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Multi–Class, Multi Residue Method for Determination of Penicillins, Cephalosporins and Quinolones in Cow Milk and Validation in Accordance with Commission Decision 2002/657/EC

emQal

European Masters in Quality in Analytical Laboratories

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February 2010

Under the supervision of Dr. José Barbosa Torralbo and Dra. Dolores Barrón Bueno in Department of Analytical Chemistry at University of Barcelona

The project entitled

Multi-Class, Multi Residue Method for Determination of Penicillins, Cephalosporins and Quinolones in Cow Milk and Validation in Accordance with Commission Decision 2002/657/EC

In partial fulfillment

Of the requirements for the degree

Masters in Quality in Analytical Laboratories

Submitted By

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February, 2010

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Acknowledgement

There are few people without whose involvement this project work would never have come to be. So I take this opportunity to thank and dedicate the following lines.

I would like to express my deepest gratitude to my supervisors, Dr. José Barbosa Torralbo (Professor, Department of Analytical Chemistry, University of Barcelona) and Dra. Dolores Barrón Bueno, for their support, guidance, and encouragement during the course of this project and helping me to develop an understanding of the subject. I am much obliged to them for sharing their ideas with me to complete the project successfully.

Thanks to Anna Jubert, Technical Director of ALLIC for providing positive samples.

I take this opportunity to thank Pili for providing me helpful suggestions.

Heartfelt thanks goes to Sandra for guiding me, for having patience to listen to me and clearing my doubts and queries from the preliminary to the concluding level.

I am very grateful to Rafa for helpful discussions, constant support and friendship.

I also wish to acknowledge Fernando, Estela for their kind cooperation and helping hands.

Thanks to Marta, Sílvia, Sergio, Raquel, Cristina, Leyre, Edyta, Gultekin and Dilara for their company, kind cooperation thus making my stay at Bioanalysis department memorable.

Finally and most importantly, I would like to thank my parents Mr. Ramhari Amatya and Mrs. Sulochana Amatya, my family and my friends for their support, concern throughout all my studies at University.

Abbreviations

ACN	-	Acetonitrile
ALLIC	-	Laboratori Interprofessional Lleter de Catalunya
AMOX	-	Amoxicillin
AMPI	-	Ampicillin
CE	-	Collision Energy
CIP	-	Ciprofloxacin
CLOX	-	Cloxacillin
CUR	-	Curtain Gas
DAN	-	Danofloxacin
DICL	-	Dicloxacillin
DP	-	Declustering Potential
ENR	-	Enrofloxacin
EP	-	Entrance Potential
FLU	-	Flumequine
FP	-	Focusing Potential
HAc	-	Acetic Acid
HFo	-	Formic Acid
HLB	-	Hydrophilic Lipophilic Balance
IS	-	Internal Standard
LEX	-	Cefalexin

LC-MS/MS	-	Liquid Chromatography coupled to Tandem Mass Spectrometry
LOD	-	Limit of Detection
LON	-	Cefalonium
LOQ	-	Limit of Quantification
MAR	-	Marbofloxacin
MeOH	-	Methanol
MRL	-	Maximum Residual Limit
MRM	-	Multiple Reaction Monitoring
NAFC	-	Nafcillin
NEB	-	Nebuliser Gas
OXAC	-	Oxacillin
PBPs	-	Penicillin Binding Protein
PER	-	Cefaperazone
PIP	-	Pipemidic Acid
PIPE	-	Piperacillin
PIR	-	Cefapirin
QUI	-	Cefquinome
RSD	-	Relative Standard Deviation
SPE	-	Solid Phase Extraction
TIO	-	Ceftiofur
UPLC-MS/MS	-	Ultra Performance Liquid Chromatography coupled to Tandem Mass Spectrometry

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Objective

The objective of this work was to develop and validate a method for multi-class, multiresidue determination of the series of Penicillins (Amoxycillin, Ampicillin, Cloxacillin, Dicloxacillin, Nafcillin, Oxacillin and PENG), Cephalosporins (Cefalexin, Cafalonium, Cefapirin, Cefaperazone, Cefquinome, Ceftiofur and Cefazolin) and Quinolones (Ciprofloxacin, Danofloxacin, Enrofloxacin, Flumequine and Marbofloxacin) regulated by European legislation 2377/90/EC in cow milk using LC-MS/MS.

Abstract

The method was developed to determine simultaneously 19 drugs of three classes (seven Penicillins and Cephalosporins, and five Quinolones) regulated by European legislation 2377/90/EC in cow milk using LC-MS/MS. To make the sample preparation as simple as possible deproteinization step using organic solvent was eliminated. SPE clean up and concentration was done using OASIS HLB cartridge. The separation of 19 antibiotics was achieved in 11 minutes using C8 column with gradient elution. MS data was acquired on MRM mode with two transitions for each compound. Validation procedure was conducted based on European directive 2002/657/EC. 89% of drugs presented recoveries higher than 65 % with exception of AMOX (57%) and DAN (38 %). Repeatability values expressed as relative standard deviation, (RSDs) were not more than 15 %. LODs values ranged from 0.03 (NAFC) to 0.5 µg/kg (PER) and LOQs from 0.1 (NAFC, PENG, CIP, DAN, and ENR) to 1.25 µg/kg (PER). The method was applied to 49 real samples. 37% of the sample was found to be non-compliant with an error probability of β. Of all samples analysed, 14 % of samples contained AMOX and 16 % contained PENG. AMOX and PENG was the most common residue found in milk sample.

1. Introduction

Antibacterial agents also categorized as anti–infective, antimicrobials or chemotherapeutics comprise synthetic and natural compounds. The latter known as antibiotics are produced by fungi and bacteria at low concentrations for inhibiting growth of other microorganisms. Strictly antibiotics should include only five classes – penicillins, tetracyclines, macrolides, aminoglycosides and Amphenicols. However currently the terms antibiotic is used synonymous with antibacterial, so synthetic drugs (e.g. quinolones or nitrofurans) and substances of high molecular weight (e.g. peptide antibiotics) also belong to this group [1].

1.1. Classification of Antibiotic

On the basis of type of activity antibiotics can be divided into two groups:

1.1.1 Bactericidal

Antibiotics that cause bacterial cell death are called bactericidal.

- **β-Lactams**: β- Lactams basically consists of two classes of thermally labile compounds, penicillins and cephalosporin. They are probably the most widely used class of antibiotics in veterinary medicine for *the treatment of bacterial infections especially in lactating cows* [2].
- **Quinolones**: Quinolones are synthetic broad spectrum antimicrobial agents used in the treatment of livestock and in aquaculture. They are used in veterinary medicine for *the treatment of pulmonary infections, urinary infections and digestive infections in cows, pigs, turkeys or chicken* [3-4].
- **Aminoglycosides**: In veterinary medicine and animal husbandry, they are widely used in the *treatment of bacterial infections, e.g. bacterial enteritis and mastitis, and have been added to feeds for prophylaxis and to promote growth*. They are currently not permitted for use as growth promoters in the European Union [5].
- **Nitroimidazoles**: Nitroimidazoles are a class of veterinary drugs used for the *treatment and prevention of certain bacterial and protozoal diseases in poultry (histomoniasis in turkeys, trichomoniasis in pigeons etc) and for swine dysentery.* Nitroimidazoles posses mutagenic, carcinogenic and toxic properties so have been prohibited in EU [3].
- **Nitrofurans**: Nitrofurans are antibacterial agents which have been widely used as *feed additives for the treatment of gastrointestinal infections (bacterial enteritis*

caused by Escherichia coli and salmonella) in cattle, pigs and poultry. Use of nitrofuran antimicrobials in food producing animals have been prohibited within EU since 1997 since they have been reported to be carcinogenic and mutagenic [3].

1.1.2 Bacteriostatic

Antibiotics that stops bacteria growth and allow host's normal defense mechanisms to combat the infection are bacteriostatic. Antibiotics that are predominantly bacteriostatic include the tetracyclines, the macrolides, chloramphenicol and trimethoprim.

- Tetracyclines: Tetracyclines are broad spectrum antibiotics effective against gram positive as well as gram negative bacteria. They are used *for promoting growth in cattle and poultry* [3].
- Sulphonamides and Trimethoprim: They exhibit a bacteriostatic rather than bactericidal effect. Sulphonamides are used as veterinary drugs for *prophylactic and therapeutic purposes; they also act as growth promoting substances* [3].
- Macrolides: They are derived from Streptomyces bacteria, and got their name due to their macrocyclic lactone chemical structure. Macrolides are an important class of antibiotics which *are widely used in veterinary practice to treat respiratory diseases or as feed additives to promote growth* [3].
- Amphenicols: They are synthetic antibiotics with similar broad-spectrum of activity. The Amphenicols class of antibiotics consists of chloramphenicol, thiamphenicol and florfenicol [6].Chloramphenicol is a broad spectrum antibiotic active against a variety of pathogen. *It was previously used in veterinary and human medicine*, however reports of plastic anemia in humans arising from its use led to its ban in the US and EU in 1994 [3]. The use of nitrofurans and chloramphenicol is not allowed in EU and therefore these compounds are classified in group A, the forbidden substances group [7].

1.2. Mechanism of Action of Antibiotics

The mechanism of action of antibiotics falls into following categories [6].

1.2.1 Antimicrobials which can act on the Bacterial Cell Wall

These interfere with synthesis of peptidoglycan layer in cell wall; they eventually cause cell lysis, bind to and inhibit activity of enzymes responsible for peptidoglycan synthesis. They interfere with cell wall synthesis by binding to penicillin binding proteins (PBPs), which are located in bacterial cell walls. (E.g. β-Lactams)

1.2.2 Antimicrobials acting on Nucleic Acid Synthesis

- *Inhibitor of RNA synthesis and function:* These antimicrobials bind to DNA-dependent RNA polymerase and inhibit initiation of RNA synthesis. They inhibit transcription. (E.g. Rifampin, rifampicin)
- *Inhibitor of DNA replication:* They inhibit bacterial topoisomerase which are necessary for DNA synthesis. (E.g. nalidixic acid, ciprofloxacin)
- *Inhibitors of precursor synthesis:* They inhibit early stages of folate synthesis. (E.g. sulphonamides)

1.2.3 Antimicrobials acting on Protein Synthesis

Antimicrobials acting on protein can be classified into those bind to 30S ribosome and 50s ribosome subunit.

Antimicrobials that bind to the 30S Ribosomal Subunit

- Aminoglycosides: The aminoglycosides irreversibly bind to the 30S ribosome and freeze the 30S initiation complex (30S-mRNA-tRNA), so that no further initiation can occur. The aminoglycosides also slow down protein synthesis that has already initiated and induce misreading of the mRNA.
- Tetracyclines: The tetracyclines reversibly bind to the 30S ribosome and inhibit binding of aminoacyl-t-RNA to the acceptor site on the 70S ribosome.
- Spectinomycin: Spectinomycin reversibly interferes with mRNA interaction with the 30S ribosome. It is structurally similar to aminoglycosides but does not cause misreading of mRNA.

Antimicrobials that bind to the 50S Ribosomal Subunit

- Chloramphenicol, lincomycin, clindamycin: These antimicrobials bind to the 50S ribosome and inhibit peptidyl transferase activity.
- Macrolides: The macrolides inhibit translocation of the peptidyl tRNA from the A to the P site on the ribosome by binding to the 50S ribosomal 23S RNA. E.g. erythromycin, azithromycin, clarithromycin

1.2.4 Inhibitors of Bacterial Folate Synthesis

These block cell metabolism by inhibiting enzymes which are needed in the biosynthesis of folic acid which is a necessary cell compound.

- Sulphonamides, sulfones: These antimicrobials are analogues of para-aminobenzoic acid and competitively inhibit formation of dihydropteric acid.

- Trimethoprim, methotrexate, pyrimethamine: These antimicrobials bind to dihydrofolate reductase and inhibit formation of tetrahydrofolic acid.

1.2.5 Antimicrobials acting on the Cell Membrane

- Amphotericin that binds to the sterol-containing membranes of fungi,
- Polymyxins that act like detergents and disrupt the Gram negative outer membrane and
- Fluconazole and itraconazole that interfere with the biosynthesis of sterol in fungi.

The diverse sites of action of antibiotics are summarized in figure 1.1.

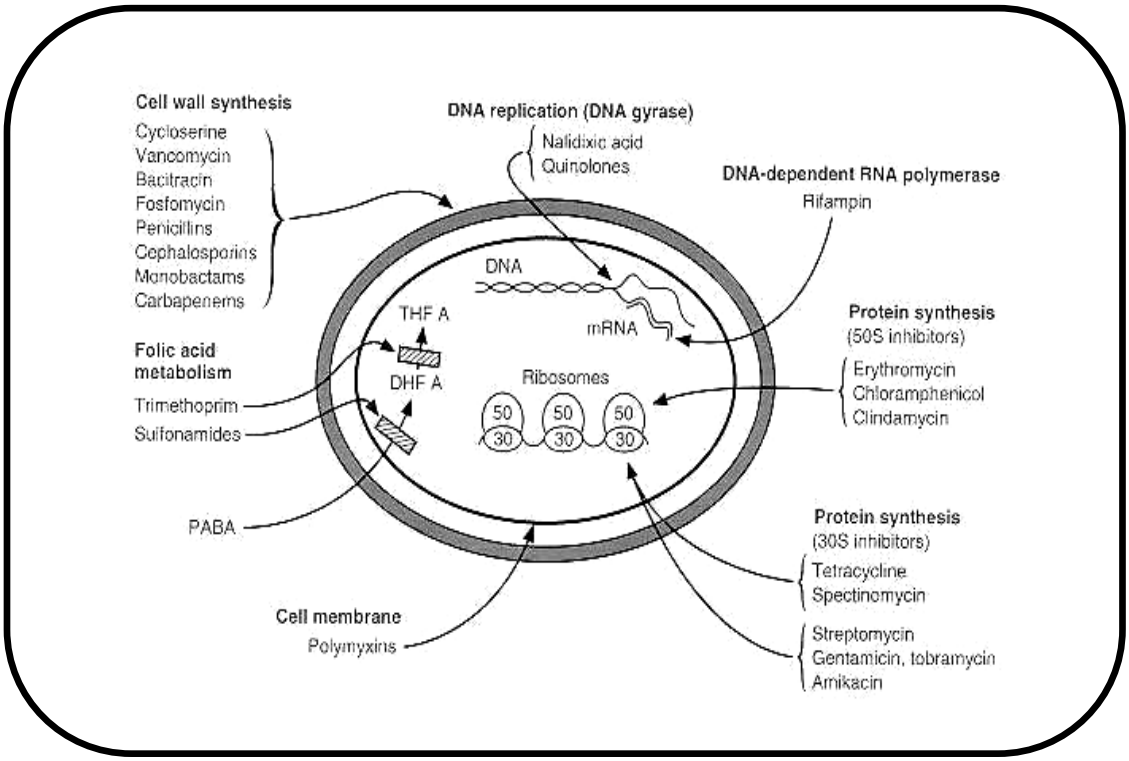


Figure (1.1): Action Sites of Antibiotics

1.3 Antibiotic Resistance Mechanism

Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are innately resistant to ≥ 1 class of antimicrobial agent. Other cases are acquired resistance. Acquired resistance that develops due to chromosomal mutations and selection is called vertical evolution. Bacteria also develop resistance through the acquisition of new genetic material from other organisms. This is termed as horizontal evolution. A resistance mutation can be expressed in several ways [8-9].

1.3.1 Production of Enzyme that destroys Active Drug

The first central mechanism by which bacteria can be resistant to an antibiotic is by production of enzymes that inactivate antimicrobials. The organism may acquire genes encoding enzymes such as β-lactamases that destroy the antibacterial agent from the cell before it can reach its target site and exert its effect.

1.3.2 Mutations in Efflux Mechanisms

Antibiotic efflux pumps are a common way for bacteria to resist the action of numerous classes of antibiotics. Bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. They are chiefly concerned with removal of waste products, but changes in their conformation can enable them to remove antimicrobials. Gram positive organisms can show resistance to macrolides by this mechanism and resistance to tetracyclines is usually mediated via efflux.

1.3.3 Bypass of Metabolic Pathway

Bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down regulation of porin genes. Resistance to sulfonamides and trimethoprim is mediated by metabolic bypass, in this case due to synthesis of altered dihydropteroate synthetase and dihydropteroate reductase. These enzymes have reduced susceptibility and affinity for sulfonamides and trimethoprim respectively.

Other mechanism of resistance may occur through one of several genetic mechanism including transformation, conjugation and transduction.

The most important vehicles for transfer of resistance genes in bacteria are plasmids, transposons and integrons.

Plasmids: Plasmids are extra chromosomal, replicable circular DNA molecules that may contain resistance genes. They replicate independently of bacterial chromosomal DNA. Plasmids are important in bacterial evolution, because they affect replication, metabolism, bacterial fertility as well as resistance to bacterial toxins, antibiotics and bacteriophages.

Transposons: Transposons (jumping genes) are short sequence of DNA that can move between plasmids, between a plasmid and the bacterial chromosome or between a plasmid and a bacteriophage (bacterial virus). Unlike plasmids transposons are not able to replicate independently and must be maintained within a functional replicon (plasmid or chromosome).

Integrons and Gene Cassettes: Integrons are naturally occurring gene expression elements. They are composed of two conserved regions and an interposed variable region, which contains gene cassettes for antibiotic resistance.

Some of the many mechanisms of resistance are indicated schematically in the figure 1.2

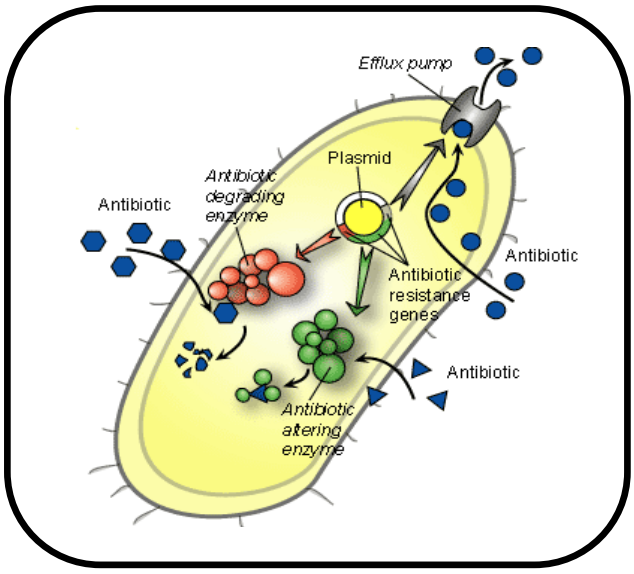


Figure (1.2): Mechanism of antibiotic resistance

1.4. Use of Veterinary Antibiotics in Dairy Cows

Antibiotic use in livestock production originated in the 1950's when waste from the fermentation process used in commercial chlortetracycline production was found to improve the growth rates of pigs, poultry, and cattle [10].

