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DISSERTATION / DOCTORAL THESIS

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„Development and validation of a routine UHPLC-MS/MS method for determination of veterinary drug residues in animal derived food stuff“

verfasst von / submitted by

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IV. LIST OF ABBREVIATIONS

6-APA	6-aminopenicillanic acid
ACN	Acetonitrile
ADME	Absorption, Distribution, Metabolism and Excretion
ATP	Adenonsine triphosphate
BVL	Federal Office of Consumer Protection and Food safety in Germany
C18EC	C18 endcapped
CAV	Cell accelerating voltage
CE	Collision energy
CFR	Code of Federal Regulations
CRM	Certified Reference Material
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DM	Dry matter
dMRM	Dynamic multiple reaction monitoring
DNA	Deoxyribonucleic acid
dSPE	Dispersive solid phase extraction
DW	Dry weight
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EMA	European Medicine Agency
EMR	Enhanced matrix removal
EMV	Electron multiplier voltage
ESI	Electro-spray-ionisation
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
FDA	US Food and Drug Organisation
GIT	Gastrointestinal tract
GyrA	Subunit A of DNA gyrase
GyrB	Subunit B of DNA gyrase
HPLC	High-performance liquid chromatography
ISTD	Internal standard

JETA-CAR	Joint Expert Technical adivisory committee on antibiotic Resistance
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MMC	Matrix matched calibration
MRL	Maximum residue level
MRM	Multiple reaction monitoring
mRNA	messenger RNA
MS	Mass spectrometer
Na_2EDTA	Disodium ethylenediaminetetraacetic acid
NC	Normal calibration
NFPA	Nonafluoropentaoic acid
PABA	4-aminobenzoic acid
PBS	Penicillin-binding proteins
PMC	Procedure matched calibration
PSA	Primary secondary amines
PTFE	Polytetrafluoroethylene
QuEChERS	Quick Easy Cheap Effective Rugged and Safe
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Retention time
SPE	Solid phase extraction
TCA	Trichloracid
THF	Tetrahydrofolate
tRNA	Transfer ribonucleic acid
UHPLC	Ultra high-performance liquid chromatography
VRE	Vancomycin-resistant enterococci
WWTP	Wastewater treatment plants
ZSep	Zirconia-coated silica

AIM OF THE THESIS

The accidental founding of penicillin by Sir Alexander Fleming and the introduction of the first commercially available antibiotic at the beginning of 1930, revolutionized the medicine in the last century. A wide range of antibiotics was found and synthetically developed during the years, which can be divided in accordance to their chemical properties and their mechanisms of action.

Due to the increasing demand of animal protein for human consumption the intensive livestock production is associated with a rising amount of veterinary drug usage in food animals. (Van Boeckel et al., 2015) The main switch in their eating habits can be observed in developing countries, covering their protein demand from animal derived products instead of formerly from cereals and other food staples. (Boland et al., 2013) Especially in Asia, in 2011 the protein supply quantity per capita was more than threefold higher than in 1961, which can be explained by the strong increase of milk production. (online source: FAOstat, accessed on: 22nd October 2016)

The officially used amount of antimicrobial substances belongs to approximately 24,000 tons the United States and in 29 European countries together, whereas the most sold groups are tetracyclines, followed by the group of penicillins, sulphonamides and ionophores. (European Medicines Agency, 2012; Food and Drug Administration - Department of Health and Human Services, 2015) The main route of administration is by addition to feed and water to either prevent diseases as well as to enhance the growth and feed efficiency. (Food and Drug Administration - Department of Health and Human Services, 2015; Sarmah, Meyer, & Boxall, 2006a)

This extensive use of antibiotics is rising the concern of developing a worldwide prevalence of antibiotic resistance. Antimicrobial substances consumed from animals and humans, are usually excreted (urine and feces) and possible entering thereby the aquatic ecosystem. Results from investigations for the occurrence of antibiotic residues in different parts of the aquatic environment demonstrate that residues were found in

the influents and effluents of wastewater treatment plants, river water, groundwater and drinking water in concentrations between ng/l to µg/l. (Carvalho & Santos, 2016)

Regarding the ability of bacteria to become resistant against one or more antibiotic substance, the more antibiotics are used in veterinary and human medicine, the higher is the risk to develop resistant strains. These antibiotic resistant strains can find different routes within the environment to spread as demonstrated in Figure 13. The main resistant strains are multiple antibiotic-resistant *salmonellae*, macrolide- or quinolone-resistant *campylobacters*, glycopeptide- or streptogramin-resistant *enterococci* and multiple antibiotic-resistant *E. coli*. The main reservoirs for antibiotic resistant strains are food producing animals and thereby resistant strains can directly be transmitted via food chain. (Newell et al., 2010; Phillips et al., 2004)

To prevent the development and spreading of antibiotic resistant strains across the food chain, the European Union set maximum residue levels (MRLs) in the Council Regulation 37/2010, regulating pharmacologically active substances and their classification regarding MRLs in foodstuffs and animal origin. (The European Commission, 2010)

Regarding these set MRLs this project aims to develop a confirmatory UHPLC-MS/MS routine method to quantify possible antibiotic drug residues in animal origin food stuff. The most important claim to this method is the ability to determine as much substances as possible in different kind of matrices. Additionally, the final sample preparation procedure must be easy to handle as well as less time and cost consuming to be suitable for routine laboratory use.

As described by Blasco et al. the three main problems concerning the determination of veterinary drug residues in animal food stuff are:

- the large number of compounds and their metabolites due to biotransformation processes
- the low analytical detection levels (MRLs are mainly set at mg/kg or µg/kg level)
- the composition and influence of the different matrices (Blasco, Picó, & Torres, 2007)

To eliminate these problems and achieve satisfying recovery rates of the substances of interest a powerful sample preparation is necessary. The main used clean-up approaches are based on the following sample preparation techniques:

- Dilute and shoot (liquid-liquid-extraction)
- QuEChERS - ***Qu****ick* ***E****asy* ***Ch****eap* ***E****ffective* ***R****ugged and* ***S****afe*
- Solid Phase Extraction (SPE) (Berendsen & Nielen, 2013)

In this work, different approaches of the previous mentioned sample preparation techniques are demonstrated, investigating different types of extraction solvents and clean-up procedures. The compounds of interest belong to the groups of quinolones, macrolides, lincosamides, ß-lactams, sulphonamides, diaminopyrimidine-derivate and tetracyclines. With the final developed method 30 different analytes can be extracted with satisfying recovery rates [%] and RSD [%] from ten different dairy matrices (raw milk, pasteurized milk, curd, sour cream, yogurt, soft and hard cheese, cream, butter and buttermilk) and ten different fish and meat matrices (chicken, prawns, salmon, trout, muscle (pig), fat, liver (pig), muscle (beef), liver (beef) and kidney (beef)). Since this work was fully conducted at the company LVA GmbH – an independent competence centre for food safety - the finalized method was validated in accordance to their in-house validation scheme to obtain the recovery rates (%), RSD (%), limit of detection (LOD) and limit of quantification (LOQ) to assess the accreditation status according to ISO 17025:9001.

1. ANTIBIOTICS

The introduction of the first commercially available sulphonamide "Prontosil" in the 1930s, as well as the accidentally discovery of the substance penicillin isolated from the mould *Penicillium notatum* by Sir Alexander Fleming (Figure 1) in 1928 revolutionized the medicine of the 20th century. In 1945 penicillin was mass produced and became widely available as a potent antibacterial substance against a wide range of bacteria. Antibiotics, deriving from the Greek terms *anti* – "against" and *bios* – "life", are both naturally produced metabolites of moulds or bacteria and synthetically produced chemotherapeutics with the ability to inhibit the growth or kill other microorganisms. In accordance to their mechanisms of action, they are separated into bacteriostatic (inhibition of cell growth without killing of the cells), bactericidal (inhibition of cell growth and killing of the cells) and bacteriolytic (cell death by lysis) antibiotics.

Figure 1: Sir Alexander Fleming [1]

1.1. Groups of antibiotics

Antibiotics can be classified in order to their chemical structures and mechanisms of action into different groups (Table 1):

	Inhibition of protein biosynthesis	Inhibition of cell wall biosynthesis	Inhibition of nucleic acid metabolism
Gram positive	Tetracyclines Macrolides Lincosamides	ß-lactams	Quinolones Sulfonamides
Gram negative	Aminoglycosides Tetracyclines		Quinolones Sulfonamides

Table 1: Groups of antibiotics

1.2. Major groups of antibiotics with their chemical properties

1.2.1. ß-Lactams

The basic chemical structure of ß-lactam antibiotics is the presence of a four-membered lactam in their molecular architecture, containing the classes of penicillins and cephalosporins. Penicillins possess a characteristic 6-aminopenicillanic acid (6-APA) core linked to the ß-lactam ring and a sidechain at position C6, and can be separated into six groups according to their activity (Figure 2). Cephalosporins contain a side-chain attached to 7-aminocephalosporanic acid core. Typical ß-lactam antibiotics are e.g. benzylpenicillin (penicillin G), amoxicillin, ampicillin, cloxacillin, ceftiofur and phenoxymethylpenicillin (penicillin V). Due to the presence of the unstable ß-lactam core ring they are susceptible to degradation by heat, in presence of alcohols and in acidic environment. ß-lactam antibiotics are bactericidal and acting by binding to the proteins in the cell membrane (PBPs - penicillin-binding proteins) which leads to a disruption of the synthesis of the peptidoglycan layer of bacteria cell walls and finally results in the lysis of growing cells. Killing activity of ß-lactams starts after a lag phase and is time-dependent, not concentration-dependent. This mechanism can only be achieved in Gram-positive bacteria, because of their large amount of peptidoglycan in their cell wall. The lipopolysaccharide layer of Gram-negative bacteria is difficult to penetrate for ß-lactams, explaining their absence effects on most Gram-negative bacteria. Due to synthetic modifications on their chemical structures, ß-lactamases resistant penicillins were developed (e.g. methicillin, cloxacillin) to become also effective against Gram-negative bacteria. ß-lactam antibiotics alone or in combination with ß-lactamase inhibitors like clavulanic acid (e.g. amoxicillin plus clavulanate) or in combination with

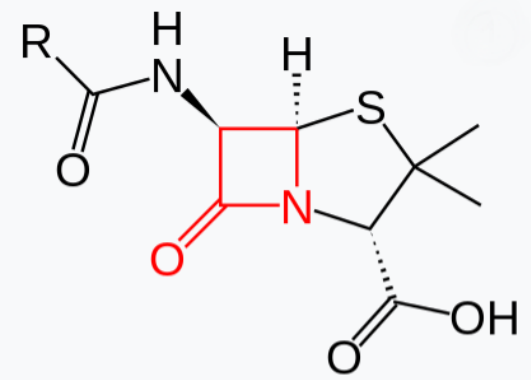

Figure 2: Basic structure of ß-lactam antibiotics. The ß-lactam ring is red marked.[14]

other antibiotics like aminoglycosides (e.g. streptomycin and penicillin G) are commonly used in the livestock for the treatment of mastitis and show no largely side effects.

1.2.2. Macrolides

The group of macrolides consists of about natural antibiotics isolated from fungi (e.g. Erythromycin A from *Saccharopolyspora erythraea*, Spiramymin from *Streptomyces ambofaciens*) and their semi-synthetic derivates. The chemical structure consists of a 12–16 carbon lactone ring to which several amino groups and/or neutral sugars are bound. Typical compounds of this group are erythromycin, spiramycin and tylosin. Macrolides bind to the ribosomal 50*S* subunit and inhibits therefore the protein synthesis and the cell growth, subsequently. They are acting predominantly bacteriostatic, whereas high concentrations are slowly bactericidal against sensitive organisms. Macrolide antibiotics are general used against infections caused by Gram-positive bacteria whereas their activity against Gram-negative bacteria is limited. Due to this spectrum of activity, which is similar to the spectrum of penicillins, macrolides are used as an alternative treatment of individuals, whose are allergic to penicillin. The dosage forms of macrolides vary from medicated feed, water-soluble powder for adding to drinking water, tablets or injections. In veterinary medicine macrolides are widely used to treat respiratory diseases and are also allowed in some countries to be used as growth promoters in pigs and chickens. Severe side effects are usually low and depend on the formulation and animal species, for example inflammatory reactions after intramammary infusion or serious gastrointestinal disturbances in horses after treatment with erythromycin.

1.2.3. Lincosamides

The compound class of lincosamides consists of the substances lincomycin, clindamycin and pirlimycin, whereas lincomycin and pirlimycin are approved for the use in food producing animals. Lincosamides derives from a galactoside containing an amino acid and sulfur. Lincomycin was the first discovered lincosamides and is isolated from *Streptomyces lincolnensis*. Clindamycin and its analogue pirlimycin are semi-syntethic derivates. Their mechanisms of action are based on the binding on the 50*S* ribosomal

subunit and interrupting therefore the protein synthesis. Lincosamides are acting concentration dependent and in order, they may be bacteriostatic or bactericidal. Their spectrum of activity involves many Gram-positive bacteria, but they are not effective against most of Gram-negative bacteria. Lincomycin is beside other bacteria useful for the treatment against *Staphylococcus* species, *Streptococcus* species (except *Streptococcus* faecalis) and *Mycoplasma* species. It is mainly administered in combination with spectinomycin or sulfadiazine in feed or drinking water for the treatment and control of respiratory diseases and for increasing weight gains. Combinations with neomycin are used for the treatment of mastitis in dairy cattle. Pirlimycin is active against *Staphylococcus aureus* and different *Streptococcus* species and is effectively used in the treatment of mastitis in lactating dairy cattle.

1.2.4. Quinolones

The group of quinolones are synthetic antimicrobial drugs which are divided into four generations according to their antibacterial spectrum. Despite there is no standardized categorization, in general, the members of earlier-generation have a narrow spectrum of activity compared to the later generations. Typical chemical structure of quinolones is the possession of a fluorine atom at the C6 position of the quinolone ring structure (Figure 3). The compounds danofloxacin, difloxacin, enrofloxacin, marbofloxacin, orbifloxacin and sarofloxacin are exclusive for veterinary use. Quinolones are antimicrobials and acting concentration-dependent, accumulating in the cytosol of macrophages and neutrophils. They are entering bacterial cells via porins and inhibit the bacterial DNA enzyme gyrase (topoisomerase II) in Gram-negative bacteria, which is responsible for introducing negative supercoils into DNA. The inhibition prevents thereby the replication and transcription of DNA due to DNA fragmentation. In Gram-positive bacteria quinolones affect topoisomerase IV resulting also in breakdown of DNA replication.

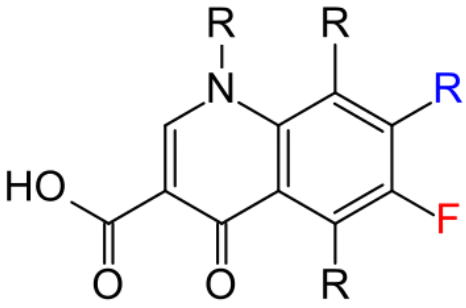

Figure 3: Basic structure of quinolones

1.2.5. Sulphonamides and Diaminopyrimidine

The class of sulphonamides comprise a large number of compounds containing the functional sulfonyl group connected to an amine group (Figure 4). Unless there is a long list of sulphonamides, there are only few compounds approved to be used in the treatment of food-producing animals. The list includes e.g. sulfadiazine, sulfamethazine, sulfamethoxazole, and for improving the efficacy, sulphonamides are often administered in combination with an antibacterial diaminopyrimidine like trimethoprim. Sulphonamides are effecting the synthesis of dihydrofolic acid, which is essential for DNA synthesis. They inhibit the enzyme dihydropteroate synthetase by competing with 4-aminobenzoate (PABA), due to their structural analogues. Combinations of sulphonamides and diaminopyrimidine are active against Gram-positive and Gram-negative microbial organisms like *Actinomyces*, *Clostridium*, *Salmonella*, *Shigella*, and *Campylobacter* species, *E. coli*, *streptococci* and *staphylococci*, whereas species of *Pseudomonas* and *Mycobacterium* are resistant to this enforced combination. Sulphonamides alone and in combination are applied to food-producing species as feed and drinking water additive, oral drugs or by intrauterine infusions. They are effective antibiotics to prevent or treat infections like mastitis, toxoplasmosis or respiratory infections showing mild reversible side effects.

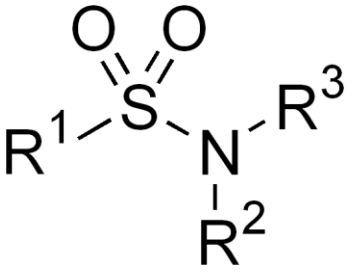

Figure 4: Basic structure of sulphonamides

1.2.6. Tetracyclines

Tetracyclines originally derived from the *Streptomyces* genus of *Actinobacteria*. Chlortetracycline, doxycycline, oxytetracycline and tetracycline are the most common compounds of this group which are applied to food-producing animals. Chlor- and oxytetracycline are isolated from *Streptomyces* species, while tetracycline is produced by hydrogenolysis of chlortetracycline and doxycycline is a semi-synthetic derivate of

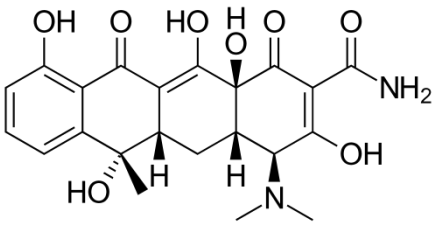

Figure 5: Basic structure of tetracyclines

oxytetracycline. The basic chemical structure of this compound group is a hydronaphthacene skeleton containing four fused rings, while the different tetracyclines mainly differ in their substitution patterns at the C5, C6 and C7 positions (Figure 5). Tetracyclines are affecting the protein synthesis by binding to the receptors of the 30*S* ribosomal subunit of susceptible bacteria. They can either act bacteriostatic but at high concentrations they are bactericidal in sensitive organisms. Tetracyclines are active against Gram-positive and Gram-negative bacteria, including e.g. *E. coli*, *Klebsiella* species, *Salmonella* species and *Streptococcus* species. Typical administration forms are medicated feeds, soluble powders, tablets, intrauterine and intramammary infusions as well as injections and are effective in the treatment of uterine infections, Clostridium diseases, pneumonia, mastitis, infectious sinusitis etc. In addition, tetracyclines are also used to enhance feed efficiency in cattle, chickens, pigs, sheep and turkeys. Severe side effects are observed after rapid intravenous administrations in most animal species, like cardiovascular dysfunction until death in horses. (Wang, MacNeil, & Kay, 2012)

1.3. Mechanisms of action and acquisition of antibiotic resistance

Beside the classification according to their chemical properties, antibiotic compound classes are divided referring to their cellular component or system they affecting. Their mechanisms of action target the bacterial cell wall synthesis, the protein-biosynthesis as well as the bacterial nucleic acids (Figure 6). (Mahon, Lehman, & Manuselis, 2011)

1.3.1. Inhibition of bacterial cell wall synthesis

Affecting the bacterial cell wall synthesis is the action mechanism of ß-lactam antibiotics to destroy bacteria. The bacterial multilayer cell wall consists of an inner cytoplasmic membrane, encased of a peptidoglycan layer, and an additionally outer membrane in Gram-negative bacteria. Synthesis of the peptidoglycan layer is divided into four major stages, whereas ß-lactams acting on the third and fourth stage. After precursors synthesis in the cytoplasm and lipid-bound precursor transportation via the cytoplasmic membrane, glycan units are insert into the cell wall and finally transpeptidation linking and maturation. (Mahon, Lehman, & Manuselis, 2011) For the maintenance of the

peptidoglycan layer, the activity of the enzymes transglycosylases and transpeptidases (also named as penicillin-binding proteins; PBPs) is responsible, which are essential for the extension of glycan strands in peptidoglycan molecules and cross-linking of immature peptidoglycan units. ß-lactam antibiotics are acting by blocking the cross-linking of peptidoglycan units due to inhibit the peptide bond formation reaction which is enhanced by PBPs. A substrate for PBP during acylation phase of cross link formation is the dipeptide D-alanyl-D-alanine. ß-lactams are analogues of the terminal dipeptide and are therefore able to bind on the active side of the PBP to achieve inhibition and kill the cells, respectively. (Kohanski, Dwyer, & Collins, 2010)

Resistance to ß-lactams occur through one or more of the following mechanisms: (1) mutations in the target PBP or development of PBPs with lower affinity to ß-lactams; (2) the increased production of ß-lactamases, which are inactivating the antimicrobials; (3) limited movement to the drug binding site through changes in the cell wall porins; and (4) active drug transport out of the cells by energy-dependent pumps. Within these mechanisms, the increased production of ß-lactamases, leading to an inactivation of ß-lactams by hydrolysis of the ß-lactam ring, is the most powerful mechanism type of resistance. (McDermott, Walker, & White, n.d.)

1.3.2. Inhibition of the protein biosynthesis

For the synthesis of functional proteins, the process of mRNA translation is necessary. The mRNA translation process consists of three sequential phases (initiation, elongation and termination) involving the ribosome and several cytoplasmic accessory factors. Ribosomes are composed of the small 30*S* and the larger 50*S* subunit and assembling during initiation phase. Since the synthesis of proteins is crucial to survive, the inhibition of the protein biosynthesis is an excellent target for antibiotics and the development of new antibiotics. This group involves the most antibiotic substances, and can be divided into 30*S* inhibitors (e.g. aminoglycosides, tetracyclines) and 50*S* inhibitors (e.g. macrolides) according to their subunit target.

Aminoglycosides are carbohydrate-containing molecules with a positive charge, which enables the interaction with a specific region of the 16*S* rRNA within the A-site of the

30*S* ribosomal subunit. This binding prevents the binding of aminoacyl-tRNA and interrupting therefore a correct translation process leading to the production of aberrant proteins. Installing of aberrant proteins into the cell wall additionally forces the cell leakage with additionally cellular penetration of antibiotics. Modifying enzymes, which are able to phosphorylate, adenylate or acetylate aminoglycosides, are responsible for the development of antimicrobial resistance to these agents. The huge number of these modifying enzymes leads to a difficult challenge to control bacterial resistance to aminoglycosides. (McDermott et al., 2003)

A further class of 30*S* inhibitors are the compound group of tetracyclines. Tetracyclines reversibly bind to the 16*S* rRNA near the aminoacyl tRNA acceptor site preventing thereby the binding of the aminoacyl-tRNA and in order the addition of new amino acids to the peptide chain. Resistance results through an increased efflux of tetracyclines from the bacterial cells, due to gene resistance encoding for a membrane protein, which is responsible for actively transport of drugs out of the cells. Further mechanism is the overexpression of proteins, which are preventing the binding of tetracyclines to bacterial ribosomes. (McDermott et al., 2003; Wang et al., 2011)

Macrolides and Lincosamides belong to the group targeting the 50*S* subunit. They are binding to the peptidyltransferase cavity and block thereby the exit tunnel of the elongating peptides, resulting in the increase of prematured peptidyl-tRNA and the fully blockage of polypeptide translation. Mutation or methylation of the 23*S* rRNA subunit leads to an increased efflux and bacteria become resistance of these antimicrobial agents, subsequently. (McDermott et al., 2003)

1.3.3. Inhibition of Nucleic Acid Metabolism

1.3.3.1. Inhibition of folate synthesis

In difference to mammalian cells, bacteria are dependent on endogenous synthesis of folic acid, which provides the essential precursor molecules for DNA synthesis. They have to synthesize folates from small molecules, whereas the pathway is mediated by two key enzymes, dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). (Mahon et al., 2011; Sköld, 2000) DHPS catalyses the transformation from PABA

to dihydropteroate and adding a glutamate moiety completes the formation of dihydrofolate (DHF). DHF gets subsequently transformed into tetrahydrofolate, catalysed by the enzyme DHFR and tetrahydrofolate (THF) is further transformed into the essential folic acid. (Bermingham & Derrick, 2002)

Sulphonamides and trimethoprim are acting on the inhibition of these key enzymes by competitive binding. Sulphonamides are structural analogues of PABA and thus they inhibit the binding of DHPS with PABA resulting in stopping the formation of dihydropteroate and dihydrofolate. To enhance the effect of sulphonamides, they are combined with trimethoprim, which prevents the formation of THF by blocking the enzyme DHFR. (McDermott et al., 2003) Antimicrobial resistance to sulphonamides are caused due to chromosomal mutations, resulting in impairing the drug penetration, production of altered forms of dihydropteroate synthetase, showing a lower affinity for sulphonamides, or an overexpression of PABA. A further and more common reason for bacterial resistance is a plasmid-mediated mechanism, causing an impaired drug penetration or the production of sulphonamide-resistant dihydropteroate synthetase. (Wang et al., 2011)

1.3.3.2. Inhibition of the DNA replication enzymes

Due to the absent cell core in prokaryotic organisms, there is no fully cell cycle like existing in eukaryotic cells, and DNA replication is immediately followed by cell division. Necessary enzymes for DNA replication are the topoisomerases I-IV, performing critical ATP-dependent functions. Quinolones are affecting DNA replication by targeting topoisomerase II, also known as DNA-gyrase, and topoisomerase IV, both comprised of two subunits. The DNA gyrase consists of the subunits GyrA and GyrB and topoisomerase IV encodes the subunits ParC/GrlA and ParE/GrlB. (Alekshun & Levy, 2007) Although DNA gyrase and topoisomerase IV show similarities in their general functions, the targets of quinolones

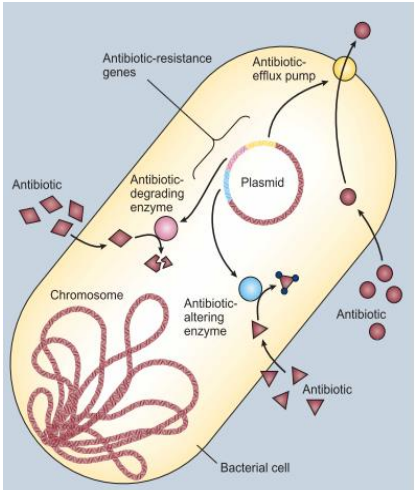

Figure 6: Different biological mechanisms of resistance. (Levy & Marshall, 2004)

are selective. In Gram-positive bacteria quinolones have a high affinity to topoisomerase IV, whereas topoisomerase II is the primary target in Gram-negative bacteria. Quinolones possess the ability to form stable interaction complexes between topoisomerases and cleaved DNA, leading to inhibition of DNA replication and finally leads immediately to bacteriostasis and eventually cell death. (Kohanski et al., 2010) Quinolones resistance can be caused due to three different mechanisms: (1) target mutations in the topoisomerases genes – topoisomerase II in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria; (2) decreased permeability of the bacterial cell wall; and (3) energy-dependent efflux pumps. (McDermott et al., 2003; Wang et al., 2011)

1.4. Production of animal products and the use of antimicrobials in livestock

1.4.1. Animal derived food production worldwide

The use of antimicrobial substances in livestock production is necessary to maintain health and productivity. In order to the globally increasing demand of animal protein for human consumption the rising livestock production is associated with a high use of veterinary pharmaceuticals in food animals. (Van Boeckel et al., 2015) Mainly responsible for the rapid growth of animal derived proteins is the dietary transformation in developing countries due to increasing incomes, populations and urbanisation. Typical dietary habits in developing countries, involving cereals and other food staples as protein source, has shifted to a food consumption similar to developed countries, where animal derived products act as a major part in diet. (Boland et al., 2013) Especially in Asia, the protein supply quantity per capita deriving from animal products was increasing from 7.3 to 25.95 g/capita/day (355 % increase compared to only 160 % worldwide) between 1961 and 2011 (Figure 7), which can be explained by the significant increase of milk production in the last 15 years (Figure 8). (online source: FAOstat, accessed on: 22nd October 2016)

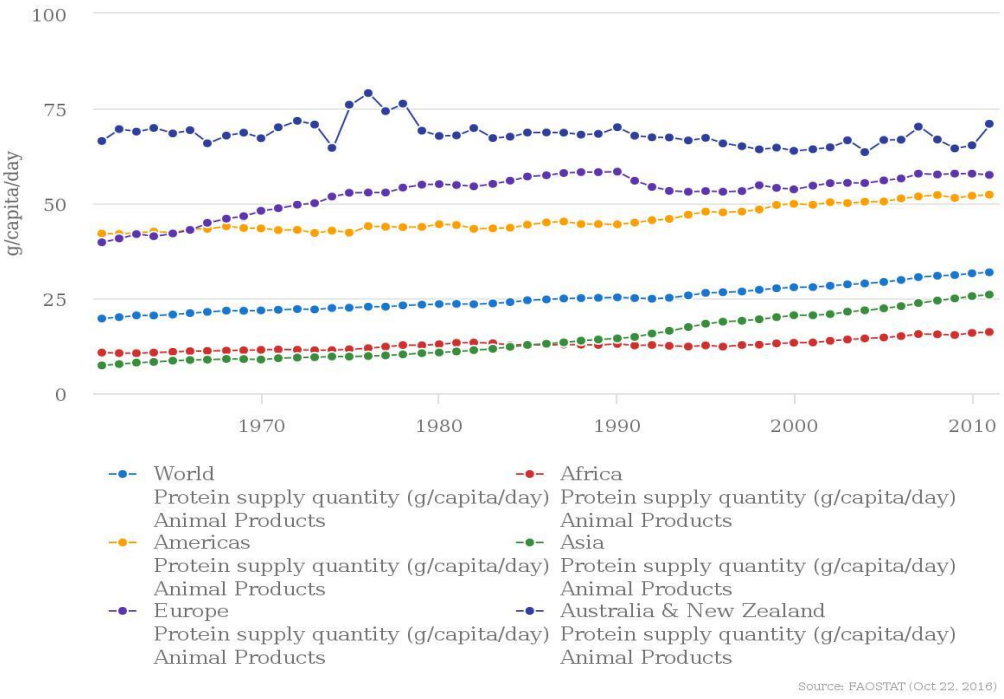

Figure 7: Development of the worldwide animal protein supply from 1961–2011.[2]

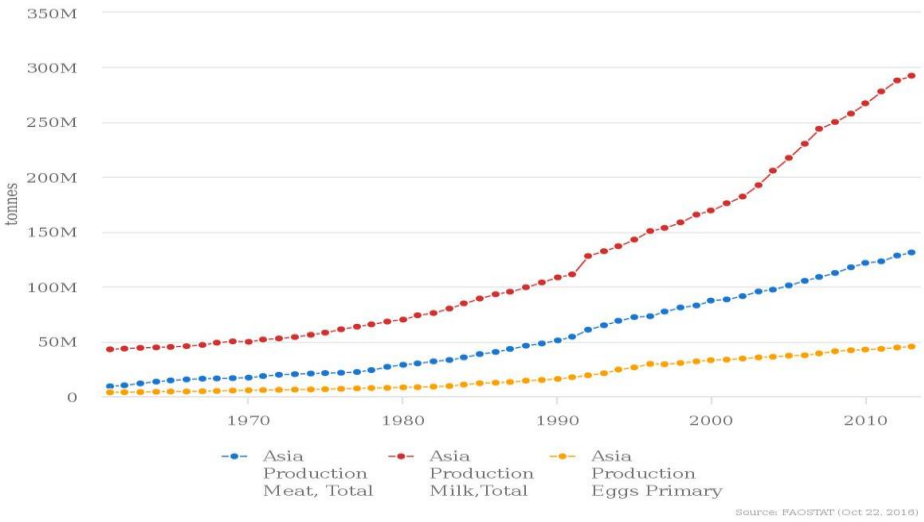

Figure 8: Development of animal derived protein production from 1961–2011 in Asia.[2]

In accordance to this rising demand of animal derived proteins in the last years, animal livestock has also been increasing, and a continued growth in consumption and livestock production can be expected (Figure 9). (Boland et al., 2013)

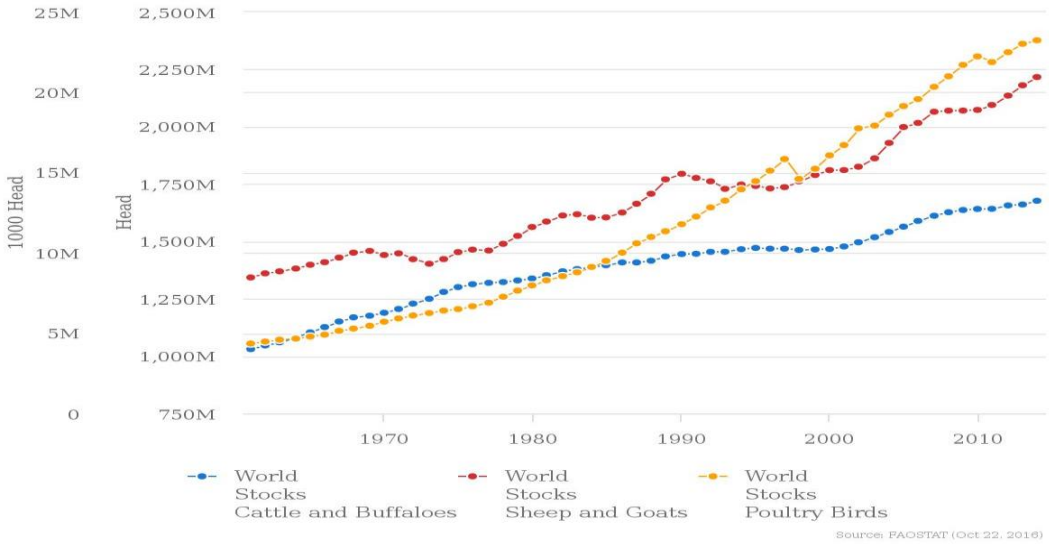

Figure 9: Development of the worldwide animal livestock from 1961–2011.[2]

To ensure controlled and stable food production, the application of several veterinary drugs and feed additives is nowadays a common practice in food-animal agriculture. This should prevent the spreading of diseases, which can easily occurs due to the close

proximity of the huge numbers of animals at these facilities. The route of administration can be in form of feed or water additive, by injection, orally, topically, paste, implant and bolus. Among the great variety of drugs and feed additives, antibiotics are the most widely administered drugs to ensure animal health care. (Sarmah et al., 2006a).

1.4.2. Usage of antimicrobial substances

1.4.2.1. The United States of America

In the US over 15 million kg antimicrobial drugs which are approved for use in food-producing animals are used in 2014, wheras 60 % of them were applied due to medically importance and 40 % for improving feed efficiency and enhancing growth. Comparing the years 2009 until 2014 an increase of 22 % of total sold antibiotics, which can be explained by the increase of 150 % for sold lincosamides. The main groups of administred drugs are tetracyclines with 43 % and ionophores with 31 % (Figure 10),

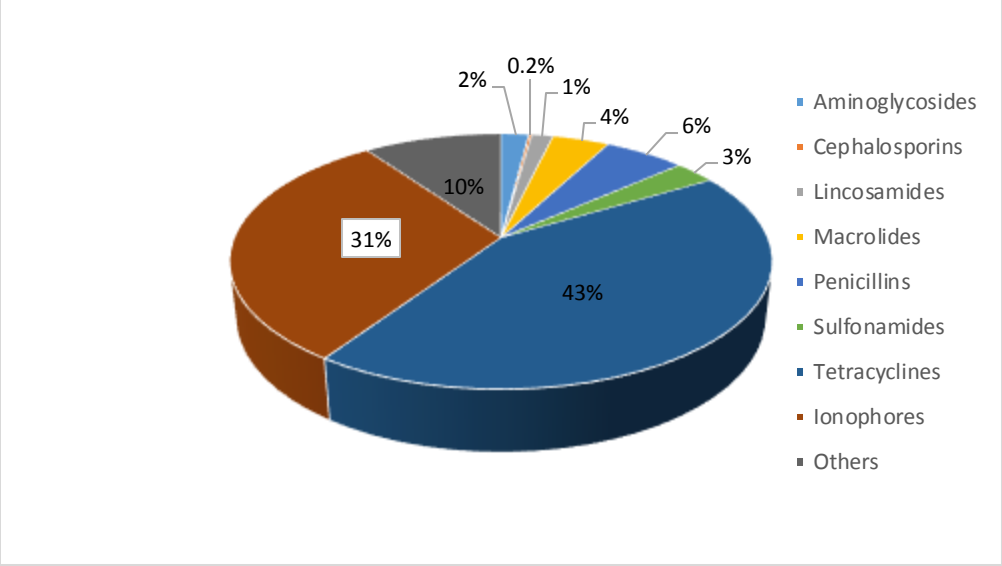

Figure 10: Annual sold antimicrobial drugs in the U.S. 2014.

whereby the main administration routes are via feed and water. (Food and Drug Administration - Department of Health and Human Services, 2015) According to a USDA survey in 1996, 93 % of all pigs in the US received antibiotics during the grower or finisher period, mainly administered by feed. The substances tylosin, chlortetracycline and bacitracin were the most common used substances in swine production, applied for 2-

2.5 months during their production cycle. (online source: USDA, accessed on: 11th November 2016)

Table 2 shows a summary of antimicrobials approved in the United States for the treatment and prevention of diseases as well as for growth promotion. Nearly all compounds are allowed to be administred to hogs for disease prevention as well as for enhancing growth and feed production. (Sarmah, Meyer, & Boxall, 2006b)

The situation in Canada concerning the antimicrobial use in food-animals as in the US, but there are no comprehensive data estimating the antibiotic consumption in food production. (Sarmah et al., 2006b)

Antibiotics	Disease prevention	Growth and feed efficiency	Type of animals
Amoxicillin	Yes	No	Swine
Ampicillin	Yes	No	Swine
Chlortetracycline	Yes	Yes	Swine, beef cattle, chicken
Oxytetracyclin	Yes	Yes	Swine
Penicillin	No	Yes	Swine, chicken, turkeys, quail, pheasant
Tetracycline	Yes	Yes	Swine
Tylosin	Yes	Yes	Swine, beef cattle, chicken
Sulfamethoxypyridazine	Yes	No	Swine
Sulfathiazole	Yes	No	Swine

Table 2: Approved veterinary drugs in the U.S. applied for disease prevention or/and to improve the growth efficiency. Modified according to Sarma et al., 2006b

1.4.2.2. Europe

With reference to the ESVAC project, aiming to collect data on sales of antimicrobial veterinary medicinal products, in 2014 about 9,000 tonnes of antibiotic substances were sold in all 29 European countries, led by Germany, Italy and the Netherlands with 14 %, 16 % and 33 % of the overall sold antimicrobials. The most sold compound groups were the class of tetracyclines (33.4 %) followed by the group of penicillins (25.5 %) and sulfonamides (11.0 %). The remaining 30 % is composed of macrolides, polymyxins, fluorquinolones, lincosamides, trimethoprim, aminoglycosides, pleuromutilins and others. Comparing the proportional total sales of different veterinary antibiotic classes of the last years, there has been no significant change in the sold antibiotic groups. Tetracyclines, penicillins and sulphonamides are the most sold veterinary drug substances in all countries (Figure 11). (European Medicines Agency, 2012, 2016b)

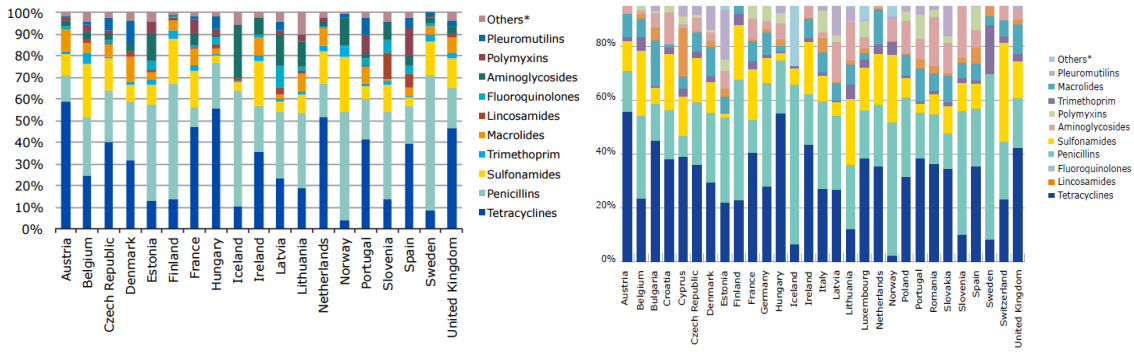

Figure 11: Distribution of sold antimicrobial classes in European countries in 2010 (left) and 2014 (right) in mg/PCU.

