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MASTER THESIS

DEVELOPMENT OF A LC-MS/MS METHOD FOR THE DETECTION OF POLYPEPTIDE ANTIBIOTICS

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ABSTRACT

Bacitracin, colistin as well as polymyxin B are well-known antibiotics that for a long time have played a minor role in human medicine due to their toxicity. In contrast they were often used in veterinary medicine. With increasing development of drug resistance pathogenic bacteria the mentioned polypeptide antibiotics regain importance and an increased control of the maximum residue limits (MRLs) established by the European Commission in foods is required. Limits are set for the sum of bacitracin A, B and C and for the sum of colistin A and B, while polymyxin B is not regulated. In this work liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was applied for the detection of bacitracin A and B, colistin A and B as well as polymyxin B_1 and B_2 in milk and animal tissue samples. Different protocols for sample preparation were evaluated, various chromatographic separation conditions tested, as well as mass spectrometric settings optimised. The developed analytical method is divided into the following stages; (1) sample preparation, based on acidic solvent extraction and followed by solid phase extraction (SPE) and purification using HR-X cartridges, (2) chromatographic separation in reversed-phase conditions based on a gradient elution program within a total run time of 15 min. and finally (3) electrospray ionization tandem mass spectrometric measurement under selective multiple reaction monitoring (MRM). Acquisition of triple charged precursor ions for all analytes was performed. The method was successfully validated by an in-house protocol according to the requirements of 2002/657/EC in these matrices. The presented method will serve as a basis for the quantitative analysis of the concerned polypeptides in foods in an analytical routine laboratory.

Keywords: polypeptide antibiotics, SPE, LC-MS/MS, milk, animal tissue, validation

KURZFASSUNG

Bacitracin und Colistin sowie Polymyxin B sind altbekannte Antibiotika, die lange in der Humanmedizin aufgrund ihrer Toxizität nur eine untergeordnete Rolle spielten. Sie wurden dagegen in der Tiermedizin vielfach eingesetzt. Mit zunehmender Resistenzbildung von pathogenen Keimen gewinnen die genannten Polypetidantibiotika wieder an Bedeutung in der Humanmedizin und eine verstärkte Kontrolle der von der Europäischen Kommission festgelegten Grenzwerte in Lebensmitteln wird erforderlich. Grenzwerte sind für die Summe von Bacitracin A, B und C sowie für die Summe an Colistin A und B festgelegt. Polymyxin B ist nicht reguliert. In dieser Arbeit wurde eine Methode zur Bestimmung von Bacitracin A und B, Colistin A und B sowie Polymyxin B_1 und B_2 in Fleisch und Milch entwickelt. Verschiedene Protokolle wurden für die Probenvorbereitung getestet, sowie die Parameter der chromatographischen Trennung und der massenspektrometrischen Detektion optimiert. Der im Rahmen dieser entwickelte analytische Prozess gliedert sich in die folgenden Schritte; (1) Probenvorbereitung mittels saurem Extraktionslösungsmittel, gefolgt durch eine Festphasenextraktion und Aufreinigung unter Verwendung von HR-X Kartuschen, (2) chromatographische Trennung in Umkehrphasenbedingungen basierend auf einer Gradientenmethode, sowie einer abschließenden (3) Elektrospray-Ionisierung und Detektion mittels Tandem-Massenspektrometrie. Dreifach geladene Vorläufer-Ionen aller Analyte wurden mittels Multiple Reaction Monitoring (MRM) gemessen. Die Methode wurde nach einem hausinternen Protokoll nach den Erfordernissen der 2002/657/EC in den genannten Matrices erfolgreich validiert. Die Methode wird als Basis für die quantitative Analyse der betroffenen Polypeptide in Lebensmitteln in einem analytischen Routinelabor dienen.

Stichwörter: Polypeptidantibiotika, LC-MS/MS, SPE, Milch, Fleisch, Validierung

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
BAC	Bacitracin
CCα	Decision limit
CCβ	Detection capability
CE	Collision energy
COL	Colistin
CXP	Cell exit potential
DP	Declustering potential
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EEC	European Economic Community
ELSD	Evaporative light scattering detector
EMEA	European Medicines Agency
EP	Entrance potential
ESI	Electrospray ionisation
EU	European Union
H_2O	Water
HCl	Hydrochlorid acid
HLB	Hydrophilic-lipophilic-balance
HPLC	High-performance liquid chromatography
IS	Internal standard
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography coupled to tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LPL	Laboratory performance level
MeOH	Methanol
MRL(s)	Maximum residue limit(s)
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
NaCl	Sodium chloride
pH	Negative decimal logarithm of the hydrogen ion activity
POL	Polymyxin
PS-DVB	Polystyrene–divinylbenzene
RSD	Relative standard deviation
SPE	Solid phase extraction
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
UV	Ultra violet

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1.1. Background to the problem

Polypeptide antibiotics such as bacitracin, colistin and polymyxin are well known in medicine as antibiotics for over 50 years. However it is assumed that they exhibit high toxicity (e.g. nephrotoxicity and neurotoxicity) and are therefore rarely applied in human medicine. Instead they are more often used in vetenary medicine (Sin *et al.*, 2005, Wan *et al.*, 2006). In this field they have been especially utilised in food producing animals in order to prevent and treat diseases, as well as to promote animal growth. However the intensified use of these antibiotics may lead to drug residues present in food, which may harm human health by absorption through the food chain (Xu *et al.*, 2012).

During the last decades the increased use of antibiotics in general has led to an increased formation of antibiotic resistant microorganisms. As a consequence, so termed 'last resort antibiotics', such as colistin, have been used more intensively. Since the formation of resistances through polypeptide antibiotics is still discussed, surveillance was increased and regulations and limits for polypeptide antibiotics were introduced.

In the EU a list of veterinary drugs is controlled under council regulation no. 2337/90 (EEC, 1990), which states the maximum residue limits (MRLs) of veterinary medicinal substances in foods of animal origin. Therefore methods have to be developed to routinely analyse foods for the presence of the concerned polypeptides and see if samples meet the required maximum limits.

In literature the determination of polypeptide antibiotics in the trace-level range constitutes a problem. Therefore the need arises to develop new selective multi-analyte methods for the detection of these peptides (Stolker and Brinkman, 2005). To date only few publications describe the analysis of both bacitracin and colistin and no publications has been found which also includes polymyxin as an analyte.

1.2. Aims

The final aim of the project is to develop and validate a method for the detection of the polypeptide antibiotics bacitracin, colistin and polymyxin B in matrices like meat and milk. These antibiotics are used amongst others as veterinary drugs. First an efficient sample preparation has to be established. This includes a sample extraction procedure together with a solid-phase clean-up step. Finally the sample can then be analysed by a LC-MS/MS measurement, whose parameter need to be optimised. The final validation is based on an in-house validation procedure and performed in accordance with the EU regulation 2002/657/EC. The proposed method is meant to serve as a method in a routine laboratory.

1.3. Objectives

The objectives of this project are to develop and validate a LC-MS/MS method for the detection of polypeptide antibiotics which meets the following requirements:

- fast and simple extraction and clean-up for matrices like meat and milk, based on liquid extraction and solid phase extraction principles,
- investigating different chromatographic systems and separation conditions,
- employing electrospray ionisation tandem mass spectrometry for the detection of the analytes and optimising the detection conditions
- and finally validating the method in accordance with 2002/657/EC, by taking into account the currently established MRL values.

1.4. Legal requirements

Veterinary drugs and more specifically antibacterial substances are listed in the Council Directive 96/23/EC under Annex I Group B 1 (EC, 2002a). In order to maintain a high level of consumer protection the European Union has introduced maximum residue limits (MRLs) for pharmacologically active substances in animal tissue and foodstuffs of animal origin. These values can be found in the Commission Regulation 37/2010 (EC, 2009). MRLs for allowed substances like bacitracin and colistin are listed in Table 1-1 and Table 1-2 respectively. For substances not mentioned, such as polymyxin, a MRL can not be established. Therefore polymyxin is not regulated and its administration to food producing animals can not be ruled out.

Similarly to trace analysis methods, also in the area of vetenary drug residues great importance is given to method validation. In the case of vetenary drugs the implementation of afore mentioned Council Directive 96/23/EC is tested according to the European Commission Decision 2002/657/EC (EC 2002b). This decision deals with the performance of an analytical method, together with the interpretation of results.

Table 1-1. EU antibiotic maximum residue limits for bacitracin (adapted from EC, 2009)

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs[a] [µg/kg}	Target Tissue	Other provisions
Bacitracin	Sum of Bacitracin A, B and C	Rabbit	150	Muscle	n.a.
			150	Fat	
			150	Liver	
			150	Kidney	
		Bovine	100	Milk	n.a.
	n.a.	Bovine	n.a.	For all tissues except milk	For intramammary use in lactating cows only

[a] Maximum residue limits

Table 1-2. EU antibiotic maximum residue limits for colistin (adapted from EC, 2009)

Pharmacologically active substance(s)	Marker residue	Animal species	MRLs [a] [µg/kg)	Target Tissue	Other provisions
Colistin	Colistin	All food producing species	150	Muscle [b]	n.a.
			150	Fat [c]	
			150	Liver	
			200	Kidney	
			50	Milk	
			300	Eggs	

[a] Maximum residue limits
[b] For fin fish this MRL relates to “muscle and skin in natural proportions”.
[c] For porcine and poultry species this MRL relates to “skin and fat in natural proportions”.

Since bacitracin consists of mainly bacitracin A, B and C, the sum of these components is taken as the marker residue during analysis (EMEA, 2001). For colistin the EU mentions only colistin as marker residue in Table 1-2. However in

EMEA (2002) it is mentioned that colistin was measured from the sum of colistin A and B with a HPLC based analytical method with fluorescence detection. Further explanations regarding the composition for both analytes will follow in section under 3.1.1

1.5. Peptide antibacterial compounds

Since the discovery of antimicrobial agents in the course of the last century, many peptide antibiotics have been isolated and described. In general these antibiotics consist of relatively few amino acid residues (12 to 100), have a positive charge of at least +2 due to the presence of the amino acids arginine and lysine, and are both hydrophilic and hydrophobic, which results in an amphiphilic character (Hancock, 1997; Jenssen *et al.*, 2006). Due to their positive charge they are often referred to as cationic antibiotics in literature (Hancock, 1997; Jenssen *et al.*, 2006; Marr *et al.*, 2006).

Peptide antibiotics can be divided into two classes, nonribosomally synthesised peptides and ribosomally synthesised ones. The former ones including substances such as gramicidins, polymyxins and bacitracins, contain drastic modifications and are mainly synthesised by bacteria. The latter group of natural ribosomally synthesised peptides are produced by all species of life (ranging from single-celled microorganisms, over plants, insects, birds and fish to mammals including humans) as part of their first line of defence (Hancock and Chapple, 1999; Jenssen *et al.*, 2006). Here the focus lies on nonribosomally synthesised peptides, while describing the other group is not within the scope of this work and is reviewed elsewhere (Hancock and Chapple, 1999).

Peptide antibiotics are mainly produced by the *Bacillus* genus. These antibiotics are mostly polypeptides and active against gram-positive bacteria, with the exception of substances like polymyxin and colistin which show activity against gram-negative microorganisms. Only some of the produced substances are used as antibacterial agents; amongst others bacitracin, polymyxin and the strongly related colistin, as well as gramicidin (Katz and Demain, 1977).

Although being entitled as ‘peptides’, these antibiotics set themselves apart from proteins through several properties (Katz, 1971; Katz and Demain, 1977):

- With a molecular weight ranging from 270 to 4500 Da polypeptide antibiotics in general are smaller in size compared to proteins.
- Peptide antibiotic producing microorganisms normally produce a family of closely related substances rather than just a single entity. These different substances differ only to a small extent from each other, generally only by one or maximal a few amino acids residues, while the rest of the molecule remains the same.
- Although some peptide antibiotics consist only of amino acids, others are composed of both amino acids and other components like fatty acids, amines or amino sugars.
- Peptide antibiotics regularly contain amino acids which are not present as such in normal proteins, for example D-amino acids.
- Antibiotics may contain cyclic structures, resulting in no free α-amino or carboxyl group, as well as possess unusual linkages and arrangements of amino acids. Bacitracin as an example is composed of a thiazoline ring (resulting from the condensation of isoleucin and cystein), an amine bond between the epsilon amino group of lysine and the ß-carboxyl group of aspartic acid, as well as a cyclic heptapeptide.
- Hydrolysis by peptidases and proteases generally does not affect peptide antibiotics. Exceptions include polymyxin B, which is vulnerable to enzymatic attack by papain and ficin.

Although the formation of allergies (Wan *et al.*, 2006) and the transference of resistances (Gibson *et al.*, 2012) is mentioned by some authors, others (Li *et al.*, 2006, EMEA, 2001, EMEA 2002) state that it is relatively improbable that microorganisms form a resistance against antimicrobial peptides. If resistances arise these are formed rather slowly and only in few cases, despite the increasing use of polypeptide antibiotics in human and vetenary medicine.

In Europe the main polypeptide antibiotics used in vetenary medicine are bacitracin and colistin (Brabander *et al.*, 2009), which together with polymyxin B will be shortly described in the following.

1.5.1. Bacitracin

Bacitracins are highly polar peptide antibiotics which are synthesised by *Bacillus licheniformis* and *Bacillus subtilis*. They exhibit activity against gram-positive bacteria, while they only have low effect against gram-negative microorganisms. Their activity is based on the inhibition of protein synthesis and cell wall synthesis, as well as interference with membrane functions of bacteria (Sin *et al.*, 2003).
The biosynthesis of bacitracins is described in various publications (Bernlohr and Novelli, 1963, Ishihara *et al.*, 1968, Katz and Demain, 1977), but will not be discussed more in detail in this work.

Structure

Bacitracin was discovered in 1943 by Johnson and its name derives from the Bacillus strain isolated from the wounds of an American girl, named Margaret Tracey. Originally it was regarded as single-component substance. Later analysis indicated that it is a mixture of similar polypeptide components, all having the same parental structure and differing only by some amino acids (Sin *et al.*, 2003). This parental structure is made up of a cyclic heptapeptide together with a thiazoline ring containing branch (Govaerts *et al.*, 2003b), as shown in Figure 1-1. According to Oka *et al.* (1998) the bacitracin complex consists of over 20 compounds, some even report numbers of more than 50 (Sin *et al.*, 2003). The biologically most active components are bacitracin A, B_1 and B_2 and C, while bacitracin F is the oxidative degradation product of bacitracin A and is microbiologically inactive. Bacitracin F is formed by oxidative deamination and contains a ketothiazole rather than an amino-thiazoline moiety (Pavli and Kmetec, 2001). Structures of all bacitracins contained in commercial samples were determined by Govaerts *et al.* (2003b) by an ion trap mass spectrometry analysis and an overview is shown in Appendix A.

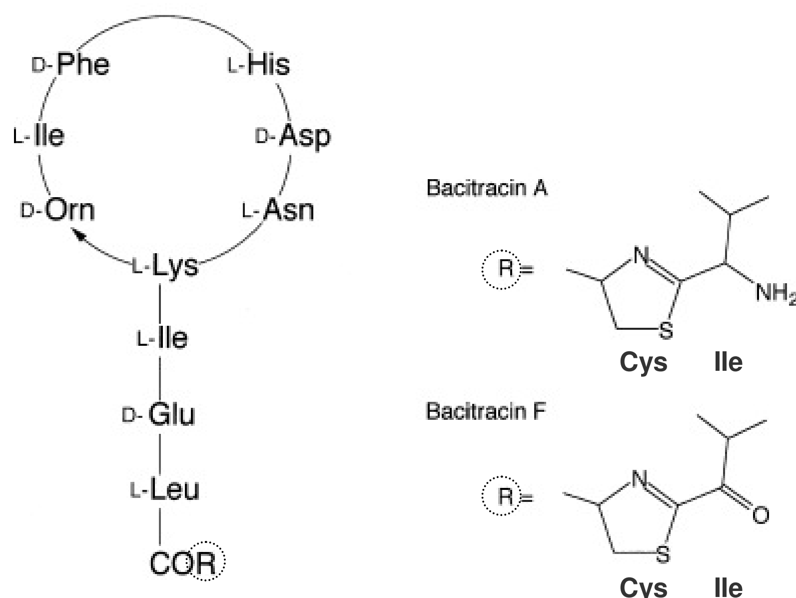

Figure 1-1. Structures of bacitracin A and F (adapted from Oka *et al.*, 1998)

According to EMEA (2002) bacitracin consist of mainly bacitracin A, B_1 and B_2, C and F in a proportion of 53%, 22%, 13%, 8% and 4% accordingly. It is not possible to identify one of these as the single marker residue in a detection method, as the ratio of these components varies depending on the producing bacterial species, as well as the employed fermentation conditions. Since bacitracin consists of mainly bacitracin A, B and C, the sum of these components is taken as the marker residue during analysis, as mentioned before in the legal requirement section under 1.4.

Afore mentioned antimicrobial activity of bacitracin (except bacitracin F) can be increased by the addition of divalent metal ions. These include Cu^{2+}>Ni^{2+}>Co^{2+}≈Zn^{2+}>Mn^{2+} in the shown order, to which most of the activity is associated (Lee *et al.*, 2011). It is thought that chelation between bacitracin and the metal ions involves the nitrogen of the thiazoline ring and the vicinal free amino-group of isoleucine. This chelation most probably stabilises the thiazoline ring and thereby inhibits the oxidative deamination to bacitracin F. On the contrary the activity of bacitracin can be inhibited by metal-chelating agents, such as EDTA (Stone and Strominger, 1971).

Applications

In its zinc form bacitracin has been used as feed additive as one of the most widely used antibiotics worldwide, due to its growth promoting effect (Capitán-Vallvey *et al.*, 2001). The use of zinc bacitracin in animal feed additives however was banned in the

EU in 1999, Council Regulation no. 2821/98 (EC, 1998), together with other antibiotics such as spiramycin, tylosin and virginiamycin. The driving force behind the ban were human health concerns, cross-resistance problems with antibiotics used in human medicine, as well as to counter the development of further antibiotic resistant bacteria strains, which can be transmitted to humans and detriment their health. Although this ban has positive effects on human health in regard to reduction of acquired infections from resistant strains, Casewell *et al.* (2003) indicate that the ban of growth-promoting antibiotics may also have adverse consequences; especially in the deterioration of animal health and thereby economic consequences for the farmers.

Feeding studies have shown that both bacitracin and its zinc complex are nearly not absorbed in the gastrointestinal tract after oral intake. It is metabolised to peptide and amino acids via its microbiologically inactive main metabolite desaminobacitracin (Frøyshow et al., 1986, EMEA, 2001).

In veterinary medicine bacitracin is applied intramammary to treat mastitis in lactating cows, generally in combination with other antibiotics like tetracycline and neomycin. Before 1998 bacitracin was also utilised in other animal species such as poultry, pigs, calves and lambs, but was then prohibited for this purpose. However an extension was given to the application of bacitracin to treat rabbits suffering from enzootic rabbit enterocolitis (EMEA, 2001).

In human medicine the zinc complex of bacitracin is used to topically treat infections, while in rabbits it is also given orally as zinc bacitracin in drinking water or feed.

1.5.2. Colistin (Polymyxin E)

Colistin is a peptide antibiotic produced by *Bacillus polymyxa* var. *colistinus* and is effective mainly against gram-negative bacteria. It belongs to the therapeutic class of polymyxins and is identical to polymyxin E. Its function relies on the destabilisation of the bacterial cell membrane, which leads to leakage of intracellular material, as well as the inhibition of the oxidative metabolism (EMEA, 2002). Colistin was discovered in the 1950s and first available for clinical use in the 1960s. Shortly after its introduction it was replaced by less toxic antibiotics, since it was thought to be neuro- and nephrotoxic. Its toxicity is still discussed (Li *et al.*, 2006), but in the 1990s with the increase of multiresistant gram-negative strains, such as *Pseudomonas aeruginosa*, it is starting to regain its importance.

Structure

Similarly to bacitracin, it is not a single-component compound, but consists of at least 30 polymyxins, whereby colistin A and B are the main ones (polymyxin E_1 and E_2 respectively). These two generally account for more than 85% by weight of total colistin. Both are constituted of a cyclic heptapeptide ring, which is attached to a tripeptide side chain and has a fatty acyl residue on the *N*-terminus (Orwa *et al.*, 2001a). The difference only lies within a terminal methyl group, while colistin A has a methyloctanoic acid, colistin B ends with a 6-methylheptanoic acid. This is illustrated in Figure 1-2. Furthermore colistin mixture also contains polymyxin E_3, E_1-I and E_1-7MOA as shown in Appendix A. In commercial colistin the composition is defined by the European Pharmacopoeia (Neagu *et al.*, 2011) as follows: the sum of polymyxins E_1, E_2, E_3, E_1-I and E_1-7MOA is at least 77.0%, while polymyxin E_1-I, E_1-7MOA and E_3 are each present at maximally 10.0 %. This is in agreement with the standard used in this work, as later shown in Table 2-1.

In this work the names colistin A and B will be used instead of polymyxin E_1 and E_2 to avoid confusion with polymyxin B_1 and B_2.