The use of antibiotics in veterinary medicine and especially in dairy cows involves [6]

1.4.1. Therapeutic use to treat infected animals against diseases e. g. mastitis of dairy cows, diarrhoea, pulmonary diseases, bacteremia, etc.

1.4.2. Prophylactic use to avoid infection of rest animals, preventing diseases. In dairy cows, antibiotics are routinely administered directly into the udder to cure and

avoid mastitis (e. g. in dry-cow therapy). The same therapeutic antibiotics are delivered directly into the fodder or drinking water.

1.4.3. Use at sub-therapeutic levels to induce growth promotion.

1.5. Antibiotic Resistance associated with Therapeutic use of Veterinary Medicine

Antimicrobials use will lead to the selection of resistant forms of bacteria which occurs with all uses including treatment, prophylaxis and growth promotion. Adverse consequences of selecting resistant bacteria in animals include:

1.5.1. An increase in the prevalence of resistant bacteria in animals, the transfer of resistant pathogens to humans via direct contact with animals or through the consumption of contaminated food and water,

1.5.2. The transfer of resistance genes to human bacteria,

1.5.3. An increase in the incidence of human infections caused by resistant pathogens,

1.5.4. Potential therapeutic failures in animals and humans. Few examples associated with resistance mechanisms are:

Fluoroquinolones use in poultry has given a dramatic rise in the prevalence of fluoroquinolones-resistant Campylobacter jejuni isolated in live poultry, poultry meat and from infected humans. Multi resistant Escherichia coli have been selected by the use of broad spectrum antimicrobials in both livestock and humans.

1.5.5. Residues of antimicrobial agents in food of animal origin in excess of the agreed acceptable minimum residue level (MRLs) may contribute to the generation of resistance in bacteria in humans [11].

1.6 Importance of determination of Antibiotic Residue

Residue analysis is concerned with food safety, as it establishes whether food is safe or unsafe for human consumption. Incorrect use of antibiotics in veterinary practice may leave residues in edible tissues. These residues may have direct toxic effects on consumer e.g. antibiotics such as penicillin can evoke allergic reactions in hypersensitive individuals even though small amount are ingested or exposed by parental routes. Or they may cause problems indirectly through induction of resistant

strains of bacteria. As a result the antibiotic resistant pathogens do not respond well to therapy by ordinary antibiotics as a result new antibiotics must be developed [3, 10].

1.7 β- Lactam Antibiotics

β-Lactam antibiotics comprise several classes of compounds such as penicillins, cephalosporins, and carbapenems, monobactams among which the penicillin and the cephalosporin are most important. These compounds contain three main parts: β-lactam ring, free carboxyl acid group and a substituted amino acid side chain. Both penicillin and cephalosporin have a β-lactam ring but in the case of penicillin it is fused to a five membered thiazolidine ring, and in the case of cephalosporin it is fused to a six membered dihydrothiazine ring.

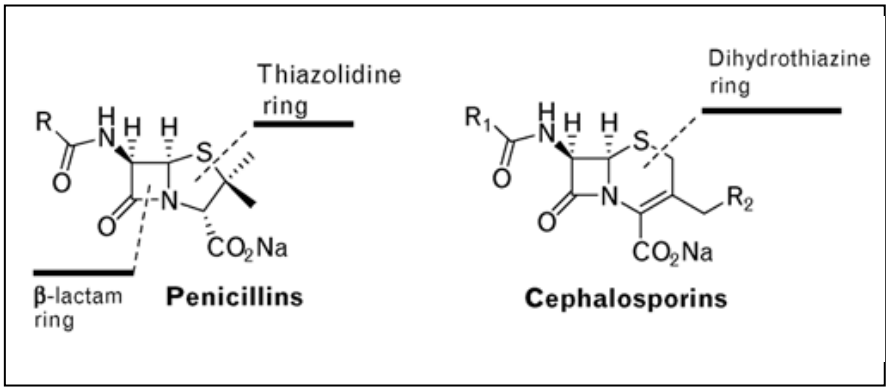


Figure (1.3): Structure of penicillin and cephalosporin

1.7.1. Penicillins

The penicillins are classified as β-lactam drugs because of their unique four-membered lactam ring. They share features of chemistry, mechanism of action, pharmacologic and clinical effects, and immunologic characteristics with other β-lactam compounds [12]. They are compounds with limited stability because of the presence of the four term ring in their structure and they are thermolabile, unstable in alcohols and isomerize in acid ambient [2].

Chemistry

All penicillins have the basic structure shown in figure 1.4. A thiazolidine ring (A) is attached to a β-lactam ring (B) that carries a secondary amino group (RNH–). Substituent - R can be attached to the amino group. Structural integrity of the 6 aminopenicillanic acid nucleus is essential for the biologic activity of these compounds. If the β-lactam ring is enzymatically cleaved by bacterial β-lactamases, the resulting product, penicilloic acid, lacks antibacterial activity. The penicillins are susceptible to

bacterial metabolism and inactivation by amidases and lactamases at the points shown below[12].

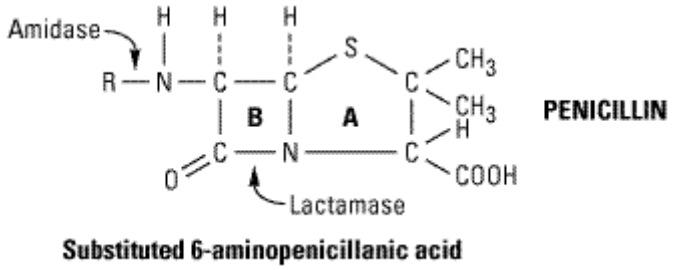


Figure (1.4): Basic structure of penicillin (6-aminopenicillanic acid)

1.7.2. Cephalosporins

Cephalosporin is similar to penicillins chemically, in mechanism of action, and in toxicity. Cephalosporins are more stable than penicillins to many bacterial -lactamases and therefore usually have a broader spectrum of activity. The cephalosporins are bactericidal and inhibit the third stage of bacterial cell wall synthesis by interfering with cross-linking of linear peptidoglycan strands [13].

Chemistry

The nucleus of the cephalosporins, 7-aminocephalosporanic acid (Figure 1.5), bears a close resemblance to 6-aminopenicillanic acid (Figure 1.4). The intrinsic antimicrobial activity of natural cephalosporins is low, but the attachment of various R1 and R2 groups has yielded drugs of good therapeutic activity and low toxicity. The cephalosporins have molecular weights of 400–450. They are soluble in water and relatively stable to pH and temperature changes [12].

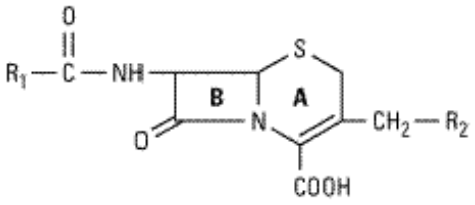


Figure (1.5): Structure of Cephalosporin

1.8 Quinolones

Quinolone are broad spectrum synthetic antimicrobial agent used in the treatment of livestock and aquaculture. The older quinolones e.g. nalidixic acid have limited activity against gram negative organisms while the more recently introduced fluoroquinolones e.g. Enrofloxacin have a wide spectrum of activity . In general Quinolone carboxylic acids are amphoteric with poor water solubility between pH 6 and 8 [13].

Chemistry

The general structure consists of quinolones consists of a 1-substituted-1, 4-dihydro-4-oxopyridine-3-caboxylic moiety combined with an aromatic or heteroaromatic ring .The carboxylic acid at position 3 and the ketone group at position 4 are necessary for DNA gyrase inhibition, where as substitution at position 1 and 7 influence the potency and the biological spectrum of activity of the drugs [4].

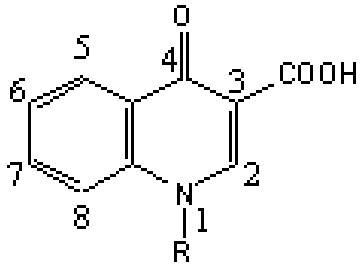


Figure (1.6): Basic structure for Quinolone

Due to the presence of carboxylic acid ($pk_a \approx 5$) and one or more amine functional groups ($pk_a \approx 8$-9), quinolones have amphoteric and zwitterionic properties; between pH 6 and 8, they exhibit poor water solubility, but are lipid soluble and able to penetrate tissues [41].

Mechanism of Action:

Quinolones block bacterial DNA synthesis by inhibiting bacterial topoisomerase II (DNA gyrase) and topoisomerase IV. Inhibition of DNA gyrase prevents the relaxation of positively super coiled DNA that is required for normal transcription and replication [12].

1.9 Multiclass, Multi Residue determination of Antibiotic in Food Samples

Multiclass, multi residue methods are gaining importance for residue control in food products. These methods are limited in comparision to single class residual methods not because that they are not needed, but because of a number of analytical challenges which have to be overcome. The first multi-class method for veterinary drugs residue in food products was reported by Yamada et.al in 2006 [14].

Form the literature review [45] we can see that multi-class, multiresidue analysis is performed in two different ways.

- Qualitative [18] or quantitative screening assays [25],
  - Screening and confirmation [15, 19, and 21],
  - Screening and quantification [26].
- Quantitative confirmatory

Table 1.1 provides a summary of recent chromatographic methods for detection of multiclass, multi residue analysis of antibiotics in wide range of sample matrices such as animal tissue, liver, kidney, egg, milk and honey.

The articles [15, 22-23, 32-33, 35-36] are based on determination of multi-class, multi residue of antibiotics in milk samples. Some papers [14, 22] include validation data but do not fully comply with the EU commission decision 2002/657/EC.

From the table 1.1, we can see that most of the papers utilizes Oasis HLB for clean up. However some papers eliminate the clean up in order to make the method fast and simple [24]. Some paper [33, 38, and 40] utilizes extraction procedure based on QuEChERS methodology.

As can be seen from table 1.1, LC-MS/MS and UPLC-MS/MS are the common detection techniques for multi-class, multi residue analysis.

This work involves development of method for Quantitative, confirmatory analysis of penicillins, cephalosporins and quinolones in milk using LC-MS/MS.

Table 1.1 Summaries of Existing Chromatographic Methodologies for Detection of Multiclass, Multi Residue Analysis of Antibiotics

No. of Residues	Analyte	Matrix	Extraction Technique	Clean up	Recovery (%)	Detection System	Application	Date [Ref]
130	Many	Bovine, Porcine, Chicken Muscle	ACN: MeOH(95:5)	-----	70-110%	LC-MS/MS	Quantitation	2006 [14]
25	βLCs,SAs,Qs,MCs	Milk	Acetonitrile	SPE (Oasis HLB)	Low for β-LCs, MCs and Qs above 70%, TCs 50-60%	LC-MS/MS	Screening, Confirmation	2008 [15]
6	TCs, PCs	Bovine and Swine, Muscle Kidney and Liver	No	No	70-115%	LC –MS/MS	Screening	2005 [16]
18	TCs, SAs, Qs	Shrimp Meat	TCA	SPE (Oasis HLB)	--------	LC-Ion trap	Screening /Confirmatory	2006 [17]
21	TCs, Qs	Pig Kidney , Liver and meat	EDTA-McIlvaine buffer	SPE (Oasis HLB)	80.2-117.8%	UPLC-MS/MS	Screening, Confirmatory	2007 [19]

19	TCs, SAs, Qs, β-LCs, MCs	Muscle (Pig , Cattle, Sheep, Horse, Deer , Reindeer) Kidney (Pig , Cattle, Sheep, Horse)	70 % Methanol	---------	--------	LC-MS/MS	Screening , Confirmation	2007 [20]
29	SAs, TCs, FQs, β-LCs	Eggs	----------	SPE (Oasis HLB)	---------	LC-MS/MS	Screening , confirmation	200 [21]
150	AVs,BZs,βagonist, β-LCs, Cs, NZs, Qs, SAs, TCs	Milk	ACN	--------	No	UPLC-TOF-MS	Screening	2007 [22]
10	PLs , TCs, Cs	Milk	Methanol, MCIlvaine Buffer	SPE (Oasis HLB)	52.1% -101.0%	UPLC	Quantification	2009 [23]
39	TCs, Qs, PCs, SAs ,TMP,MCs	Chicken Muscle	MeOH:H_2O(70:30) Containing EDTA	No clean up	60-96.5%	UPLC-MS/MS	Quantification, Confirmation	2008 [24]
100	BZs, CH,DV, LI,MCs, NZs,PCs, SAs,TCs, Ts	Muscle , Liver , Kidney	Liquid – Liquid Extraction	SPE (Oasis HLB)	Muscle:14-118% Liver:16- 121% Kidney:13-141%	UPLC-TOF	Quantitative	2008 [25]

100	BZs,MCs,PCs,Qs, SAs,PYs,TCs,NZs , Ts,Ns,Is,As,	Milk	ACN	SPE (Strata- X)	80-120%	UPLC-TOF-MS	Screening, Quantification, Confirmation	2008 [26]
15	PCs ,Cs	Bovine Muscle, Kidney and Milk	ACN/Water	SPE (Oasis HLB)	Milk : 57-115% Muscle:65-111% Kidney:46-90%	LC-MS-MS	Screening / Quantification/ Confirmation	2004 [28]
47	TCs,Qs,MCs ,SAs ,DPs ,LCs	Milk	TCA, MCIlvaine buffer	SPE Oasis HLB	94-112%	LC-MS/MS	Quantification	2009 [32]
18	Qs, SAs, MCs, As, TCs	Milk	Liquid Extraction with ACN	No cleanup	70-110%	UPLC – MS/MS	Quantification, Confirmation	2008 [33]
19	TCs, SAs, TMP, Ds	Muscle (Bovine, Ovine, Porcine and Poultry)	0.1M EDTA and ACN	No SPE clean up	94-102.1%	UPLC – MS/MS	Quantification Confirmation	2009 [34]
7	TCs, SAs ,CPs	Bovine Milk	TCA	SPE (Oasis HLB)	83-112%	HPLC-DAD	Quantitative	2009 [35]

10	SAs, TCs, Ps	Cow Milk	TCA, MCIlvain buffer	SPE (Oasis HLB)	72-97%	HPLC-MS	Quantitative	2007 [36]
8	FQs, TCs	Catfish muscle	ACN and Citrate Buffer	-----	60-92%	LC–Fluorescence	Quantitation	2007 [37]
25	TCs, MCs, Qs, SAs, As	Eggs	ACN+Citric acid+Na_2EDTA	QuEChERS	60-119%	UPLC – MS/MS	Quantitation	2009 [38]
15	TCs , FQs , MCs , LCs ,AGs , SAs, PLs	Honey	-------	SPE (Strata X 60mg, 3ml)	65-104%	LC-MS/MS	Quantification ,Confirmation	2008 [39]
41	NZs, SAs, FQs, Qs, Is	Chicken muscle	QuEChERS	Dispersive SPE	Varies	LC-MS/MS	Screening	2009 [40]

Acronyms: PCs: Penicillins , Cs : Cephalosporins, TCs : Tetracyclines , Qs : Quinolones, MCs: Macrolides, SAs: Sulfonamides, DPs:diaminopyrimidine , LCs: Lincosamides, PLs : Phenicol , TMP: Trimethoprim, Ps: Pyrimethamine, Ts : tranquilizers , As : Antihelminthes , PYs : Pyrimidine, NZs: Nitroimidazoline, CAP: chloramphenicol , Ds: dapsone , Ns : NSAIDS, AVs: avermectin , COs: corticoids, Is: Ionophores, As: Amphenicols, β-LCs: β-Lactams, FQs: Fluoroquinolones, AGs: Aminoglycosides

1.10 European Regulation for the Use of Veterinary Drugs

In EU the use of veterinary drugs is regulated through council regulations 2377/90/EC. This regulation describes the procedure for the establishment of maximum residue limits (MRLs) for veterinary medicinal products in foodstuffs of animal origin. The primary consumer safety consideration is addressed through Maximum Residue Limits (MRL). The MRL defines the maximum level of residues of any component of a veterinary medicine that may be present in foodstuffs of animal origin without presenting any harm to the consumer [28]. MRLs are fixed at the mg/kg level (part per million) or even at the µg/kg level (parts per billion). Residues below MRL are considered safe and samples are considered non compliant [6].

The council regulation 2377/90/EC has established lists of compounds that have a fixed MRL (Annex I), that need no MRL (Annex II) or which have a provisional MRL (Annex III). According to directive 96/23/EC, β-lactam and quinolones belong to group B, and the MRL values for these compounds milk have been established and are shown in Tables 1.2, 1.3 and 1.4 for bovine tissues and milk.

