Development and Validation of an HPLC-MS/MS Multi-Class Method with Special Emphasis on Pharmacologically Active Agents in Milk and Chicken Feed

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FACULTY OF FOOD TECHNOLOGY OSIJEK

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DEVELOPMENT AND VALIDATION OF AN HPLC-MS/MS MULTI-CLASS METHOD WITH SPECIAL EMPHASIS ON PHARMACOLOGICALLY ACTIVE AGENTS IN MILK AND CHICKEN FEED

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Pomoć pri izradi:	Michael Sulyok, Dipl.inž. Dr.techn.

Razvoj i validacija HPLC-MS / MS metode za višeklasnu analizu s posebnim naglaskom na farmakološki aktivne tvari u mlijeku i hrani za perad

Lidija Kenjerić, 0009071964

Sažetak: U ovom je radu razvijana višeklasna metoda visokotlačne tekućinske kromatografije tandemske masene spektrometrije (HPLC-MS/MS) za analizu farmakološki aktivnih tvari, odnosno različitih klasa ostataka veterinarskih lijekova u mlijeku i hrani za perad, koja je naposljetku i validirana. Metoda korištena u ovoj studiji bila je modifikacija pristupa koji su opisali Malachová i sur. (2014). Ova je metoda prenesena i utvrđeni su LC-MS parametri antibiotskih i antimikrobnih spojeva. Omjer m/z za sve optimizirane analite određen je u pozitivnom i u negativnom ionizacijskom načinu. Optimizirani parametri implementirani su u „novu“multi metodu. Potom je testirana stabilnost standarda, dok je za potrebe optimizacije ekstrakcijske procedure ispitana uporaba različitih otapala. Prije analize, uzorci mlijeka i hrane za perad podvrgnuti su ekstrakciji u kiselim i neutralnim ekstrakcijskim uvjetima. Metoda je validirana ispitivanjem sljedećih parametara izvedbe: linearnost, LOD, LOQ, preciznost, točnost i unutarlaboratorijska ponovljivost. Rezultati validacije metode pokazuju da usprkos velikog opsega ispitivanih analita u smislu polarnosti, stabilnosti i topljivosti, velik dio analita (80-90%) udovoljava SANTE kriterijima prema kojima je validacija provedena.

Ključne riječi:	ostaci veterinarskih lijekova, HPLC-MS/MS, hrana za perad, mlijeko, validacija
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Development and validation of an HPLC-MS / MS multi-class method with special emphasis on pharmacologically active agents in milk and chicken feed

Lidija Kenjerić, 0009071964

Summary: In this work, a multi-class high-pressure liquid chromatography - tandem mass spectrometry (HPLC-MS / MS) method was developed and validated for the analysis of pharmacologically active substances, i.e., different classes of veterinary drug residues in milk and poultry feed. The method used in this study was a modification of the approach described by Malachová et al. (2014). The m/z ratio for all optimized analytes were detected both in the ESI positive and negative mode and the more sensitive mode was selected. The m/z ratio for all optimized analytes was determined in both positive and negative ionization modes. Optimized parameters have been implemented in a "new" multimethod. The stability of standards was tested, and different solvents were tested for the purpose of optimizing the extraction procedure. Prior to analysis, milk and poultry feed samples were subjected to extraction under acidic and neutral extraction conditions. The method was validated through examination of the following performance parameters: linearity, LOD, LOQ, precision, accuracy and within laboratory repeatability. The results of the method validation show that despite the large range of tested analytes in terms of polarity, stability and solubility, a large part of the analytes (80-90%) meet the SANTE validation criteria.

Key words: veterinary drug residues, HPLC-MS/MS, poultry feed, milk, validation

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Posvećeno mami, tati, Marku i Josipu.

Neizmjerno hvala Michaelu, Davidu, Sashi i Filippu.

Circumstances do not make the man; they only reveal him to himself.

Epictetus

Table of Contents

1.OBJECTIVES

Veterinary drugs are pharmacologically active substances that are administered as therapeutic, prophylactic and growth-promoting agents to animals (Stolker and Brinkman, 2005).

Before application of veterinary medicine, it is important to understand how this will affect the entire food chain. In addition to animal health, proper use of veterinary medicines is essential for the successful production and ultimately the health of the consumers themselves. Challenges such as residual levels, intrinsic toxicity, antimicrobial resistance, and environmental contamination from animal waste arise here. A substantial number of veterinary drugs is available on the market. They are divided into several major classes, depending on the target of their action. Veterinary drug residues are metabolites of a drug and remaining traces of the drug in the edible tissues and organs of animals intended for slaughter and subsequently intended for consumption. Carcinogenicity, disruption of intestinal microbiota, and development of multidrug resistance are just some of the complications that can occur due to the consumption of foods high in residues (Rana et al., 2019). Observing the path of residues and maintaining them within set limits is thus crucial for the safe food chain. Consequently, the need to develop methods that will properly eliminate or at least minimize these issues is an ongoing argument throughout the world. Accordingly, this work aimed to establish a validated high pressure liquid chromatography tandem mass spectrometry (HPLC-MS/MS) multi-class method with special emphasis on pharmacologically active agents (such as different classes of veterinary drugs) in animal-based foodstuff and plant-based feedstuff. Initially, the method was optimized, built and full method validation for a selected number of analytes was performed, together with a solvent testing study and isochronous stability study of investigated analytes.

2.INTRODUCTION

2.1. CLASSIFICATION OF VETERINARY DRUGS

When a new drug is manufactured, it must be classified in a particular class. The World Health Organization (WHO) has implemented a classification system that is called The Anatomical Therapeutic Chemical (ATC) Classification System (WHO, 2020c). The present system provides a scheme which classifies active factors according to chemical and therapeutic properties as well as targeted organs or body systems. Substance classes covered by this work are anthelmintic, antiprotozoal, macrocyclic lactones, sulfonamides, aminoglycosides, macrolides, tetracyclines, quinolones, nitroimidazole, polymyxin, pleuromutilin, ß-lactams (penicillins and cephalosporins), coccidiostats, amphenicols, non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids. Overall, 157 veterinary drugs were investigated in different food and feed matrices.

2.1.1. Antiparasitics

Parasites are organisms that live on or in the organism that is called the host. Major parasite groups include helminths, protozoa, ectoparasites, and others (Kappagoda and Singh, 2011). Their adaptation and reproduction phases are carried out in the host system that may trigger pathological changes in different host tissues, although these rarely prove fatal. Control of parasitic diseases plays an important role for small and large producers of livestock or poultry and for veterinary medicine itself, as it impairs the health, reproduction, and productivity of the animal (Holmes, 1993). Parasite agents are the target of antiparasitic drugs, which act by controlling growth or causing destruction of the parasite. Anthelmintic drugs are subdivided according to the class of worms they act on, but also based on their chemical structure. There are anticestodal, antinematodal and antitrematodal anthelmintics. Ectoparasites are organisms that live on the skin of a host, for example, lice or scabies. Ectoparasiticides are subdivided into scabicides and pediculicides. Nematodes (roundworms), cestodes (tapeworms), and trematodes (flukes) are considered as helminths. Anthelmintic drugs affect the neuromuscular transmission of nematodes (Martin, 1997). One of the most frequently used anthelmintic is levamisole (Dasenaki et al., 2017). Levamisole (**Figure 1**) is used in veterinary medicine for the treatment of lungworms and gastrointestinal nematodes. This drug is considered to be an acetylcholine antagonist since the nervous system of the parasite is its target site. Levamisole has agonistic activity toward the L-subtype nicotinic acetylcholine receptors in the nematode muscle producing contraction and spastic paralysis (Martin, 1997; McHugh et al., 2020).

Chemical structure of benzimidazoles include heterocyclic aromatic compound that consists of a benzene ring fused to an imidazole ring. It is used in veterinary medicine for the treatment of gastrointestinal nematode and trematode infections. Benzimidazoles bind to the β-tubulin of nematodes causing inhibition of further polymerization of α- and β-tubulin subunits which ultimately results in a lethal effect (Martin, 1997). The most important representatives of this subclass of anthelmintics are albendazole (**Figure 1**), cambendazole, fenbendazole and thiabendazole.

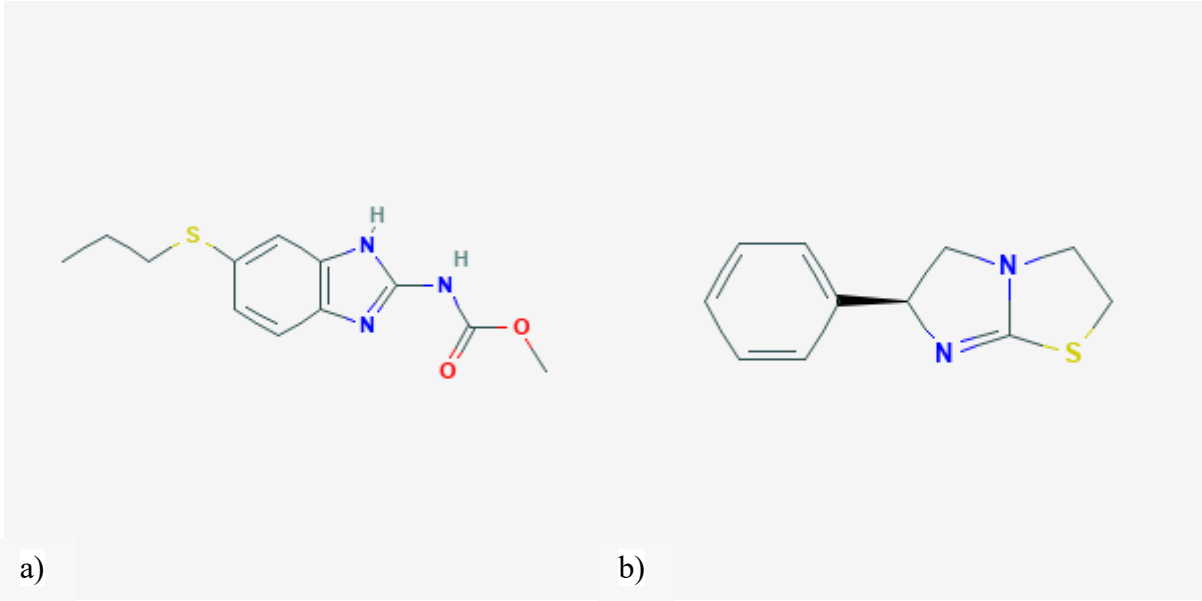


Figure 1 Chemical structure of albendazole (a) and levamisole (b), (PubChem, 2021)

2.1.2. Macrocyclic lactones

Milbemycins and avermectins belong to the family of macrocyclic lactones (MLs). These two subclasses are derived by fermentation from soil microorganisms of the genus *Streptomyces*. MLs have a broad antiparasitic application in animal medicine. All subclasses of MLs belong to the same structural chemical family, but each differs in biological activities. Avermectins and milbemycins are most commonly used to treat parasitic diseases. Avermectins are characterized by a macrocyclic lactone ring which involves glycosidic linkages. Milbemycin's have a structurally similar macrocyclic lactone ring characterized by the absence of disaccharide groups (Danaher et al., 2006). Macrocyclic lactones cause starvation and paralysis and kill parasites by irreversibly binding to glutamate-gated chloride channels thus inhibiting electrical activity and transmission of neural signals in nerve and muscle cells of invertebrates (Danaher et al. 2006). One of the most popular MLs, which is classified as milbemycin, is

moxidectin (**Figure 2**). Moxidectin is used for infections by gut nematodes, heartworms and ectoparasites (Prichard et al., 2012).