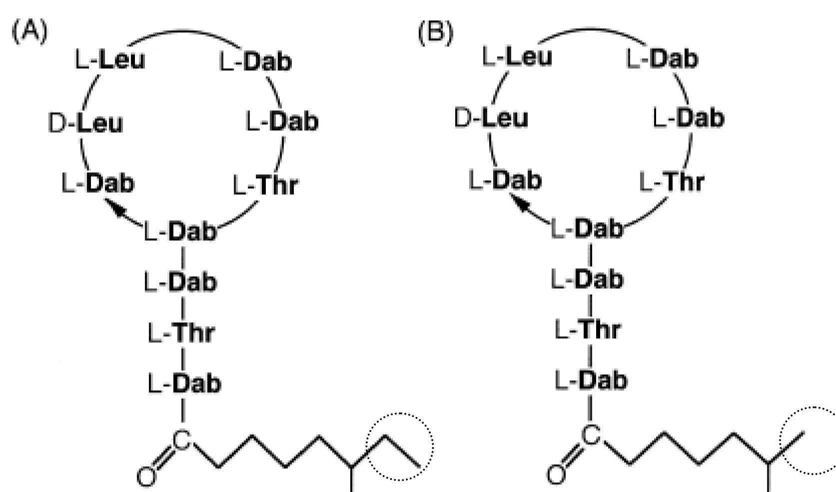

Figure 1-2. Structures of (A) Colistin A and (B) Colistin B (adapted from Oka *et al.*, 1998)

Application

Commercially colistin is available either as methanesulfonate or sulphate salt. While colistin methanesulfonate is mainly used in human medicine as parenteral administration, colistin sulphate is more applied in vetenary medicine in oral preparations. Here it used both for the prevention and treatment of diseases in mainly rabbits, pigs, poultry, cattle, sheep and goats. Colistin is used for poultry producing eggs, as well as milk producing animals (cattle, sheep, goats), whereby both

products are intended for human consumption. Its administration generally takes place orally in form of feed and drinking water, while parenteral and intramammary use is also possible. Absorption after oral administration is relatively poor. This could be one of the causes why is thought to be improbable that colistin assists in the formation of resistant strains (EMEA, 2002). Colistin was also used as part of gas mixtures to treat chick before housing, while this treatment has been banned in the mean time.

1.5.3. Polymyxin B

Polymyxin B is structurally very similar to colistin, as it also part of the polymyxin antibiotic family. The difference mainly lies within the cyclic ring, where a D-phenylalanine is replaced by D-leucin in colistin. Polymyxin B is again a mixture of closely related components, produced by *Bacillus polymyxa* and is active against gram-negative bacteria (Orwa *et al.*, 2001b). It has the same mechanism of action as colistin. Figure 1-3 shows the main components polymyxin B_1 and B_2, while the other standard components which differ from each other only in the fatty acyl moiety are listed in Appendix A.

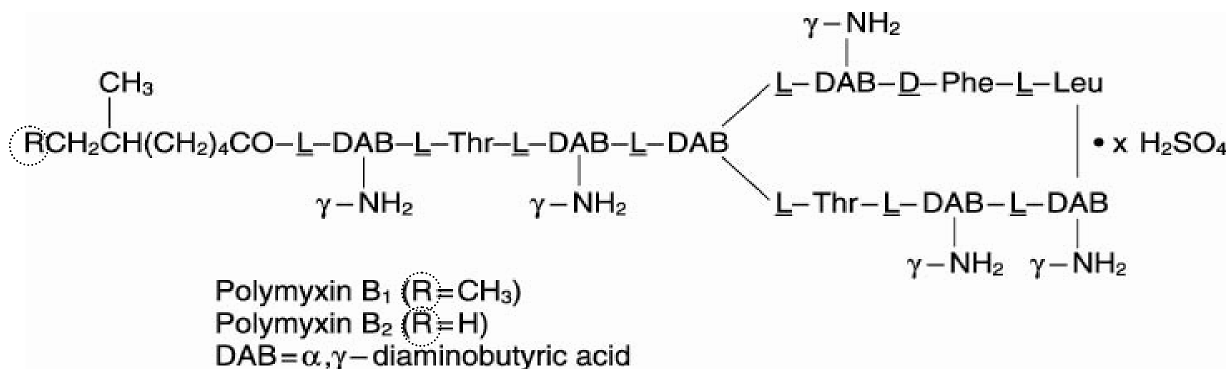

Figure 1-3. Structures of Polymyxin B_1 and B_2 (adapted from Drugs.com[1])

The biosynthesis of both colistin and polymyxin is described by Katz and Demain (1977), but will not be further elaborated in this work.

It is applied both in human and vetenary medicine, mainly against gram-negative bacteria and in the treatment of superficial bacterial infections.

[1] http://www.drugs.com/pro/pediotic.html (accessed on 04.01.2013)

1.6. Analytics of polypeptide antibiotics – state of the art

In literature a few methods have been published for the detection of polypeptide antibiotics. However most of the methods either focus on the detection of colistin in food (Decolin *et al.*, 1997, Stappen, 2010, Suhren *et al.*, 2005, Xu *et al.*, 2012,), as well as plasma samples (Dotsikas *et al.*, 2011, Gobin *et al.*, 2010, Li *et al.*, 2001, Ma *et al.*, 2008) or rather on the detection of bacitracin in food (Lee *et al.*, 2011, Turnipseed *et al.*, 2008) or feed samples (Boscher *et al.*, 2010, van Poucke *et al.*, 2003). Only Wan *et al.* (2006) and Sin *et al.* (2005) analyse both analytes in a combined methods. Since polymyxin B is not regulated yet, there are only few studies where it is analysed. Cao *et al.* (2008) used HPLC with fluorescence detection, while Cheng *et al.* (2010) developed a LC-MS/MS method to detect polymyxin, both studies however analysed plasma samples. In studies concerned with the analysis of food or feed samples to the best knowledge of the author polymyxin B has not been included as an analyte yet, however was used as an internal standard in several methods (Dotsikas *et al.*, 2011, Gobin *et al.*, 2010, Ma *et al.*, 2008, Sin *et al.*, 2005, Wan *et al.*, 2006).

Before presenting the present state of the art when it comes to polypeptide antibiotic analysis, the general problematic of the standard composition will be described.

Reference standards

Polypeptide antibiotics are natural fermentation products and as already mentioned complex mixtures of structurally similar compounds. Therefore pure standards of single components are not always commercially available and their purity and stoichiometry has to be determined. These two values are needed in order to develop a new reliable analytical method and accurately quantify the residues in biological matrices. The purity of the substance is often specified on the certificate of analysis, while the exact stoichiometry is not provided. In literature so far mainly UV an MS/MS methods have been employed for the purpose of stoichiometry. Both detection methods however assume that the instrumental response factor for the single components in a mixture is the same. Since the difference between the components, e.g. an alkyl amino group in the case of colistin A and B or polymyxin A and B, is relatively low, the detector response can be regarded as similar for the compounds.

In literature the problematic of the calculation of analyte based on the reference standard composition is often described (Decolin *et al.*, 1997, Gmur *et al.*, 2003, Wan *et al.*, 2006). In the following for each analyte the found literature in regard to standard composition will be presented.

Bacitracin

Bacitracin consists of components A, B, C and traces of bacitracin F in a ratio of 53%, 35%, 8% and 4%. However this ratio depends upon the bacterial species and the correspondent fermentation conditions (EMEA, 2002).

Sin *et al.* (2005) measured the composition of both bacitracin and colistin by MS/MS detection. He found that the percentage of bacitracin is 81.2% A, 15.4% B and 3.4% C, while colistin was composed of 80.8% A and 19.2% B. Since bacitracin A and colistin A had the highest proportion, they were regarded as the representative compounds of the two antibiotics and were thus quantified in the analysis.

Colistin

Ma *et al.* (2008) used UV detection to analyse her colistin reference substance, which was indicated to have 87% of purity and was composed of 31.6% colistin A and 55.8% B.

Decolin *et al.* (1997) compared the composition of colistin obtained from different vendors. Purity ranged from 91.7% to 86.2%, while colistin A proportions went from 43.9% to 70.6% and colistin B from 47.8% down to 15.6%. Therefore he recommends quantifying colistin by taking the sum of the peaks of A and B.

Dotsikas *et al.* (2011) indicates that colistin consists of at least 30 compounds, with colistin A and B representing the main components and accounting together for >85% of colistin by weight. In their work the results of three different types of detectors for the determination of stoichiometry are compared, namely an ELSD (Evaporative Light Scattering Detector), UV and MS/MS detector. They conclude that all three detectors can be used for the analysis of complex stoichiometry. However in their opinion ELSD is the detector of choice due to its specificity. Results of both purity and ratio of components can be directly used, without that assumptions have to be made. In regard to purity determination, a MS/MS detector can not estimate the purity, while for a UV detector one can not assume that the instrumental response is comparable for analytes of interest and structurally unknown impurities.

The assumption by Wan *et al.* (2006) that colistin is composed exclusively of pure colistin A and B is false, purity values are rather in the range of 85-90% in commercial reference standards.

Gmur *et al.* (2003) indicated that he used pure polymyxin E_1 (colistin A) in his work. The source of his supplier could however not be located.

Stappen (2010) mentions the presence of a certified HPLC-standard for colistin, available from the European Directorate for the Quality of Medicines. The percentage of five components of this standard are stated, whereby the sum of these is 86.9%. The main components colistin A and B are present in 44.2% and 33.7% respectively, summing up together to 77.9%. However as all colistin standards, this one is available in its sulphate salt form.

Polymyxin B

Only Cheng *et al.* (2010) analysed the proportion of both polymyxin B_1 and B_2 in his reference standard and found a composition of 78% and 17% respectively. Furthermore his colistin was composed of 71% colistin A and 24% B.

Summing up it can be said that the proportion values for each analyte vary depending on its source. Moreover colistin and polymyxin are often available in form of their sulphate salt, whereby the ratio of salt and pure analyte is also often not exactly known. All of the found literature does not consider the percentage of sulphate present. Stappen (2010) mentions this problematic, but does not consider the sulphate salt in her work. In this project the proportion of sulphate was considered in order to calculate the active colistin ratio, as described more in detail in section 3.1.1.

Furthermore some studies have been made to try to identify the compounds and impurities in reference standards of both bacitracin (Govaerts *et al.*, 2003a), as well as colistin and polymyxin B (van den Bossche *et al.*, 2011).

In the following a short overview on the sample preparation procedure and analysis in the field of polypeptide antibiotics during the last decade is given.

1.6.1. Sample preparation

Polypeptide antibiotics are polar and relatively stable in acidic conditions. Therefore generally acid buffers, diluted acid solutions or polar organic solvents are employed to extract the analytes from the sample. This is based on liquid-liquid extraction principles Sin *et al.* (2003). In literature the use of methanol-water mixtures (Gibson *et al.*, 2012) together with HCl (Xu *et al.*, 2012) or HFA (Lee *et al.*, 2011) is reported, as well as ACN in combination with TCA (Sin *et al.*, 2005), but also methods with solely diluted HCl solution (Wan *et al.*, 2006).
An aliquot is taken after this extraction step and diluted with water to increase the aqueous proportion. In all cases this aliquot was then treated by a solid-phase-extraction and clean-up procedure, whereby Oasis HLB (Gibson *et al.*, 2012, Lee *et al.*, 2011, Sin *et al.*, 2005), Phenomenex Strata-X (Wan *et al.*, 2006) and ProElut^{TM} PLS columns (Xu *et al.*, 2012) were employed. All of these cartridges are based on a highly cross-linked polystyrene-divinylbenzene (PS-DVB) copolymer resin. SPE protocols employed were comparable, since they were all based on the same copolymer.
Clean-up was followed by a sample concentration step and dried analytes were filled up with solutions consisting of acidic (HFA or TCA) organic solvent – water mixtures (methanol or ACN).

1.6.2. Chromatography

Eluents for chromatography were normally composed of HFA or TFA in both water and ACN. Chromatographic columns were mainly reversed-phase C_{18} (Gobin *et al.*, 2010, Sin *et al.*, 2005, Wan *et al.*, 2006, Xu *et al.*, 2012) or C_8 (Dotsikas *et al.*, 2011, Stappen, 2010) ones. Gradient elution programs were mainly reported, although also studies an isocratic method was found (Ma *et al.*, 2008).

1.6.3. Detection

Initial methods for the qualitative and quantitative determination of both bacitracin and colistin include the use of microbiological methods. These however have the disadvantage that they lack specificity and short time of analysis (Bell, 1992). Further

developments include thin layer chromatographic, capillary electrophoretic, immunological and HPLC methods. When employing an UV detector the problem arises that polypeptide antibiotics have a very weak ultraviolet absorption and no native fluorescence (Li *et al.*, 2001). Therefore derivatisation methods were developed to be able to detect the analytes by fluorescence measurement. UV detection in general however lacks selectivity and sensitivity in complex matrices (Decolin *et al.*, 1997). During the last years tandem mass spectrometry measurements were increasingly used for analysis. Their ability to precisely detect low levels of analytes, while having a high selectivity most probably makes them the method of choice in the future monitoring in residue analysis of milk samples and animal tissues (Sin *et al.*, 2003).

1.7. Project overview

For the determination of polypeptide antibiotics the mass-spectrometric measurement has to be developed as a first step. For this purpose, the substances listed in Table 2-1 were measured with the MS/MS system while optimising ionisation conditions and formation of fragments. The successful detection of these analytes was followed by the establishment and later improvement of a liquid chromatographic separation. Therefore different chromatography columns and separation conditions were tested in order to obtain a sufficient, but also fast separation. This instrumental part was followed by the development of an appropriate sample preparation protocol. Both liquid/liquid-extraction alone and in combination with solid-phase-extraction and clean-up were tested, whereby the latter technique showed more promising results after initial tests and was therefore pursued. During sample preparation initial experiments were conducted using matrix-free analyte solutions, while further tests were carried out using spiked meat and milk samples. Finally the optimised overall method was validated for milk and meat based on the European Commission regulation 2002/657/EC.

CHAPTER II: MATERIAL AND METHODS

2.1. Material

Unless stated differently, eluents, solutions and dilutions were made using high purity deionised water (18.2 MΩ.cm) produced with a Synergy UV system (Merck Millipore Darmstadt, Germany), while normal deionised water was used during sample preparation for control samples.

2.1.1. Chemicals and Reagents

98-100% formic acid was purchased from Merck Millipore (Darmstadt, Germany), methanol, acetonitrile, ethylene glycol, 99.9% ethanol, sodium chloride and 37% concentrated hydrochloride acid from VWR BDH Prolabo (Darmstadt, Germany), while trichloroacetic acid was obtained from Sigma-Aldrich (Steinheim, Germany). Solvents used were of HPLC gradient grade.

Bacitracin A standard (mainly A, but mixture contains also bacitracin B, C and others) and Polymxin sulphate (mixture of mainly B_1 and B_2) were purchased from Sigma-Aldrich (Steinheim, Germany), while colistin sulphate (mixture of colistin A, B and others) was acquired from Dr. Ehrenstorfer (Augsburg, Germany). Internal standard bacitracin F was obtained from Toku-E (Bellingham, USA). An overview of the substances used in this work is shown in Table 2-1. More detailed explanation of the calculation of concentrations will follow in chapter 3.1.

Table 2-1. Substances used in this work

Substance (in mixture)	Purity (%)	Molecular formula	Molecular weight (Da)	CAS
Bacitracin A	74.6	$C_{66}H_{103}N_{17}O_{16}S$	1421.75	22601-59-8
Bacitracin B	n.a.	$C_{65}H_{101}N_{17}O_{16}S$	1407.73	1402-99-9
Bacitracin C	n.a.	$C_{64}H_{99}N_{17}O_{16}S$	1393.72	1403-00-5
Bacitracin F (IS)	99.06	$C_{66}H_{98}N_{16}O_{17}S$	1418.70	22601-63-4
Colistin A [a, b]	68.70	$C_{53}H_{100}N_{16}O_{13}$	1169.5	7722-44-3
Colistin B [a, b]	68.70	$C_{52}H_{98}N_{16}O_{13}$	1155.4	7239-48-7
Polymyxin B1 [b]	60-70	$C_{56}H_{98}N_{16}O_{13}$	1203.49	1405-20-5
Polymyxin B2 [b]	n.a.	$C_{55}H_{98}N_{16}O_{13}$	1189.47	34503-87-2

[a] The sum of polymyxins (E1, E2, E3, E1-I, E1-7MOA) was indicated with 78.3 %; while the mixture contains 8.3% of polymyxin E1-I, 0.0% polymyxin E1-7MOA and 1.3% polymyxin E3 which were deducted from the sum.
[b] available as sulphate salt.

Internal standard

The initial idea was to use polymyxin B as an internal standard as suggested by several studies in literature. However it was then decided to include polymyxin B in the list of target analytes. In literature single papers suggest the use of the macrolide antibiotic josamycin (Gibson *et al.*, 2012), the aminoglycoside antibiotic netilmicin (Li et al., 2001) or fibrinopeptide B (Cheng *et al.*, 2010) as internal standards. These substances however are structurally not closely related to the chemical species of interest in this project and thus might behave differently during sample preparation compared to the target analytes. Ideally the internal standard and the target analytes should be very similar, but not identical and effects of sample preparation on signal intensities should be comparable for both of them. Therefore the idea was to use a deuterated internal standard. However with the exception of deuterated bacitracin, deuterated polypeptide antibiotics are generally not produced. Due to the high prices and limited choice of suppliers for deuterated bacitracin, bacitracin F as biologically inactive compound of bacitracin was chosen instead. Since bacitracin F was obtained towards the end of the project, prior to validation, it is not included in initial experiments in chapter 3.

2.1.2. Biological samples

Animal muscle (poultry, bovine, porcine) samples and bovine milk samples were obtained from local supermarkets and in the case of meat, homogenised in domestic blenders and stored in several containers. All samples were stored at -20℃ prior to analysis and defrosted in small batches over night in the fridge at 4℃. Defrosted samples were stored in the fridge at 4℃ for several days, if sample preparation and analysis took place during consecutive days.

2.1.3. Apparatus

Sample preparation

Homogenisation of samples was carried out using an automated multi-sample homogeniser FASTH21 together with disposable homogenising vessels FOODcon7 (Omni International, Kennesaw, USA) in the case of solid samples and with a Multi Reax shaking device (Heidolph, Schwabach, Germany) in the case of liquid samples, which were filled into 30 ml polypropylene tubes. Centrifugation was carried out on a Heraeus Multifuge X1R centrifuge (Thermo Scientific, Waltham, USA). An analytical

balance Nr. 1702 004 was used for weighing of chemicals and reagents, while a laboratory balance Nr. LA620P was employed in the case of sample weighting, both from Sartorius (Göttingen, Germany). Eppendorf Research (Hamburg, Germany) micropipettes of 0.1–10,000 µl were used for volumetric analysis and a table vortex mixer MS3 basic (IKA, Staufen, Germany) was used for mixing. Organic solvents were evaporated under a gentle stream of nitrogen at 40℃ using a block heater SBH130S D/3 together with a sample concentration unit, both from Stuart (Staffordshire, UK).

Various SPE columns were tested in the course of the sample preparation. Oasis HLB (3 ml, 60 mg) from Waters (Eschborn, Germany), Strata-X (3 ml, 60 mg) from Phenomenex (Aschaffenburg, Germany) and Chromabond HR-X (3 ml, 60 mg) as well as Chromabond OH Diol (3 ml, 200 mg) both from Macherey-Nagel (Düren, Germany) were used for solid-phase extraction.

Liquid Chromatography

An Agilent Technologies 1200 series HPLC system (Waldbronn, Germany) consisting of an autosampler, a degasser, a two-channel binary pump, and a temperature control oven (set at 30℃) was employed. Chromatographic separation was performed on various HPLC columns which were tested in the course of the method development; analytical columns used are listed in Table 2-2 and were tested for optimal chromatographic performance. All analytical columns were connected to a 4.0 mm×3.0 mm C_8 guard column from Phenomenex (Aschaffenburg, Germany). A mixture of mobile phases, (A) 0.1% HFA in H_2O and (B) 0.1% HFA in ACN, was delivered at a flow rate of 0.25 ml/min for columns with an ID of 2 mm (0.5 ml/min for ID of 3 mm respectively) under a gradient elution program. The optimised gradient program shown in Table 2-3 was the developed in the course of this work. After its establishment it was used for all further experiments and the final validation. The gradient program starts with an initial mobile phase ratio of 95% A and 5% B and ends with an 8 minute equilibration at the same ratio in order to restore the initial conditions before the next injection.

Table 2-2. Chromatographic columns tested in this work

Producer	Name	Dimension	Particle size	Phase
Phenomenex (Aschaffenburg, Germany)	Luna	150 mm×2 mm	5 µm	C_8
	Luna	150 mm×2 mm	3 µm	C_{18}
	Luna	150 mm×3 mm	3 µm	C_{18}
	Aqua	150 mm×3 mm	5 µm	C_{18}
Thermo Scientific (Waltham, USA)	HyPurity	150 mm×3 mm	5 µm	C_{18}
Agilent (Waldbronn, Germany)	Zorbax Eclipse	150 mm×4.6 mm	5 µm	C_{18}

Table 2-3. Optimised HPLC gradient program

Time [min.]	Mobile phase A [%]	Mobile phase B [%]
0	95	5
1	95	5
5	50	50
5.5	5	95
6.5	5	95
7	95	5
15	95	5

Electrospray ionization mass spectrometry

For identification and detection the HPLC system was interfaced with an API 4000™ Q-Trap mass spectrometer (Applied Biosystems, Darmstadt, Germany) with an ESI turbo ion source operated at positive mode. High-purity nitrogen was used as the collision gas and cleaned air as the sheath and auxiliary gas for tandem mass spectrometry. The dwell time was set to 30 ms for each MRM transition during all experiments. During the tuning process analyte solutions were infused separately through a syringe pump together with an eluent stream from the HPLC into the mass spectrometer. For this purpose the syringe pump 11 plus from Harvard Apparatus (Holliston, USA) was employed together with a 1 ml Hamilton Gastight 1001 syringe (Bonaduz, Switzerland).