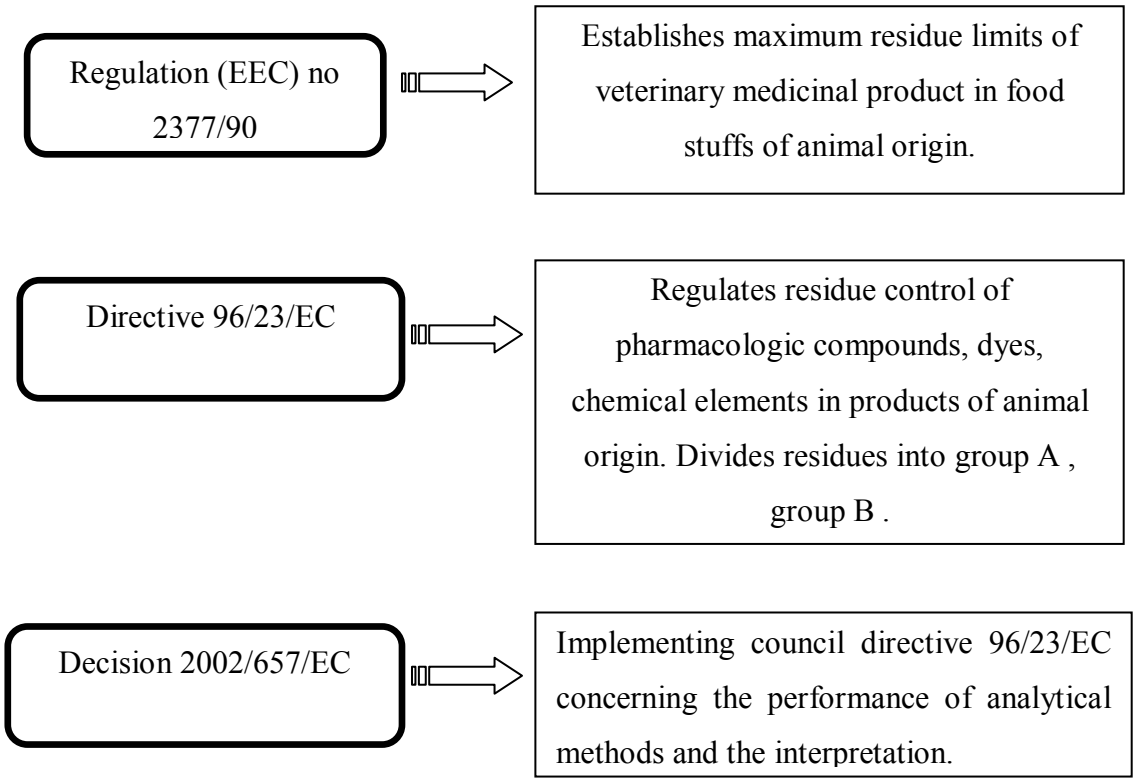
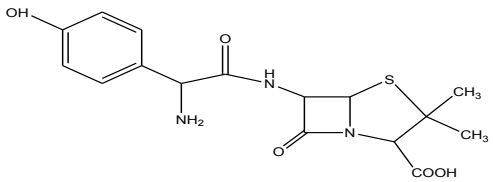
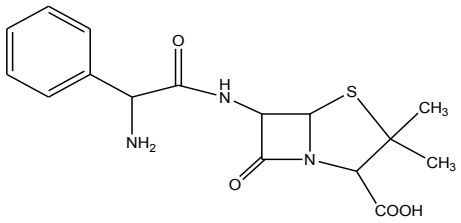
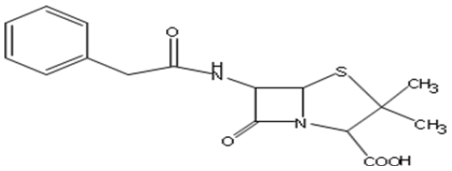
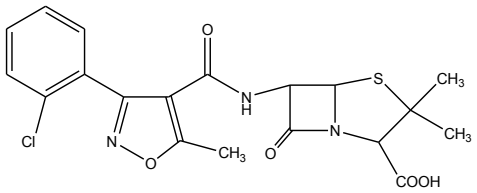
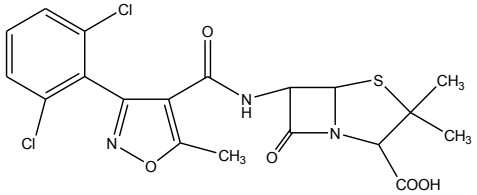


Table: 1.2. List of Penicillins that are regulated by EU in Milk

Name and Chemical Structure	Animal Species	MRL	Target Tissue
Amoxycillin (AMOX) 	All food producing species	50 µg/kg 50 µg/kg 50 µg/kg 50 µg/kg 4 µg/kg	Muscle Fat Liver Kidney Milk
Ampicillin (AMPI) 	All food producing species	50 µg/kg 50 µg/kg 50 µg/kg 50 µg/kg 4 µg/kg	Muscle Fat Liver Kidney Milk
Benzylpenicillin (PEN G) 	All food producing species	50 µg/kg 50 µg/kg 50 µg/kg 50 µg/kg 4 µg/kg	Muscle Fat Liver Kidney Milk
Cloxacillin (CLOX) 	All food producing species	300 µg/kg 300 µg/kg 300 µg/kg 300 µg/kg 30 µg/kg	Muscle Fat Liver Kidney Milk
Dicloxacillin (DICL) 	All food producing species	300 µg/kg 300 µg/kg 300 µg/kg 300 µg/kg 30 µg/kg	Muscle Fat Liver Kidney Milk

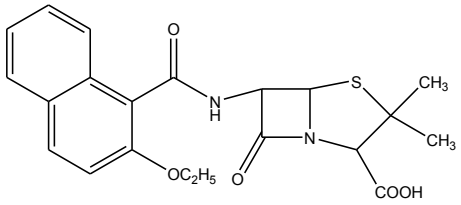
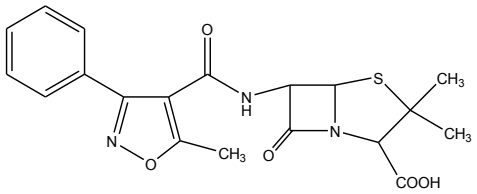
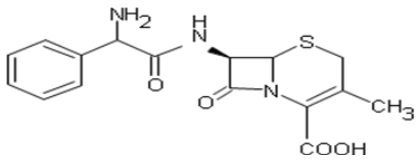
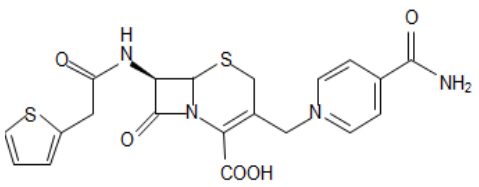
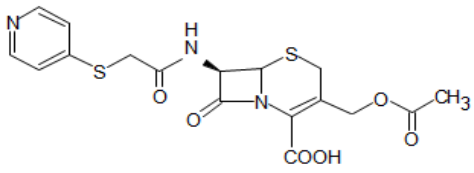
Nafcillin (NAFC) 	All ruminants	300 µg/kg 300 µg/kg 300 µg/kg 300 µg/kg 30 µg/kg	Muscle Fat Liver Kidney Milk
Oxacillin (OXAC) 	All food producing species	300 µg/kg 300 µg/kg 300 µg/kg 300 µg/kg 30 µg/kg	Muscle Fat Liver Kidney Milk

Table: 1.3. List of Cephalosporins that are regulated by EU in milk

Name and Chemical Structure	Animal species	MRL	Target tissue
Cefalexin (LEX) 	Bovine	200 µg/kg 200 µg/kg 200 µg/kg 1000µg/kg 100µg/kg	Muscle Fat Liver Kidney Milk
Cefalonium (LON) 	Bovine	20µg/kg	Milk
Cefapirin (PIR) 	Bovine	50 µg/kg 50 µg/kg 100µg/kg 60 µg/kg	Muscle Fat Kidney Milk

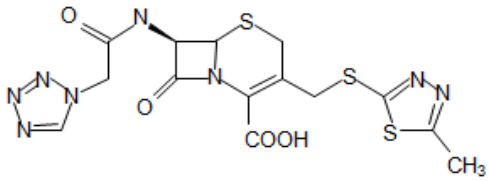
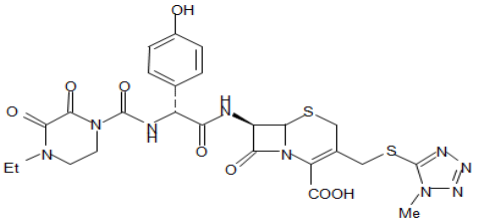
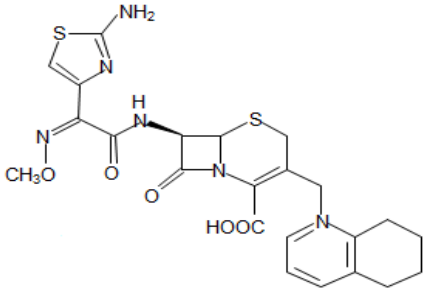
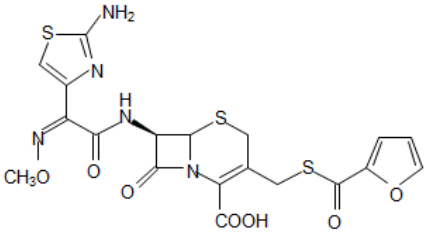
Cefazolin (ZOL) 	Bovine , ovine , caprine	50 µg/kg	Milk
Cefaperazone (PER) 	Bovine	50 µg/kg	Milk
Cefquinome (QUI) 	Bovine	50 µg/kg 50 µg/kg 100µg/kg 200µg/kg 20 µg/kg	Muscle Fat Liver Kidney Milk
Ceftiofur (TIO) 	All mammalian food producing species	1000 µg/kg 2000 µg/kg 2000 µg/kg 6000 µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk

Table: 1.4. List of Quinolones that are regulated by EU in milk

Name and Chemical Structure	Animal species	MRL	Target tissue
Danofloxacin (DAN)	Bovine, ovine, caprine	200 µg/kg 100 µg/kg 400 µg/kg 400 µg/kg	Muscle Fat Liver Kidney

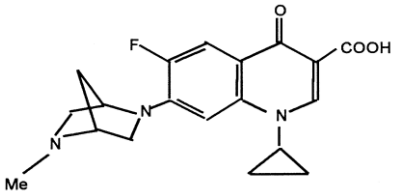
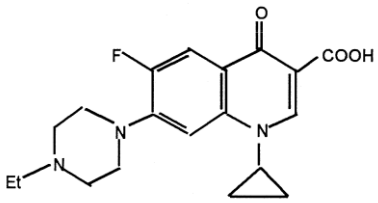
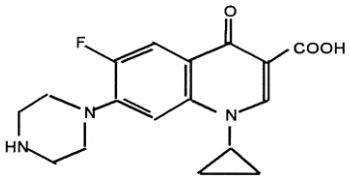
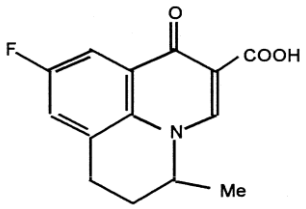
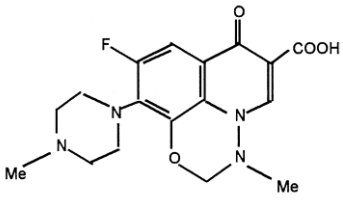
		30 µg/kg	Milk
Sum of : Enrofloxacin (ENR)  Ciprofloxacin (CIP) 	Bovine , ovine , caprine	100 µg/kg 100 µg/kg 300 µg/kg 200µg/kg 100 µg/kg	Muscle Fat Liver Kidney Milk
Flumequine (FLU) 	Bovine ,Porcine Ovine , Caprine	200 µg/kg 300 µg/kg 500 µg/kg 1500 µg/kg 50 µg/kg	Muscle Fat Liver Kidney Milk
Marbofloxacin (MAR) 	Bovine	150 µg/kg 50 µg/kg 150µg/kg 150µg/kg 75 µg/kg	Muscle Fat Liver Kidney Milk

Figure 1.7 and 1.8 shows the chemical structure for internal standard

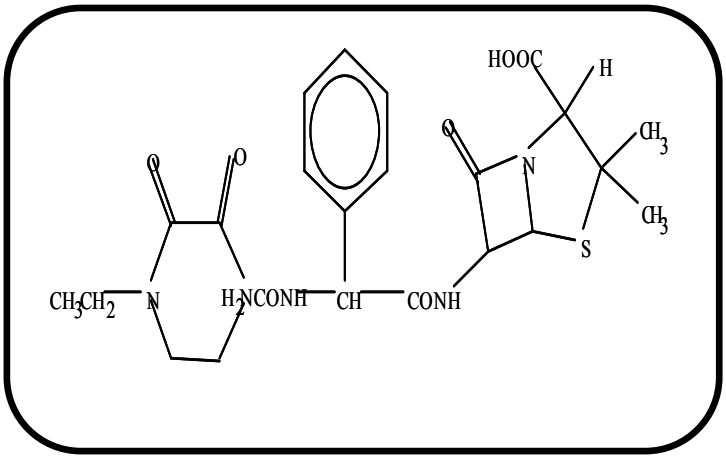


Figure (1.7)PIPE (Piperacillin)

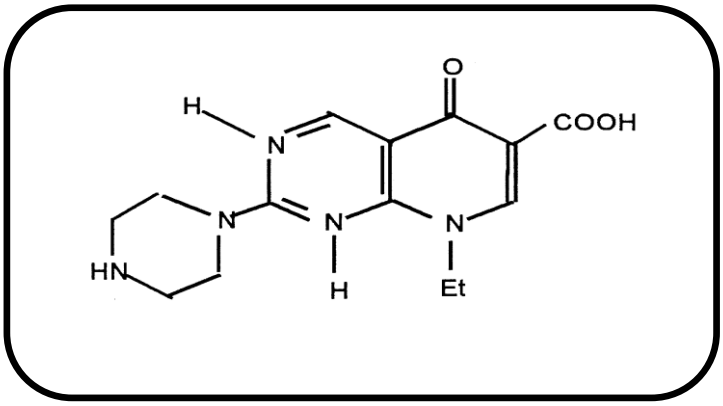


Figure (1.8) PIP (Pipemidic acid)

The determination of single class of antibiotics has been carried out previously in our research group [29-31]. This method involves combination of these works for simultaneous determination of penicillins, cephalosporins and quinolones in milk samples by LC-MS/MS.

The work begins with preliminary study to choose the type of milk to develop the method. After selection of type of milk further a comparative study was done for optimization of extraction method for better recovery of analytes. We have then proceeded to validation of the method according to European commission decision 2002/657/EC and some parameters from FDA guidelines using raw milk. Finally the method was applied for real samples (positive milk samples) supplied by *Laboratori Interprofessional Lleter de Catalunya (ALLIC)*.

Experimental

2.1 Reagents and Materials

2.1.1. Standards

- The penicillin's standards Ampicillin (AMPI), Dicloxacillin (DICL), Benzylpenicillin (PENG) were supplied by the European pharmacopeia (Strasbourg Cedex, France), Amoxycillin (AMOX), Nafcillin(NAFC) and Oxacillin (OXAC) were obtained from Sigma-Aldrich (St. Louis, MO, USA), and Cloxacillin (CLOX) were obtained from Fluka (Buchs, Switzerland).

- The cephalosporin standard Cephalexin (LEX),Cefalonium (LON), Cefapirin (PIR), Cefazolin (ZOL) and Cefoperazone (PER) were obtained from Sigma Aldrich, Cefquinome (QUI) from A.K Scientific, and Ceftiofur (TIO) from Riedel-de Haën.

- The Quinolone standard Danofloxacin (DAN) were purchased from Pfizer (Karlsruhe, Germany) , Enrofloxacin (ENR) (Cenavisa Reus, Spain) , Flumequine (FLU), Marbofloxacin (MAR), Ciprofloxacin (CIP) from (Sigma, St. Louis ,MO,USA),

- The internal standard Piperacillin (PIPE) and Pipemidic acid (PIP) were obtained from Fluka (Buchs, Switzerland) and (Prodesfarma) respectively.

2.1.2. Reagent and Solvent

- All reagents were LC grade. Methanol, MeOH (99.9%), Acetonitrile, ACN (99.9%), Formic acid, HFO (98%), Acetic acid (glacial), HAC 99.9 % were obtained from Merck, Darmstadt, Germany.

- Water of LC grade with resistivity 18.2 MΩcm was obtained using a water purifier Purelab plus.

- Sodium dihydrogen phosphate, NaH_2PO_4 > 99.0 % was obtained from Merck Darmstadt, Germany.

2.1.3 Preparation of Standard and Stock Solutions

- Individual Stock Solutions: Stock standard solution of each antibiotics were prepared at a concentration of 100 ppm by dissolving the quantity of each compound (AMOX, AMPI, PENG, CLOX, DICL, NAFC, OXAC, LEX, LON,

PER, QUI, TIO, ZOL and PIR) in water, (CIP, DAN, ENR, and MAR) in 50 Mm acetic acid and FLU in acetonitrile.

- Working Solutions: 100 MRL of the working standard mixture solutions used to spike the milk samples were prepared by mixing the individual solution and stored under refrigeration (-8°C) until use. These solutions were prepared separately for penicillins, cephalosporins and quinolones. 100 MRL solution was prepared in following way.20 MRL solution was prepared by diluting 100 MRL solution.
  - → For penicillins 40µl of AMOX, AMPI, PENG and 300 µl of CLOX, DICL, OXAC and NAFC in 10 ml of water.
  - → For cephalosporins 600µl of PIR, 200 µl of QUI, LON, 500 µl of ZOL, PER and 1000 µl of LEX and TIO in 10 ml of water.
  - → For quinolones 300µl of DAN, 1000 µl of ENR and CIP, 500 µl of FLU and 375 of MAR in 10 ml of water.
- A solution of PIPE and PIP was prepared at concentration of 100 mg L^{-1} in water.
- 0.1M sodium dihydrogen phosphate solution at pH 10 was prepared by dissolving in water adjusting pH by 5M NaOH.

2.1.4. Solid Phase Extraction Cartridge

The solid phase extraction (SPE) cartridge used in this study was Oasis HLB (3 ml, 60 mg) obtained from Waters, Milliford; USA. Oasis HLB is a macro porous copolymer consisting of two monomers, the lipophilic N-divinylbenzene and the hydrophilic N-vinylpyrrolidone. The hydrophilic N-vinylpyrrolidone increases the water wettability of the polymer and the lipophilic divinylbenzene provides the reversed phase retention necessary to retain analytes [3].

The characteristics, properties and structure of the Oasis HLB sorbent are detailed in figure 2.1 and table 2.1.

Table: 2.1. Properties of Oasis HLB sorbent

	Specification
Specific surface area (m^2/g)	727-889
Average pore diameter (A°)	73-89
Total pore volume (cm^3/g)	1.18-1.44
Average particle diameter (µm)	25.0-35.0
Fines content	≤ 1.0 %

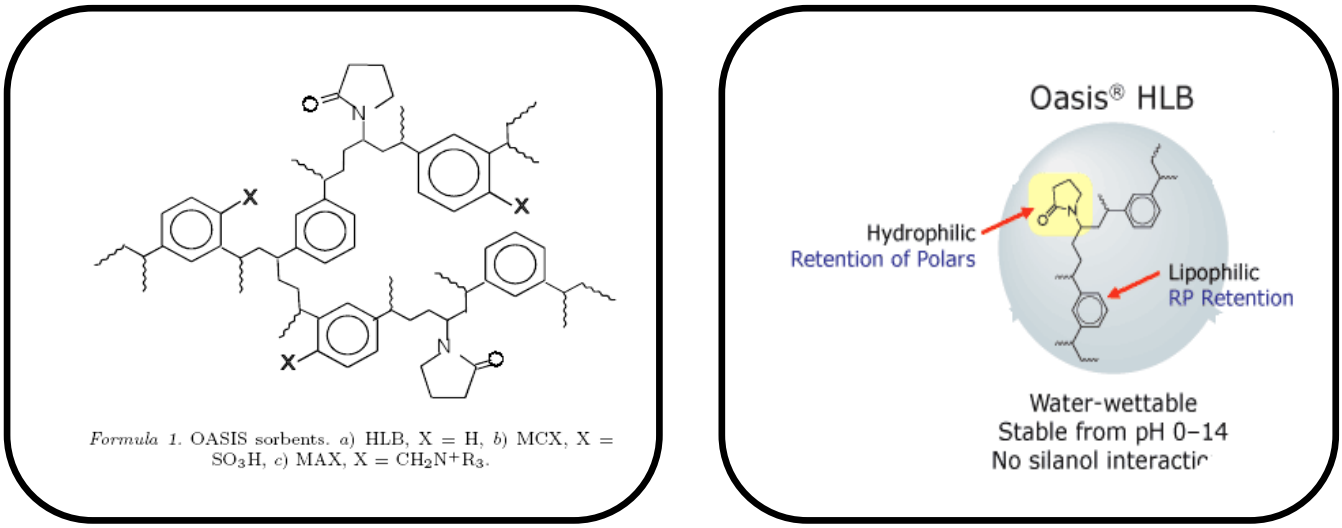


Figure (2.1): Structure of Oasis HLB cartridge

Oasis HLB cartridges was choosen for this experimental procedure because it allows to extract all kinds of antibiotics studied at the same time. From the literature review we can see that Oasis HLB has been successful in isolating residues of interest in milk [15,23,32,36] and also have been successfully utilized in many multiclass residue methods [17,19,21,25,27].It has proven to provide efficient extraction with optimal recovery, equal retention and also history of batch to batch reproducibility [25].