1.4.2.3. Africa

Official information about the sale and use of antimicrobial substances in Africa are lacking. (Sarmah et al., 2006b) Nevertheless, Darwish et al. reviewed the problematically existance of antibiotic residues in feedstuff in Africa. The review describes published studies presenting the evalutiation results on antimicrobial use on farms in different African countries. In order to these results

Country	Antibiotic	Foodstuff	Reference
Egypt	Tetracyclines	Chicken meat	Salama *et al.*, 2011
		Bovine carcasses	Morshdy *et al.*, 2013
	β-lactams	Eggs	Khattab *et al.*, 2010
	Cephalosporines	Rabbit meat	AbdEl-Aty *et al.*, 2001
		Rabbit liver	
		Rabbit kidney	
	Macrolides	Milk	Goudah *et al.*, 2007
Sudan	Quinolones	Animal-derived foods	El-tayeb *et al.*, 2012
	Tetracyclines	,,	
Kenya	Tetracyclines	Beef, liver & kidney	Muriuki *et al.*, 2001
	β-lactams	milk	Shitandi and Sternjo, 2001
Ethiopia	Tetracyclines	Meat & edible tissues	Myllyniemi *et al.*, 2000
Tanzania	Tetracyclines	Milk	Kurwijila *et al.*, 2006
		Eggs	Nonga *et al.*, 2010
	Chloramphenicol	,,	
Nigeria	Tetracyclines	Meat	Olufemi and Agboola, 2009
		Eggs	Ezenduka *et al.*, 2011
	Nitrofurans	Animal-derived foods	
	Clhoramphenicol	Eggs	Omeiza *et al.*, 2012
	β-lactams	Cattle meats	Ibrahim *et al.*, 2010
Ghana	Tetracyclines	Milk	Addo *et al.*, 2011
South Africa	Tetracyclines	Milk	Bester and Lombard, 1979

Table 3: Antibiotic residues in various animal-derived foods in African countries. (Sarmah et al., 2006b)

tetracyclines and ß-lactams are the most common used groups of antibiotics in foodstuff. The most affected products are milk, eggs and different kinds of meat, summarized in Table 3. (Darwish, Eldaly, El-abbasy, & Ikenaka, 2013)

1.4.2.4. Asia

China is the biggest producer and consumer of poultry and pig in the world. Due to the lack of available data , the estimated amounts of annually used antibiotics varies between 25,000 tons (Wei, Ge, Huang, Chen, & Wang, 2011) and 110,000 tons (Collignon & Voss, 2015). According to the results of conducting studies, investigating antibiotic residues in water and manure in different provinces in China, it can be assumed which types of antibiotics are mainly applied to livestock. Wei et al. described tetracyclines and sulfonamides as the most detected substances in animal wastewater and surface water around farms in Jiangsu Province. (Wei et al., 2011) Zhao et al. analysed manure samples from pig, chicken and cow farmes of eights provinces of China. The main compounds they found belonged to the group of quinolones and tetracyclines in pig and cow dung, and in chicken dung the detected quinolones and sulfonamides. (Zhao, Dong, & Wang, 2010)

In other developing Asian countries like India, Thailand, Indonesia there is no control of antibiotic use in food animals, consequently there are no data available on the types or amounts of used antimicrobials in food-producing animals. (Sarmah et al., 2006b)

1.4.2.5. Australia and New Zealand

Before the year 2000, a number of antimicrobials were registered in Australia as growth promoters and were available for livestock owners, feed millers and feed mixtures. The use of such growth promoting agents was adapted from the Australian Government after the report of the Joint Expert Technical adivisory committee on antibiotic Resistance (JETA-CAR), by forbidden substances like glycopeptides, amphenicols or gentamicin. (Sarmah et al., 2006b) Although there are no data available about the quantities of antibiotic agents used in animals, in 2015 the Department of Health and the Department of Agriculture released their first National Antimicrobial Resistance

Strategy guide to defend antibiotic misuse and resistance. (Department of Health and Department of Agriculture, 2015)

In New Zealand the data of sold and used antibiotics from 2004 until 2009 were collected from their Ministry of Agriculture and Forestry. In line with these data the amount of sold active substances slightly increased from 50,032 kg in 2004/05 up to 55,809 kg in 2008/09. The main administration routes are via feed, containing oxytetracycline and tylosin, and injections, for principally applying penicillins, tetracyclines, macrolies and aminoglycosides. Beside bacitracin (39%), penicillins (28%), macrolides/lincosamides (9.7%), sulfonamides (9.2%) and tetracyclines (8%) are the the most sold active substances in 2008/09. (Ministry of Agriculture and Forestry of New Zealand, 2010)

1.5. Pharmacokinetic of antibiotics

Pharmacokinetic of pharmacologically active substances describes the movement of these compounds in organisms, independent of their biological activity. For evaluating the interaction between an active substance and a specific organism, different kinds of compartment models have been developed, where the compartments are divided into the ADME (**A**bsorption, **D**istribution, **M**etabolism and **E**xcretion) scheme. Referring to this scheme, the way how a substance entering the blood circulation until its removal, including its dissemination and transformation in the body, can be described for a better understanding of dose-response relationship of substances in different organsims.

1.5.1. ß-lactams

Compounds belonging to the group of ß-lactams possess carboxylic acid groups, confering them a strongly acidic character. Thereby they are completely ionized at the neutral pH of all body fluids, except in the acidic gastric juice. The ionisation prevents the diffusion into the cells, resulting in high plasma concentrations. Concerning the bioavailability, the absorbtion of benzylpenicillin from the GIT is very low (1–2 %), which is explained by its instability in aqueous solutions and the acidic conditions in the gastric. However, stability is improved in basic amino group containing substances like phenoxymethylpenicillin, amoxicillin and ampicillin. After parenteral administration ß-

lactams reach their maximum plasma concentration within 0.25–1h, and they are eliminated in origin form with the urine. Distribution is referred to the tissues, where high concentrations are observed in the kidney and low concentrations occur in fat and muscle tissue. (Wang et al., 2011)

1.5.2. Lincosamides

Lincosamides are able to achieve high concentrations in intracellular fluids as well as in milk, in order to their properties of being lipophilic and weak aliphatic. These compounds are applied by adding them to the drinking water of the livestock or parenterally to pigs, achieving a fast absorption from the GIT but only with a bioavailability between 20–50 %.

Lincomycin is distributed into different tissues, showing high concentrations in liver and kidney, in contrast to low concentrations in muscle and skin. It is eliminated through hepatic metabolism, and approximately 20 % of the administered dose occurs in parent drug form in the urine. (Wang et al., 2011)

1.5.3. Macrolides

The antibiotic group of macrolides are only partially ionized at physiological pHs, due to their weak aliphatic chemistry. Non-ionized molecules get easily absorbed and diffusing into intra- and transcellular fluids. Additionally, they are lipophilic substances, which enables them to enter into milk where they are cleared slowly. For example, tilmicosin was measured at a concentration of 0.8 mg/l milk for 8–9 days after a single sucutaneous dose of 10 mg/kg. (Wang et al., 2011)

1.5.4. Quinolones

In aspect to their chemical structures, quinolones are amphoteric by possesing carboxylic acid as well as basic amino groups, existing as zwitterions at physiological pH. Quinolones are lipophilic, and bioavailability after intramuscular dosing is very high in all species. Oral administration achieves also high bioavailability but this type of administration is not used in ruminants. Fluorquinolones are distributed into lung, liver

and kidney, whereas low concentrations are observed in plasma and elimination route is via kidney. (Wang et al., 2011)

1.5.5. Sulfonamides and Diaminopyrimidines

Sulfonamides are weak organic acids and therefore they are mainly unionized in body fluids. In un-ionized conditions sulfonamides are usually lipophilic. They are easily diffusing into cells and fluids which are more basic than plasma, but reaching fluids below plasma pH, like milk very poor.The lipophilic diaminopyrimidines are in contrast to sulfonamides weak organic bases, which is responsible for ionization at physiological pH enabling them to cross the cell membranes. Absorption rate of sulfonamides is generally well after oral administration, but varying within species and they are distributed into extra- and transcellular fluids. Sulfonamides are either eliminated via urine in parent form or via metabolized products resulting from acetylation of the amino group on the N-4 position of the benzene ring. The acetylated forms tend to become cristallized and can therefore cause crystalluria with damaging the kidneys. Highest tissue concentrations are observed in liver and kidney in food producing species. (Wang et al., 2011)

1.5.6. Tetracyclines

The representatives of the tetracycline group are amphoteric and forming salts with acids and bases. Oxytetracycline and chlortetracycline are moderate lipid soluble, while the synthetic doxycycline is high lipid soluble. After oral administration the bioavailability of tetracyclines is except for doxycycline very low, which could be explained by ionization at pHs within GIT. Further reason could be the complex binding properties with Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{2+} ions within feed. Doxycyclin has additionally a greater affinity to zinc than to calcium, resulting in a decreased bioavailability if zinc is supplemented in the feed. Tetracyclines are mainly formulated for the addition to feed and water and are used in poultry, pigs, fish and cattle. Approximately 40 % of administered dose is eliminated in feces, but this depends on drug and administration route and doxycycline is mainly excreted biliary. Tetracyclines (except of doxycyclin) can also be metabolized to their inactive epimers, which has to be considered during

quantification. Residues of tetracyclines can be bound in bones for months after treatment, possible contaminating therefore meat or bonemeal. Otherwise residues of tetracyclines are mainly detect in liver, kidney and musce in descending row. (Wang et al., 2011)

1.6. Antibiotic residues in the environment and the development of antibiotic resistance

The main growing concern of the intensive use of antimicrobial substances is about the development of a worldwide prevalence of antibiotic resistance. Antibiotics entry into the environment via different routes, whereas farm soil and groundwater are acting as the primary reservoirs of residues. (Tasho & Cho, 2016) Consumed antibiotics from both animals and human, are generally entering the ecosystem through excretion (urine and feces) affecting primarely the aquatic environment. Discharge of effluents from wastewater treatment plants, leaking sewers and manure storage tanks, fertilizing fields with antibiotics containing manure as well as the deployment of antimicrobials in aquacultures are possible sources attaining the aquatic environment (Figure 12). (Carvalho & Santos, 2016)

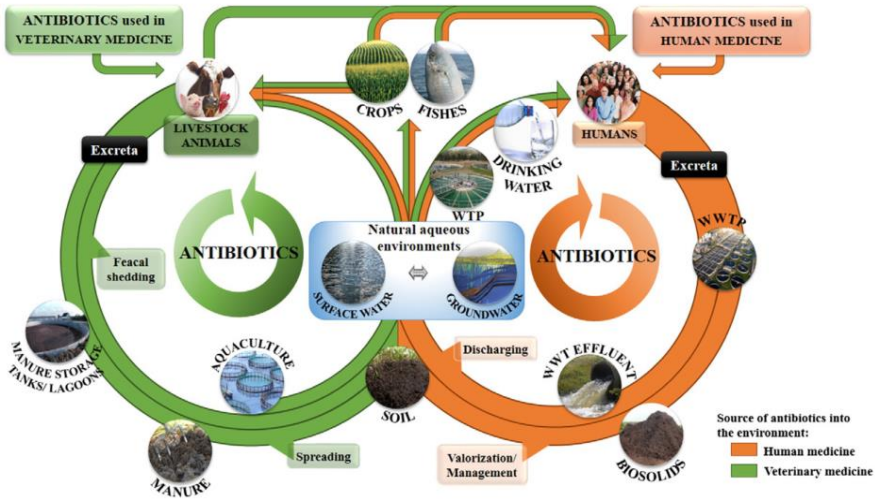

Figure 12: Environmental pathways for distribution of antibiotics in humans and livestock (Carvalho & Santos, 2016).

In Europe intensive investigations for the occurrence of antibiotic residues in different parts of the aquatic environment were conducted during the last years which were reviewed by Carvalho and Santos, 2016. The outcomes of the reviewed studies demonstrate that drug residues were found in the influents and effluents of wastewater treatment plants (WWTPs), river water, groundwater and drinking water in concentrations between ng/l to µg/l. In accordance to the used amount of different antibiotic compounds, the most prevalent detected substances belongs to the

quinolones and sulfonamides including trimethoprim. The highest concentrations were found in WWTPs and low concentrations could be detected in drinking water. (Carvalho & Santos, 2016) Due to the fast and easy hydrolysation of ß-lactams and the ability of tetracyclines to form complexes, this explains the unlikely occurance of these groups in the aquatic environment. (Kemper, 2008) The Austrian Ministry of Health, together with the Environment Agency Austria and the Austrian Agency for Health and Food Service, conducted a monitoring programme about pharmaceuticals and waste water indicators in Austrian ground and drinking water. For antibiotics in ground water they found the substances erythromycin, lincomycin, sulfadimidin, sulfamethoxazol and sulfathiazol from four different places with concentration over limit of detection. The highest amount could be detected for sulfamethoxazole with 21 ng/l. In drinking water positive results showing the substance sulfamethoxazole up to a concentration of 5.6 ng/l on five different places. (Bundesministerium für Gesundheit (BMG), 2015)

Beside the possible antimicrobial contamination of ground- and drinking water resulting from effluents of WWTPs, a further source is the direct application of antibiotics containing manure on the fields as organic soil fertilizer. Approximately 70 % of the used pharmacologically active compounds are excreted unchanged, which let to assume the used amount of active substances when investigated manure samples are showing concentrations of antibiotics in the mg/l range. (Kümmerer, 2009) Christian et al. conducted investigations of collected liquid manure samples from swine and cattles. In contrast to cattle manure, sulfadimidine was found in swine manure at concentration levels of around 1 mg/kg. (Christian et al., 2003) During a screening study on a conventional pig farm in Germany, the application patterns of antibiotics and the occurrence of the applied substances in manure were surveyed. At pig fattening farms the main compound they detected was tetracycline with a mean value of 152 mg/kg dry weight (DW) and a maximum value of 300 mg/kg DW. Chlortetracycline and doxycycline were analyzed with mean values of 26.9 mg/kg DW and 20.3 mg/kg DW, respectively. Comparing with the results from breeding farms, doxycycline was the most frequent detected substance with a mean value of 19.8 mg/kg DW, while tetracycline and oxytetracycline were the substances showing the highest amount of over 200 mg/kg

DW. Among the group of administered sulfonamides they only found sulfadimidine with a maximum of 23 mg/kg DW and sulfadimethoxine at a concentration of 0.5 mg/kg DW. (Widyasari-Mehta, Hartung, & Kreuzig, 2016) Zhoa et al. evaluated the occurrence of antibiotics collected manure samples from swine and cattle farms in China. In all feces samples from the swine farms tetracyclines were detected with maximum conentrations of 97.6 ± 1.7 mg/kg chlortetracycline, 9.3 ± 0.3 mg/kg tetracycline, 2.2 ± 0.09 mg/kg doxycycline and 1.7 ± 0.06 mg/kg oxytetracycline. Sulfonamides and quinolones were only found in low concentrations, whereas lincomycin were nearly analyzed in all samples with a maximum concentration of 17 ± 0.8 mg/kg. In the manure samples from the dairy cattle farms only chlortetraycline could be detected at a high value of 1.5 ± 0.2 mg/kg. (Zhou et al., 2013) Martínez-Carballo et al. investigated manure samples from Austrian pig and chicken/turkey farms on the groups tetracyclines, sulfonamides, trimethoprim and quinolones. Their results for pig manure demonstrated the occurrence of chlortetracycline, oxytetracycline and tetracycline at maximum concentrations of 46 mg/kg, 29 mg/kg and 23 mg/kg, respectively. Among the group of sulfonamides, sulfadimidine could be observed at a concentration up to 20 mg/kg in pig manure and sulfadiazin in chicken and turkey feces at a maximum concentration of 91 mg/kg. Trimethoprim was only detected in chicken and turkey dung up to 17 mg/kg. Chinolones could only be detected in low concentrations in pig manure, whereas in chicken and turkey dung levels up to 8.3 mg/kg enrofloxacin were analyzed. In addition they investigated soil samples previously fertilized with manure, observing significant amounts of chlortetracycline, enrofloxacin and ciprofloxacin. (Martínez-Carballo, González-Barreiro, Scharf, & Gans, 2007) During an 11-months targeted monitoring study for veterinary medicines in the environment, maximum concentrations of lincomycin with 8.5 µg/kg and oxytetracyclin with 305 µg/kg were detected in soil samples after application of slurry. Sulfadiazine and trimethoprim were found at maximum concentrations of 0.8 µg/kg and 0.5 µg/kg, respectively. Samples were analyzed in regular time intervals, founding the highest concentrations for lincomycin, oxytetracycline and sulfadiazine within the first two weeks after slurry application, followed by a degredation within the next two months. Interestingly, the maximum

concentration for trimethoprim was found 28 days after application. (Boxall et al., 2005) Hamscher et al. investigated the different behaviour of sulfonamides and tetracyclines within a 3-year long-term fieldstudy. Their data provide the observation of average tetracycline concentrations of over 150 µg/kg dry matter (DM) in mainly soil samples drawn from 20–30 cm depth, but without evidence that tetracyclines are seeping into deeper soil segments or groundwater. Although sulfamethazine was detected in soil samples in low concentrations (< 2 µg/kg), concentrations above 0.1 µg/l were observed in groundwater samples, assume the leaching of sulfamthatzine into the groundwater. (Hamscher et al., 2005) Data for Austria are available from a conducted study performed from the Environment Agency Austria. They observed in blank soils samples maximum values for residues of erythromycin (35 µg/kg DM) and trimethoprim (6.5 µg/kg DM) after application of manure. (Umweltbundesamt GmbH, 2016) In a former study they found the compounds oxytetracyclin and enrofloxacin with concentrations of 120 µg/kg DM and 5.7 µg/kg DM in fertilized soil samples. (Umweltbundesamt GmbH, 2010)

Due to the presence of signigficant amounts of antibiotic residues in soil through repeated manure application, the uptake of these residues into plants were investigated in severeal studies. Within a greenhouse study they were able to demonstrate the presence of chlortetracycline in corn, green onion and cabbage, grown in antibiotic containing manure treated soil. The measured concentrations were between 2–17 µg/kg fresh weight, increasing with the amount of antibiotics present in the manure. (Kumar, Gupta, Baidoo, Chander, & Rosen, 2005) In a study performed by Kang et al. the uptake of five antibiotics (chlortetracycline, monensin, sulfamethazine, tylosin and virginiamycin) by 11 vegetable crops were investigated, showing an uptake of antibiotics in almost all vegetables after manure treatment. The main found compounds were chlortetracycline, monensin and sulfamethazine but with concentrations generally below LOQ. (Kang et al., 2013)

As described above (see chapter 1.3) bacteria are able to develop resistance against one or more antibiotic agents. The more antibiotics are used in human and veterinary medicine the higher is the possibility to form resistant bacteria. Antibiotic-resistant infections can affect everybody, resulting in difficult, sometimes toxic treatment

options. Resistant bacteria strains find different routes within the environment to spread, as demonstrate in Figure 13.

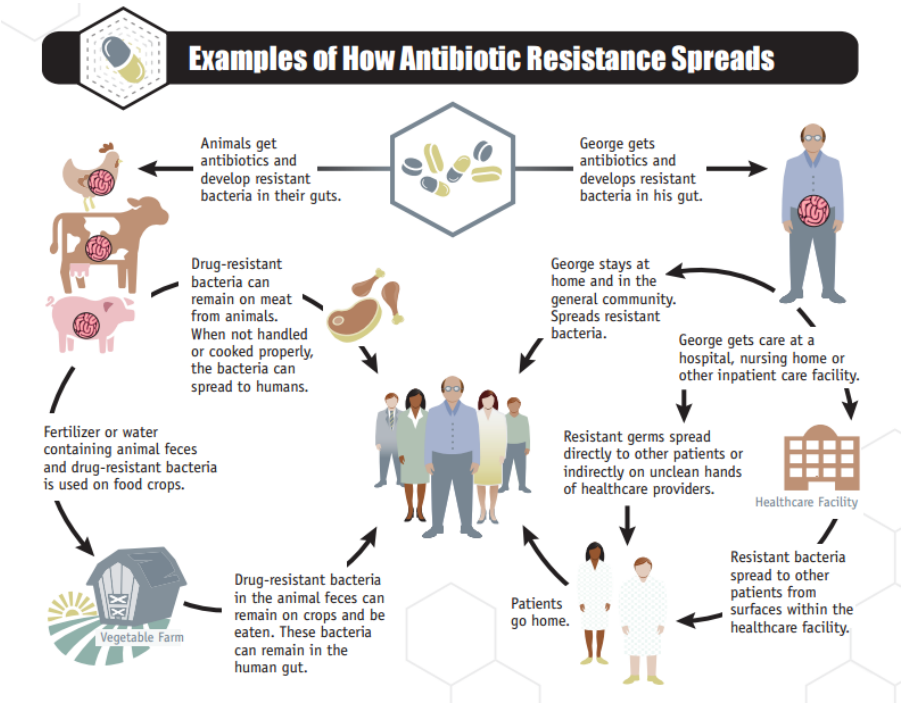

Figure 13: Possible routes of how antibiotic resistant bacteria strains can spread.[3]

In context between antibiotic use in animals and antibiotic resistance in humans, the important resistant strains are the multiple antibiotic-resistant *salmonellae*, macrolide- or quinolone-resistant *campylobacters*, glycopeptide- or streptogramin-resistant *enterococci* and multiple antibiotic-resistant *E. coli*. The main route of transmission for these strains is via food chain. (Phillips et al., 2004) In food occuring resistant *Salmonella* spp. or *Campylobacter* spp. can directly cause infections after ingestion or food handling. *E. coli* and *Enterococcus* spp. are indirect hazards due to transfering resistant genes to a for human pathogenic bacterium. (Newell et al., 2010)

Resistant Salmonella strains are generally occuring in beef, pork, poultry, dairy products and eggs (Figure 14). Strains are transmitted from the animal to the food and subsequently to the human. Resistance depends on the serotype and can be typically observed to those antibiotics which are frequently used in animal livestock. In contrast to the decreasing incidence of resistance to nalidixic acid, ciprofloxacin resistance is increasing (Figure 15). (Newell et al., 2010)

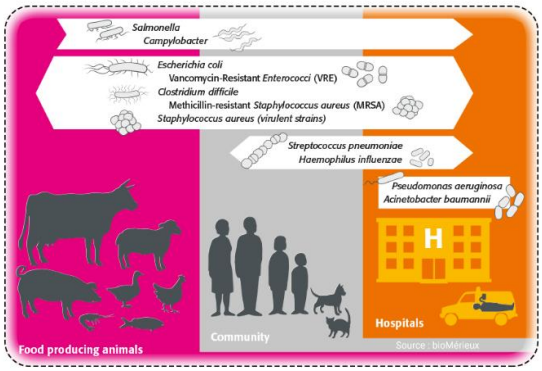

Figure 14: Reservoirs of antibiotic-resistant bacteria.[4]

The most common reservoir for resistant *Campylobacter* strains is poultry meat, whereas strains are mainly resistant against quinolones. First observerations of quinolone resistant *Campylobacter jejuni* infections in humans in the Netherlands was coincided with the introduction of enrofloxacin in poultry therapy. (van den Bogaard & Stobberingh, 2000) *Vancomycin-resistant enterococci* (VRE) is a serious problem, since enterococci are important pathogens of nosocomial infections. Resulting from the former intensive use of the glycopeptide antimicrobial avoparcin as growth promoter, VRE strains can be isolated from animal products as well as from farmers. (Newell et al., 2010)

Spreading of resistance genes *from E. coli* by transferring from animal to human has already been described, whereby the role of food has to be more investigated. In several studies the transfer of resistance genes from E. coli to Salmonella has been demonstrated experimentally in poultry intestinal tract and there are also findings indicating the acquisition of resistance plasmids in the human gut. (Newell et al., 2010)

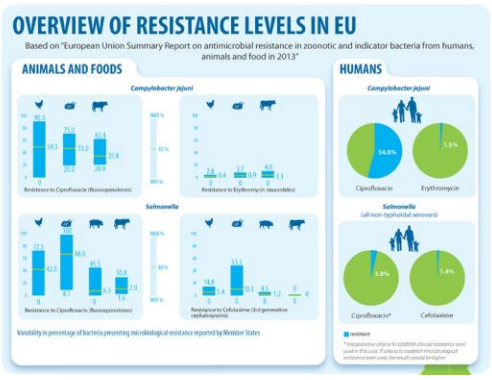

Figure 15: Antimicrobial resistance levels in the European Union.[5]

1.7. Legislation of veterinary drugs and action plans

In the European Union the use of veterinary drugs are regulated in the Council Regulation 37/2010, regulating pharmacologically active substances and their classification regarding MRLs in foodstuffs and animal origin (Figure 16). (The European Commission, 2010). This regulation is based on Regulation (EC) No 470/2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin. (The European Parliament and of the Council, 2009) Table 1 of (EC) No 37/2010 lists all allowed substances including their MRLs in different animal species further differing the target tissues and table 2 includes all prohibited substances for which no MRL has been established. In addition to these Regulation the European Comission released 2010 the Commission Notice 2015/C 299/04 containing guidelines for the prudent use of antimicrobials in veterinary medicine aiming a reduction of unnecessary used antimicrobials and therefore resulting increase of antimicrobial resistance. (The European Commission, 2015)

Pharmacologically active Substance	Marker residue	Animal Species	MRL	Target Tissues	Other Provisions (according to Article 14(7) of Regulation (EC) No 470/2009)	Therapeutic Classification
Enrofloxacin	Sum of enrofloxacin and ciprofloxacin	Bovine, ovine, caprine	100 µg/kg 100 µg/kg 300 µg/kg 200 µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk	For fin fish the muscle MRL relates to 'muscle and skin in natural proportions'. MRLs for fat, liver and kidney do not apply to fin fish. For porcine species the fat MRL relates to 'skin and fat in natural proportions'. Not for use in animals from which eggs are produced for human consumption.	Anti-infectious agents/Antibiotics
		Porcine, rabbit	100 µg/kg 100 µg/kg 200 µg/kg 300 µg/kg	Muscle Fat Liver Kidney		
		Poultry	100 µg/kg 100 µg/kg 200 µg/kg 300 µg/kg	Muscle Skin and fat Liver Kidney		
		All other food producing species	100 µg/kg 100 µg/kg 200 µg/kg 200 µg/kg	Muscle Fat Liver Kidney		

Figure 16: Example from the Council Regulation 37/2010 regulating the MRL for enrofloxacin.

In the United States the US Food and Drug Organisation (FDA) is responsible for setting MRLs of veterinary drug residues in animal derived food stuff. The specific tolerances for residues are established in the "Code of Federal Regulations (CFR)" Title 21 – Chapter

I – subchapter E p part 556. (online source: GPO – U.S. Government Publishing Office, accessed on 09th November 2016)

For reducing the use of antimicrobials and its related increase of antimicrobial resistance, the European Commission started 2001 together with all European Countries a strategy to combat the threat of antimicrobial resistance. This includes the ban of hormones and growth promoting agents in animal feed in January 2006. The first EU Summary Reports on antimicrobial resistance in zoonotic bacteria in animals and food was published in 2010, containing data from the years 2004–2008. In 2015 together with the agencies ECDC and EMA they published a report concluding the use of certain antimicrobials are associated with the occurence of resistance. (online source: EFSA, accessed on: 09th November 2016)

The European Medicine Agency, based on the request from the European Commission developed the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) strategy 2016–2020, aiming to collect information on how antimicrobial medicines are used in animal across the European Union. The collected data are essential for the identification of possible risk factors which could lead to the development and spread of antimicrobial resistance in animals. (European Medicines Agency, 2016a)

1.8. Analytical strategies for determination of veterinary drugs in animal foodstuff

Blasco et al. described the three main problems concerning the determination of veterinary drug residues in animal food stuff:

- the large number of compounds and their metabolites due to biotransformation processes
- the low analytical detection levels (MRLs are mainly set at mg/kg or µg/kg level)
- the composition and influence of the different matrices (Blasco et al., 2007)

For elimination of these problems and achieving satisfying recovery rates of the substances of interest a powerful sample preparation is necessary. The following discussed extraction and clean-up approaches are frequently used in the determination of veterinary drug residues:

- Dilute and Shoot
- QuEChERS – ***Qu****ick* ***E****asy* ***Ch****eap* ***E****ffective* ***R****ugged and* ***S****afe*
- Solid Phase Extraction *(SPE)* (Berendsen & Nielen, 2013)

1.8.1. Dilute and Shoot

This technique offers the simplest sample preparation, aiming to reduce matrix effects by dilution of the sample extracts (Table 4). Extraction of the compounds of interest from the matrix is mainly performed with methanol, acetonitrile or acetone, often acidified with formic or acetic acid. The main disadvantage of this fast and simple extraction strategy is the extensive maintenance of the LC-MS system like intensive column cleaning and regeneration or MS ion source cleaning. (Wang et al., 2011)

Compound groups	Matrix	Extraction solvent	Dilution	Detection system	Ref.
Ma, Qu, SAM, TC, Tr, ß-lactams	Chicken musde	MeOH:H_2O (70:30, v/v) containing EDTA	1:4 with H_2O	UHPLC-MS/MS	(Chico et al., 2008)
Ma, Qu, SAM, TC, ß-lactams	Muscle & kidney	MeOH:H_2O (70:30, v/v) containing EDTA	1:5 with H_2O	LC-MS/MS	(Granelli & Branzell, 2007)
Co, Ni, Ma, Qu, SAM, TC, ß-lactams	Meat, milk, honey, eggs	H_2O/acetonitrile or Acetone with 1 % HCOOH	No dilution – small LC injection volume	UHPLC-MS/MS	(Mol, Plaza-Bolanos, Zomer, de Rijk, & Stolker, 2008)

Table 4: Examples of applications using "Dilute and Shoot" to extract veterinary drug compounds. *Co=Coccidiostats, Ni=Nitroimidazoles, Ma=Macrolides, Qu=Quinolones, SAM=Sulphonamides, TC=Tetracyclines, Tr=Trimethoprim.*

1.8.2. QuEChERS - Quick Easy Cheap Effective Rugged and Safe

QuEChERS sample preparation is based on liquid-liquid extraction procedure and was originally developed for the analyses of hundreds of pesticides in fruits and vegetable samples. (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003) The extraction is performed by adding organic solvent (acetonitrile, ethylacetate, or acetone) to the sample and due to the addition of salts (anhydrous $MgSO_4$, Na_2SO_4, NaCl, and/or buffering agents) the extract is separated into a polar H_2O and a less polar solvent phase. The analytes of interest migrates into the organic phase whereas possible matrix interferences go into the aqueous phase. For determination of veterinary drug residues QuEChERS extraction method has been modified by different working groups (Table 5). (Wang et al., 2011)

Compound groups	Matrix	Extraction solvent and clean-up salts	Further clean up	Detection system	Ref.
A, Av, B, Ma, Qu, SAM, TC	Milk	1 % acetic acid in ACN + 0.1 M Na_2EDTA 4 g $MgSO_4$ 1 g NaOAc	Dilution (1:1) Filtration	UHPLC-MS/MS	(Aguilera-Luiz, Vidal, Romero-González, & Frenich, 2008)
Co, Ni, Qu, SAM	Chicken muscle	1 % acetic acid in ACN 5 g Na_2SO_4 500 mg Bondesil NH_2	Evaporation Filtration	LC-MS/MS	(Stubbings & Bigwood, 2009)
A, Ma, Qu, SAM, TC	Eggs	1 % acetic acid in ACN + 0.1 M Na_2EDTA 4 g $MgSO_4$ 1 g NaOAc	Dilution (1:1) Filtration	UHPLC-MS/MS	(Frenich, del Mar Aguilera-Luiz, Vidal, & Romero-González, 2010)

Table 5: Examples of applications using QuEChERS based methods to extract veterinary drug compounds.
A=Anthelmintics, Av=Avermectins, B=Benzimidazoles, Co=Coccidiostats, Ma=Macrolides, Ni=Nitroimidazoles, Qu=Quinolones, SAM=Sulphonamides, TC=Tetracyclines, Tr=Trimethoprim

1.8.3. Solid Phase Extraction – SPE

Solid phase extraction (SPE) is the most common clean-up technique for the simultaneous analysis of different veterinary drug classes in animal derived products. The use of SPE technique is characterized by significant reduction of matrix effects. There are a lot of different sorbents (e.g. C8, C18, NH_2) with different retention mechanisms (e.g. reverse phase, normal phase, weak/strong cation/anion exchange). The choice of interaction mechanism is limited due to the different chemical and physical properties of the compounds of interest. In **Table 6** applications using SPE for the determination of veterinary drug residues are summarized. (Berendsen & Nielen, 2013)

Compound groups	Matrix	Extraction solvent	SPE cartridge	Further clean-up	Detection system	Ref.
Li, Ma, Qu, SAM, TC, Tr	Milk	100 µl TCA (20%) 8 ml MI-buffer Second extraction with 6 ml MI-buffer	Oasis HLB (200 mg, 6 ml) =reverse phase	Evaporation Filtration	UHPLC-MS/MS	(Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), 2011)
Amp, Ma, Qu, SAM, Sulfons, TC,	Honey	5 ml HCl (2 M) 30 ml H_2O 5 ml n-hexane (twice)	StrataX-C (200 mg, 6 ml) =strong cation exchange	Evaporation Filtration	HPLC-MS/MS	(Galarini, Saluti, Giusepponi, Rossi, & Moretti, 2015)
ß-lactams, Qu, SAM, TC	Eggs	18 ml sodium-succinate buffer	Oasis HLB (60 mg, 3 ml)	Evaporation Filtration	LC-MS/MS	(Heller, Nochetto,Rummel, & Thomas, 2006)

Table 6: Examples of applications using SPE sample clean-up for analysis of veterinary drug compounds. *Amp=Amphenicols, Li=Lincosamides, Ma=macrolides, Qu=Quinolones, SAM=sulphonamides, TC=Tetracyclines, Tr=Trimethoprim, MI-buffer=McIlvaine buffer consisting of 0.1 M citric acid-hydrate-solution/0.2 M Na_2EDTA-dihydrate-solution (60/40, v/v)*

1.9. Quantification of the analytes using an HPLC-MS/MS system

High performance liquid chromatography (HPLC) – mass spectrometry combines the physical separation with the mass analysis of analytes providing a powerful technique with high sensitivity. By using this analytical approach, the criteria for a confirmatory method which are demand by European Commission Decision 2002/657/EC can be fulfilled.

1.9.1. Chromatographic separation by HPLC

"Chromatography" is defined as the separation process of analytes, based on two non-miscible phases – the solid stationary phase and the liquid (in case of liquid chromatography) mobile phase. The analytes must be soluble in liquids and are separated in order to their chemical properties and their different interactions with the chromatographic phases.

There are a lot of different stationary phases with different properties, but fundamentally there are two types of HPLC:

- *Normal phase (NP) – HPLC:* this type of separation describes the use of a polar stationary phase combined with an apolar mobile phase. The higher the polarity of the analytes the longer they are getting retained on the stationary phase. By using a polar mobile phase the analytes are eluted from the stationary phase.

- *Reversed phase (RP) – HPLC:* reversed phase chromatography uses an apolar stationary phase together with a polar mobile phase to achieve retention of apolar substances. Separation mechanism is performed by different interactions (hydrophobic, polar, ionic or steric), which can be solved by using an apolar solvent to elute the analytes from the stationary phase.

1.9.2. Chromatographic resolution

The most important factor in chromatography is aiming to obtain the optimum resolution of individual compounds in minimum time. A resolution value of at least 1.5 between two peaks describes the (baseline) separation of two compounds enabling the accurate measurement of each peak area or height Figure 17. Calculation of chromatographic resolution (R_s) is performed by the following equation:

$$R_s = \left(\frac{RT_B - RT_A}{0.5\ (W_A + W_{B)}} \right)$$

RT_A...retention time substance A
RT_B...retention time substance B
W_A ... peak width at baseline substance A
W_B ... peak width at baseline substance B

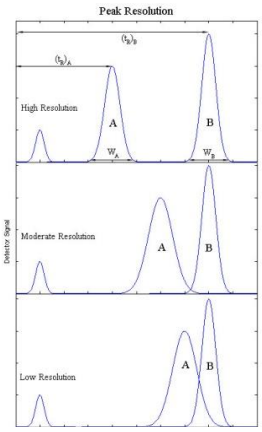

Figure 17: Examples of chromatographic resolutions.[6]

According to the "Fundamental Resolution Equation" chromatographic resolution is affected by three important parameters – ***Efficiency (N), Selectivity (α) and Retention (k)***. These parameters can be influenced to control the resolution obtained from a chromatographic separation, whereas the most effective changes can be obtained by altering the selectivity (Figure 18).

$$R_s = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k}{k+1} \right)$$

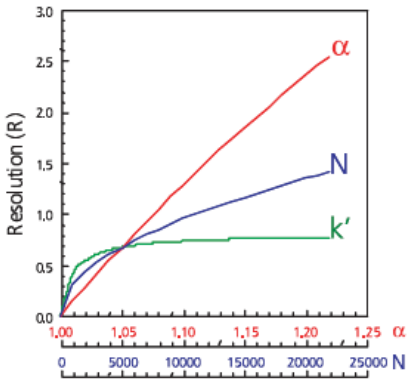

Figure 18: Important parameters on chromatographic resolution.[7]

1.9.2.1. *Efficiency (N)*

The efficiency of a chromatographic peak describes the dispersion of the analyte band as it diffuses through the HPLC system and column. This parameter is therefore influenced from the plate number (N) of a chromatographic column and indicates its performance. Efficiency can be calculated as follows:

$$N = 5.54 \left(\frac{t_r}{w_{\frac{1}{2}}} \right)^2$$

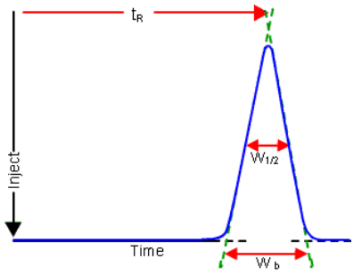

Figure 19: Peak width.[13]

A higher plate number results in better separation due to the increasing number of analyte equilibrations between stationary and mobile phase. Columns with a high plate number providing sharp peak shapes, whereas peaks from columns with a low plate number are wide and decreased in their quality (Figure 19). This decrease can be explained by the Van-Deemter-equation , involving the three main factors leading to this quality lost:

$$HETP = A\left(d_p\right) + \frac{B}{\mu} + C\left({d_e}^2\right)\mu$$

multiple paths

longitudinal diffusion (flow rate)

mass transfer

HETP = height equivalent to a theoretical plate, a measure of the resolving power of the column [m]

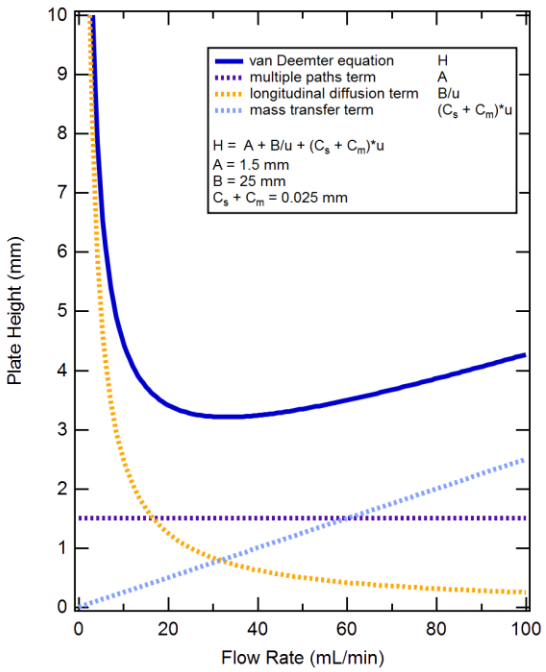

Figure 20: Van Deemter equation.[11]

The first term of this equation expresses the plate number of the column. The smaller the particle size, the higher is the efficiency due to the increasing number of plates. But with the increase of particles within the column the pressure of the liquid mobile phase increases. Another possibility to increase the plate number is to use a longer column, showing for a given particle size a direct proportional relation to the column length, however pressure and running time are increasing direct proportional, too (Figure 20). By changing the efficiency through using columns with other particle sizes and lenghts, the chromatographic resolution improves by a multiple factor of 1.4 in order of duplicating the plate numbers (Figure 21).

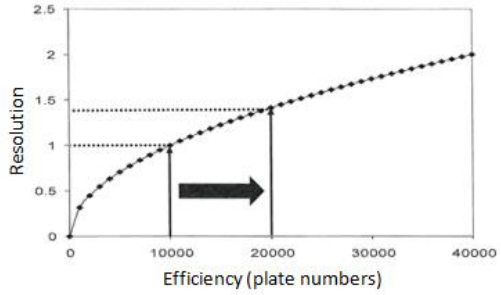

Figure 21: Influence of the efficiency to the resolution. Modified according to [12].

1.9.2.2. Selectivity (α)

The term selectivity or separation factor describes the ability of a chromatographic system to distinguish between analytical compounds in order to their different chemical interactions with the stationary phase. The selectivity between two analytes of interest can be expressed and visualized with the following equation (Figure 22):

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

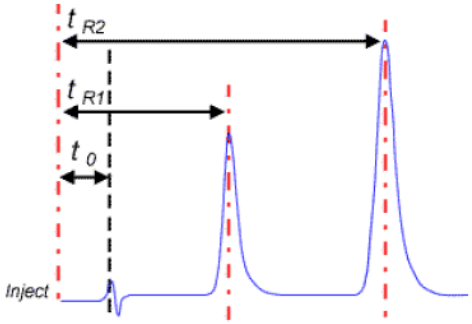

Figure 22: Selectivity of two analytes.[13]

For a powerful separation, the value for selectivity must always be greater than one. The greater the selectivity, the greater is the distance between the two peak apices. In case of α is equal to one, both analytes are co-eluting showing no separation of their peaks. Selectivity can be altered by changing the stationary phase as well as the composition of the mobile phase (Figure 23), or by changing the column temperature.