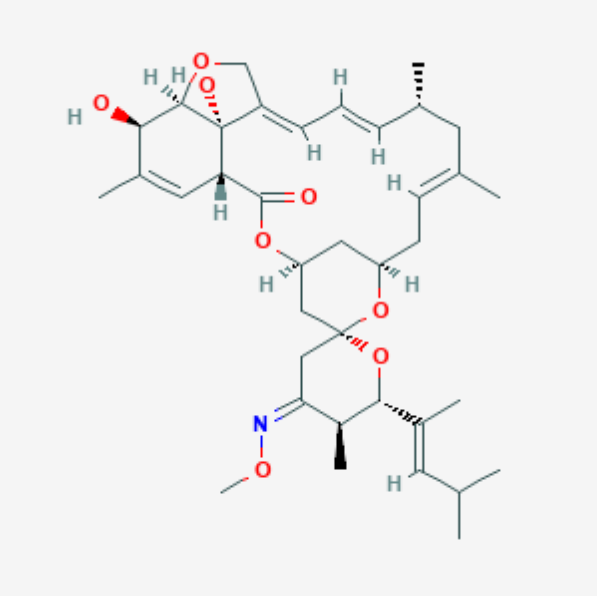


Figure 2 Chemical structure of moxidectin, (PubChem, 2021)

2.1.3. Antimicrobials

Bacteria and fungi (yeasts and moulds) visible under the microscope are considered microorganisms that can occur in a single-cell form or as a colony of cells. Based on the interactions with the host organism (human, animal, or plant), microbes are classified into certain groups: symbionts ("useful"), parasites ("harmful"), or free-living (Olano et al., 2011). Those who induce the disease are called pathogens and can enter the body in a variety of ways: mouth, eyes, nose, urogenital openings, or through wounds or bites that breach the skin barrier. Antimicrobials are agents that inhibit the growth of microorganisms or cause a lethal outcome. The main purposes of antimicrobial drugs are to cure affected animals, expedite recovery, and prevent the incidence of infections. For an antimicrobial drug to be efficient in speeding up the recovery process, proper utilization is considered the key factor. There are several ways to classify antimicrobials, the most frequent certainly being based on the type of microorganisms they primarily target: bacteria (antibacterial), fungi (antifungal), protozoa (antiprotozoal), and viruses (antiviral) (Asif, 2017). However, it is important to highlight antibiotics as antibacterial since they are widely used in the treatment of bacterial infections in humans and animals. On the other hand, overuse of antibiotics and antimicrobials represents one of the major medical challenges that the world is currently facing. According to the FDA data from 2014, as much as 80% of the total antibiotics produced in the United States (US) were intended for use on

farms (FDA, 2017). One of the reasons why this is happening is certainly the fact that antibiotics play a large role in the modern agriculture and livestock industries. Not surprisingly, they can also be found in a variety of, primarily animal, foods used for human consumption, e.g., raw and cooked meat, fish, milk, and drinking water (Li et al., 2017; Kennedy et al., 1998). The classes of antibiotics most commonly used in veterinary medicine are tetracyclines, sulfonamides, aminoglycosides, quinolones, penicillins and cephalosporins. Chemical structures of some of the most widely used antibiotics in veterinary medicine are given in **Figure 3**.

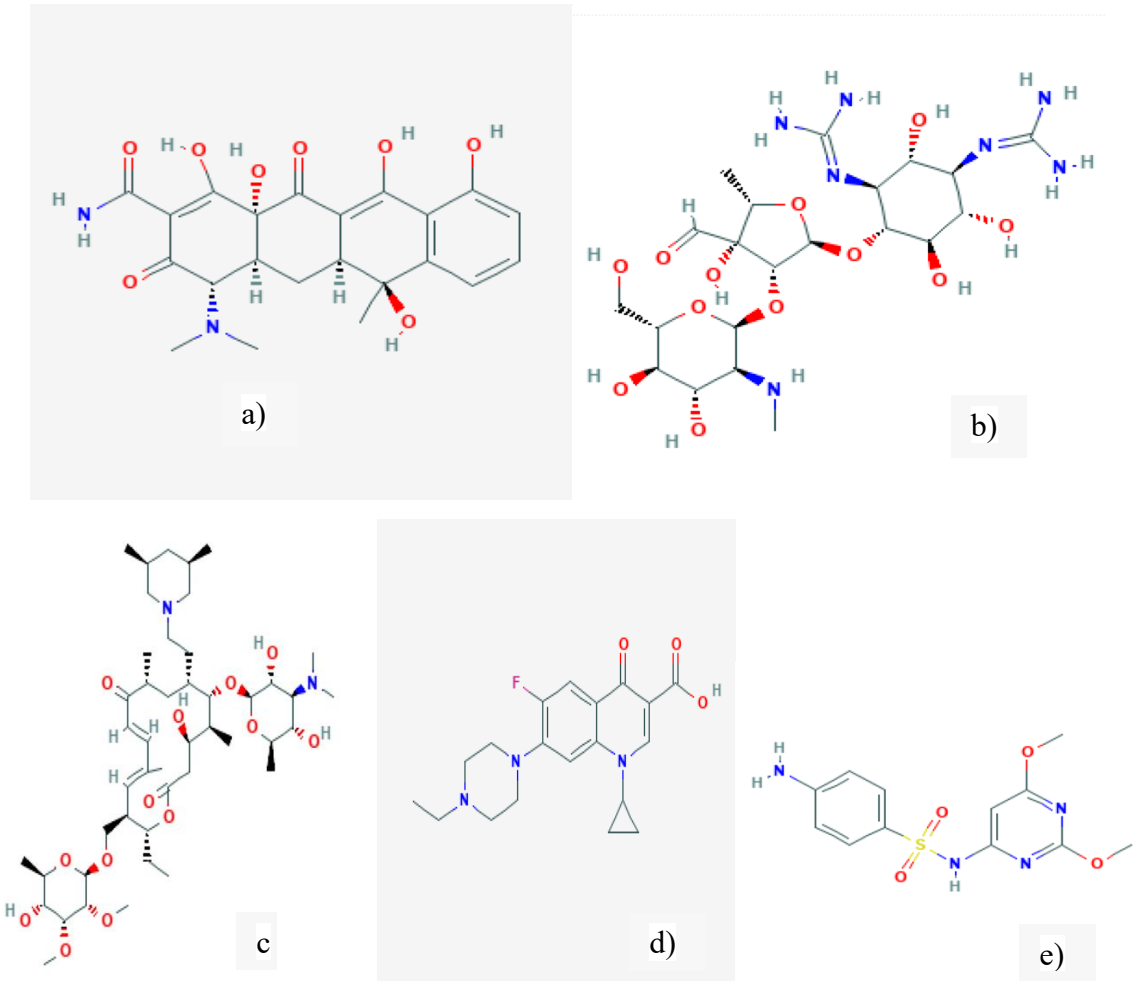


Figure 3 Chemical structures of tetracycline (a), streptomycin (b), tilmicosin (c), enrofloxacin (d), and sulfadimethoxine (e), (PubChem, 2021)

2.1.3.1. Tetracycline antibiotics

Tetracyclines act successfully against gram (+) and gram (-) bacteria. They are produced by the *Streptomyces* genus of *Actinobacteria*. The tetracycline structure includes four hydrocarbon rings to which various functional groups are bound (**Figure 3, a**). A number of structural

features were identified that determine the pharmacological properties of a tetracycline (Chopra and Roberts, 2001). The mode of action is explained through the binding of a drug to the bacterial 30S ribosomal subunit, thereby disturbing the codon-anticodon interactions between tRNA and mRNA causing inhibition of protein synthesis. Tetracycline, oxytetracycline, and chlortetracycline are some of the most frequently used drugs belonging to the tetracycline antibiotics. In veterinary medicine, they are most commonly used in the treatment of infections caused by mycoplasma, chlamydia, spirochetes, tick-borne and other pathogens (Chopra and Roberts, 2001).

2.1.3.2. Sulfonamides

Sulfonamides are broad-spectrum synthetic antimicrobials. Sulfonamides are a group of compounds that are derived from sulfonic acid by replacing a hydroxyl group with an amine group (**Figure 3, e**). A characteristic moiety of antibiotic sulfonamide drugs, as opposed to diuretic, antidiabetic and antiretroviral sulfonamides, is an arylamine group (-Ph-NH_2) at the N4 position. They are thus structural analogs of para-aminobenzoic acid (PABA) and inhibit folic acid synthesis in bacteria. Competing with PABA for bacterial enzyme dihydropteroate synthase, antibiotic sulfonamides prevent synthesis of dihydrofolic acid, a precursor of folic acid. Folic acid in bacteria is essential for the synthesis of pyrimidines and purines and hence in nucleic acid synthesis. In veterinary medicine, sulfonamides are used to treated coccidiosis, inflammatory bowel disease, mastitis, and numerous other infections (Campbell, 1999).

2.1.3.3. Aminoglycosides

Aminoglycosides are a group of antibiotics used against different Gram (+) and Gram (-) organisms. Structurally, their core is made of amino sugars linked with glycosidic bridges. They act by inhibiting protein synthesis in bacteria by irreversibly binding to the 16S ribosomal RNA of the 30S ribosome (Kotra et al., 2000). Streptomycin (**Figure 3, b**) is one of the well-known aminoglycoside antibiotics. It is used as a sulfate salt to treat sheep, poultry, cattle, and other animals.

2.1.3.4. Quinolones

Quinolones are antibiotics with wide use in animal and human medicine. They are effective against Gram (+) and Gram (-) bacteria along with mycobacteria, and anaerobes (Pham et al., 2019). Five different classes of quinolones are available, and an additional class of antibiotics

called fluoroquinolones have also been developed. Fluoroquinolones are synthetic antibacterial agents obtained from quinolones by modifying the chemical structure of a 4-quinolone molecule with a fluorine atom at the C-6 position (**Figure 3, d**). The target of quinolone drugs is DNA synthesis, halted by inhibition of bacterial topoisomerase type II, DNA gyrase, and topoisomerase IV. The function of these enzymes is considered crucial in bacterial chromosome replication, segregation, transcription, recombination, or repair (Pham et al., 2019).

2.1.3.5. Macrolides

Macrolides are a broad group of antibiotics consisting of natural members, pro-drugs, and semi-synthetic derivatives. They contain a macrocyclic lactone ring that is 14-16 membered to which sugar molecules are attached (**Figure 3, c**). Macrolides bind to bacterial 50S ribosomal subunit and block protein synthesis by inhibiting peptidyltransferase. They also interfere with elongation of the polypeptide chain (Kanoh and Rubin, 2010).

Macrolides are used to treat infections caused by aerobic Gram (+) cocci and bacilli and they are moderately active in vitro against Gram (-) organisms such as *Mannheimia haemolytica*, *Pasteurella multocida, and Histophilus somni*. They are most commonly used in veterinary medicine to treat bovine respiratory infections (Anadón and Reeve-Johnson, 1999).

2.1.3.6. β-lactams

ß-lactams are a large class of antibiotics with a broad spectrum of antimicrobial activity used in human and animal medicine. All substances in this family have a common chemical arrangement based on the 3-carbon and 1-nitrogen ring or ß-lactam ring (**Figure 4**) β-lactam antibiotics interfere with the formation of the peptidoglycan layer of bacterial cell wall in Gram (-) and Gram (+) bacteria causing inhibition of cell division and ultimately cell death. The ß-lactam subclasses comprise cephalosporins, penicillins, monobactams, carbapenems, and ß-lactamase inhibitors (Fernandes et al., 2013).

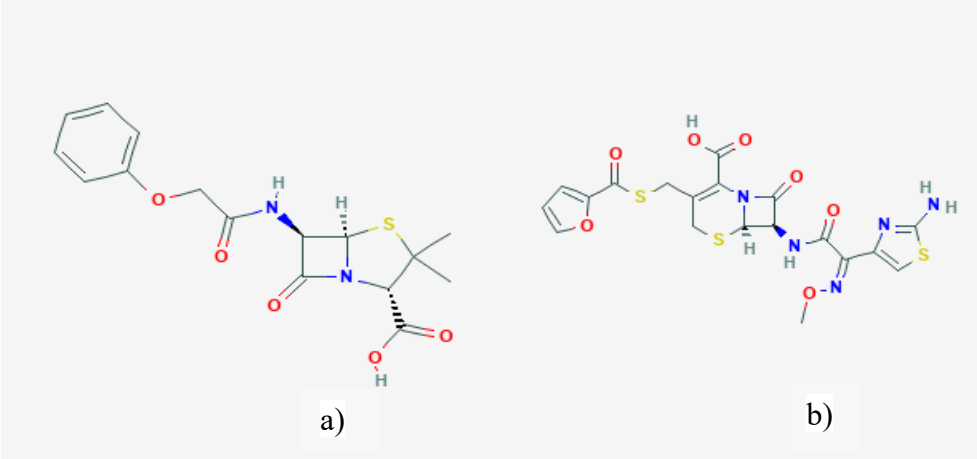


Figure 4 Chemical structures of penicillin V (a), and ceftiofur (b), (PubChem, 2021)

2.1.3.6.1. Cephalosporins

Cephalosporins (**Figure 4, b**) are widely used antibiotics due to their broad spectrum of activity and low toxicity. Cephalosporins act by inhibiting bacterial cell wall synthesis. Five generations of cephalosporin drugs have been developed, which can be divided according to their action against Gram (-) or Gram (+) bacteria, or both (Bui and Preuss, 2017). The use of cephalosporins in veterinary medicine, unlike human medicine, is limited, prompting development of the third and fourth generations of cephalosporins with the intention of veterinary medicine use (Hornish et al., 2002).

2.1.3.6.2. Penicillins

Penicillins are β-lactam antibiotics obtained from molds belonging to the genus *Penicillium.* They can be divided into naturally occurring penicillins and semisynthetic penicillins. The naturally occurring penicillins are produced by fermentation while the semisynthetic penicillins are made through modification of 6-aminopenicillamic acid, the core moiety of all penicillins. It is the side chain attached to the 6-amino group which determines the spectrum of activity and pharmacological properties of the component (Oshiro, 1999). Penicillin G and V (**Figure 4, a**) are the longest known naturally occurring penicillins.