2.2 Instrumentation

- Technical balance from Sartorius laboratory (model LP2200P, sensitivity 0.01-0.05g) was used to weigh the samples and NaH_2PO_4 buffer.

- Analytical balance from Mettler Toledo (Model AB204, precision 0.1 mg) was used for weighing standards.
- Selecta ultrasound system was used to dissolve the standard solutions.
- The pH of the buffer and mobile phase was measured using Crison-micro pH 2002 Potentiometer (± 0.1 mV) (Crison, Barcelona, Spain). A 5203 combinated pH electrode from Crison was used and the electrode was soaked in water when not in use.
- A vortex mixer Heidolph, model Reax 2000 was used for samples agitation.
- Supelco visidry 24 port was used for solid phase extraction
- Additive Visidry desiccant, designed for the Supelco Visiprep vacuum distributor, using a stream of compressed nitrogen was used for sample evaporation.
- Hettich Mikro 20 Mini centrifuge, to centrifuge the samples after being reconstructed

2.2.1. LC-MS/MS

Chromatographic analysis was performed using a HP Agilent Technologies 1100 LC system equipped with an auto sampler. Separations were achieved using Zorbax Eclipse XDB-C8 (150 mm x 4.6 mm, 5µm particle size , Agilent Technologies) with a Kromasil C8 guard column (20 mm x 4.5 mm, Aplicaciones Analíticas).

Mass spectrometry was performed on API 3000 triple quadrupole mass spectrometer (PE SCIEX) using turboionspray ionization in positive mode. Each transition was detected in MS/MS multiple reaction monitoring mode with 90ms dwell time.

2.2.2. UPLC-MS/MS

Chromatographic analysis was performed using Acquity – Ultra Performance LC – Waters system equipped with an auto sampler. The chromatographic separations were evaluated using two columns: Acquity UPLC BEH shield RP 18 (50 mm x 2.1 mm, 1.7 µm particle diameter) Waters, Ireland and Kinetex 2.6 U C18 100 A (150 x 4.60 mm, Phenomenex, Kinetex –USA).

Mass spectrometry was performed on API 3000 triple quadrupole mass spectrometer (PE SCIEX) using turboionspray ionization in positive mode. 40 ms dwell time was used for each transition.

2.2.3. Data Process

For both instruments, instrument control and data acquisition were performed with the help of the software "Analyst" version 1.4.2 by Applied Biosystem.

2.3. Experimental Procedure

2.3.1. Milk Samples

Different kinds of milk samples were analysed. Raw milk samples (not pasteurized) were used for method validation which was kindly supplied by *Laboratori Interprofessional Lleter de Catalunya (ALLIC)*. The raw milk sample was stored in frozen state at -20°C until analysis. The commercial milk samples (whole milk) were obtained from local supermarket. Positive milk samples were obtained from different cows specimen orally medicated with β-Lactams antibiotics which were supplied by *ALLIC*.

2.3.2. Sample Preparation

Sample preparation is one of the critical steps for multiclass, multi residue determination of antibiotics in food sample because antibiotics with different physicochemical properties (pk_a, polarity, solubility) have to be determined simultaneously. To establish a suitable method for extraction of antibiotics from raw milk different extraction methods were applied represented in table 2.2.

Table: 2.2 Nomenclature of Extraction Procedure

Method	Extraction Procedure	Procedure
1	E1	→ Weigh, → Spike with WS and IS → No centrifugation of the sample
2	E2	→ Centrifuge the sample, → Weigh, → Spike with standards
3	E3	→ Weigh, → Spike → Centrifuge the sample before passing through SPE cartridge

The steps of sample preparation are listed below:

- 2 g of milk was weighed into a polypropylene centrifuge tube in case of E1 and E3. However, in case of E2 the sample was centrifuge before weighing.
- 2 ml of Milli–Q water was added into individual test tubes.
- The sample was spiked with working standard and internal standard.
- 0.5 ml of 0.1 M NaH_2PO_4 (pH 10) solution was added and the mixture was vortex for 40 seconds.
- Conditioning of the sorbent: Before the sample was passed through the SPE cartridge it was precondition with 1 ml of MeOH, 1 ml of H_2O and 1 ml of NaH_2PO_4 solution at pH10.
- Application of sample to the sorbent: Retention of analyte and matrix occurs in sorbent. In case of E2 the sample was centrifuge after addition of standards, before passing through SPE cartridge. Care was taken so that supernatant was transferred to cartridge avoiding any visible fat layer. Positive pressure was applied with the help of syringe when needed.
- Rinsing the sorbent bed: After the sample is passed through the cartridge to decrease the matrix interference it was cleaned with 3 ml of water.
- Elution of the retained compounds: In order to disrupt the analyte – sorbent interaction, 2 ml of methanol was added and substances were eluted.
- The eluate was evaporated to dryness by nitrogen stream and the residue was reconstituted with 200 µl of water.
- The tube containing the extract was vortex for 1 min and was transferred to eppendorf which was centrifuged at 10000 rpm for 4 minutes and supernatant is stored in glass vials in freezer at -8°C until analysed by LC-MS/MS.
- For UPLC-MS/MS analysis, samples were further filtered by using a Millipore ultra free -MC centrifugal filter device. (Centrifuge filter device, 0.22 µm filter unit) before injection.

General Steps involved in the use of SPE are represented schematically in the figure 2.2

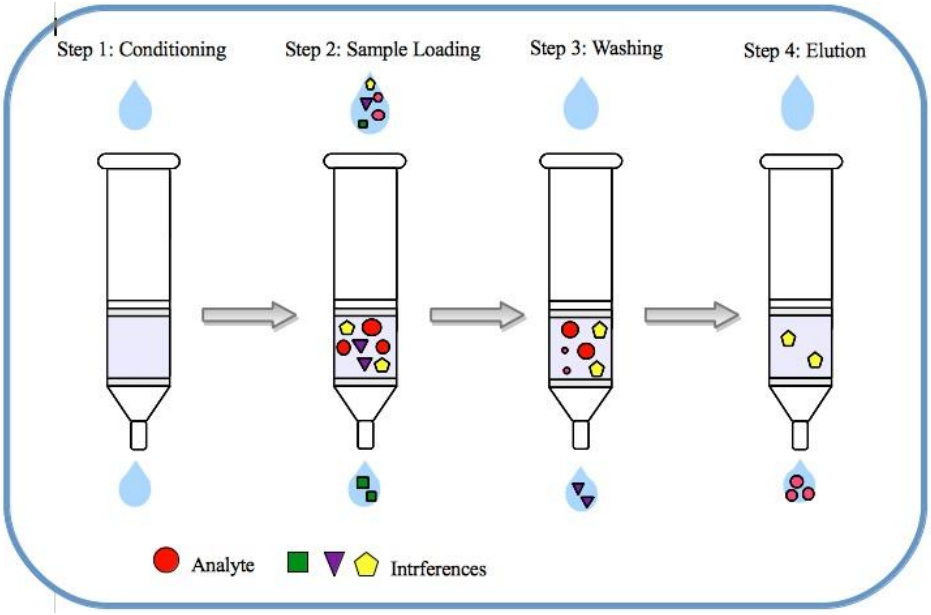


Figure (2.2): General SPE procedure

Flow chart diagram of the process is represented schematically in Fig 2.3

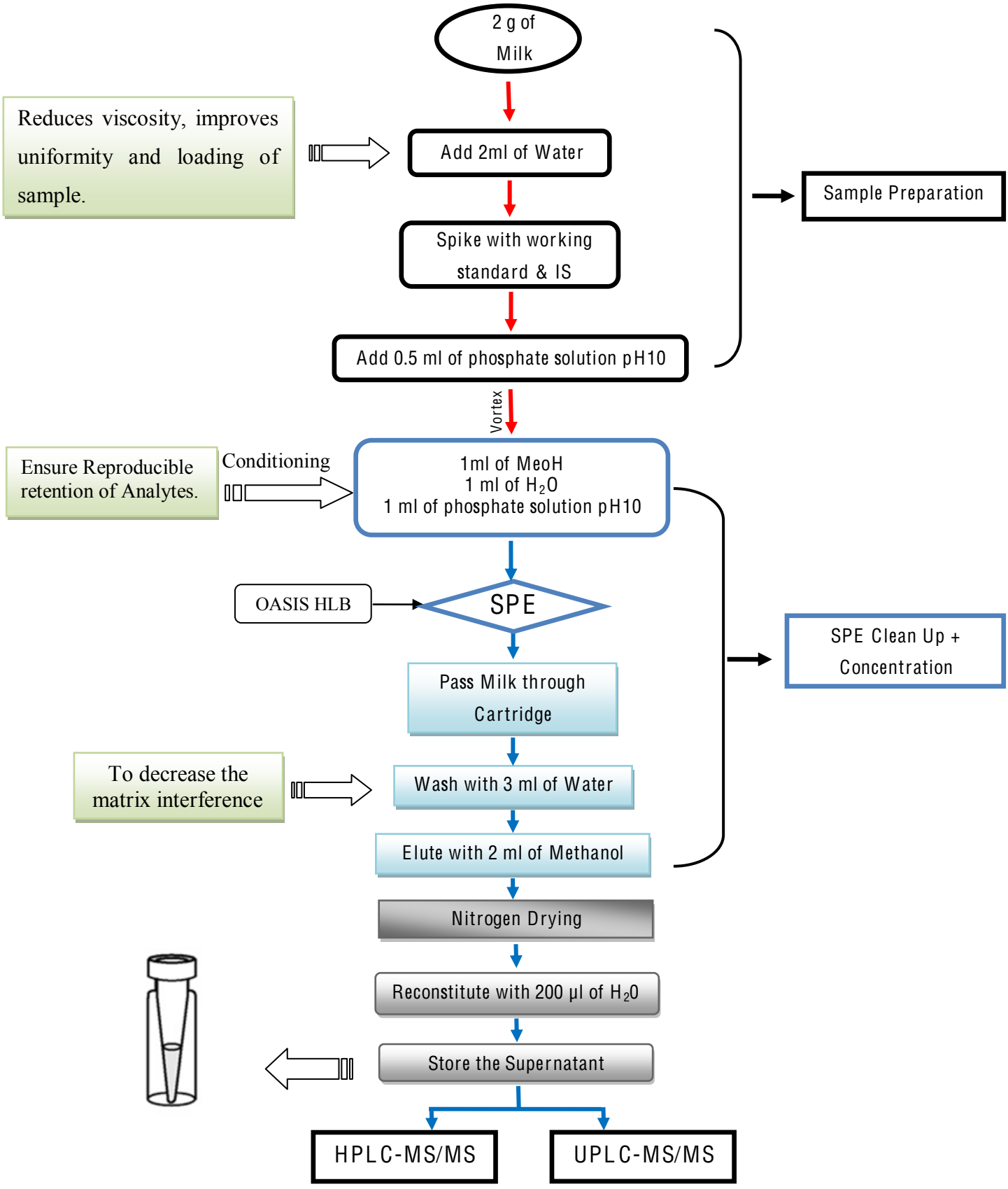


Figure (2.3): Flow chart for extraction of Analyte

2.3.3. Chromatographic Condition

2.3.3.1. LC-MS/MS

Separation was performed on a gradient mode with mobile phase A consisting of 0.1 % formic acid in H_2O and mobile phase B consists of 0.1% formic acid in ACN. Both A and B contains 0.1 % formic acid as mobile phase additive. Addition of formic acid in mobile phase can change the pH value of mobile phase and affect the chromatographic separation. The flow rate was set at 1ml/min and the injection volume was 20µl.The total run time for each injection was 10 min. Table 2.3 shows the gradient used for the separation using LC-MS/MS system and E3 extraction method.

Table 2.3 LC-MS/MS gradient

Time	A(H_2O)	B(ACN)
0	85	15
2	85	15
4	55	45
7	44	56
8.5	85	15
11	85	15

Figure 2.4 shows the separation of antibiotics studied

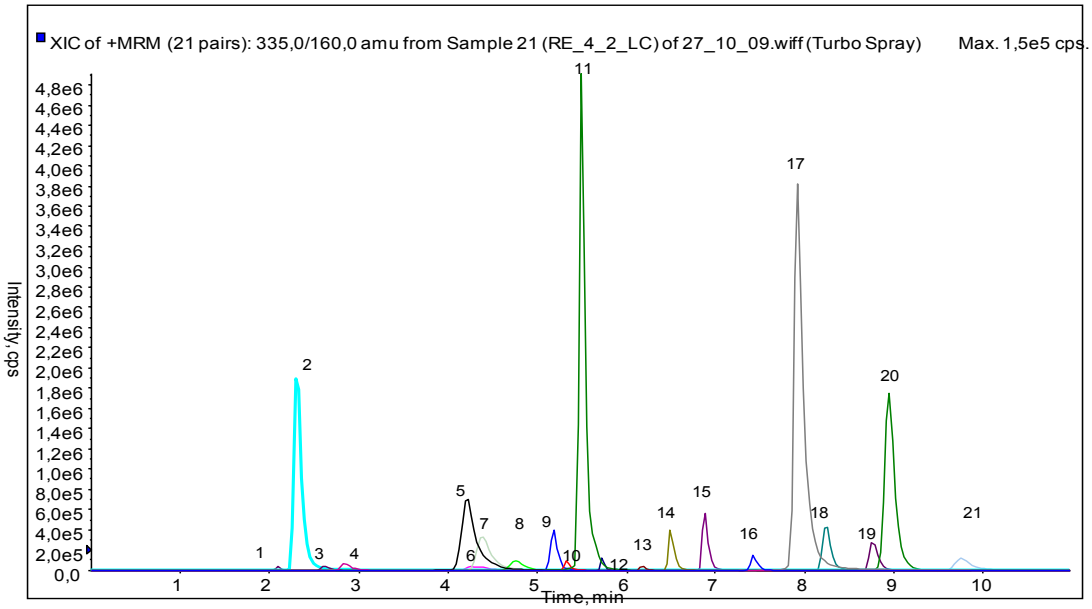


Figure (2.4): Chromatogram obtained from a milk sample fortified to the 4MRL concentration using LC-MS/MS where 1)AMOX, 2) PIR, 3) QUI, 4) PIP, 5) MAR, 6) AMPI, 7) LEX, 8) LON, 9) CIP, 10) DAN, 11)ENR, 12) ZOL, 13) PER, 14) TIO,15)PIPE 16) PENG 17) FLU 18) OXAC 19) CLOX 20) NAFC, and 21) DICL.

2.3.3.2. UPLC-MS/MS System

The mobile phase used for UPLC-MS/MS is similar to that used for LC-MS/MS as described in 2.3.3.1. Two columns were checked for the separation efficiency of analytes. Properties for packing for both column are given below.

A) Acquity UPLC BEH (Bridged ethylsiloxane/silica hybrid particle) Shield RP 18

The column chemistry is shown in figure 2.5 and the properties of the packing material of the column is described in table 2.4

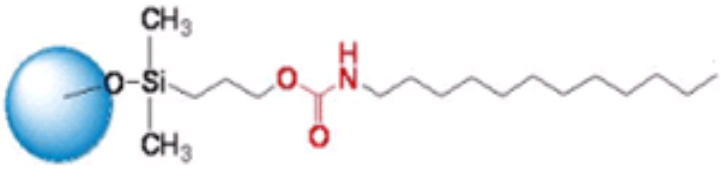


Figure (2.5) ACQUITY UPLC BEH Shield RP 18

Table (2.4) Acquity UPLC BEH Chemistry

Bonded phase	Ligand type	Monofunctional embedded polar group
	Ligand Density	3.3 µmol/m^2
	Carbon Load	17%
	Endcap style	TMS
	pH range	2-12
BEH Particle	Pore Diameter	130°A
	Pore Volume	0.7 mL/g
	Surface Area	185 m2/g

b) Kinetex 2.6 µm Core-Shell Particle

Kinetex core shell particle consists of porous shell and core shell. The material characteristics of Kinetex column is shown in table (2.5)

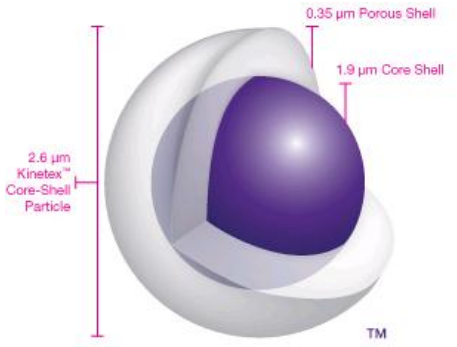


Figure (2.6) Kinetex 2.6 µm Core-Shell Particle

Table (2.5) Kinetex 2.6 µm Core-Shell Particle

Total Particle size (µm)	2.6
Porous Shell (µm)	0.35
Solid core (µm)	1.9
Pore Size (°A)	100
Effective surface area (m^2/g)	200
Effective carbon load %	12
pH stability	1.5-10
Pressure stability	600 bar

Initially the flow rate was set to 0.3ml/min for both columns but for Kinetex column peaks were overlapping so was changed to 1 ml/min. The injection volume was 6µl. Several gradient were studied, the better separation were obtained with the gradient showed in Table 2.4.

Table 2.6 Gradient for two columns

Acquity			Kinetex		
Time	% A(ACN)	% B (H_20)	Time	% A(ACN)	% B (H_20)
0	15	85	0	20	80
0.28	15	85	1.67	20	80
0.74	45	55	3.06	50	50
1.44	56	44	5.14	61	39
1.78	56	44	6.18	61	39
2.36	15	85	7.92	20	80
3.5	15	85			

2.3.3.3. Optimization of the Operational Parameters of the Mass Spectrometer

In a previous study [29-31] the MS conditions were optimized by direct infusion of each compound at a concentration of 10 mg/L at a flow rate of 0.005 ml min^{-1}. In this study same potential was used to obtain the optimum parameters for each compound. The optimized potential used for each antibiotic in order to detect antibiotics with higher signals is shown in table 2.6.

Declustering potential (DP): This is the potential applied to the orifice plate. This potential has the greatest effect on the amount of fragmentation of the ions in the orifice skimmer region of the source area. The declustering potential is the difference between the orifice voltage and the skimmer voltage. The higher the voltage the greater is the amount of declustering. The working range of the DP is typically 0 to 100 V although it may be set as high as 200 V.

Focussing potential (FP): The FP helps to focus the ions through the skimmer. It also effects the declustering of ions in the orifice – skimmer region. The FP is difference between the voltage on the focussing ring and the skimmer voltage. A typical value is 200- 300 V.

Entrance potential (EP): The EP is the difference between the voltage on the high pressure entrance quadrupole and the skimmer voltage. It is typically set at – 10 V and affects selling of all other instrument voltage.