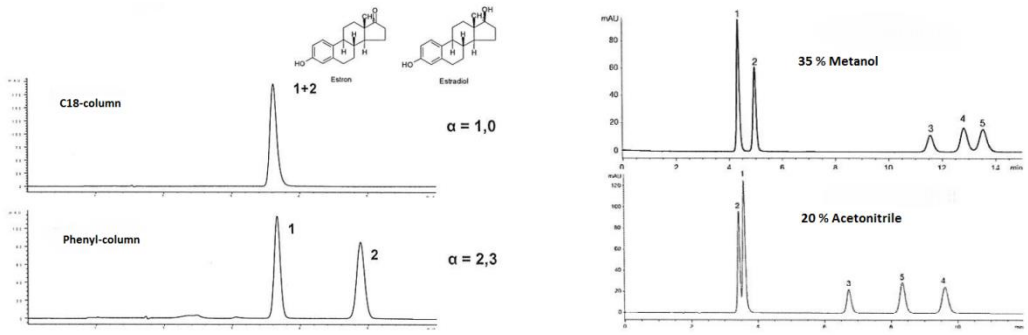

Figure 23: Influence of the stationary phase (left) and mobile phase (right) on the selectivity.[12]

1.9.2.3. Retention factor (k)

The retention factor *k* describes the ratio of retention time of the analyte on the column compared to the retention time of a non-retained comound. The non-retained compound explains the "dead-time" of the column, meaning that all analytes eluting before this substance have no affinity to the stationary phase. The retention factor value should be between 1 and 10 to achieve a good separation. High k values indicate a high retention, whereas too high values resulting in broad peaks and decreased efficiency (Figure 24). For improving the retention factor alterations of the strong solvent in the mobile phase is the most effective way (Figure 25).

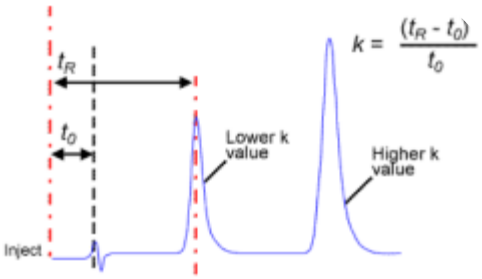

Figure 24: Influence of different k values on the retention time.[13]

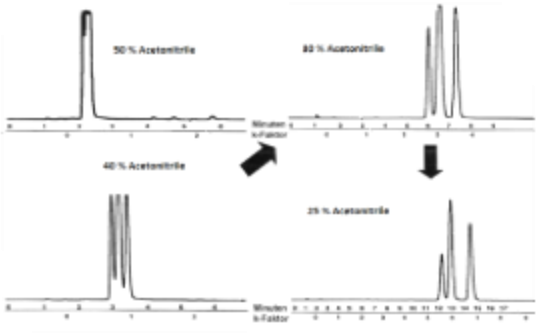

Figure 25: Influence of mobile phase composition on the k value.

1.10. Analytical detection by tandem mass-spectrometry

The principle work of tandem mass-spectrometry, or also known as MS/MS or MS^2, is to form ions and break them down into selected precursor ions and their fragmented product ions. Ions are formed by using different types of ionization sources

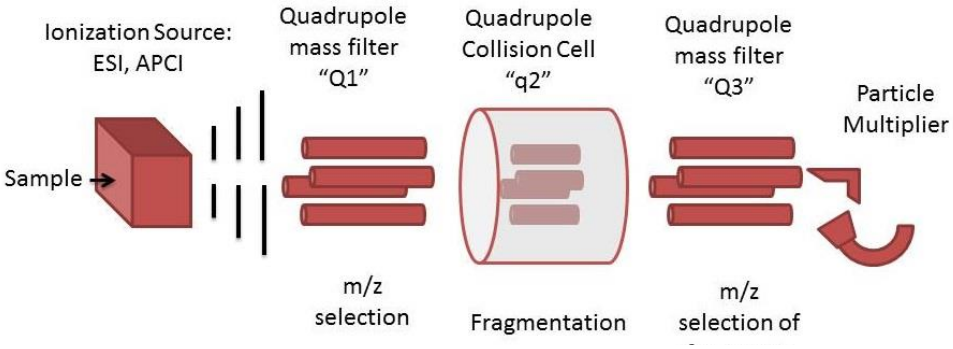

Figure 26: Scheme of a triple quadrupole mass spectrometer.[8]

(e.g. electrospray ionisation, electron impact, chemical ionisation etc.) after transferring the molecules into the gas phase. In tandem mass-spectrometry two mass analyzers are coupled, whereas in the first stage (MS1) precursor ions of a specific mass-to-charge ratio (*m/z*) are selected. After fragmentation the resulting product ions are selected in the second mass analyzer (MS2) in accordance to their *m/z* values. For selecting the specific ions and fragments the most used technique is the use of quadrupole mass filters, mainly consisting of three quadrupoles which are lined up in a row (Figure 26).

Quadrupoles are consisting of four cylindrical rods, organized parallel to each other (Figure 27). Two opposite rods are applying opposite charges generating therefore an oscillating electric field within the quadrupole. In order to the stability of their trajectories within these electric fields, ions are getting separated by following their flight path.

The second quadrupole, which is located between mass filter one and three acts as the collision cell, is responsible for ion fragmentation, occuring in the presence of an inert gas (e.g. Ar, He or N_2). Ions are fragmented into their typical product or daughter ions and are further transferred into the third quadrupole (MS2).

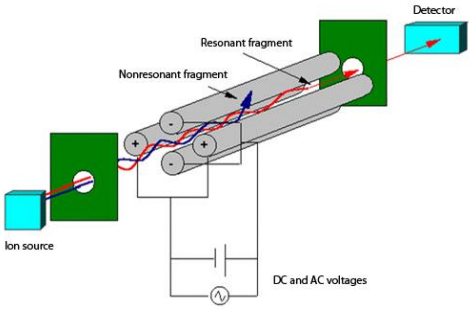

Figure 27: Schematic of a quadrupole filter.[9]

After selecting the fragments of interest in this mass filter, they are sent to the detector. The mainly used detector which is used in triple-quadropole technology is the highly sensitive electron multiplier detector.

For this detection type the analytes have to be converted into electrons by using a conversion dynode. Signal amplifaction is performed by a cascade of dynodes, which are accelerating the electrons up to a speed enables them to generate more electrons before hitting the next dynode (Figure 28).

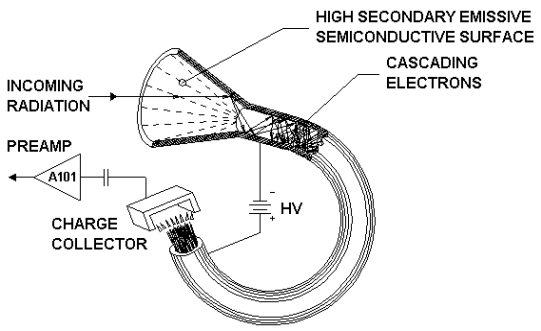

Figure 28: Schematic of an electron multiplier.[10]

By using the technique of triple quadrupole mass spectrometry, measurements with high sensitivity can be performed within short time. Beside the information about mass-to-charge ratio, it allows to obtain structural information by the unique fragmentation pattern of each molecule.

2. MATERIALS AND METHODS

2.1. Reagents

2.1.1. Chemicals

- **Acetone min**. 99,70 % (C3H6O) –VWR Chemical (Fontenay-sous-Bois, France)
- **Acetonitrile HPLC grade** (H3CCN) –VWR Chemical (Fontenay-sous-Bois, France)
- **Methanol HPLC grade** (CH3OH) – Chem-Lab NV (Zedelgem, Belgium)
- **Water for LC/MS – Milli-Q®**; Milli-Q water purification system; 0.22 µm; Lot: F4CA66816; Merck Millipore (Darmstadt, Germany)
- **Ammonium formate (HCO_2NH_4)** for HPLC ≥ 99.0% – Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Citric acid monohydrate ($HOC(COOH)(CH_2COOH)_2 \cdot H_2O$)** ≥ 99.0% - Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Disodium hydrogen phosphate (Na_2HPO_4)** - Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Disodium ethylenediaminetetraacetate dihydrate (Na_2EDTA)** - Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Formic acid 99-100 % (CH_2O_2)** - VWR Chemical (Fontenay-sous-Bois, France)
- **Hydrochloric acid 25%** - Merck Chemicals GmbH (Darmstadt, Germany)
- **Chloroform** - Merck Chemicals GmbH (Darmstadt, Germany)
- **Dimethylsulfoxid (DMSO)** - Merck Chemicals GmbH (Darmstadt, Germany)
- **Phosphoric acid ≥ 85%** - Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **TCA (20%)**- Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **C18 Endcapped bulk sorbent –** Agilent Technologies (Waldbronn, Germany)
- **BEKOlut® Citrate-Kit-01 –** buffered citrate salts, BEKOlut GmbH & Co KG, Hauptstuhl, Germany
- **BEKOlut® Citrate-Kit-02 – $PSA/MgSO_4$ -** BEKOlut GmbH & Co KG, Hauptstuhl, Germany

- **Supel™QuE – Zsep Tube**, 500 mg ZSep - Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- **Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$) -** Merck Chemicals GmbH (Darmstadt, Germany)
- **Sodium sulfate anhydrous (Na_2SO_4) -** Merck Chemicals GmbH (Darmstadt, Germany)

2.1.2. Solutions

- **Na2EDTA-McIlvaine buffer solution (0.1 M, pH 4.0):** 12.6 g citric acid monohydrate, 14.24 g Na_2HPO_4 and 37.2 g Na_2EDTA were dissolved in 1 l Milli-Q water and placed in an ultrasonic bath until complete dissolution

- **Mobile phase A (H_2O, 5 mM ammoniumformate, 0.1 % HCOOH):** was prepared by dissolving 0.3153 g ammoniumformate in 1 l Milli-Q water with 1 mL HCOOH

- **Mobile phase B (MeOH, 5 mM ammoniumformate, 0.1 % HCOOH):** was prepared by dissolving 0.3153 g ammoniumformate in 1 l MeOH with 1 mL HCOOH

- **Initial mobile phase solution (95% mobile phase A/5% mobile phase B):** was prepared by mixing 950 mL of mobile phase A with 50 mL of mobile phase B.

2.2. Materials

2.2.1. Equipment

- **Agilent Technologies LC-QQQ-MS liquid-chromatograph**

  1290 Infinity UHPLC combined with a 6490 Triple Quadrupole Mass Spectrometer; Model: G6490A; (Waldbronn, Germany)

- **1290 sampler; Model: G4226A; Serial: DEBAP02121**
- **1290 Bin Pump; Model: G4220A; Serial: DEBAA02564**
- **1260 Iso Pump; Model: G1310B; Serial: DEAB903902**
- **ALSTherm; Model: G1330A; Serial: DE82203645**
- **1290 TCC; Model: G1316C; Serial: DEBAC02955**

- **Agilent Technologies RRHD-column Zorbax Eclipse Plus C18 2.1*100mm; 1.8 µm**

- **Agilent MassHunter workstation software**

  Quantitative Analysis (B.07.00)

  Qualitative Analysis (B.07.00)

- **Centrifuge 5430**; max. speed: 17,500^{-1}, Serial: 5427AL013297; Eppendorf AG (Hamburg, Germany)

- **Collomix**; Type: VIBA 300; Serial: 892014; Rühr- und Mischgeräte GmbH (Gaimersheim, Germany)

- **Grindomix**; Type: GM200; Serial: 129240218G; Retsch GmbH (Haan, Germany)

- **Sartorius laboratory scale**; max. 820 g, d = 0.01 g; Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)

- **Sartorius laboratory scale**; max. 250 g, d = 0.01 g; Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)

- **Polytetrafluorethylene (PTFE) membrane filter**; diameter 0.45 µm; Sartorius Lab Instruments GmbH & Co KG (Goettingen, Germany)

- **Centrifugation tubes**, volume 50 ml and 15 ml; PP; Eppendorf AG (Hamburg, Germany)

- **Sonorex ultrasonic bath**; Bandelin electronic (Berlin, Germany)

- **Shaker**; max. Speed: 2,500 rpm; REAX control; Heidolph (Schwabach, Germany)

- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 20 – 200 µl; incl. epT.I.P.S®-box; yellow; Eppendorf AG (Hamburg, Germany)

- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 100 – 1,000 µl; incl. epT.I.P.S®-box; blue; Eppendorf AG (Hamburg, Germany)

- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 0.5 – 5 ml; incl. epT.I.P.S®-sample bags; purple; Eppendorf AG (Hamburg, Germany)

- **Piston stroke pipette;** Eppendorf Research® plus; single channel; variable; 1 – 10 ml; incl. epT.I.P.S®-sample bags; green; Eppendorf AG (Hamburg, Germany)

- **Nitrogen evaporator;** TurboVap® LV Concentration Evaporator; Automated Evaporation System; Biotage (Uppsala, Finland)

- **QuEChERS dSPE EMR – Lipid;** Agilent Technologies; (Waldbronn, Germany)

- **Strata-X – Polymeric Reversed Phase SPE cartridges**, 200mg/6ml, Phenomenex Inc. (Aschaffenburg, Germany)

Miscellaneous: beaker glass, bulkhead bottle, ground-glass stoppers, hopper, chromatographic sample vials, spatula, volumetric flask, pasteur pipettes

2.3. Solid Standard Substances

Compound name	Empirical Formula	CAS-no.	Lot-no.	Exp. Date	Producer
Amoxicillin trihydrate	$C_{16}H_{19}N_3O_5S \cdot 3H_2O$	61336-70-7	SZBB264XV	09/2016	Sigma-Aldrich
Ampicillin trihydrate	$C_{16}H_{19}N_3O_4S \cdot 3H_2O$	7177-48-2	SZBA083XV	03/2015	Sigma-Aldrich
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	80370-57-6	SZBC270XV	09/2016	Sigma-Aldrich
Chlortetracycline hydrochloride	$C_{22}H_{23}ClN_2O_8 \cdot HCl$	64-72-2	SZBB129XV	05/2016	Sigma-Aldrich
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	85721-33-1	SZBA347XV	12/2015	Sigma-Aldrich
Cloxacillin sodium salt monohydrate	$C_{19}H_{17}ClN_3NaO_5S \cdot H_2O$	7081-44-9	SZBC109XV	04/2017	Sigma-Aldrich
Danofloxacin	$C_{19}H_{20}FN_3O_3$	112398-08-0	SZBA019XV	05/2015	Sigma-Aldrich
Doxycycline hyclate	$C_{22}H_{24}N_2O_8 \cdot HCl \cdot 0.5H_2O \cdot 0.5C_2H_6O$	24390-14-5	SZBA203XV	07/2014	Sigma-Aldrich
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	93106-60-6	SZBA336XV	12/2014	Sigma-Aldrich
Erythromycin A dihydrate	$C_{37}H_{67}NO_{13} \cdot 2H_2O$	59319-72-1	SZBD105XV	05/2016	Sigma-Aldrich
Flumequine	$C_{14}H_{12}FNO_3$	42835-25-6	SZBA014XV	01/2015	Sigma-Aldrich
Lincomycin hydrochloride monohydrate	$C_{18}H_{34}N_2O_6S \cdot HCl \cdot H_2O$	7179-49-9	SZB8329XV	11/2014	Sigma-Aldrich
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	115550-35-1	SZBC248XV	09/2015	Sigma-Aldrich
Oxytetracycline hydrochloride	$C_{22}H_{24}N_2O_9 \cdot HCl$	2058-46-0	SZB9287XV	10/2014	Sigma-Aldrich
Penicillin G potassium salt	$C_{16}H_{17}KN_2O_4S$	113-98-4	SZBD003XV	01/2018	Sigma-Aldrich

Penicillin V potassium salt	$C_{16}H_{17}N_2O_5SK$	132-98-9	SZBD101XV	04/2018	Sigma-Aldrich
Pirlimycin hydrochloride	$C_{17}H_{31}ClN_2O_5S$	Ready-made solution:	Pirsue™, 5mg/ml	11/2015	Pfizer
Spiramycin	$C_{43}H_{74}N_2O_{14}$	8025-81-8	071M1865V	08/2014	Sigma-Aldrich
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	68-35-9	SZBB137XV	05/2016	Sigma-Aldrich
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	122-11-2	SZBB028XV	01/2016	Sigma-Aldrich
Sulfadoxin	$C_{12}H_{14}N_4O_4S$	2447-57-6	SZBB090XV	03/2016	Sigma-Aldrich
Sulfamerazine	$C_{11}H_{12}N_4O_2S$	127-79-7	SZBA309XV	11/2015	Sigma-Aldrich
Sulfamethazine	$C_{12}H_{14}N_4O_2S$	57-68-1	SZBB193XV	07/2016	Sigma-Aldrich
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	723-46-6	SZBC066XV	03/2017	Sigma-Aldrich
Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	80-35-3	SLBD6074V	12/2013	Sigma-Aldrich
Sulfathiazole	$C_9H_9N_3O_2S_2$	72-14-0	SZBB294XV	10/2016	Sigma-Aldrich
Tetracycline hydrochloride	$C22H24N2O8 \cdot HCl$	64-75-5	SZBA140XV	05/2015	Sigma-Aldrich
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	108050-54-0	SZBA088XV	03/2015	Sigma-Aldrich
Trimethoprim	$C_{14}H_{18}N_4O_3$	738-70-5	SZB9352XV	12/2014	Sigma-Aldrich
Tylosin tartrate	$C_{46}H_{77}NO_{17} \cdot C_4H_6O_6$	74610-55-2	SZBC271XV	09/2015	Sigma-Aldrich

Table 7: Solid Standard substances

2.4. Internal standard substances

Compound name	Empirical Formula	CAS-no.	Lot-no.	Exp. Date	Producer
Ciprofloxacin-d8 hydrochloride hydrate	$C_{17}D_8H_{10}FN_3O_3 \cdot HCl \cdot xH_2O$	1216659-54-9 (anhydrous)	SZBE094XV	04/2017	Sigma Aldrich
Danofloxacin-(methyl-d3)	$C_{19}D_3H_{17}FN_3O_3$		SZBD274XV	10/2016	Sigma Aldrich
Demeclocycline hydrochloride hydrate	$C_{21}H_{21}ClN_2O_8 \cdot HCl \cdot xH_2O$	64-73-3 (anhydrous)	SZBD101XV	04/2018	Sigma Aldrich
Doxycycline-d3 hyclate			24-GHZ-100-1		TRC
Enrofloxacin-d5 hydrochloride	$C_{19}D_5H_{17}FN_3O_3 \cdot HCl$		SZBE297XV	10/2017	Sigma Aldrich
Erythromycin-13C- d3					TRC
Flumequine-1,2 carboxy-(13C3)	$^{13}C_3C_{11}H_{12}FNO_3$	1185049-09-5	SZBE174XV	06/2017	Sigma-Aldrich
Lincomycin-d3			4-CWA-72-2		TRC
Marbofloxacin-d8			5-LIU-159-4		TRC
Penicillin G- d7-N-ethylpiperidinium salt	$C_{23}D_7H_{26}N_3O_4S$	1217445-37-8	SZBE300XV	10/2017	Sigma-Aldrich
Spiramycin I-d3			2-HTW-117-4		
Sulfadiazine-(phenyl-13C6)	$^{13}C_6C_4H_{10}N_4O_2S$	1189426-16-1	SZBE310XV	11/2017	Sigma-Aldrich

Sulfadimethoxine- d6	$C_{12}D_6H_8N_4O_4S$	73068-02-7	SZBD063XV	03/2016	Sigma-Aldrich
Sulfadoxin-d3	$C_{12}D_3H_{11}N_4O_4S$	1262770-70-6	SZBC173XV	06/2015	Sigma-Aldrich
Sulfamerazine-(phenyl-13C6)	$^{13}C_6C_5H_{12}N_4O_2S$	1196157-80-8	SZBD003XV	01/2016	Sigma-Aldrich
Sulfamethazine-(phenyl- 13C6) hemihydrates	$^{13}C_6C_6H_{14}N_4O_2S \cdot 0.5H_2O$	1196157-77-3	SZBE310XV	11/2017	Sigma-Aldrich
Sulfamethoxazole -phenyl-13C6	$^{13}C_6C_4H_{11}N_3O_3S$	1196157-90-0	SZBE016XV	01/2017	Sigma-Aldrich
Sulfamethoxypyridazine- d3	$C_{11}D_3H_9N_4O_3S$	1172846-03-5	SZBE113XV	04/2017	Sigma-Aldrich
Sulfathiazole-(phenyl-13C6)	$^{13}C_6C_3H_9N_3O_2S_2$	1196157-72-8	SZBE094XV	04/2017	Sigma-Aldrich
Trimethoprim-d9	$C_{14}D_9H_9N_4O_3$	1189460-62-5	SZBE141XV	05/2017	Sigma-Aldrich

Table 8: Internal Standard substances

2.5. Standard Working Solutions

Before mixing the standard working solutions for spiking the samples as well as for calibration, a stock solution of each solid standard substance must be prepared. These stock solutions were prepared at a concentration of 1000 mg/l of each compound by exactly weighing and dissolving in their individual solvent solution and stored at -18 °C in the dark. The compounds tylosin, tilmicosin and spiramycin are soluble in ACN, the ß-lactams in H_2O:ACN (50:50), the sulphonamides, ceftiofur and ciprofloxacin in MeOH and the remaining quinolones in alcalized MeOH hydroxide.

To prepare the working antibiotic mix standard solution five minimixes (quinolone-mix; macrolide/lincosamide-mix; ß-lactam-mix; sulphonamide-mix; tetracycline-mix) were previously mixed according to their substance groups and subsequently 100 µl of each minimix were diluted in 10 mL mobile phase initial conditions (95% mobile phase A, 5 % mobile phase B). The concentration of the antibiotic-mix standard ranged between 20 – 400 µg/L (Table 9).

Compound	**Minimix**	**Concentration antibiotic mix-standard (µg/l)**
Ciprofloxacin	QU MIX	100
Danofloxacin	QU MIX	60
Enrofloxacin	QU MIX	100
Flumequine	QU MIX	100
Marbofloxacin	QU MIX	150
Erythromycin A dihydrate	MALI MIX	80
Lincomycin hydrochloride monohydrate	MALI MIX	300
Pirlimycin Hydrochloride	MALI MIX	200
Spiramycin	MALI MIX	400
Tilmicosin	MALI MIX	100
Tylosin tartrate	MALI MIX	100

Amoxicillin trihydrate	PEN MIX	80
Ampicillin trihydrate	PEN MIX	80
Ceftiofur	PEN MIX	200
Cloxacillin sodium salt monohydrate	PEN MIX	60
Penicillin G potassium salt	PEN MIX	40
Penicillin V potassium salt	PEN MIX	100
Sulfadiazin	SAM MIX	20
Sulfadimethoxine	SAM MIX	20
Sulfadimidin = Sulfamethazine	SAM MIX	20
Sulfadoxin	SAM MIX	20
Sulfamerazine	SAM MIX	20
Sulfamethoxazole	SAM MIX	20
Sulfamethoxypyridazine	SAM MIX	20
Sulfanilamide	SAM MIX	20
Sulfathiazole	SAM MIX	20
Trimethoprim	SAM MIX	100
Chlortetracycline hydrochloride	TC MIX	200
Doxycyclin hyclate	TC MIX	200
Oxytetracycline hydrochloride	TC MIX	200
Tetracycline hydrochloride	TC MIX	200

Table 9: Antibiotic-mix standard. *QU...quinolones, MALI...macrolides/lincosamides, PEN...penicillins/ß-lactams, SAM...sulphonamides, TC...tetracyclines.*

For establishing a standard straight calibration of all substances the previous described working antibiotic mix standard solution was diluted according to the following scheme (Table 10), to obtain different concentration levels. All levels are diluted in 10 ml initial mobile phase conditions and transferred into chromatographic vials and stored at -18°C.

Compound	L1 [µg/l]	L2 [µg/l]	L3 [µg/l]	L4 [µg/l]	L5 [µg/l]	L6 [µg/l]	L7 [µg/l]	L8 [µg/l]	L9 [µg/l]	L10 [µg/l]
Amoxicillin trihydrate	0.08	0.31	0.63	1.25	2.50	5.00	10.00	20.00	40.00	80.00
Ampicillin trihydrate	0.08	0.31	0.63	1.25	2.50	5.00	10.00	20.00	40.00	80.00
Ceftiofur	0.20	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00	200.00
Chlorotetracycline hydrochloride	0.20	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00	200.00
Ciprofloxacin	0.10	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
Cloxacillin sodium salt monohydrate	0.06	0.23	0.47	0.94	1.88	3.75	7.50	15.00	30.00	60.00
Danofloxacin	0.06	0.23	0.47	0.94	1.88	3.75	7.50	15.00	30.00	60.00
Doxycyclin hyclate	0.20	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00	200.00
Enrofloxacin	0.10	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
Erythromycin A dihydrate	0.08	0.31	0.63	1.25	2.50	5.00	10.00	20.00	40.00	80.00
Flumequine	0.10	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
Lincomycin hydrochloride monohydrate	0.29	1.17	2.34	4.69	9.38	18.75	37.50	75.00	150.00	300.00
Marbofloxacin	0.15	0.59	1.17	2.34	4.69	9.38	18.75	37.50	75.00	150.00
Oxytetracycline hydrochloride	0.20	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00	200.00
Penicillin G potassium salt	0.04	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00	40.00
Penicillin V potassium salt	0.10	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
Pirlimycin Hydrochloride	0.98	3.91	7.81	15.63	31.25	62.50	125.00	250.00	500.00	1000.00
Spiramycin	0.39	1.56	3.13	6.25	12.50	25.00	50.00	100.00	200.00	400.00

Sulfadiazin	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfadimethoxine	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfadimidin = Sulfamethazine	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfadoxin	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfamerazine	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfamethoxazole	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfamethoxypyridazine	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfanilamide	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Sulfathiazole	0.02	0.08	0.16	0.31	0.63	1.25	2.50	5.00	10.00	20.00
Tetracycline hydrochloride	0.20	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00	200.00
Tilmicosin	0.10	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
Trimethoprim	0.10	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
Tylosin tartrate	0.10	0.39	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00

Table 10: Preparation scheme of antibiotic mix calibration levels.

2.6. Internal Standards mixture

Individual stock solutions of the ISTD compounds were also prepared at a concentration of 1000 mg/l and stored at –18 °C in the dark. Penicillin G-d7-N-ethylpiperidinium salt was dissolved in H_2O:ACN (50:50), marbofloxacin-d8 and erythromycin-13C-d3 in chloroform, ciprofloxacin-d8 hydrochloride hydrate and enrofloxacin-d5 hydrochloride in H_2O. The remaining substances were dissolved in MeOH. An ISTD mix solution was prepared reaching concentrations between 20 – 1000 µg/L (Table 11).

Compound	concentration ISTD-mix-solution (µg/L)
Ciprofloxacin-d8 hydrochloride hydrate	60
Danofloxacin-(methyl-d3)	100
Enrofloxacin-d5 hydrochloride	100
Flumequine-1,2,carboxy-13C3	100
Marbofloxacin-d8	150
Erythromycin-13C, d3	300
Lincomycin-d3	400
Spiramycin I-d3	1000
Penicillin G-d7 N-ethylpiperidinium salt	400
Sulfadiazine-phenyl-13C6	20
Sulfadimethoxine-d6	20
Sulfamethazine-(phenyl-13C6)hemihydrate	20
Sulfadoxin-d3	20
Sulfamerazine-(phenyl-13C6)	20
Sulfamethoxazole-phenyl-13C6	20
Sulfamethoxypyridazine-d3	20
Sulfanilamide-13C6	20
Sulfathiazole-(phenyl-13C6)	20
Trimethoprim-d9	100
Demeclocyclin hydrochloride hydrate	200
Doxycycline-d3 hyclate	200

Table 11: Internal standard (ISTD) mix solution.

2.7. Optimization of MS/MS parameters

The optimization of the MS parameters (precursor and daughter ions, collision energy (CE) and cell accelerating voltage (CAV)) was conducted by injecting a standard solution of 500 µg/L of each antibiotic compound diluted in initial mobile phase solvent. To avoid a possible retention of the compounds, no analytical column was used, and thereby the analytes got directly injected into the mass spectrometer. Additionally, the mobile phases are used without any gradient and are mixed with 50% mobile phase A and 50% mobile phase B during whole optimization process.

2.7.1. Scan – Precursor ion

For determination of the precursor ion, a full-scan spectrum of each substance was collected in order to select the most abundant m/z value. After direct injection of 10 µl of each substance, the precursor ion was selected after positive and negative electro-spray-ionisation (ESI), whereas for all substances protonated $[MH]^+$ ions were detected with the highest abundance. After ionisation the charged ions are transferred into the mass spectrometer, consisting of three quadrupoles, whereas the first quadrupole is during scan mode permeable and the third quadrupole is responsible for the measurement. By adjusting the EMV (electron multiplier voltage) the signal can be intensified, but for optimisation work an EMV of 300 was considered to be usually sufficient. In contrast to mass spectrometers without iFunnel technology, the fragmentor was not necessary to be tuned. This revolutionary technology is focused to enhance the nebulisation and desolvation in order to increase the number of ions transferred to the mass analyzer, while simultaneously the used gas amount can be reduced.

2.7.2. Product ions

The previous obtained precursor ions were used to determine at least two typical fragments after inducing different collision energies (CE). The collision energy is a parameter influencing the second quadrupole (hexapole), of the mass spectrometer and is necessary to get the typical transition ions from each substance, by accelerating

product ions through an electric field and through a collision with neutral nitrogen-gas-molecules. To identify the optimal collision energy and product ions of each substance, the molecules were fragmented with 5, 10, 15, 20, 25, 30, 35 and 40 Volt. In case of very stable substances the voltage must sometimes be increased, to reach a successful fragmentation.

2.7.3. Collision Cell Accelerator Voltage

The collision cell accelerator voltage (CAV) influences the transfer from the second to the third quadrupole in the mass spectrometer. It defines the resting time of the molecule in the hexapole which is sufficient to get fragments before the fragments are transmitted to the next quadrupole. If this parameter would not be maintained, the hexapole would not stop the fragmentation. For optimization of this parameter, the substances were tested with 1, 3, 5 and 8 Volt.

2.7.4. Retention time

Beside the typical transitions of each compound, the retention time is a further decision criterion for substance characterisation. To obtain the retention time for each substance one total HPLC-gradient run as described below (Table 12) was conducted in multiple reaction monitoring (MRM) mode. In this measurement mode, the previous specified transitions for the compound is measured during the whole run time, allowing to find out the specific retention time.

After determining all necessary parameters, for this work a dynamic MRM measurement method was used to improve sensitivity of the measurements by reducing the impact of concurrent ions, which is thereby generally used for methods including a high number of analytes. In contrast to the usual MRM mode, the dynamic MRM mode provides to define a retention time window for each compound, meaning that only specific transitions are measured at a specified retention time window. Further difference is the utilization of a constant cycle time instead of the dwell time. This technology enhances the data points per peak and improves the peak shape, subsequently.

For evaluation of the results for all parameters the MassHunter Workstation Software for LC/MS Data Acquisition B.07.00, Qualitative Analysis B.07.00 and QQQ Quantitative Analysis B.07.00 software from Agilent Technologies Inc. were used. All MS/MS transitions with their CEs, CAVs and RTs are shown in appendix II.

In addition to these previous determined parameters, the following settings for the MS/MS were used:

- *Gas temperature:* 200 °C
- *Gas flow:* 15 l/min
- *Nebulizer:* 30 psi
- *Sheath gas temperature:* 375 °C
- *Sheath gas flow:* 11 l/min
- *Capillary voltage:* 3500 V (positive ionization)
- *Nozzle voltage:* 300 V (positive ionization)

2.8. UHPLC measurement conditions

As described in chapter 1.9. the most powerful parameter for achieving a high chromatographic resolution is the selectivity and depends mainly by the used analytical column and the composition of the mobile phases. In order to the chemical structures of the compounds of interest in this work, the analytical column should provide retention for apolar substances. Therefore, a typical reversed phase column with endcapped C18 retention mechanism was chosen to obtain retention of the analytes. For eluting the substances from the stationary phase an aqueous mobile phase combined with methanol as the second mobile phase, both containing ammonium formate and formic acid, were used to proceed a gradient separation. The analytical conditions were thereby performed as follows:

- *Analytical column:* Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 x 100 mm, 1.8 µm (Agilent Technologies, Inc.)

- *Mobile phase A:* Milli-Q water, 5 mM ammonium formate, 0.1% formic acid
- *Mobile phase B:* Methanol (HPLC-grade), 5 mM ammonium formate, 0.1% formic acid

- *Separation gradient:*

t (min)	Mobile Phase (%A)	Mobile Phase (%B)
0	95	5
2	95	5
3	83	17
6	30	70
8	30	70
9	0	100
11	0	100
15	95	5

Table 12: Separation gradient. *Total run time: 15 minutes per sample.*

- *Further HPLC parameters:*
  - *Injection volume:* 20 µl
  - *Column temperature:* 40 °C
  - *Gradient flow:* 0.25 ml/min
  - *Autosampler temperature:* 5 °C

2.9. Sample preparation

For this work, different kinds of dairy and meat products, as well as the matrix fish were investigated. The matrices of choice for dairy products were defined as follows and bought in organic quality:

- Raw milk, *3.5 % fat content*
- Pasteurized milk, *3.5 % fat content*
- Cream, *36 % fat content*
- Butter, *80 % fat content*
- Curd, *20 % fat in dry matter*

- Sour cream, *15 % fat content*
- Yogurt, *3.5 % fat content*
- Buttermilk, *1 % fat content*
- Soft cheese, *covered with white mould and red cultures, 55 % fat content*
- Hard cheese, *contains 50 % fat in dry matter*

In addition, we defined the following meat and fish products for analyses and were bought in non-organic, or if possible in organic quality:

- Chicken – lean mat
- Pig – lean meat, fat and liver
- Beef – lean meat, liver and kidney
- Shrimps
- Salmon (with skin)
- Trout (with skin)

In routine laboratory work it is very important to provide homogenous samples for subsequent extraction and analysis to ensure equal distribution of possible containing analytes to obtain identically results. Matrices with a soft texture, like yogurt or whipped cream, need to be carefully shaken or stirred with a spoon. Matrices with an inhomogeneous texture, like cheese or meat were homogenized together with dry ice using a mixer to achieve a powdery matter. During whole sample preparation procedure, it is necessary to avoid possible contamination or degradation of the analytes. Therefore, clean and with acetonitrile rinsed equipment was used for homogenisation and the homogenized samples were stored in a freezer at -18°C (Figure 29).

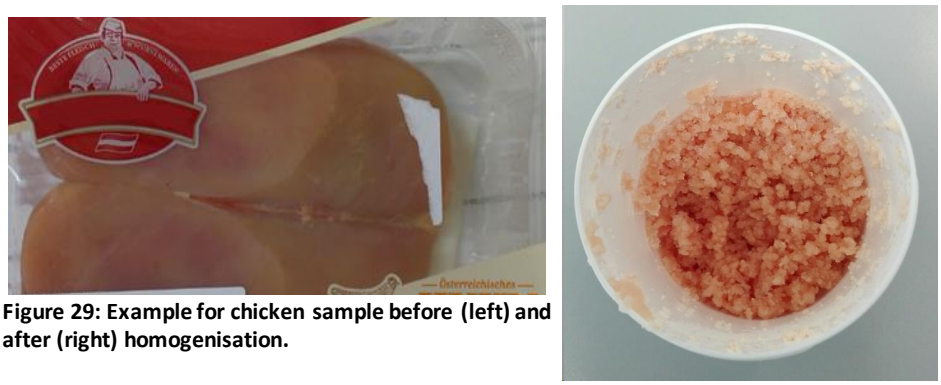

Figure 29: Example for chicken sample before (left) and after (right) homogenisation.

2.10. Extraction and clean-up techniques

As described in chapter 1.8 an efficient sample preparation technique is required to achieve satisfying results. The main sample preparation techniques for veterinary drugs are described by conducting dilute-and-shoot, modified QuEChERS (***Qu****ick* ***E****asy* ***Ch****eap* ***E****ffective* ***R****ugged and* ***S****afe*) approaches and Solid Phase Extraction (SPE). Our final method is aiming to be easy, cheap and fast to be suitable in routine use ensuring a high sample throughput. The following section describes the different sample extraction procedures which were performed during method development process.

2.10.1. Dilute and shoot (Liquid-Liquid Extraction)

After homogenizing the samples as described in 2.9, 2.0 ± 0.1 g of sample were weight into 50 ml centrifugation tubes. The analytes were extracted by using acetonitrile alone or in combination with formic acid or McIlvaine buffer. After extraction by shaking the samples using an overhead-shaker the samples were centrifuged for 5 minutes at 6,000 rpm. Finally, the obtained supernatant was filtered into an HPLC-glass vial for HPLC measurement by using a 0.45 µm PTFE filter (Figure 30).

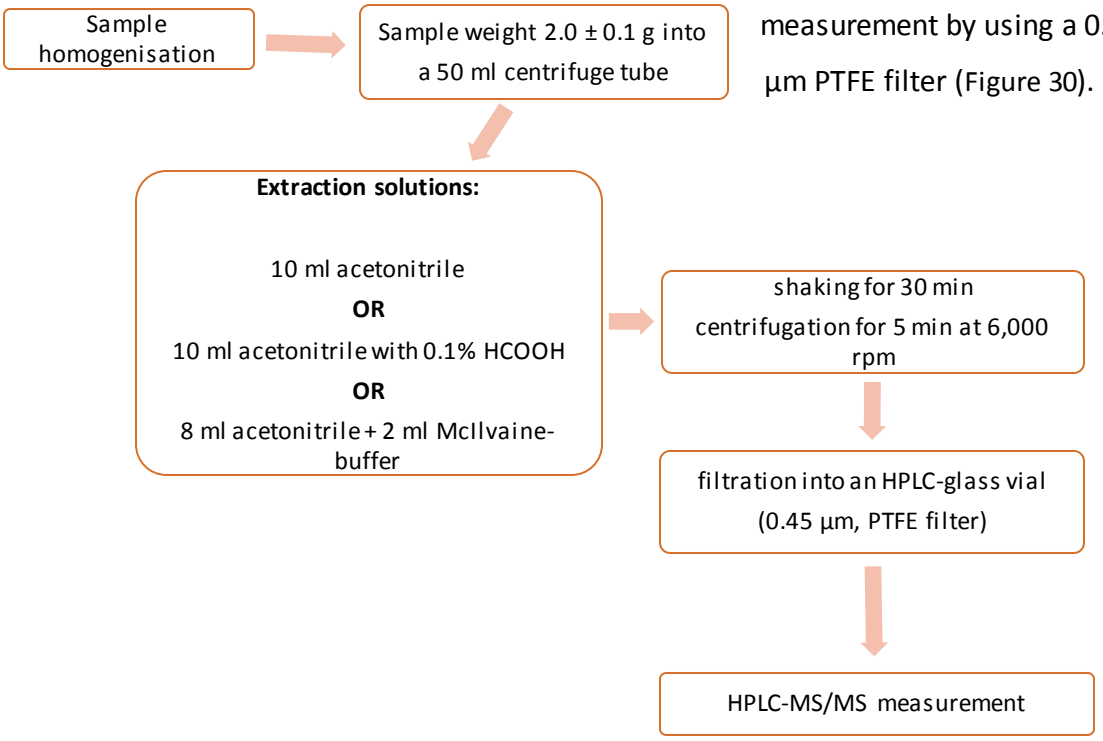

Figure 30: Sample preparation scheme for dilute and shoot (liquid-liquid) extractions.

2.10.2. QuEChERS (Quick Easy Cheap Effective Rugged and Safe) approaches

In accordance to the principle of QuEChERS sample clean-up by addition of different extraction salts to remove possible matrix influences, different modified approaches were conducted. 2.0 ± 0.1 g of homogenized sample were weight into 50 ml centrifuge tubes and 10 ml of acidified acetonitrile mixed with water or McIlvaine-buffer were added to obtain an ACN:H_2O ratio of 1:1. To extract the analytes different extraction salts were added followed by shaking the samples for 2 minutes using the Collomix device. Subsequently the samples were centrifuged for 5 minutes at 6,000 rpm resulting in a separation into an ACN upper layer containing the analytes and a lower H_2O layer (Figure 31).

Additionally, different types of dispersive SPE salts were tested aiming to obtain better matrix removals and thereby achieving better recovery results. Therefore an aliquot of the supernatant was transferred into 15 ml tubes containing different clean-up salts and the samples were once more shaken for 2 minutes and centrifuged for 5 minutes at 6,000 rpm. As a further step to improve the results, the resulting supernatant was evaporated to dryness under gentle nitrogen steam at 45 °C and reconstituted with 2 ml of initial mobile phase and filtered into an HPLC-glass vial by using a 0.45 µm PTFE filter.

In (**Figure 32**) the different approaches are summarized, demonstrating the different combinations of extraction solvents, extraction salts and dispersive SPE salts.