Instrumental settings, data acquisition and processing were managed via the internal software package (Analyst version 1.5.1), while various calculations and the final validation was done using Excel 2003 (Microsoft Office).

2.2. Methods

2.2.1. Preparation of solutions

Extraction solution

Methanol is mixed with water in a 1:1 (v/v) ratio, whereby 500 ml of methanol are mixed in a bottle with 500 ml of water.

Washing solution

Washing solution consists of 10% methanol in water. Therefore, 10 ml of methanol are filled into a 100 ml volumetric flask and made up to the mark with water.

Ethylene glycol solution

10 g of ethylene glycol are dissolved in 100 ml of ethanol, which results in a 10% (w/v) solution.

Elution solution

70 ml of methanol and 200 µl of formic acid are filled into a 100 ml volumetric flask and made up to the mark with water.

Injection solution

10 ml of methanol and 100 µl of formic acid are filled into a 100 ml volumetric flask and made up to the mark with water.

Eluent A

Eluent A is a 0.1% formic acid solution in water, whereby 1 ml of formic acid is made up to the mark with water in a 1 l volumetric flask.

Eluent B

Eluent B is a 0.1% formic acid solution in acetonitrile, whereby 1 ml of formic acid is made up to the mark with acetonitrile in a 1 l volumetric flask.

Both eluents are sonicated for 15 minutes prior to use in order to be gas-free.

2.2.2. Preparation of analyte and spiking solutions

Stock solutions for each analyte consisted of approximately 1000 mg/l of bacitracin A, bacitracin F, colistin A and B and polymyxin B_1 and B_2 in H_2O/MeOH (80/20) and were stored at -20 °C. Working mixture solution (1 mg/l) for spiking was prepared by 1:1000 dilutions out of the four stock solutions in 0.1% HFA together in one volumetric flask. Bacitracin F as internal standard was diluted 1:2000 in 0.1% HFA, resulting in a 0.5 mg/l solution. Working and internal standard solutions were stored at 4 °C in the fridge.

Five point calibration curves in the range of 50-250 µg/l were constructed by appropriate dilutions with 0.1% HFA in a 10% MeOH solution (v/v). Spiking of the recovery solutions was done in a range of 25-1000 µg/kg, while the internal standard was added at 25-300 µg/kg.

2.2.3. Mass spectrometry

Tuning of the reference substances

Initial tuning of the reference substances on the mass spectrometer was performed in order to determine the dominant precursor ions of the substances. This was conducted with separate solutions of bacitracin, colistin and polymyxin in 0.1% FA with a concentration of 5 mg/l. The syringe pump was operated at a flow rate of 10 µl/min in combination with an eluent flow (50% A, 50% B) of 150 µl/min. The applied potential to the ESI, the desolvation temperature, the declustering (DP) and entrance potential (EP) were optimised in regard to the intensities of precursor ions. Each selected precursor ion was then further fragmented into characteristic mass fragments, namely its product ions. These were determined in multiple reaction monitoring (MRM). Optimal voltage values for collision energy (CE) and collision cell exit potential (CXP) were determined with respect to the relative intensity of the chosen product ion. Furthermore the ionisation conditions, such as voltage, temperature and gas flows, were optimised. Unit resolution was used during all experiments and dwell times of 30 ms during later analysis.

Results of the tuning process, together with transitions for each substance and the optimised voltage values are presented in Table 2-4, while Table 2-5 lists the optimal ionisation conditions used for all substances.

Table 2-4. Selected mass spectrometric parameters of the reference substances

Substance	Ion	Precursor	MRM ion	DP [V]	EP [V]	CE [V]	CXP [V]
Bacitracin A	$[M+3H]^{3+}$	475.3	86.1	70	12	60	10
			356.2*	70	12	26	10
			670.1*	70	12	20	10
	$[M+2H]^{2+}$	712.5	670.0*	110	12	32	10
Bacitracin B	$[M+3H]^{3+}$	470.3	662.6	70	12	20	10
			669.6	70	12	20	10
	$[M+2H]^{2+}$	705.0	669.5	100	12	34	10
Bacitracin C	$[M+3H]^{3+}$	465.4	227.0	70	12	31	10
			662.4	70	12	19	10
	$[M+2H]^{2+}$	697.9	662.5	140	11	32	10
Bacitracin F	$[M+2H]^{2+}$	710.6	309.2	100	11	43	10
			280.9	100	11	53	10
Colistin A	$[M+3H]^{3+}$	390.7	241.3*	70	13	20	10
			384.9*	70	13	17	11
			465.6	70	13	21	10
	$[M+2H]^{2+}$	585.5	535.5	130	12	27	10
Colistin B	$[M+3H]^{3+}$	386.1	101.0*	75	12	26	10
			374.5*	75	12	19	10
			380.2*	75	12	17	10
	$[M+2H]^{2+}$	578.6	569.8	115	12	25	10
Polymyxin B_1	$[M+3H]^{3+}$	402.1	101.2*	75	12	26	10
			390.5*	75	11	20	10
			396.1*	75	11	18	12
	$[M+2H]^{2+}$	602.6	241.5*	120	11	32	10
Polymyxin B_2	$[M+3H]^{3+}$	397.2	385.6	70	10	20	10
			391.8	70	10	18	10
	$[M+2H]^{2+}$	595.6	586.5	100	11	24	10

DP – Declustering Potential, EP – Entrance Potential, CE – Collision Energy, CXP – Cell Exit Potential
*Marked MRM transitions are also employed in methods found in literature.

Table 2-5. Optimal ionisation conditions

Parameter	Value
Curtain gas	20 psi
Collision gas	10 psi
Ion Spray Voltage	5500 V
Temperature	500°C
Ion Source Gas 1	35 psi
Ion Source Gas 2	45 psi

Determination of standard composition

As described in section 1.7.2 the exact stoichiometry of compounds in the available standards is not always indicated on the certificate of analysis. In order to estimate the proportion of the components in the mixture, the double and triple charged precursor ions were measured in a pseudo-MRM experiment (same ions were chosen as product ions). Hereby mean potentials (DP, EP, CE and CXP) were used for each group of substances, in order to obtain comparable results. The proportion was determined by the ratio of peak areas of one of the components to the total peak area of all components. Settings used for measurement are listed in Table 2-6.

Table 2-6. Mass spectrometric parameters for standard composition determination

Substance	DP [V]	EP [V]	CE [V]	CXP [V]
Bacitracin A 475.3 / 475.3	70	12	5	10
Bacitracin A 712.5 / 712.5	110	12	5	10
Bacitracin B 470.3 / 470.3	70	12	5	10
Bacitracin B 705.0 / 705.0	110	12	5	10
Bacitracin C 465.4 / 465.4	70	12	5	10
Bacitracin C 697.9 / 697.9	110	12	5	10
Colistin A 390.7 / 390.7	70	12	5	10
Colistin A 585.5 / 585.5	110	12	5	10
Colistin B 386.1 / 386.1	70	12	5	10
Colistin B 578.6 / 578.6	110	12	5	10
Polymyxin B1 402.1 / 402.1	70	12	5	10
Polymyxin B1 602.6 / 602.6	110	12	5	10
Polymyxin B2 397.2 / 397.2	70	12	5	10
Polymyxin B2 595.6 / 595.6	110	12	5	10

2.2.4. Liquid chromatography

During the method development the columns listed in Table 2-2 were tested. For this purpose the same gradient was run on these columns and based on retention time, signal-to-noise ratio and peak shape the optimal column was chosen. On this column different chromatographic conditions and gradients were then checked in order to optimise the chromatogram in regard to peak-to-peak baseline separation, as well as sufficient partition of analyte and interfering matrix peaks. This included:

- column temperatures of 20°C, 30°C and 40°C,
- initial isocratic gradient for 1 minute at different proportions of organic ACN eluent (70%, 80%, 90%),
- slope of the gradient program by varying the percentage of organic solvent (50%, 60%, 70%, 80% and 90%) during the gradient program.

Furthermore initial experiments were conducted to determine the influence of the injection solution on chromatographic separation. For this purpose analytes were injected at a concentration of 50 µg/l in different mixtures of HFA, H_2O, MeOH and ACN.

2.2.5. Sample preparation

Different sample preparation methods were adapted from literature. In the following the final optimised method is described under method 1. Method 2 was an initial approach in order to see if a solid phase extraction is necessary. Method 3 was compared to method 1 in regard to recovery and amount of matrix interferences. In the following chapter under section 3.2 the experimental modifications during method development will be described in more detail.

Method 1

Animal muscles samples were weighted in representative portions of 1 g into FOODcon7 vessels, while 2.5 g of milk samples were weighted into polypropylene tubes. Working solution in the range of 25-1000 µg/kg was added. After the addition of 10 ml of MeOH/H_2O (1:1) extraction solution the samples were homogenised for 45 seconds in the case of solid samples, while milk was shaken for 5 minutes. Then 0.5 ml of 6 M HCl was added and another homogenisation took place. The mixture was centrifuged at 4600 rpm for 5 minutes at room temperature. 5 ml of supernatant

were taken and mixed with 15 ml of H_2O prior to clean-up and solid phase extraction. SPE columns were first conditioned with 3 ml MeOH and then 3 ml H_2O. The whole 20 ml water sample mixture was then loaded on to the column, using a 25 ml column-attachment. This was followed by a washing step using 3 ml 10% MeOH solution. Elution took place with 2 ml 0.2/70/30 HFA/MeOH/H_2O. 500 µl of 10% ethylene glycol in ethanol was added as a keeper solution. Samples were concentrated through organic solvent evaporation using a gentle stream of nitrogen at 40℃ and a block heater. The remaining leftover of the sample was filled up to 1 ml of final volume with 950 µl of 0.1/10/90 HFA/MeOH/H_2O. These extracts were then filled into vials for LC/MS/MS analysis.

Method 2

A second process employing a different organic solvent and acid was tested. 2.5 g of sample was weighted into a tube, mixed with 5 ml 4% TCA in ACN and homogenised for 45 seconds. After the addition of 12.5 ml H_2O further homogenisation took place and 10 ml supernatant was taken. This was mixed with another 10 ml of H_2O before loading it on a pre-conditioned SPE column. Further steps of solid phase extraction and clean-up were just as described in method 1.

Method 3

A sample preparation and extraction method not employing SPE columns was tested. 2 g of sample were weighted, 5 ml of H_2O, as well as 10 ml ACN added. This was followed by the addition of 500 µl of working solution. Homogenisation for 45 seconds was followed by the addition of 2 g of NaCl. After a further homogenisation, 5 ml of supernatant were decanted into a tube for sample concentration. Organic solvent was evaporated to dryness using a gentle stream of nitrogen at 40 ℃. The evaporated samples were finally made up to a final volume of 1 ml with 0.1/10/90 HFA/MeOH/H_2O. These extracts were finally filled into vials for LC/MS/MS analysis.

Solid-phase extraction cartridges

Experiments to find the optimal SPE cartridge, from the ones listed in 2.1.3, were conducted based on the solid phase extraction steps described in method 1. For this purpose 10 ml of 0.1% HFA solution spiked with 10 µg/l were loaded onto the preconditioned columns (3 ml MeOH and 3 ml H_2O). Then the cartridges were

washed with 3 ml of 5% MeOH solution and the analytes eluted with 2 ml 0.2/70/30 HFA/MeOH/H_2O. 500 µl of 10% ethylene glycol in ethanol were added and samples were concentrated using a gentle stream of nitrogen at 40℃. To the remaining leftover 950 µl of 0.1/10/90 HFA/MeOH/H_2O were added.

2.2.6. Calculations used for results

Results shown in chapter 3 were calculated in various ways. Initially only peak areas as well as their ratios were used to evaluate results. In order to obtain more comparable results, during later experiments a set of standards was measured along with the prepared samples and results in µg/l were obtained though external calibration.

Spiking levels in µg/kg

Spiking levels used in this work are expressed in µg analytes per kg of sample. These were calculated using Equation 2-4, whereby c is the sample weight dependent concentration, x represents the measured concentration in µg/l (obtained through external calibration), m is the sample weight in g and V the final volume in the analysed vial.

Equation 2-1. Calculation of µg/kg values

$$c = \frac{x}{m} \times V$$

Apparent process recovery (uncorrected)

Apparent process recovery values presented in this work were calculated according to Equation 2-2. Here the ratio between c_{act}, which represents the actual concentration in µg/l obtained through external calibration of the measured fortified sample, and c_{exp}, which corresponds to the maximal possible recovery, is calculated. For this purpose the expected concentration is calculated as shown in Equation 2-3. It includes the concentration and volume of initially added spiking solution (c_{spike} and V_{spike} respectively) and the factor f which considers the dilution and concentration steps throughout the sample preparation. Therefore the volume of aliquot $V_{aliquot}$ loaded onto the solid phase extraction cartridges is divided by the total volume at the beginning of the sample preparation V_{total} and the final volume in the vial V_{final}.

Equation 2-2. Calculation of recovery

$$Recovery(\%) = 100 \times c_{act} / c_{\exp}$$

Equation 2-3. Calculation of the concentration of maximal expected recovery

$$c_{\exp} = \frac{1000 \times c_{spike} \times V_{spike}}{f} \; ; \; f = \frac{V_{aliquot}}{V_{total} \times V_{final}}$$

Furthermore standard addition was done in order to evaluate if the analytical signal is enhanced or suppressed by the presence of matrix compounds, also known as matrix effects. For this purpose analyte-free samples were processed according to the described method and a defined volume of working standard was added to the samples prior to sample reconstitution for LC-MS/MS analysis. Generally 100 µl of working standard were added to 900 µl of sample, resulting in an expected concentration of 100 µg/l. This, together with the actual measured concentration was used to estimate the matrix effects through Equation 2-2.

Moreover to evaluate the extraction efficiency of the process, analyte-free samples were extracted and prior to loading the sample on the SPE column, they were spiked with a defined volume of working standard to yield an expected concentration. Again the recovery, for the process without the extraction, could be calculated according to Equation 2-3. From this the extraction efficiency could be deducted.

2.2.7. Method validation

The method was validated by an in-house protocol based on the European Commission decision 2002/657/EC. During validation analyte stability in solution and matrix, specificity, selectivity, instrument and method linearity, repeatability, within-laboratory reproducibility, trueness, LOD and LOQ, as well as decision limit (CCα) and detection capability (CCβ) were determined.

Values obtained during method validation, where indicated, were corrected by both recovery, as well as a combination of recovery and internal standard. Both corrections were done since initially it was not yet clear if the internal standard was suitable for the proposed method in this work.

Stability

Stability of analytes in solution

Stability of analytes in solution was tested at a concentration of 100 µg/l in 0.1/10/90 HFA/MeOH/H_2O. The used composition was the same as the final analysis solution employed for standard and working solutions. Sufficient aliquots of the selected concentration were prepared from diluting a fresh stock solution. Vials with 1 ml of solution were labelled and stored according to the scheme in Table 2-7. The analyte concentration was measured directly after preparation and then on a regular basis after 1, 3, 7, 14, 21, 30 and 42 days.

Table 2-7. Scheme for the determination of stability of analytes in solution

Storage temperature	20℃ (RT)	15℃ (auto sampler)	4℃ (fridge)	-20℃ (freezer)
Brown vials	7	7	7	7
Transparent vials	7	-	-	-

The remaining concentration of the analytes was calculated according to Equation 2-4, whereby A_t represents the area measured at the correspondent time point and A_f the area of the solutions stored in the freezer at -20℃.

Equation 2-4. Stability of analyte in solution

$$Anaylte\ remaining\ (\%) = A_t \times 100 / A_f$$

Stability of analytes in matrix

Similarly the stability of analytes in matrix was assessed. For this purpose 900 µl of blank matrix sample (both milk and poultry) were fortified with 100 µl of working standard (resulting in a concentration of 100 µg/l). Half of this sample was stored in a vial in the fridge at the 4℃ and the other half in the freezer at -20℃. Measurements took place immediately after preparation, followed by analysis after 3 and 8 days. The percentage of remaining analyte was also calculated with the help of Equation 2-4.

Specificity and Selectivity

The specificity and selectivity of the method is based on the measurement of one precursor ion and two to three daughter ions. The 2002/657/EC indicates that for a quantitative method for the analysis of substances, regulated in Annex 1 96/23/EC

group B, 3 identification points have to be recorded for methods not recording a full-ion scan. Each precursor ion earns 1 identification point per ion, while a transition product obtains 1.5 points. Therefore in this work where 1 precursor and 2-3 daughter ions are used for quantification, identification points are 4 and 5.5 respectively and therefore in accordance with the performance criteria stated in the Commission decision.

Instrument linearity and repeatability

Instrument linearity was assessed through a five point calibration curve in the range of 50-250 µg/l, measured in duplicate. Coefficients of determination R^2 higher than 0.995 together with a relative standard deviation for the method lower than 4.1% were regarded as good. Instrument repeatability was determined by a 7-fold measurement of a 100 µg/l calibration standard. As a rule of thumb the coefficient of variation should be below 10% for LC-MS/MS methods. Furthermore blank values were established by 3-fold measurement of injection solution.

Method linearity

Both bovine milk samples as well as poultry muscle samples were treated and stored as described in 2.1.2. The validation of the method is done according to the sample preparation described in section 2.2.4 method 1. For milk validation 1 ml of HCl was used during sample preparation instead of the mentioned 0.5 ml.

Samples were processed in duplicate on three different days (4 in the case of milk). Each of the sample series included a non-spiked matrix blank, as well as four spiking levels in the case of meat and five in the case of milk. Spiking was generally performed at equidistant concentrations as shown in Table 2-8. Exact fortification levels for each analyte (due to different proportions in the reference standard) are listed later on in Table 3-10 and Table 3-11 in section 3.3.

Table 2-8. Spiking levels used for validation

	Spiking level [µg/kg]	Volume spiking solution [µl] – 1000 µg/l
Meat (1 g of sample)	0, 75, 150, 225, 300	0, 75, 150, 225, 300
Milk (2.5 g of sample)	0, 25, 50, 75, 100, 150	0, 63, 125, 188, 250, 375

Calculation of µg/kg values

Spiking values for the validation were calculated as described above according to Equation 2-1. During the validation recovery-corrected as well as recovery and internal standard corrected values were calculated. In order to obtain non-idealised values for the recovery-correction, an average of all recoveries of spiked samples of the respective day's sample preparation was calculated and then the single values were corrected by this mean recovery value. These mean recovery values were also used for the combined recovery and internal standard correction.

Corrected recovery values

Corrected recovery values were calculated according to Equation 2-2, with the difference that in this case c_{act} represents the spiking values in µg/kg (corrected by mean-recovery or mean-recovery and internal standard) as just described, while c_{exp} is the spiking level employed as listed in Table 2-8.

Repeatability

In order to determine the repeatability of the method seven to eight samples, spiked with the same volumes, were processed on the same day under identical conditions (1 sample, 1 person, 1 instrument, same chemicals). The values used were corrected using the mean recovery, as well as mean recovery and internal standard. The repeatability is then calculated according to Equation 2-5, whereby s is the standard deviation of the parallel determinations and x is the average value.

For meat samples this was tested at a spiking level of 75 µg/kg and for milk samples at 50 µg/kg. The maximal acceptable coefficient of variation was calculated according to Horwitz (Kromidas, 1999).

Equation 2-5. Calculation of the coefficient of variation for repeatability / reproducibility

$$V_K(\%) = \frac{s}{x} \times 100$$

Within-laboratory reproducibility

Reproducibility was calculated out of six values, whereby the day of the sample preparation, the person and the chemicals, more precisely the amount of added hydrochloric acid, as well as the way of sample homogenisation were varied, in order to obtain a measure for the method robustness. The value was calculated according to Equation 2-5 and again the decision limit was calculated according to Horwitz (Kromidas, 1999).

Trueness of quantitative method

The corrected µg/kg values used for the repeatability of the method were also used to determine the trueness of the method. The recovery corrected and recovery and internal standard corrected values were expressed as percentage of the calculated expected spiked values.

LOD and LOQ

The limit of detection (LOD) was calculated through the method linearity, which was determined as described above. The value for the LOD was obtained as shown in Equation 2-6. It considers the slope of the linear regression *b* and the residual standard deviation s_y (standard deviation of the predicted and observed values), which together represent the method standard deviation s_{x0}. The limit of quantification (LOQ) was then calculated as 3 times the LOD (Leiterer, 2008).

$$LOD = 4 \times s_{x0} = 4 \times \frac{s_y}{b}$$

$$LOQ = 3 \times LOD$$

Equation 2-6. Calculation of LOD and LOQ

CCα and CCβ

The decision limit CCα is the amount of analyte at or above which the sample is regarded as positive, considering an error probability of 5% (α=0.05). In other words at CCα with a probability of 95% one has a true positive finding. The alpha-value is the probability for a false positive result.

The detection capability CCβ on the other hand is the smallest amount of the substance in a sample of interest which can be detected, identified and/or quantified with an error probability of β, in this case β = 5% (van Loco *et al.*, 2007). The beta-value is the probability of a false negative result.

CCα and CCβ were calculated according to Equation 2-7, whereby the allowed limits are taken into account. The calculation considers the *MRL* (maximum residue limit) and the standard deviation *s* of the multiple analysis of a spiked sample at a level around the MRL.