Table 2.6 shows the optimal values of the potentials

Table 2.7 Optimal values of the potentials

Family	Antibiotic	DP	FP	EP
Penicillins	AMOX	40	150	6
	AMPI	65	150	6
	CLOX	40	140	7
	DICL	50	150	8
	OXAC	50	120	9
	NAFC	40	160	9
	PENG	65	220	7
Cephalosporin	LEX	30	125	5
	LON	30	125	5
	PIR	40	150	5
	PER	50	200	11
	QUI	40	175	5
	TIO	50	200	5
	ZOL	40	175	5
Quinolones	CIP	45	200	10
	DAN	45	200	10
	ENR	45	200	10
	FLU	38	200	10
	MAR	45	200	10
IS	PIP	50	200	10
	PIPE	40	175	5

The turboion spray was in positive mode with following settings:

Ion spray voltage

This is the voltage applied to the needle that ionizes and nebulises the sample. The ion spray voltage parameter depends on the polarity and affects the stability of the spray and sensitivity. The ion spray voltage used is 4500V.

Nebuliser gas (NEB)

This is the gas that focuses the spray in the nebuliser. The nebuliser gas parameter affects the stability of the spray and sensitivity. The Nebulizer gas used is 10 arbitrary units.

Curtain gas (CUR)

This is the gas between the curtain plate and the orifice plate. The main function of the curtain gas is to prevent the contamination of the ions optics. The curtain gas depends on the solvent flow rate. The curtain gas used is 12 arbitrary units.

Auxiliary Gas

The nebuliser and auxiliary gas affects the stability of the spray and sensitivity. These gases have different names in different instruments. For turbo ion spray sources the auxiliary gas is the turbo (heater) gas. Auxiliary gas flow is 4500 cm^3/min and Auxiliary gas temperature is 400 º C.

Two MRM transitions were studied for each analyte. The most intense one was used for the quantification and the minor one for confirmation of the analyte. The quantification and identification transition that was optimized in a previous study [29-31] was applied in this study.

European commission decision 2002/657/EC establishes identification (IPs) in order to confirm the residues of antibiotics in food stuffs of animal origin. For the confirmation of antibiotics studied (listed in group B of Annex I of directive 96/23/EC), a minimum of 4 IPs is required. With two transitions, 1 precursor and two daughter ions, we achieve total of 4 IPs using LC-MS/MS.

Table 2.7 shows MS/MS transitions for quantification and confirmation as well as collision energy values optimized for each of the studied antibiotics.

Table 2.8 MS/MS Parameters for the antibiotics studied

Family	Antibiotic	HPLC* t_R(min)	UPLC# t_R(min)	Quantification Transition	Collision Energy (CE)	Identification Transition	Collision Energy (CE)
Penicillins	AMOX	2.1	0.65	366 →114	28	366→208	19
	AMPI	4.26	0.86	350→106	26	350→192	21
	CLOX	8.76	2	436→160	20	436→277	20
	DICL	9.75	2.21	470→160	21	470→311	22
	NAFC	8.94	2.05	415→199	19	415→256	21
	OXAC	8.23	1.89	402→160	18	402→243	18
	PENG	7.42	1.68	335→160	16	335→176	16

Cephalosporins	LEX	2.31	0.85	348→140	35	348→158	15
	LON	6.18	1.12	459→152	30	459→337	20
	PIR	4.38	0.68	424→292	20	424→181	35
	PER	6.49	1.43	646→290	35	646→530	20
	QUI	4.76	0.74	529→134	20	529→396	20
	TIO	5.73	1.45	524→285	30	524→241	25
	ZOL	2.61	1.34	455→323	15	455→295	25
Quinolones	CIP	5.19	1.08	332→314	32	332→ 288	27
	DAN	5.33	1.11	358→340	31	358→283	31
	ENR	5.5	1.14	360→316	29	360→ 342	29
	FLU	7.92	1.75	262→244	26	262→202	45
	MAR	4.22	0.88	363→320	22	363→345	30
I.S.	PIPE	2.85	1.54	518→143	25	518→359	15
	PIP	6.88	0.71	304→286	30	304→261	25

* Column used for separation is Zorbax Eclipse XDB-C8 (150mm x 4.6mm, Agilent Technologies)

Column used for separation is Acquity UPLC BEH shield RP 18.

3. Results and Discussion

3.1. Preliminary Study

3.1.1. Comparision between Raw and Commercial Milk

Milk consists of fat which have a very different composition in raw and commercial milk. In order to optimize the extraction method a preliminary study was done using commercial whole milk (CM) and comparing the recoveries obtained with the raw milk (RM). In order to compare the behaviour of the antibiotics in both milk samples, the easier method i.e. E1 was applied as described in experimental section 2.3.2.

Figure 3.1.shows the recoveries obtained from both milk samples. We can see that half of the antibiotics present significantly different recovery in both milk samples. Recovery for antibiotics obtained with commercial milk is better. We can say that may be the presence of high fat content in raw milk makes the recovery worse than commercial milk. From this we can draw a conclusion that to optimize the extraction method it is necessary to use the raw milk not the commercial milk.

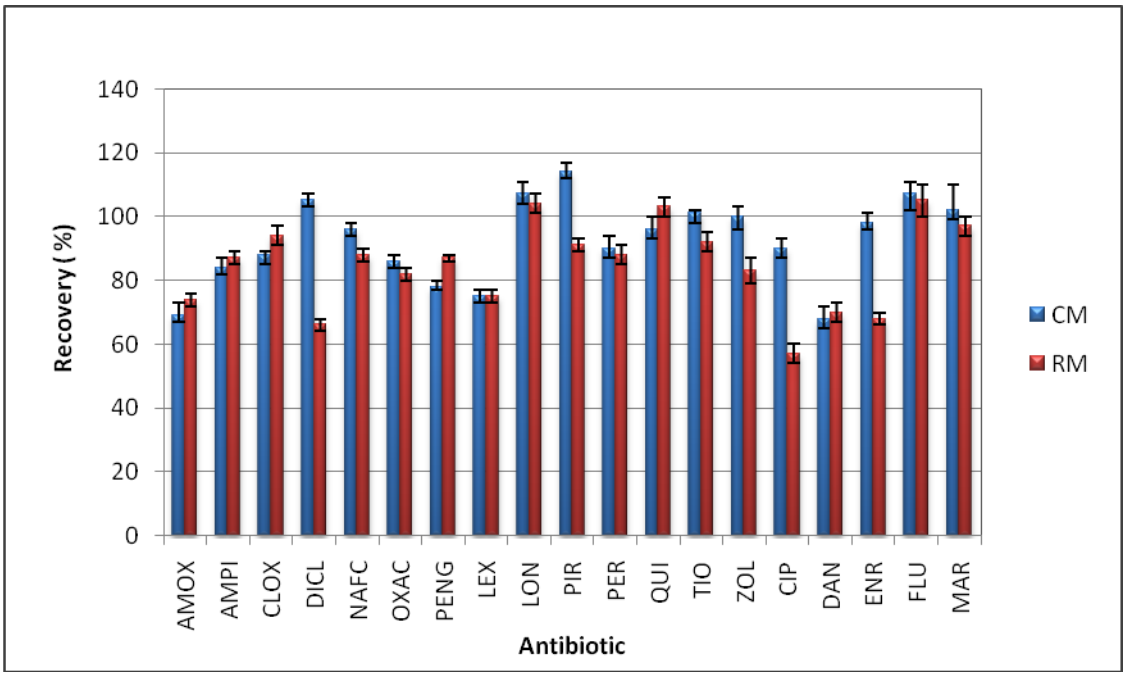


Figure (3.1) Recovery obtained from raw and commercial milk.

3.1.2. Comparison between Air and Nitrogen Drying

Evaporation step is a critical part of the sample preparation process. A study was done comparing (%) recovery of each analyte using commercial milk to see if there is difference between the nitrogen and air drying of the sample. 6 samples were

prepared at 2 MRL level for each antibiotic and extraction was performed as described in section 2.3.2. The elution fraction obtained from SPE was evaporated to dryness under a stream of air or nitrogen.

The recoveries for quinolones and penicillins are similar using two different drying techniques. However, the recoveries for cephalosporins are slightly higher with nitrogen drying as can be observed in figure 3.2. So nitrogen drying was preferred for subsequent studies to be made with raw milk.

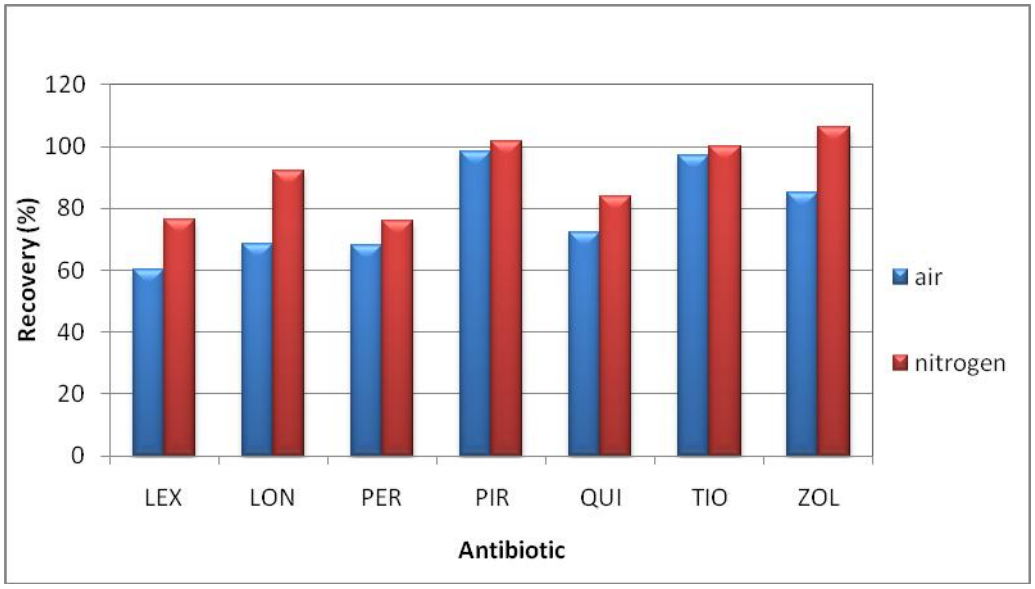


Figure (3.2) Recovery (%) obtained for cephalosporin using air and nitrogen drying.

3.2. Optimization of the Extraction Method

Three different extraction methods were compared in analysing raw milk to obtain maximum recovery. As can be observed in experimental section, table 2.2, methods E2 and E3 have a centrifugation step that is made at different points of the sample preparation process while E1 do not have their centrifugation step.

Figure 3.3 show the recovery of the penicillins studied using three different extraction methods (E1, E2 and E3).

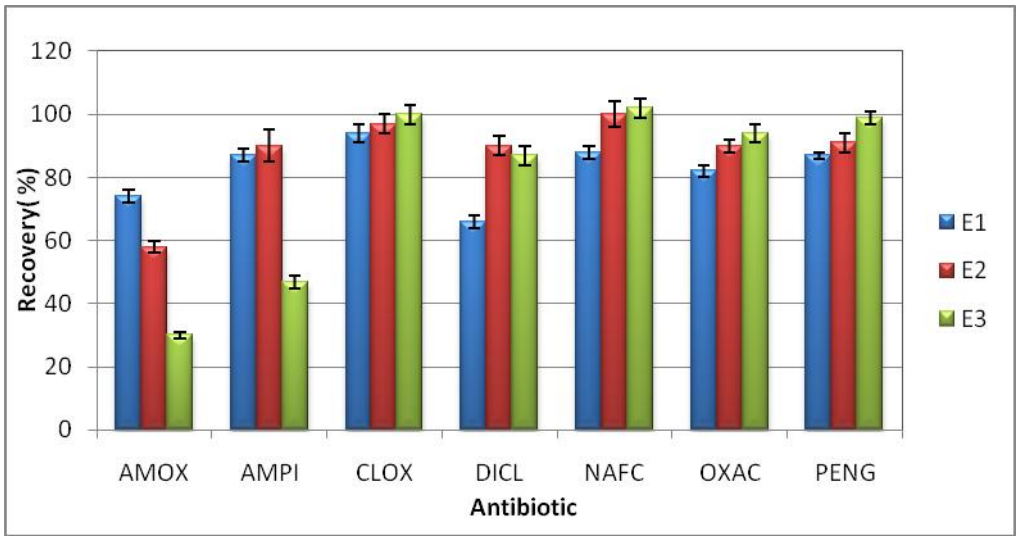


Figure (3.3) Recoveries (%) of penicillins using three different extraction methods

We can see that except for AMOX and AMPI in E2 and E3 the recoveries of penicillins are higher than 80 %.

Figure 3.4 show the recovery of cephalosporins studied using the three methods. As can be observed in the figure, the recoveries for cephalosporins are approximately 70 % except for LEX and QUI.

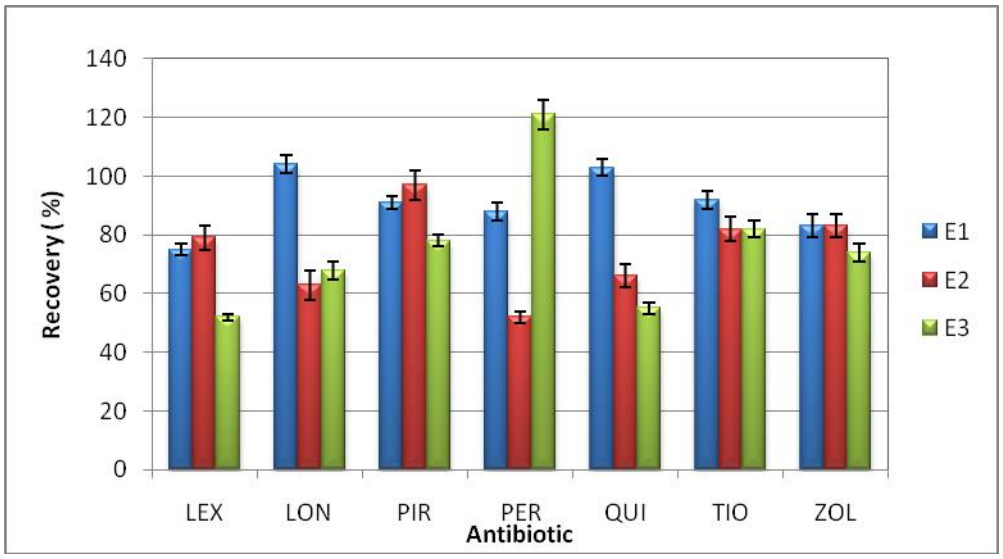


Figure 3.4 Recoveries (%) of cephalosporins using three different extraction methods

Figure 3.5 shows the recovery of quinolones using three methods. Quinolones present higher recoveries than 60 % except for DAN in method E3.

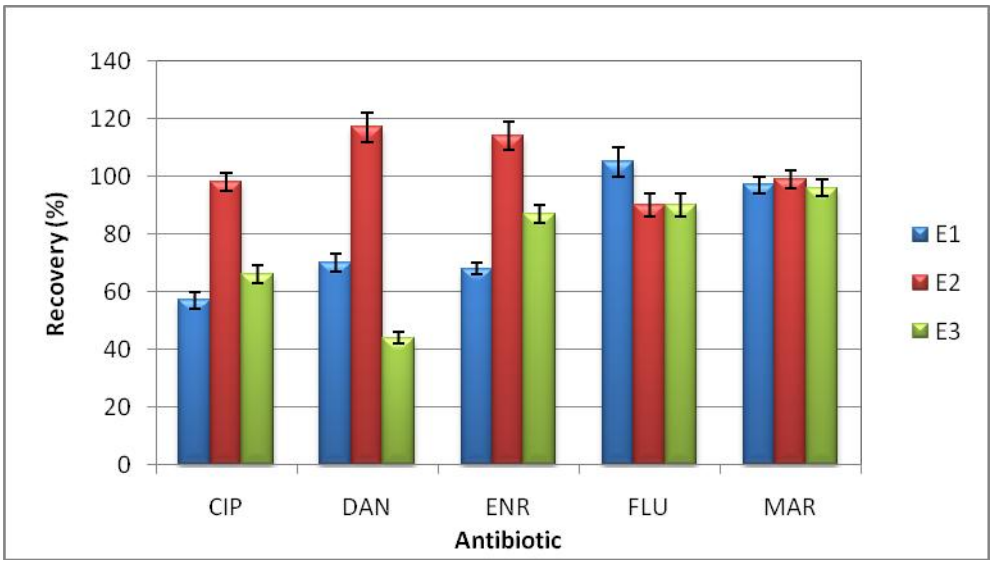


Figure 3.5 Recoveries (%) of quinolones using three different extraction methods

Following conclusions are drawn from the result obtained using three (E1, E2 and E3) extraction method. E1 was not choosen as due to higher amount of fat present in raw milk it was difficult to pass the sample through the SPE cartridge without centrifugation. E2 and E3 have a centrifugation step. E2 begins with this centrifugation step and E3 spike with antibiotics and then centrifuged. Although the results of E2 are better than those obtained with E3, the E3 method was choosen for subsequent studies because the procedure is more similar to the procedure used for positives samples. The E3 method would be given lower results because drugs would be lost during the centrifugation step in the same way as positive samples. Thus E3 was choosen for performing validation study.

3.3. UPLC-MS/MS Optimization

The method that was developed in LC was applied to UPLC. When we change from LC to UPLC analysis, the MS parameters need to be altered to obtain the highest MS signals. One of the parameters that need to be adjusted is dwell time. Dwell time was changed to obtain enough data points for each peak considering that we analyse 21 antibiotics simultaneously. This parameter measures the period of time that is spend collecting data, at a particular mass , and usually ranges 10 ms to 200 ms. Variations in dwell time (90 msec, 40 msec, 20 msec and 10 msec) was made with mixture of standards. Appropriate dwell time balance the improvement of signal to noise ratio with sufficient data points to characterize the chromatographic peaks. With 40ms

dwell time the baseline noise is reduced and the peaks are well separated .So 40 ms dwell time was selected for carrying out the experimental work.

3.4. Comparative Study of Two UPLC Columns

In order to achieve better separation and retention of analytes, main chromatographic condition such as chromatographic column and gradient elution profile was studied using two different columns .The column chemistry for two are different as shown in experimental section table 2.4 and 2.5 so the separation achieved with two columns are different.

3.4.1. Acquity UPLC BEH shield RP 18 (50 x 2.1 mm, 1.7µm particle diameter)

Acquity BEH incorporates an embedded polar group (carbamate group) into the bonded phase ligand. Features of embedded polar group include alternate selectivity to that of alkyl reversed phase columns, excellent peak shape for bases reducing peak tailing and aqueous mobile phase compatibility. The organic-inorganic hybrid, with ethylsiloxane bridges both on the surface and throughout the body of the material, provides a broader range of chemical stability, especially the pH operating range (pH1-12), while minimizing interactions of the matrix with any analyte functionalities.