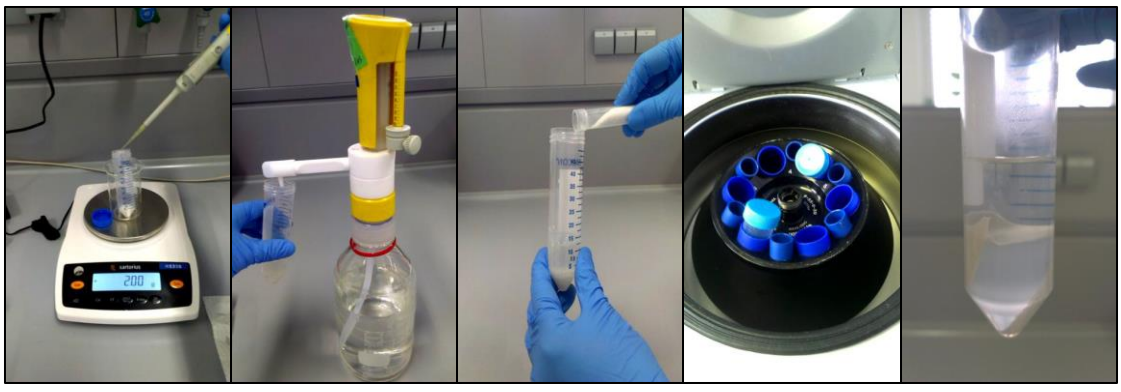

Figure 31: QuEChERS sample preparation scheme.

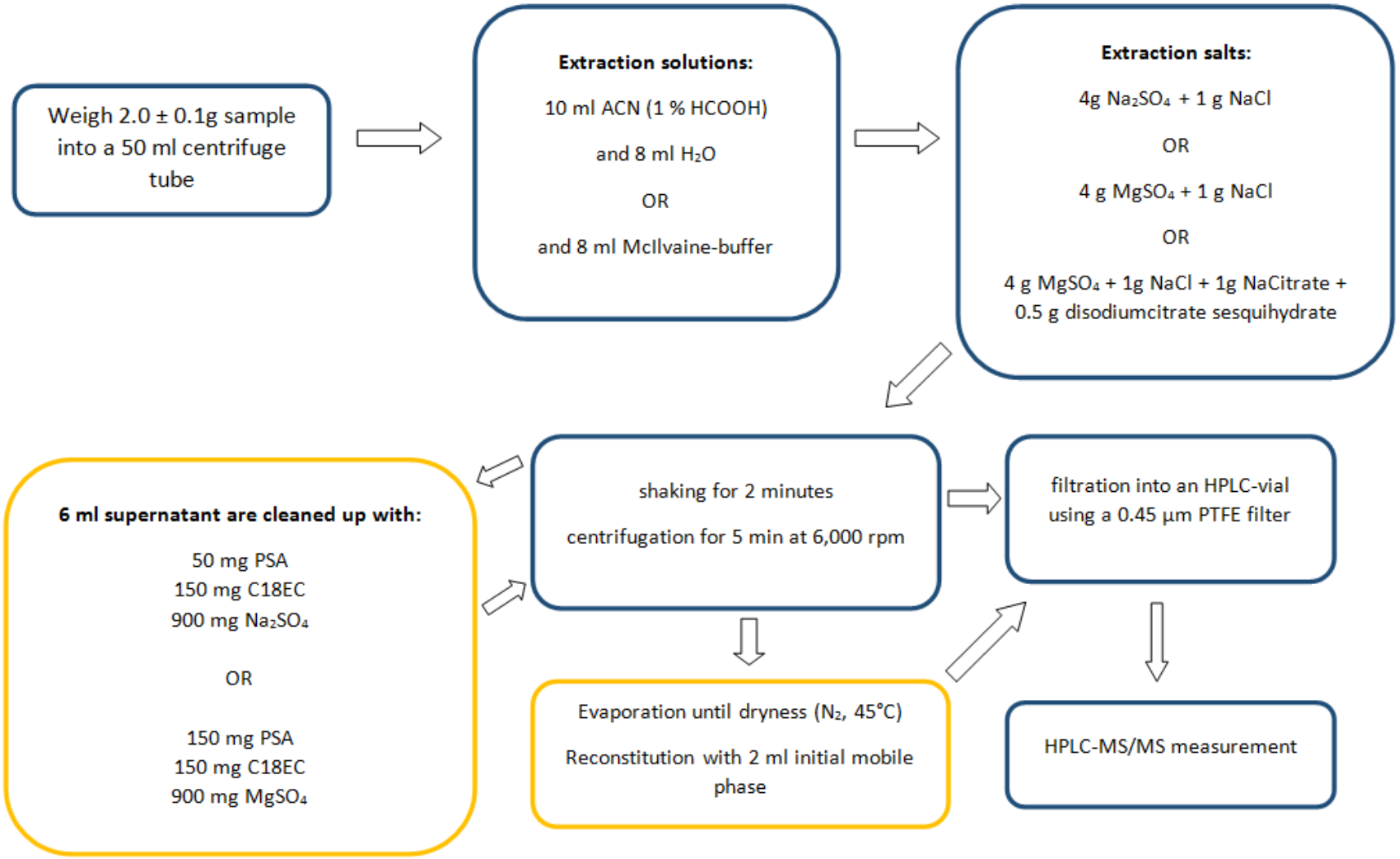

Figure 32: Scheme of conducted QuEChERS approaches. *Sample preparation steps in yellow boxes demonstrate the additional clean-up steps.*

2.10.3. Solid Phase Extraction (SPE)

Solid phase extraction (SPE) is an effective clean-up procedure to remove disturbing matrix influences achieving clean injection solutions. Despite there are many different sorbent types, providing several kinds of retention interactions with the compounds of interest, we decided to perform our tests by using a typical reversed phase mode. The principle of this kind of clean-up is based on hydrophobic interactions between the sorbent and the analytes (Figure 33).

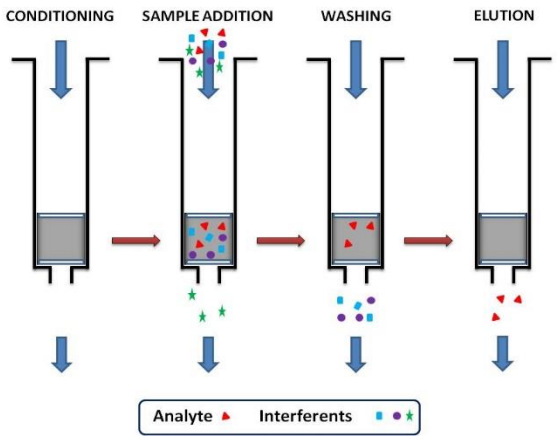

Figure 33: Principle of solid phase extraction (SPE) procedure.[15]

Our experiments were conducted by weighing 2.0 ± 0.1 g of homogenized sample into a 50 ml centrifuge tube and the analytes were extracted by acetonitrile alone or in combination with McIlvaine buffer, formic acid or H_2O. After shaking for 2 minutes by using the Collomix device the samples were centrifuged for 5 minutes at 6,000 rpm. An aliquot of the obtained apolar supernatant was diluted with H_2O to ensure a maximum of 20% acetonitrile in the solution. This step is necessary to retain the analytes on the sorbent. Before this extract can be applied on the SPE cartridges, the sorbent must be activated by previously conditioning with 3 ml methanol followed by 3 ml H_2O for equilibration. After this conditioning step, the extract with the analytes were load and interferences with less retention then the analytes were removed by a followed washing step using H_2O. Afterwards the sorbent was dried down by applying vacuum and the analytes were subsequently eluted with 3 ml methanol. It must be noticed that it is crucial to control the flow rate during all these steps by approximately 1 drop per second to ensure enough time for interactions between the analytes in the solvent and the SPE sorbent. The cleaned extract was additionally evaporated until dryness under nitrogen steam at 45 °C and afterwards reconstituted with 2 ml initial mobile phase. After filtration with a 0.45 µm PTFE filter into an HPLC-glass vial the sample was ready for HPLC-MS/MS measurement (Figure 34).

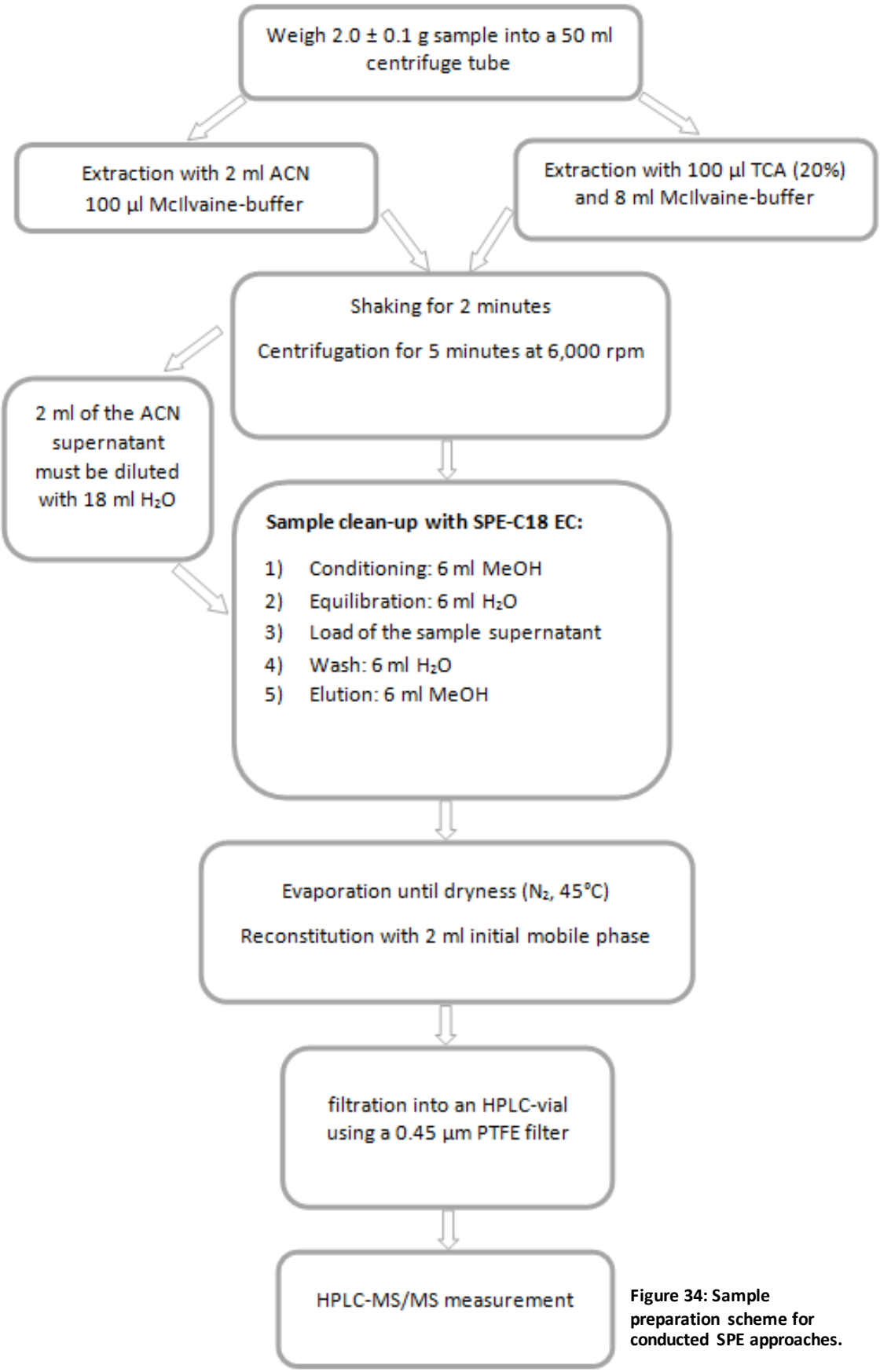

Figure 34: Sample preparation scheme for conducted SPE approaches.

2.10.4. Dispersive Solid Phase Extraction (dSPE)

Dispersive Solid Phase Extraction (dSPE) provides a combination of QuEChERS and SPE sample preparation techniques. In contrast to QuEChERS no extraction salts for phase fractioning is used and instead of using SPE cartridges different kinds of bulk sorbents are used for effective sample clean-up by providing different types of interactions for matrix removal.

For investigating this sample preparation procedure 2.0 ± 0.1 g homogenized sample were weight into 50 ml centrifuge tubes. Extraction of the analytes from the matrices was conducted with acetonitrile mixed with H_2O or McIlvaine-buffer to obtain a ACN:H_2O ratio of 4:1. After shaking for 2 minutes using the Collomix device, the samples were centrifuged for 5 minutes at 6,000 rpm. The received supernatant was subsequently transferred to a 15 ml centrifuge tube containing C18EC dSPE bulk sorbent either alone or in combination with PSA or ZSep. In addition, the especially developed for Enhanced Matrix Removal - EMR bulk sorbent was investigated. The samples were immediately shaken for 2 minutes followed by centrifugation for 5 minutes at 6,000 rpm. An aliquot of 5 ml from the obtained supernatant was transferred to a new 15 ml centrifuge tube and placed into the N_2-evaporator at a temperature of 45 °C until dryness. The analytes were reconstituted with 2 ml of initial mobile phase and filtered into an HPLC glass vial by using a 0.45 µm PTFE-filter (Figure 35).

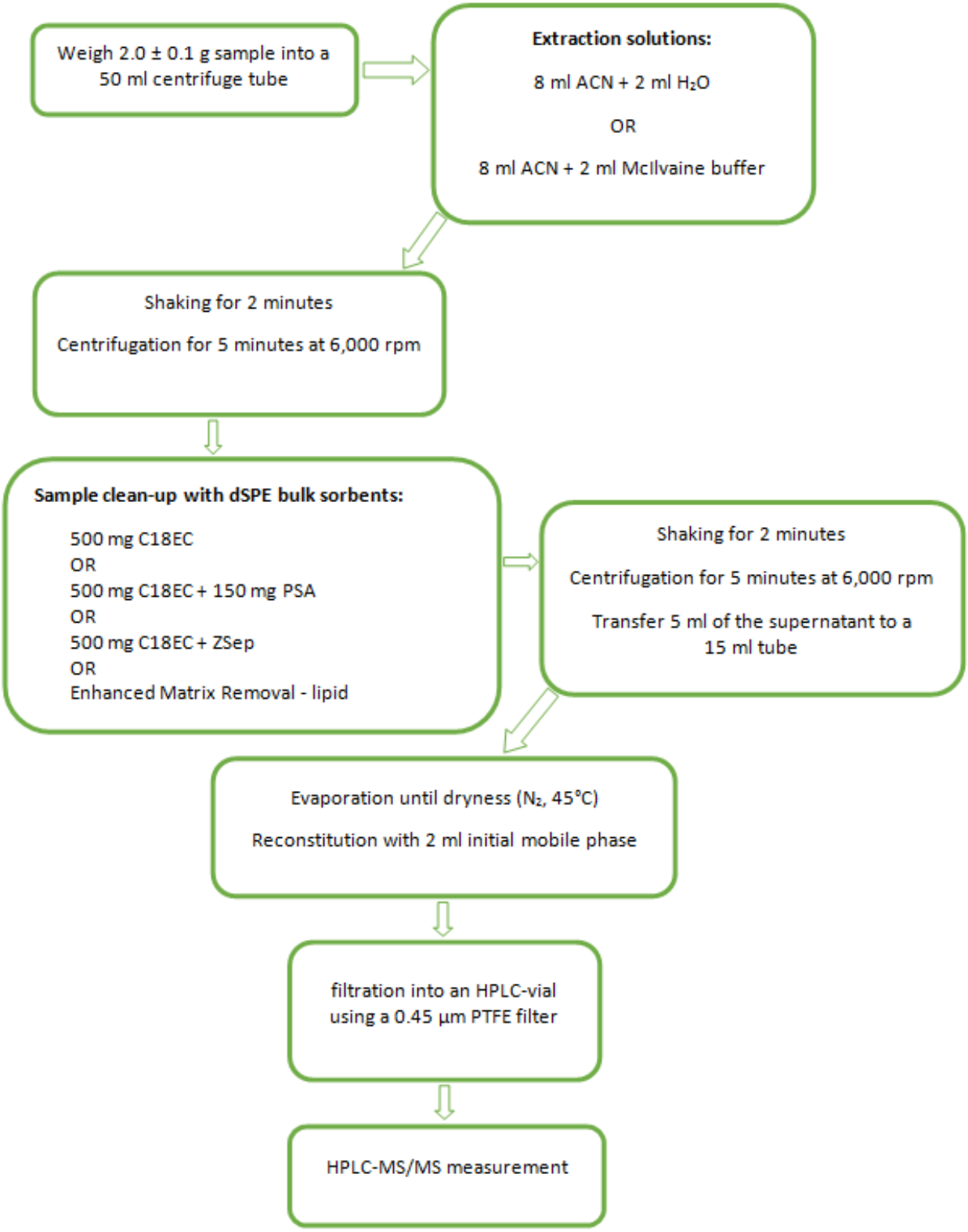

Figure 35: Sample preparation schema for conducted experiments using dSPE clean-up.

2.11. Analytical method validation

To achieve the accreditation status in accordance to ISO/IEC 17025 for the developed method, it is necessary to meet the following requirements. Method validation is defined in ISO/IEC 17025 as the "*confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled*". (Magnusson, 2014) This means that an analytical method validation is a powerful tool to provide quality assurance and demonstrate if a method is fit for purpose. (Kromidas, 2011) The extend and implementation of a validation must be aligned with the objectives of the method, whereas the following performance characteristics are investigated for validation:

- Selectivity
- Limit of detection (LOD) and limit of quantification (LOQ)
- Trueness (bias, recovery)
- Precision (repeatability, intermediate precision and reproducibility)

Further it must be decided if the validation should be performed according to the interlaboratory comparison approach or to the single-laboratory approach. In our case, the single-laboratory approach is appropriate, because the validated method should only be used in our laboratory and should not become a published standard. (Magnusson, 2014)

2.11.1. Selectivity

The term selectivity is defined as *"the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour."* (Vessman et al., 2001) This definition means that the selectivity of a method must be investigated in order to its ability to detect the compound of interest in samples including matrix dependent interferences. The most practical way to assess the selectivity is to compare a chromatogram of the analytes with a chromatogram including the analytes and interferences. The obtained results can

additionally be supported by considering chromatographic parameters, like retention time or peak shape. (Kromidas, 2011)

2.11.2. Limit of detection (LOD) and limit of quantification (LOQ)

The term limit of detection (LOD) defines the smallest concentration of a compound which can be detected by the method at a specific level of confidence. In contrast the limit of quantification (LOQ) provides the value of the smallest concentration of a compound which can quantitative be measured, in regard to precision and trueness. (Kromidas, 2011)

In accordance to regulatory authorities, like the United States Pharmacopoeia (USP), Foods and Drugs Administration (FDA), International Union of Pure and Applied Chemistry (IUPAC), International Conference on Harmonisation (ICH) and Association of Analytical Communities (AOAC) there are different guidelines for determining the parameters LOD and LOQ. (Shrivastava & Gupta, 2011)

The several approaches are describing the calculation of LOD and LOQ by using:

- Visual definition
- Signal-to-noise ratio
- Calculation from the standard deviation of the blank
- Calculation from the calibration line at low concentrations. (Shrivastava & Gupta, 2011)

2.11.3. Accuracy

Accuracy is the general term in the validation process for assessing systematic and random errors, expressed with the performance characteristics *precision* and *trueness*. (Kromidas, 2011) By measuring the accuracy the closeness of a single result to a known reference value can be expressed.

2.11.3.1. Trueness

Trueness is defined as the term describing the deviation of a measurement compared to the reference value. Practically the trueness can be assessed by three different approaches:

- ***Measurement of certified reference material (CRM)*** usually for 10 times and comparing the mean value $\bar{x}$ with the reference value x_{ref}. With these results, bias can be determined which can be expressed in absolute terms

$$b = \bar{x} - x_{ref}$$

or relative in per cent

$$b\ (\%) = \frac{\bar{x} - x_{ref}}{x_{ref}}\ x\ 100$$

or the relative recovery

$$R(\%) = \frac{\bar{x}}{x_{ref}}\ x\ 100$$

- ***Assessing the recovery rates by using spiked samples*** and calculate the relative spike recovery *R' (%)* at the various concentrations:

$$R'\ (\%) = \frac{\bar{x}' - \bar{x}}{x_{spike}}\ x\ 100$$

$\bar{x}'$ … mean spiked value
$\bar{x}$ … mean value
x_{spike} …added concentration

- ***Measuring CRM and comparing the results with results obtained using an alternative method*** and calculate absolute, relative and relative recovery as described above. (Magnusson, 2014)

2.11.3.2. Precision

The precision of an analytical measurement describes the closeness of agreement between a series of measurements received from several samplings of the same homogeneous sample performing the same conditions and should be used at three levels: repeatability, intermediate precision and reproducibility. Repeatability describes the precision under the same operating conditions, also named as intra-assay precision. Intermediate precision describes within-laboratories variations (e.g. different days, different analysts, etc.) and reproducibility expresses the precision between laboratories. By calculating the variance, standard deviation or coefficient of variation of a series of measurement, the precision of an analytical procedure can be expressed. (ICH, 2005)

2.11.4. Validation performance

The final developed method (Figure 36) was validated according to our in-house validation procedure. In order to the set maximum residue levels (MRLs) for pharmacologically active substances in animal derived food stuff in the European Regulation 37/2010 (The European Commission, 2010) , we aimed to establish a limit of quantification 1/10 of the referenced MRLs. Therefore, the different chosen matrices were spiked at a concentration level of 1/10 and ½ of the MRLs. Since there are no existing MRLs for processed dairy products, for the different dairy matrices the MRL set for bovine milk was taken.

For the different kind of meat matrices different MRLs provide the regulation. For more easiness, the lowest MRLs within the matrices were taken for validation of all meat matrices. A table with all specific MRLs is attached in appendix I.

After exactly weighing of 2.0 ± 0.1 g of sample into a 50 ml centrifuge tube the samples were spiked with 100 µl “low spike antibiotic mix” to assess 1/10 of MRLs concentration level. For ½ of MRLs concentration level the samples were spiked with 100 µl of “high spike antibiotic mix” (Table 13 and Table 15).

For each matrix one blank sample and 7 replicates of each spike level were processed according to the sample preparation scheme on different days (Table 14 and Table 16). For quality control assessing both possible disturbances during sample preparation and measurement a certified reference material was prepared and measured together with the samples on each validation day. The obtained results were evaluated by using Agilent Mass Hunter Quantitative software version B07.00 and processed in MS Excel to assess the previous described validation performance parameters.

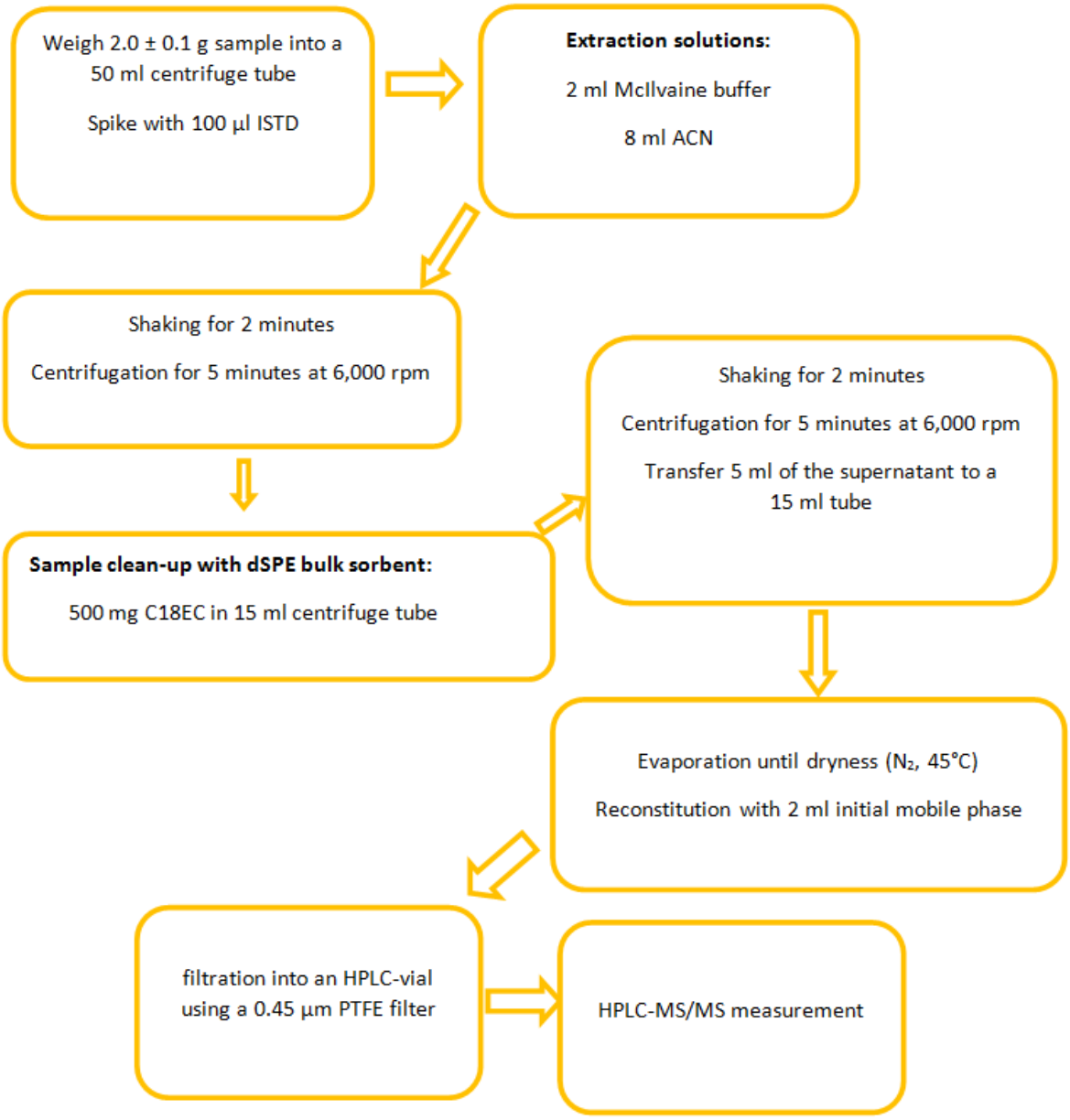

Figure 36: Scheme of the final sample preparation procedure.

Compound	MRL [µg/kg] Bovine milk	1/10 x MRL [µg/kg]	1/2 x MRL [µg/kg]	Concentration "low spike antibiotic mix" [µg/kg]	Concentration "High spike antibiotic mix" [µg/kg]
Ciprofloxacin	50	5	25	100	500
Danofloxacin	30	3	15	60	300
Enrofloxacin	50	5	25	100	500
Flumequine	50	5	25	100	500
Marbofloxacin	75	7.5	37.5	150	750
Erythromycin A	40	4	20	80	400
Lincomycin	150	15	75	300	1500
Pirlimycin	100	10	50	200	1000
Spiramycin	200	20	100	400	2000
Tilmicosin	50	5	25	100	500
Tylosin	50	5	25	100	500
Amoxicillin	4	0.4	2	80	400
Ampicillin	4	0.4	2	80	400
Ceftiofur	100	10	50	200	1000
Cloxacillin	30	3	15	60	300
Penicillin G	4	0.4	2	40	200
Penicillin V	No MRL			100	500
Sulfadiazine	10	1	5	20	100
Sulfadimethoxine	10	1	5	20	100
Sulfadimidin = Sulfamethazine	10	1	5	20	100
Sulfadoxine	10	1	5	20	100
Sulfamerazine	10	1	5	20	100
Sulfamethoxazole	10	1	5	20	100
Sulfamethoxypyridazine	10	1	5	20	100
Sulfanilamide	10	1	5	20	100
Sulfathiazole	10	1	5	20	100
Trimethoprim	50	5	25	100	500
Chlortetracycline	100	10	50	200	1000
Doxycyclin	No MRL			200	1000
Oxytetracycline hydrochloride	100	10	50	200	1000
Tetracycline hydrochloride	100	10	50	200	1000

Table 13: Spike concentrations for validation of dairy products.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Raw milk	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Pasteurized milk	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Whipped cream	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Butter	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Curd	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Sour cream	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Yogurt	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Buttermilk	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Soft cheese	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Hard cheese	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7

Table 14: Validation plan for dairy matrices. L1-L7 means 7 replicates fortified with 100 µl of low spike concentration mix. H1-H7 means 7 replicates fortified with 100 µl of high spike concentration mix.

Compound	Lowest MRL [µg/kg]	1/10 x MRL [µg/kg]	1/2 x MRL [µg/kg]	Concentration "low spike antibiotic mix" [µg/kg]	Concentration "High spike antibiotic mix" [µg/kg]
Ciprofloxacin	50	5	25	100	500
Danofloxacin	50	5	25	100	500
Enrofloxacin	1000	100	500	2000	10000
Flumequine	100	10	50	200	1000
Marbofloxacin	50	5	25	100	500
Erythromycin A	300	30	150	600	3000
Lincomycin	50	5	25	100	500
Pirlimycin	100	10	50	200	1000
Spiramycin	50	5	25	100	500
Tilmicosin	200	20	100	400	2000
Tylosin	200	20	100	400	2000
Amoxicillin	50	5	25	100	500
Ampicillin	50	5	25	100	500
Ceftiofur	100	10	50	200	1000
Cloxacillin	50	5	25	100	500
Penicillin G	25	2,5	12,5	50	250
Penicillin V	100	10	50	200	1000
Sulfadiazine	200	20	100	400	2000
Sulfadimethoxine	10	1	5	20	100
Sulfadimidin = Sulfamethazine	10	1	5	20	100
Sulfadoxine	10	1	5	20	100
Sulfamerazine	10	1	5	20	100
Sulfamethoxazole	10	1	5	20	100
Sulfamethoxypyridazine	10	1	5	20	100
Sulfanilamide	10	1	5	20	100
Sulfathiazole	10	1	5	20	100
Trimethoprim	10	1	5	20	100
Chlortetracycline hydrochloride	100	10	50	200	1000
Doxycyclin	50	5	25	100	500
Oxytetracycline hydrochloride	50	5	25	100	500
Tetracycline hydrochloride	100	10	50	200	1000

Table 15: Spike concentrations for validation of meat matrices.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Chicken muscle	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Pig muscle	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Beef muscle	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Pig fat	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Pig liver	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Beef liver	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Beef kidney	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Shrimps	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Salmon	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7
Trout	Blank	L1 H1	L2 H2	L3 H3	L4 H4	L5 H5	L6 H6	L7 H7

Table 16: Validation plan for meat matrices. L1-L7 means 7 replicates fortified with 100 µl of low spike concentration mix. H1-H7 means 7 replicates fortified with 100 µl of high spike concentration mix.

3. RESULTS & DISCUSSION

3.1. Standards optimization

As described in chapter 2.7, for each compound the typical precursor ion with at least two daughter ions must be determined to enable characterisation of each substance. To achieve best peak abundances the specific mass spectrometric parameters collision energy and cell accelerating voltage are additionally detected. Based on the following example with the substance tetracycline the optimization process will be demonstrated:

3.1.1. Scan – Precursor ion

Measured on the molecular mass of tetracycline with 444.44 g/mol a full-scan spectrum between 200–600 m/z was applied to identify the precursor ion. This broad spectrum is chosen in case of possible forming adducts with components (from e.g. the mobile phases) or in case of separating components, e.g. bonded water. Due to the missing column, the total ion chromatogram (TIC) provides a peak within the first seconds after injection. To get the information about the most abundant ion, this chromatogram must be extracted into a spectrum showing all measured ions. In case of tetracycline the ion with the mass of 445.2 g/mol shows the highest abundance and is therefore chosen as the precursor ion (Figure 37).

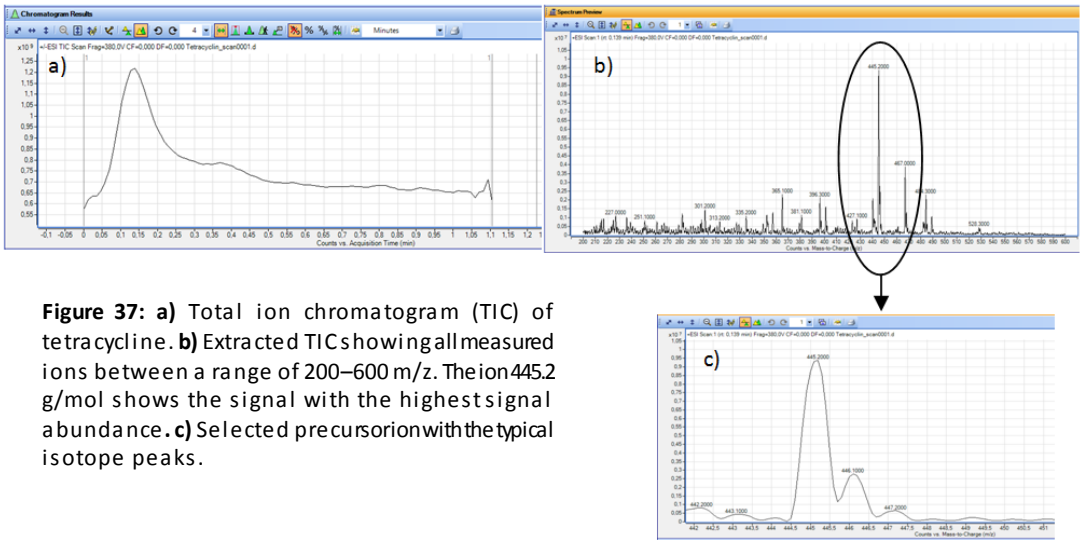

Figure 37: a) Total ion chromatogram (TIC) of tetracycline. **b)** Extracted TIC showing all measured ions between a range of 200–600 m/z. The ion 445.2 g/mol shows the signal with the highest signal abundance. **c)** Selected precursor ion with the typical isotope peaks.

3.1.2. Product ions

By inducing different collision energies (CE) at least two typical fragments are obtained from the previous determined precursor ion. As described above the precursor ion gets fragmented by applying different collision energies from 5–40 Volts obtaining previously a fully chromatogram for each energy level. By extracting each chromatogram into its spectrum, the product ions with their specific collision energy will be determined by choosing the peak with the highest abundance. In the example for tetracycline the ions with the highest mass-to-charge ratio are 427.1 and 410.1 m/z and show the highest abundance after inducing 10 and 20 V, respectively. (Figure 38)

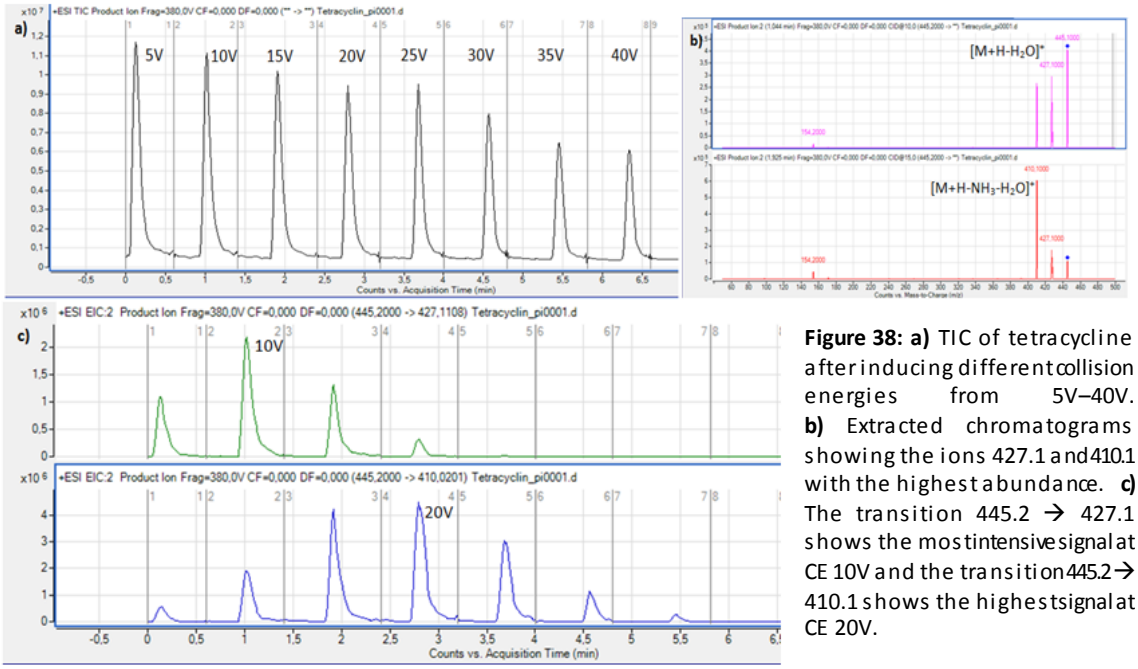

Figure 38: a) TIC of tetracycline after inducing different collision energies from 5V–40V. **b)** Extracted chromatograms showing the ions 427.1 and 410.1 with the highest abundance. **c)** The transition 445.2 → 427.1 shows the most intensive signal at CE 10V and the transition 445.2 → 410.1 shows the highest signal at CE 20V.

3.1.3. Collision Cell Accelerator Voltage (CAV)

To determine the specific CAVs, each transition was tested with four different energies from 1–8 V, showing a full chromatogram for each energy level and transition. By extracting the chromatograms, the spectrum shows the peaks for each transition with each CAV. The peak showing the most abundance determines the specific CAV for the chosen transition. In case of tetracycline both transitions are showing peaks with the highest intensity when a CAV of 5 V for transition 445 → 427 and 8 V for transition 445 → 410 is applied. (Figure 39)

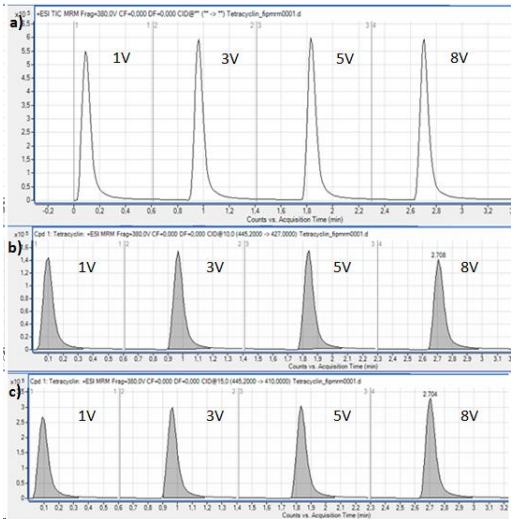

Figure 39: ***a)*** *TIC of tetracycline after inducing different CAVs.* ***b)*** *For transition 445.2 → 427.0 the highest signal was obtained after inducing 5V and* ***c)*** *shows the most abundance signal for transition 445.2 → 410.0 after inducing 8V.*

3.1.4. Retention time

The retention time is evaluated by running a total HPLC-gradient for each substance in MRM mode, and is for tetracycline at 6.3 min. (Figure 40)

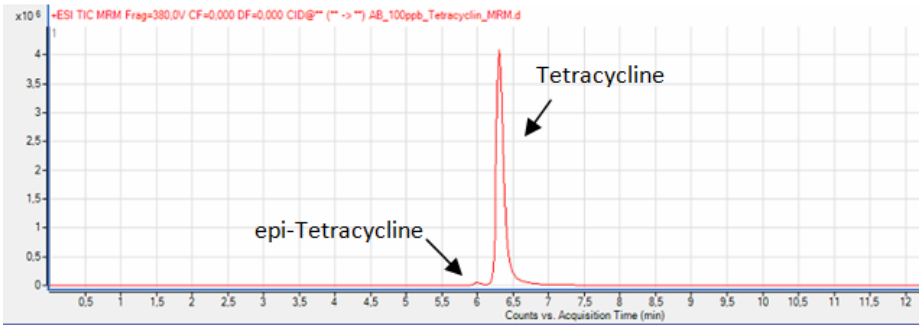

Figure 40: Retention time for tetracyline and its 4- epimer.

3.2. Extraction and clean-up techniques

3.2.1. Dilute and shoot ("Liquid-liquid-extraction")

Dilute and shoot sample preparation technique is based on the liquid-liquid-extraction principle. Our experiments were in the first instance conducted with the matrix milk, because it represents the most important matrix. 2.0 ± 0.1 g of organic milk sample were fortified with 100 µl of antibiotic mix (Table 9) to obtain a concentration 1/10 of their MRLs. For efficient extraction of the analytes from the matrix different organic solvents were examined, whereas the compound groups have good solubility in polar organic solvents (Figure 41). (Dasenaki & Thomaidis, 2010) (Hammel, Mohamed, Gremaud, & Guy, 2008) The high protein content in milk is described as the main problem associated with the subsequent chromatographic determination of antibiotics. Therefore acetonitrile alone or in combination with acids are generally used for protein precipitation. (Freitas, Barbosa, & Ramos, 2014) Tetracyclines tend to form complexes with divalent cations like Mg^{2+} or Ca^{2+}-ions, which are substantially present in milk. The addition of EDTA should avoid the formation of these chelates and thereby increasing the recovery of tetracyclines. (Chico et al., 2008) For investigation of this sample preparation procedure, we figured out the extraction with methanol, acetonitrile, acidified acetonitrile (containing 0.1 % HCOOH) and acetonitrile combined with Na_2EDTA (McIlvaine-buffer). In contrast to the obtained clean extract when using acetonitrile, the extraction with methanol provided a cloudy supernatant, which was not suitable for LC-MS/MS-injection.