2.1.4. Coccidiostats

Coccidiostats, also called anticoccidial drugs, are antiprotozoal substances used for the treatment of coccidiosis caused by protozoan parasites. Coccidiostats are divided into polyether ionophores and non-polyether ionophores according to their structure (**Figure 5**) (Anadón et al., 2014). Veterinary medicine use is mostly limited to livestock and poultry production. They

act by inhibiting the reproduction and obstructing the development of parasites in the gut of the host (Kan, 2004). Coccidiostats are commonly used as feed additives in the EU and occasionally high levels of residues are detected in food generated by non-compliance with the withdrawal period. Consequently, the problems of parasite resistance and inevitable carryover of these drugs into other animal feeds arise (EC, 2012).

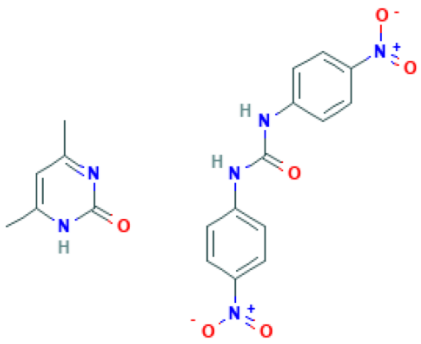


Figure 5 Chemical structure of nicarbazin, (PubChem 2021)

2.1.5. Amphenicols

Amphenicols are antibiotics used in the treatment of Gram (+) and Gram (-) bacteria. They have a phenylpropanoid structure described as an aromatic ring with a three-carbon propane tail (**Figure 6**). The target of their mechanism is microbial protein synthesis inhibition. Amphenicols bind to the 50S ribosomal subunit of the bacteria and thus block the enzyme peptidyltransferase leading to the prevention of protein chain elongation (Guidi et al., 2017). Amphenicols are used to treat and control of bacterial infections like skin infections, wound infections, bone infections, intestinal, and respiratory tract infections.

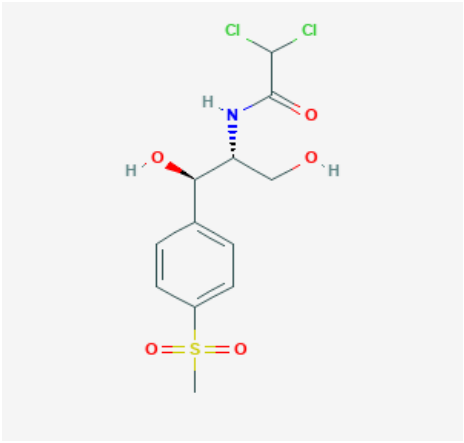


Figure 6 Chemical structure of thiamphenicol, (PubChem 2021)

2.1.6. Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a drug class used to control pain and inflammation in human and animal medicine. They can be divided according to their chemical structure and selectivity into acetylated salicylates (e.g., aspirin), non-acetylated salicylates (diflunisal), propionic acids (naproxen, ibuprofen, acetic acids (diclofenac, indomethacin), enolic acids (meloxicam, piroxicam), anthranilic acids (meclofenamicone, methylecenamethylamine), and selective COX-2 inhibitors (firocoxib (**Figure 7**), celecoxib, etoricoxib). NSAIDs inhibit cyclooxygenase enzymes (COX-1 or COX-2) thus affecting the metabolism of arachidonic acid and its conversion to proinflammatory thromboxanes, prostaglandins, and prostacyclins (Gunaydin et al., 2018).

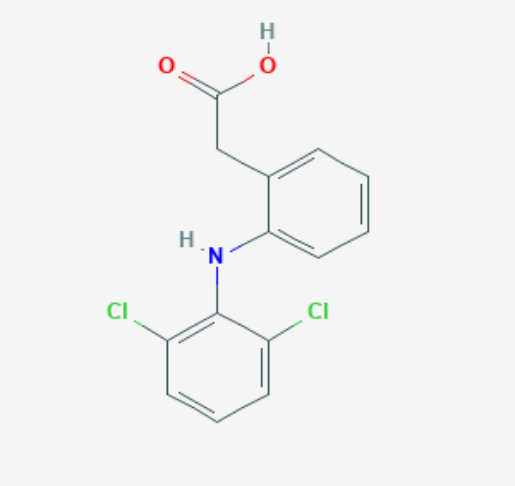


Figure 7 Chemical structure of firocoxib, (PubChem, 2021)

2.1.7. Corticosteroids

Corticosteroids represent steroid hormones produced in the adrenal cortex of vertebrates. Synthetic analogues of these hormones (**Figure 8**) are manufactured by the pharmaceutical industry based on their naturally occurring structures. Mineralocorticoids and glucocorticoids are two classes of corticosteroids used in veterinary medicine. Glucocorticoids with cortisol as a major representative are involved in the regulation of carbohydrate, fat and protein metabolism. They have an anti-inflammatory effect and generally weaken immune responses by several different mechanisms. Mineralocorticoids with aldosterone as a major agent are involved in the regulation of water and electrolyte levels by promoting renal water retention. This drug class possesses diverse pharmacological functions. Corticosteroid medications have powerful anti-inflammatory and immunosuppressive effects and are therefore used widely for

the treatment of inflammation and immune function diseases (Narang and Singh Preet, 2019). Corticosteroids enter the cytoplasm where they bind to specific intracellular receptor proteins. The resulting hormone-receptor complex enters the cell nucleus, whereby the transcription factor binds to specific DNA sequences within the promoter region of corticosteroid-sensitive genes. The binding process then changes the transcription level of messenger RNA (mRNA) (Ramamoorthy and Cidlowski, 2016).

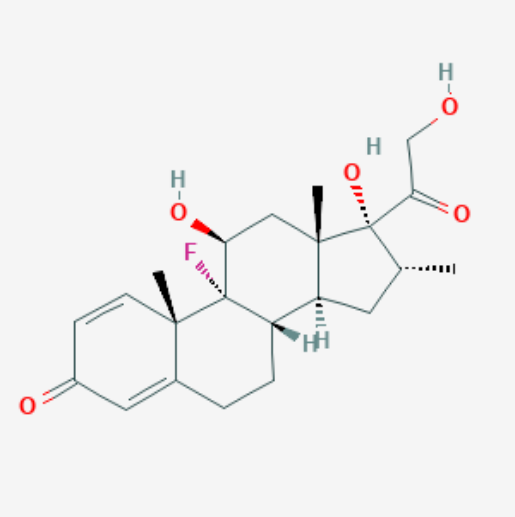


Figure 8 Chemical structure of dexamethasone, (PubChem, 2021)

2.1.8. Other veterinary drugs

Nitroimidazoles are antimicrobial agents that are successful against anaerobes and protozoal infection. The main representative of nitroimidazole is metronidazole which inhibits nucleic acid and in turn deoxyribonucleic acid (DNA) synthesis in microbial cells, causing loss of helical DNA structure and strand breakage (Lamp et al., 1999). Polymyxins B and E (colistin) are antibiotics that are effective against Gram (-) bacterial infections. Colistin acts by disrupting the bacterial cell membrane through the displacement of calcium and magnesium ions leading to an increase in the permeability of the cell membrane, leakage of cell contents, and ultimately cell death (Conly and Johnston, 2006). Pleuromutilins, mainly tiamulin and valnemulin, are primarily used is in veterinary medicine to treat pigs and to a lesser extent for poultry and rabbits. They bind to the 50S subunit of the ribosome at the peptidyl transferase center, resulting in suppression of translation and ultimately inhibition of bacterial protein synthesis (Paukner and Riedl, 2017).

2.2. ANTIMICROBIAL RESISTANCE

Frequent and uncritical administration of antibiotics drives the development of resistance in bacteria. Antimicrobial resistance (AMR) represents the main threat to public health and food chain and is gaining in importance worldwide (WHO, 2020a). Increasing numbers of multidrug-resistant bacteria result in a lack of effective antibiotic agents necessary in human and veterinary medicine. There are various ways in which AMR evolves, one of them is a transmission of antibiotics to the environment, through agricultural manure and sludge-fertilized soils. Also, livestock waste and domestic wastewater are significant sources of antimicrobial resistance in the environment. Considering the major problem of wastewater from different industries, the environment is a tremendous and complex medium for spreading resistance.

Agricultural producers commonly use antibiotics as growth promoters and for preventative purposes, not only for the treatment of diseased animals (WHO, 2017). In animals, antibiotics and other antimicrobials are used for three different purposes: therapeutic for the treatment of diseases, prophylactic to prevent the disease, and as subtherapeutic to improve animal performance (Landers et al., 2012). Association between antibiotic use in food-producing animals and AMR in humans is undeniable (WHO, 2020b; EFSA, 2021), and any nontherapeutic use present the public health threat related to AMR. For example, in dairy cows, mastitis is treated with antibiotics (penicillins, aminoglycosides and macrolides; oxytetracycline, chloramphenicol, trimethoprim and several sulfonamides). However, the prophylactic use of a dry-cow therapy after the lactation period to prevent and control future mastitis is a common treatment (Kvist, 2016). A particular problem in veterinary medicine is metaphylaxis. Metaphylaxis is the administration of antibiotics for the prevention of disease appearance in other healthy individuals who are in contact with the animal that has a diagnosed clinical infection. The minimization of metaphylaxis is demanding since antibiotics are generally given to animals via food and water which they consume together. For example, one of the diseases, where metaphylaxis has been proven to be an effective treatment is bovine respiratory disease (González-Martín et al., 2011). AMR may occur through food, direct contact between animals and humans, or shared environmental sources, e.g. swimming water (Tang et al., 2017). European Commission Notice (EC, 2015) for the prudent use of antimicrobials in the veterinary sector was adopted to encourage the vigilant use of antimicrobials in veterinary medicine. The best and most effective way to reduce AMR and antimicrobial use is to prevent and reduce the need for medication. In the veterinary sector, this can be achieved by maintaining

hygienic conditions and bio-safety measures, including infection prevention protocols and continuous health control programs, by improving animal breeding systems and care, and ensuring proper animal nutrition (Givens, 2005; Rana et al., 2019). Pursuant to its consideration of the AMR concern, the WHO, in collaboration with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (OIE), has developed a Global Action Plan (GAP) (WHO, 2015). The main objectives of GAP are broadening knowledge of the AMR through communication, education and training, enhancing the knowledge on surveillance and research; carefully administering antibiotics, discovering new drugs / therapies, influencing the preservation of existing antibiotics and trying to control the spread of resistant bacteria in the environment, developing a sustainable economic solution that will cover all countries and increase investment in new medical solutions and healthcare purposes etc.

2.3. ANALYTICAL METHODS FOR THE DETERMINATION OF VETERINARY DRUG RESIDUES

Due to increased food production, the need for veterinary drugs is also growing. As use increases so does the number of challenges posed by veterinary drugs, antimicrobial resistance certainly being the most important one. Consequently, there is an increasing number of laws and regulations that ensure food safety and quality. Thus, there is a necessity for appropriate analytical techniques and methods to demonstrate adherence to established legislation for food and feed in order to maintain quality and safety. Preferably, the methods for analysis of veterinary drug residues should be selective, fast, robust, economical, etc. Analysis in food and feed is a process consisting of sampling, sample preparation, extraction to separate residues from the matrix, and residue identification and quantification. Many methods can be used for both qualitative and quantitative analysis. Depending on the specific requirements of the food production market or national and international food safety authorities, the methods can be divided into routine or rapid analysis. Accordingly, there are many different analytical techniques on the market for monitoring residues of veterinary drugs, some of which include liquid chromatography (LC) and gas chromatography (GC) or LC, GC coupled with different detectors, enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis (CE), micellar electrokinetic capillary chromatography (MEKC), etc. (Wang et al., 2021). One of the most used approaches for quantitative residue determination of veterinary drugs in food and feed is liquid chromatography tandem mass spectrometry (LC-MS/MS) which uses

electrospray ionization (ESI). Table 1 shows the advantages and disadvantages of some analytical methods used for veterinary drug residues (Wang et al., 2021; Injac et al., 2009).