Due to different nature of analytes and in order to elute all analytes in a reasonably short time different gradient profile was studied. Initially, the flow was set to 0.20 ml/min. But the flow rate was small for chromatographic separation so with the same gradient condition, flow rate was changed to 0.3 ml/min. The optimized gradient is shown in experimental section table 2.4 and figure 3.6 shows the separation.

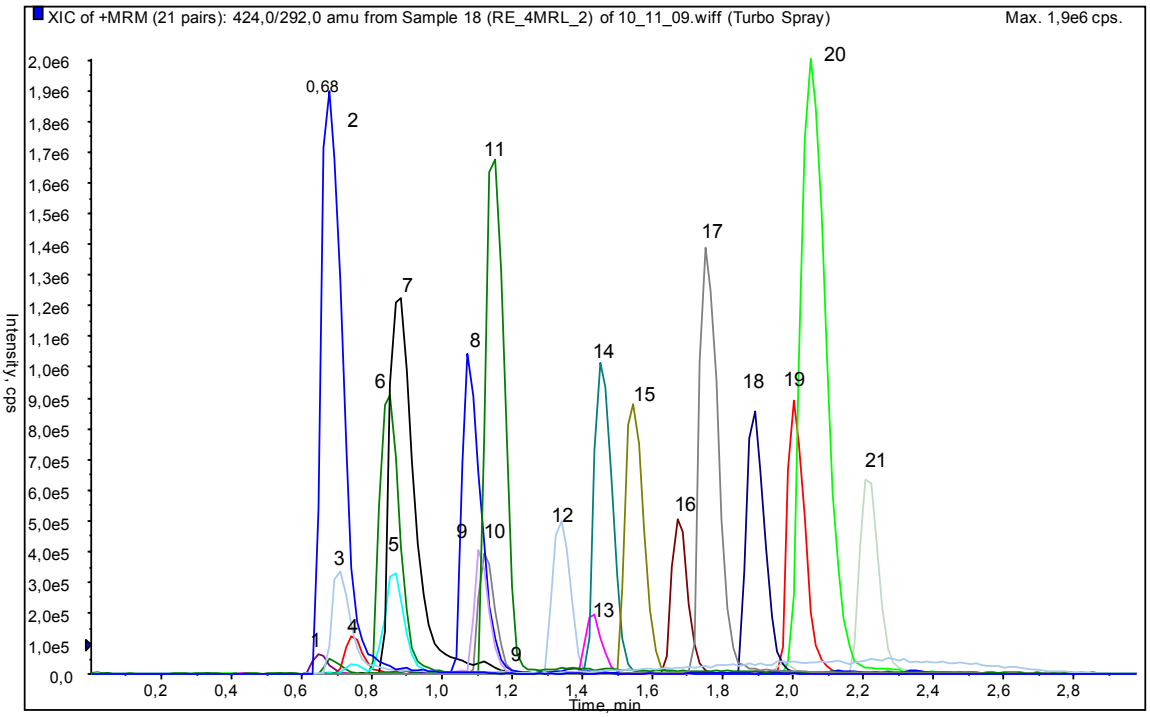


Figure (3.6) Separations obtained from a milk sample fortified to the 4MRL concentration for all antibiotics using Acquity UPLC BEH shield RP 18 column, where 1)AMOX, 2) PIR, 3) PIP, 4) QUI, 5) LEX, 6) AMPI, 7) MAR, 8) CIP, 9) DAN, 10) LON,11) ENR, 12) ZOL, 13) PER 14) TIO, 15) PIPE 16) PENG, 17) FLU,18) OXAC, 19) CLOX, 20) NAFC, and 21) DICL.

3.4.2. Kinetex 2.6 U C18 (150 x 4.6 mm)

The Kinetex core-shell particle is not fully porous. Using sol-gel processing techniques, a durable, homogenous porous shell is grown on a solid silica core. This highly optimized growth process produces an extremely narrow particle size distribution and dramatically reduces two major sources of peak dispersion- Eddy Diffusion and Resistance to Mass Transfer. This reduces all major sources of band broadening that allows for ultra-high performance on any LC system

Kinetex C18 column was applied in order to obtain the separation for the antibiotics studied. The flow rate was changed from 0.3 to 1 ml/min to obtain better separation. The best result was obtained with the gradient shown in table 2.4 and the separation in Figure 3.7.

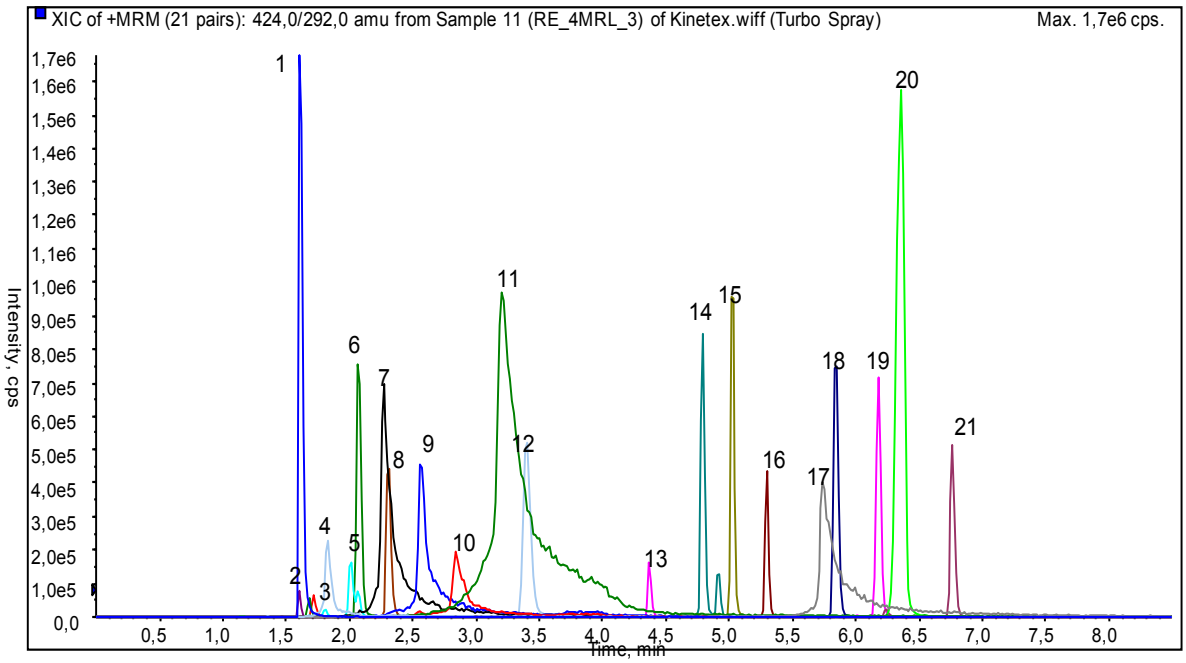


Figure (3.7) Separations obtained from milk sample fortified to the 4MRL conc. using Kinetex column where 1) AMOX, 2) PIR 3) QUI, 4) PIP, 5) AMPI, 6) LEX, 7) MAR, 8) LON, 9) CIP, 10) DAN, 11) ENR, 12) ZOL, 13) PER, 14) TIO, 15) PIPE, 16) PENG, 17) FLU, 18) OXAC, 19) CLOX, 20) NAFC, and 21) DICL.

From the figure 3.6 and 3.7 we can see that the separation of antibiotics with Kinetex column is not good because the bands are too broad especially for quinolones. Comparatively better results were obtained with the Acquity UPLC BEH RP 18 column. However desirable, complete separation of the 21 drugs is a secondary requirement for multi class multi residue analysis because the quantification and identification transition are different for all substances. Moreover the analysis by Acquity UPLC column is achieved in less than 3 minutes which is beneficial to be applied in routine analytical laboratories as it helps to decrease analysis time and solvent consumption. This column was used for carrying out (%) recovery and for analysis of positive samples.

3.5. Quality Parameters

The performance of the developed method was validated according to the European Union regulation 2002/657/EC [43] and some parameters from the FDA guidelines for bio analytical procedure [44]. The quality parameter established were

- Limit of detection (LOD),
- Limit of quantification (LOQ),
- Linearity (calibration curve),
- Recovery (%),
- Precision
- Decision limit (CC_α), and
- Detection capability (CC_β)

3.5.1. Limit of detection (LOD) and Limit of quantification (LOQ)

LOD is the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise [44] and the limit of quantification (LOQ) is the smallest analyte concentration that can be quantified with a given confidence level.

To estimate LOD and LOQ, milk samples were spiked at 8 different concentration level ranging from 0.001 to 0.1MRL (0.001, 0.003, 0.005, 0.01, 0.025, 0.05, 0.08, and 0.1)

Limits of detection (LODs) was calculated at a signal to noise ratio of 3 while the LOQ value was calculated by using a signal to noise ratio of 10.

The results obtained for LODs and LODs are shown in table 3.1.

As can be seen from table 3.1, LODs ranged from 0.03 (NAFC) to 0.5 µg/kg (PER) and LOQs from 0.1 (NAFC, PENG, CIP, DAN, and ENR) to 1.25 µg/kg.

Table 3.1. LOD and LOQ of antibiotics studied in milk using LC-MS/MS

			LC-MS/MS	
	Antibiotic	MRL	LOD (µg/kg)	LOQ (µg/kg)
Penicillins	AMOX	4	0.1	0.3
	AMPI	4	0.1	0.3
	CLOX	30	< 0.1	0.15
	DICL	30	0.15	0.5
	OXAC	30	< 0.1	0.15
	NAFC	30	0.03	0.1
	PENG	4	0.04	0.1
Cephalosporins	LEX	100	0.3	1
	LON	20	0.3	1
	PER	50	0.5	1.25
	PIR	60	0.1	0.3
	QUI	20	0.2	0.5
	TIO	100	0.3	0.5
	ZOL	50	0.15	0.3
Quinolones	CIP	100	< 0.1	0.1
	DAN	30	0.03	0.1
	ENR	100	< 0.1	0.1
	FLU	50	0.05	0.15
	MAR	75	0.2	0.5

3.5.2. Linearity and Calibration curve (CC)

The calibration curve is the relationship between the instrument response and known concentration of analyte [43].

Calibration curves are prepared by plotting the area ratio between the analyte and the internal standard versus ratio of analyte concentration and the internal standard concentration

The linearity of the method was evaluated at eight different concentration levels in milk samples. The working range of the curve was from 0.1 to 3 MRL (0.1, 0.3, 0.5, 1, 1.5, 2, 2.5, and 3 MRL). For each level of concentration two duplicates were prepared. PIPE and PIP was used as internal standard for Quinolones and β-Lactams respectively at a concentration of 100 mg L^{-1}.

The samples prepared for calibration curve were analysed using both techniques, LC-MS/MS and UPLC-MS/MS. Table 3.2 summarizes the calibration curve using both techniques.

Using LC-MS/MS, the calibration curves for all analytes are linear in given range with a correlation coefficient of 0.999 with exception of AMOX and ENR (0.990). Although good correlation coefficient was obtained using UPLC-MS/MS, the correlation coefficients are slightly lower than LC-MS/MS.

Table 3.2 Calibration curve of each studied analyte together with their correlation coefficient

Family	Antibiotic	LC-MS/MS	r	UPLC-MS/MS	r
Penicillins	AMOX	y = 0.383x-0.000177	0.990	y= 0.181x+ 0.000478	0.981
	AMPI	y = 3.22x-0.00115	0.993	y=1.48x + 0.00342	0.990
	CLOX	y = 0.74x-0.00144	0.995	y=0.515x+ 0.00211	0.988
	DICL	y = 0.727x-0.0176	0.995	y= 0.341x-0.0166	0.960
	NAFC	y = 5.49x+0.00881	0.992	y= 3.54x+0.192	0.985
	OXAC	y = 1.38x+0.00807	0.996	y= 0.589x +0.0044	0.990
	PENG	y = 1.96x+0.00368	0.995	y= 1.48x+0.00384	0.997

Cephalosporins	LEX	y = 0.506x-0.0123	0.995	y=0.16x+0.0106	0.982
	LON	y = 0.953x+0.00578	0.994	y=0.769x+0.0179	0.983
	PIR	y = 3.09x+0.00693	0.993	y=1.18x+0.142	0.984
	PER	y = 0.0262x+0.00196	0.993	y= 0.0103x+0.000619	0.980
	QUI	y = 0.431x-0.0033	0.991	y=0.114x -0.00156	0.990
	TIO	y = 0.407x+0.00892	0.993	y=0.116x+ 0.0136	0.990
	ZOL	y = 0.248x+0.0192	0.990	y=0.212 x+0.0149	0.986
Quinolones	CIP	y = 1.36x-0.00526	0.992	y=1.36x+ 0.0915	0.986
	DAN	y = 0.185x-0.00886	0.991	y=1.1x-0.0701	0.985
	ENR	y = 15.2x-1.3	0.990	y= 4.05x-0.0632	0.990
	FLU	y = 40x+3.16	0.996	y=3.34x+0.419	0.983
	MAR	y = 5.65x-0.569	0.995	y=2.99x-0.189	0.986

Where y * = instrumental response (A analyte / A IS)

x * = analyte concentration / IS concentration.

3.5.3. Recovery (%) Study

The accuracy of the method was assessed by recovery test. The recovery of an analytical method is defined as the parameter that measures the efficiency that method has in the analytes extraction process. Recovery experiments are performed by comparing the analytical result of sample in which working standard and internal standard are added before the extraction procedure with unextracted standard in which samples are spiked with working standard and internal standard after the SPE procedure at the same concentration (representing 100 % recovery).

The recovery (%) is calculated through calibration curve and an external curve.

- For both calibration and external curve, 8 concentration levels between 0.1 and 3 MRL were prepared.
- For the external curve, samples are prepared using the same procedure as for calibration samples but the only difference is working solution and internal standard are spiked directly to the extracts after the SPE
- From the external curve equation and the area ratio between antibiotics and internal standard obtained in the calibration curves, we have determined the recovery of each antibiotic studied.

Table 3.3 shows the recovery for each antibiotic studied using two different techniques. The standard error for each recovery is also shown in the same table.

Using LC-MS/MS, it can be observed that out of 19 drugs analysed 17 drugs presented recoveries higher than 65 % with exception of AMOX (57%) and DAN (38 %). Using UPLC-MS/MS, recovery (%) is approximately 70 % with exception of AMOX (48%), PER (59 %) and DAN (42%).

Comparing the recoveries obtained from both techniques, it can be observed that most of the antibiotics present differences lower than 10 %, except for NAFC, PER, QUI and TIO. The significant difference in recovery in these antibiotics may be due to some variation in procedure. Before we inject the sample in UPLC-MS/MS, we pass the sample through centrifugal filter device (0.22 μm). This may be one of the reasons behind low recovery of some analyte. The other reason may be the sample

volume was insufficient to be injected into UPLC-MS/MS as same sample that was finished analyzing in LC-MS/MS was injected in UPLC-MS/MS.

Table 3.3 Recovery of Antibiotic studied in Milk samples using LC-MS/MS and UPLC-MS/MS

		LC-MS/MS	UPLC-MS/MS
Family	Antibiotic	Recovery (%)	Recovery (%)
Penicillins	AMOX	57(4) *	48(3)*
	AMPI	78(2)	69(2)
	CLOX	90(2)	99(4)
	DICL	101(2)	100(13)
	NAFC	93(3)	122(7)
	OXAC	86(2)	95(3)
	PENG	78(2)	88(1)
Cephalosporins	LEX	83(2)	82(4)
	LON	112(2)	110(4)
	PIR	117(3)	100(5)
	PER	76(3)	59(3)
	QUI	105(3)	94(4)
	TIO	97(3)	77(3)
	ZOL	112(4)	108(5)
Quinolones	CIP	67(2)	77(4)
	DAN	38(2)	42(2)
	ENR	69(3)	77(3)
	FLU	106(3)	116(7)
	MAR	86(3)	82(4)

*% Error associated with recovery (Standard error)

3.5.4. Precision Study

The precision of the method was evaluated in terms of repeatability (single day – intraday precision) and intermediate precision (different days - interday precision).

The repeatability of an analytical method is defined as the ability of the method to generate the same results in repeated analysis of the same sample, performed by the same operator under the working conditions of the equipment. The procedure was repeated on 3 different days in order to determine inter-day precision.

The intra-day and inter-day precision of the methods were evaluated at three concentration levels (0.5 MRL, 1 MRL and 2 MRL). 5 replicates were prepared for each concentration. To quantify the concentration of samples, calibration curve was prepared that was injected along with the samples. Finally, the relative standard deviation (%) of the results was calculated. The acceptance criteria for RSD are 15 % (except for LOQ 20 %) which is based according to the FDA guidance for validation of analytical methods. [44]

Tables 3.4 and 3.5 shows the values obtained for intra-day and inter-day repeatability for antibiotics studied in milk using LC-MS/MS.

As can be observed from the table 3.4, the repeatability values expressed as RSD % are lower than 15 % which is within the acceptance criteria. Similar results are obtained in other two consecutive days.

Table 3.4 Intra-day repeatability values for antibiotic studied in milk using LC-MS/MS.

Family	Antibiotic	0.5 MRL (n=5)		MRL (n=5)		2 MRL (n=5)	
		Conc.	%RSD	Conc.	% RSD	Conc.	% RSD
Penicillins	AMOX	1.8	3	3.9	3	7.8	2
	AMPI	1.4	10	3.6	8	7.5	7
	CLOX	15.1	1	29.2	3	59.2	2
	DICL	14.9	3	29.9	4	59.4	2
	OXAC	14.3	4	29.0	7	59.9	2
	NAFC	13.3	3	26.6	10	57.3	4
	PENG	1.7	9	3.9	7	7.9	2
Cephalosporins	LEX	45.9	6	85.0	5	167.8	13
	LON	8.1	9	16.7	11	35.4	14
	PER	16.6	7	50.7	9	103.1	14
	PIR	23.5	10	44.8	8	97.9	6
	QUI	9.0	9	16.7	9	35.5	10
	TIO	40.5	8	79.8	7	163.5	2
	ZOL	17.9	13	41.0	11	85.4	11
Quinolones	CIP	44.7	11	96.8	4	190.5	6
	DAN	15.0	10	35.6	10	68.1	11
	ENR	43.4	7	101.4	4	188.0	6
	FLU	22.5	11	44.7	11	89.7	6
	MAR	35.2	8	82.2	10	144.6	5

Table 3.5 Inter-day repeatability values for antibiotic studied in milk using LC/MS/MS

Family	Antibiotic	0.5 MRL(n=15)		MRL(n=15)		2 MRL(n=15)	
		Conc.	% RSD	Conc.	%RSD	Conc.	%RSD
Penicillins	AMOX	1.6	14	3.8	10	7.2	10
	AMPI	1.5	13	3.8	10	7.1	12
	CLOX	14.6	7	29.0	4	60.1	10
	DICL	15.0	8	29.5	7	59.1	10
	OXAC	14.0	5	30.6	8	59.8	8
	NAFC	12.3	10	27.4	9	53.3	11
	PENG	1.7	12	3.8	6	7.9	7
Cephalosporin	LEX	48.6	8	95.2	10	189.2	10
	LON	7.8	11	17.1	13	36.5	14
	PER	22.7	14	47.5	13	96.9	15
	PIR	22.9	13	46.8	13	90.9	10
	QUI	8.6	14	17.3	15	32.0	12
	TIO	39.3	11	86.3	9	158.5	8
	ZOL	19.1	13	42.1	11	92.2	12
Quinolone	CIP	45.3	14	99.4	7	200.9	11
	DAN	15.3	14	28.9	14	61.3	13
	ENR	48.6	13	102.5	8	196.5	8
	FLU	23.0	14	46.0	11	94.5	7
	MAR	34.9	10	85.9	14	139.1	12

As can be observed from table 3.5 all the values are within acceptance criteria.