For assessing the recovery rates [%] of the analytes, the obtained calculated values were corrected by the following sample preparation factor:

$$correction\ factor = \frac{2\ (sample\ weight)}{10\ (extraction\ solvent)} = \mathbf{0.2}$$

Results are demonstrating that this kind of sample preparation is not efficient enough to achieve satisfying recovery rates for all compounds in the investigated matrix. According to the obtained recoveries, the matrix shows suppressing as well as enhancing effects to the analytes. Especially the group of quinolones showed an increasing effect comparing the recovery rates obtained with the different extraction solvents. While the extraction with acetonitrile alone shows recovery rates up to 270%, the addition of Na_2EDTA increased the recovery up to 1100%. As described by Herrera-Herrera et al., quinolones are forming complexes with EDTA which can improve the extraction from the matrix. (Herrera-Herrera, Hernández-Borges, Rodríguez-Delgado, Herrero, & Cifuentes, 2011)

Within the group of macrolides and lincosamides erythromycin A was not detectable. Best results for this group was obtained by extraction with acidified acetonitrile achieving a mean recovery rate of 63%. The extraction with acetonitrile alone let to assume, that the matrix is not precipitated and enhancing the signals of the analytes.

According to the group of ß-lactams, this kind of sample preparation could only extract three out of six substances, whereas the extraction with acetonitrile alone enhancing the signal up to 800 % recovery rates.

The best extraction solution for the group of sulfonamides was the combination of acetonitrile with Na_2EDTA, achieving a mean recovery rate of 98%. Extraction with acetonitrile alone demonstrate an strong enhancing effect of the matrix on the recovery.

In contrast to the expectations to prevent chelate formation by using Na_2EDTA, tetracyclines could not be extracted with this combination. The direct injection of the non-volatile Na_2EDTA salt and the resulting ion suppression within the mass spectrometer could be responsible for these results. Comparing the results of extraction with acetonitrile and combined with HCOOH, the last extraction solution lead to acceptable recovery rates for all 4 substances, whereas the extraction with ACN alone increases the recoveries up to 340%. This could be explained by a more efficient protein precipitation when an acidified solution is used (Figure 42).

In conclusion, despite this sample preparation technique provides a simple and quick procedure, the matrix could not be removed sufficiently to achieve satisfying results of the analytes for implementation into routine work. Therefore an additionally clean-up step is crucial for developing a successful routine method.

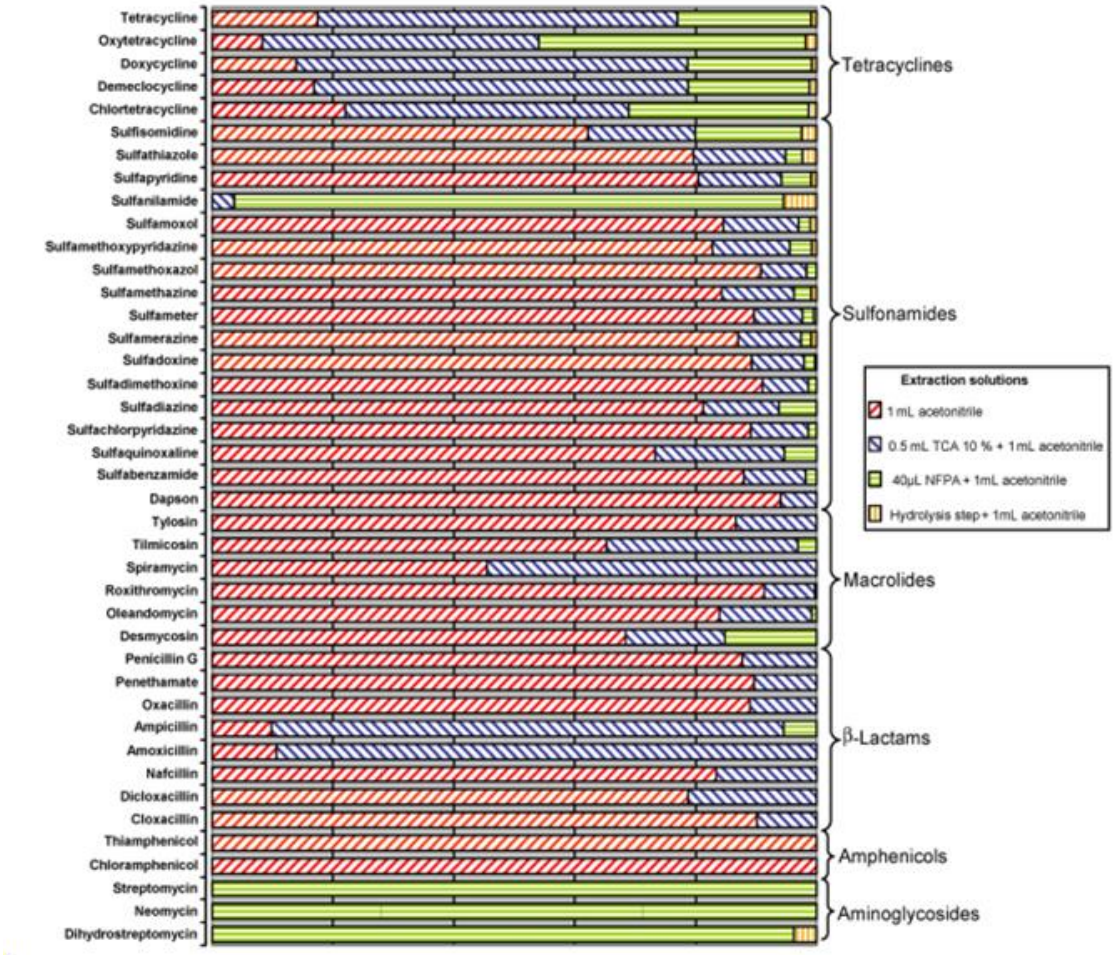

Figure 41: Relative recovery of each substance after extraction with different extraction solutions from spiked honey samples. (Hammel et al., 2008)

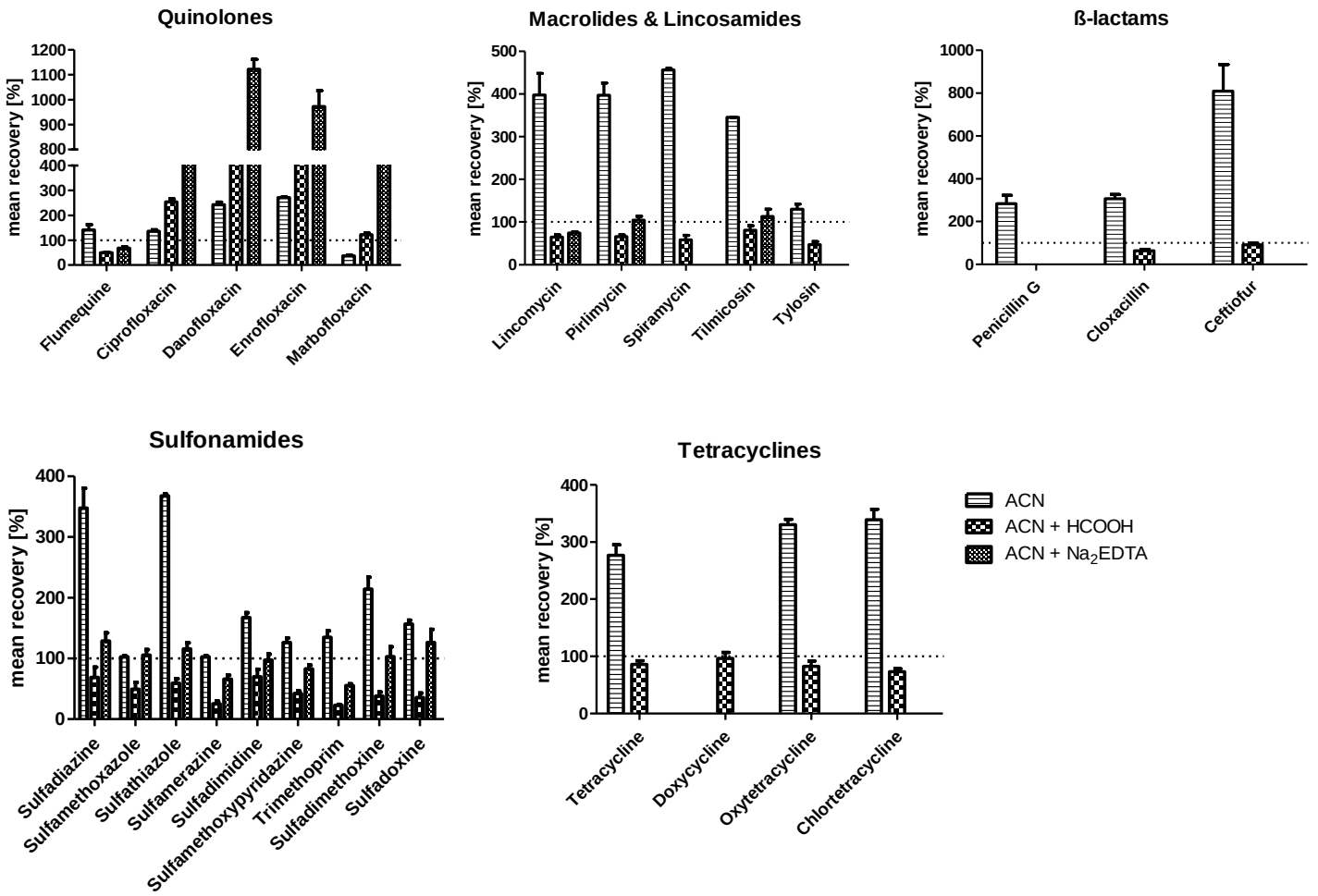

Figure 42: Mean recovery rates [%] ± SD of the analytes extracted from spiked bovine milk samples (n=5) after conducting liquid-liquid sample preparation using different extraction solutions.

3.2.2. QuEChERS (Quick Easy Cheap Effective Rugged and Safe) approaches

QuEChERS sample preparation revolutionized the analysis of pesticide residues in fruits and vegetables, since it provides a fast and effective extraction procedure for hundreds of pesticides. (Anastassiades et al., 2003) But this application is not limited to pesticides and is also developed for the determination of other substances, like acrylamide in various types of food matrices. (Mastovska and Lehotay, 2006). Further, several studies with modified QuEChERS approaches were published for the detection of veterinary drugs in different kind of animal derived food stuff. (Stubbings & Bigwood, 2009) (Aguilera-Luiz et al., 2008) (Aguilera-Luiz et al., 2008; Pérez, Romero-González, Vidal, & Frenich, 2013) The origin principle of QuEChERS describes a single-phase extraction with acetonitrile, followed by a liquid-liquid partitioning induced by addition of anhydrous $MgSO_4$ and NaCl. For removing residual water and possible matrix components the acetonitrile extract can be complemented by subsequent clean-up with $MgSO_4$ and dSPE salts. (Anastassiades et al., 2003) Especially fatty samples are cleaned-up with primary secondary amines (PSA) sorbent and C18 salts to remove possible fat residues from the acetonitrile extract. (Restek, 2012) PSA and C18 sorbents also effectively remove polar interferences like organic acids, colour pigments and sugars, providing thereby a more purified sample extract to inject into the HPLC-MS/MS system. (Anastassiades et al., 2003) (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), 2011)

Comparison of extraction solution – influence of Na_2EDTA on the recovery rate

Our experiments were conducted by fortifying 2.0 g of organic bovine milk samples with 100 µl of the antibiotic-multistandard mix to obtain a concentration level of 1/10 of their MRLs. For protein precipitation 10 ml of acidified acetonitrile was used to extract the analytes efficiently from the matrix. In regard to achieve a H_2O:ACN ratio of 1:1 to prevent agglutination of the extraction salts 8 ml H_2O or 8 ml of McIlvaine-buffer were added. Because the natural water content of bovine milk is approximately 90 % only 8 ml of water is necessary to achieve this ratio. By the addition of Na_2EDTA containing

McIlvaine-buffer we wanted to investigate the possible positive effect on quinolones and tetracyclines. As already described both compound groups tend to form complexes with divalent cations, which should be prevented by the addition of Na_2EDTA.

To assess the recovery rates [%] the obtained results must be corrected in order to the following correction factor, resulting from the dilution during sample preparation:

$$correction\ factor = \frac{2\ (sample\ weight\ in\ g)}{10\ (extraction\ solvent\ in\ ml)} = \mathbf{0.2}$$

As the results are demonstrating, Na_2EDTA is significantly influencing the extraction rate and thereby the recovery rates of all quinolones, which is explainable by the reduced formation of complexes with cations in the milk. (Figure 43)

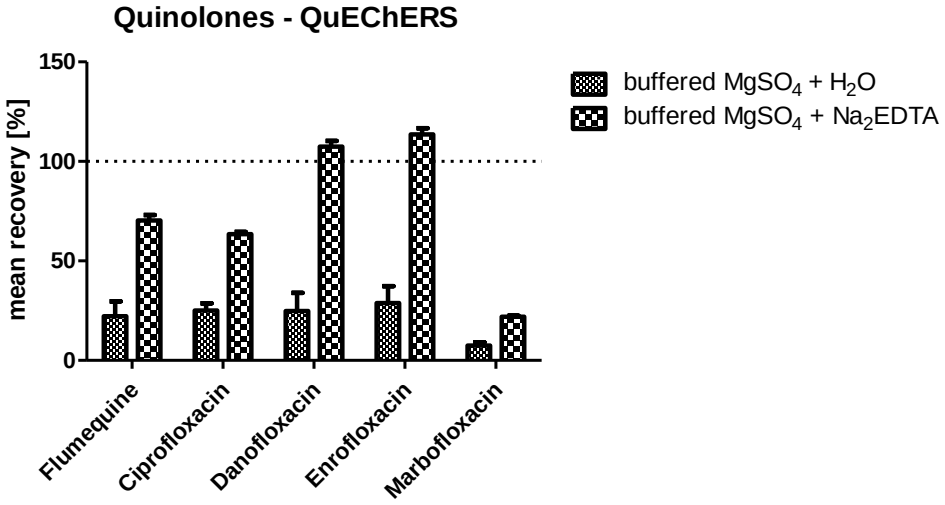

Figure 43: Comparison of H_2O and the influence of Na_2EDTA on the recovery rates [%] within the group of quinolones after QuEChERS sample preparation. *Shown are the mean recovery rates [%] ± SD, n = 5.*

To assess possible influences of the matrix in order to the recovery rates we calculated the obtained signals from the samples comparing to a matrix matched calibration curve. Therefore a calibration curve was prepared in extraction solvent containing matrix interferences instead of solvent solution without impurities. The results demonstrate that the matrix has a strong enhancing effect on the recovery rates especially to the

substances ciprofloxacin, danofloxacin and enrofloxacin, which can be reduced by applying a matrix matched calibration (Figure 44).

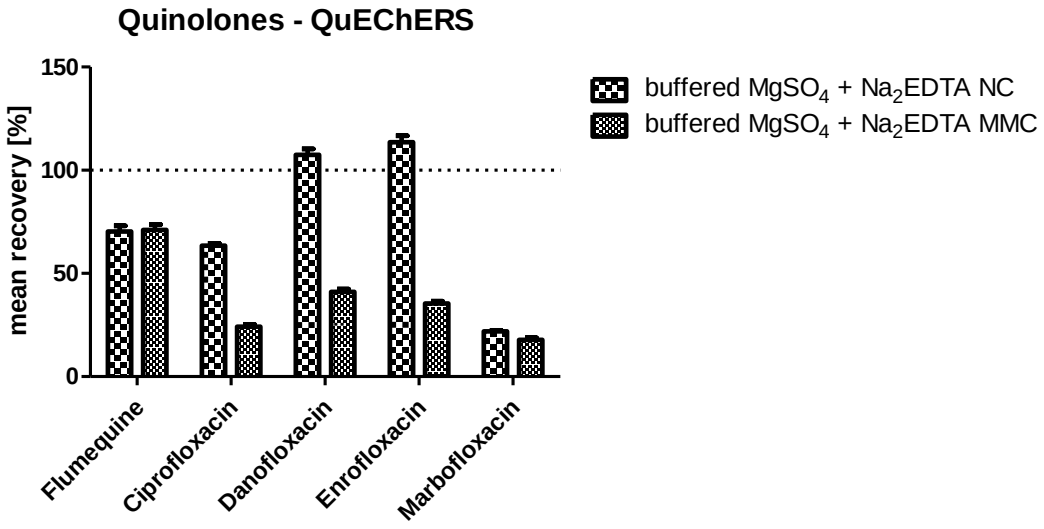

Figure 44: Quinolones quantified by normal calibration (NC) compared to matrix matched calibration (MMC) after QuEChERS sample preparation. *Strong matrix influences are observed, especially for the substances danofloxacin and enrofloxacin.*

This enhancing effect can also be demonstrated by comparing the two different 5 point calibration curves, showing a steep curve when matrix interferences are present in the solvent. (Figure 45)

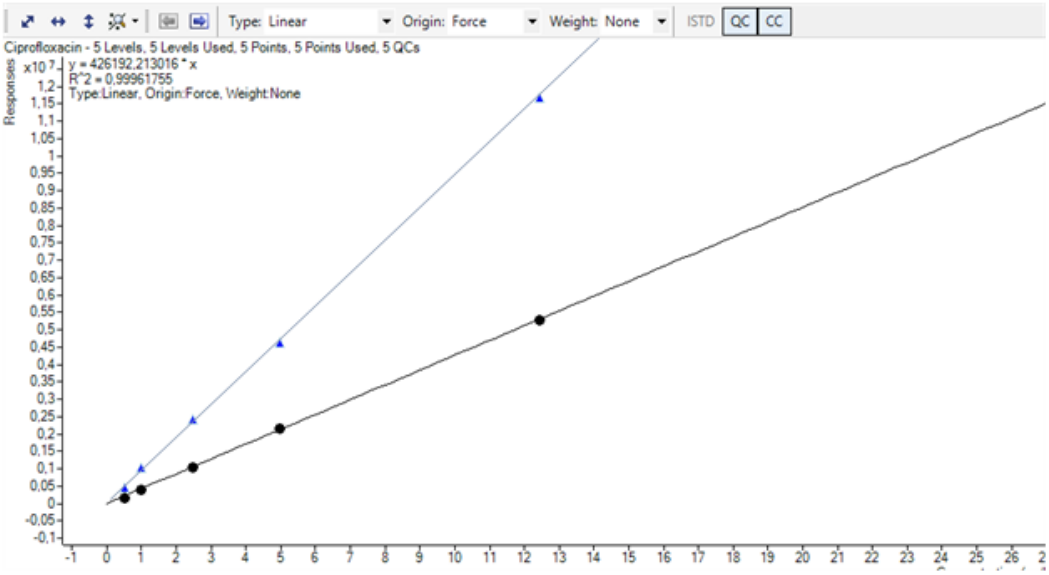

Figure 45: Five point calibration curve for ciprofloxacin in solvent (black) and in matrix containing solvent (blue).

In case of the group of sulphonamides there is a slight decrease of recovery rates observable, when Na_2EDTA is added to the extraction solution. Only in case of sulfadoxin there is a significantly increase when Na_2EDTA is used, compared to the use of H_2O alone in the extraction solvent (Figure 46). Additionally, it must be noted that these compounds are showing asymmetric peak shapes, which can be explained by the different solvents in the calibration and the samples, where the analytes are solved (Figure 47).

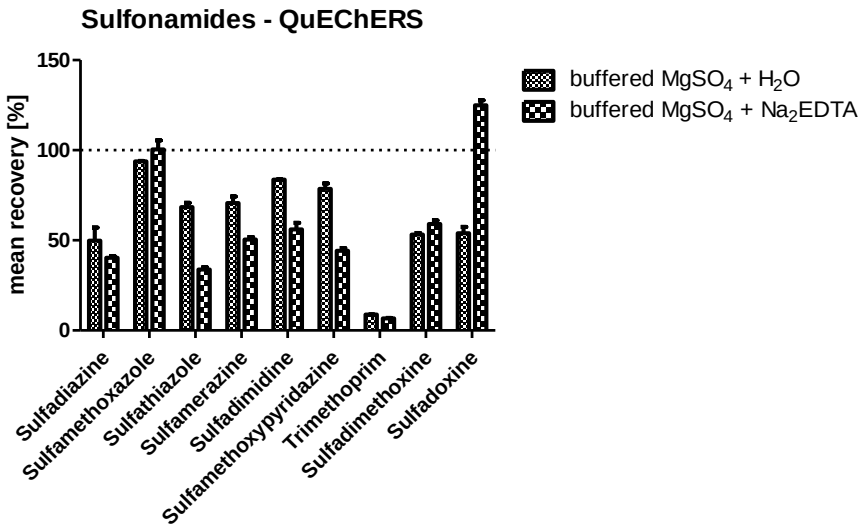

Figure 46: Comparison of H_2O and the influence of Na_2EDTA on the recovery rates [%] within the group of sulfonamides after QuEChERS sample preparation. *Shown are the mean recovery rates [%] ± SD, n = 5.*

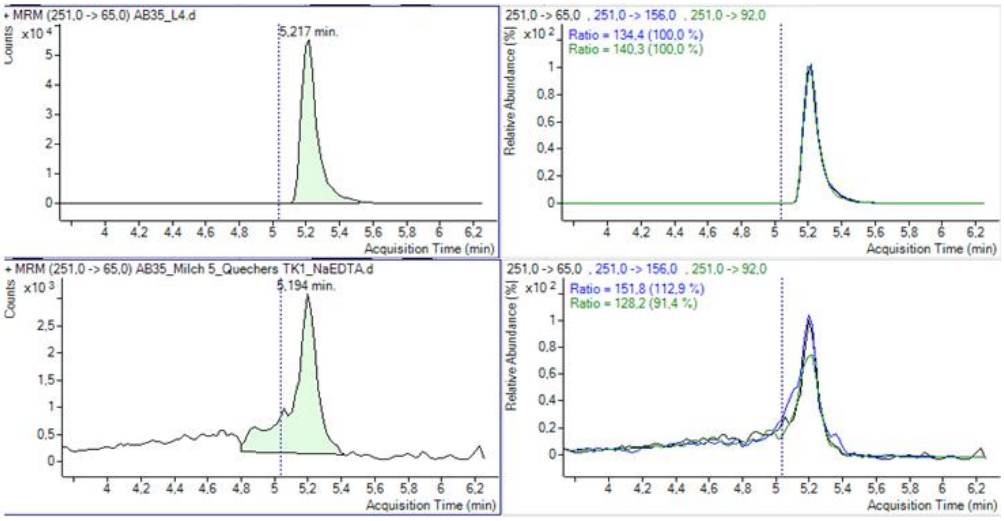

Figure 47: Sulfadiazine in **a)** calibration solvent and **b)** in acetonitrile after QuEChERS sample preparation, resulting in asymmetric peak shapes.

Substances from the other compound groups were not detectable after this type of extraction. This could either be explained by the separation of the polar phase by using the buffered $MgSO_2$ or by the present of matrix components, which are suppressing and disturbing the signal of the analytes.

3.2.2.1. Difference between buffered $MgSO_4$, $MgSO_4$ and Na_2SO_4

In this step we wanted to investigate the difference between using various extraction salts. Therefore we prepared organic milk samples as described above and separated the water by using the following different extraction salts:

- 4g $MgSO_4$ + 1g NaCl + 1g NaCitrate + 0.5 g disodiumcitrate sesquihydrate
- 4g $MgSO_4$ + 1g NaCl
- 4g Na_2SO_4 + 1g NaCl

As already described in the results of the previous experiment, also this experiment provided results only for the compounds of the quinolones and sulphonamides. All other substances could not be detected after this kind of sample preparation.

In case of the quinolones, extraction with Na_2SO_4 compared to $MgSO_4$ significantly increasing the recovery rates for all substances, except of flumequine. This could be explained by the chelate formation with divalent cations. Nevertheless, there is still a lot of matrix present in the sample, enhancing therefore the recovery rates, which is demonstrated in Figure 48 by comparing the sample results against solvent and matrix matched calibration.

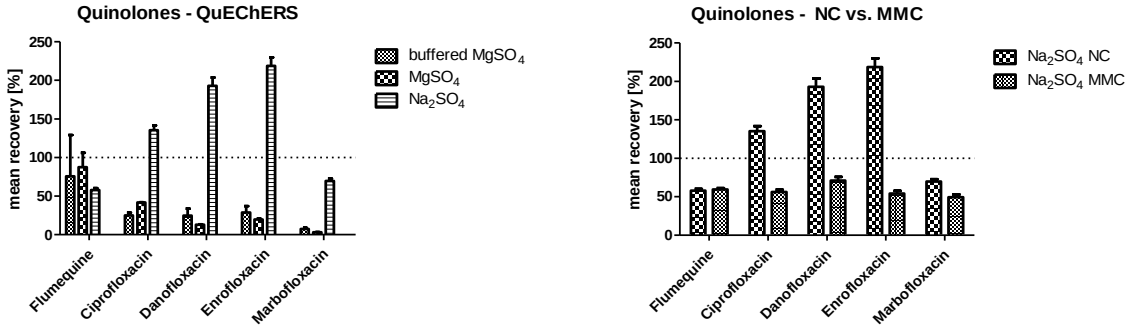

Figure 48: a) Comparison of recovery rates [%] for quinolones after using different extraction salts. ***Shown are the mean values ± SD (n=5).*** **b)** Evaluation of matrix influences by calculating the recovery rates against solvent and matrix matched calibration.

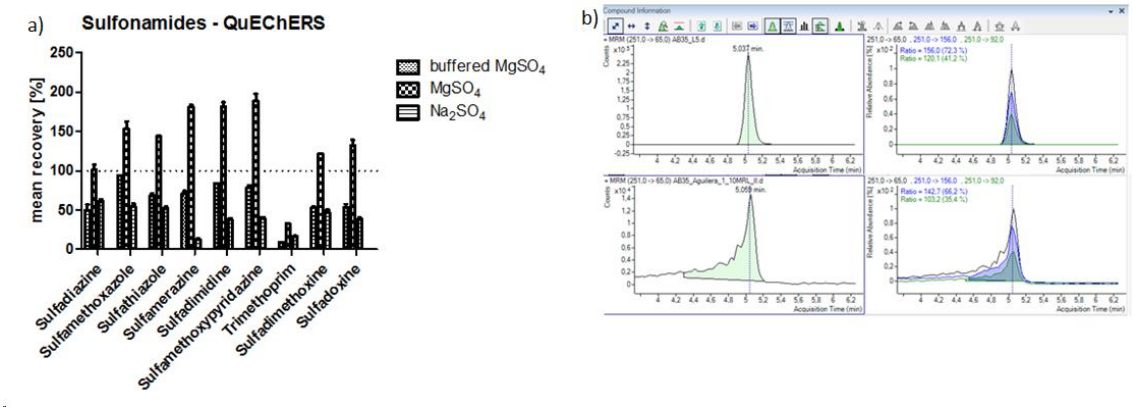

Figure 49: a) Comparison of recovery rates [%] for sulfonamides after using different extraction salts. *Shown are the mean values ± SD (n=5).* **b) Example for different peak shapes of sulphonamides after sample extraction.** *Chromatograms of sulfadiazine in calibration solvent and after sample preparation.*

For sulphonamides the best results were assessed after extraction with buffered $MgSO_4$ for all substances within this group. Additionally, the peak shapes are asymmetric due to the different solvents used for the calibration and the samples (Figure 49).

3.2.2.2. Additional clean-up with dispersive SPE sorbents

To reduce matrix interferences, especially proteins and fat, the samples were additionally cleaned up with PSA sorbent in combination with C18EC sorbent and $MgSO_4$, after extraction with buffered $MgSO_4$ salt as described above. In addition we

investigated if the amount of PSA influences the recovery rates of the analytes. Therefore the combinations were prepared as follows:

- 50 mg PSA + 150 mg C18EC + 900 mg Na_2SO_4
- 150 mg PSA + 150 mg C18EC + 900 mg Na_2SO_4

Further an aliquot of the cleaned supernatant was evaporated until dryness under a gentle nitrogen stream and subsequent reconstitution with initial mobile phase for improving the peak shapes. Therefore the correction factor for this sample preparation must be calculated as follows:

$$correction\ factor = \frac{2\ (sample\ weight\ in\ g)}{10\ (extraction\ solution\ in\ ml)}\ x\ \frac{4\ (supernatant\ in\ ml)}{2\ (initial\ mobile\ phase\ in\ ml)} = \mathbf{0.4}$$

As the obtained results are demonstrating there is no significant difference between the used amounts of PSA for better sample clean-up (Figure 50). However, the application of PSA sorbent indicates the absorption of the analytes, especially the compounds belonging to the sulphonamides group, due to the reduced recovery rates compared to the sample preparation without PSA. Within the group of quinolones, the substances danofloxacin and enrofloxacin are showing high recovery rates, assuming that the matrix is still influencing and thereby enhancing their signals. In order to prove this effect, the samples were also calculated using a matrix matched calculation, where a significantly decrease of their mean recovery rates can be observed (Figure 51).

The additional evaporation step improves the peak shapes for the sulphonamides, assuming, that this step is crucial for further sample preparation experiments to obtain symmetric peak shapes. (Figure 52). Despite these modified QuEChERS approaches provided a simple and fast preparation technique, only two out of five substance groups could be analyzed. Further, to achieve acceptable recovery rates the analytes would have to be quantified by using a matrix matched calibration, which is not useful in routine laboratories.

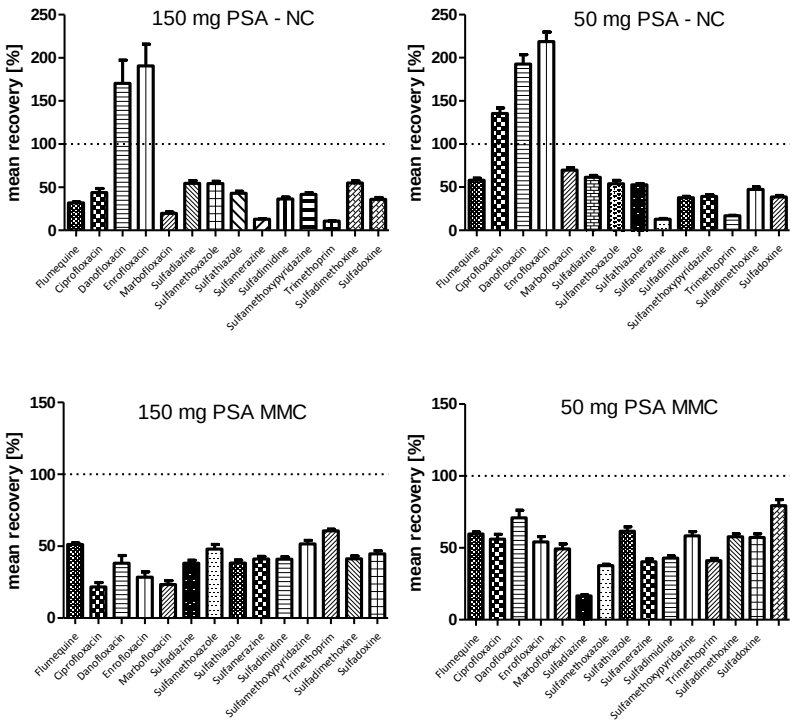

Figure 50: Comparison of different amounts of PSA sorbent. *Shown are the mean recovery rates [%] ± SD (n = 10)* quantified by calculating against solvent calibration (NC) and matrix matched calibration (MMC).

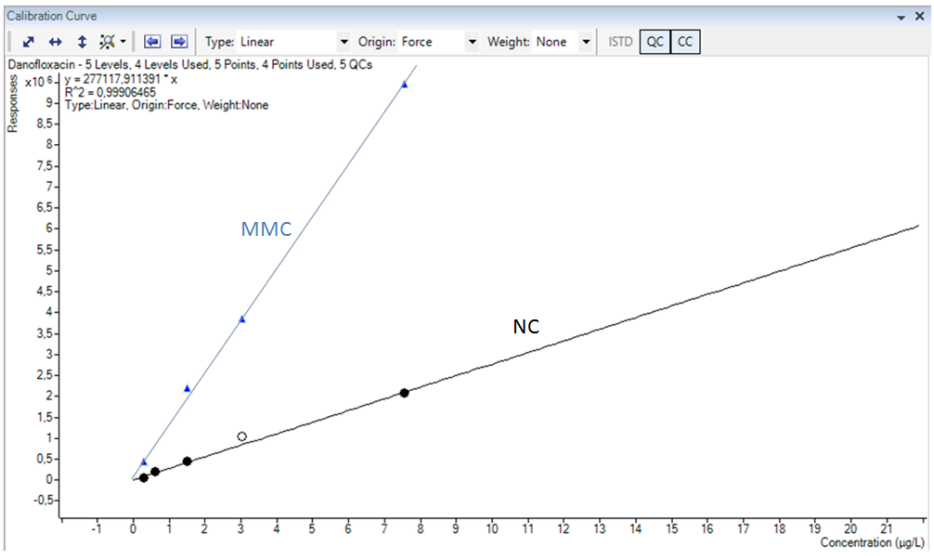

Figure 51: ***Five point calibration curve for ciprofloxacin in solvent (black) and in matrix containing solvent (blue).***

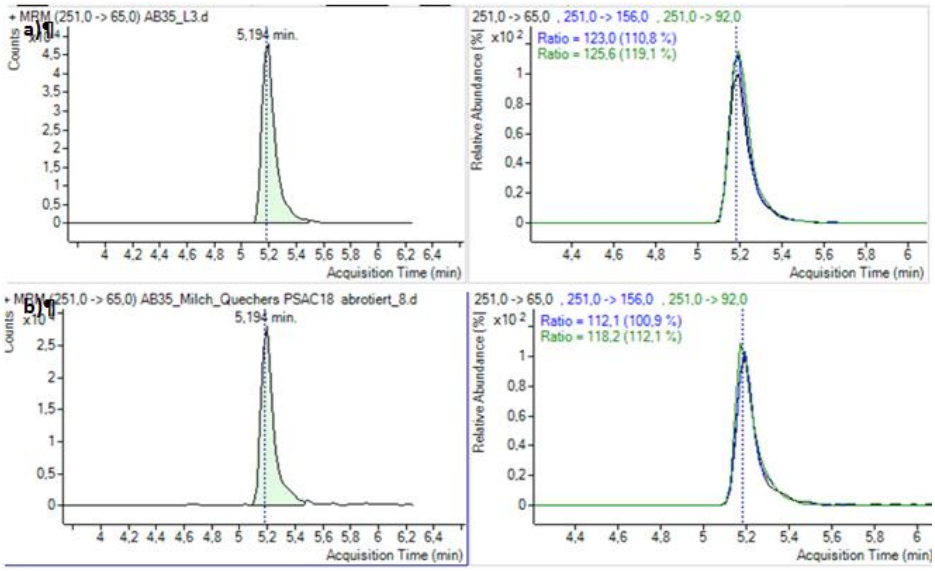

Figure 52: Improvement of the peak shape of sulfadiazine in a) calibration solvent and **b)** after sample clean-up with additional evaporation.

3.3. Solid Phase Extraction (SPE)

Solid phase extraction clean-up improves usually the matrix removal from the samples, thus should increases the recoveries of the analytes. In order that our compounds of interest are show retention on a C18 reverse phase analytical HPLC column, we conducted our experiments by using a SPE cartridge containing 500 mg endcapped hydrophobic C18 sorbent. For activation of the sorbent, the cartridge had to be conditioned with 6 ml MeOH and equilibrated with 6 ml H_2O. After loading the sample supernatant possible interferences should be removed by an additional washing step with 6 ml H_2O. The analytes get eluted from the sorbent by using 6 ml MeOH which was evaporated and reconstituted for injection into the HPLC-MS/MS system. Because these steps were not variable, we investigated different extraction solutions in regard to achieve satisfying recovery rates.

3.3.1. Extraction with TCA (20%) and McIlvaine-buffer

For our experiments 2.0 ± 0.1 g of organic milk samples were weight in and subsequently spiked with 100 µl of the antibiotic-multistandard to achieve a concentration level of 1/10 of their MRLs. In order to the standardized method provided from the Federal Office of Consumer Protection and Food safety (BVL) in Germany, for achieving accreditation level, we were committed to perform their sample preparation guideline, using TCA (20%) for protein precipitation and McIlvaine-buffer for extraction of the analytes. (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), 2011) To assess the recovery rates [%] the obtained measured results must be adjusted with the following correction factor:

$$correction\ factor = \frac{2\ g\ (sample\ weight)}{8.1\ ml\ (extraction\ solvent)}\ x\ \frac{8.1\ ml\ (supernatant)}{2\ ml\ (initial\ mobile\ phase)} = 1$$

The results obtained by this type of extraction and additional SPE clean-up provides chromatograms demonstrating good peak shapes for all substances (Figure 53). Except of the substance erythromycin A and the whole group of ß-lactams, all other substances were detectable. For the groups of macrolides/lincosamides and tetracyclines recovery

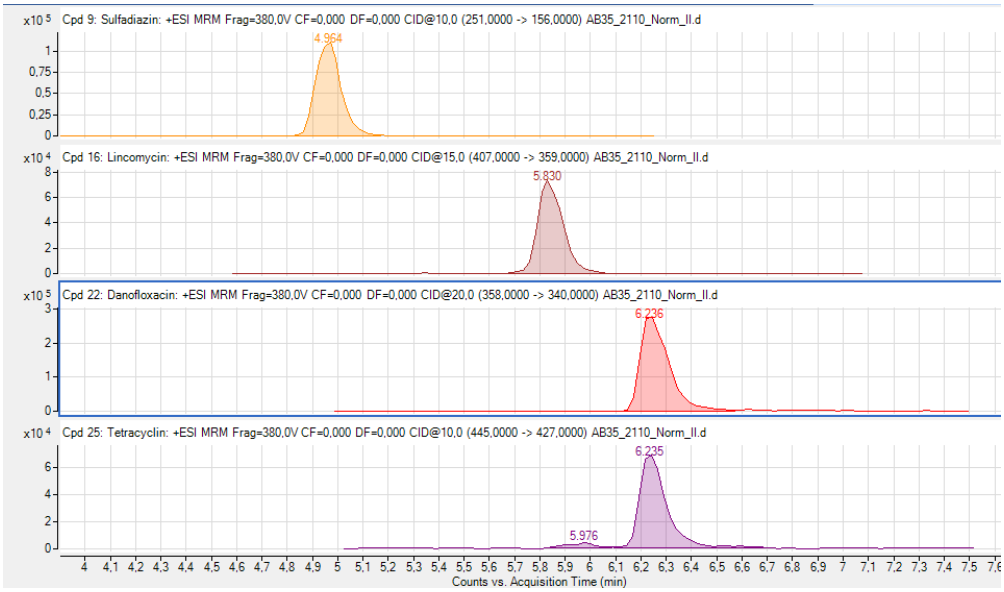

Figure 53: Examples of peak shapes after SPE extraction from each compound group.

rates between 70 – 120 % were obtained, whereas the substance tilmicosin shows a recovery rate of nearly 300 %. Nevertheless, the group of quinolones only achieved recovery rates between 10 and 50 %, and most of the substances belonging to the sulphonamides are below a recovery rate of 50 % (Figure 54).

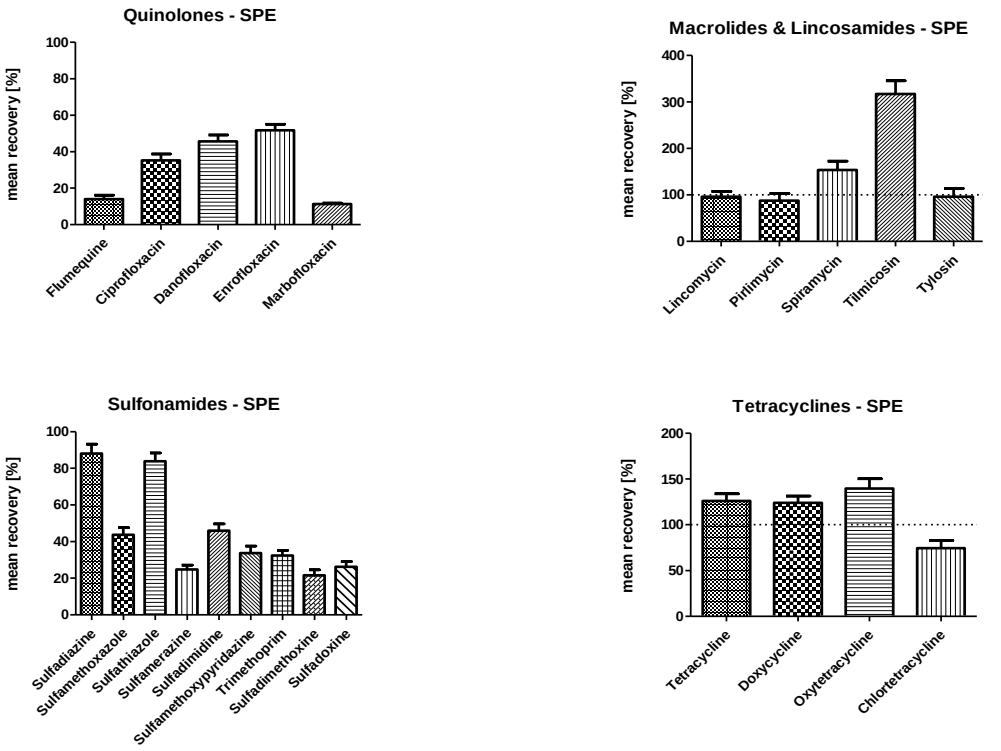

Figure 54: Mean recovery rates [%] ± SD (n = 9) after extraction with TCA and McIlvaine buffer and SPE clean-up.