In the case of polymyxin B where no permitted limit has been established to date, a laboratory performance limit (LPL) in accordance to colistin of 50 µg/kg for milk and 150 µg/kg for meat was set.

$$CC\alpha = MRL + (1.64 \times s)$$
$$CC\beta = CC\alpha + (1.64 \times s)$$

Equation 2-7. Calculation of CCα and CCβ

CHAPTER III: RESULTS AND DISCUSSION

Before presenting the results of this work and discussing them, the overall strategy for the method development is presented shortly as follows:

Initially the mass spectrometric detection method was set-up and the reference substances were tuned on the mass spectrometer, in order to be able to precisely detect the analytes of interest with high intensities. This step was followed by the selection of a suitable column and the development of a chromatographic gradient program. The purpose of the optimisation of the gradient program was the establishment of sufficient, but also fast separation of the analytes. This ideally results in a chromatogram with separately eluting peaks and an acceptable peak shape. Developing a sample preparation protocol was the next step, first employing matrix-free solutions and then matrices of interest such as meat and milk. Different extraction solutions were tested, together with different pH values and acids as well as extraction times. Furthermore liquid-liquid extraction was compared to the use of solid-phase extraction and clean-up cartridges. The steps during solid-phase extraction, such as washing and elution, were further evaluated. The sample preparation protocol was optimised in regard to optimal recovery values, especially in regard to critical steps like analyte extraction, sample clean-up and finally sample concentration. In order to assess the performance of the developed analytical method and the reliability of the obtained results, the method was validated according to the criteria stated in 2002/657/EC.

The approach during the method development may appear to be in reverse order, since it starts from the last step in an analysis, namely the detection and ends with the sample preparation, normally the first step in a method. However this order is necessary to assure that the analytes after sample preparation can be properly analysed with the LC-MS/MS system.

3.1. LC-MS/MS method

3.1.1. Mass spectrometry

As mentioned the method development started with the tuning process of the reference substances on the mass spectrometer, as described in 2.2.3. Substances were measured in positive ESI-mode, while ESI-negative measurements were not conducted. In literature the former one is the method of choice and the ESI-negative mode yields weak or no signals at all and is not recommended by van Poucke *et al.* (2003). Only one paper by Jansson *et al.* (2009) employs ESI-negative for the measurement of colistin A and B in plasma samples.

Optimal ionisation conditions varied between the substances, due to their different chemical structures; while curtain and collision gas were set at the same value, optimal ion spray voltage was in the range of 5000-5500 V, optimal temperature varied between 500-550 °C, ion source gas 1 and 2 were optimal from 30-35 psi and 45-50 psi respectively. Hence the different conditions had to be compared in order to find an overall optimum. Since the intensity of colistin and polymyxin were about a factor 10 lower compared to the one of bacitracin, the ionisation conditions were adapted to the ones of the former mentioned compounds. The chosen ionisation settings are shown in Table 2-5.

Full Ion Scan

The initial full scan of the bacitracin reference standard is shown in Figure 3-1. While the formation of single-charge ions in the range of *m/z* 1393-1422 is hardly visible, double $[M+2H]^{2+}$ and triple-charge $[M+3H]^{3+}$ ions are rather formed. The peaks of bacitracin A, B and C, double and triple-charge, are each identified in the enlarged areas. In regard to relative intensity of both double and triple-charge ions, the reference standard contains mainly bacitracin A, followed by B and bacitracin C as a minor component. This is as expected, since the purchased standard was a purified bacitracin A standard (74.6% bacitracin A). More detailed analysis of the standard composition will follow in later on in this chapter.

The Figure also indicates that the triple-charge ions of bacitracin are roughly as double intense as the double-charge ones. The ratio of these two ions was analysed

in both standard and matrix containing solutions for all analytes over time in order to evaluate the constancy of this ratio. Attempts to direct the formation of ions into one of the two directions were not made. However the ratio between double and triple-charge ions over time in both matrix and non-matrix containing samples was analysed in order to evaluate if the formation process is stable. Results indicate that there is a certain variation between the measurements on different days and various types of samples. The ratio of double to triple-charge ions for bacitracin A in a standard solution varies between 0.05-0.11, while in the range of 0.05-0.22 when analysing different sample types. Similarly for the other analytes the ratio seems to be more constant in standard solutions compared to matrix containing samples. Fluctuations of the ratio between the different charged ions are visible. The constancy of the ratio however has to be further observed over longer time periods in order to be able to make a founded assumption.

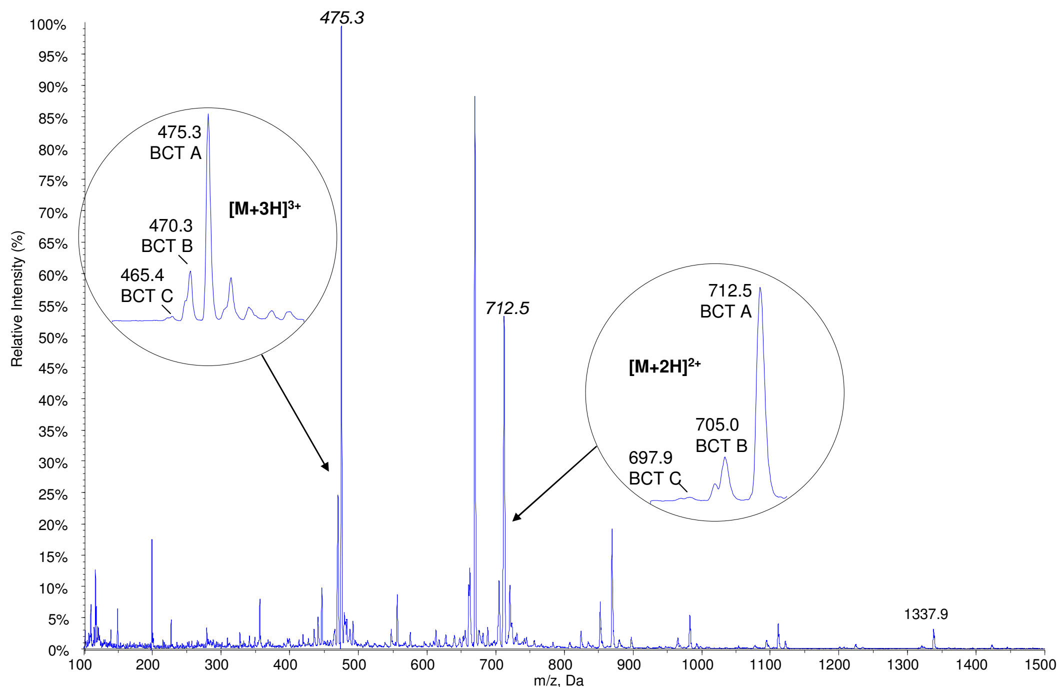

Figure 3-1. Full scan mass spectrum of bacitracin (5 mg/l) into the API4000 mass spectrometer; Ion clusters around m/z 475 showing the triple-charge ions $[M+3H]^{3+}$ and at around 712 the double-charge ions $[M+2H]^{2+}$ of bacitracin A, B and C, measured under positive electrospray ionisation conditions.

In Figure 3-2 the full scan of the later bought internal standard bacitracin F is represented. The scan shows singly, doubly and triply charged mother ions. In the case of bacitracin F the double charged ions are by far the most intense ones, followed by triple and single charged ones formed in low abundance. Therefore double charged ions where used in this case. Furthermore triple charged ions of bacitracin F have a calculated m/z 473.9, while the ones of bacitracin A are in relative vicinity with m/z 474.2. A mass resolution of around 1500 is needed to distinguish these two peaks. Hence the triple charged ions in the Figure could also be a bacitracin A impurity in the reference standard.

The Figure also shows several peaks in the lower *m/z* region, around 100 – 200. This could either indicate the presence of several single amino acids in the reference substance, as well as the formation of these in the injection chamber of the mass spectrometer.

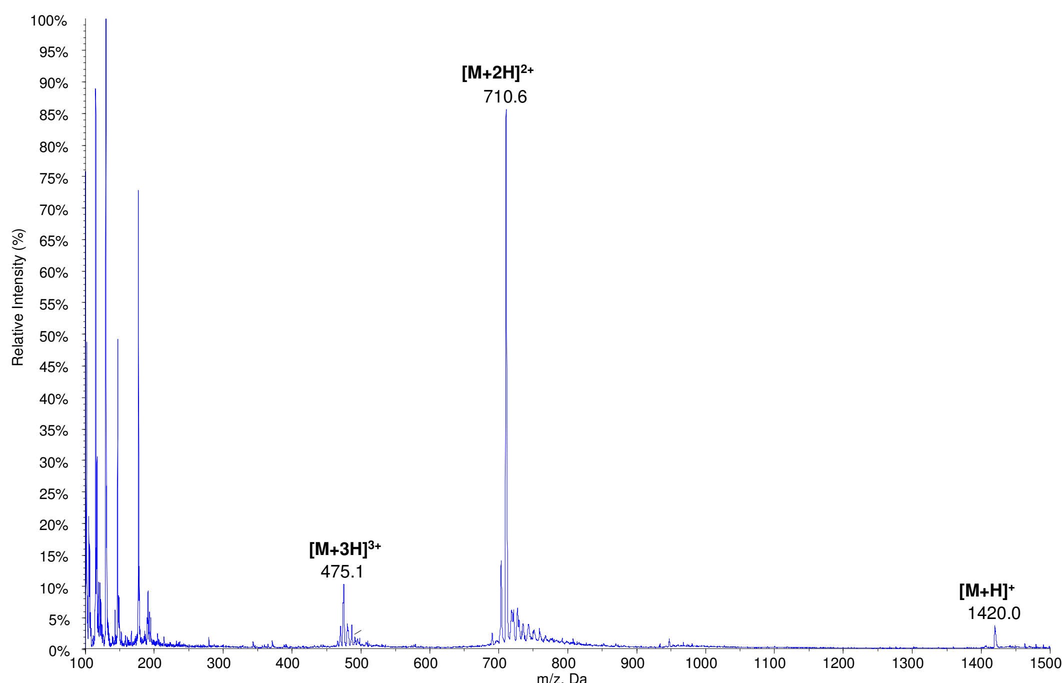

Figure 3-2. Full scan mass spectrum of bacitracin F (5 mg/l) into the API4000 mass spectrometer; Ion clusters at m/z 475 showing the triple-charge ions $[M+3H]^{3+}$, at 710 the double-charge ions $[M+2H]^{2+}$ and at 1420 the $[M+H]^{+}$ ion, measured under positive electrospray ionisation conditions.

Figure 3-3 represents the full ion scan of the colistin reference standard. Both double and triple molecular ions are visible, whereas single charged are not formed. Double and triple charged ions indicate that in regard to intensity more colistin A is present in the standard compared to B. Similarly as with bacitracin A, B and C, the triple charged ions are about a factor 2-3 more intense when compared to the double charged ones.

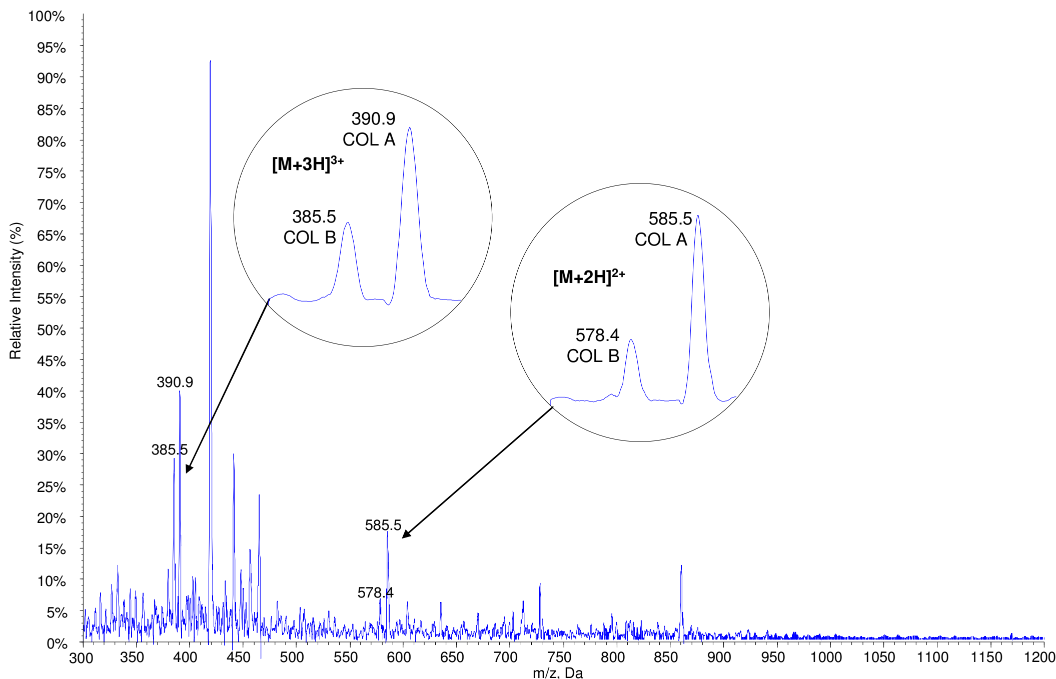

Figure 3-3. Full scan mass spectrum of colistin (5 mg/l) into the API4000 mass spectrometer; Ion clusters around m/z 390 showing the triple-charge ions $[M+3H]^{3+}$ and at around 585 the double-charge ions $[M+2H]^{2+}$ of colistin A and B, measured under positive electrospray ionisation conditions.

In Figure 3-4 the full ion scan of polymyxin B is shown, where again the double and triple charged molecular ions are visible, while the single charged one is not present. Polymyxin B1 and B2 can be seen in the enlarged areas, together with polymyxin B3, which was not analysed in this work. The information of the vendor that polymyxin B1 accounts for 60-70% of the total product is in accordance with the scan shown in the Figure. The intensity of polymyxin B1 is slightly higher compared to the one of B2. Again the intensity of triple charged ions is larger than the one of double charged ions, for polymyxin around a factor 3.

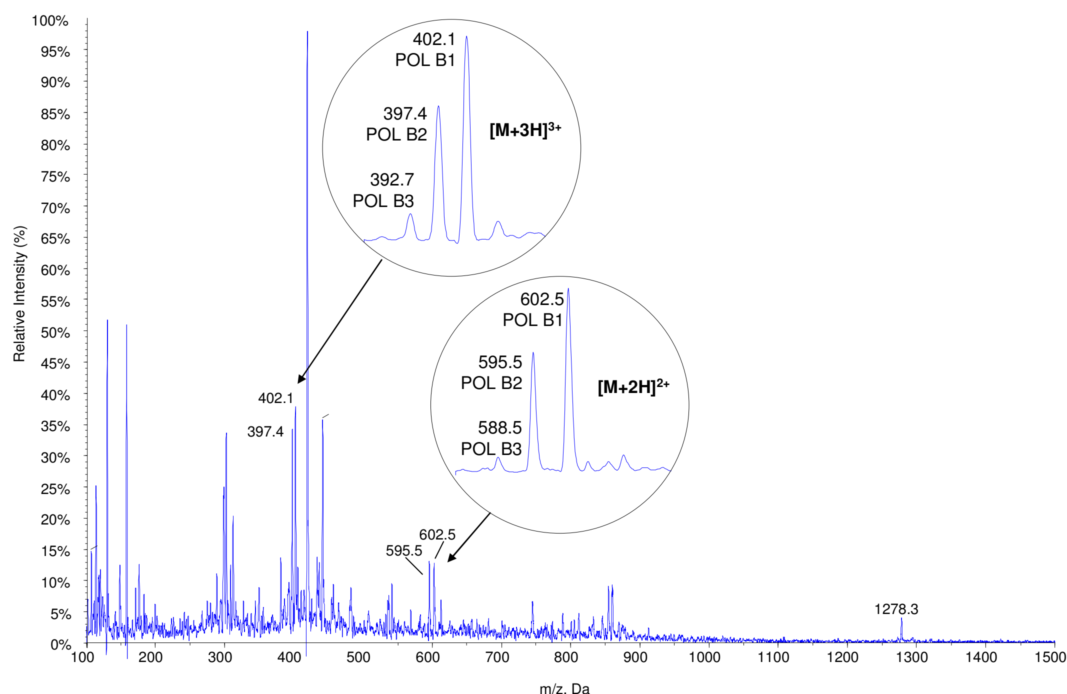

Figure 3-4. Full scan mass spectrum of polymyxin (5 mg/l) into the API4000 mass spectrometer; Ion clusters around m/z 402 showing the triple-charge ions $[M+3H]^{3+}$ and at around 602 the double-charge ions $[M+2H]^{2+}$ of polymyxin B1, B2 and B3, measured under positive electrospray ionisation conditions.

The formation of single, double and triple charged mother ions when analysing small proteins, such as polypeptide antibiotics, is discussed in literature. Wan *et al.* (2006) also found that double and triple-charge ions are the dominant species in their mass spectra, measured on a TSQ Quantum Discovery mass spectrometer. Ratios for doubly and triply charged ions were 1:3 for both bacitracin A and colistin B, as well as 1:2 for colistin A. Moreover van Poucke *et al.* (2003) confirmed the formation of triply charged ions on a Quattro LC mass spectrometer. Dotsikas *et al.* (2011) also concluded that the most intense signals in his work for colistin, employing an AB Sciex API 3000 mass spectrometer, were obtained from triple charge ions.

In contrast to the formation of triply charged ions that have been reported above, Ma *et al.* (2008) using the same API 3000 instrument reported that doubly charged ions of colistin are the dominant species in their analysis. This was also confirmed by Sin *et al.* (2005), who utilised an API 4000 instrument and analysed both bacitracin and colistin. Lee *et al.* (2011) also report that rather double-charge ions are formed in their experiments employing a Waters ZQ mass spectrometer. They state that under certain experimental conditions also triple-charge ions are formed, without going more into detail.

In conclusion it can be said that in literature the formation of both double and triple charged ions is reported and there is no evidence for a certain tendency. Experimental conditions, such as the type of interface and mass analyser used, as well as the composition of the used mobile phase and the injection solution could favour the formation of one ion over the other.

Product Ion Scan

After optimising the DP and EP values for the selected precursor ions, product ions were measured for each analyte, as listed in Table 2-4.

For overview purposes only product scans of the triple-charge precursor ions for each analyte will be shown and discussed. These were used for quantification in this work. For bacitracin F however the double-charge precursor ion was analysed, since it showed higher intensities than the triple-charge one. Alongside also double-charge ions were analysed and measured during all experiments to obtain information about the ratio as mentioned in the beginning of this chapter. The corresponding product scans of double-charge ions can be found in Appendix B.

In the initial phase of the project several product ions per precursor were measured during the tuning process and initial experiments. In the course of the method development some of these MRM transitions were found to be strongly influenced by matrix interferences, as well as resulted in peaks with low signal-to-noise ratio and the formation of double peaks. Consequently these were taken out of the measurement method and for reasons of clarity are not included in the following Figures. A list with all MRM transitions can be found in Appendix B.

The EU regulation 2002/657/EC instructs that quantification with LC-MS/MS should be based on the measurement of the two most intense product ions. The most intense ion (quantifier ion) is used for quantification, while the second most intense one (qualifier ion) is taken for confirmatory and identification purposes, by comparing the ratio of the peak areas of the two ions to each other. In this work for some analytes however a third ion was selected, since the qualifier ions were equally intense and a decision will be taken after evaluating further measurements in various matrices.

Figure 3-5, Figure 3-6 and Figure 3-7 show the product ion scan of bacitracin A, B and C accordingly. The spectra of the triple-charge precursor ions along with the product ions used for quantification are illustrated. The molecular structure, along with the mass losses during product ion formation were assigned based on Govaerts *et al.* work on sequence analysis of bacitracin A (2003b).

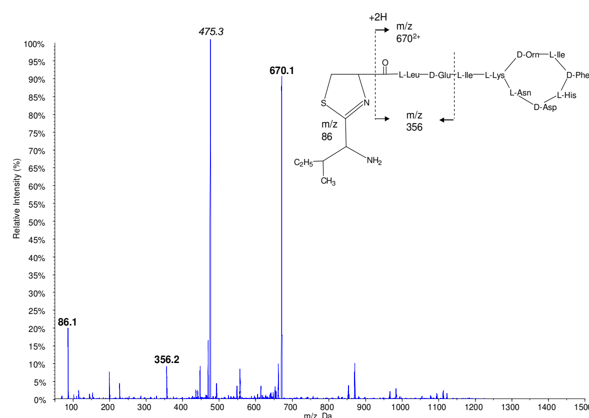

Figure 3-5. Product ion spectrum of bacitracin A; triple-charge precursor ion with m/z 475.3; product ions are 86.1, 356.2 and 670.1; mass losses and the corresponding structures are indicated through arrows.

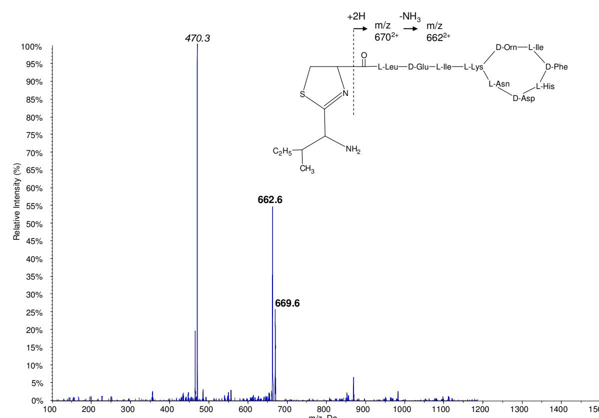

Figure 3-6. Product ion spectrum of bacitracin B; triple-charge precursor ion with m/z 470.3; product ions are 662.6 and 669.6; mass losses and the corresponding structures are indicated through arrows.