Table 1 Advantages and disadvantages of some analytical methods used in determining veterinary drug residues

Analytical technique	Advantages	Disadvantages
LC-MS	high recovery, high selectivity, good reproducibility, low interference	expensive equipment, experts needed
GC	good recovery, precision and reproducibility	derivatization and specific capillary columns needed, expensive equipment, experts needed
ELISA	easy operation, convenience, high efficiency and sensitivity, strong specificity, low detection cost, selective and efficient	recovery and precision lower in comparison with LC
CE	efficient, fast, automated separation technology, low reagent consumption, high separation efficiency	small injection volume, small capillary diameter, low sensitivity caused
MEKC	high separation power, ability to separate both ionic and neutral compounds, simple operation, few sample pre-processing steps, and low instrumentation	lower sensitivity in comparison with LC method

3.MATERIALS AND METHODS

3.1. CHEMICALS

LC gradient-grade acetonitrile and methanol as well as MS-grade glacial acetic acid (p.a.) and ammonium acetate were purchased from Sigma-Aldrich (Vienna, Austria). Reference standards were purchased from Sigma-Aldrich (Vienna, Austria), Dr. Ehrenstorfer (Augsburg, Germany), European Union Reference Laboratory (Berlin, Germany) or were obtained as gifts from various research groups. Reference standards (>CL), divided into classes (XVIII) according to their chemical properties, analyzed in this work were:

I. anthelmintic: fenbendazole, fenbendazole sulfone, triclabendazole sulfone, triclabendazole sulfoxide, triclabendazole, albendazole, albendazole sulfone, albendazole sulfoxide, albendazole-2-aminosulfone, rafoxanide, closantel, oxyclozanide, clorsulon, cambendazole, oxibendazole, praziquantel, niclosamide, levamizole, flubendazole, mebendazole, mebendazole amine, morantel, nitroxynil, pyrantel pamoate, thiabendazole
II. antiprotozoal: ronidazole, dimetridazole, ornidazole, carnidazole, ipronidazole
III. macrocyclic lactones: doramectin, eprinomectin, moxidectin
IV. sulfonamides: sulfasalazine, sulfaethoxypyridazin, dapsone, sulfacetamide, sulfaguanidin, sulfadiazine, sulfathiazole, sulfapyridin, sulfamerazine, sulfamoxole, sulfisoxazolel, sulfameter, sulfamethazine, sulfamethizole, sulfamethoxazoleol, sulfaclozine, sulfaphenazole, phtalylsulfathiazole, trimethoprim (antifolate antibacterial agent-acts synergistically with sulfonamides), sulfachloropyridazine, sulfadimethoxine, sulfadimidine, sulfadoxine, sulfamethoxypyridazine, sulfamonomethoxine
V. aminoglycosides: streptomycin, apramycin, dihidrostreptomycin, kanamycin, neomycin B, sisomycin
VI. macrolides: spiramycin, oleandomycin, josamycin, lincomycin, clindamycin, erythromycin A, roxithromycin tulathromycin, tilmicosin, tylosin
VII. tetracyclines: demeclocycline, meclocycline, methacycline, minocycline, chlortetracycline, oxytetracycline, doxycycline
VIII. quinolones: marbofloxacin, norfloxacin, ofloxacincin, ciprofloxacin, danofloxacin, enrofloxacin, orbifloxacin, difloxacin, nalidixic acid, flumequine, oxolinic acid, perfloxacin, fleroxacin, lomefloxacin, sarafloxacin, pipemidic acid, cinoxacin
IX. nitroimidazoles: metronidazole
X. polymixin: colistin
XI. pleuromutilin: valnemulin, tiamulin

XII. β-lactams: amoxicillin, ampicillin, dicloxacillin, oxacillin, tazobactam, piperacillin, ticarcillin, sulbactam, clavulanic acid

XIII. penicillins: penicillin G, penicillin V, cloxacillin, aspoxicilin

XIV. cephalosporins: cefadroxil, ceftizoxime, ceftriaxone, cefuroxime, desfuroylceftiofur, cephapirin, cefalonium, cefazolin, cefoperazone, ceftiofur, cefacetrile, cefquinome, cephalexin

XV. coccidiostats: clazuril, diclazuril, nicarbacin, clopidol, halofuginone, ethopabat, robenidyn, decoquinate, monensin, salinomycin, lasalocid, maduramicin, nequinate, amprolium, dinitrocarbanilide

XVI. amphenicols: thiamphenicol, florfenicol, chloramphenicol

XVII. NSAIDs: ketoprofen, naproxen, meloxicam, flunixin, carprofen, diclofenac, ibuprofen, mefenamic acid, tolfenamic acid, firocoxib, celecoxib

XVIII. corticosteroids: dexamethasone, flumethasone, methylprednisolone, betamethasone, prednisolone, triamcinolone.

3.2. FOOD AND FEED SAMPLES

Models of five artificial chicken feed samples were prepared by mixing different proportions of soy, distillers' dried grain with solubles (DDGS), rapeseed, and maize (**Table 2**), as described by Steiner et al. (2020a). Heterogeneous individual raw samples were provided by the following companies: LVA (Klosterneuburg, Austria), Bipea (Paris, France), Biomin (Getzersdorf, Austria), and Garant-Tiernahrung (Pöchlarn, Austria). Milk samples with fat contents of 0.5%, 0.9%, 1.5%, 3.2% and 3.6% were purchased at a nearby store (**Table 3**).

Table 2 Composition of in-house prepared samples of artificial chicken feed expressed in percentages (%)

Artificial chicken feed (ACF)					
Ingredient:	ACF 1	ACF 2	ACF 3	ACF 4	ACF 5
Maize	68	69	71	67	74
DDGS	-	3	-	-	5
Rapeseed	2	3	6	5	2
Soy	30	25	28	28	19

Table 3 Composition of milk samples

Cow milk (M)	
	Fat content
M1	0.5% milk fat
M2	0.9% milk fat
M3	1.5% milk fat
M4	3.2% milk fat
M5	3.5% milk fat

3.3. METHOD

3.3.1. Instrumental parameters and equipment

The analytical procedure for this experiment was described by Malachová et al. (2014), the existing method was transferred, and new transitions were added following optimization. Briefly, a QTrap 5500 MS/MS system (Sciex, Foster City, CA, USA) equipped with a Turbo V electrospray ionization (ESI) source was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini C18-column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4 × 3 mm i.d. (both Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode with a flow rate of 1000 µL/min. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B), respectively. For further purification of reverse osmosis water, a Pure-lab Ultra system (ELGA Lab Water, Celle, Germany) was used. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100% within 9 min was followed by a hold time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The injection volume was 5 µL. ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarity in two separate chromatographic runs. The settings of the ESI source settings were as follows: source temperature 550°C, curtain gas 30 psi (206.8 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying

gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage −4500 V and +5500 V, respectively, collision gas (nitrogen) - medium. The column temperature was set at 25°C. The target cycle time was 1000 ms, the MS pause time was 3 ms, and the detection window width was 40 and 52 s in the positive and negative ESI mode, respectively. According to the SANTE/11813/2017 validation guidelines the two MRM transitions per analyte are acquired for confidence (EC, 2019.).

3.3.2. Calibration solutions

Stock standard solutions were prepared with respect to analyte solubility in a particular solvent as described by Desmarchelier et al. (2018). Solid substance was weighed (minimum weight of 1 mg) and the liquid level was adjusted with appropriate solvents to obtain a targeted concentration of 1000 μg/mL. Solutions were sonicated until complete dissolution. Respecting the solubility of compounds, six different solvents were used: water, methanol, methanol + water (1:1), methanol + dimethyl sulfoxide (DMSO) (1:1), water + acetonitrile (1:1), 1 mM sodium hydroxide in methanol. Overall, six intermediate mixtures (each substance at 10 μg/mL) were prepared by combining individual stock solutions dissolved in the same solvent. All solutions were stored at -20°C. The final working solution was freshly prepared by mixing the intermediate mixtures. Detailed overview related to the preparation of the 174 individual stock solutions and intermediate mixtures is given in the Supplementary materials. Furthermore, long duration of the study caused by the global COVID-19 pandemic was responsible for partial degradation of the compounds over the 10-month storage period. As a result of acquired knowledge related to solvents and storage, new stock solutions (1000 μg/mL) and only three intermediate mixtures (10 μg/mL) in different solvents were prepared. More details regarding new solubilities and intermediate mixtures also can be found in the Supplementary materials.

3.3.2.1. Calibration

External neat calibration was performed by serial dilutions of the final working solution with acetonitrile/water (1:1): 1:1, 1:3: 1:10, 1:30, 1:100. To check the linearity of the response, linear 1/x weighted calibration curves were constructed for the neat solvent standards. The construction of calibration curves and peak integration were performed using MultiQuant 2.0.2 software (Sciex, Foster City, CA, USA).

3.3.3. Sample preparation

3.3.3.1. Dilute and shoot

Feed samples were extracted both with and without the use of acid. Acidic procedure was investigated in order to assess the feasibility of a combined method for antibiotics and mycotoxins, the latter requiring acidic conditions, while milk extraction was carried out without acid. For method validation purposes, chicken feed samples and milk samples were spiked at two levels with the appropriate amount of the final working solution. Spiked feed samples were then left overnight at 4°C to achieve solvent evaporation and equilibration between the matrix and the analytes. Extraction of spiked feed and milk samples and post extraction spikes were performed with respective volumes of acetonitrile/water (80:20) (**Figure 9**), while acidic extraction of feed samples was preformed using acetonitrile/water/acetic acid mix (79:20:1, v/v/v). The samples were then placed in a horizontal position on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and agitated for 90 min. This was followed by centrifugation on a GS-6 centrifuge (Beckman Coulter, Fullerton, CA, USA) at 3500 rpm for 10 min. After this, 500 µL of the supernatant was aliquoted to an HPLC vial. Each aliquot was diluted with an equal amount of a solvent mix: acetonitrile/water (20:80, v/v) in the case of acid-free extraction, i.e., acetonitrile/water/acetic acid (20:79:1, v/v/v) for acidic extraction. After appropriate mixing, 5 µL of the diluted extract was injected into the LC-MS/MS system without further pre-treatment. The whole procedure (**Figure 9**) was miniaturized only for validation purposes and for the economic use of standards. Routine analysis uses larger amounts of samples: 5 (or 20) g extracted using 20 (or 80) mL of solvent. For post-extraction spiking, raw extracts (blank extracts) of each model matrix were fortified with an appropriate amount of the working solution. Again, mix of acetonitrile/water/acetic acid (20:79:1, v/v/v) was used in the case of acidic extraction, while acetonitrile/water (20:80, v/v) mix was used for acid-free extraction.

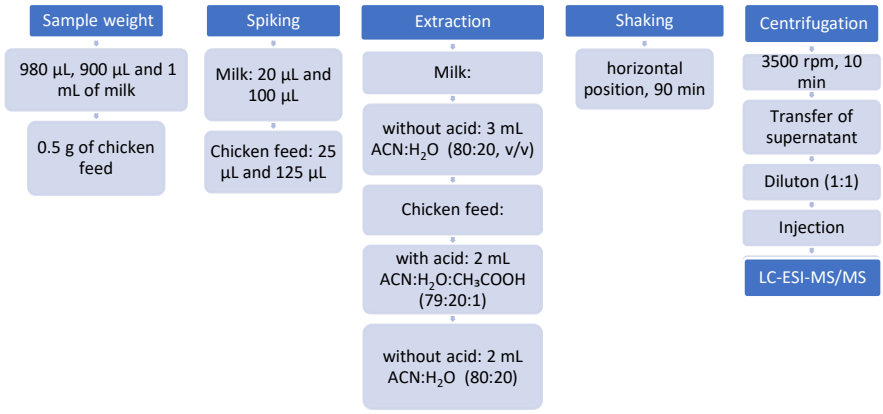


Figure 9 Sample preparation scheme

3.3.3.2. Solvent testing

Five different solvents were tested: acetonitrile/water/acetic acid (79:20:1): acetonitrile/water/acetic acid (20:79:1), acetonitrile:water (1:1), pure cow milk, milk:extraction solvent (1:1), milk:acetonitrile (1:1). Tested solvents were spiked at three different concentration levels: 30, 100, and 300 µg/mL. Extraction:dilution solvent (1:1) was used as a baseline for calculation, since recovery was calculated by comparing the area of extraction: dilution solvent (1:1) with the area of the other tested solvents.

3.3.3.3. Stability study

Isochronous measurements were performed to examine the stability of veterinary drug standards. The measurements involved storing standards over different periods at different temperatures, in a way which allowed all measurements to be performed simultaneously (Lamberty et al., 1998). The isochronous measurement approach predicts that all samples are stored at temperatures considered to prevent any degradation (-80° and -20°C in this experiment) during the desired test interval, and then transferred to different temperatures for different time periods tested. In this work the stability of the veterinary drug standards during a long storage period (three months, intermediate mixes) and a short period (one-week, individual stock solutions) was examined (**Table 4**). Applied storage solvents were selected on the basis of internal knowledge and search of various literature sources with the work by Desmarchelier et al. (2018) being the most important. Replicates of the final working solutions were prepared without the presence of acid in acetonitrile:water (1:1, v/v), and in acidified conditions with acetonitrile/water/acetic acid (79:20:1). Short-term stability testing included storing standards for 1, 2, 4, and 7 days at -20°C as a baseline, and the additional testing conditions were: refrigerator (4°C), room temperature (23°C) -dark, room temperature-light.