3.3.2. Extraction with ACN

As described in literature, acetonitrile is the most prevalent solution to extract veterinary drugs from food matrices, due to its ability to precipitate the containing proteins to ensure a clean supernatant. For retaining the analytes on the SPE sorbent, the organic part of the supernatant should not exceed 20% and must therefore be diluted with H_2O. For our experiments 2.0 ± 0.1 g of organic milk samples were spiked with 100 µl of antibiotic-multistandard solution and subsequently extracted with 2 ml

of acetonitrile alone or in combination with 100 µl of McIlvaine buffer. The received supernatant was diluted with 18 ml of H_2O and applied on the previous conditioned SPE cartridge and eluted with 6 ml MeOH. The eluate was evaporated until dryness and reconstituted with 2 ml of initial mobile phase mix and filtered for injection into the HPLC-MS/MS system.

For assessing the recovery rates the obtained results must be corrected by calculating with the following correction factor:

$$correction\ factor = \frac{2\ g\ (sample\ weight)}{2\ ml\ (extraction\ solvent)}\ x\ \frac{2\ ml\ (supernatant)}{2\ ml\ (initial\ mobile\ phase)} = 1$$

The obtained results are demonstrating that the addition of EDTA slightly increases the recovery rates of the quinolones, as well as the recoveries for tetracycline and oxytetracycline. Also the sulphonamides sulfadimethoxine and sulfadoxin are positively influenced by the addition of EDTA. Nevertheless, most of all substances are below the optimum recovery rate range between 70–120% and only two compounds of the tetracycline group were detectable. Additionally erythromycin A and the group of ß-lactams could not be detected, too (Figure 55).

In order to these results we investigated possible losses during sample preparation, by analysing the eluate directly after sample application and the washing solution. With this step we observed that the compound sulfadiazine is getting lost during the washing step, since the analyte was detected in this solution. Tetracyclines were also found after second elution indicating that tetracyclines are retained in the stationary phase maybe explaining the low recoveries.

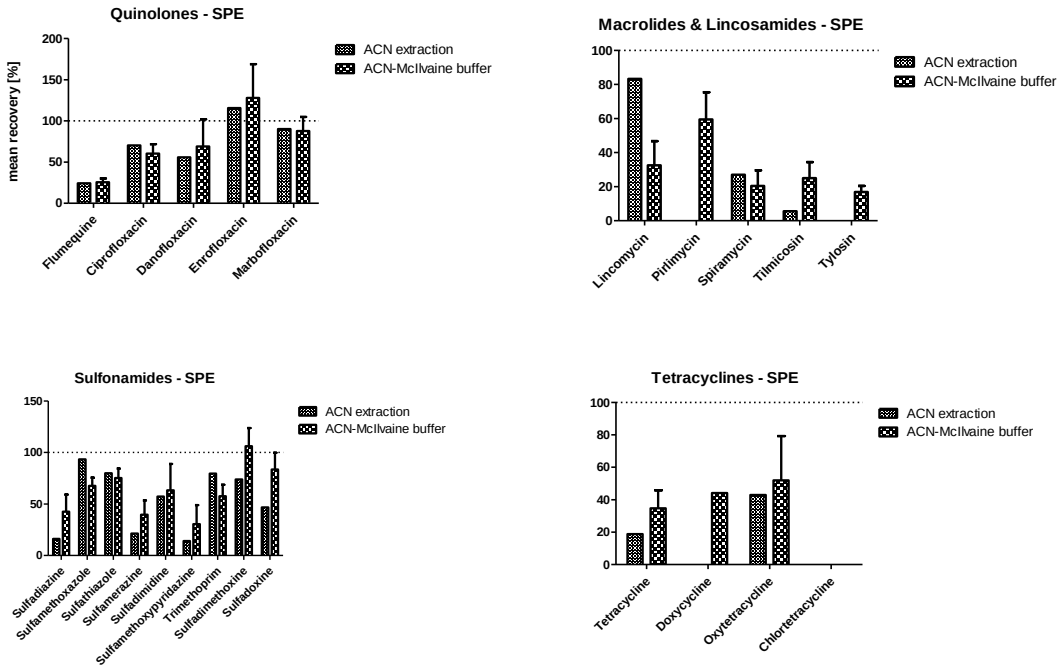

Figure 55: Comparison of mean recovery rates [%] ± SD (n = 9) after extraction with ACN alone and in combination with McIlvaine buffer and subsequent SPE clean-up.

3.4. Dispersive Solid Phase Extraction (dSPE)

Dispersive solid phase extraction (dSPE) is a combination of the easy and fast sample preparation provided by QuEChERS and the SPE principle to bind matrix co-extractives onto sorbents while the compounds of interest are remaining in the extract. Based on recent publications, in this work we investigated the sample clean-up with C18EC bulk sorbent alone as well as in combination with PSA or zirconia (ZSep) sorbents and dSPE-EMR salts.

3.4.1. Dispersive SPE with C18EC bulk sorbent

Initial experiments were conducted by investigating sample preparation with C18EC bulk sorbent in organic bovine milk samples. Therefore the 2.0 ± 0.1 g of the homogenized sample were weight into a 50 ml centrifuge tube and the analytes were extracted by

using 8 ml acetonitrile combined with 2 ml McIlvaine buffer. Subsequently, the complete supernatant was transferred to a centrifuge tube filled with 500 mg C18EC bulk sorbent and after shaking and centrifugation 5 ml of the obtained supernatant is transferred into a 15 ml tube. This supernatant was evaporated by using N_2 until dryness and reconstituted with 2 ml initial mobile phase.

The obtained results must be corrected with the following correction factor, to calculate the recovery rates:

$$correction\ factor = \frac{2\ g\ (sample\ weight)}{10\ ml\ (extraction\ solvent)} * \frac{5\ ml\ (transferred\ supernatant)}{2\ ml\ (initial\ mobile\ phase)} = 0.5$$

First results demonstrated that nearly all substances could be detected, except penicillin V, amoxicillin and ceftiofur, assuming that these substances are getting rapidly degraded. Unfortunately only 12 substances obtained good recovery rates between 70–120% (Figure 56). Therefore matrix effects were evaluated by quantifying with matrix

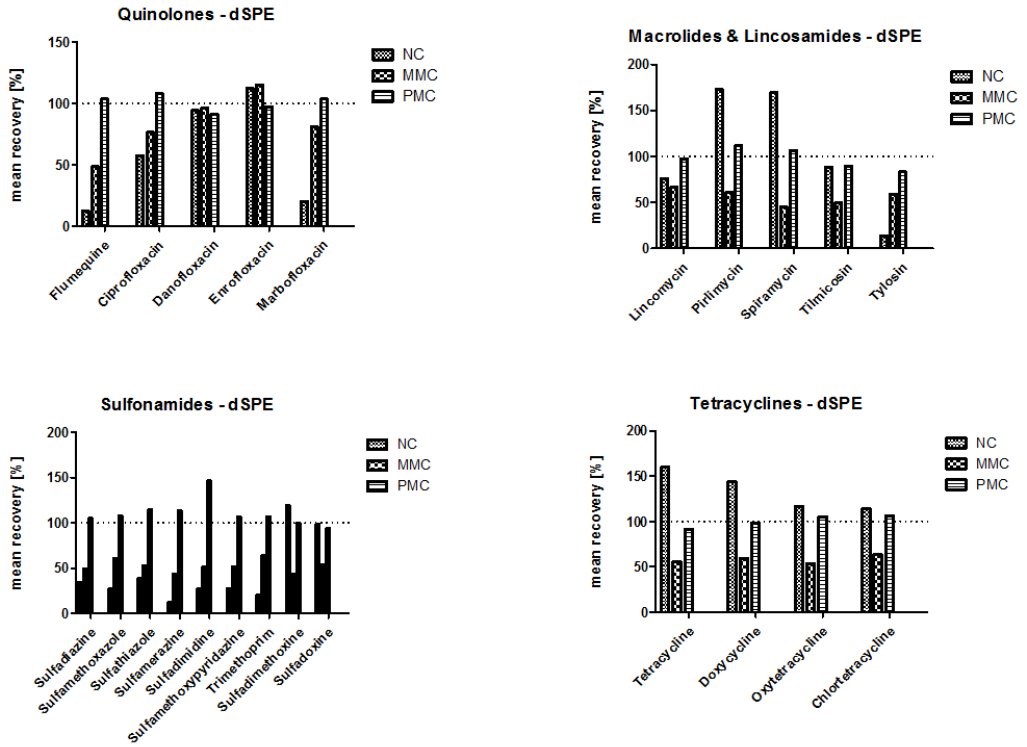

Figure 56: Comparison of mean recovery rates [%] after extraction with ACN alone and in combination with McIlvaine buffer and subsequent C18EC-dSPE clean-up. *Recovery rates were quantified by calculating against normal calibration (NC), matrix matched calibration (MMC) and procedure matched calibration (PMC).*

matched calibration demonstrating increasing but also decreasing adjustments of recovery rates. This knowledge let to assume that the matrix is not the most important influence factor, subsequently performing quantification by procedure matched calibration to eliminate possible influences from the matrix and sample preparation. In accordance with this quantification acceptable recovery rates were obtained for all substances (Figure 57).

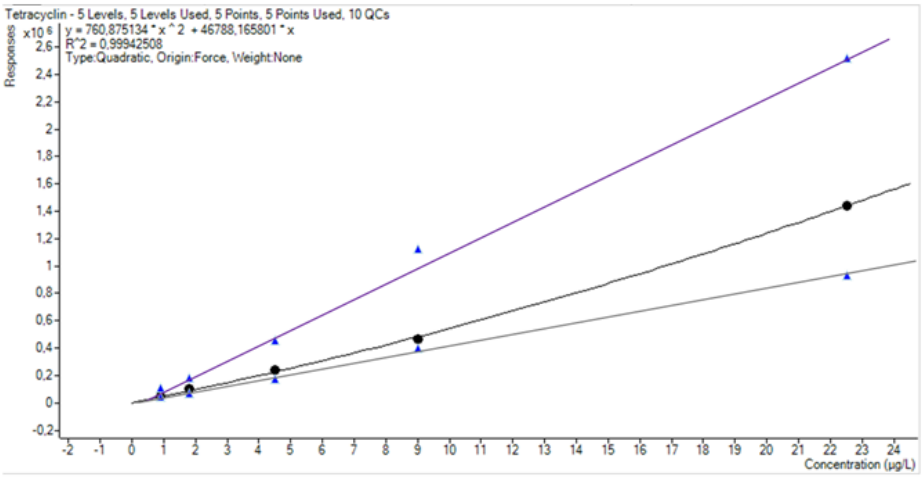

Figure 57: *Five point calibration curve for tetracycline in solvent (black), in matrix containing solvent (pink) and after sample preparation procedure (grey).*

However, the application of procedure matched calibration for various kinds of matrices is not suitable in a routine laboratory, indicating that the use of internal standard substances is essential.

In order to confirm these findings this sample preparation technique was additionally tested in further dairy products using ISTD mix solution to correct procedure and matrix effects (Figure 58). For optimization of the extraction solution, the already described positive effect of Na_2EDTA on tetracyclines was tested, receiving better results for all tetracyclines compared to the extraction only with water (Figure 59).

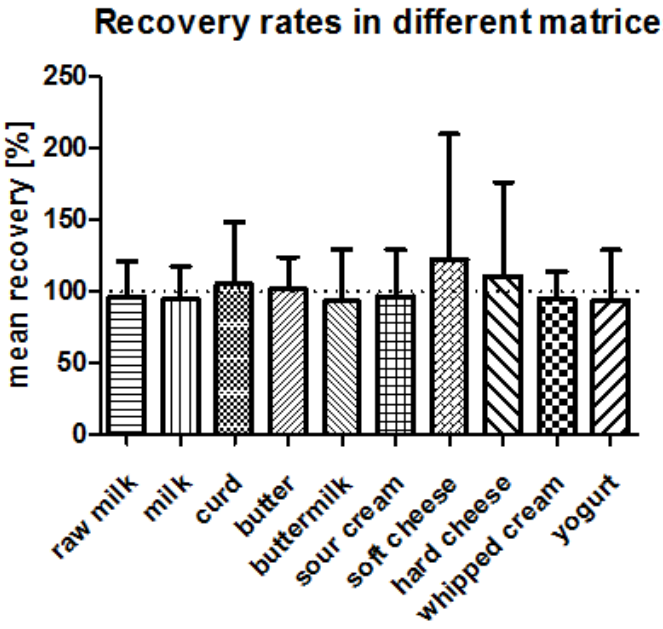

Figure 58: Mean recovery rates of all compounds in different types of matrices using ISTD to correct possible procedure and matrix interferences.

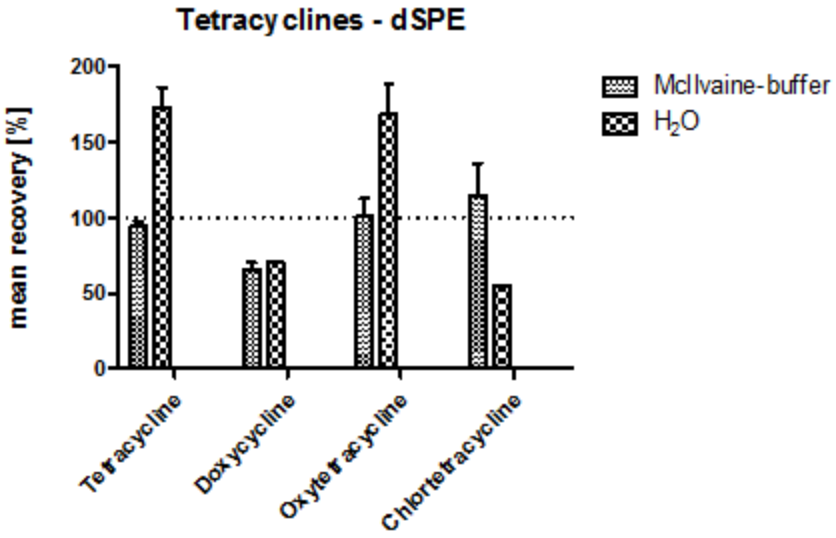

Figure 59: Influence of Na_2EDTA to the recovery of tetracyclines. Shown are the mean recovery rates [%] ± SD (n = 3).

3.4.2. Dispersive SPE with C18/ZSep, C18/PSA and EMR bulk sorbents

With reference to the main components of dairy products, experiments were additionally carried out with C18/ZSep (usually used for fatty samples), C18/PSA (for removing proteins) and dSPE-EMR (for fat content > 5%) in expectation to reduce matrix interferences more effectively.

Sample clean up with the combination C18/ZSep showed recovery rates near 100% for sulphonamides and quinolones, but low recovery rates for macrolides/lincosamides and tetracyclines (Figure 60). Furthermore the quality of the symmetric peak shapes for quinolones and tetracyclines were decreased (Figure 61).

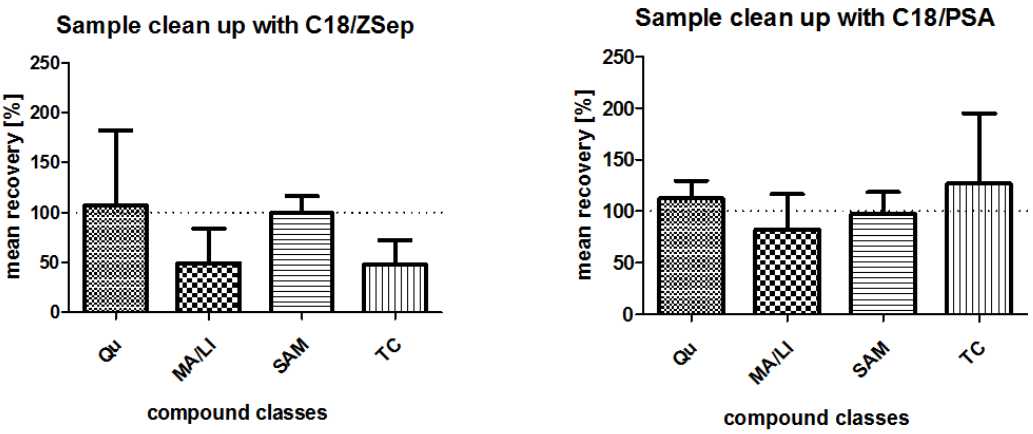

Figure 60: Comparison of the mean recovery rates [%] ± SD (n=5) within the compound groups, after sample clean-up using C18 bulk sorbent combined with ZSep or PSA. *Qu=quinolones, MA/LI=macrolides/lincosamides, SAM=sulphonamides, TC=tetracyclines.*

The combination C18/PSA indicated mean recovery rates of nearly 100% for all compounds in all matrices (Figure 60). Nevertheless, quinolones and tetracyclines showed a decrease in their peak shapes and it was not possible to analyze doxycycline.

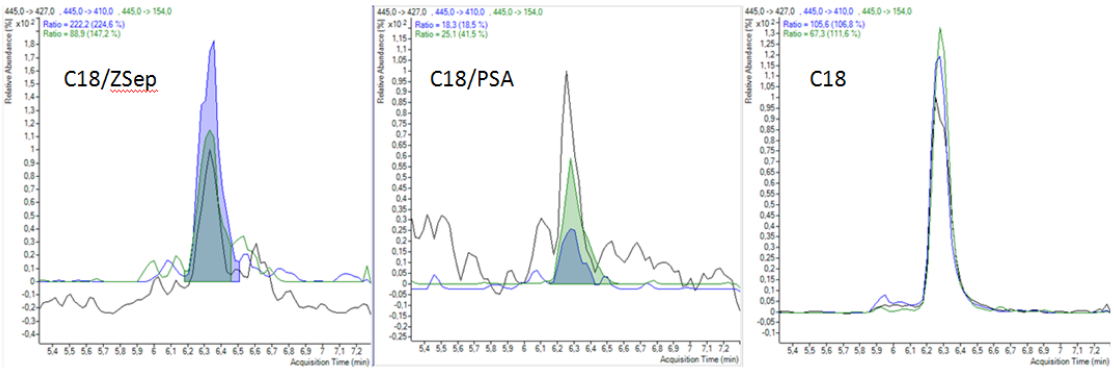

Figure 61: Peak shape of tetracycline after using C18/ZSep, C18/PSA or C18 bulk sorbent for sample clean-up.

According to the published application from Agilent Technologies (Agilent Technologies Inc., 2015) EMR-lipid-dSPE salts should be used in combination with final-EMR-lipid-polish salt to improve recovery rates of the compounds of interest. Corresponding to conducted experiments the best results for all substance groups were obtained without using final-EMR-lipid-polish salt, consequently further tests were examined only with EMR-lipid-dSPE. Recovery rates in different matrices (milk, cream, butter, cheese) after clean-up with C18EC and EMR-lipid-dSPE were compared pointing out significant differences in the group of tetracyclines (**Figure 62**).

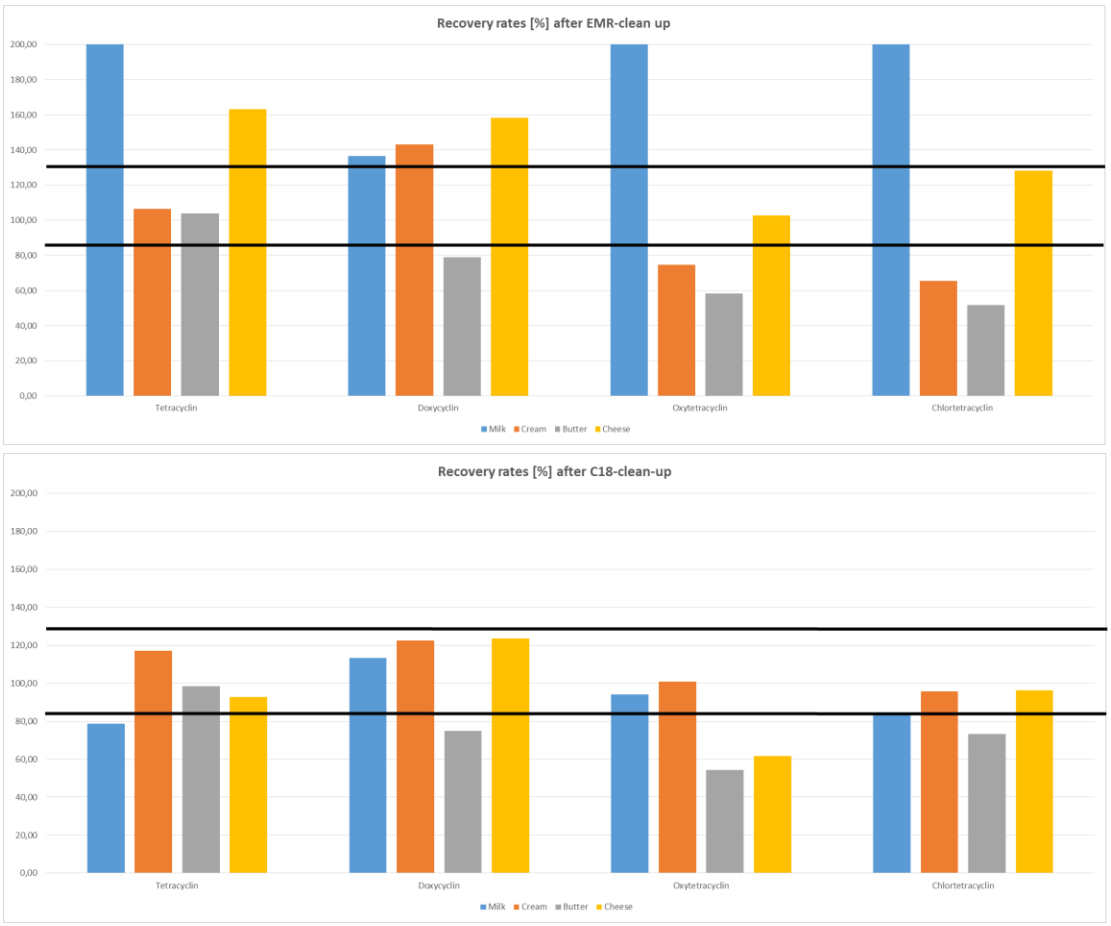

Figure 62: Mean recovery rates [%] (n=3) after EMR clean-up and C18 clean-up.

Additionally the reproducibility and robustness of the different clean-up salts was tested by injecting the same samples (milk) 20 times (scan type: dMRM):

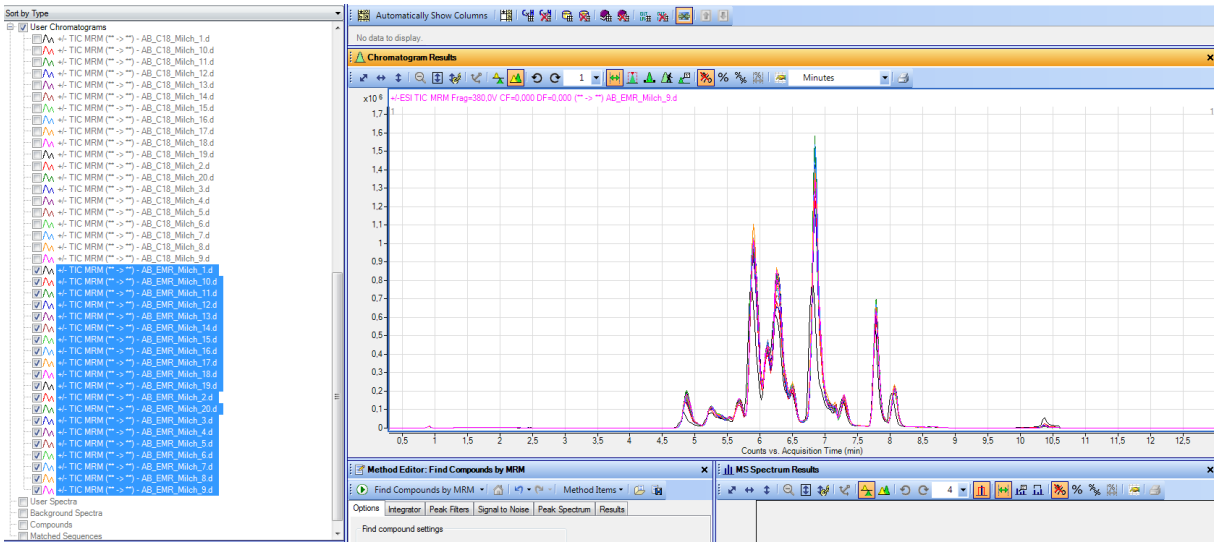

Figure 63: Robustness of EMR clean-up.

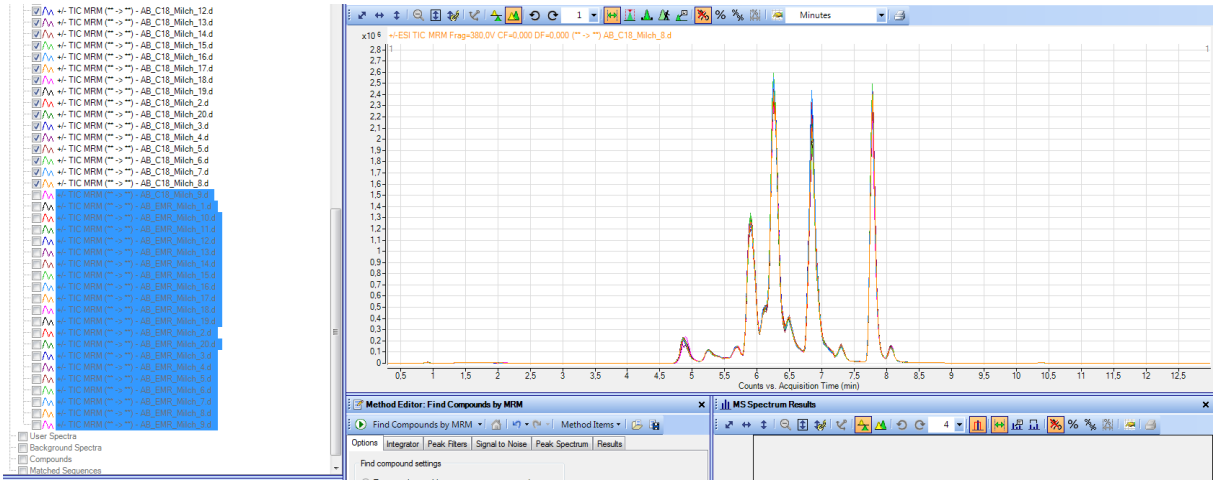

Figure 64: Robustness of C18 clean-up.

These chromatograms indicate better responses, reproducibility and precision for samples with C18-clean-up (Figure 63 and Figure 64).

Regarding all these experiments the best results for all compounds in all matrices were achieved with Na_2EDTA-ACN extraction and C18EC clean up as described above (chapter 3.4.1).

3.5. Method validation

3.5.1. Selectivity

The selectivity of an analytical method is necessary to determine if any interferences in the sample which could overlay the signals from the analytes or maybe provide 30.00 positive signals. For determination of the selectivity of a method a chromatogram containing all analytes is compared to a chromatogram containing all analytes and possible interferences.

To assess the selectivity of our method, a total ion chromatogram from the analytes in solvent is compared to total ion chromatograms containing different matrix interferences. Regarding the obtained chromatograms, there are no severe influences caused by matrix interferences, meaning that there are no interferences leading to signal suppression of the analytes or producing 30.00 positive signals (Figure 65 and Figure 66).

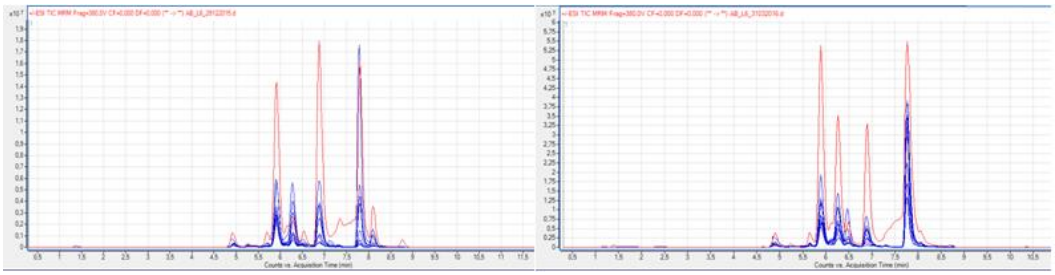

Figure 65: Total ion chromatograms in solvent (red) compared to the TICs in matrices (blue). *Left are shown the chromatograms in dairy matrices, and right are demonstrated the chromatograms in meat matrices.*

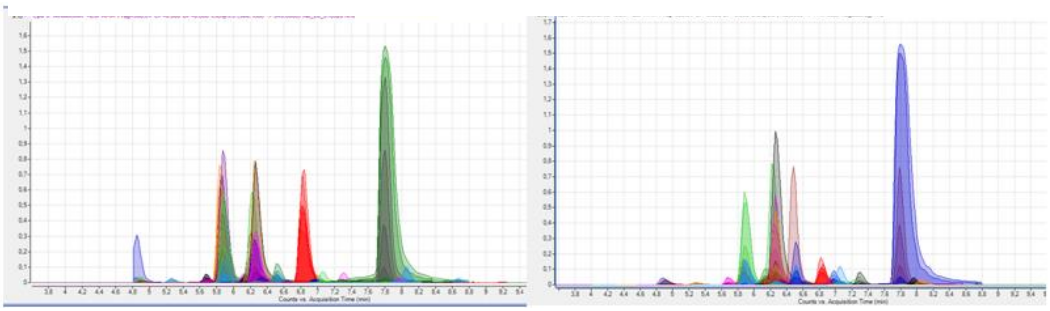

Figure 66: Extracted chromatograms showing the analytes in the solvent (left) and in the matrix salmon (right).

3.5.2. Limit of detection (LOD) and limit of quantification (LOQ)

By using the terms LOD and LOQ the capacity of a method can be described, whereas the LOD defines the smallest concentration of an analyte which can be detected in regard of a specific level of confidence and the LOQ describes the smallest concentration of a compound which can be measured in regard of precision and trueness. (Kromidas, 2011)

LOD and LOQ of the analytes were determined by calculation the signal-to-noise ratio, whereas a signal-to-noise ratio of > 3 is necessary for establishing the LOD and for the LOQ a ratio of > 10 is needed. To obtain the signal-to-noise ratio of an analyte in a matrix, the signal of the blank is determined as "noise" and is calculated against the signal of the analyte in the matrix (Figure 67).

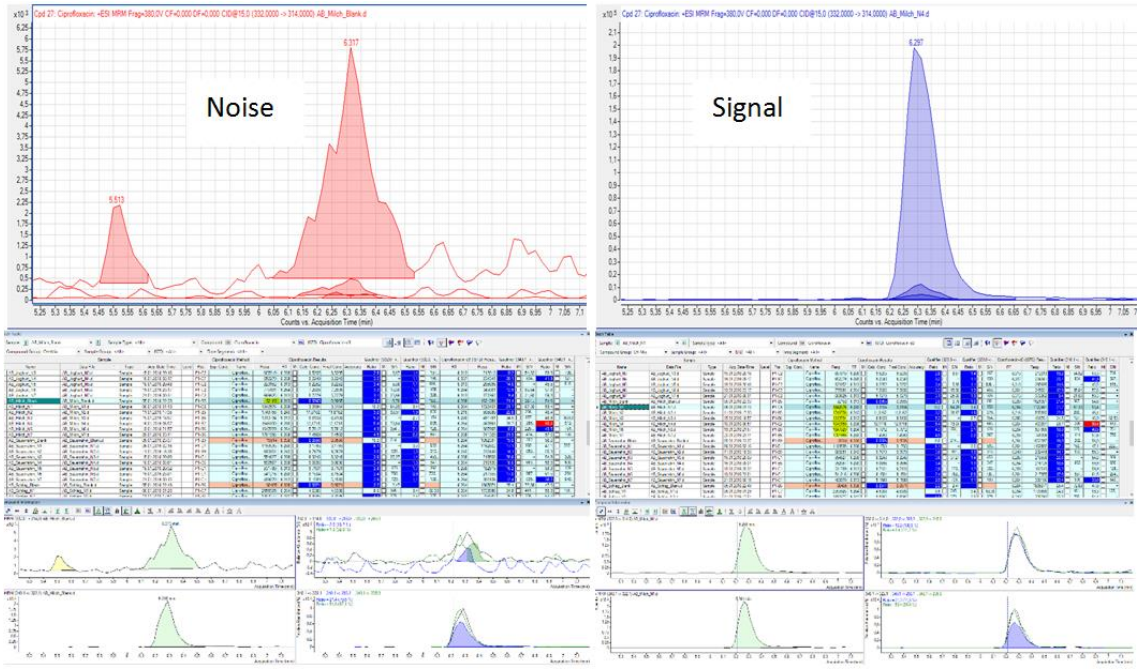

Figure 67: Example for determination of the signal-to-noise ratio for ciprofloxacin.

For example, the signal-to-noise ratio of ciprofloxacin in milk is obtained by dividing the average of the low spiked response values by the response value of the blank sample:

$$signal - to - noise = \frac{1.4\ mio}{52{,}180} = 26.83$$

Because the signal-to-noise ratio is higher than 10 for the low spiked concentration, this concentration can be set as LOQ and LOD can thereby be assessed by dividing this concentration by the factor 3.

LODs and LOQs for all substances in all matrices are listed in appendix III.

3.5.3. Accuracy

3.5.3.1. Trueness

Trueness describes a part of the term accuracy and is defined as the deviation of a measurement compared to the reference value.

There are different ways existing to determine the trueness of a method, whereby we examined the trueness by measuring certified reference materials (CRM) together with the spiked samples for validation.

- *Measurement of CRM*

  The certified reference materials were for veterinary drug residues were obtained from Test Veritas S.r.l. The matrix was lyophilized partially defatted raw bovine milk naturally incurred with antibiotic substances. Each CRM was reconstituted with double distilled water according to their instruction and subsequently prepared simultaneously with the validation samples.

  Each CRM was prepared and measured five times and to determine the trueness of this method the relative bias and recoveries were calculated (Table 17).

CRM-no.	Matrix	Compound	Relative bias (%)	Relative recovery (%)
1414-1	Incurred raw bovine milk	Doxycycline	19,48	119,48
1321-1	Incurred raw bovine milk	Oxytetracycline	16,78	116,78
1414-2	Incurred raw bovine milk	Enrofloxacin	12,13	113,80
1414-3	Incurred raw bovine milk	Ciprofloxacin	23,57	80,93
1320-2	Incurred raw bovine milk	Sulfamethazine	13,52	115,64
1320-3	Incurred raw bovine milk	Sulfamerazine	18,98	123,42
1320-4	Incurred raw bovine milk	Sulfadiazine	26,96	136,91

Table 17: Summary of the relative bias and recoveries.

- *Recovery rates from spiked samples*

  The obtained recovery rates resulting from the validation are listed in appendix III. As acceptable recovery rate a range between 70–120% is set, otherwise a correction must be performed if the substance is present in a sample.

3.5.3.2. Precision

The precision of an analytical method is defined as the closeness of agreement between a series of measurements. For our method, we examined the repeatability of the method, by preparing the samples on different days under same operation conditions. Precision is expressed by calculation the standard deviation and is listed for all matrices and compounds in appendix III. A relative standard deviation below 20% is determined to be an acceptable value.

4. CONCLUSION

The discovery of penicillin by Sir Alexander Fleming and the introduction of the first commercially available antibiotic “Prontosil” at the beginning of 1930 revolutionized the medicine in the last century. A large number of antibiotics was found and introduced into the market during the decades, and they can be separated in accordance to their chemical properties and their mechanisms of action.

Since the demand of animal protein for human consumption is increasing worldwide, especially due to the transformation of the eating habits in developing countries, the intensive livestock production requires an increasing amount of veterinary drugs. (Boland et al., 2013; Van Boeckel et al., 2015) In order to the increasing milk production in Asia, the protein supply quantity per capita raised more than threefold from 1961 to 2011. (online source: FAOstat, accessed on: 22nd October 2016)

The officially used amount of antimicrobial substances belongs to approximately 15,000 tons in the United States and 9,000 tons 29 European countries, whereas the most sold groups are tetracyclines, followed by the group of penicillins, sulphonamides and ionophores. (European Medicines Agency, 2012; Food and Drug Administration - Department of Health and Human Services, 2015) Adding pharmaceutical substances to feed and water represents the main application route for animals for preventing diseases as well as for enhancing the growth and feed efficiency. (Food and Drug Administration - Department of Health and Human Services, 2015; Sarmah et al., 2006a)

The main concern about the misuse and overuse of antibiotics is the development of a worldwide antibiotic resistance. Antibiotics can enter into the aquatic system because they are generally excreted by urine and feces. Residues can already be determined in the influents and effluents of wastewater treatment plants, river water, groundwater and drinking water in concentrations between ng/l to µg/l. (Carvalho & Santos, 2016)

The more antibiotics are used in veterinary and human medicine, the higher is the risk that bacteria are developing resistant strains, which can enter different routes to spread within the environment (Figure 12). The main resistant strains, e.g. the multiple antibiotic-resistant *salmonellae*, macrolide- or quinolone-resistant *campylobacters*, or the multiple antibiotic-resistant *E. coli* are present in food producing animals and can thereby directly be transmitted via food chain to the human. (Newell et al., 2010; Phillips et al., 2004)

To protect customers from possible further uptake of antibiotics from animal food stuff, the European Union set maximum residue levels (MRLs) in the Council Regulation 37/2010, regulating pharmacologically active substances and their classification regarding MRLs in foodstuffs and animal origin. (The European Commission, 2010)

In accordance to these MRLs this work describes a confirmatory UHPLC-MS/MS routine method to quantify 30 antibiotic substances belonging to the compound groups of quinolones, macrolides, lincosamides, ß-lactams, sulphonamides, diaminopyrimidine-derivate and tetracyclines. For achieving accreditation level for this method, the substances were chosen regarding the standardized method provided from the Federal Office of Consumer Protection and Food safety (BVL) in Germany (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), 2011) and extended with the group of ß-lactams. To offer a broad spectrum of matrices to our customers the final method was validated in ten dairy matrices (raw milk, pasteurized milk, curd, sour cream, yogurt, soft and hard cheese, cream, butter and buttermilk) and ten different fish and meat matrices (chicken, prawns, salmon, trout, muscle (pig), fat, liver (pig), muscle (beef), liver (beef) and kidney (beef)).

One critical claim for this work was to develop one extraction method for all analytes and all matrices. As described by Berendsen & Nielen, the main sample preparation procedures for the extraction of antibiotics from different matrices are:

- Dilute and shoot (liquid-liquid-extraction)
- QuEChERS - ***Qu****ick* ***E****asy* ***Ch****eap* ***E****ffective* ***R****ugged and* ***S****afe*
- Solid Phase Extraction (SPE) (Berendsen & Nielen, 2013)

In our work, we investigated the different sample preparation techniques by using organic bovine milk sample fortified with an antibiotic-mix resulting in a concentration of 1/10 of their MRLs.

Dilute and shoot

This easy and quick approach was conducted by using methanol, acetonitrile, acidified acetonitrile (containing 0.1 % HCOOH) and acetonitrile combined with Na_2EDTA (McIlvaine-buffer). While the supernatant obtained from methanol extraction was cloudy and not suitable for LC-MS/MS injection, the acetonitrile alone and in combination provided a clean supernatant for injection. This could be explained by the ability of acetonitrile to precipitate proteins, which are highly containing in milk. (Freitas et al., 2014)

Results are demonstrating that the extraction with acetonitrile alone provides recovery rates up to 400 % within all different groups, which could be explained by still present matrix interferences, which are able to enhance the recovery rates. In contrast to these results, the combination with formic acid let to assume that the protein precipitation is more sufficient, except within the group of quinolones, where recovery rates up to 400 % were obtained. By the addition of EDTA the formation of chelates between divalent cations and tetracyclines should be avoided. (Chico et al., 2008) In contrast to our expectations, tetracyclines could not be detected anymore and the recovery of the quinolones increases up to 1100%. One explanation therefore could be the complex forming ability of quinolones with EDTA. (Herrera-Herrera et al., 2011) Within the group of ß-lactams, only three out of six substances were detectable.

These findings demonstrate that this easy and fast sample preparation approach is not sufficient to achieve satisfying recovery rates, due to the strong matrix influences.

