25

	%Recovery	R^2	Matrix Factor	% Matrix Effect	LOD (µg/kg)	LOQ (µg/kg)
Flumequine	89.7	0.998	1.93	92.8	0.03	0.09
Marbofloxacin	79.4	0.991	1.40	40.0	1.29	3.86
Ofloxacin	74.6	0.992	2.58	158.4	0.64	1.92
Oxolinic acid	103	0.993	2.03	103.2	0.09	0.27
Sarafloxacin	84.5	0.994	1.18	17.8	3.83	11.50
Tiamulin	104	0.993	1.16	15.6	0.08	0.25
Albendazole	82.6	-	-	-	1.49	4.46
Febantel	89.6	0.999	1.08	7.5	0.15	0.44
Flubendazole	93.4	0.996	2.09	109.4	0.17	0.52
Mebendazole	71.0	0.998	2.87	187.1	0.19	0.56
Oxfendazole	101	0.996	1.27	26.8	0.33	1.00
Thiabendazole	96.2	1.000	0.75	-24.6	0.39	1.18
Triclabendazole	51.0	0.996	1.05	5.4	1.49	4.47
Fenbendazole	68.3	0.921	0.00	-100.0	2.42	7.26
Levamisol	12.5	0.997	1.09	9.4	0.68	2.03
Colchicine	114	0.950	1.87	87.1	0.39	1.18
Trimethoprim	107	0.993	0.82	-18.2	0.58	1.74
Rifaximin	93.0	0.996	1.33	32.6		
Amoxicillin	25.0	0.956	7.00	600.0	6.48	19.43
Oxacillin	92.2	0.9770	0.67	-33.3	35.40	106.21
Penicillin V	87.8	0.998	1.67	66.7	112.16	336.47
Penicillin G	90.8	0.997	1.28	28.1	39.05	117.16
Dicloxacillin	96.6	0.972	1.00	0.0	78.90	236.69
Clarithromycin	101	0.981	1.21	21.4	4.35	13.05
Erythromycin	84.0	0.993	91.00	9000.0	7.35	22.06
Tilmicosin	110	0.993	0.50	-50.0	24.19	72.58
Tylosin	90.5	0.9812	0.50	-50.0		
Carbadox	89.1	0.978	0.92	-8.0	4.03	12.09
Olaquindox	11.7	0.994	1.25	25.0	25.83	77.50
Bromohexine	8.3	0.958	2.26	125.6	7.37	22.12
Morantel	35.1	0.967	0.65	-35.3	6.12	18.35
Novobiocin	69.1	0.995	0.35	-65.0	0.91	2.73
Lincomycin	5.3	0.984	3.26	226.2	22.69	68.06
Emamectin	98.8	0.993	1.18	18.4	9.98	29.95
Metronidazole	17.4	0.989	0.80	-20.2	12.96	38.87
Ternidazole	34.3	0.985	1.13	12.5	10.28	30.83
Ronidazole	35.2	0.998	0.89	-11.4	6.83	20.50
Cefadroxil	-	-	-	-	35.72	107.15
Cefapirin	58.5	-	-	-	28.69	86.08
Ceftiofur	90.8	-	-	-	79.98	239.93
Imidocarb	173	0.995	0.33	-66.7	165.05	495.15

	%Recovery	R^2	Matrix Factor	% Matrix Effect	LOD (µg/kg)	LOQ (µg/kg)
Chlortetracycline	52.5	0.983	5.00	400.0	10.26	30.79
Doxycycline	105	0.990	0.00	-100.0	0.72	2.17
Oxytetracycline	48.1	1.000	2.44	144.4	8.75	26.26
Tetracycline	47.2	0.998	2.88	187.5	8.18	24.53
Minocycline	68.6	0.980	-	-	1.37	4.12
Florfenicol	29.9	0.9751	2.55	154.6	4.15	12.45
Thiamphenicol	4.4	0.9934	2.15	114.6	11.09	33.27
Chloramphenicol	29.7	0.9849	1.69	69.1	8.95	26.86
Oxyclozanide	0.7	0.9873	2.39	138.7	6.77	20.30

7.2.5 Selectivity/Specificity

The selectivity of the method was evaluated by analyzing blank poultry feed samples. No background peaks, above a signal-to-noise ratio, were present at the same elution time as the target compounds, ensuring the method's acceptable selectivity for the studied compounds.