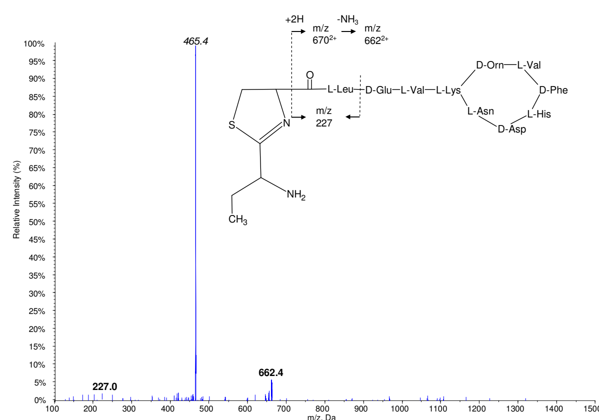

Figure 3-7. Product ion spectrum of bacitracin C; triple-charge precursor ion with m/z 465.4; product ions are 227.0 and 662.4; mass losses and the corresponding structures are indicated through arrows.

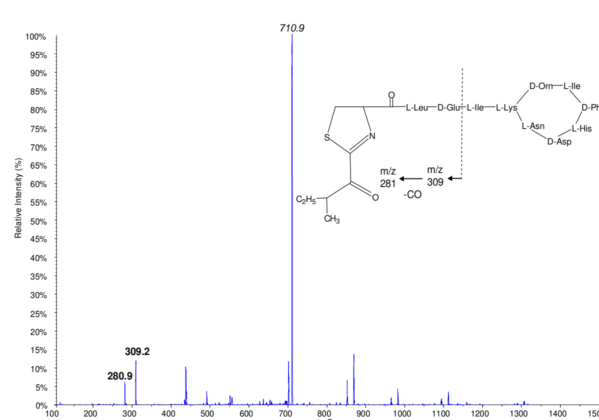

Figure 3-8. Product ion spectrum of bacitracin F; double-charge precursor ion with m/z 710.9; product ions are 280.9 and 309.2; mass losses and the corresponding structures are indicated through arrows.

Figure 3-8 shows the mass spectra of the product ion scan of bacitracin F for its double-charge precursor ion and the two resulting product ions. Information on the structural processes during mass loss was again taken from Govaerts *et al.* (2003b).

In literature to the best knowledge of the author there is no method describing the analysis of bacitracin F as an analyte, most probably due to the fact that it is a biologically inactive oxidation product and therefore analytically of minor relevance. However bacitracin F is mentioned in different papers when it comes to sequencing of bacitracin and its components (Ikai *et al.*, 1995, Siegel *et al.*, 1994, Morris *et al.*, 1994, Govaerts *et al.*, 2003b).

Figure 3-9 and Figure 3-10 illustrate the product ion spectrum of colistin A and B respectively. Molecular structures were adapted from Orwa *et al.* (2001) wherein the structural characterisation of colistin is described. For colistin A the formation of *m/z* 241 can be structurally explained as shown in the Figure and is mentioned in Sin *et al.* (2005) work. The fragment of *m/z* 385 is utilised by Wan *et al.* (2006) during their analysis and corresponds to the triple charged ion with the loss of a water molecule $[M+3H-H_2O]^{3+}$.

The product ions found for colistin B are also mentioned in literature; *m/z* 101 in the work of Dotsikas *et al.* (2011) and both 374 and 380 in the analytical method of Wan *et al.* (2006). The latter two fragments can be explained by the loss of water molecule(s) from the triply charged ions, $[M+3H-2H_2O]^{3+}$ and $[M+3H-H_2O]^{3+}$ respectively.

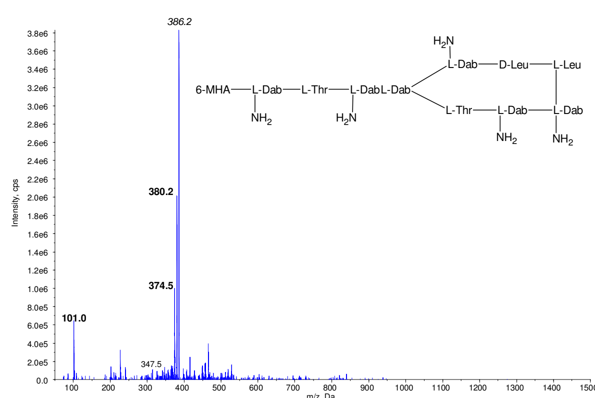

Figure 3-9. Product ion spectrum of colistin A; double-charge precursor ion with m/z 390.7; product ions are 241.3, 384.9 and 465.6; mass losses and the corresponding structures are indicated through arrows.

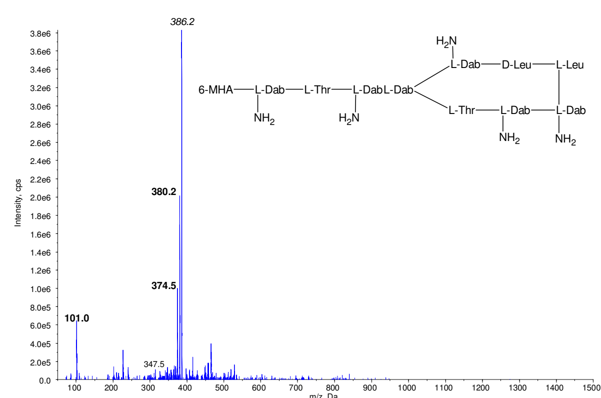

Figure 3-10. Product ion spectrum of colistin B; double-charge precursor ion with m/z 386.2; product ions are 101.0, 374.5 and 380.2.

Figure 3-11 and Figure 3-12 show the spectrum for the product ions of polymyxin B_1 and B_2 correspondingly. Structures were based on a study by Orwa *et al.* (2001 a),

which dealt with the structural analysis of polymyxins. All methods found in literature only focus on polymyxin B_1 as internal standard, disregarding B_2. Therefore product ions of polymyxin B_1 can be found in literature, namely *m/z* 101 in Dotsikas *et al.* (2011) and 390 as well as 396 in Wan *et al.* (2006). MRM transitions for triple charged polymyxin B_2 ions are not mentioned in literature, since it is not commonly analysed.

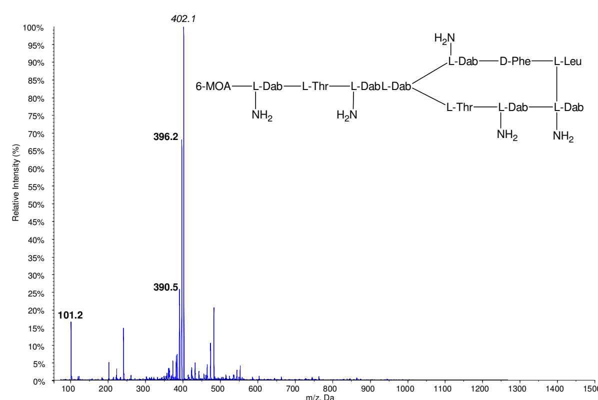

Figure 3-11. Product ion spectrum of polymyxin B1; double-charge precursor ion with m/z 402.1; product ions are 101.2, 390.5 and 396.2.

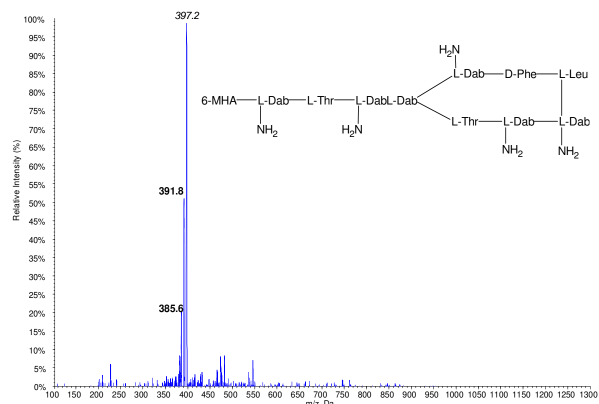

Figure 3-12. Product ion spectrum of polymyxin B2; double-charge precursor ion with m/z 390.7; product ions are 385.6 and 391.8.

Determination of standard composition

In the beginning of the project only the purity and the relevant proportion of the standards (see Table 2-1) was taken into account when calculating the analyte concentrations in solution. For colistin and polymyxin which were bought in the form of their sulphate salts, the amount of sulphate present was deducted in order to calculate the relevant proportion of the analyte in the entire molecule. For colistin A and B the sulphate proportion was not indicated on the certificate of analysis, since the value varies between batches. Upon request a value of 16.4% for the employed standard in this work was disclosed, which results in a relevant proportion of 83.6%. For polymyxin, which is available as disulphate salt, a relevant proportion of 83.4% was calculated.

The values indicated in the following “Liquid Chromatography” and “Sample preparation” chapter however do not consider the proportion of the single substances (bacitracin A, B, C; colistin A, B; polymyxin B_1, B_2) in the reference standard mixture. They are based on the wrong assumption that each analyte is contained in equal

proportion in the mixture. The determination of substance composition was not conducted at this point of the work.

For the validation however (see chapter 3.3) the different proportions as listed in Table 3-1 for triply charged ions were considered for concentration calculations. As described in section 2.2.3 intensity of single MRM transitions for each analyte, both double and triple charge, were analysed. From this measurement the proportions of the standard composition could be estimated. Furthermore the purity of polymyxin B_1 is between 60 and 70%. The initial assumption taken that the purity is 70%, seems to be wrong when looking at the Table and is rather around 60%. Therefore this was considered in the calculations during validation.

Table 3-1. Determination of substance composition

Substance	Proportion [%] (n=2)	Proportion used for calculation [%]
Bacitracin		
A	75 ± 1.5	75
B	20 ± 1.3	20
C	5 ± 0.2	5
Colistin		
A	74 ± 2.7	75
B	26 ± 2.7	25
Polymyxin		
B_1	58 ± 5.4	60
B_2	42 ± 5.4	40

As described in the introduction of this work, it is often suggested to employ HPLC-UV in order to determine the substance composition. An attempt to develop an isocratic gradient which was able to separate the single peaks in order to determine the substance composition with a UV detector was not successful. Most probably due to the fact that the concentration of the analytes used was not high enough for the UV detector, since polypeptide antibiotics exhibit relatively low UV absorbance (Decolin *et al.*, 1997). Furthermore the isocratic method had to be developed on the LC-MS/MS, because the LC-UV instrument was only available for limited time. Therefore eluents generally employed in UV spectrometry for the analysis of polypeptide antibiotics, such as phosphate buffers, could not be tested on the mass spectrometer since they are non-volatile and can clog the MS inlet capillary.

3.1.2. Liquid Chromatography

After the mass spectrometric method was established, a chromatographic elution program was developed. Analytical methods for the detection of polypeptide antibiotics in literature often report the use of either C_{18} or C_8 reversed phase columns as reviewed by Sin *et al.* (2003), while using a gradient elution program (Sin *et al.*, 2005, Wan *et al.*, 2006, Xu *et al.*, 2012). Therefore the selection was limited to the use of these two types of columns and gradient instead of isocratic elution programs.

Chromatograms of a 100 µg/l standard on a Luna C_8 column however did not show a chromatographic separation of the analytes. Therefore the use of C_8 columns was no longer pursued in this work. Instead various C_{18} columns were tested.

The initial HPLC gradient elution program was adapted from Wan *et al.* (2006) and is listed in Table 3-2. In the present work the length of the isocratic run at high organic mobile phase B was shortened from 4 min. to 1 min. and after that the initial conditions were restored within 0.5 minute instead of 4 minutes, compared to the version in the paper. This led to a longer equilibration time at the end of the gradient program. Experiments showed that an equilibration time of 3 minutes (as stated in the paper) led to retention time shifts in subsequent analysis. Therefore and based on past experience an equilibration time of 7-10 minutes is needed to restore initial conditions before the following injection.

Table 3-2. Initial HPLC gradient elution program (adapted from from Wan *et al.*, 2006)

Time [min.]	Mobile phase A [%]	Mobile phase B [%]
0	95	5
4	30	70
5 [a]	30	70
5.5 [b]	95	5
15	95	5

[a] originally 8 minutes [b] originally 12 minutes

Figure 3-13 shows the chromatogram of a 100 µg/l standard under the gradient elution program in Table 3-2. Since bacitracin F was not available at this point it is not shown. The chromatogram indicates that bacitracin (A, B, C) elutes at around 4.8

minutes and is baseline separated from colistin and polymyxin, which elute after 4.3-4.5 minutes. However the aim was to also achieve a baseline separation between the structural analogues of colistin and polymyxin. Furthermore the objective included that elution of the peaks does not occur too early in the chromatogram, as interfering matrix peaks may impair integration results.

In order to evaluate the chromatographic performance of different chromatographic columns these were tested as indicated in section 2.2.4.

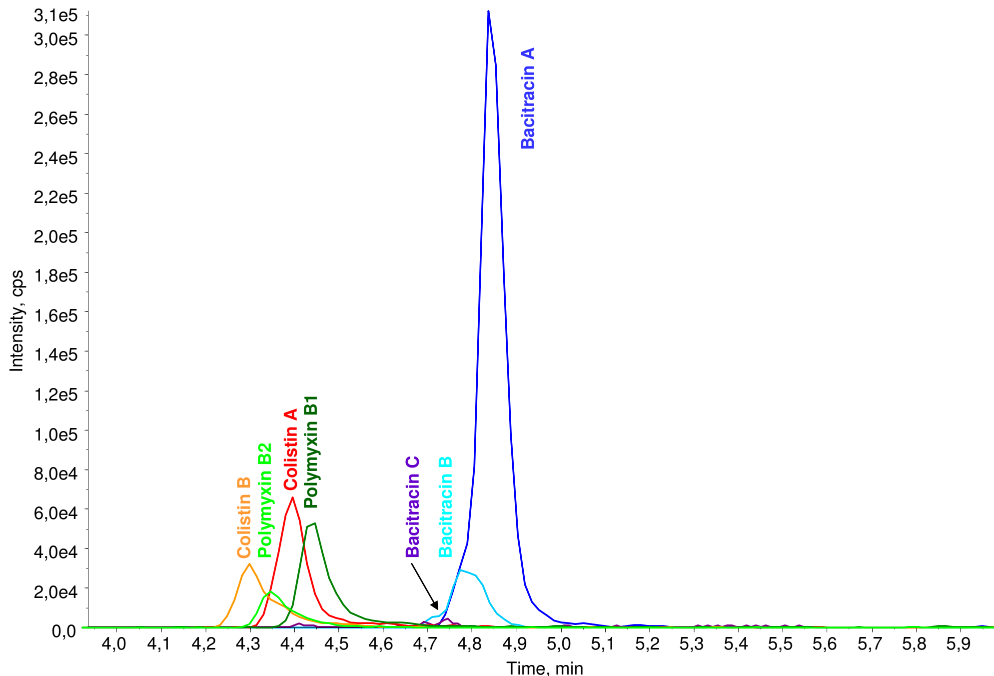

Figure 3-13. Chromatogram (relevant excerpt) of the analytes of interest (100 µg/l standard) on a Phenomenex Luna C_{18} 150x3, 3µ column following the initial gradient elution program. Peaks are based on the sum of triple-charged ion transitions.

Figure 3-14 combines four chromatograms, each of them showing a bacitracin A peak resulting from the measurement of a 100 µg/l standard on the indicated chromatographic column. The Figure shows that the retention time, the signal-to-noise ratio as well as the intensity of the peak varies according to the chosen column.

For the sake of clarity only bacitracin A is shown in the Figure. The other analytes however showed similar results on the various columns and their results were also incorporated in the decision-making process.

All of the peaks have a small shoulder prior to the main peak, which probably is substance specific. Retention times of the Luna, Aqua and HyPurity are around 7 minutes, while the bacitracin elutes on the Zorbax column at around 8.5 minutes. The

signal-to-noise ratio and the intensities of the bacitracin peak are the highest on the HyPurity and the Luna column.

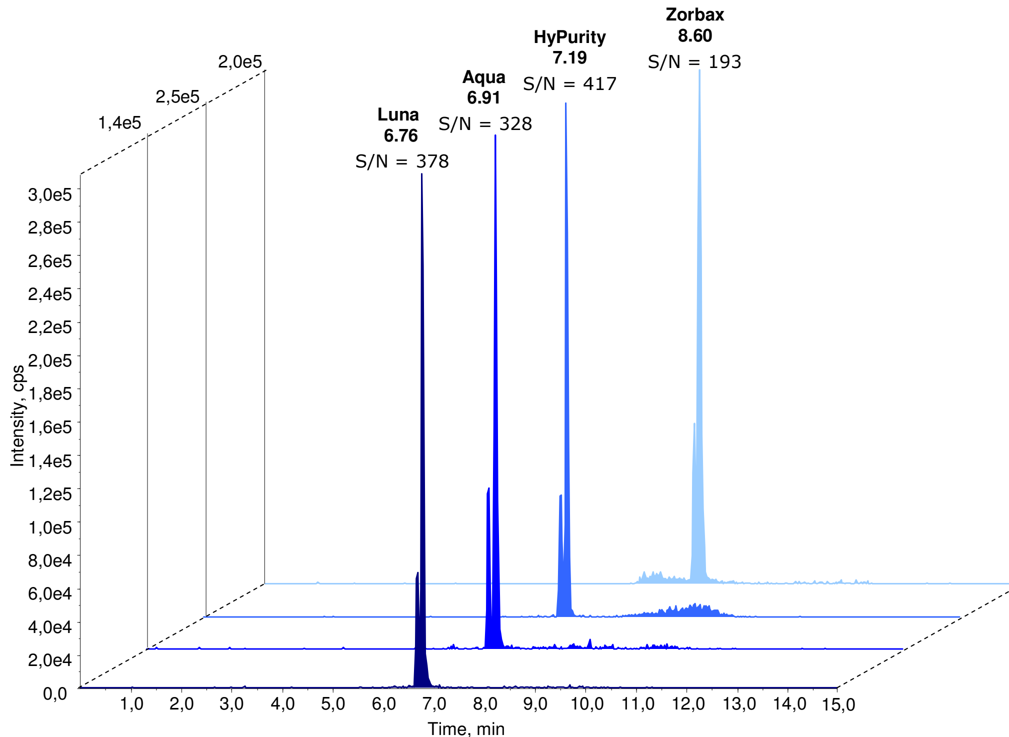

Figure 3-14. Chromatogram of bacitracin A (100 µg/l standard) on four different chromatographic columns: Luna – Phenomenex Luna C_{18} 150x3, 3µ; Aqua – Phenomenex Aqua C_{18} 150x3, 5µ; HyPurity – Thermo Scientific HyPurity C_{18} 150x3, 5µ; Zorbax – Agilent Zorbax Eclipse XDB-C_{18} 150x4.6, 5µ. Peaks are based on the sum of triple-charged ion transitions.

Figure 3-15 and Figure 3-16 show again the chromatograms of bacitracin A in a 100 µg/kg spiked milk and meat sample respectively, measured on the four columns of choice. The samples were prepared according to method 1 described in section 2.2.5. While there is no matrix peak visible for milk in Figure 3-15, spiked meat samples in Figure 3-16 indicate the presence of a relatively high matrix peak, which elutes right before bacitracin. For later integration ideally the analyte peak is spatially separated from the matrix peak in order to facilitate peak integration and calculation of results. In regard to signal-to-noise ratio and intensity the Luna and HyPurity column perform the best.

Furthermore it becomes clear that meat is a more demanding matrix compared to milk, when it comes to sample clean-up procedures and the presence of interfering matrix compounds, such as small proteins.

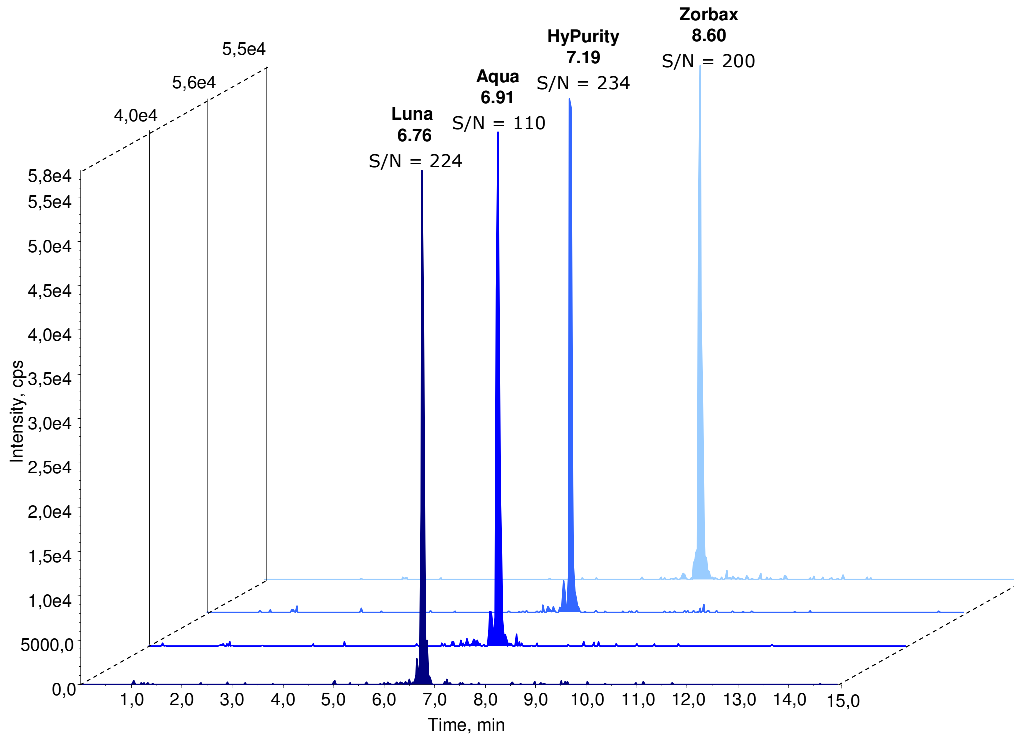

Figure 3-15. Chromatogram of bacitracin A (in a 100 µg/kg fortified milk sample) on four different chromatographic columns: Luna – Phenomenex Luna C_{18} 150x3, 3µ; Aqua – Phenomenex Aqua C_{18} 150x3, 5µ; HyPurity – Thermo Scientific HyPurity C_{18} 150x3, 5µ; Zorbax – Agilent Zorbax Eclipse XDB-C_{18} 150x4.6, 5µ. Peaks are based on the sum of triple-charged ion transitions.