The day after the last time point all standards deriving from the different storing regimes, as well as the control vial (-20°C), were brought to room temperature and measured in a randomized sequence to avoid the occurrence of any trend induced by the measurement. Long-term stability was tested by preparing six intermediate mixes in the respective solvents. In the end, there were 17 sets (six vials). Tested conditions were -20°C, refrigerator (4°C), room temperature-dark and room temperature-light, and control temperature at -80°C while the tested period was 2, 4, 8, and 12 weeks. Control set (last remaining set at -80°C) was taken out on the last day and each mix/point (10 µg/mL) was put together and diluted, with and without addition of acid to a obtain final analyte concentration of 200 ng/mL. This was carried out in a way that from all six vials in individual set (17 sets in total) 20 µL was transferred to a new vial and mixed together (120 µL) and then filled with 880 µL of solvent to bring the total volume to 1 mL. The results are presented as recovery, obtained by dividing the peak area of the standard kept at the baseline temperature (-20°C for short-term and -80°C for long-term stability), and the area of the standard stored at the tested temperatures.

Table 4 Stability testing scheme

Short term stability				
Conditions:	1 day	2 days	4 days	7days
-20 °C	1 multimix / 500 µL→Ready to measure			
4 °C				
23 °C, light				
23 °C, dark				
Long term stability				
Conditions:	2 weeks	4 weeks	8 weeks	12 weeks
80 °C	6 intermediate mixtures (17 sets)-200 µL (10 µg/mL)→Further dilution			
-20 °C				
4 °C				
23 °C, light				
23 °C, dark				

3.3.4. Validation

Method validation was conducted in accordance with SANTE/12682/2019 validation guideline criteria (EC, 2019). To determine the performance of the method, both matrices were fortified with a working solution covering all the target analytes. All spiked concentrations were chosen to be in the middle of the calibration range. For milk, the lower concentration ranges are determined to cover the appropriate detection limits of each compound as well as the legal limits of pharmacologically active substances regarding maximum residue limits (MRL) in foodstuffs of animal origin as stated in Commission Regulation (EC) No 37/2010 (EC, 2010). Regulation 1831/2003 on additives used in animal nutrition (EC, 2003) has been considered concerning feed matrices, although the document does not specify any legal limits (MRLs). Fortified samples were extracted and diluted according to the protocol described in the Sample preparation section. Afterwards, diluted samples were placed in a sequence along with external neat calibration standards, and post extraction spiked samples. To determine within laboratory repeatability, validation of the method was performed over three different days at a high concentration level. Examination of the matrix effect, which is expressed as signal suppression/enhancement (SSE) and extraction efficiencies was performed by fortification of diluted blank extracts of each model matrix at the concentration range corresponding to the external standards at a high concentration level. Limit of quantification (LOQ) and limit of detection (LOD) were determined according to the EURACHEM guide (Eurachem Working group, 2014). LOQ represents the lowest level at which the performance is acceptable for a typical application. The LOQ evaluation comprises replicate measurements (n = 5) of individual samples spiked with a low concentration of analytes to determine the standard deviation S_o expressed in concentration units. Determination of LOQ and LOD values was performed by multiplying the standard deviation by a factor of 10 and 3, respectively. Extensive validation data set is provided in Supplementary data.

3.3.5. Data evaluation

The construction of calibration curves and peak integration were performed using MultiQuant 2.0.2 software (Sciex, Foster City, CA, USA). Further data evaluation, such as the calculation of the method performance parameters, was carried out in Microsoft Excel 2013. The recovery of the extraction step (RE), the apparent recovery (RA), and the signal suppression/enhancement (SSE) were calculated from the peak areas of the samples spiked

before extraction, the samples spiked after extraction, and the neat solvent standards, respectively, as follows:

$$R_E(\%) = \frac{\text{area (sample spiked before extraction)}}{\text{area (sample spiked after extraction)}} \times 100$$

$$R_A(\%) = \frac{\text{area (sample spiked before extraction)}}{\text{area (neat solvent standard)}} \times 100$$

$$SSE(\%) = \frac{\text{area (sample spiked after extraction)}}{\text{area (neat solvent standard)}} \times 100$$

4. RESULTS AND DISCUSION

4.1. LC-MS/MS OPTIMIZATION

The method used in this study was a modification of the approach described by Malachová et al. (2014). This method was transferred, and LC-MS parameters of the antibiotic and antimicrobial compounds were determined. For analysis of all analytes by LC-tandem mass spectrometry optimal conditions (available in Supplementary materials) include precursor ion (Q1), product ion (Q3), dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE), cell exit potential (CXP), and retention time (RT). The m/z ratio for all optimized analytes were detected both in the ESI positive and negative mode and the more sensitive mode was selected. Each analyte, for which no MRM transitions were identified by literature search, was scanned individually and the precursor ion was identified. Within the range, given the molecular weight of the component in which the precursor ion could be expected to appear, a scan was made, always taking into account modifiers that may appear in the form of, for example, acetate adducts. After the precursor ion was determined and the declustering potential optimized to yield a maximum signal, the product ions were selected. For each product ion, the optimum collision energy and cell exit potential were determined to yield maximum signal strength. These optimized MS/MS transitions were scanned during the whole chromatographic run to identify retention time of each analyte. Finally, the optimized parameters were introduced into the "new" multimethod.

The next step was to create a calibration curve that contained five levels for each component to check the linearity and to estimate the instrumental LOD for all the compounds. For each tested analyte, the dependence of the area of the obtained peak after MRM (multiple reaction monitoring) analysis and the concentration of the veterinary drug was shown, and the obtained curves were corrected by linear regression. Based on the deviation of the curve from the direction of linear regression, the linearity of the method was determined. To determine the instrumental LOD, signal-to-noise ratio values were determined for each veterinary drug based on calibration curves. All optimized data for analysed substances can be found in the Supplementary materials. Electrospray ionisation (ESI)-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarity in two separate chromatographic runs. Scheduled multiple reaction monitoring (sMRM) mode measures each analyte in a defined time window while dwell times are automatically generated by the software. Thus, the time required to complete all transitions is significantly reduced, a

better signal-to-noise ratio (S / N) and a higher number of data points per peak are obtained (Schreiber and Pace, 2010).

4.2. METHOD OPTIMIZATION

In order to establish good laboratory practice, planning, analysis, archiving, and reporting are required to establish management over the analytical standards used in the analysis. Given that the aim of this work is to develop an LC-MS/MS multimethod (different classes of veterinary drugs) to analyze more than 150 residues of veterinary drugs in food and feed, good laboratory practice was the necessary prerequisite. As veterinary drugs are an extremely complex class of compounds with respect to polarity, stability, solubility and other properties, solubility and stability were tested first. This was followed by pre-validation as proof of principle. The last experiment performed was method validation. Validation is a crucial step in the development / conversion of any new method as its purpose is to determine the performance characteristics and limitations of the method (Eurachem Working group, 2014).

4.2.1. Solvent testing

Through a comparison with other toxicants such as mycotoxins, plant toxins, and pesticides, it was observed that the vast majority of veterinary drugs require special conditions in terms of stability and solubility (Steiner et al., 2020b). Due to the wide range of the polarities and consequently solubilities/stabilities of examined veterinary drugs, using knowledge gained while setting up the calibration curve, efficacies of different extraction solvents were tested. The main ambition was to find a solvent which will allow the development of an extraction system suitable for both polar and non-polar compounds. The rationale behind this experiment was the well-known chemical rule "like dissolves like", for example polar solvents tend to dissolve polar solutes. The extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v): dilution solvent (acetonitrile/water/acetic acid 20/79/1, v/v/v), 1:1, was used as a basis (100%) and the efficiency of the other solvents was calculated compared to the extraction: dilution solvent. The results were presented as recovery, according to the criteria from the validation guideline SANTE/12682/2019 (EC, 2019). With milk as solvent, it is necessary to pay attention given the high fat content and the possibility of a long-term contamination of the system. Therefore, smaller injection volumes combined with higher flow rates are required. Recovery calculations showed that substance classes and particular compounds from some classes do not

exhibit acceptable recovery in the presence of solvent with acid. On the other hand, pure milk or solvent containing milk are certainly not an option for polar classes of drugs (**Table 5**), such as cephalosporines, quinolones, corticosteroids and even some sulfonamides, since the calculated recoveries do not fall within the desired range of 70-120%. Nevertheless, milk matrix showed stabilizing effect for some of the compound classes: coccidiostats, NSAIDs, macrocyclic lactones and some ß-lactams. Therefore, based on these results, the possibility of applying matrix matched calibration using one sample can be considered in the routine analysis laboratories.

Table 5 Results of solvent testing for selected analytes

Substance class:	Substance:	ACN:H20, (1:1)	Pure cow milk	Pure cow milk:Extraction solvent, (1:1)	Pure cow milk:ACN, (1:1)
Cephalosporines	Cephacetrile				
	Ceftiofur				
	Cefapirin				
	Cefalonium				
	Cefaperazone				
	Cefquinome				
	Cefazolin				
Quinolones	Nalidixic acid				
	Difloxacin				
	Danofloxacin				
	Enrofloxacin				
	Ofloxacin				
	Flumequine				
	Marbofloxacin				
	Orbifloxacin				
	Ciprofloxacin				
	Norfloxacin				
	Oxolinic acid				
Glucocoritcoids	Methylprednisolone				
	Flumethasone				
	Dexamethazone				
Sulfonamides	Sulfamethoxazole				
	Sulfadoxine				
	Sulfadimethoxine				
	Sulfaclozine				
	Sulfachlorpyridazine				
	Sulfaethoxypiridazine				
	Sulfaphenazole				
	Phthalylsulfathiazole				
Recovery range:	70-120	50-70	120-140	<50	>140

Considering the data given in Table 5, no significant difference was observed between neutral and acidic conditions. However, in case of a short-term stability prolonged stability under neutral storage conditions was demonstrated (see section **Stability study**). Furthermore, the obtained results indicate better outcome and consequently less disturbances in the measuring process when solvent mixtures without acid (**Figure 10**) were applied with as much as 96% of the analytes reaching yields between 70-120%. The reason for this is the influence of the milk matrix, its constituents interfering with the ionization process. Accordingly, the experiment showed that dilution of milk without the presence of acid reduces the matrix effect as opposed to the presence of acid. This outcome greatly influenced design of the following optimizations,

since stability study and validation of the method were carried out with two different solvents, in the presence and without the presence of acid.