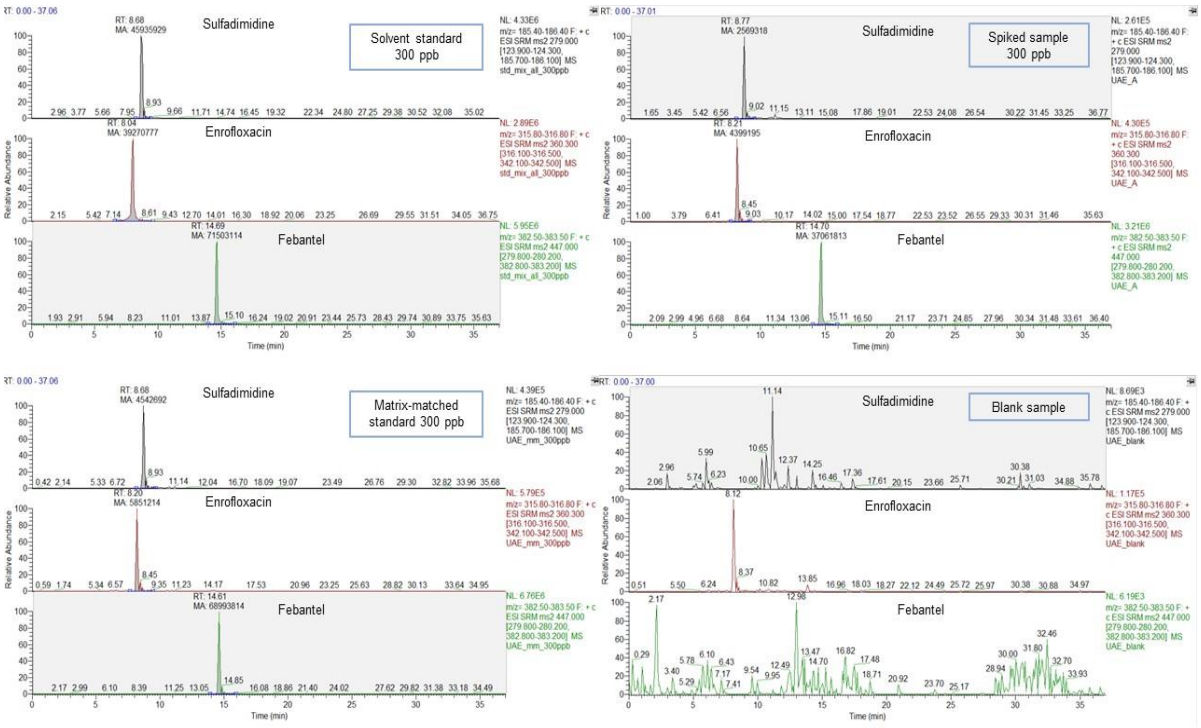


Figure 16. SRM chromatogram of selected compounds in standard solution, matrix-matched standard, spiked sample and blank sample.

7.2.6 Matrix Effect

Matrix effects higher than 20% or lower than -20% are indicative of a strong matrix effect. The % ME calculated as described in 8.1.7 are presented in Table 7 and as expected, taking into consideration the complexity of the matrix and the many potentially impeding agents, strong signal suppression or enhancement can be noted for all of the studied compounds.

7.3 Conclusions

Veterinary drugs are widely used in animal husbandry either to prevent or treat the diseases that afflict them. Since veterinary drugs in animal feed, with the exception of coccidiostats and histomonostats, are unregulated by a legal framework, they are also often used illegally as growth promoters for economical gain.

The goal achieved through this study was the development of a sensitive and reliable multi-class methodology for the simultaneous determination of multiple veterinary drug residues spanning many antibiotic and anthelmintic groups in an extremely challenging and complex matrix. The final sample preparation protocol comprised of a solid-liquid extraction of the matrix facilitated by ultrasound waves, followed by cleanup with protein precipitation, hexane partitioning and SPE. This method yielded the best results recovery- and matrix effect- wise and was validated according to the EC 2002/657 Regulation. The obtained %RSD, LOD and LOQ values were deemed acceptable for a total of 70 veterinary drugs. Still, as evidenced by these parameters, the developed methodology is not suitable for certain analytes, for whom perhaps more specific and extensive sample treatment must be applied.

ABBREVIATIONS-ACRONYMS

EU	European Union
HPLC	High pressure liquid chromatography
EC	European Council
SPE	Solid Phase Extraction
MS/MS	Tandem Mass Spectrometry
UV	Ultraviolet
FL	Fluorescence
VA	Veterinary antibiotic
CE	Collision energy
QuEChERS	Quick Easy Cheap Effective Rugged Safe
HLB	Hydrophilic-Lipophilic Balance
IP	Identification Point
HRMS	High Resolution Mass Spectrometry
QTOF	Quadrupole Time of Flight
ACN	Acetonitrile
F.A	Formic acid
MeOH	Methanol
ESI	Electrospray Ionization
PSA	Primary Secondary Amine
EDTA	Ethylenediaminetetraacetic acid
LOD	Limit of Detection
LOQ	Limit of Quantification
MRM	Multiple Reaction Monitoring

EFSA	European Food Safety Authority
WHO	World Health Organisation
OIE	World Organisation for Animal Health
AGISAR	Advisory Group on Integrated Surveilance of Antimicrobial Resistance
WWTPS	Wastewater Treatment Plants
USDA	United States Department of Agriculture
FSIS	Food Safety Inspection Service
FAO	Food and Agriculture Organization
JECFA	Joint Expert Committee for Food Additives
FDA	Food and Drugs Administration
CIA	Critically Important Antibiotic
FAI	Food Allergen Initiative
MRL	Maximum Residue Limit
CGMPR	Current Good Manufacturing Practice Regulation
ML	Maximum Limit
SRM	Selected Reaction Monitoring
ME	Matrix Effect
IS	Internal standard
d-SPE	dispersive-Solid Phase Extraction
UAE	Ultrasound Assisted Extraction
MAE	Microwave Assisted Extraction
LC-MS/MS	Liquid Chromatography – tandem Mass Spectrometry

QqQ	Triple quadrupole
RSD	Relative Standard Deviation
RT	Retention time
SD	Standard Deviation
UHPLC	Ultra-high performance liquid chromatography

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