*QuEChERS - **Qu**ick **E**asy **Ch**eap **E**ffective **R**ugged and **S**afe*

In the first step of QuEChERS sample preparation we investigated the influence of EDTA by using McIlvaine-buffer instead of H_2O on the chelate forming tetracyclines and quinolones. The results from this experiment demonstrate that the use of EDTA positively influences the recovery rates of quinolones, which could be explained by their reduced formation of complexes. By assessing the matrix influences by quantifying the recovery rates using a matrix matched calibration a strong enhancing effect can be demonstrated for the compounds ciprofloxacin, danofloxacin and enrofloxacin. In case of the sulphonamides, a slight decrease on their recovery rates was observed when EDTA is used. Additionally, the analytes showed asymmetric peak shapes due to the different solvents in the calibration and the samples, where the analytes are solved. All other substances were not detectable after this type of sample preparation, explainable due to the present of matrix components or the separation of the polar phase.

Further, we investigated if there is a difference between the extraction salts Na_2SO_4, $MgSO_4$ and buffered $MgSO_4$. The obtained results let to assume, that the quinolones are forming complexes with Na_2^{2+}, since the recovery rates are rising over 200%. Within the group of sulphonamides, the best results were achieved by using buffered $MgSO_4$.

For better matrix removal in addition to this extraction, the supernatants were additionally cleaned up with various amounts of PSA to remove protein and fat residues. With these experiments, we found that there is no difference when the amount of PSA varies and by quantifying with MMC the present of matrix interferences can be demonstrated.

Solid Phase Extraction (SPE)

For these experiments the analytes were extracted from the matrix either by using TCA combined with McIlvaine buffer or by using acetonitrile alone or in combination with McIlvaine buffer and subsequent the supernatant was cleaned up by using a reversed phase SPE cartridge containing 500 mg C18EC sorbent. The combination of TCA and

McIlvaine buffer provided good recovery rates for all tetracyclines as well as within the group of macrolides, while the groups of quinolones and sulphonamides are showing recovery rates below 50%. Better results were achieved for these compound groups by using acetonitrile for analytes extraction. Nevertheless, macrolides/lincosamides and tetracyclines were detected only at a recovery rate below 50%. The loss of tetracyclines were observed due to investigation of the eluate and washing solution, where these compounds were still found, let to assume, that these compounds are retaining on the C18EC material. Further, the group of ß-lactams was not detectable after this type of sample preparation.

Dispersive Solid Phase Extraction (dSPE)

This type of sample preparation combines the easy and fast sample preparation provided by QuEChERS and the SPE principle to bind matrix interferences. In this work we investigated the sample clean-up with C18EC bulk sorbent alone as well as in combination with PSA or zirconia (ZSep) sorbents and dSPE-EMR salts. While the sample clean up with PSA and ZSep sorbents were resulting in good recovery rates, the quality of the symmetric peak shapes for tetracyclines and quinolones were decreased. Satisfying recoveries (70–120%) were obtained for all compounds using C18EC bulk sorbent after extraction with ACN in combination with McIlvaine buffer and quantification with procedure matched calibration. This indicates that the addition of internal standards is essential to correct matrix interferences as well as losses during sample preparation procedure, since the use of MMC or PMC is not useful in routine laboratory.

The final developed method was validated in accordance to our in-house validation scheme in ten different dairy matrices (raw milk, pasteurized milk, curd, sour cream, yogurt, soft and hard cheese, cream, butter and buttermilk) and ten different fish and meat matrices (chicken, prawns, salmon, trout, muscle (pig), fat, liver (pig), muscle (beef), liver (beef) and kidney (beef)). From the validation data recovery rates (%), RSD

(%), limit of detection (LOD) and limit of quantification (LOQ) were assessed for all substances in each matrix, listed in appendix III.

For evaluation of the correctness of the method, we already successfully (z-score < ± 2.00) participated on different proficiency tests, provided by Test Veritas S.r.I. (Table 18)

Finally, the method was reviewed from the accreditation service "Akkreditierung Austria" and received the status of an accredited method in accordance to ISO 17025:9001. The method is offered as a routine method for the determination of antibiotic residues in food matrices by the company LVA GmbH.

Parts of this work was presented on two conferences (ANAKON 2015, RAFA 2015) in form of poster presentations (Appendix IV), as well as a paper submitted to the journal "Food analytical methods" where the manuscript is still under revision.

Proficiency test no.	**Matrix**	**Compound**	**z-Score**
MI 1623	Lyophilized bovine milk	Oxytetracycline	**1.26**
FI1641	Lyophilized fish	Chlortetracycline	**-0.80**
M1642	Lyophilized turkey muscle	Danofloxacin	**-1.30**
M1643	Lyophilized turkey muscle	Chlortetracycline	**-0.50**
		Tetracycline	**0.50**
M1644	Lyophilized swine muscle	Sulfadiazine	**1.60**
		Sulfadimethoxine	**0.30**
M1715	Lyophilized bovine milk	Oxytetracycline	**1.11**
		Danofloxacin	**1.50**

Table 18: Summary of the results from the participated proficiency tests.

5. SUMMARY

In line with the worldwide increasing demand of animal protein for the human consumption, the intensive livestock production requires an increasing amount of veterinary drugs. (Boland et al., 2013; Van Boeckel et al., 2015) The main risk of the severe overuse of antibiotics is a worldwide development of antibiotic resistance. Resistant bacterial strains may be present in food producing animals and can thereby directly be transmitted via food chain to the human. (Newell et al., 2010; Phillips et al., 2004)

To protect the customers from the uptake of antimicrobial substances by animal derived food stuff, the European Union set maximum residue levels (MRLs) in the Council Regulation 37/2010. (The European Commission, 2010)

In accordance to these MRLs this work aimed to develop a confirmatory UHPLC-MS/MS routine method to quantify 30 antibiotic substances belonging to the compound groups of quinolones, macrolides, lincosamides, ß-lactams, sulphonamides, diaminopyrimidine-derivate and tetracyclines. Since the finalized method will be offered as an accredited routine test method for the company LVA GmbH, a wide range of different kind of matrices were chosen (ten dairy matrices and ten different fish and meat matrices) for validation.

During method development phase different sample preparation approaches were investigated (Dilute-and-Shoot, QuEChERS and SPE) by using organic bovine milk samples fortified with an antibiotic-mix resulting in a concentration of 1/10 of their MRLs.

Results are demonstrating that satisfying recovery rates (70–120%) were only obtained by using C18EC bulk sorbent for sample clean-up after extraction with ACN in combination with McIlvaine buffer. Quantification of chosen substances were performed by using internal standards to correct matrix interferences as well as losses during sample preparation procedure.

The final developed method was validated in accordance to our in-house validation scheme, assessing recovery rates (%), RSD (%), limit of detection (LOD) and limit of quantification (LOQ) for all substances in each matrix, listed in appendix III. To proof the accuracy of the method, we successfully (z-score < ± 2.00) participated on different proficiency tests, provided by Test Veritas S.r.I. (Table 18)

Finally, the method was reviewed from the accreditation service "Akkreditierung Austria" and received the status of an accredited method in accordance to ISO 17025:9001.

6. ZUSAMMENFASSUNG

Aufgrund des weltweit steigenden Konsums von tierischen Proteins, ist die intensive Landwirtschaft darauf angehalten, den Einsatz von Antibiotika dementsprechend zu erhöhen. (Boland et al., 2013; Van Boeckel et al., 2015) Das größte Risiko des übermäßigen Einsatzes von Antibiotika in der Tierzucht, ist die Entwicklung von resistenten Bakteriensträngen, welche direkt über tierische Lebensmittel in den Menschen aufgenommen werden und somit eine mögliche Antibiotikaresistenz auslösen können. (Newell et al., 2010; Phillips et al., 2004)

Um die Konsumenten vor dieser Exposition zu schützen, wurden von der Europäischen Union im Rahmen der Verordung EU (VO) 37/2010 maximal zulässige Höchstwerte von pharmakologisch wirksamen Substanzen in tierischen Rohstoffen festgelegt. (The European Commission, 2010)

Basierend auf dieser Verordnung war das Ziel dieser Arbeit eine UHPLC-MS/MS Methode für die quantitative Bestimmung von 30 Analyten diverser Wirkstoffklassen (Quinolone, Makrolide, Lincosamide, ß-Laktame, Sulfonamide, Diaminopyrimidin-Derivate und Tetrazykline) zu entwickeln und anschließend zu validieren. Da die entwickelte Methode als akkreditierte Routineuntersuchungsmethode in der Firma LVA GmbH angeboten werden soll, wurde ein breites Spektrum an Matrizen (10 Milchprodukte, 10 Fleisch- und Fischprodukte) für die Validierung ausgewählt.

Zur Entwicklung der Methode wurden verschiedene Ansätze zur Probenaufabeitung (Dilute-and-Shoot, QuEChERS and SPE) mit Milchproben biologischer Qualität, welche mit einem Antibiotika-Mix auf eine Konzentration von 1/10 der MRLs dotiert wurden, durchgeführt.

Wie aus den Ergebnissen ersichtlich, konnten zufriedenstellende Wiederfindungsraten (70–120%) mittels der Kombination von ACN und McIlvaine-Puffer zur Proteinfällung und der anschließenden Aufreinung mit C18EC bulk Material erzielt werden. Die Quantifizierung erfolgt für ausgewählte Substanzen über den Zusatz eines internen Standards, um sowohl Matrixeffekte, als auch möglichen Analytenverlust während des Aufarbeitungsprozesses zu kompensieren.

Diese Methode wurde gemäß der in-House Validierungs Richtlinien validiert, wobei Wiederfindungsraten (%), Standardabweichung (%), Nachweis- (LOD) und Bestimmungsgrenze (LOQ) der Methode in allen Matrizen ermittelt wurde. Zur Überprüfung der Richtigkeit der Methode wurde erfolgreich (z-score < ± 2.00) an verschiedenen Ringversuchen des Anbieters Test Veritas S.r.l. teilgenommen. (Table 18)

Abschließend wurde die neu entwickelte und validierte Methode zur Überprüfung an das Akkreditierungsservice „Akkreditierung Austria“ eingereicht und erhielt nach erfolgreichem Audit den Status einer akkreditierten Methode nach ISO 17025:9001.

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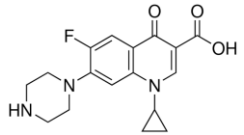
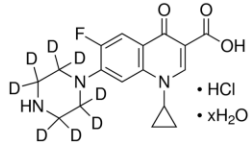
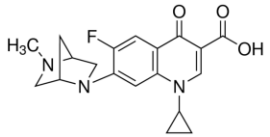
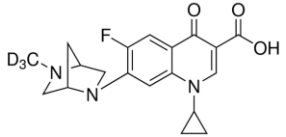
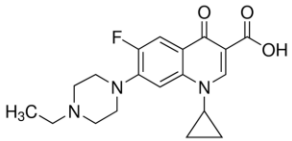
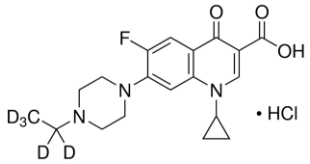
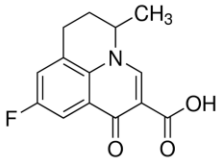
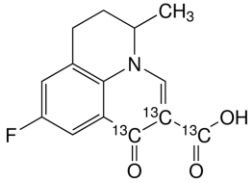
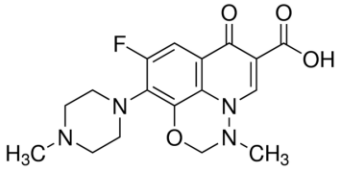
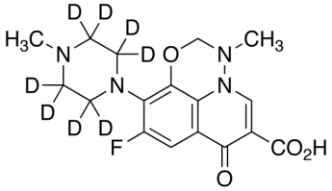
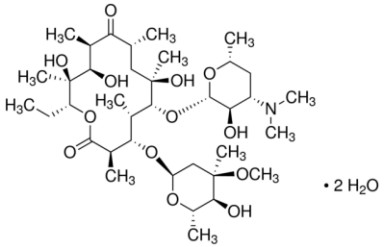
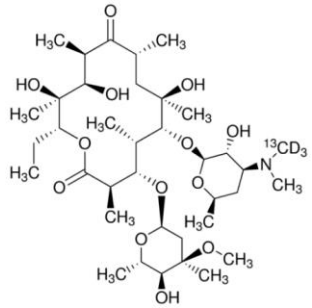
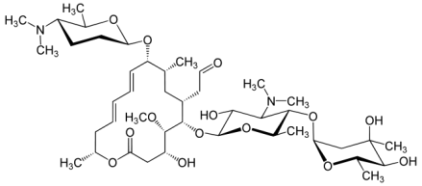
APPENDIX I – MAXIMUM RESIDUE LEVELS (MRLS) IN DIFFERENT MATRICES ACCORDING TO EC 37/2010 IN µG/KG

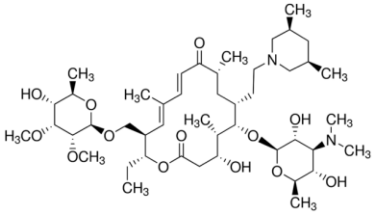
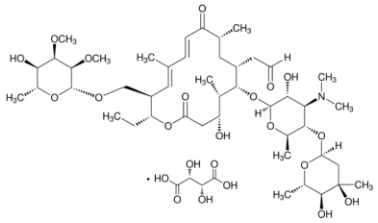
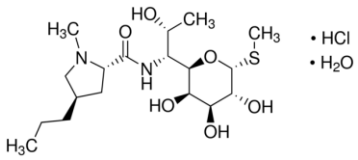
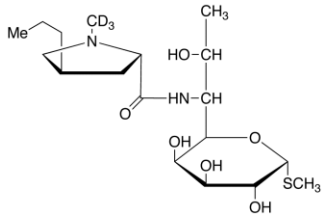
	Bovine	Muscle				Fat				Liver			Kidney		
	Milk	**Bovine**	**Pig**	**Chicken**	**Fish**	**Bovine**	**Pig**	**Chicken**	**Fish**	**Bovine**	**Pig**	**Chicken**	**Bovine**	**Pig**	**Chicken**
Amoxicillin	4	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Ampicillin	4	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Ceftiofur	100	1000	1000	1000	1000	2000	2000	2000	2000	2000	2000	2000	6000	6000	6000
Chlortetracycline	100	100	100	100	100					300	300	300	600	600	600
Ciprofloxacin	100	100	100	100	100	100	100	100	100	300	200	200	200	300	300
Cloxacillin	30	300	300	300	300	300	300	300	300	300	300	300	300	300	300
Danofloxacin	30	200	100	200	100	100	50	100	50	400	200	400	400	200	400
Doxycyclin	no MRL	100	100	100		300	300	300	300	300	300	300	600	600	600
Enrofloxacin	100														
Erythromycin A	40	200	200	200	200	200	200	200	200	200	200	200	200	200	200
Flumequine	50	200	200	400	600	300	300	250	250	500	500	800	1500	1500	1000
Lincomycin	150	100	100	100	100	50	50	50	50	500	500	500	1500	1500	1500
Marbofloxacin	75	150	150			50	50			150	150		150	150	
Oxytetracycline	100	100	100	100	100					300	300	300	600	600	600
Penicillin G	4	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Penicillin V	no MRL		25	25				25			25	25		25	25
Pirlimycin	100	100				100				1000			400		
Spiramycin	200	200	250	200		300	300			300	2500	400	300	1000	
Sulfadiazin	100	100													
Sulfadimethoxine															
Sulfadimidin															
Sulfadoxin															
Sulfamerazine															
Sulfamethoxazole															
Sulfamethoxypyridazine															
Sulfathiazole															
Tetracycline	100	100	100	100	100					300	300	300	600	600	600
Tilmicosin	50	50	50	75	50	50	50	75	50	1000	1000	1000	1000	1000	250
Trimethoprim	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Tylosin	50	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.															

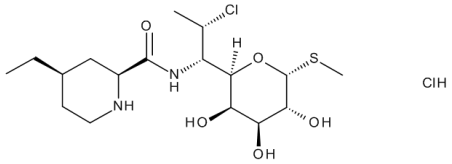
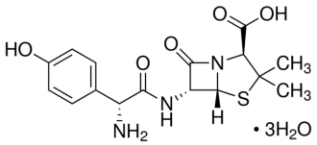
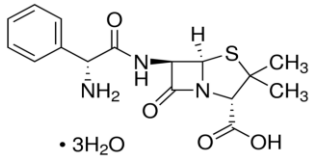
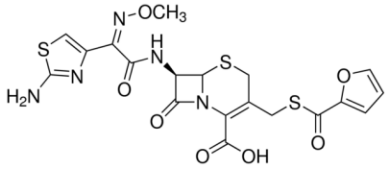
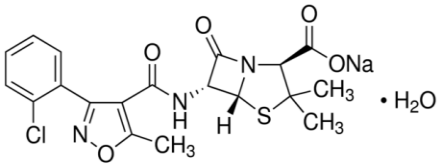
APPENDIX II – SUMMARY OF ALL MS/MS TRANSITIONS AND PARAMETERS

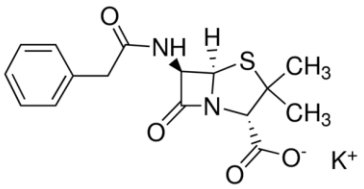
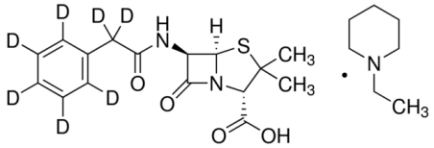
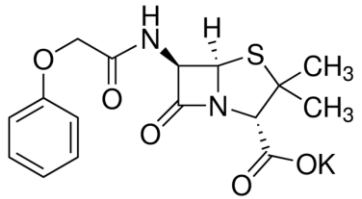
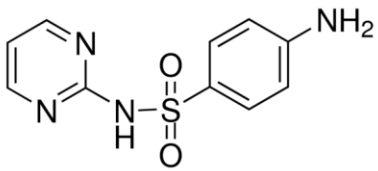
Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Quinolones							
Ciprofloxacin		331.4	332	314	6.3	15	1
				245		20	1
Ciprofloxacin-d_8		339.4	340	322	6.3	25	1
				235		45	1
Danofloxacin		357.4	358	340	6.3	20	1
				314		15	3
Danofloxacin-d_3		360.4	361	343	6.3	20	1
				317		15	3
Enrofloxacin		359.4	360	342	6.3	15	1
				245		20	1

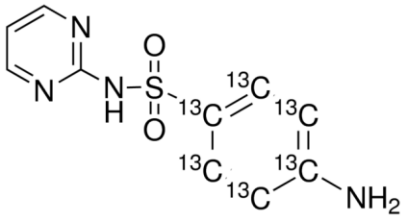
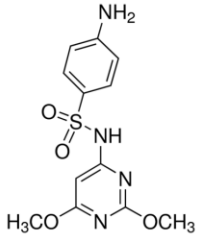
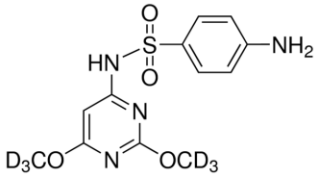
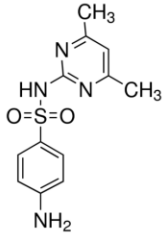
Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Enrofloxacin-d_5		364.4	365	347	6.3	20	1
				245		30	1
Flumequine		261.3	262	244	7.8	25	3
				202		35	5
Flumequine-$^{13}C_3$		264.2	265	247	7.8	15	1
				205		35	1
Marbofloxacin		362.4	363	320	6.0	15	1
				345		10	3
Marbofloxacin-d_8		370.4	371	79	6.0	25	1
				353		20	1

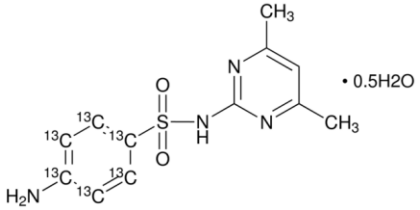
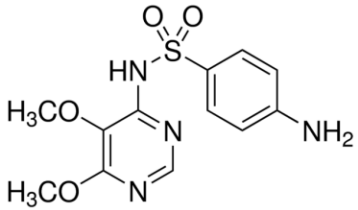
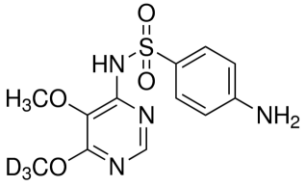
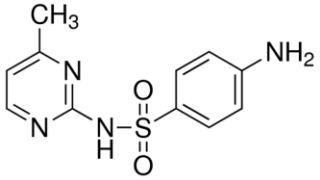
Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Macrolides							
Erythromycin A		733.9	734	158	8.0	35	1
				576		20	1
Erythromycin A – ^{13}C-d_3		737.9	739	580	8.0	15	1
				162		30	3
Spiramycin		843.1	844	174	6.9	40	1
				101		45	1

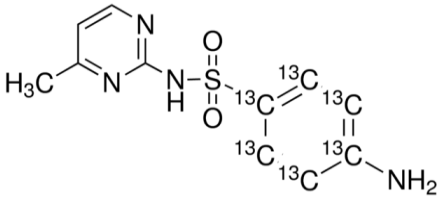
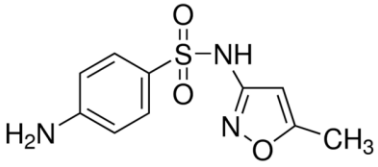
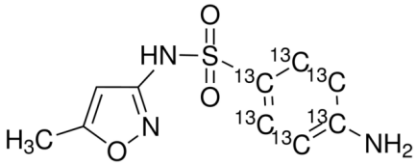
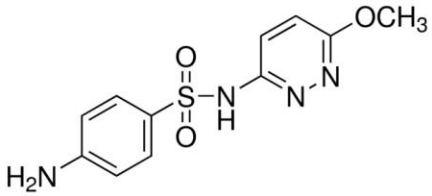
Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Tilmicosin		869.2	870	174	7.3	45	1
				696		45	5
Tylosin		916.1	916	174	7.9	40	3
				101		55	3
Lincosamides							
Lincomycin		406.5	407	126	5.9	30	1
				359		15	1
Lincomycin-d_3		409.5	410	129	5.9	30	3
				392		15	1

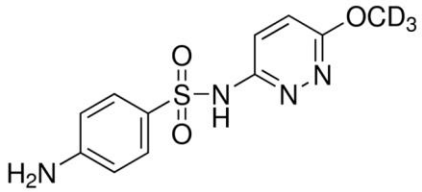
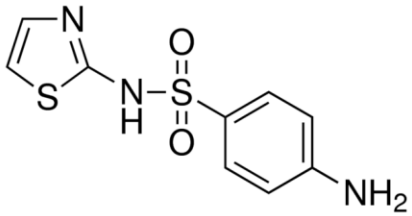
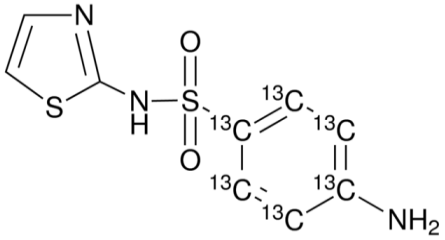
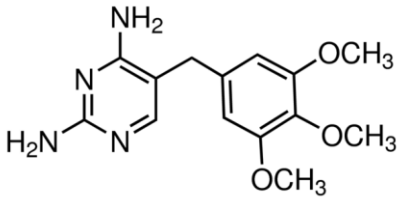
Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Pirlimycin		410.2	411	112	7.4	15	1
				56		45	3
ß – lactams							
Amoxicillin		365.4	366	208	3.3	10	3
				114		20	5
Ampicillin		349.4	350	192	6.3	15	5
				174		10	5
Ceftiofur		523.6	524	241	7.1	15	3
				126		35	5
Cloxacillin		435.9	436	277	8.0	15	1
				160		15	5

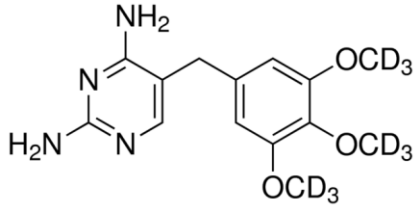
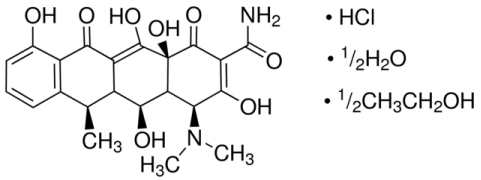
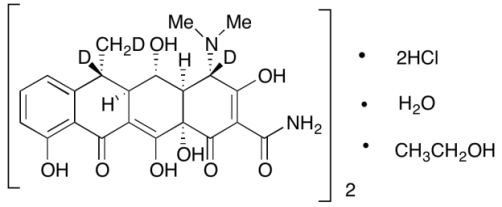
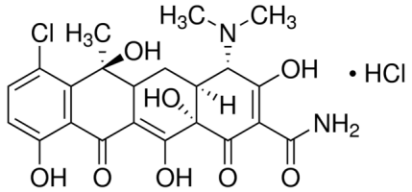
Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Penicillin G		334.4	335	176	7.6	10	1
				160		10	1
Penicillin G-d_7		341.4	342	183	7.6	5	5
				160		1	8
Penicillin V		350.4	351	160	7.9	10	3
				192		15	1
Sulphonamides							
Sulfadiazine		250.3	251	92	4.8	25	3
				156		10	1

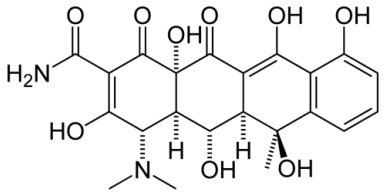
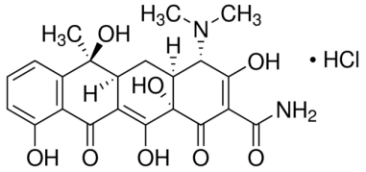
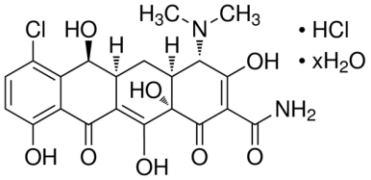
Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Sulfadiazine-$^{13}C_6$		256.2	257	98	4.8	30	3
				162		15	3
Sulfadimethoxine		310.3	311	156	7.0	15	3
				108		30	3
Sulfadimethoxine-d_6		316.3	317	156	7.0	20	1
				108		25	3
Sulfamethazine		278.3	279	186	6.1	15	3
				156		20	1

Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Sulfamethazine-$^{13}C_6$		284.3	285	186	6.1	20	3
				162		20	1
Sulfadoxine		310.3	311	156	6.5	10	3
				108		25	5
Sulfadoxine-d_3		313.3	314	156	6.5	15	1
				92		30	1
Sulfamerazine		264.3	265	156	5.7	15	1
				92		25	3

Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Sulfamerazine-$^{13}C_6$		270.3	271	98	5.7	35	1
				172		15	5
Sulfamethoxazole		253.3	254	156	6.4	10	5
				92		25	3
Sulfamethoxazole-$^{13}C_6$		259.3	260	162	6.4	15	1
				114		20	1
Sulfamethoxypyridazine		280.3	281	156	6.2	10	1
				92		35	1

Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Sulfamethoxypyridazine-d_3		283.3	284	156	6.2	15	3
				108		30	5
Sulfathiazole		255.3	256	156	5.3	5	1
				92		15	3
Sulfathiazole-$^{13}C_6$		261.3	262	162	5.3	10	1
				114		20	5
Diamino-pyrimidine-derivate							
Trimethoprim		290.3	291	230	6.0	20	3
				261		25	3

Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Trimethoprim-d_9		299.3	300	234	6.0	25	1
				264		30	1
Tetracyclines							
Doxycycline		444.4	445	428	7.3	15	5
				154	6.3 (4-epi)	35	1
Doxycycline-d3		447.4	448	431	7.3	20	1
				413		25	3
Chlortetracycline and its 4-epimer		478.9	479	444	7.0	20	5
				462	6.7 (4-epi)	15	5

Compound	Chemical structure	Molecular mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)	RT (min)	CE (eV)	CAV (eV)
Oxytetracycline and its 4-epimer		460.4	461	426	6.4	15	5
				443	6.2 (4-epi)	5	5
Tetracycline and its 4-epimer		444.4	445	410	6.3	15	5
				427	6.0 (4-epi)	10	5
Demeclocycline (ISTD for all three remaining TCs)		464.9	465	448	6.6	5	8
				430		15	8

APPENDIX III: VALIDATION DATA

Dairy products

Matrix: bovine raw milk. 3.5 % fat content

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	118	122	4	5	2.00	4.00
Ampicillin	4	4	20	71	74	13	8	2.00	4.00
Ceftiofur	100	10	50	111	94	7	3	3.00	10.00
Chlortetracycline	100	10	50	60	65	4	10	3.00	10.00
Ciprofloxacin	100	5	25	96	115	18	9	2.00	5.00
Cloxacillin	30	3	15	79	84	16	5	1.00	3.00
Danofloxacin	30	3	15	83	73	7	15	1.00	3.00
Doxycyclin	no MRL	10	50	113	96	7	4	3.00	10.00
Enrofloxacin	100	5	25	88	105	8	16	2.00	5.00
Erythromycin A	40	4	20	182	179	37	11	7.00	20.00
Flumequine	50	5	25	78	153	10	9	2.00	5.00
Lincomycin	150	15	75	85	74	12	12	5.00	15.00
Marbofloxacin	75	7.5	37.5	100	107	11	6	2.50	7.50
Oxytetracycline	100	10	50	67	62	15	19	3.00	10.00
Penicillin G	4	2	10	157	169	14	7	0.70	2.00
Penicillin V	no MRL	5	25	75	83	5	8	2.00	5.00
Pirlimycin	100	10	50	85	82	7	6	3.00	10.00
Spiramycin	200	20	100	144	171	52	19	6.00	20.00
Sulfadiazin	100	1	5	79	83	7	4	0.33	1.00
Sulfadimethoxine		1	5	91	86	8	16	0.33	1.00
Sulfadimidin		1	5	96	94	8	12	0.33	1.00
Sulfadoxin		1	5	94	98	10	9	0.33	1.00
Sulfamerazine		1	5	91	92	9	11	0.33	1.00
Sulfamethoxazole		1	5	58	81	13	13	0.33	1.00
Sulfamethoxypyridazine		1	5	93	85	11	7	0.33	1.00
Sulfathiazole		1	5	84	75	8	9	0.33	1.00
Tetracycline	100	10	50	93	95	11	7	3.00	10.00
Tilmicosin	50	5	25	64	90	22	14	2.00	5.00
Trimethoprim	50	5	25	98	120	4	4	2.00	5.00
Tylosin	50	5	25	47	54	20	14	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: pasteurized bovine milk. 3.5 % fat content

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
		low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	58	67	16	20	2.00	4.00
Ampicillin	4	4	20	104	88	24	18	2.00	4.00
Ceftiofur	100	10	50	93	84	6	14	3.00	10.00
Chlortetracycline	100	10	50	85	78	11	16	3.00	10.00
Ciprofloxacin	100	5	25	79	94	21	16	2.00	5.00
Cloxacillin	30	3	15	91	94	19	18	1.00	3.00
Danofloxacin	30	3	15	104	85	7	16	1.00	3.00
Doxycyclin	no MRL	10	50	83	77	19	21	3.00	10.00
Enrofloxacin	100	5	25	106	116	9	18	2.00	5.00
Erythromycin A	40	4	20	58	68	14	53	1.50	4.00
Flumequine	50	5	25	89	118	9	10	2.00	5.00
Lincomycin	150	15	75	92	90	7	17	5.00	15.00
Marbofloxacin	75	7.5	37.5	77	97	21	17	2.50	7.50
Oxytetracycline	100	10	50	88	77	19	21	3.00	10.00
Penicillin G	4	2	10	130	164	37	37	0.70	2.00
Penicillin V	no MRL	5	25	93	93	13	15	2.00	5.00
Pirlimycin	100	10	50	85	98	20	17	3.00	10.00
Spiramycin	200	20	100	80	110	22	21	6.00	20.00
Sulfadiazin	100	1	5	98	109	15	21	0.33	1.00
Sulfadimethoxine		1	5	95	91	7	14	0.33	1.00
Sulfadimidin		1	5	94	77	13	20	0.33	1.00
Sulfadoxin		1	5	94	93	15	22	0.33	1.00
Sulfamerazine		1	5	87	95	15	15	0.33	1.00
Sulfamethoxazole		1	5	67	72	19	19	0.33	1.00
Sulfamethoxypyridazine		1	5	82	93	20	15	0.33	1.00
Sulfathiazole		1	5	109	108	31	20	0.33	1.00
Tetracycline	100	10	50	102	93	13	19	3.00	10.00
Tilmicosin	50	5	25	123	117	53	33	2.00	5.00
Trimethoprim	50	5	25	114	116	16	12	2.00	5.00
Tylosin	50	5	25	40	44	21	16	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: cream. 36% fat content

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	43	60	21	20	2.00	4.00
Ampicillin	4	4	20	85	75	28	20	2.00	4.00
Ceftiofur	100	10	50	93	92	8	14	3.00	10.00
Chlortetracycline	100	10	50	86	80	21	16	3.00	10.00
Ciprofloxacin	100	5	25	81	100	13	13	2.00	5.00
Cloxacillin	30	3	15	90	88	14	18	1.00	3.00
Danofloxacin	30	3	15	101	101	20	23	1.00	3.00
Doxycyclin	no MRL	10	50	91	88	16	15	3.00	10.00
Enrofloxacin	100	5	25	96	107	14	17	2.00	5.00
Erythromycin A	40	4	20	73	54	19	21	1.50	4.00
Flumequine	50	5	25	83	115	11	11	2.00	5.00
Lincomycin	150	15	75	92	94	16	19	5.00	15.00
Marbofloxacin	75	7.5	37.5	68	100	18	19	2.50	7.50
Oxytetracycline	100	10	50	81	70	20	21	3.00	10.00
Penicillin G	4	2	10	127	152	31	23	0.70	2.00
Penicillin V	no MRL	5	25	94	88	13	18	2.00	5.00
Pirlimycin	100	10	50	87	92	19	15	3.00	10.00
Spiramycin	200	20	100	130	114	21	19	6.00	20.00
Sulfadiazin	100	1	5	98	114	8	18	0.33	1.00
Sulfadimethoxine		1	5	94	91	6	9	0.33	1.00
Sulfadimidin		1	5	86	84	10	18	0.33	1.00
Sulfadoxin		1	5	80	87	9	15	0.33	1.00
Sulfamerazine		1	5	84	90	13	14	0.33	1.00
Sulfamethoxazole		1	5	79	74	15	14	0.33	1.00
Sulfamethoxypyridazine		1	5	80	98	15	15	0.33	1.00
Sulfathiazole		1	5	104	120	18	16	0.33	1.00
Tetracycline	100	10	50	109	83	16	18	3.00	10.00
Tilmicosin	50	5	25	118	113	60	38	2.00	5.00
Trimethoprim	50	5	25	107	108	9	9	2.00	5.00
Tylosin	50	5	25	56	51	20	11	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: butter. 80% fat content

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	33	78	31	58	2.00	4.00
Ampicillin	4	4	20	49	50	41	21	2.00	4.00
Ceftiofur	100	10	50	78	75	9	20	3.00	10.00
Chlortetracycline	100	10	50	83	76	16	13	3.00	10.00
Ciprofloxacin	100	5	25	91	111	12	18	2.00	5.00
Cloxacillin	30	3	15	45	61	16	17	1.00	3.00
Danofloxacin	30	3	15	100	84	8	20	1.00	3.00
Doxycyclin	no MRL	10	50	100	113	24	19	3.00	10.00
Enrofloxacin	100	5	25	93	104	17	10	2.00	5.00
Erythromycin A	40	4	20	80	72	22	15	1.50	4.00
Flumequine	50	5	25	84	105	11	12	2.00	5.00
Lincomycin	150	15	75	89	80	20	19	5.00	15.00
Marbofloxacin	75	7.5	37.5	87	98	19	16	2.50	7.50
Oxytetracycline	100	10	50	80	72	20	19	3.00	10.00
Penicillin G	4	2	10	128	139	48	36	0.70	2.00
Penicillin V	no MRL	5	25	63	65	18	19	2.00	5.00
Pirlimycin	100	10	50	80	87	16	19	3.00	10.00
Spiramycin	200	20	100	89	118	19	22	6.00	20.00
Sulfadiazin	100	1	5	89	90	21	16	0.33	1.00
Sulfadimethoxine		1	5	95	91	10	15	0.33	1.00
Sulfadimidin		1	5	87	85	13	16	0.33	1.00
Sulfadoxin		1	5	85	84	8	12	0.33	1.00
Sulfamerazine		1	5	80	85	14	7	0.33	1.00
Sulfamethoxazole		1	5	78	77	8	13	0.33	1.00
Sulfamethoxypyridazine		1	5	88	95	16	15	0.33	1.00
Sulfathiazole		1	5	78	83	17	11	0.33	1.00
Tetracycline	100	10	50	90	90	20	21	3.00	10.00
Tilmicosin	50	5	25	156	119	46	34	2.00	5.00
Trimethoprim	50	5	25	110	100	9	8	2.00	5.00
Tylosin	50	5	25	57	60	21	33	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: buttermilk. 1% fat content

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	60	57	17	21	2.00	4.00
Ampicillin	4	4	20	40	30	18	22	7.00	20.00
Ceftiofur	100	10	50	34	34	8	7	3.00	10.00
Chlortetracycline	100	10	50	118	100	11	18	3.00	10.00
Ciprofloxacin	100	5	25	78	91	16	19	2.00	5.00
Cloxacillin	30	3	15	14	16	32	18	1.00	3.00
Danofloxacin	30	3	15	86	71	19	7	1.00	3.00
Doxycyclin	no MRL	10	50	98	81	20	20	3.00	10.00
Enrofloxacin	100	5	25	125	118	14	14	2.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.				
Flumequine	50	5	25	90	110	25	8	2.00	5.00
Lincomycin	150	15	75	80	89	17	16	5.00	15.00
Marbofloxacin	75	7.5	37.5	49	68	21	15	2.50	7.50
Oxytetracycline	100	10	50	94	62	14	23	3.00	10.00
Penicillin G	4	2	10	n.d.	n.d.				
Penicillin V	no MRL	5	25	n.d.	n.d.				
Pirlimycin	100	10	50	34	35	22	12	3.00	10.00
Spiramycin	200	20	100	94	120	20	19	6.00	20.00
Sulfadiazin	100	1	5	117	118	18	22	0.33	1.00
Sulfadimethoxine		1	5	94	86	21	12	0.33	1.00
Sulfadimidin		1	5	85	84	18	17	0.33	1.00
Sulfadoxin		1	5	74	80	16	18	0.33	1.00
Sulfamerazine		1	5	86	92	12	19	0.33	1.00
Sulfamethoxazole		1	5	70	72	20	19	0.33	1.00
Sulfamethoxypyridazine		1	5	93	102	19	19	0.33	1.00
Sulfathiazole		1	5	164	217	62	35	0.33	1.00
Tetracycline	100	10	50	125	98	10	10	3.00	10.00
Tilmicosin	50	5	25	83	82	33	20	2.00	5.00
Trimethoprim	50	5	25	103	105	17	14	2.00	5.00
Tylosin	50	5	25	7	7	21	24	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: sour cream. 15% fat content

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	56	59	12	14	2.00	4.00
Ampicillin	4	4	20	58	56	16	21	2.00	4.00
Ceftiofur	100	10	50	36	35	17	19	3.00	10.00
Chlortetracycline	100	10	50	100	82	12	21	3.00	10.00
Ciprofloxacin	100	5	25	69	103	21	21	2.00	5.00
Cloxacillin	30	3	15	11	9	20	16	1.00	3.00
Danofloxacin	30	3	15	103	97	15	17	1.00	3.00
Doxycyclin	no MRL	10	50	94	92	21	14	3.00	10.00
Enrofloxacin	100	5	25	119	119	13	8	2.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.				
Flumequine	50	5	25	88	112	17	18	2.00	5.00
Lincomycin	150	15	75	83	86	17	19	5.00	15.00
Marbofloxacin	75	7.5	37.5	63	84	19	15	2.50	7.50
Oxytetracycline	100	10	50	79	65	19	16	3.00	10.00
Penicillin G	4	2	10	n.d.	n.d.				
Penicillin V	no MRL	5	25	n.d.	n.d.				
Pirlimycin	100	10	50	39	34	15	21	3.00	10.00
Spiramycin	200	20	100	110	132	32	20	6.00	20.00
Sulfadiazin	100	1	5	106	118	14	21	0.33	1.00
Sulfadimethoxine		1	5	98	89	17	16	0.33	1.00
Sulfadimidin		1	5	85	84	14	10	0.33	1.00
Sulfadoxin		1	5	70	82	21	20	0.33	1.00
Sulfamerazine		1	5	86	83	13	17	0.33	1.00
Sulfamethoxazole		1	5	42	60	17	13	0.33	1.00
Sulfamethoxypyridazine		1	5	96	96	11	16	0.33	1.00
Sulfathiazole		1	5	131	183	44	32	0.33	1.00
Tetracycline	100	10	50	125	107	14	15	3.00	10.00
Tilmicosin	50	5	25	76	85	38	19	2.00	5.00
Trimethoprim	50	5	25	113	101	20	10	2.00	5.00
Tylosin	50	5	25	5	7	14	20	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: yogurt. 3.5% fat content