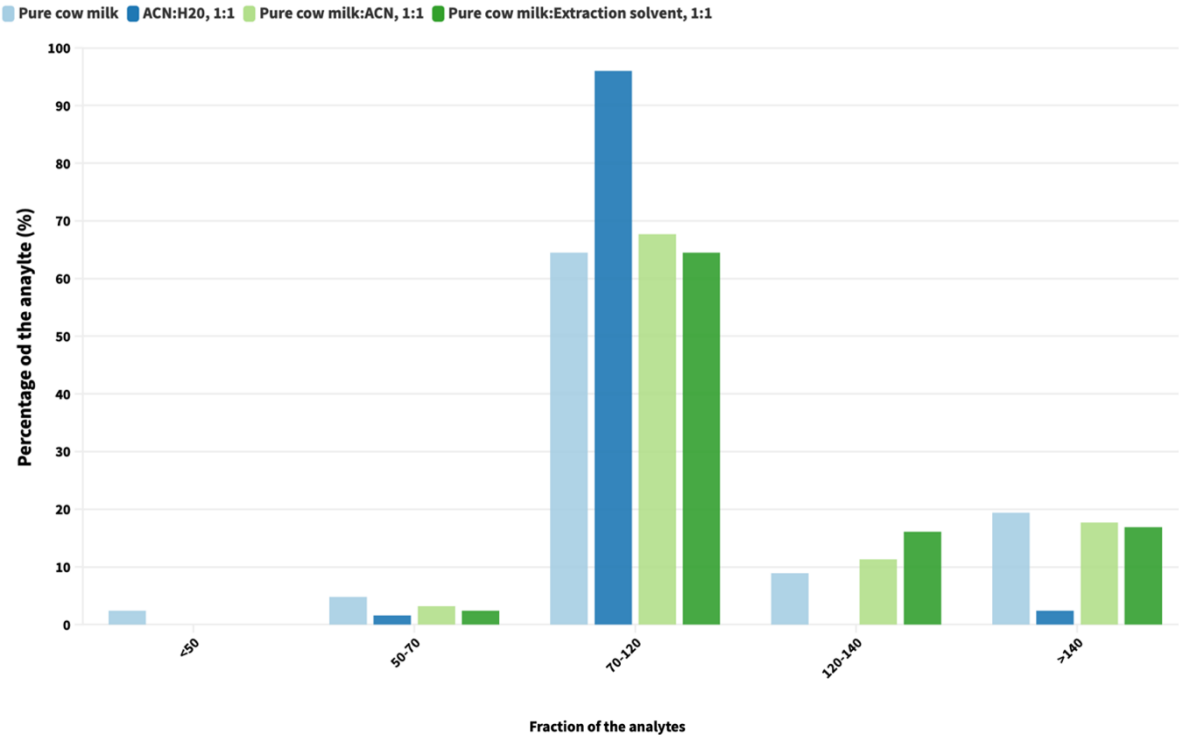


Figure 10 Comparison of all recoveries obtained for tested solvents

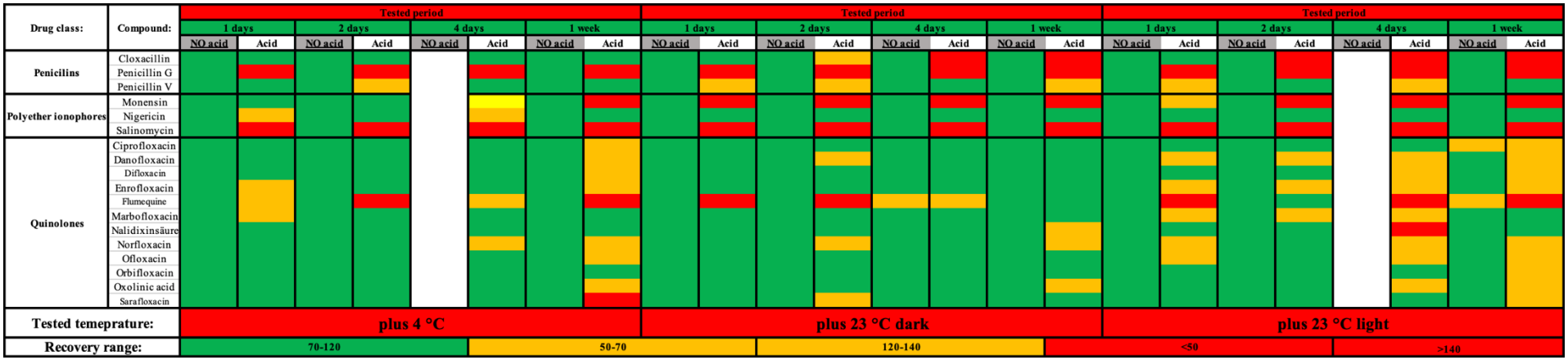
4.2.2. Stability study

4.2.2.1. Short term stability study

As expected, temperature 4°C and time period of 1-2 days proved to be the optimal conditions for storage of multi-mix standards. When it comes to tested solvents, acidified conditions proved to be significantly worse for the storage of a final working solution of particular veterinary drugs classes. Penicillins, polyether ionophores and quinolones in particular (**Table 6**) showed an absolute preference for an acid free solvent, for "4 days" period, for measurement without the presence of acid data are not available due to instrument operation problems. Moreover, all compounds remained reasonably stable in neutral conditions even at room temperature, without any significant influence of light exposure. This indicates that stability under neutral storage conditions is acceptable in cases where larger batch samples (requiring an analysis time of a few days) are supposed to be tested. All the presented results indicate that it is feasible to use a serial dilution of a single multi-component mix for external calibration

instead of different sets of external calibrants in routine analysis, as already confirmed by other authors (Mol et al., 2015).

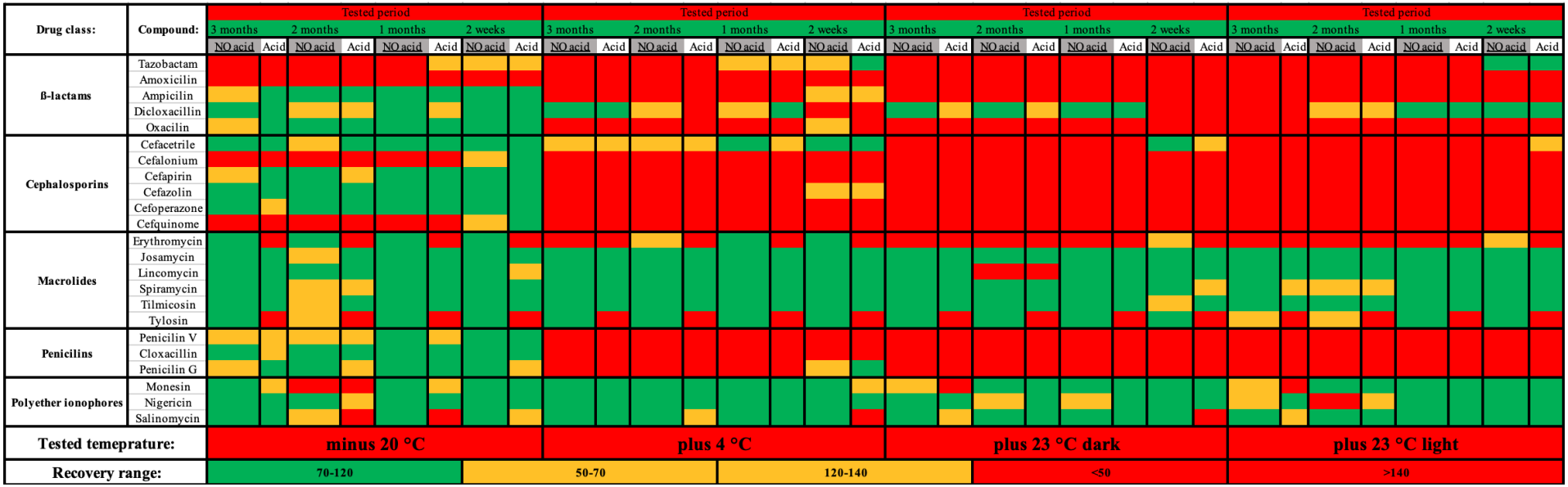
Table 6 Results of short-term stability study for selected analytes



4.2.2.2. Long term stability study

Long term stability study again proved acidic conditions to be worse for several classes of compounds such as penicillins, cephalosporins, macrolides, polyether ionophores. For example, penicillin V and G (**Table 7**) can only be stored for 2 weeks in acid-free conditions at minus 20°C. Storage at higher temperatures or for prolonged periods did not meet the set criteria. Any temperature other than minus 20°C led to almost complete loss for 90% of the analytes belonging to ß-lactams or cephalosporins, after 1 month of storage. In contrast, most of the other compounds showed an acceptable stability at minus 20°C and even 4°C for a prolonged period of time. For example, most compounds from the classes including sulfonamides, coccidiostats, glucocorticoids and NSAIDs (Supplementary materials) were observed to maintain the desired stability even at room temperature and in the presence of light. These results suggest that, whenever possible, longer storage of penicillins, cephalosporins and ß-lactams should use a freezing temperature of minus 20°C or, even better, of minus 80°C, as it was also recommended by Desmarchelier et al. (2018).

Table 7 Results of long-term stability study for selected analytes



4.3. METHOD VALIDATION

Prior to the validation itself, pre-validation was performed as a proof of principle. Method validation was performed according to SANTE / 12682 / 2019 validation guideline (EC, 2019), since there is no directive or guideline for the implementation of method validation covering several classes of compounds in complex matrices nor specifically veterinary drug residues (Malachova et al., 2014).

4.3.1. Method accuracy

The validation procedure was based on an external calibration prepared in neat solvent. Spiking was performed both before and after extraction to distinguish between matrix effects and analyte loss during extraction. The recovery criterion was set in the range from 70-120% according to the applied SANTE / 12682 / 2019 guideline (EC, 2019). However, it should not be forgotten to emphasize that the concepts of the term “recovery” in various validation guidelines are still not sufficiently specified (Sulyok et al., 2020). Consequently, in this work, the accuracy of the method was assessed on the basis of apparent recovery (RA), which, according to IUPAC, is defined as “observed value derived from an analytical procedure by means of a calibration graph divided by a reference value” (Burns et al., 2002) or briefly explained as "a combined measure of matrix effects and losses during extraction" (Steiner et al., 2020b). Satisfactory apparent recovery values were determined for 56% of the analytes in milk, while for extraction efficiencies (or recovery of extraction, RE) 85% of the analytes were in the desired range of 70-120% (**Figure 11**). However, if the recovery criteria are expanded to 60-140% as is suggested for routine analysis by the SANTE guidelines (EC, 2019), larger fractions of compliant analytes would be obtained. In the case of chicken feed, apparent recoveries were in the range of 43% for acidic and 46% for neutral extraction conditions, while, similar to milk, this share of complying compounds for extraction efficiencies increases to 72% in acidic and to 80% in neutral extraction conditions (**Figure 12**).