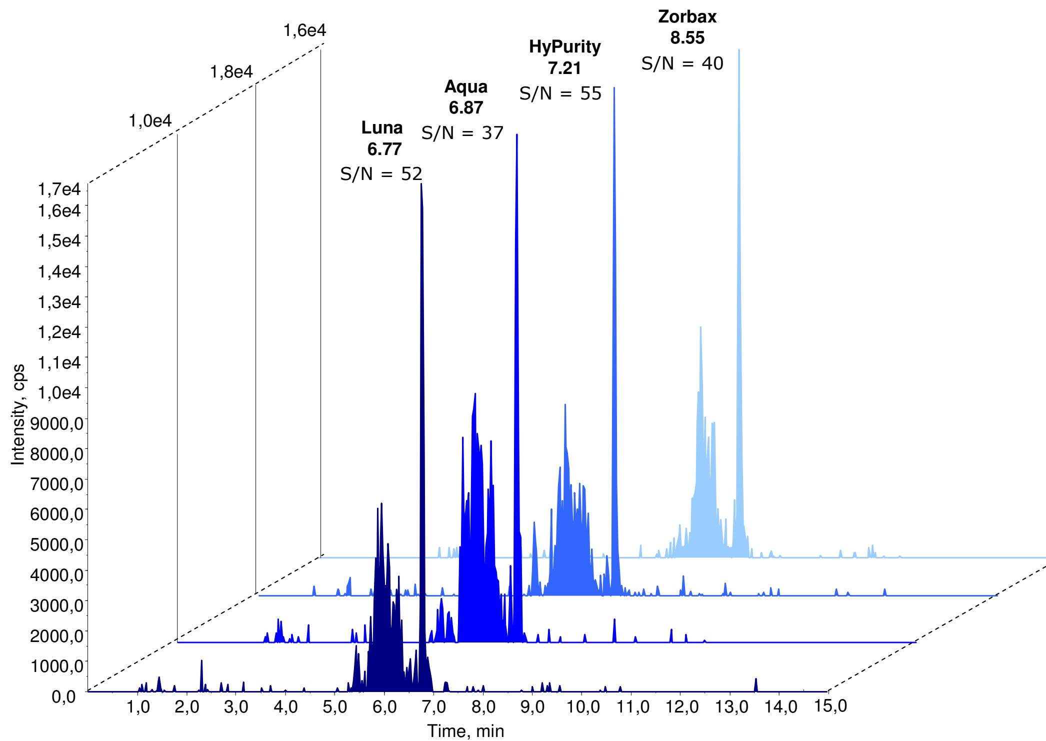

Figure 3-16. Chromatogram of bacitracin A (in a 100 µg/kg fortified meat sample) on four different chromatographic columns: Luna – Phenomenex Luna C_{18} 150x3, 3µ; Aqua – Phenomenex Aqua C_{18} 150x3, 5µ; HyPurity – Thermo Scientific HyPurity C_{18} 150x3, 5µ; Zorbax – Agilent Zorbax Eclipse XDB-C_{18} 150x4.6, 5µ. Peaks are based on the sum of triple-charged ion transitions.

Furthermore the criterion of selectivity and robustness of the method is proven by comparing Figure 3-14 with Figure 3-15 and Figure 3-16. Ideally the retention time of the analytes should be identical in the standard solutions and prepared samples. When looking at all four columns one can recognise that the retention times of bacitracin A in the standard solution and the milk sample is the same, while it slightly differs between standard and meat sample (0.1-0.6%). According to the 2002/657/EC regulation however differences of ± 2.5% are within the permitted tolerance range.

Consequent chromatographic experiments were conducted on a Thermo Scientific HyPurity C_{18} column. Although the Phenomenex Luna C_{18} column chromatographic performance was acceptable as well, high counter pressures arose during measurements after several runs. Employing an older HyPurity column with several thousand injections furthermore showed that the performance did not diminish over time.

The temperature of the column oven during the elution program did not seem to affect the chromatography and intensity of the peaks too much, as illustrated in Figure 3-17. This is exemplary shown for colistin A and bacitracin A. Therefore the temperature was left at the initially set 30°C, since an augmentation did not yield better results.

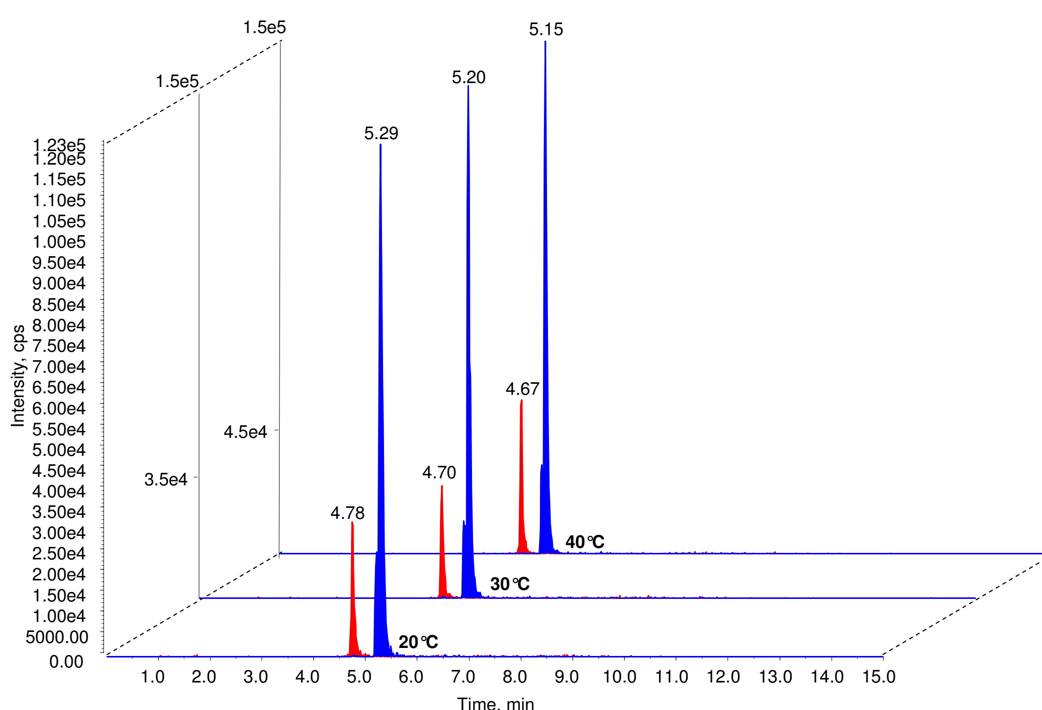

Figure 3-17. Chromatogram of colistin A (a) and bacitracin A (b) (100 µg/l standard) recorded at column oven temperatures of 20°C, 30°C and 40°C during the gradient elution program. Peaks are based on the sum of triple-charged ion transitions.

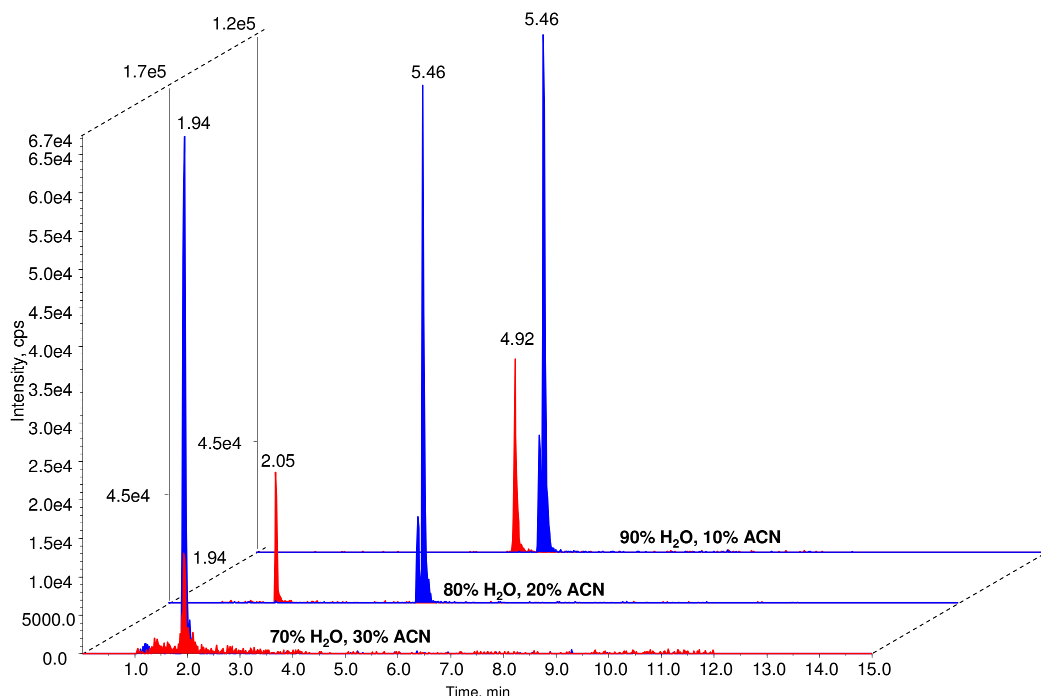

Figure 3-18. Chromatogram of colistin A (a) and bacitracin A (b) (100 µg/l standard) registered at different percentages (70%, 80%, 90%) of initial content of 0.1% HFA in H_2O as eluent B. Peaks are based on the sum of triple-charged ion transitions.

Further experiments on the same column involved the determination of optimal ratio between organic and aqueous eluent at the beginning of the gradient, shown in Figure 3-18, and the examination of the gradient slope, displayed in Figure 3-20.

Figure 3-18 indicates that by increasing the water content of the initial gradient conditions, the analyte peaks elute towards the middle of the run. Less water content leads to earlier elution of both bacitracin and colistin. This effect could be caused by the fact that the initial eluent composition should be similar to the used injection solution. As also stated by Sin *et al.* (2005) differences in organic fraction between the injection solution and the initial eluent mixture can lead to peak distortion and non reproducible retention times.

Furthermore a too early elution of the peaks generally is not desired, since matrix compounds could interfere with the analytes of interest.

Table 3-3 and Figure 3-19. visualise how the influence of the slope of the gradient was tested, whereby results are only shown for the bold marked values in the table. As visible in Figure 3-20 the slope influences the distance between the elution of bacitracin and colistin. The steeper the slope (by increasing the organic part of the eluent mixture) the closer the peaks elute and vice versa. At the same time no effect was visible on peak width.

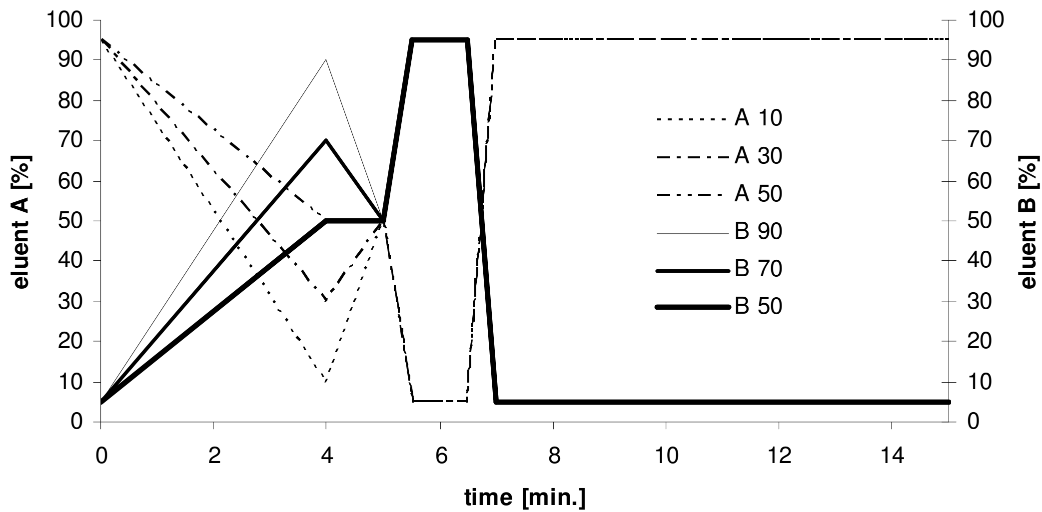

Figure 3-19. Schematic of gradient program for different initial slopes.

Table 3-3. Gradient programs for optimal slope identification

Time [min.]	Mobile phase A [%]	Mobile phase B [%]
0	95	5
4	**10** / 20 / **30** / 40 / **50**	**90** / 80 / **70** / 60 / **50**
5	50	50
5.5	5	95
6.5	5	95
7	95	5
15	95	5

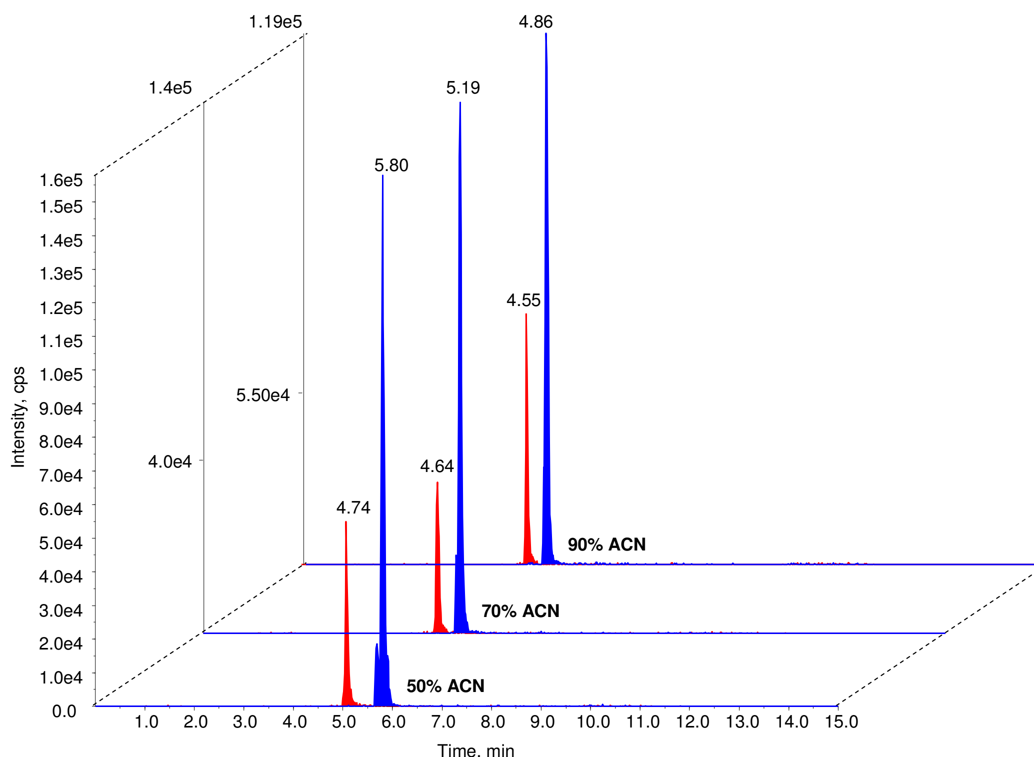

Figure 3-20. Chromatogram of colistin A (a) and bacitracin A (b) (100 µg/l standard) registered at different slopes (50%, 70% and 90% 0.1% HFA in ACN) after 4 min. of gradient program. Peaks are based on the sum of triple-charged ion transitions.

After these steps the optimised gradient program, as listed in Table 3-4 was established for all further experiments. The initial 1st minute of isocratic program was introduced in order to lightly separate the peaks from each other during further elution. High water content was chosen for this purpose, which was similar to the injection solution. The following slow increasing slope was intended to enhance this separation effect between the peaks. High amount of organic mobile phase B is then needed to detach any bound organic matrix compounds and thereby increase the lifetime of the column. Final equilibration time is needed to re-establish the initial conditions.

Table 3-4. Optimised HPLC gradient program

Time [min.]	Mobile phase A [%]	Mobile phase B [%]
0	95	5
1	95	5
5	50	50
5.5	5	95
6.5	5	95
7	95	5
15	95	5

Figure 3-21 demonstrates the final chromatogram of a 100 µg/l standard recorded under the optimised elution program as indicated above. Although bacitracin A, B and C are not baseline separated, colistin A and B, as well as polymyxin B_1 and B_2 nearly are. Furthermore all the peaks elute later during the run, compared to the initial chromatogram shown in Figure 3-13, which lowers the risk of co-elution of analytes and matrix interferences.

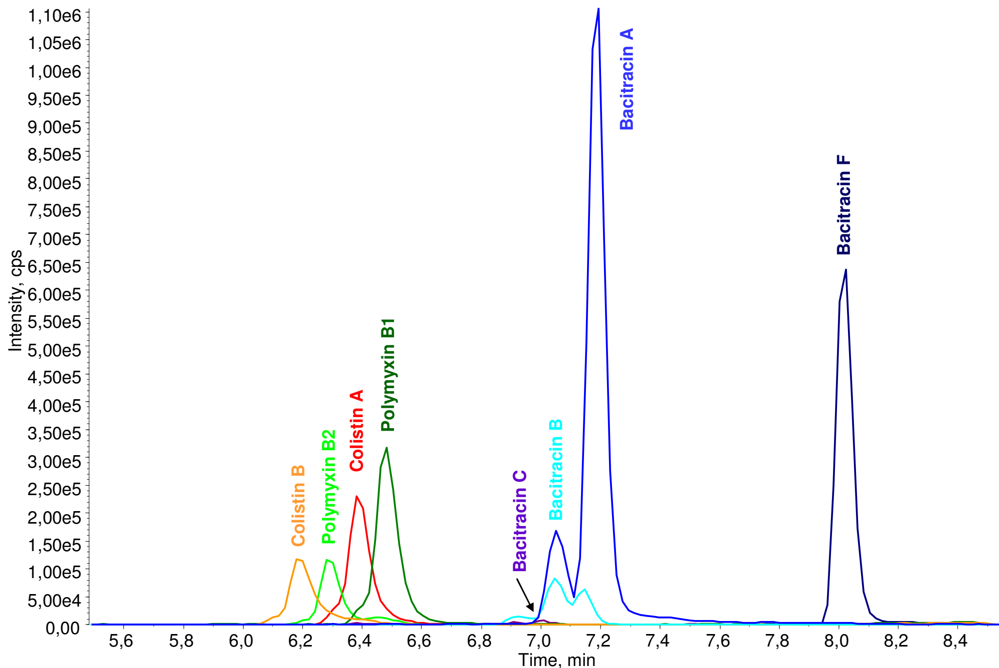

Figure 3-21. Chromatogram (relevant excerpt) of the analytes of interest (100 µg/l standard) on a ThermoScientific HyPurity C_{18} 150x3, 5µ column following the optimised gradient elution program. Peaks are based on the sum of triple-charged ion transitions.

Optimal injection solution

The optimal injection solution was determined by preparing 100 µg/l of analyte standard solution in various solutions in duplicate. A list of possible solutions is shown in Table 3-5.

Table 3-5. Injection solutions tested

0.2% HFA in H_2O	HFA/ACN/H_2O (0.2/10/90)	MeOH/H_2O (2:1)
HFA/MeOH/H2O (0.05/10/90)	H_2O/ACN (50/50)	MeOH/H_2O (1:2)
HFA/MeOH/H2O (0.1/10/90)	HFA/ACN/ H_2O (0.2/50/50)	MeOH/H_2O (10/90)
HFA/MeOH/H2O (0.2/10/90)		HFA/MeOH/H_20 (0.2/50/50)

Results indicated that all of the analytes had the highest peak area and at the same time the lowest standard deviation when measured in a HFA/MeOH/H2O (0.1/10/90) injection solution. Therefore this solution was used for further experiments and for the dilution of the calibration standards. As discussed before the initial eluent composition, especially the organic fraction should be comparable to the one of the injection solution to obtain reproducible results.

Analytes measured in solutions without the addition of acid, such as H_2O/ACN or MeOH/H_2O, had a factor 3-4 less intensities compared to the chosen solution, probably due to the fact that acids, as proton donators facilitate the formation of positive ions, when measuring in ESI-positive mode.

Further considerations

The two mobile phases, namely 0.1% HFA in H_2O and 0.1% HFA in ACN, were chosen based on information found in literature. Most of the analytical methods described employ these eluents.

Furthermore no carry-over between the injections was observed when measuring blank injection solution or the ACN-wash solution after a run of several samples. Therefore there was no need for an automated exterior needle wash between the injections.