3.5.5. Decision Limit and Detection Capability

In accordance with the 2002/657/EC decision, the validation procedure includes the determination of CC_α (Limit of decision) and CC_β (Capability of detection). This two statistical limits allow to evaluate the critical concentration above which the method reliably distinguish and quantify a substance taking into account the variability of the method and the statistical risk to take a wrong decision [45].

3.6.5.1. Decision limit (CC_α)

CC_α is defined as the limit at and above which it can be concluded with an error probability of α that a sample is non- compliant [43].

3.6.5.2. Detection capability (CC_β)

Detection capability (CCβ) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 – β. In the case of substances with an established permitted limit (MRL), this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1 – β [43] as in the case of antibiotics studied.

For the measurement of CC_α and CC_β, 20 blank milk samples were spiked at the MRL concentration and were analyzed by the corresponding calibration curves.

CC_α is calculated as the concentration at the MRL plus 1.64 times the corresponding standard deviation.

$$CC_\alpha = C_{MRL} + 1.64 \times SD_{MRL}$$

In a previous work conducted in the research group in tissue of pig, we found that the $SD_{CC\alpha}$ for all the analytes is similar to the SD_{MRL}, so supposing $SD_{CC\alpha} = SD_{MRL}$, CC_β parameter could be calculated as

$$CC_\beta = CC_\alpha + 1.64 \times SD_{MRL}$$

Table 3.6 shows the values of CC_α and CC_β obtained using LC-MS/MS.

Table 3.6 CC_α and CC_β obtained from LC-MS/MS

			LC-MS/MS	
Family	Antibiotic	MRL	CC_α (μg/kg)	CC_β (μg/kg)
Penicillins	AMOX	4	5.0	6.1
	AMPI	4	4.6	5.3
	CLOX	30	33.8	37.7
	DICL	30	33.5	36.9
	OXAC	30	33.3	36.6
	NAFC	30	33.5	37.0
	PENG	4	4.52	5.04
Cephalosporins	LEX	100	109.5	119.0
	LON	20	24.0	28.1
	PER	50	57.3	64.6
	PIR	60	67.2	74.4
	QUI	20	24.5	29.0
	TIO	100	109.5	119.1
	ZOL	50	55.4	60.8
Quinolones	CIP	100	111.3	122.6
	DAN	30	38.3	46.6
	ENR	100	112.0	124.1
	FLU	50	58.9	67.7
	MAR	75	90.3	105.5

Thus when samples are analysed we can say that samples with values higher than CC_β are non compliant with an error probability of β.

3.6. Applicability of the Method

Samples that were tested positive in the β-lactam screening test were obtained from ALLIC in four different lots. The effectiveness of the developed method was checked by analyzing these samples. The samples were labelled as M126-138, M142-156, M157-167, M170-178 and M184, M189, M190. Most part of the samples were analysed in duplicate.

For samples labelled as M126-138, analysis was done using LC-MS/MS and UPLC-MS/MS. The results are summarized in table 3.7. From a total of 13 samples analysed all the samples contain β-lactam. As can be observed in table 3.7 , using both techniques , M128, M130, M131, M132, M133, M138 were non compliant as the concentration calculated in these samples were higher than CC_β values obtained. The results obtained with both techniques are comparable except for M135 which is non compliant with UPLC technique.

Table 3.7 Results obtained in the analysis of Positive Milk Samples

	LC-MS/MS		UPLC-MS/MS	
	Positive in	Concentration (µg/kg)	Positive in	Concentration (µg/kg)
M126	AMOX[a], PER[b]	1.3(0.1)[a],39(4)[b]	AMOX[a], PER[b]	1.1(0.1)[a],55(8)[b]
M127	AMOX[a], PER[b]	2.0(0.3)[a],6.2(3)[b]	AMOX[a], PER[b]	2.2(0.1)[a],17(1)[b]
M128	PENG	5.9(0.2)	PENG	6.9(0.7)
M129	AMOX	1.2(0.2)	AMOX	1.2(0.4)
M130	AMOX	7(0.2)	AMOX	7.1(0.2)
M131	PENG	19(0.3)	PENG	20(0.01)
M132	PENG	14(0.4)	PENG	14(0.1)
M133	PENG	29(9)	PENG	28(2)
M134	AMOX	3(0.5)	AMOX	3.2(0.5)
M135	AMOX	4.8(0.6)	AMOX	6.2(1.8)
M136	LEX	36(12)	LEX	37(18)
M137	LEX	66(4)	LEX	60(13)
M138	LEX	128(15)	LEX	136(41)

Figure 3.8 and 3.9 show the chromatograms obtained by LC-MS/MS and UPLC-MS/MS for positive sample M138 that present a high concentration of LEX.

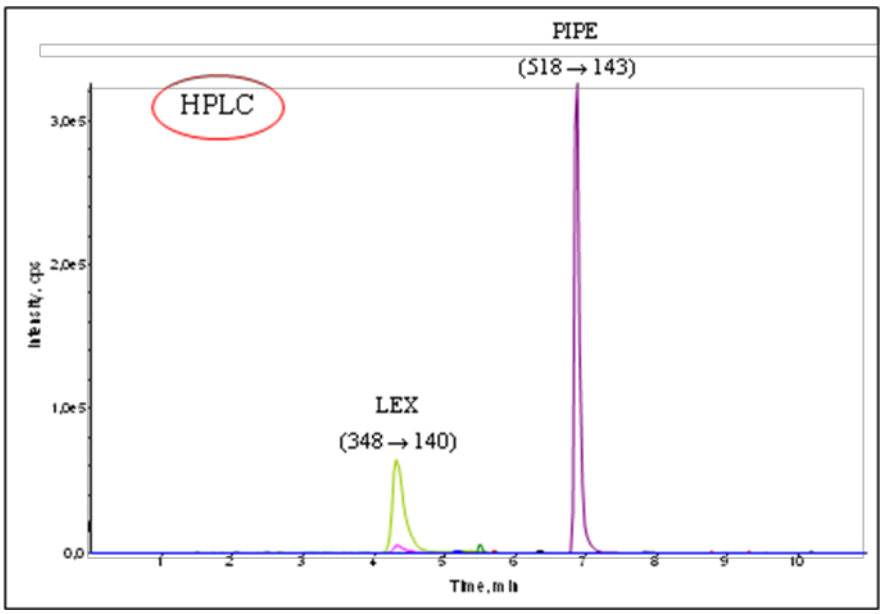


Figure 3.8 Chromatogram obtained in the analysis of non compliant sample M138 using LC-QqQ technique.

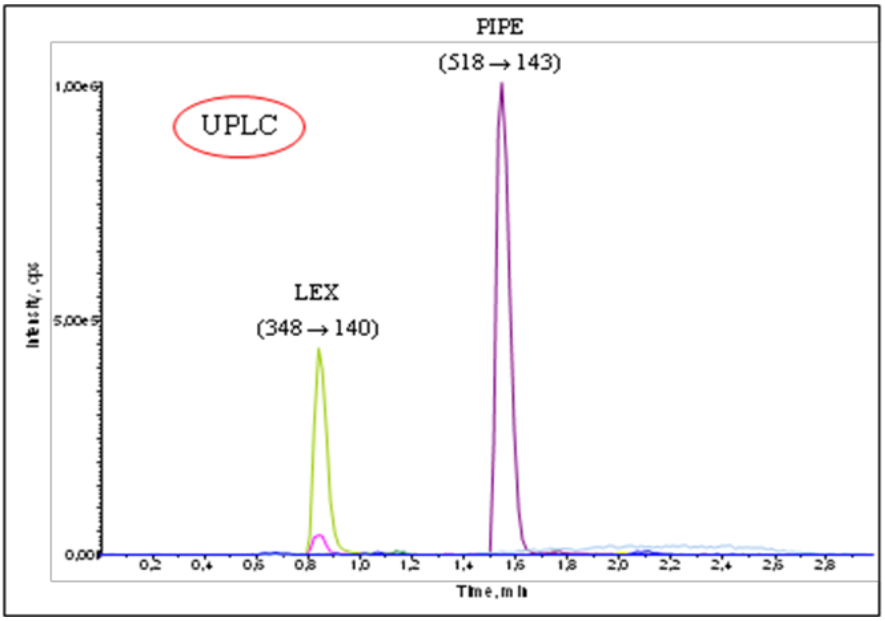


Figure 3.9 Chromatogram obtained in the analysis of non compliant sample M138 using UPLC-QqQ technique

The results for (M142-M190) are summarised in table 3.8 with the antibiotics present in each sample and their concentration.

Table 3.8 Results obtained in the analysis of Positive Milk Samples

	Positive in	Concentration (µg/kg)
M142	AMOX	7.2(1.6)
M143	AMOX	4.7
M144	AMOX	46
M145	CLOX[a], LON[b]	51.5 (0.9) [a], 4.4 (0.2)[b]
M146	PIR	11.3 (0.7)
M147	PENG	3.5 (0.4)
M148	PIR	7.5(0.8)
M149	AMPI[a], DICL[b]	2.9 (0.3)[a], 2.5(0.2)[b]
M150	CIP[a], ENR[b]	22.7(0.9) [a],11.1[b]
M151	CIP[a], ENR[b]	24.1(0.5) [a],11.3(0.4)[b]
M154	PIR	6.3(0.6)
M155	PIR	5.6(0.2)
M156	PIR	8.4(0.6)
M157	PENG	4.1(0.3)
M158	PIR	8(0.4)
M159	PENG	1.6
M160	AMOX	19(4)
M161	AMOX	10(1)
M162	AMPI[a], DICL[b]	6(0.02)[a] 6.2(0.4)[b]
M163	AMOX[a],PER[b]	6.5(1.3)[a],7.7(0.4)[b]
M164	PIR	9.6
M165	PENG	6.5
M166	PIR	22.5(0.8)
M167	PENG	1(0.05)

M170	PENG	3.7(0.2)
M171	PIR	15(2)
M172	PENG	3.4(0.6)
M173	PENG	2.7(0.2)
M174	PENG	3.8(0.2)
M175	PENG	3.8(0.2)
M176	PENG	3.5(0.1)
M177	PENG	5.1(0.1)
M178	AMOX	1.9(0.4)
M184	PENG	14.5
M189	PENG	12.7(1.4)
M190	AMOX	42.3(4.4)

As can be observed from the table 3.8, all the samples contain at least one antibiotic. Sample M142, M144, M145, M160, M161, M162, M163, M165, M177, M184, M189 and M190 were non compliant with an error probability of β, as the concentration calculated in these samples were higher than CC_β values obtained. From the tested samples, M145 and M163 contain one penicillin and one cephalosporin (CLOX and LON) which indicates multiple uses of β-lactam. Two samples were positive in the quinolones ENR and its metabolite CIP.

Figures 3.10 and 3.11 shows as an example the chromatogram obtained for positive samples by LC-MS/MS, with the corresponding confirmatory chromatogram. Figures 3.10 corresponds to a sample of AMOX with very high concentration of 42.3 µg/kg, and Figures 3.11 contains PENG with concentration of 12.7µg/kg.

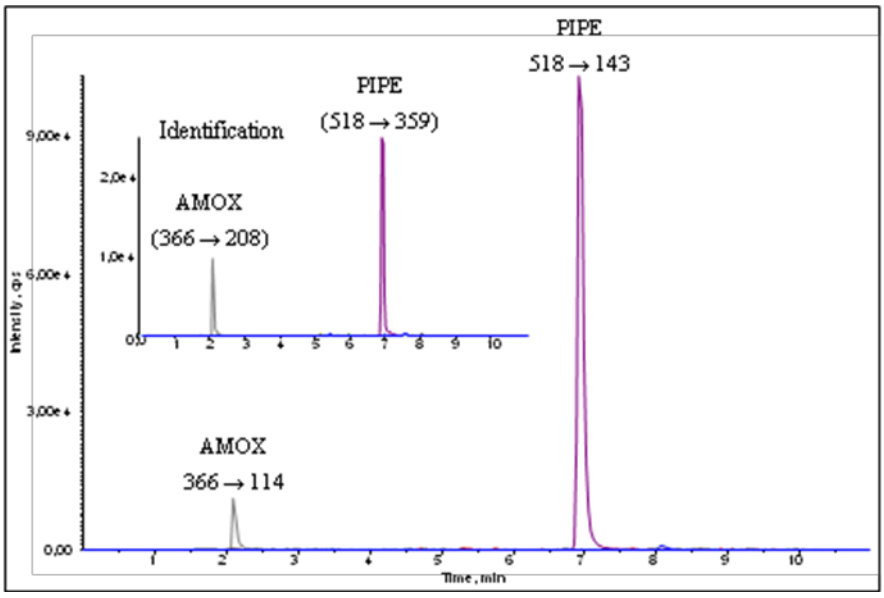


Figure 3.10 Chromatogram obtained in the analysis of non compliant sample M190 with AMOX using LC-QqQ technique.

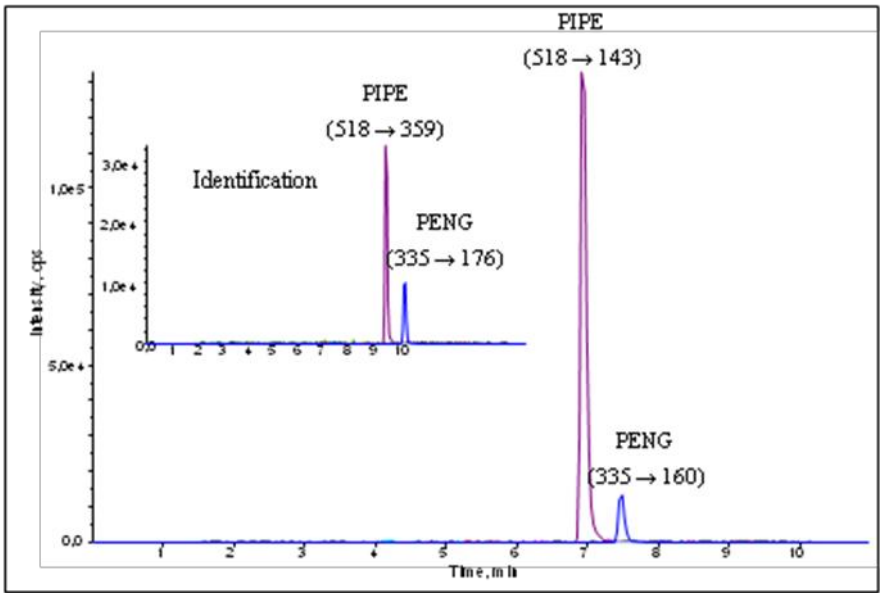


Figure 3.11 Chromatogram obtained in the analysis of non compliant sample M189 with PENG using LC-QqQ technique

From the results obtained from table 3.7 and 3.8, we make a conclusion that AMOX and PENG are the most common residues found in milk samples. 14 % of samples contained AMOX and 16 % contained PENG .Other samples contain AMPI, CLOX and LEX. The presences of these residues were quantified and confirmed .Thus the results confirms the applicability of the method for the determination of positive samples.

4. Conclusions

A multi-class, multi residue method was developed for the simultaneous determination of 19 antibiotics regulated by European legislation 2377/90/EC in cow milk using LC-MS/MS. The method was optimised and following conclusions are drawn.

1. Extraction Procedure

   Several studies were conducted to optimize the extraction method. Following conclusions were drawn from the studies.

   - Raw milk is preferred over commercial milk for method development.
   - Drying method affects the recovery of some analytes. Different recoveries were obtained for cephalosporins using two different drying techniques (air drying or nitrogen drying). Better recoveries are obtained with nitrogen drying so it was choosen.
   - For extraction of antibiotics from the milk sample it is preferred to weigh, spike and centrifuge in order to simulate the procedure that happens in positive samples.

2. Separation of antibiotics:

   - For LC-MS/MS, with the optimized gradient, and using C8 column (150 mm x 4.6 mm, 5µm) separation of 19 antibiotics was obtained in 11 minutes.
   - UPLC-MS/MS technique was applied to separate antibiotics using two different columns. It was found that with the use of Acquity UPLC BEH shield RP 18 (50 mm x 2.1 mm, 1.7 µm particle diameter) ,19 antibiotics can be analysed in less than 3 minutes

3. Quality Parameters:

   The developed method was validated. Quality parameters proved this method to be efficient.

   - LODs ranged from 0.03 (NAFC) to 0.5 µg/kg (PER).
   - LOQs from 0.1 (NAFC, PENG, CIP, DAN, and ENR) to 1.25 µg/kg (PER).The LOQs obtained for each antibiotic are much lower than the MRLs (regulation 2377/90).

- The calibration curves for all analytes are linear with a correlation coefficient of 0.999 with exception of AMOX and ENR (0.990).
- The majority of antibiotics showed recoveries higher than 65 % with exception of AMOX (57%) and DAN (38 %).
- The repeatability values expressed as RSD % are lower than 15 % which are in accordance with FDA regulation.

4. Positive Samples:

   The developed method was applied for 49 real samples supplied by ALLIC. Among all samples analysed, 37% of the sample was found to be non-compliant with an error probability of β. AMOX and PENG was the most common residue found. 14 % of non compliant sample contained AMOX and 16 % contained PENG.

Thus a simple and efficient method was developed and validated for simultaneous determination of 19 antibiotics regulated by European legislation in milk samples using LC-MS/MS.

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Appendix 1: Antibiotic use in Europe

The current available data on antibiotic use in the EU/EEA Member States collected by the European Surveillance of Antimicrobial Consumption (ESAC) project is shown in figure 1.It shows antibiotic use measured in Defined Daily Doses (DDD) per 1000 inhabitants and per day. Each bar refers to a specific country while the different antibiotic classes used in that country. The reported data mainly refer to antibiotic use outside hospitals (outpatient use) which accounts for the largest proportion of human consumption. However, comparison of data between countries report hospital use and outpatient use together. The total outpatient antibiotic use ranged from 11.0 DDD per 1000 inhabitants and per day in The Netherlands to 28.6 DDD per 1000 inhabitants and per day in France. Penicillins were the most frequently prescribed antibiotic class in all countries, whereas the proportion of use of other antibiotic classes varied among the countries.