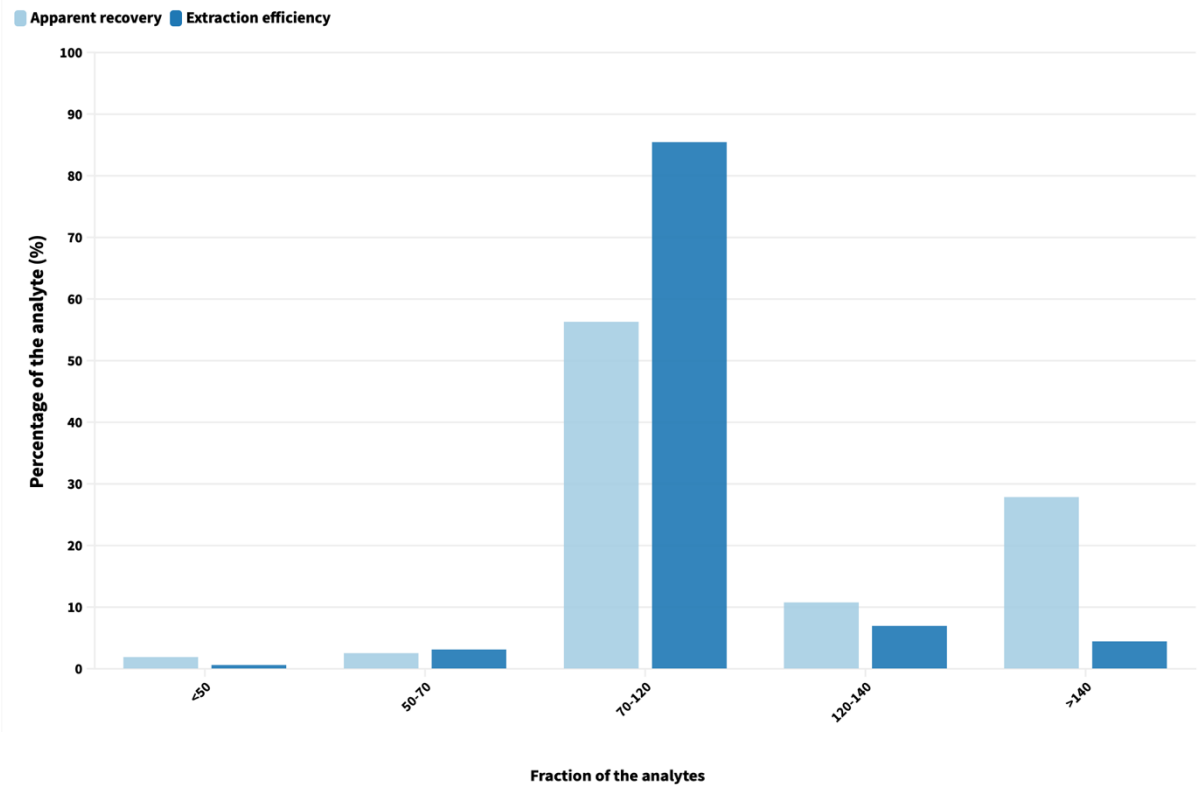


Figure 11 Apparent recoveries and extraction efficiencies obtained for milk

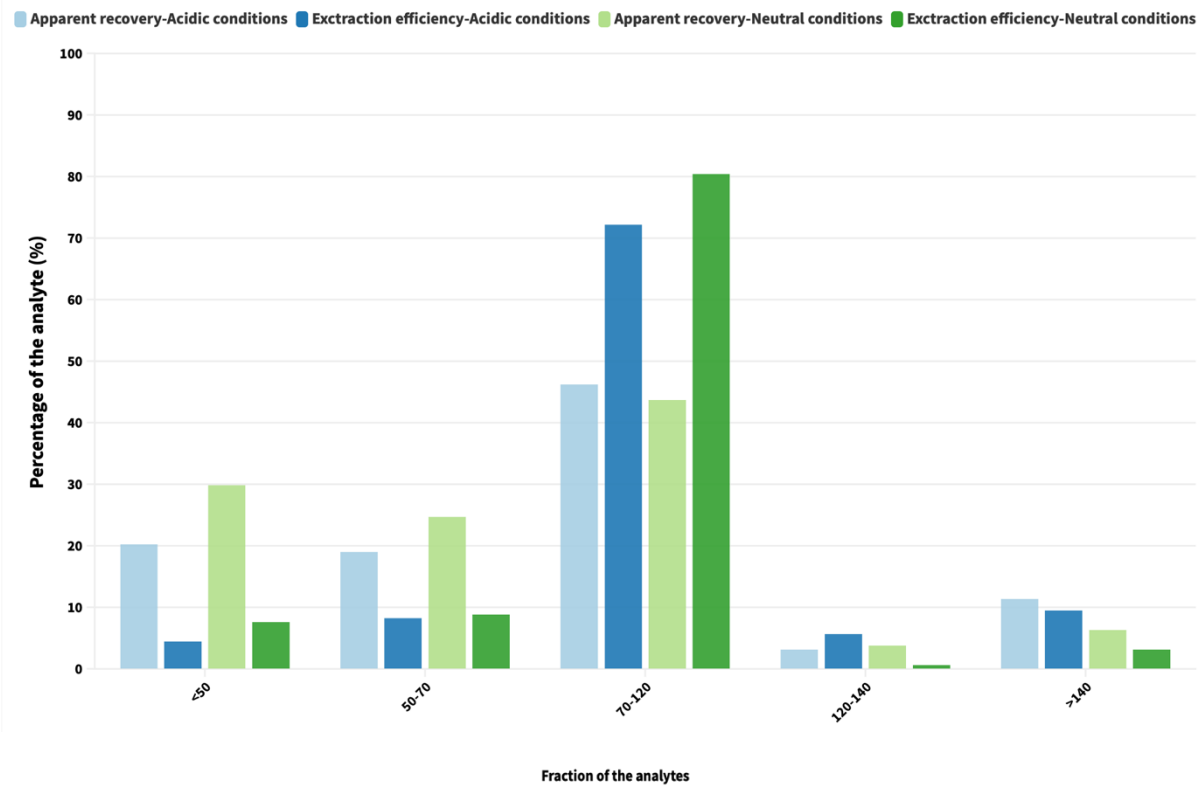


Figure 12 Apparent recoveries and extraction efficiencies obtained for chicken feed

4.3.2. Matrix effect

The main cause for the distinction between apparent recovery and extraction efficiency are matrix effects caused by the presence of co-eluting components. Matrix effects decrease apparent recovery but do not affect extraction efficiency. This is the principal reason why a revision of the term recovery is needed, as different guidelines use contradicting definitions (including and excluding matrix effects, respectively) (Codex Alimentarius, 2009; Burns et al., 2002). The matrix effect in the HPLC-ESI-MS/MS system occurs when components that elute together with the analyte of interest interfere with the ionization process in the MS detector. As a result, competition occurs between these components and the desired analyte in the ionization process, which results in signal suppression or (more rarely) enhancement (thus the abbreviation SSE). This phenomenon is defined as absolute matrix effect and apparently causes a decrease (or more rarely an increase) of the recovery related to external solvent-based calibration. In addition to the absolute matrix effect, Matuszewski et al. (2003) introduced the term relative matrix effect, which is a variation of the absolute matrix effect observed in different lots (or varieties, brands etc.) of the same matrix. These relative effects are the most important limitation of multi-analyte approaches as they cannot be compensated by the widely applied concept of matrix matched calibration (preparation of serial dilution in blank extract instead of neat solvent) (Sulyok et al., 2020). There is still no official advice on an acceptable measure of matrix effects, whereas in case of relative matrix effects certain criteria for the RSD values of the absolute effect determined in different lots have been set in biomedical analysis (Viswanathan et al., 2007; EMA, 2019; Sulyok et al., 2020).

The main reference in classifying the influence of the matrix (in terms of absolute matrix effect) in this work was a paper by Ferrer Amate et al. (2010.). Therefore, for milk matrix signal enhancement was stronger overall with 25% of analytes above 120%. In contrast, strong signal suppressions were especially visible in chicken feed, which is a much more complex matrix, with 39% and 41% analytes below 70% for both procedures (with and without acid; **Figure 13**).

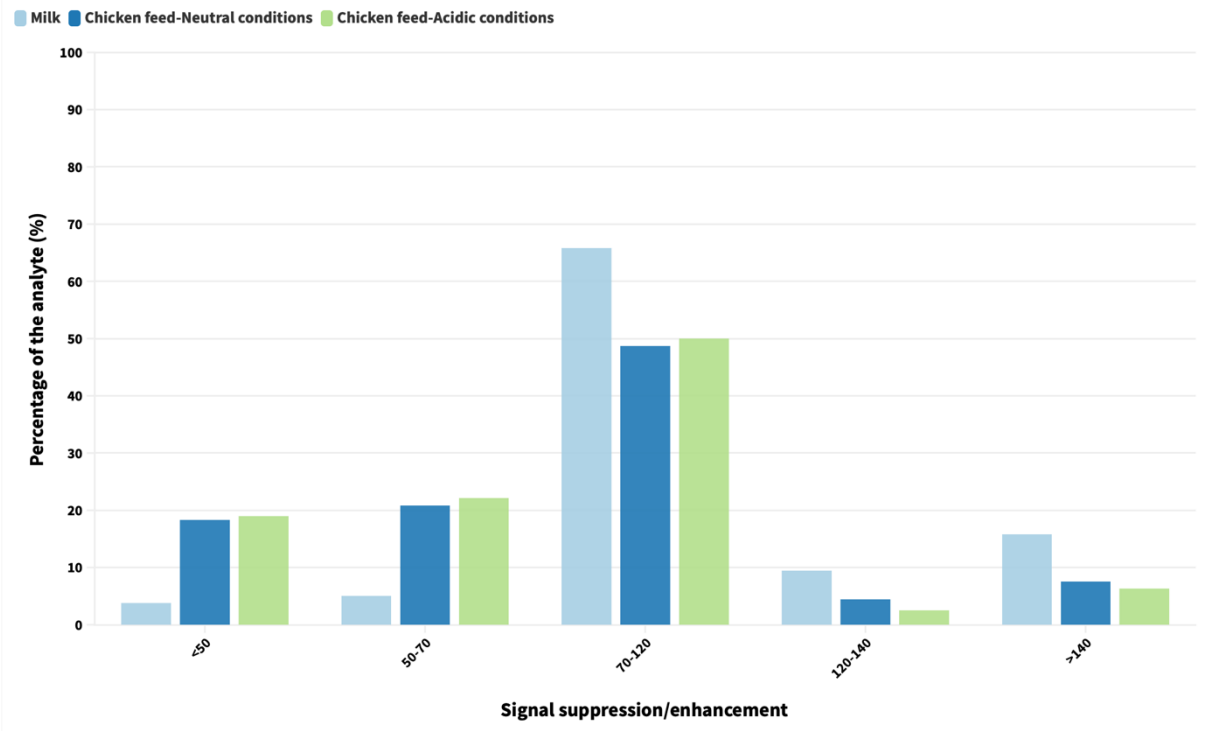


Figure 13 Signal suppression / enhancement obtained for both investigated matrices

4.3.3. Method precision

Precision of the method and within laboratory repeatability were determined by spiking a set of five different samples at high concentration level per matrix contrary to the "identical test items" as is recommended in the CEN/TR 16059:2010 (CEN, 2010). SANTE / 12682 / 2019 guideline criterion for precision as well as within-laboratory repeatability is RSD% < 20 (EC, 2019). The method precision in milk matrix was similarly influenced by relative matrix effects with a median RSDSSE of 4% and the variability of the extraction (RSDRE) with a median of 6% (**Figure 14**). When it comes to chicken feed for neutral extraction conditions, median of RSD for all calculated parameters was between 4% and 5% (**Figure 15**). Merely a few compounds were exceeding the 20% criterion limit for precision, and this might be, as already mentioned before, of greater importance for multi analysis when obtained from different samples instead of the identical material (Steiner et al., 2020b). On the other hand, chicken feed with acidic extraction conditions showed worse results for the variability of the extraction with median RSDRE of 13.4% compared to the median of RSDSSE and RSDRA between 7.2 and 7.8% (**Figure 16**). Surprisingly low RSDSSE in the case of milk and chicken feed with neutral extraction indicate the obvious benefit of having milk matrix in calibration due to stabilizing matrix properties for some compound classes, and related use of matrix-matched calibration. Moreover, solvent testing conducted in method optimization also indicated potential benefits

of matrix-matched calibration, which is in line with some other authors who observed stabilizing effect upon the addition of vitamin C that possess antioxidant activity (Diaz et al., 2010).

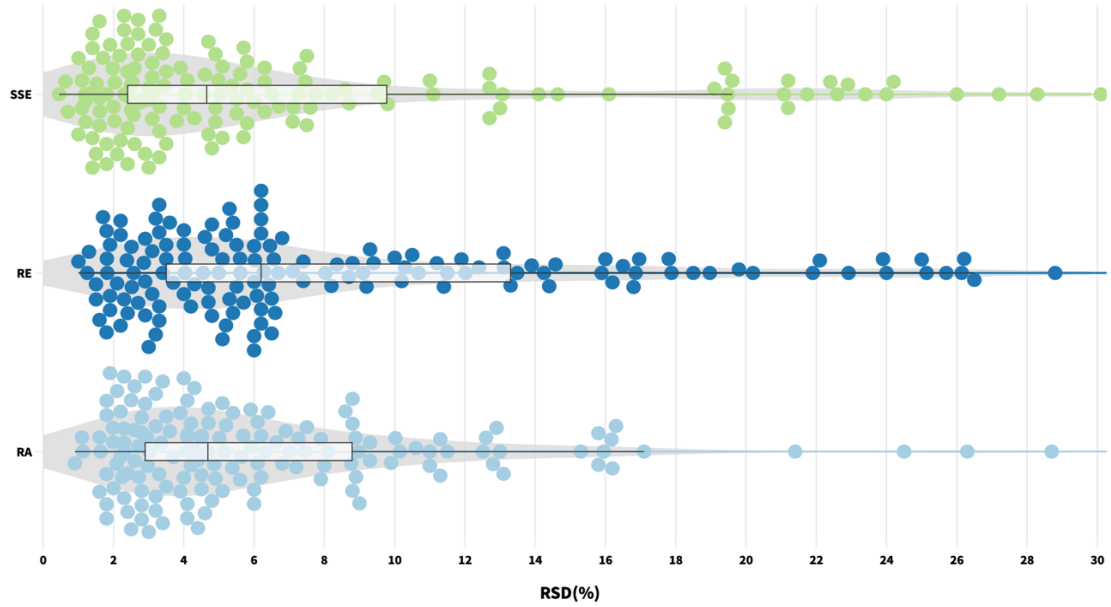


Figure 14 Relative standard deviation (RSD) of apparent recoveries (RA), extraction efficiencies (RE) and signal suppression enhancements (SSE) obtained for milk

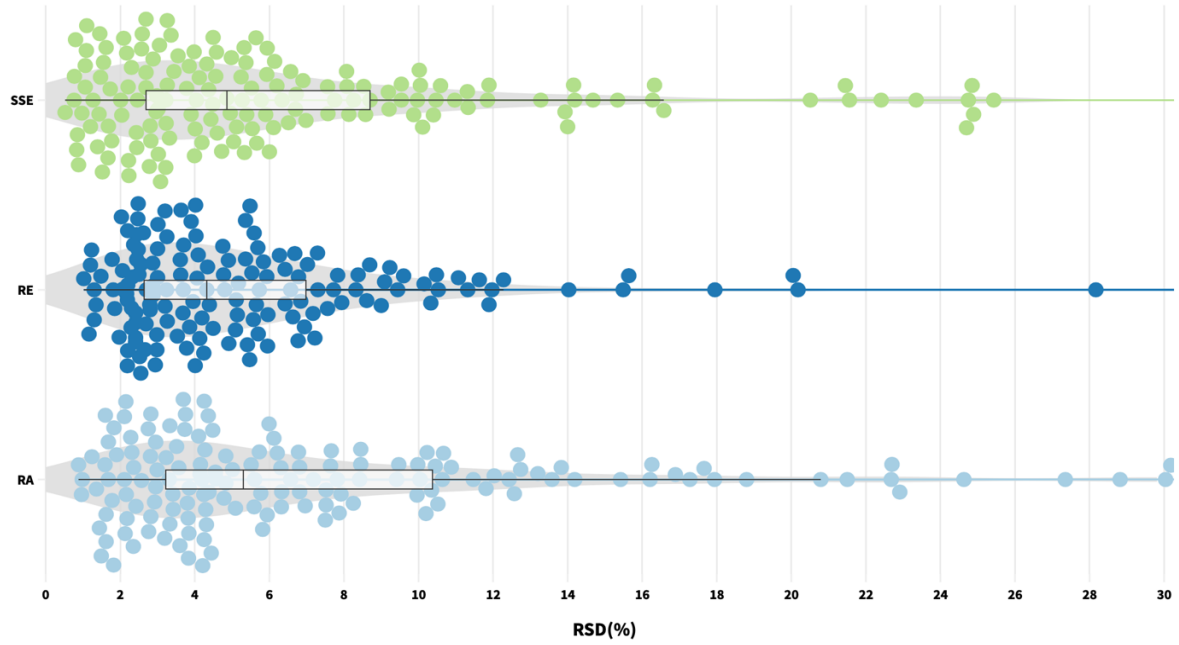


Figure 15 Relative standard deviation (RSD) of apparent recoveries (RA), extraction efficiencies (RE) and signal suppression enhancements (SSE) obtained for chicken feed with neutral extraction