3.2. Sample preparation

After the establishment of a chromatographic separation method, a sample preparation protocol was developed. Initially three different protocols for analyte extraction and clean-up were tested and compared.

3.2.1. Liquid-Liquid sample extraction (method 3)

A short preliminary experiment was conducted in order to determine if the analytes can be simply extracted through liquid-liquid extraction from a spiked matrix without the need to use SPE cartridges. Thereby time and money could be saved during sample preparation. Based on established protocols of other analysis methods in the laboratory, the liquid-liquid extraction was done according to the method 3 as described in 2.2.5.

Results with spiked milk and meat samples however indicated that no analytes were visible in the chromatogram. Only spiked water samples showed the presence of bacitracin at low intensities, but no colistin. A possible explanation amongst others could be a poor extraction capacity and high ion suppression due to matrix interferants, which was reviewed by Annesley in 2003. This also accounts for the fact that to the best knowledge of the author there is no protocol mentioned in literature which does not employ SPE cartridges when analysing polypeptide antibiotics with a LC-MS/MS method. Sin *et al.* group (2005) describe that analytes were strongly interfered by endogenous substances when analysing meat samples. This resulted in low recovery values due to suppression of interferences. Therefore a solid phase extraction is needed as cleanup step.

3.2.2. Liquid-Liquid extraction and solid-phase clean-up (method 1 and 2)

Method 1, as described in 2.2.5, was based on the different methods found in literature for colistin (Xu *et al.*, 2012) and bacitracin (Lee *et al.*, 2011), as well as multi analyte methods (Wan *et al.*, 2006). The initial experimental protocol was developed combining aspects of the published sample preparation methods.
Method 2 on the other hand was adapted from Sin *et al.* (2005) which analysed both colistin and bacitracin in food samples and is described in 2.2.5.

Several experiments with 100 µg/kg spiked meat samples, employing both HR-X and Strata-X SPE columns, following the protocol of method 1 and 2 were conducted. Results indicate that the analyte recovery for samples following the method 1 protocol generally was higher compared to the other method. Although for bacitracin no relevant differences were visible (recoveries of 38-47% for method 1 compared to values of 41-47% for method 2), the methods yielded differing values for both colistin and polymyxin (59-71% for method 1 versus 15-33% for method 2). There were only slight differences between the two SPE columns employed; the choice of an appropriate cartridge will be discussed later in this chapter.

A possible explanation for the discrepancies between the methods could be the protein precipitation potential of different acids used (HCl and TCA), as well as organic solvents employed (MeOH and ACN). During sample preparation, after the centrifugation step, the supernatant of method 2 samples was still slightly turbid compared to the supernatant of method 1 samples. This could be caused by the suspension of very fine particles in solution, originating from insufficient deproteinization of the milk and meat samples. Cheng *et al.* (2010) reported that TCA concentrations of 30% were optimal for recovery of colistin and polymyxin in rat plasma.

Further experiments and method optimisation steps were based on method 1.

3.2.3. Extraction solutions

Continuative experiments employing various kinds of organic acids were conducted. Acids are needed to precipitate proteins in samples and thereby facilitate the extraction of analytes with an organic solvent. In addition to HCl as described in method 1, HFA was also used in a final concentration of 0.2% in the extract solution. While recovery for bacitracin where comparable for HCl and HFA, colistin and polymyxin recoveries were only in the range of 1-15% when using HFA. Low recovery values could be also caused by the fact that these two acids have different pK_s values and the solubility (and thereby extractability) of the analytes is dependent on the degree of protonation of their amino acids groups.

Following experiments whereby the flow-through solution of HFA treated and spiked samples, which was loaded on to the SPE column, was collected showed that it contained around 30% of originally spiked colistin and polymyxin. This explains that a part of the analytes is not retained by the SPE column when using HFA. The SPE

flow-through solution of HCl treated samples could not be directly analysed, since chloride might precipitate as salt in the mass spectrometer. HFA on the other hand is a more volatile acid.

Sin *et al.* group (2005) also performed experiments with formic acid as well as acetic acid and concluded that they are not suited for bacitracin and colistin analysis in milk samples.

3.2.4. Sample quantity

Initially samples quantities of 5 g meat and 2.5 g milk were weighed for sample preparation, as indicated in most publications. However the quantity of meat compared to other analysis methods in the laboratory seemed quite high and more amount of extraction solvent was needed. Therefore initial experiments were also conducted with 2.5 g of meat.

During the course of the method development experiments with meat quantities ranging from 0.5 to 2.5 g (spiked at the same µg/kg level, while adjusting the spiking volume) were conducted in order to evaluate if the sample quantity can be reduced for meat. A lower sample weight might facilitate the sample preparation procedure, especially during solid-phase extraction the cartridge gets less clogged up. Also chromatography results already indicated that meat, in comparison to milk, is a more challenging matrix when it comes to matrix interferences. On the other hand a low sample weight bears the risk of having a low recovery when spiking the sample and not being able to detect positive samples as such in later routine analysis.

Results showed that recovery values decreased with increasing amount of sample used, while spiking levels of 75 µg/kg were visible in all the samples. Hence during the final validation 1 g of meat was used.

3.2.5. Solid-phase extraction cartridges

Four different solid-phase extraction cartridges were tested, as listed in 2.1.3, in order to find the one with the best price/performance ratio. The protocol for the solid-phase extraction was based on the standard procedure for Chromabond® HR-X cartridges (Application Nr. 304310) found on the Macherey-Nagel website, which was in accordance with protocols used in literature (Gibson *et al.*, 2012, Lee *et al.*, 2011, Wan *et al.*, 2006).

Evaluation of performance was mainly based on recovery values, but also on repeatability of the values and ease of handling during sample preparation. Experiments were conducted in duplicate as described in 2.2.5 and all four cartridges were treated the same way. The expected final concentration in the vials was 100 µg/l and was used for the calculation of the recovery shown in Figure 3-22, Figure 3-23 and Figure 3-24. These Figures indicate that the recovery is in the range of 75-98% for the first three cartridges, namely the Oasis HLB, Phenomenex Strata-X and Macherey-Nagel HR-X (all 3 ml and 60 mg). Only the Macherey-Nagel OH Diol (3 ml, 200 mg) shows lower recoveries for bacitracin (45-49%), while nearly no recovery for colistin and polymyxin (2-5%). The OH Diol column was used since it was mentioned in the application note 300750 from the Macherey-Nagel website when looking for SPE cartridges for the analysis of bacitracin. A possible explanation for the low recoveries using the OH Diol resin is the fact that not the recommended conditions and solutions were used. However since in literature mainly Oasis HLB and Phenomenex Strata-X cartridges are reported when analysing polypeptide antibiotics, the OH Diol cartridges were not further tested. The other three cartridges are all based on polystyrene divinyl benzene (PS-DVB) polymer resins.

For further experiments the Macherey-Nagel HR-X cartridge was chosen, since recovery values were comparable, results reproducible and handling during sample preparation was easy and fast (no blocking and high sample flow rate). Furthermore their price was 2-3 times less compared to the Oasis HLB and Strata-X tubes.

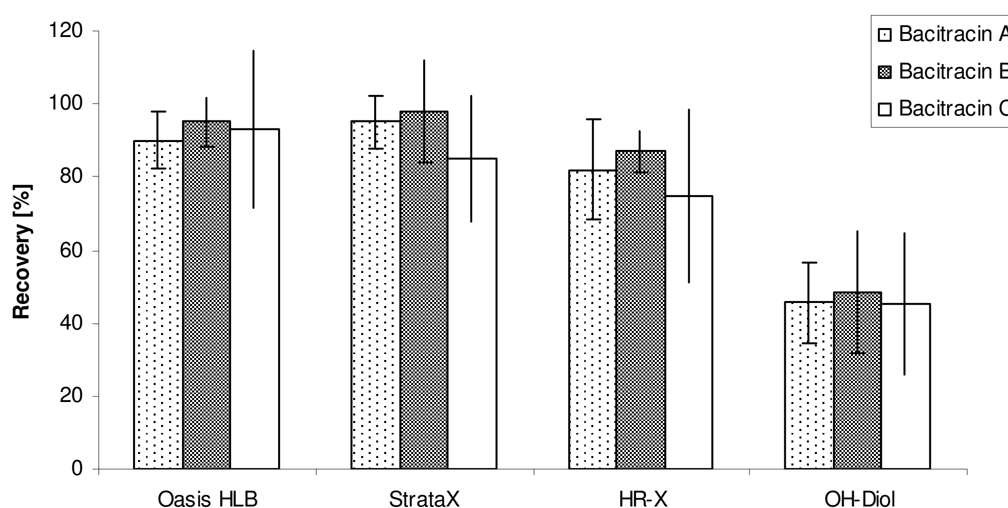

Figure 3-22. Recovery values of a spiked water sample for bacitracin on four different solid-phase extraction columns (n=2).

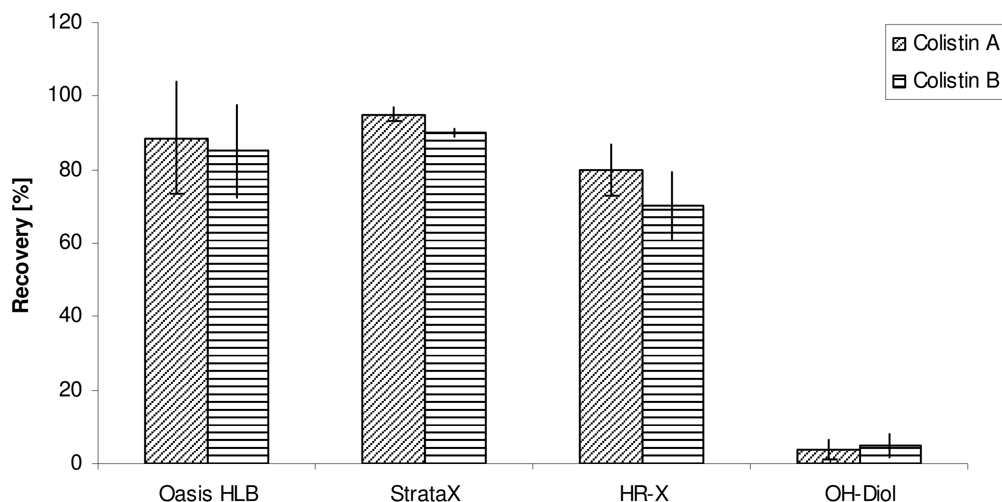

Figure 3-23. Recovery values of a spiked water sample for colistin on four different solid-phase extraction columns (n=2).

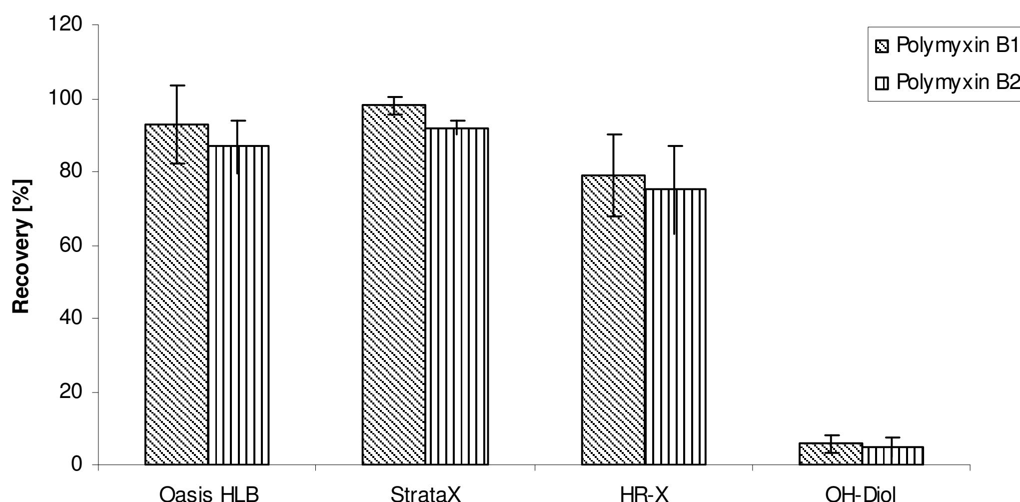

Figure 3-24. Recovery values of a spiked water sample for polymyxin on four different solid-phase extraction columns (n=2).

Experiments also indicated that recoveries were about 50% higher when diluting the 5 ml aliquot from the extraction step with 15 ml of water prior to loading it on the conditioned SPE column. Most probably the SPE cartridge yields better results when the sample composition is aqueous rather than organic.

Washing

The protocol found on the Macherey-Nagel website indicated that a 5% MeOH solution should be used for column washing. In the course of the method development a 10% solution was employed and tested, in order to wash out more parts of retaining matrix interferences.

In order to evaluate if analyte loss occurs during the washing of the SPE column, five 1 ml fractions of the 10% MeOH washing solution were collected and 10 µl 10% HFA was added before analysis in order to obtain a composition comparable to the one of the injection solution. Meat samples, as well as water samples, were spiked with 1000 µg/kg. This high value was chosen in order to facilitate the detection of possible washing losses. Results for both samples and all analytes indicated that the summed up washing fractions showed only between 0 and 0.8% loss of analytes. This points out that the washing conditions used were most probably appropriate.

Elution

Following the washing step, the quantity of elution solution needed to elute all the analytes retained on the column was tested. For this purpose the same 1000 µg/kg spiked samples as for the washing experiments were used. Figure 3-25 indicates that more than 95% of the analytes elute in the first two millilitres. The third millilitre of elution solution contain between 1% and 5% of analytes, while the fourth fraction held less than 1%. Subsequent sample concentration experiments however showed that evaporation of 3 ml of sample led to prolonged concentrations times, especially due to present water content. Therefore the loss of analyte on the third millilitre was accepted in the protocol development.

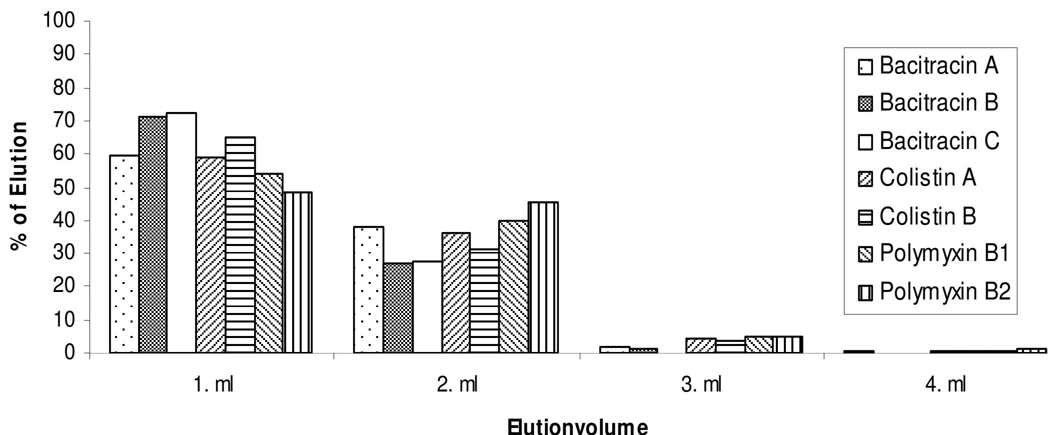

Figure 3-25. Solid-phase extraction elution experiment with four 1 ml fractions of 0.2/30/70 HFA/H_2O/MeOH, of a 1000 µg/kg spiked meat sample (n=1).

Experiments conducted towards the end of the development, after the validation, indicate that the water content of the elution solution can be reduced and therefore sample concentration time minimised. This is demonstrated in Figure 3-26 where recovery values are comparable for the three different elution solutions, containing 10, 20 and 30% of water.

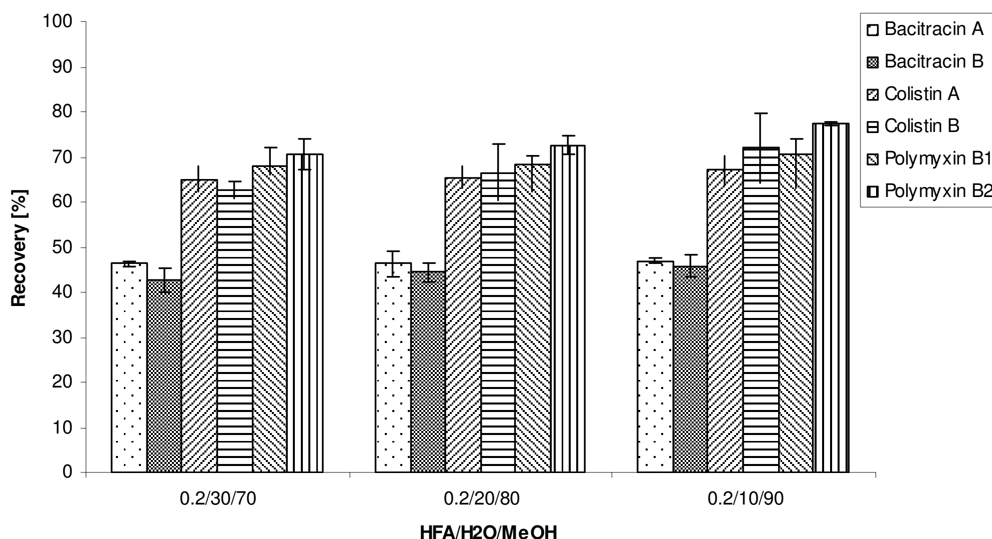

Figure 3-26. Solid-phase extraction elution experiment employing elution solutions with different water/methanol ratio (n=2).

3.2.6. Sample concentration

Sample concentration was needed on the one hand to change the elution solvent, containing high proportions of methanol, into the more aqueous injection solution. The eluents coming from the SPE were evaporated under a gentle stream of nitrogen at 40℃. However precaution has to be taken since bacitracin slowly decomposes when heating it for prolonged time as described by Sin *et al.* (2005) and Capitán-Vallvey *et al.* (2001). This could be caused by either temperature instability, as well as the presence of acids in the extract, which might degrade acid-labile analytes when solvent volume approaches dryness through acid hydrolysis.

Therefore eluents in duplicate were evaporated to dryness and less than 1 ml respectively, with and without ethylene glycol. When evaporating to dryness in the presence of ethylene glycol, which acts as a keeper substance, a small amount remains in the test tube. Adding 500 µl of a 10% ethylene glycol solution to the eluents led to a leftover of 50 µl in this work.

Results indicate that the highest recovery values were obtained when evaporating the eluents to dryness in the presence of ethylene glycol. These were 30-40% higher

for bacitracin compared to samples evaporated to dryness without the keeper solution. Recovery values for colistin and polymyxin behaved similarly with and without keeper. Evaporating to less than 1 ml and then adding injection solution to fill up to 1 ml final volume did not give higher recoveries for any of the analytes. Directly evaporating to dryness with ethylene glycol makes the work in a routine laboratory more smooth compared to evaporating to less than 1 ml which might hinder efficient working.

3.2.7. Recovery values

Overall process recovery values (apparent recovery) for the method are listed in Table 3-6 for milk and meat samples respectively. These values are not corrected by the mean recovery or an internal standard. Recoveries for colistin and polymyxin are generally higher than bacitracin for both milk and meat samples.

Table 3-6. Process recovery values (uncorrected) for spiked meat and milk samples

Analyte	Recovery (meat) [%]	Recovery (milk) [%]
Bacitracin A	57.3	65.0
Bacitracin B	50.0	55.0
Colistin A	74.0	82.0
Colistin B	76.0	75.0
Polymyxin B_1	81.0	80.0
Polymyxin B_2	45.0	70.0

Table 3-7. Standard addition recovery values for meat and milk samples

Analyte	µg/l (meat)	µg/l (milk)
Bacitracin A	70.9	78.6
Bacitracin B	58.3	55.7
Colistin A	75.5	81.0
Colistin B	75.0	89.2
Polymyxin B_1	86.5	83.0
Polymyxin B_2	68.8	72.8

Results of the standard addition (spiking of the prepared non-analyte containing samples before measurement as described in 2.2.6) is shown in Table 3-7. This

shows that ions of all analytes are suppressed during mass spectrometric measurement since found values are lower than the spiked 100 µg/l. Signal enhancement, which is also a possible effect, does not apply in this case.
Furthermore recoveries of fortified samples before and after extraction procedure (before SPE) were compared to evaluate the extraction loss (described in 2.2.6). Depending on the matrix (meat, milk or water samples) the extraction loss was between 20-40% for bacitracin, while in the range of 10-20% for colistin and polymyxin. As expected, losses were higher for meat and lower for milk and water samples.

When adding the loss of extraction and ion suppression to the recovery values listed in Table 3-6 values around 100% are obtained. Further analyte loss can be assigned to washing procedure during solid phase extraction, as well as final sample concentration.

3.2.8. Robustness of the method

Final experiments were conducted to evaluate the robustness of the method in regard to extraction time and amount of HCl used. This was done to find out if there are any critical steps within the method which have to be considered when routinely analysing samples. For example letting samples stand for a while or adding the wrong amount of acid.

Extraction time

Meat sample were spiked at 100 µg/kg and prepared either in one go or with extraction times of 30 and 60 minutes. In the latter case samples were either mechanically shaken or allowed to stand for the indicated time. Results indicate that best recoveries (60% for bacitracin A) were obtained for samples treated without any excess of extraction time, while shaken and non-shaken samples showed slightly lower recoveries (47% and 50% respectively). Colistin and polymyxin had comparable results to bacitracin.
Experiments with extraction time, use of ultrasonication as well as mechanical shaking when analysing zinc bacitracin were conducted by Capitán-Vallvey *et al.* (2001). The group obtained different results and stated that the best recoveries were obtained with mechanical shaking, while low recoveries were obtained for

ultrasonicated samples. Extraction time was varied between 5 and 45 minutes, while a 20 minute extraction time was finally chosen.
However since extraction time did not seem to be a crucial step in this work, which strongly influences the final recovery, no extra extraction time was included in the method. This facilitates the use of the method in a routine laboratory in regard to time and instrumental requirements.