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	61	65	14	16	2.00	4.00
Ampicillin	4	4	20	58	61	31	17	2.00	4.00
Ceftiofur	100	10	50	69	70	12	12	3.00	10.00
Chlortetracycline	100	10	50	88	86	22	11	3.00	10.00
Ciprofloxacin	100	5	25	73	89	9	18	2.00	5.00
Cloxacillin	30	3	15	55	56	17	19	1.00	3.00
Danofloxacin	30	3	15	105	94	7	13	1.00	3.00
Doxycyclin	no MRL	10	50	84	82	20	15	3.00	10.00
Enrofloxacin	100	5	25	113	114	18	10	2.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.				
Flumequine	50	5	25	86	110	11	8	2.00	5.00
Lincomycin	150	15	75	86	89	20	17	5.00	15.00
Marbofloxacin	75	7.5	37.5	66	93	22	21	2.50	7.50
Oxytetracycline	100	10	50	107	80	19	16	3.00	10.00
Penicillin G	4	2	10	125	198	42	86	0.70	2.00
Penicillin V	no MRL	5	25	63	64	22	11	2.00	5.00
Pirlimycin	100	10	50	78	82	18	21	3.00	10.00
Spiramycin	200	20	100	107	105	38	25	6.00	20.00
Sulfadiazin	100	1	5	102	113	18	20	0.33	1.00
Sulfadimethoxine		1	5	93	94	12	15	0.33	1.00
Sulfadimidin		1	5	86	84	18	9	0.33	1.00
Sulfadoxin		1	5	73	75	13	16	0.33	1.00
Sulfamerazine		1	5	87	91	13	13	0.33	1.00
Sulfamethoxazole		1	5	63	66	10	16	0.33	1.00
Sulfamethoxypyridazine		1	5	95	93	14	20	0.33	1.00
Sulfathiazole		1	5	149	153	44	37	0.33	1.00
Tetracycline	100	10	50	118	91	11	12	3.00	10.00
Tilmicosin	50	5	25	79	86	42	17	2.00	5.00
Trimethoprim	50	5	25	103	97	12	7	2.00	5.00
Tylosin	50	5	25	20	23	10	19	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: curd. 20% fat in dry matter

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	47	53	10	9	2.00	4.00
Ampicillin	4	4	20	72	56	16	15	2.00	4.00
Ceftiofur	100	10	50	63	55	8	18	3.00	10.00
Chlortetracycline	100	10	50	96	93	16	9	3.00	10.00
Ciprofloxacin	100	5	25	69	87	17	19	2.00	5.00
Cloxacillin	30	3	15	5	5	42	17	1.00	3.00
Danofloxacin	30	3	15	101	77	10	17	1.00	3.00
Doxycyclin	no MRL	10	50	87	81	21	15	3.00	10.00
Enrofloxacin	100	5	25	111	117	21	18	2.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.				
Flumequine	50	5	25	86	116	11	9	2.00	5.00
Lincomycin	150	15	75	81	83	11	16	5.00	15.00
Marbofloxacin	75	7.5	37.5	76	82	20	11	2.50	7.50
Oxytetracycline	100	10	50	84	72	12	13	3.00	10.00
Penicillin G	4	2	10	27	56	162	145	0.70	2.00
Penicillin V	no MRL	5	25	14	13	32	38	2.00	5.00
Pirlimycin	100	10	50	45	50	16	12	3.00	10.00
Spiramycin	200	20	100	84	114	23	17	6.00	20.00
Sulfadiazin	100	1	5	97	116	18	21	0.33	1.00
Sulfadimethoxine		1	5	91	81	14	8	0.33	1.00
Sulfadimidin		1	5	82	87	16	18	0.33	1.00
Sulfadoxin		1	5	82	76	14	15	0.33	1.00
Sulfamerazine		1	5	89	83	21	17	0.33	1.00
Sulfamethoxazole		1	5	52	72	18	20	0.33	1.00
Sulfamethoxypyridazine		1	5	87	96	19	13	0.33	1.00
Sulfathiazole		1	5	134	144	47	29	0.33	1.00
Tetracycline	100	10	50	117	103	7	16	3.00	10.00
Tilmicosin	50	5	25	73	86	44	18	2.00	5.00
Trimethoprim	50	5	25	98	105	13	11	2.00	5.00
Tylosin	50	5	25	8	9	18	16	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: soft cheese. 55% fat in dry matter

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	n.d.	n.d.				
Ampicillin	4	4	20	n.d.	n.d.				
Ceftiofur	100	10	50	n.d.	n.d.				
Chlortetracycline	100	10	50	80	81	20	10	3.00	10.00
Ciprofloxacin	100	5	25	75	80	21	16	2.00	5.00
Cloxacillin	30	3	15	n.d.	n.d.				
Danofloxacin	30	3	15	93	71	17	17	1.00	3.00
Doxycyclin	no MRL	10	50	123	92	22	18	3.00	10.00
Enrofloxacin	100	5	25	173	123	28	13	2.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.				
Flumequine	50	5	25	89	113	17	14	2.00	5.00
Lincomycin	150	15	75	87	93	16	17	5.00	15.00
Marbofloxacin	75	7.5	37.5	56	80	22	15	2.50	7.50
Oxytetracycline	100	10	50	74	83	20	18	3.00	10.00
Penicillin G	4	2	10	n.d.	n.d.				
Penicillin V	no MRL	5	25	n.d.	n.d.				
Pirlimycin	100	10	50	11	18	15	20	3.00	10.00
Spiramycin	200	20	100	107	59	36	11	6.00	20.00
Sulfadiazin	100	1	5	103	117	16	19	0.33	1.00
Sulfadimethoxine		1	5	89	76	21	18	0.33	1.00
Sulfadimidin		1	5	87	76	22	15	0.33	1.00
Sulfadoxin		1	5	87	77	22	14	0.33	1.00
Sulfamerazine		1	5	89	94	16	15	0.33	1.00
Sulfamethoxazole		1	5	72	74	17	19	0.33	1.00
Sulfamethoxypyridazine		1	5	83	94	20	20	0.33	1.00
Sulfathiazole		1	5	173	214	47	34	0.33	1.00
Tetracycline	100	10	50	99	95	18	15	3.00	10.00
Tilmicosin	50	5	25	69	87	54	21	2.00	5.00
Trimethoprim	50	5	25	94	108	15	13	2.00	5.00
Tylosin	50	5	25	5	5	12	32	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: hard cheese. 55% fat in dry matter

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	4	4	20	n.d.	n.d.				
Ampicillin	4	4	20	n.d.	n.d.				
Ceftiofur	100	10	50	n.d.	n.d.				
Chlortetracycline	100	10	50	83	95	9	21	3.00	10.00
Ciprofloxacin	100	5	25	58	78	21	21	2.00	5.00
Cloxacillin	30	3	15	n.d.	n.d.				
Danofloxacin	30	3	15	93	67	21	10	1.00	3.00
Doxycyclin	no MRL	10	50	94	95	17	18	3.00	10.00
Enrofloxacin	100	5	25	107	118	10	5	2.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.				
Flumequine	50	5	25	85	113	9	9	2.00	5.00
Lincomycin	150	15	75	94	99	12	17	5.00	15.00
Marbofloxacin	75	7.5	37.5	69	79	20	16	2.50	7.50
Oxytetracycline	100	10	50	100	88	28	22	3.00	10.00
Penicillin G	4	2	10	n.d.	n.d.				
Penicillin V	no MRL	5	25	n.d.	n.d.				
Pirlimycin	100	10	50	38	56	13	25	3.00	10.00
Spiramycin	200	20	100	100	73	23	37	6.00	20.00
Sulfadiazin	100	1	5	95	110	21	16	0.33	1.00
Sulfadimethoxine		1	5	112	102	15	25	0.33	1.00
Sulfadimidin		1	5	77	82	17	19	0.33	1.00
Sulfadoxin		1	5	78	73	15	15	0.33	1.00
Sulfamerazine		1	5	90	89	18	18	0.33	1.00
Sulfamethoxazole		1	5	99	77	14	19	0.33	1.00
Sulfamethoxypyridazine		1	5	95	92	20	15	0.33	1.00
Sulfathiazole		1	5	218	207	57	9	0.33	1.00
Tetracycline	100	10	50	100	102	23	138	3.00	10.00
Tilmicosin	50	5	25	107	85	38	44	2.00	5.00
Trimethoprim	50	5	25	107	101	18	21	2.00	5.00
Tylosin	50	5	25	10	11	15	20	2.00	5.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Fish and meat matrices

Matrix: chicken

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	26	28	18	17	1.67	5.00
Ampicillin	50	5	25	58	44	20	12	1.67	5.00
Ceftiofur	1000	100	500	76	70	15	13	33.33	100.00
Chlortetracycline	100	10	50	79	87	16	12	3.33	10.00
Ciprofloxacin	100	5	25	98	quadratic	20	quadratic	1.67	5.00
Cloxacillin	no MRL	30	150	79	84	19	16	10.00	30.00
Danofloxacin	300	5	25	96	quadratic	18	quadratic	1.67	5.00
Doxycyclin	200	10	50	75	75	37	29	10.00	30.00
Enrofloxacin	100	5	25	95	120	11	10	1.67	5.00
Erythromycin A	200	20	100	89	130	16	11	6.67	20.00
Flumequine	400	20	100	62	73	15	7	6.67	20.00
Lincomycin	100	5	25	99	98	17	13	1.67	5.00
Marbofloxacin	no MRL	5	25	85	99	9	9	1.67	5.00
Oxytetracycline	100	10	50	99	106	19	9	3.33	10.00
Penicillin G	50	5	25	62	80	21	32	1.67	5.00
Penicillin V	25	2.5	12.5	89	92	11	12	0.83	2.50
Pirlimycin		10	50	57	61	10	14	3.33	10.00
Sulfadiazin	100	1	5	91	96	15	11	0.33	1.00
Sulfadimethoxine		1	5	62	77	18	13	0.33	1.00
Sulfadimidin		1	5	83	96	11	19	0.33	1.00
Sulfadoxin		1	5	85	95	13	7	0.33	1.00
Sulfamerazine		1	5	95	97	9	11	0.33	1.00
Sulfamethoxazole		1	5	81	85	13	15	0.33	1.00
Sulfamethoxypyridazine		1	5	90	99	17	12	0.33	1.00
Sulfathiazole		1	5	87	94	15	17	0.33	1.00
Tetracycline	100	10	50	91	102	9	11	3.33	10.00
Trimethoprim	50	5	25	109	quadratic	12	quadratic	1.67	5.00
Tylosin	100	10	50	37	31	15	20	3.33	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: prawns

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	52	55	29	19	1.70	5.00
Ampicillin	50	5	25	60	53	21	13	1.70	5.00
Ceftiofur	1000	100	500	63	63	10	15	33.30	100.00
Chlortetracycline	100	10	50	87	93	17	15	3.30	10.00
Ciprofloxacin	100	5	25	104	quadratic	18	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	72	78	16	10	10.00	30.00
Danofloxacin	300	5	25	103	quadratic	16	quadratic	1.70	5.00
Doxycyclin	200	10	50	61	73	29	27	10.00	30.00
Enrofloxacin	100	5	25	92	126	19	20	1.70	5.00
Erythromycin A	200	20	100	129	179	22	35	6.70	20.00
Flumequine	400	20	100	61	72	16	10	6.70	20.00
Lincomycin	100	5	25	75	74	22	11	1.70	5.00
Marbofloxacin	no MRL	5	25	93	95	6	11	1.70	5.00
Oxytetracycline	100	10	50	100	114	13	9	3.30	10.00
Penicillin G	50	5	25	44	49	21	27	1.70	5.00
Penicillin V	25	2.5	12.5	83	87	13	8	0.80	2.50
Pirlimycin		10	50	65	70	13	10	3.30	10.00
Sulfadiazin	100	1	5	86	97	12	9	0.30	1.00
Sulfadimethoxine		1	5	82	90	17	13	0.30	1.00
Sulfadimidin		1	5	86	92	15	16	0.30	1.00
Sulfadoxin		1	5	88	102	11	12	0.30	1.00
Sulfamerazine		1	5	80	96	10	10	0.30	1.00
Sulfamethoxazole		1	5	80	80	15	12	0.30	1.00
Sulfamethoxypyridazine		1	5	94	97	12	9	0.30	1.00
Sulfathiazole		1	5	92	97	15	14	0.30	1.00
Tetracycline	100	10	50	107	106	15	9	3.30	10.00
Trimethoprim	50	5	25	108	quadratic	5	quadratic	1.70	5.00
Tylosin	100	10	50	37	54	10	25	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: salmon

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	39	42	18	14	1.70	5.00
Ampicillin	50	5	25	51	48	15	12	1.70	5.00
Ceftiofur	1000	100	500	76	72	7	13	33.30	100.00
Chlortetracycline	100	10	50	73	81	20	11	3.30	10.00
Ciprofloxacin	100	5	25	94	quadratic	20	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	76	86	17	15	10.00	30.00
Danofloxacin	300	5	25	94	quadratic	22	quadratic	1.70	5.00
Doxycyclin	200	10	50	64	72	21	36	10.00	30.00
Enrofloxacin	100	5	25	99	114	19	14	1.70	5.00
Erythromycin A	200	20	100	104	133	24	15	6.70	20.00
Flumequine	400	20	100	67	72	17	5	6.70	20.00
Lincomycin	100	5	25	93	102	15	12	1.70	5.00
Marbofloxacin	no MRL	5	25	91	104	9	9	1.70	5.00
Oxytetracycline	100	10	50	68	80	13	16	3.30	10.00
Penicillin G	50	5	25	73	111	30	48	1.70	5.00
Penicillin V	25	2.5	12.5	92	91	20	12	0.80	2.50
Pirlimycin		10	50	48	51	10	12	3.30	10.00
Sulfadiazin	100	1	5	96	100	14	7	0.30	1.00
Sulfadimethoxine		1	5	85	89	17	15	0.30	1.00
Sulfadimidin		1	5	86	90	11	9	0.30	1.00
Sulfadoxin		1	5	83	100	14	22	0.30	1.00
Sulfamerazine		1	5	86	98	15	5	0.30	1.00
Sulfamethoxazole		1	5	76	86	10	8	0.30	1.00
Sulfamethoxypyridazine		1	5	92	96	16	15	0.30	1.00
Sulfathiazole		1	5	98	99	12	5	0.30	1.00
Tetracycline	100	10	50	72	83	19	15	3.30	10.00
Trimethoprim	50	5	25	116	quadratic	12	quadratic	1.70	5.00
Tylosin	100	10	50	21	39	109	63	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: trout

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	36	37	16	14	1.70	5.00
Ampicillin	50	5	25	49	50	21	17	1.70	5.00
Ceftiofur	1000	100	500	85	79	7	12	33.30	100.00
Chlortetracycline	100	10	50	82	85	13	12	3.30	10.00
Ciprofloxacin	100	5	25	87	quadratic	21	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	81	85	20	15	10.00	30.00
Danofloxacin	300	5	25	100	quadratic	19	quadratic	1.70	5.00
Doxycyclin	200	10	50	73	69	29	30	10.00	30.00
Enrofloxacin	100	5	25	103	128	16	9	1.70	5.00
Erythromycin A	200	20	100	94	141	21	2	6.70	20.00
Flumequine	400	20	100	69	72	12	8	6.70	20.00
Lincomycin	100	5	25	89	101	12	15	1.70	5.00
Marbofloxacin	no MRL	5	25	86	99	14	6	1.70	5.00
Oxytetracycline	100	10	50	77	83	9	15	3.30	10.00
Penicillin G	50	5	25	70	85	37	27	1.70	5.00
Penicillin V	25	2.5	12.5	95	91	15	12	0.80	2.50
Pirlimycin		10	50	61	61	14	7	3.30	10.00
Sulfadiazin	100	1	5	123	105	9	5	0.30	1.00
Sulfadimethoxine		1	5	80	97	11	19	0.30	1.00
Sulfadimidin		1	5	81	85	11	9	0.30	1.00
Sulfadoxin		1	5	83	99	8	10	0.30	1.00
Sulfamerazine		1	5	94	99	14	12	0.30	1.00
Sulfamethoxazole		1	5	78	85	13	11	0.30	1.00
Sulfamethoxypyridazine		1	5	84	93	16	8	0.30	1.00
Sulfathiazole		1	5	95	95	19	12	0.30	1.00
Tetracycline	100	10	50	82	87	13	10	3.30	10.00
Trimethoprim	50	5	25	105	quadratic	12	quadratic	1.70	5.00
Tylosin	100	10	50	18	33	116	82	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: muscle (pig)

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	28	29	15	14	1.70	5.00
Ampicillin	50	5	25	50	53	14	14	1.70	5.00
Ceftiofur	1000	100	500	77	76	13	9	33.30	100.00
Chlortetracycline	100	10	50	73	78	16	9	3.30	10.00
Ciprofloxacin	100	5	25	93	quadratic	18	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	80	85	13	10	10.00	30.00
Danofloxacin	300	5	25	104	quadratic	19	quadratic	1.70	5.00
Doxycyclin	200	10	50	77	63	13	24	10.00	30.00
Enrofloxacin	100	5	25	100	117	9	8	1.70	5.00
Erythromycin A	200	20	100	102	148	20	14	6.70	20.00
Flumequine	400	20	100	62	77	17	12	6.70	20.00
Lincomycin	100	5	25	100	103	14	18	1.70	5.00
Marbofloxacin	no MRL	5	25	88	110	11	12	1.70	5.00
Oxytetracycline	100	10	50	96	97	13	6	3.30	10.00
Penicillin G	50	5	25	84	110	42	52	1.70	5.00
Penicillin V	25	2.5	12.5	92	96	11	14	0.80	2.50
Pirlimycin		10	50	60	62	9	14	3.30	10.00
Sulfadiazin	100	1	5	83	97	12	6	0.30	1.00
Sulfadimethoxine		1	5	82	90	11	17	0.30	1.00
Sulfadimidin		1	5	83	88	15	10	0.30	1.00
Sulfadoxin		1	5	82	98	13	15	0.30	1.00
Sulfamerazine		1	5	90	100	13	7	0.30	1.00
Sulfamethoxazole		1	5	81	84	10	10	0.30	1.00
Sulfamethoxypyridazine		1	5	90	96	11	14	0.30	1.00
Sulfathiazole		1	5	104	101	12	21	0.30	1.00
Tetracycline	100	10	50	107	109	10	12	3.30	10.00
Trimethoprim	50	5	25	109	quadratic	4	quadratic	1.70	5.00
Tylosin	100	10	50	44	42	21	18	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: fat

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	116	129	18	32	1.70	5.00
Ampicillin	50	5	25	51	56	19	19	1.70	5.00
Ceftiofur	1000	100	500	89	81	11	12	33.30	100.00
Chlortetracycline	100	10	50	75	82	11	9	3.30	10.00
Ciprofloxacin	100	5	25	94	quadratic	11	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	75	80	20	20	10.00	30.00
Danofloxacin	300	5	25	104	quadratic	21	quadratic	1.70	5.00
Doxycyclin	200	10	50	109	81	27	40	10.00	30.00
Enrofloxacin	100	5	25	102	122	15	20	1.70	5.00
Erythromycin A	200	20	100	160	197	26	27	6.70	20.00
Flumequine	400	20	100	63	73	11	9	6.70	20.00
Lincomycin	100	5	25	99	97	15	13	1.70	5.00
Marbofloxacin	no MRL	5	25	86	107	15	15	1.70	5.00
Oxytetracycline	100	10	50	86	99	10	12	3.30	10.00
Penicillin G	50	5	25	48	22	54	73	1.70	5.00
Penicillin V	25	2.5	12.5	90	86	22	14	0.80	2.50
Pirlimycin		10	50	79	83	14	13	3.30	10.00
Sulfadiazin	100	1	5	83	93	9	5	0.30	1.00
Sulfadimethoxine		1	5	92	101	16	14	0.30	1.00
Sulfadimidin		1	5	84	88	12	12	0.30	1.00
Sulfadoxin		1	5	84	101	9	10	0.30	1.00
Sulfamerazine		1	5	88	99	10	9	0.30	1.00
Sulfamethoxazole		1	5	80	86	12	11	0.30	1.00
Sulfamethoxypyridazine		1	5	88	90	17	19	0.30	1.00
Sulfathiazole		1	5	92	103	16	12	0.30	1.00
Tetracycline	100	10	50	100	102	14	7	3.30	10.00
Trimethoprim	50	5	25	105	quadratic	12	quadratic	1.70	5.00
Tylosin	100	10	50	67	65	17	18	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: liver (pig)

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	32	35	16	18	1.70	5.00
Ampicillin	50	5	25	33	26	17	18	1.70	5.00
Ceftiofur	1000	100	500	37	38	15	13	33.30	100.00
Chlortetracycline	100	10	50	70	78	10	16	3.30	10.00
Ciprofloxacin	100	5	25	91	quadratic	17	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	61	66	16	13	10.00	30.00
Danofloxacin	300	5	25	95	quadratic	20	quadratic	1.70	5.00
Doxycyclin	200	10	50	81	64	23	39	3.30	10.00
Enrofloxacin	100	5	25	121	129	8	4	1.70	5.00
Erythromycin A	200	20	100	102	150	18	20	6.70	20.00
Flumequine	400	20	100	62	75	17	12	6.70	20.00
Lincomycin	100	5	25	102	103	17	9	1.70	5.00
Marbofloxacin	no MRL	5	25	79	94	16	16	1.70	5.00
Oxytetracycline	100	10	50	78	88	11	13	3.30	10.00
Penicillin G	50	5	25	76	65	89	63	1.70	5.00
Penicillin V	25	2.5	12.5	48	55	20	20	0.80	2.50
Pirlimycin		10	50	26	29	16	19	3.30	10.00
Sulfadiazin	100	1	5	83	90	18	15	0.30	1.00
Sulfadimethoxine		1	5	56	68	24	16	0.30	1.00
Sulfadimidin		1	5	89	87	14	15	0.30	1.00
Sulfadoxin		1	5	80	107	11	18	0.30	1.00
Sulfamerazine		1	5	89	96	12	6	0.30	1.00
Sulfamethoxazole		1	5	84	88	21	14	0.30	1.00
Sulfamethoxypyridazine		1	5	91	104	16	16	0.30	1.00
Sulfathiazole		1	5	127	102	16	12	0.30	1.00
Tetracycline	100	10	50	73	82	19	17	3.30	10.00
Trimethoprim	50	5	25	98	quadratic	17	quadratic	1.70	5.00
Tylosin	100	10	50	6	7	44	29	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: muscle (beef)

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	25	29	10	18	1.70	5.00
Ampicillin	50	5	25	54	54	15	17	1.70	5.00
Ceftiofur	1000	100	500	72	69	15	8	33.30	100.00
Chlortetracycline	100	10	50	61	68	18	12	3.30	10.00
Ciprofloxacin	100	5	25	93	quadratic	12	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	69	78	11	16	10.00	30.00
Danofloxacin	300	5	25	87	quadratic	20	quadratic	1.70	5.00
Doxycyclin	200	10	50	68	60	22	33	3.30	10.00
Enrofloxacin	100	5	25	99	117	18	9	1.70	5.00
Erythromycin A	200	20	100	116	124	17	19	6.70	20.00
Flumequine	400	20	100	65	72	19	10	6.70	20.00
Lincomycin	100	5	25	97	98	22	15	1.70	5.00
Marbofloxacin	no MRL	5	25	94	105	9	13	1.70	5.00
Oxytetracycline	100	10	50	83	84	17	11	3.30	10.00
Penicillin G	50	5	25	52	82	16	42	1.70	5.00
Penicillin V	25	2.5	12.5	84	86	7	12	0.80	2.50
Pirlimycin		10	50	58	61	17	12	3.30	10.00
Sulfadiazin		1	5	80	97	13	8	0.30	1.00
Sulfadimethoxine		1	5	81	91	17	11	0.30	1.00
Sulfadimidin		1	5	79	92	12	10	0.30	1.00
Sulfadoxin	100	1	5	75	95	15	12	0.30	1.00
Sulfamerazine		1	5	92	101	21	7	0.30	1.00
Sulfamethoxazole		1	5	71	78	17	16	0.30	1.00
Sulfamethoxypyridazine		1	5	89	93	10	15	0.30	1.00
Sulfathiazole		1	5	93	96	15	20	0.30	1.00
Tetracycline	100	10	50	88	95	10	9	3.30	10.00
Trimethoprim	50	5	25	111	quadratic	17	quadratic	1.70	5.00
Tylosin	100	10	50	43	36	16	20	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

Matrix: liver (beef)

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	26	29	15	15	1.70	5.00
Ampicillin	50	5	25	37	28	20	20	1.70	5.00
Ceftiofur	1000	100	500	55	50	19	14	33.30	100.00
Chlortetracycline	100	10	50	76	85	14	9	3.30	10.00
Ciprofloxacin	100	5	25	112	quadratic	18	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	64	69	13	11	10.00	30.00
Danofloxacin	300	5	25	101	quadratic	12	quadratic	1.70	5.00
Doxycyclin	200	10	50	69	53	33	37	10.00	30.00
Enrofloxacin	100	5	25	103	120	19	7	1.70	5.00
Erythromycin A	200	20	100	113	154	20	15	6.70	20.00
Flumequine	400	20	100	64	72	20	12	6.70	20.00
Lincomycin	100	5	25	104	101	17	5	1.70	5.00
Marbofloxacin	no MRL	5	25	84	98	16	11	1.70	5.00
Oxytetracycline	100	10	50	74	80	11	9	3.30	10.00
Penicillin G	50	5	25	70	70	26	48	1.70	5.00
Penicillin V	25	2.5	12.5	55	57	17	15	0.80	2.50
Pirlimycin		10	50	37	38	16	13	3.30	10.00
Sulfadiazin	100	1	5	90	104	13	9	0.30	1.00
Sulfadimethoxine		1	5	81	80	21	11	0.30	1.00
Sulfadimidin		1	5	82	87	11	13	0.30	1.00
Sulfadoxin		1	5	87	104	20	17	0.30	1.00
Sulfamerazine		1	5	99	95	21	10	0.30	1.00
Sulfamethoxazole		1	5	56	83	50	13	0.30	1.00
Sulfamethoxypyridazine		1	5	92	84	18	14	0.30	1.00
Sulfathiazole		1	5	121	114	41	26	0.30	1.00
Tetracycline	100	10	50	86	96	7	10	3.30	10.00
Trimethoprim	50	5	25	109	quadratic	21	quadratic	1.70	5.00
Tylosin	100	10	50	13	11	34	35	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

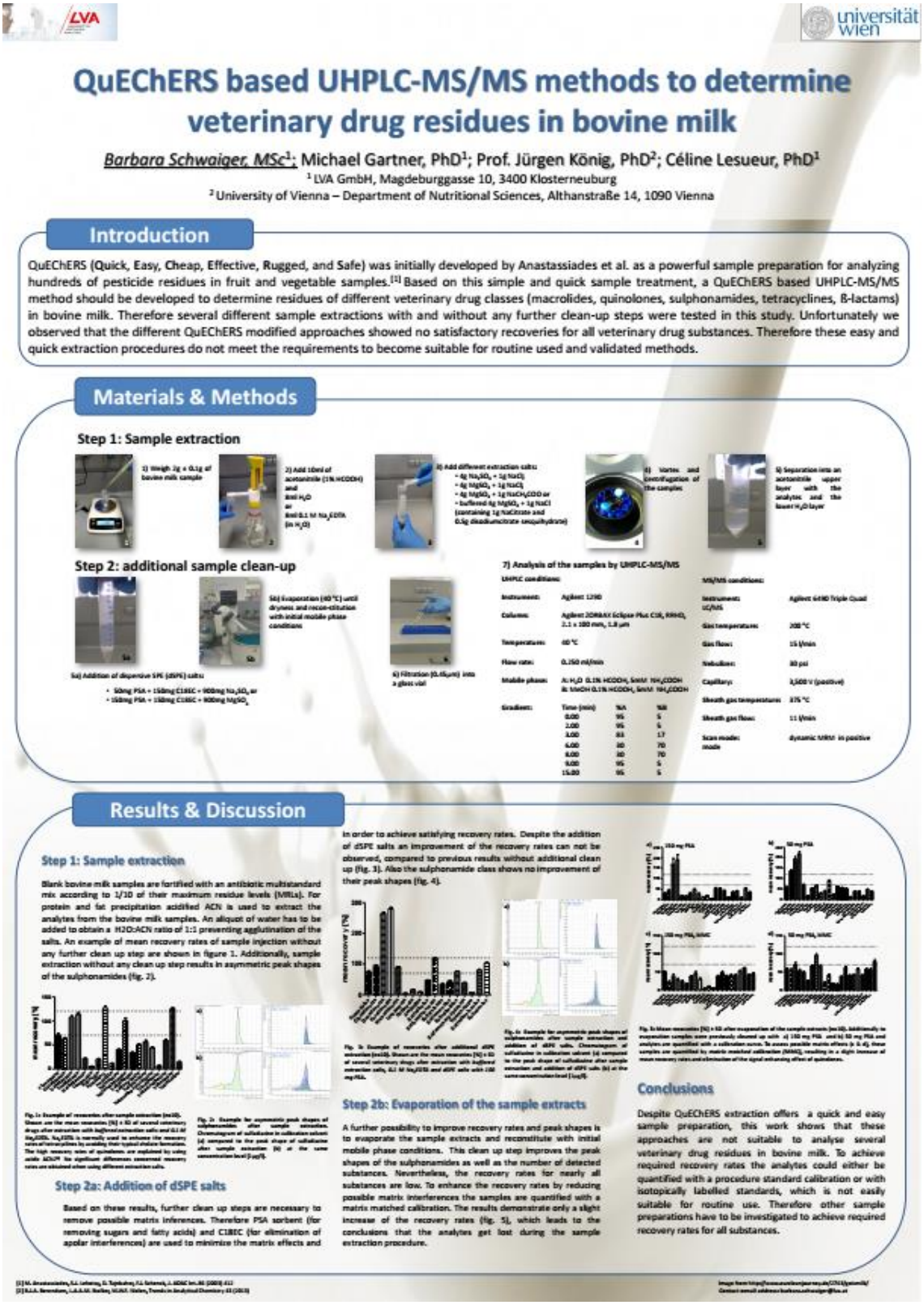
Matrix: kidney (beef)

Compound		spike concentration (µg/kg)		Recovery (%)		RSD (%)		LOD	LOQ
	MRL (µg/kg)	low level	high level	spike low concentration	spike high concentration	spike low concentration	spike high concentration	(µg/kg)	(µg/kg)
Amoxicillin	50	5	25	49	54	19	13	1.70	5.00
Ampicillin	50	5	25	62	59	4	10	1.70	5.00
Ceftiofur	1000	100	500	19	17	108	128	33.30	100.00
Chlortetracycline	100	10	50	112	114	9	7	3.30	10.00
Ciprofloxacin	100	5	25	83	quadratic	19	quadratic	1.70	5.00
Cloxacillin	no MRL	30	150	86	87	13	12	10.00	30.00
Danofloxacin	300	5	25	88	quadratic	21	quadratic	1.70	5.00
Doxycyclin	200	10	50	83	70	22	28	10.00	30.00
Enrofloxacin	100	5	25	103	119	16	11	1.70	5.00
Erythromycin A	200	20	100	96	151	22	5	6.70	20.00
Flumequine	400	20	100	66	71	18	10	6.70	20.00
Lincomycin	100	5	25	98	102	15	18	1.70	5.00
Marbofloxacin	no MRL	5	25	88	98	9	18	1.70	5.00
Oxytetracycline	100	10	50	158	156	9	8	3.30	10.00
Penicillin G	50	5	25	99	83	58	76	1.70	5.00
Penicillin V	25	2.5	12.5	83	77	26	11	0.80	2.50
Pirlimycin		10	50	60	62	16	10	3.30	10.00
Sulfadiazin	100	1	5	81	92	10	8	0.30	1.00
Sulfadimethoxine		1	5	52	66	10	13	0.30	1.00
Sulfadimidin		1	5	88	87	14	11	0.30	1.00
Sulfadoxin		1	5	81	102	9	15	0.30	1.00
Sulfamerazine		1	5	93	103	19	8	0.30	1.00
Sulfamethoxazole		1	5	76	85	16	13	0.30	1.00
Sulfamethoxypyridazine		1	5	78	99	10	18	0.30	1.00
Sulfathiazole		1	5	107	106	13	22	0.30	1.00
Tetracycline	100	10	50	133	147	5	11	3.30	10.00
Trimethoprim	50	5	25	117	quadratic	7	quadratic	1.70	5.00
Tylosin	100	10	50	24	22	12	18	3.30	10.00

Recoveries between 70–120 % and RSD < 20 % are marked green, n.d. = not detectable, Cipro- and enrofloxacin are regulated in sum, Sulfonamides are regulated in sum.

APPENDIX IV – POSTER PRESENTATIONS

- **ANAKON 2015 – March 23rd–26th 2015, Graz, Austria**



QuEChERS based UHPLC-MS/MS methods to determine veterinary drug residues in bovine milk

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Introduction

QuEChERS (**Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, and **S**afe) was initially developed by Anastassiades et al. as a powerful sample preparation for analyzing hundreds of pesticide residues in fruit and vegetable samples.[1] Based on this simple and quick sample treatment, a QuEChERS based UHPLC-MS/MS method should be developed to determine residues of different veterinary drug classes (macrolides, quinolones, sulphonamides, tetracyclines, ß-lactams) in bovine milk. Therefore several different sample extractions with and without any further clean-up steps were tested in this study. Unfortunately we observed that the different QuEChERS modified approaches showed no satisfactory recoveries for all veterinary drug substances. Therefore these easy and quick extraction procedures do not meet the requirements to become suitable for routine used and validated methods.

Materials & Methods

Step 1: Sample extraction

Step 2: additional sample clean-up

7) Analysis of the samples by UHPLC-MS/MS

UHPLC conditions:

Instrument:	Agilent 1290
Column:	Agilent ZORBAX Eclipse Plus C18, RRHD, 2.1 x 100 mm, 1.8 µm
Temperature:	60 °C
Flow rate:	0.350 ml/min
Mobile phase:	A: H_2O 0.1% HCOOH, 5mM NH_4COOH B: MeOH 0.1% HCOOH, 5mM NH_4COOH

Gradient: Time (min)	%A	%B
0.00	95	5
2.00	95	5
3.00	83	17
6.00	30	70
8.00	30	70
9.00	95	5
15.00	95	5

MS/MS conditions:

Instrument: LC/MS	Agilent 6490 Triple Quad
Gas temperature:	200 °C
Gas flow:	15 l/min
Nebulizer:	30 psi
Capillary:	3,500 V (positive)
Sheath gas temperature:	375 °C
Sheath gas flow:	11 l/min
Scan mode:	dynamic MRM in positive mode

Results & Discussion

Step 1: Sample extraction

Blank bovine milk samples are fortified with an antibiotic multistandard mix according to 1/10 of their maximum residue levels (MRLs). For protein and fat precipitation acidified ACN is used to extract the analytes from the bovine milk samples. An aliquot of water has to be added to obtain a H2O:ACN ratio of 1:1 preventing agglutination of the salts. An example of mean recovery rates of sample injection without any further clean up step are shown in figure 1. Additionally, sample extraction without any clean up step results in asymmetric peak shapes of the sulphonamides (fig. 2).

Step 2a: Addition of dSPE salts

Based on these results, further clean up steps are necessary to remove possible matrix inferences. Therefore PSA sorbent (for removing sugars and fatty acids) and C18EC (for elimination of apolar interferences) are used to minimize the matrix effects and in order to achieve satisfying recovery rates. Despite the addition of dSPE salts an improvement of the recovery rates can not be observed, compared to previous results without additional clean up (fig. 3). Also the sulphonamide class shows no improvement of their peak shapes (fig. 4).

Step 2b: Evaporation of the sample extracts

A further possibility to improve recovery rates and peak shapes is to evaporate the sample extracts and reconstitute with initial mobile phase conditions. This clean up step improves the peak shapes of the sulphonamides as well as the number of detected substances. Nevertheless, the recovery rates for nearly all substances are low. To enhance the recovery rates by reducing possible matrix interferences the samples are quantified with a matrix matched calibration. The results demonstrate only a slight increase of the recovery rates (fig. 5), which leads to the conclusions that the analytes get lost during the sample extraction procedure.

Conclusions

Despite QuEChERS extraction offers a quick and easy sample preparation, this work shows that these approaches are not suitable to analyse several veterinary drug residues in bovine milk. To achieve required recovery rates the analytes could either be quantified with a procedure standard calibration or with isotopically labelled standards, which is not easily suitable for routine use. Therefore other sample preparations have to be investigated to achieve required recovery rates for all substances.

- **7th International Symposium on Recent Advances in Food Analysis (RAFA) November 3rd – 6th, 2015, Prague, Czech Republic**

Dispersive solid phase extraction for quantification of antibiotic residues in dairy products using UHPLC-MS/MS

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Overview

food safety – antibiotic residues in dairy products – dispersive SPE salts – multiclass UHPLC-MS/MS method – bacterial influence to the recovery rates

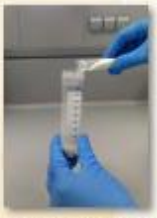
Introduction

This project aims to develop a confirmatory UHPLC-MS/MS multi-class method for determination of antibiotic residues in milk and different kinds of dairy products. The final method should be used in routine laboratory work to protect customers from additionally uptake of antibiotic drugs, due to misuse in animal livestock.

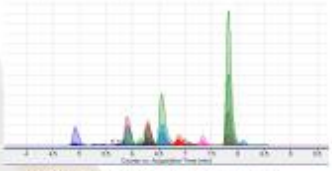
Materials & Methods

Matrices of interest:
e.g. cheeses, curd, butter, whipped cream, yogurt etc.

Extraction solutions:
- ACN/H_2O 4:1
- ACN/Na_2EDTA-McIlvaine buffer 4:1



Sample clean-up with various dispersive SPE salts:
- 500 mg C18 endcapped
- 500 mg C18EC/150 mg PSA
- 500 mg C18/ZSep



UHPLC-MS/MS quantification of following drug classes:
- quinolones
- macrolides
- lincosamides
- sulfonamides
- tetracyclines

Results & Conclusion

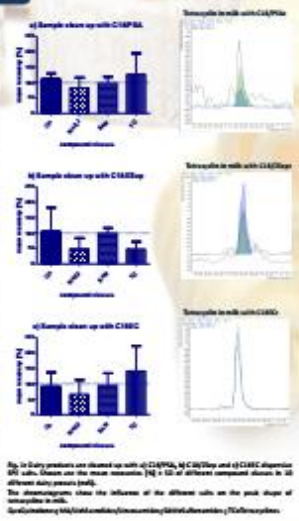
2g ± 0,1g of homogenized samples are fortified with an antibiotic multiclass standard mix according to 1/10 of their maximum residue levels. To reduce matrix effects internal standards are used.

Antibiotic residues are extracted with ACN/H2O 4:1 from and samples are cleaned-up with different dispersive SPE salts to remove fatty components from the matrices.

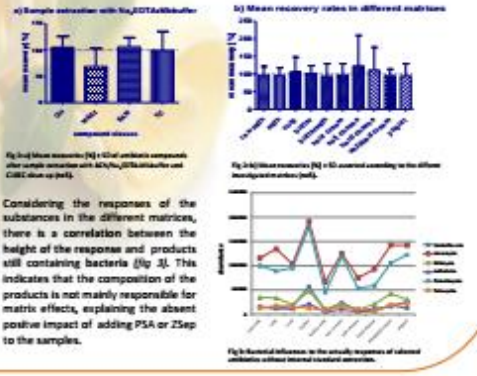
The combination C18/PSA indicates a mean recovery rate of nearly 100% for all compounds in all matrices. Nevertheless quinolones and tetracyclines shows a decrease in their peak shapes. Doxycycline is not able to be detected (fig 1a).

Sample clean up with C18/ZSep results in a low mean recovery rates for tetracyclines, macrolides/lincosamides. Quinolones show a recovery rate of 100%, but with a high standard deviation. The usage of ZSep is accompanied with a decrease of peak shape quality for quinolones and tetracyclines (fig 1b).

Best results in mean recovery and peak shapes are achieved by conducting sample clean up with C18EC dispersive SPE salt alone. Only Tetracyclines are showing a too high recovery rate as well as a high standard deviation (fig. 1c).



The high recovery rates for tetracyclines can be explained by their characteristic ability to form complexes with cations like Mg^{2+} or Ca^{2+}, which are naturally present in dairy products.
For reducing these complex buildings a Na_2EDTA-McIlvaine buffer solution is used instead of water for extraction.
With this combined extraction solution, followed by sample clean up with C18EC a mean recovery rate between 70-110% for all substances in all matrices can be achieved (fig 2a and b). Only cheesy products show a high standard deviation assuming this method is not reproducible for these matrices.



Considering the responses of the substances in the different matrices, there is a correlation between the height of the response and products still containing bacteria (fig 3). This indicates that the composition of the products is not mainly responsible for matrix effects, explaining the absent positive impact of adding PSA or ZSep to the samples.

Outlook

For better understanding the bacterial influence, these products have to be tested on their microbiological profile.
Nevertheless, with this method it is possible to achieve satisfying recovery rates for over 20 antibiotic residues in dairy products. Additionally, the easy and fast sample preparation allows a high sample-throughput, consequently this method can be validated and used in routine laboratory work.