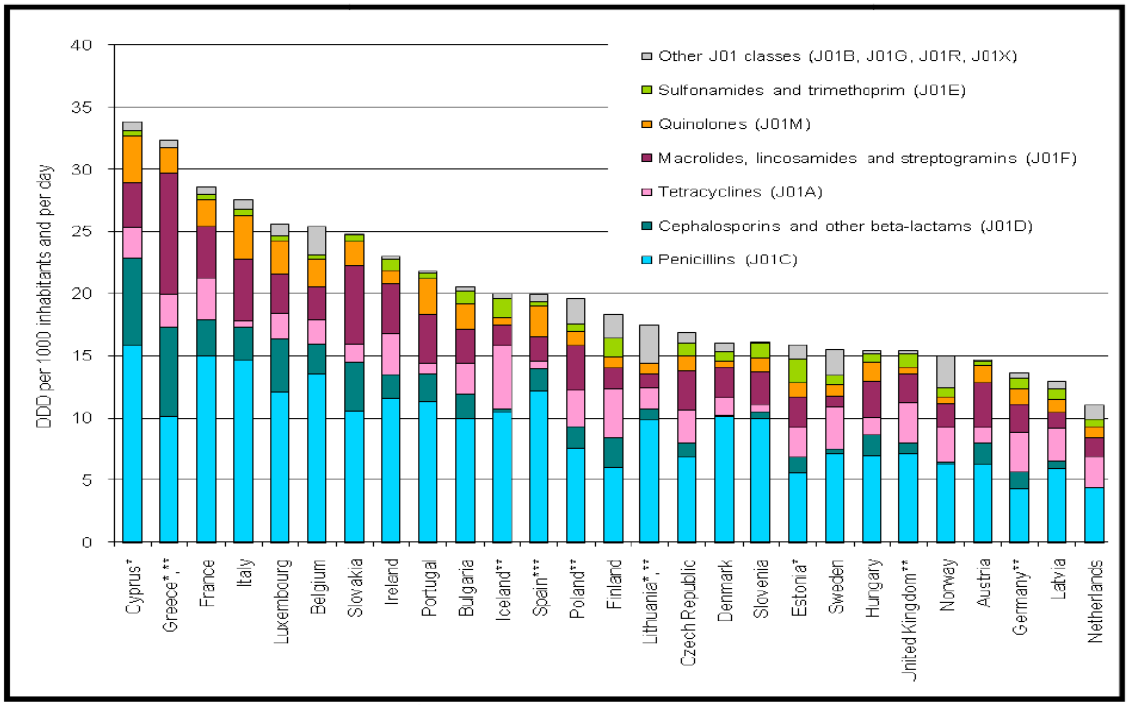

Figure (1) Total outpatient antibiotic use in EU/EEA countries

* Total use, i.e. including inpatients, for Cyprus, Estonia, Greece and Lithuania.

** 2006 data for Germany, Greece, Iceland and Lithuania; 2005 data for

*** Reimbursement data, which do not include over the counter sales without prescription. *Data source:* ESAC 2007.

Appendix 2: Usage of Antibiotic in Human and Animals

According to a study by the European Federation of Animal Health (FEDESA), farm animals in 1999 consumed 4,700 tonnes (35 percent) of all the antibiotics administered in the European Union, while humans consumed 8,500 tonne (65 percent). Of the antibiotics that were given to animals, 3,900 tonne (29 percent of total usage) were administered to help sick animals recover from disease, while 786 tonnes (6 percent of total usage) were fed to farm animals as growth promoters. The survey estimated that the amount of antibiotics used as growth promoters had fallen by half since 1997, when animals consumed around 1,600 tonne as feed additives. [1]

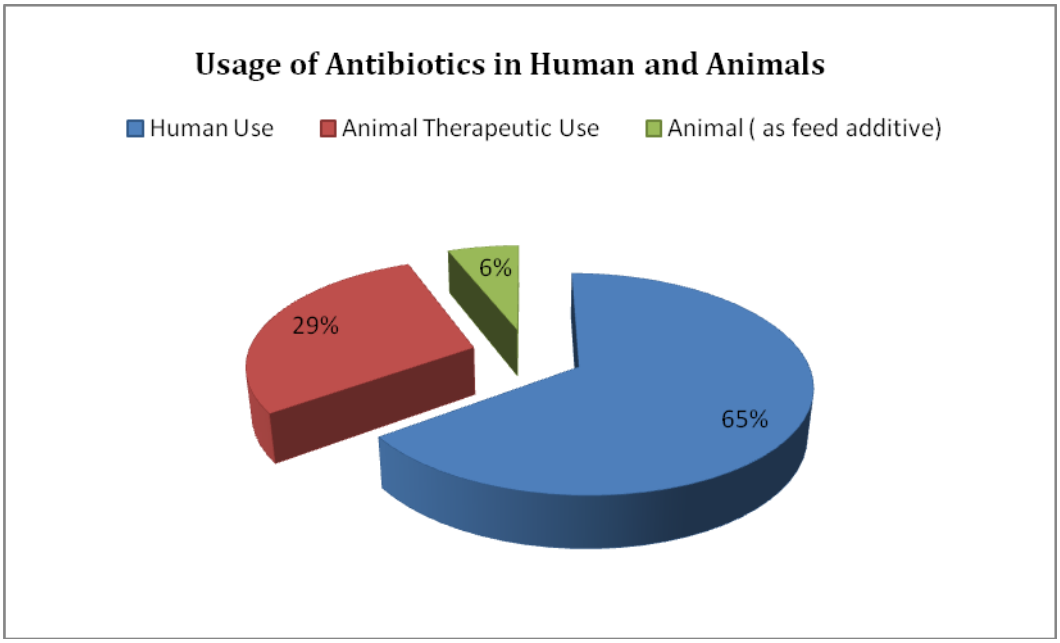

Table 1 : Overview of published use data of several chemical groups of antibiotics for several European countries, presented in metric tonnages (t = 1000 kg) and the relative proportion (% of total antibiotic use) per country [2]

Antibiotic group	Denmark	Finland	France	Netherlands	Sweden	UK
Aminoglycosides	11.6 (10 %)	0.3(2%)	77(6%)	9(2%)	0.6(4%)	22(5%)
Amphenicols, Phenicol	0.3 (0.3%)	n.s.	5.2 (0.4)	n.s.	n.s.	n.s.
β-Lactams and cephalosporins	34.5 (30%)	8.7 (62%)	112 (9%)	45 (10%)	9.6 (60%)	63 (13%)
Macrolides and lincosamides	16 (14%)	0.5 (3.7%)	96 (8%)	24 (5%)	1.1 (7%)	59 (12%)
Fluoroquinolones and Quinolones	0.4(0.3%)	0.1(0.6%)	20(1.6%)	7(1.5%)	0.2(1%)	1.1(0.2%)
Sulfonamides and trimethoprim	12.7 (11%)	2.3 (16%)	240 (19%)	93 (21%)	2.9 (18)	77 (16)
Tetracyclines	30 (26%)	1.3 (8.9%)	638 (50%)	269 (59)	1.3 (8)	243 (51%)
Other antibiotics	8.4 (7%)	1 (7 %)	82 (6.5%)	6 (1.3%)	0.4 (2)	11 (2.3)
Totals	114	14.2	1270	453	16.1	476

n.s.,not specified

Reference

[1]ftp://ftp.fao.org/docrep/fao/009/a0282e/a0282e00.pdf

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Appendix 3: Antibiotic Resistance in Europe

E. coli is the most frequent cause of bacteraemia by gram-negative bacteria, as well as community and hospital-acquired urinary tract infections. The Europe-wide increase of resistance in E. coli to all antibiotic classes under surveillance is continuing and the speed by which important antibiotics like fluoroquinolones are losing their activity against E. coli is alarming. This trend is highlighted by the shift towards red which is evident comparing the maps of 2003 and 2008 (Fig. 1a and 1b).

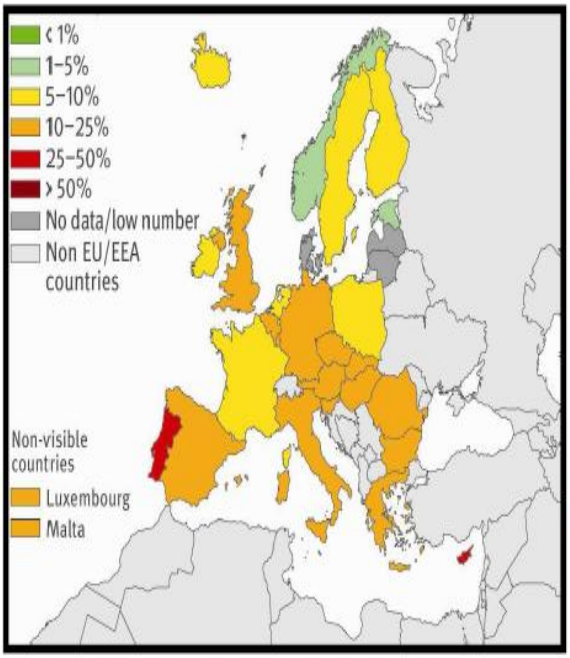

Figure 1a) Proportion of fluoroquinolone-resistant *Escherichia coli* in EU/EEA countries in 2003 *("low number" = less than 10 isolates reported)*
Data source: EARSS

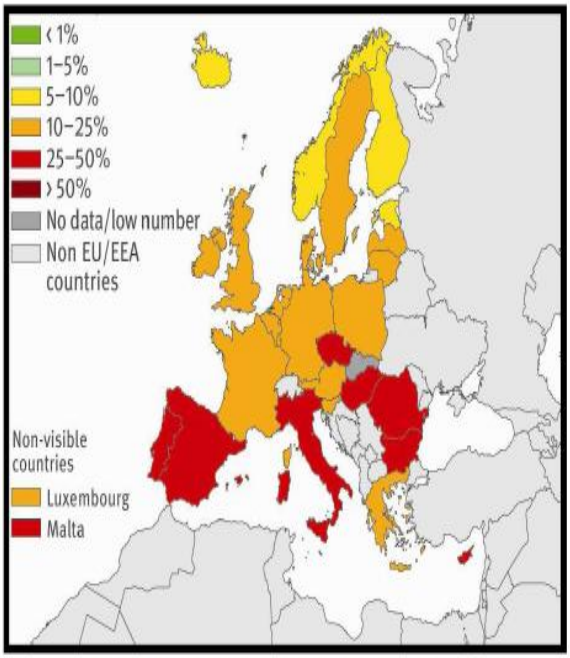

Figure 1b) Proportion of fluoroquinolone-resistant *Escherichia coli* in EU/EEA countries in 2008 *("low number" = less than 10 isolates reported)*
Data source: EARSS

(Note: The country colours range from green to dark red according to the frequency of resistance).

Staphylococcus aureus, in its methicillin-resistant form (MRSA), is the most important cause of antibiotic-resistant healthcare-associated infections worldwide. In 2008, more countries showed decreasing MRSA proportions, thus the MRSA problem seems to have stabilised, or even decreased for most countries. Nevertheless, MRSA proportions are still above 25% in one third of countries (Fig. 2).

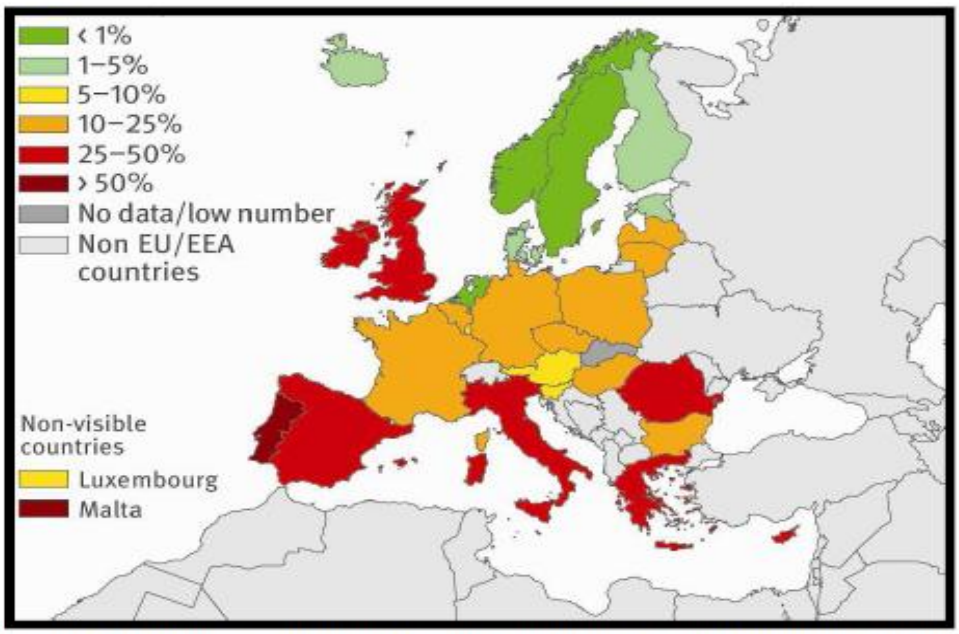

Figure 2b) Proportion of methicillin-resistant *Staphylococcus aureus* in EU/EEA countries in 2008 *("low number" = less than 10 isolates reported)*
Data source: EARSS

Pseudomonas aeruginosa (P. aeruginosa) is an important cause of infection among patients with localised and systemic immune defects. Resistance to carbapenems in P. aeruginosa are high all over Europe, as almost three quarters of the countries (23 of 32) reported more than 10% carbapenems resistance (Fig. 3). In Europe, multi-drug resistance is the dominant threat posed by invasive P. aeruginosa.

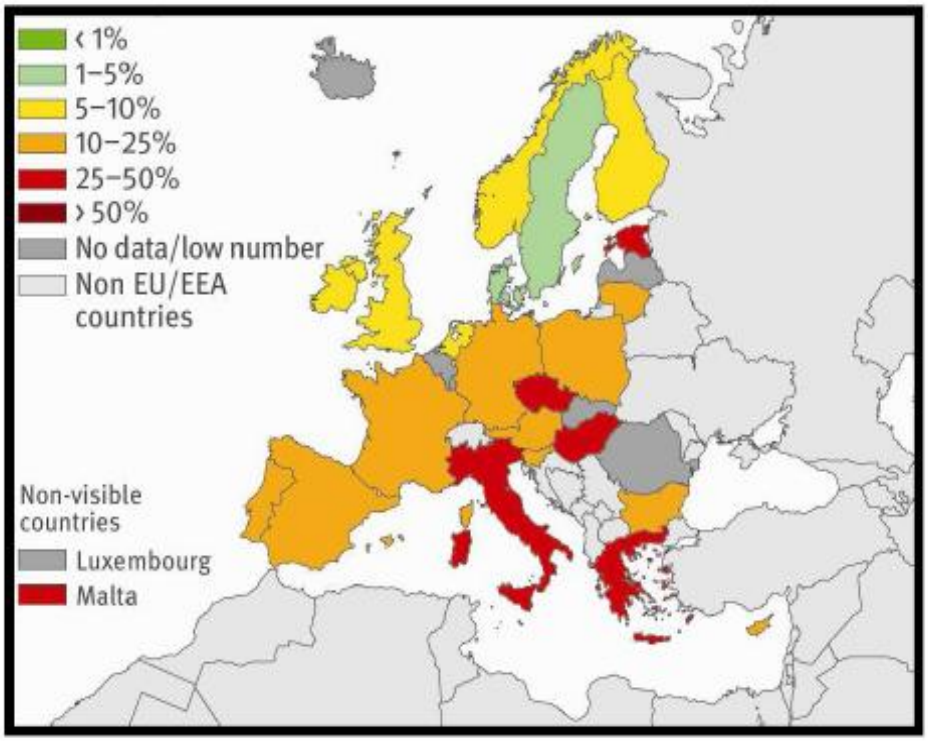

Figure 3) Proportion of carbapenem-resistant *Pseudomonas aeruginosa* in EU/EEA countries in 2008 *("low number" = less than 10 isolates reported)*
***Data source:* EARSS**

Klebsiella pneumoniae (K. pneumoniae) is an important cause of urinary and respiratory tractinfections, especially in individuals with impaired immune systems, such as people with diabetes and hospitalised patients with invasive devices, such as urinary catheters and drips. With regard to K. pneumoniae, a high frequency of resistance to 3rd generation cephalosporins (Fig. 4), fluoroquinolones and aminoglycosides is evident in Central and South-eastern Europe. Many of these strains, including the most frequent one, show resistance to all the above mentioned antibiotic classes.

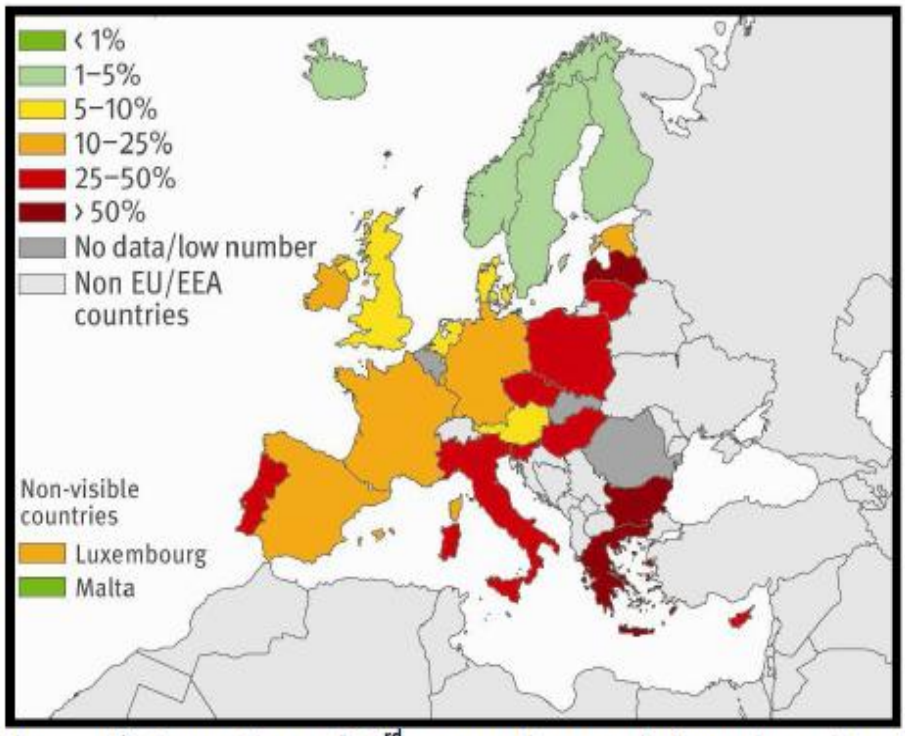

Figure 4) Proportion of 3rd generation cephalosporin-resistant *Klebsiella pneumoniae* in EU/EEA countries in 2008 *("low number" = less than 10 isolates reported)*
***Data source:* EARSS**