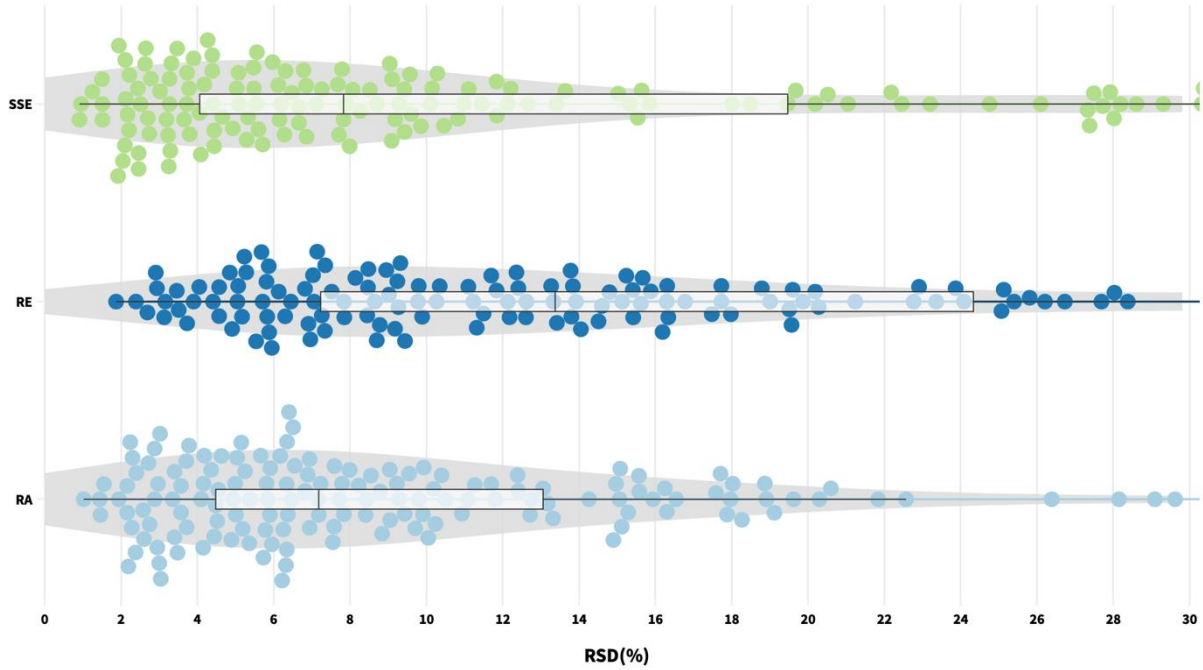


Figure 16 Relative standard deviation (RSD) of apparent recoveries (RA), extraction efficiencies (RE) and signal suppression enhancements (SSE) obtained for chicken feed with acidic extraction

4.3.4. Within laboratory repeatability

Within laboratory repeatability, as already mentioned, was proven "by spiking a set of five different sample at high concentration level per matrix" over three days. For milk and chicken feed with neutral extraction conditions, 86% of all analytes met the SANTE criterion of ≤ 20% (EC, 2019) (**Figure 17**). This percentage drops to 83% of all investigated analytes in the case of chicken feed with acidic extraction. Based on these results it can be seen that in the end there is actually no significant difference in extraction procedures contrary to what the results of solvent testing and determination of method accuracy showed. Either way, this also demonstrates that matrix effects and extraction efficiencies remain unaltered with time. This is of great importance, as in routine analysis of samples from different matrices physically matching the standards to each matrix would consume a lot of time.

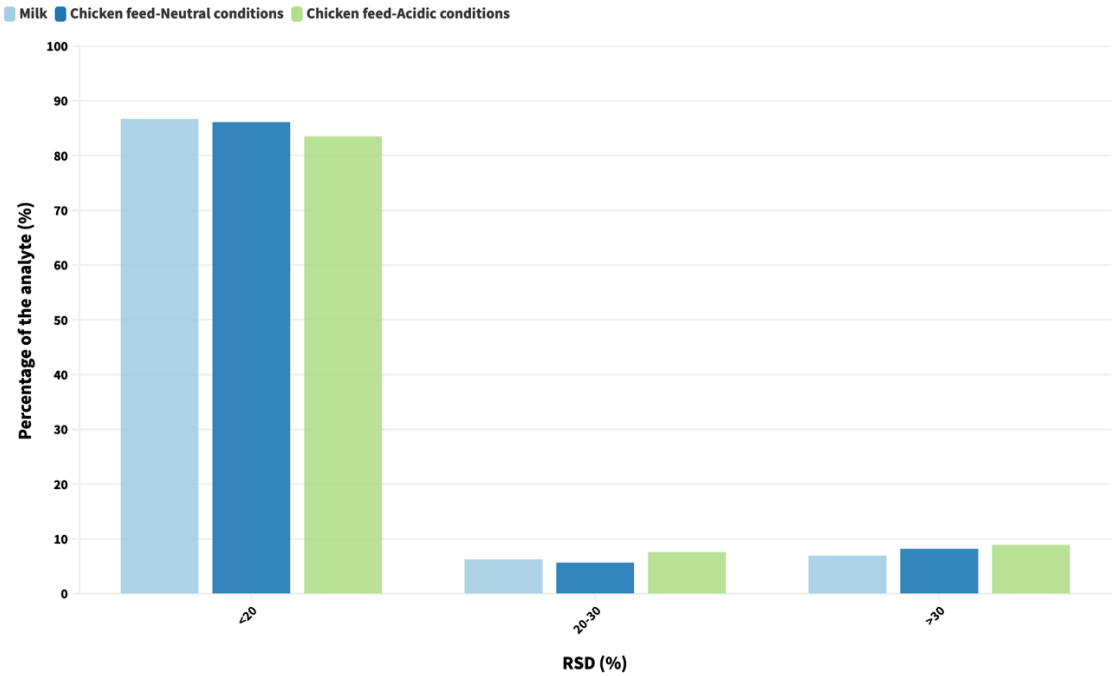


Figure 17 Within laboratory repeatability obtained for both investigated matrices

4.3.5. Limit of quantification

The limits of quantification were calculated according to the EURACHEM guideline (Eurachem working group, 2014). This approach defines the LOQ as the obtained standard deviation expressed in absolute concentration units multiplied by a factor of 10. This corresponds to a relative standard deviation of 10% for LOQ, which is in contrast to the vast majority of other guidelines having a RSD $\leq$ 20% as the limit (EC, 2019). Most analytes had the LOQ in milk between 10 and 50 ug/kg, which in turn means that levels of these analytes were lower than the maximum residue limits for veterinary drug residues in milk. Respective results obtained for chicken feed included the LOQ range for most analytes between 10-50 µg/kg (**Figure 18**). Analytes which were not below the given limits still resulted in very large peaks at the lower spiking levels. For instance, chloramphenicol is a prohibited substance and the MRL for amoxicillin is 4 µg/kg, thus leading to a significant over-estimation of the LOQ. The LOQ has been determined based on the signal to noise ratio (**Table 8**), although this approach has been discouraged by the European reference laboratories recently (Wenzl et al., 2016). Numerical values for analyte LOQs calculated based on the S/N ratio are given in the Supplementary data.

As regards chicken feed, regulations on MRLs for veterinary drug residues still do not exist. If factors such as MRLs for milk and edible animal tissue, and most importantly, veterinary drug

administration routes (subcutaneous, intravenous, intramuscular, oral), are considered, collected data certainly meet the criteria (Baron et al., 2014; Reeves et al., 2011).

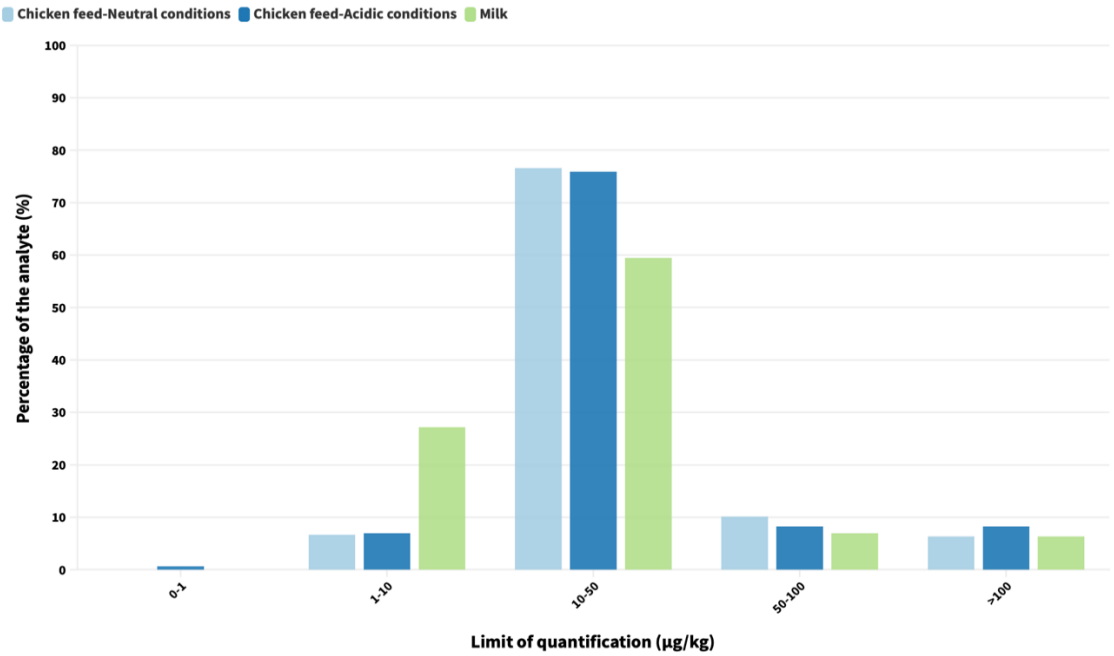


Figure 18 Limits of quantification for all tested analytes in milk and chicken feed

Table 8 LOQ values based on S/N compared to EURACHEM guideline calculation suggestion, calculated for analytes exhibiting large signal on the lower spiking level

Substance class:	Substance:	MRL (µg/kg)	LOQ (µg/kg) [1]	LOQ (µg/kg) [2]
ß-lactam	Dicloxacillin	30	11.6	1.24
ß-lactam	Amoxicillin	4	11.88	4.30
Amphenicols	Chloramphenicol	Prohibited	533.78	0.16
Cephalosporins	Cefalonium	20	84.2	3.87
Glucocorticoids	Prednisolone	6	40.43	3.89
Anthelmintics	Albendazole sulfone	100	12.2	0.46

[1] Calculated based on Eurachem guideline (2014)

[2] Calculated based on signal to noise ratio (S/N)

5. CONCLUSIONS

Based on the results of research conducted in this master thesis, the following conclusions can be drawn:

The use of milk as a solvent indicates the stabilizing effect of this matrix on certain classes of veterinary drugs, such as coccidiostats, NSAIDs, macrocyclic lactones and some ß-lactams.
When a multi-mix of veterinary drugs including penicillins, polyether ionophores and quinolones is stored for one week, it is favourable to do it at a temperature of +4°C degrees under neutral conditions. However, the stability at room temperature for the typical duration of an analytical sequence is sufficient.

When intermediate solutions of veterinary drugs are stored for a period of 1 month, it would be best to apply a temperature of -20°C. In case of storage longer than one month even at a temperature of -20°C, use of freshly prepared intermediate solutions containing classes of veterinary drugs such as penicillins, cephalosporins and ß-lactams is recommended.

Most analytes had the LOQ in milk and chicken feed between 10 and 50 μg/kg, which means that levels of these analytes were lower than the maximum residue limits for veterinary drug residues in milk, while for feed regulations on MRLs for veterinary drug residues still do not exist. Within laboratory repeatability for milk and chicken feed was ≤ 20% (according to the SANTE criterion) for 86% of all analytes.

The results of the method validation show that, although the properties of the tested analytes were extremely broad in terms of polarity, stability and solubility, a large fraction of analytes (80-90%) complies to the SANTE recovery criterion of 70-120% and RSD <20%.

The developed method does not meet the criteria in the case of streptomycin, sulbactam, colistin, nequinate, ticarcillin, clavulanic acid, desfuroylceftiofur, vancomycin and baquiloprim.

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SUPPLEMENTARY DATA

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