Amount of acid

In the end of the work, after the validation, experiments with different amounts of HCl were conducted, as listed in Table 3-8 together with the appropriate pH values in milk and meat. Measurement of pH values was done in the diluted solution (aliquot and water) which was then loaded on the SPE column. It can be recognised that milk in comparison to meat has a higher buffer capacity, since pH values are slightly higher.

Resulting recovery values for all analytes in milk and meat are illustrated in Figure 3-27 for milk and Figure 3-28 for meat samples. Both Figures indicate that with increasing amount of HCl and concurrent lower pH recoveries for bacitracin decline, while they rise for colistin and polymyxin. Although the pH does not change too much, influence on recovery is considerable. A compromise between bacitracin and colistin/polymyxin had to be found and 1 ml of HCl was chosen for milk samples, while 500 µl for meat samples.
One can also recognise that generally error bars for meat samples are higher compared to milk samples, which could indicate that meat is a more complex matrix and yields less reproducible results.

In their studies Capitán-Vallvey *et al.* (2001) also stated that best recoveries were obtained for an extraction solution of pH 2, while in a solution around pH 1, matrix interferences hindered the analysis. Also pH values >3 led to drastically decreased recovery values. When using a 6M HCl solution in this work, it was difficult to set the pH at a determined value, since it a relatively strong acid. Furthermore depending on the type of sample and its buffer capacity the amount of acid needed might change. Therefore in a routine laboratory when analysing large amounts of sample it is easier to add a defined amount of acid, instead of measuring the pH for each sample individually.

The results of these experiments however show that the pH is an important factor in the course of the sample preparation and continuative experiments might be necessary.

Table 3-8. Influence of HCl quantity on pH of meat and milk samples

HCl [µl]	pH (meat)	pH (milk)
300	1.53	1.61
500	1.28	1.41
700	1.15	1.24
1000	0.94	1.11

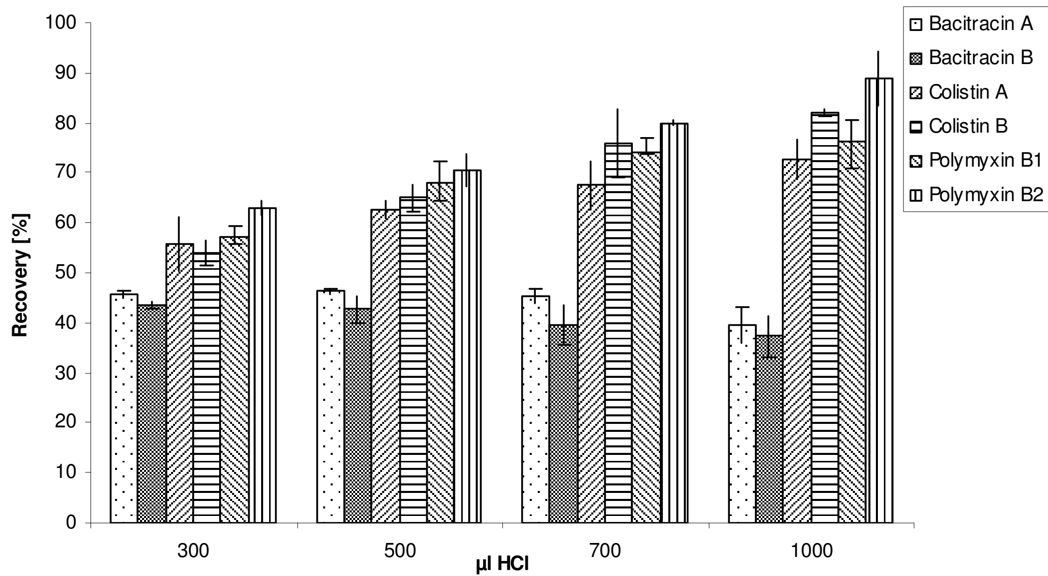

Figure 3-27. Influence of HCl quantity used during milk sample preparation on recovery (n=2).

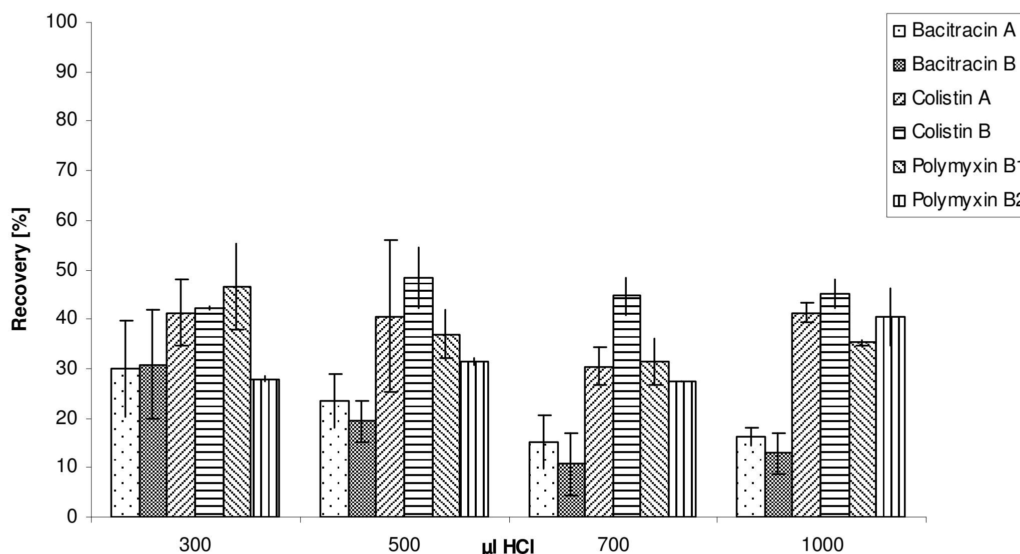

Figure 3-28. Influence of HCl quantity used during meat sample preparation on recovery (n=2).

3.2.9. Internal standard

As described earlier in section 2.1.1 bacitracin F was used as an internal standard in the final method validation (see section 3.3). Internal standards are needed to compensate for influences during sample preparation on the analytes, as well as a possible decline of intensity in the mass spectrometer when analysing large amounts of samples in one run. Concerning the first aspect, in routine analysis spiked and non-spiked samples are measured in parallel. Therefore values can also be corrected with the recovery of the spiked sample. Regarding the intensity decline, measurements of a 100 µg/l standard at the beginning and the end of a sample queue (without any ACN wash run in-between) was done, in order to evaluate if the intensity does decline or remains constant. Results showed that the peak area of the standard measured at the end of the queue was between 95-105% of the initially measured standard. Hence the use of an internal standard in this case is not urgently needed; its use however would give the method certain reliability.

As mentioned in the introduction bacitracin F is the oxidative degradation product of bacitracin A. Therefore it was found to be present also in the bacitracin reference solution in amounts of 5-10%. Degradation mechanisms need to be understood to find out if the reaction reaches equilibrium at a certain point or all bacitracin A is degraded after prolonged storage. Therefore its use as internal standard should be considered with caution.

3.3. Method validation

The validation of the method was conducted according to the requirements stated by the European Commission in the 2002/657/EC guidelines. These guidelines were especially designed for the validation of methods analysing veterinary residues. In contrast to other validation methods, this guideline includes the calculation of decision limits CCα and CCβ. The validation for all analytes will be presented in the following.

Results for the validation were calculated according to section 2.2.7. Values were both corrected with the mean recovery, as well as in combination with the internal standard. Values corrected by both internal standard and recovery however showed higher deviation values and less linearity. Therefore they are not presented in the following.

Since bacitracin C was only present in minor amounts in the reference standard and therefore spiking levels were relatively low (see Table 3-10 and Table 3-11), no reliable validation results could be obtained for this analyte. Therefore it was not included in the validation. Future re-validation should include higher spiking levels to also take into account bacitracin C.

Stability

Stability of analytes in solution

Stability analysis of analytes in solution was conducted as described in 2.2.7. The solution they were stored in had a pH of 3. Figure 3-29 shows the results of samples stored in the autosampler at 15°C for a period of 42 days. This experiment simulates the case when the standards are kept in the autosampler for prolonged time when several analyses per week are done. Both colistin and polymyxin seem to be stable for the indicated period of time, although values are subjected to strong fluctuations. For bacitracin (with the exception of bacitracin F) a small decrease to about 90% remaining activity can be observed after 42 days.

Normally however standard solutions were kept in the fridge at 4°C. For this case results indicate that both bacitracin and colistin/polymyxin are stable when stored in the fridge for 42 days. When keeping the samples at room temperature (20°C) the activity of bacitracin solution decreases to 61-65%, while when storing it at room temperature in transparent vials activities of 56-62% remained. Colistin and polymyxin on the other hand seem to be stable even at room temperature in dark

vials for the indicated time period, while a decrease to 49-53% in transparent vials is visible. Therefore standard vials were generally kept in the fridge at 4℃ in dark vials until needed for measurements and placed back there after analysis.

In literature several stability studies for polypeptide antibiotics can be found. Stability of bacitracin is described in the product information sheet of Sigma-Aldrich[2]. The substance is relatively unstable in aqueous solutions at room temperature; in acidic solution stability is given, while pH above 9 leads to degradation. Its instability most probably is related to the transformation of bacitracin A into bacitracin F, which exhibits low antimicrobial activity. Studies concerning the stability of bacitracin F were not found in literature.

Furthermore studies show that bacitracin solution is stable at -20℃ in the freezer for six months in aqueous solution (Wan *et al.*, 2006), as well as when dissolved in 0.1% formic acid in the fridge at 4℃ (Lee *et al.*, 2011, Sin *et al.*, 2005, Turnipseed *et al.*, 2008).

For colistin studies of Li *et al.* (2003) indicate that colistin is not stable at pH above 7. This was also confirmed by an experiment in this work, whereby a sample of a customer was analysed on the presence of colistin. No colistin was found, but the pH of the solution was around 7. Fortification with 100 µg/l and analyses both immediately and the next day (stored at room temperature) showed that 90% of colistin was degraded. Also Orwa et al. (2002) report that colistin solutions degrade faster above pH 5 and polymyxin has its maximum degradation at pH 7.

Further studies report that colistin is stable at -20℃ in the freezer in aqueous solution (Gobin *et al.*, 2010, Wan *et al.*, 2006), as well as when dissolved in 0.1% formic acid in the fridge at 4℃ (Sin *et al.*, 2005). For polymyxin similar results are reported.

[2]www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/b0125pis.Par.0001.File.tmp/b0125pis.pdf (accessed on 04.01.2013)

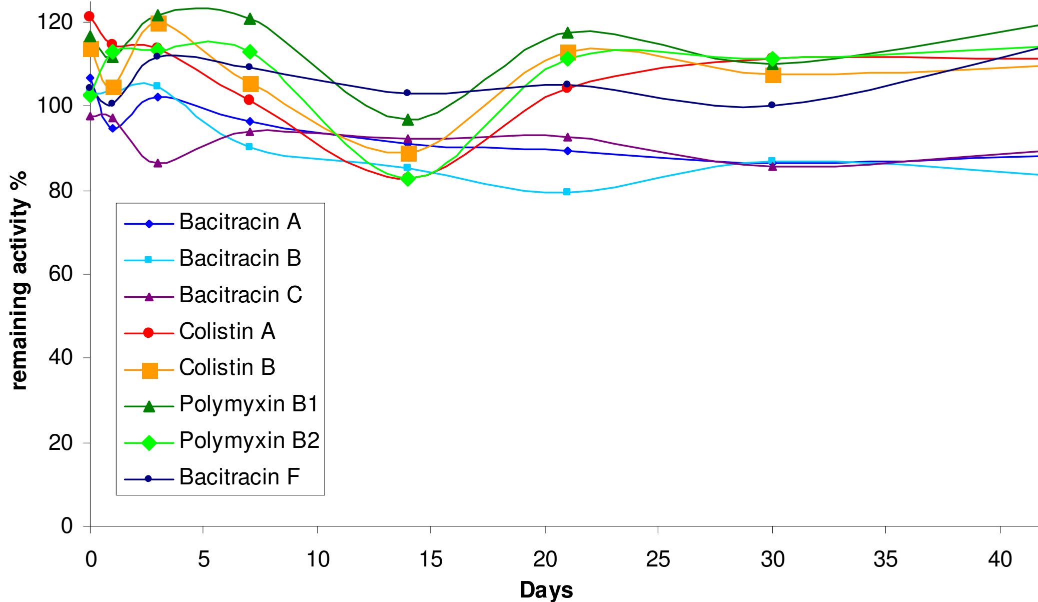

Figure 3-29. Analyte stability over a period of 40 days at 15°C in the autosampler

Stability of analytes in matrix

Measurement procedure of analyte stability of prepared spiked milk and meat samples is also described in 2.2.7. Results indicate that the analytes are stable for one week in the fridge at 4°C in case they need to be reanalysed.

Selectivity and Specificity

In LC-MS/MS analysis substances can be identified based on mass transitions and retention time. Ideally the retention time of the analytes should be identical in the standard solutions and prepared samples, which is an indication for the selectivity of the method, as well as its robustness. The comparison of retention times was already discussed in section 2.2.4. Specificity on the other hand is based on the mass selectivity of the detector. It describes the ability to distinguish between the analyte and possible interfering substances. The mass selective transitions of the method are listed in 2.2.3.

Instrument linearity and repeatability

Initially the instrument linearity and repeatability was determined as described in section 2.2.7, at equidistant concentrations in the range between 50-250 µg/l. Results are shown in Table 3-9. Acceptance criteria for a linear calibration function were defined according to the in-house validation and include:

- a linear coefficients of determination higher than 0.995,
- a relative standard deviation below 4.1%,
- as well as a y-axis intercept of 0 ± 5% of the target concentration (calculated according to Equation 3-1).

Experience has furthermore demonstrated that repeatability values for LC-MS/MS methods below 10% are satisfactory.

Equation 3-1. Calculation of target concentration

Max. target concentration = 0.05 x ((mean value of work area x slope) + axis intercept))

Table 3-9 indicates that the instrument linearity for some of the analytes is not ideal and has high relative standard deviations. In most cases the axis intercept has an elevated value. This has to be further observed during routine analysis

Table 3-9. Validation results for instrument linearity and repeatability

Analyte	Linearity R^2	RSD [%]	Axis intercept	Repeatability [%]
Bacitracin A				
475 → 86	0.999	1.71	Elevated	9.42
475 → 356	0.991	5.74	Good	4.53
475 → 670	0.964	11.76	Elevated	7.57
Bacitracin B				
470 → 662	0.998	2.67	Good	5.66
470 → 669	0.996	3.95	Good	6.34
Bacitracin C				
465 → 227	0.999	1.8	Good	12.74
465 → 662	0.986	6.36	Elevated	14.91
Bacitracin F				
710 → 280	0.990	6.07	Elevated	7.72
710 → 309	0.948	14.22	Elevated	10.37
Colistin A				
390 → 241	0.996	4.07	Elevated	10.96
390 → 384	0.983	8.03	Elevated	6.87
390 → 465	0.977	9.30	Elevated	10.15
Colistin B				
386 → 101	0.986	7.34	Elevated	9.79

386 → 374	0.996	3.72	Elevated	6.80
386 → 380	0.997	3.49	Elevated	9.90
Polymyxin B_1				
402 → 101	0.977	9.41	Good	9.50
402 → 390	0.973	10.13	Elevated	6.24
402 → 396	0.963	11.88	Elevated	18.80
Polymyxin B_2				
397 → 385	0.988	6.77	Elevated	5.24
397 → 391	0.998	3.01	Elevated	8.63

Blank values for all analytes were determined by 3-fold measurement of injection solution. For all substances the resulting area was substantially lower compared to the area of the lowest standard used.

Method linearity

Method linearity was determined as described in 2.2.7. The acceptance criteria are the same as mentioned above for the instrument linearity. Table 3-10 and Table 3-11 list the theoretically calculated spiking level for each analyte in milk and meat samples respectively, considering its proportion in the reference standard (as described in 3.1.1). Samples quantities were weighed with a small deviation of 2.5 g ± 0.5% for milk and 1.0 ± 1.0% for meat in order to be able to take the calculated spiking levels as actual levels in the sample for linearity calculations.

The 2002/657EC indicates to use the following levels of fortification:

- 0, 0.5, 1, 1.5 and 2 times the MRL for substances with a MRL,
- while 0, 1, 1.5, 2, and 2.5 times the LPL for non regulated substances.

Since polymyxin is not regulated with a MRL, LPL values were determined based on colistin values (50 µg/kg in milk and 100 µg/kg in meat samples).
Since MRL values for bacitracin and colistin are different for milk and LPL regulated substances have different levels of fortification, a compromise was chosen in order avoid the analysis of too many spiking levels. Similarly for meat spiking levels for polymyxin were adapted to the ones of bacitracin and colistin.

Table 3-10. Calculated spiking levels in µg/kg for all analytes in milk samples

Spiking level [µg/kg]	Bacitracin			Colistin		Polymyxin	
	A	*B*	*C*	*A*	*B*	*B1*	*B2*
25	25.0	6.7	1.5	17.0	5.7	21.4	14.3
50	50.0	13.3	3.0	34.0	11.3	42.9	28.6
75	75.0	20.0	4.6	51.0	17.0	64.3	42.9
100	100.0	26.7	6.1	68.0	22.7	85.7	57.1
150	150.0	40.0	9.1	101.9	34.0	128.6	85.7

Table 3-11. Calculated spiking levels in µg/kg for all analytes in meat samples

Spiking level [µg/kg]	Bacitracin			Colistin		Polymyxin	
	A	*B*	*C*	*A*	*B*	*B1*	*B2*
75	75.0	20.0	4.6	51.0	17.0	64.3	42.9
150	150.0	40.0	9.1	101.9	34.0	128.6	85.7
225	225.0	60.0	13.7	152.9	51.0	192.9	128.6
300	300.0	80.0	18.3	203.9	68.0	257.1	171.4

Table 3-12 and Table 3-13 present the results for the method linearity for meat and milk samples respectively. They were calculated by combining the results of the duplicate sample preparation of all three days. Prepared meat samples, at spiking levels of 0, 75, 125, 175, 225 and 300 µg/kg, show good linear coefficients of determination and a y-axis intercept within the permitted range for all analytes. Only relative standard variations for polymyxin are augmented. Nevertheless the method for meat samples overall can be regarded as having a linear relationship between the employed spiking level and the resulting signal, under the influence of effects from sample preparation and sample measurement. Therefore the method can yield reliable results in the mentioned concentration range.

Table 3-12. Validation results for method linearity (meat samples)

Analyte	Linearity R^2	RSD [%]	Axis intercept
Bacitracin A	0.999	1.7	Good
Bacitracin B	0.998	4.0	Good
Colistin A	0.998	3.9	Good
Colistin B	0.999	2.1	Good
Polymyxin B_1	0.995	6.4	Good
Polymyxin B_2	0.995	6.7	Good

For milk samples at spiking levels of 0, 25, 50, 75, 100 and 150 µg/kg analytes showed good linearity and relative standard deviation, with the exception of bacitracin B, which however still in an acceptable range. Elevated axis intercepts seem to be a problem in milk samples and further routine analysis has to show if this is a persistent problem.

Table 3-13. Validation results for method linearity (milk samples)

Analyte	Linearity R^2	RSD [%]	Axis intercept
Bacitracin A	0.998	4.0	Elevated
Bacitracin B	0.993	7.7	Good
Colistin A	0.999	3.1	Elevated
Colistin B	0.999	2.8	Elevated
Polymyxin B_1	0.999	3.4	Elevated
Polymyxin B_2	0.997	4.9	Good

It must be noted however that the results for linearity and relative standard deviation for each single day partially were outside the acceptance criteria. These deviations nevertheless balance each other out when regarding the results over three days.

Figure 3-30 and Figure 3-31 demonstrate a blank milk sample, together with a 25 µg/kg spiked milk sample, which corresponds to ½ the MRL. The blank milk sample was spiked with the internal standard bacitracin F. Figure 3-30 shows that there are relatively few matrix interferences visible, which do not interfere with the analytes displayed in Figure 3-31.

Figure 3-32 and Figure 3-33 illustrate the blank together with a 75 µg/kg spiked meat sample, again corresponding to the ½ MRL. It becomes clear that polymyxin B_2

interfere with matrix compounds at this fortification level and colistin B is on top of a matrix peak.

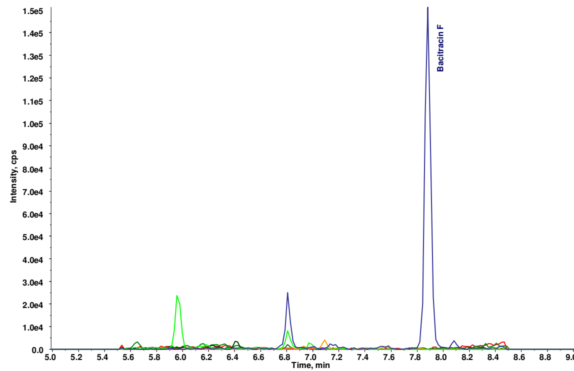

Figure 3-30. Blank milk sample spiked with 25 µg/kg of internal standard (bacitracin F).

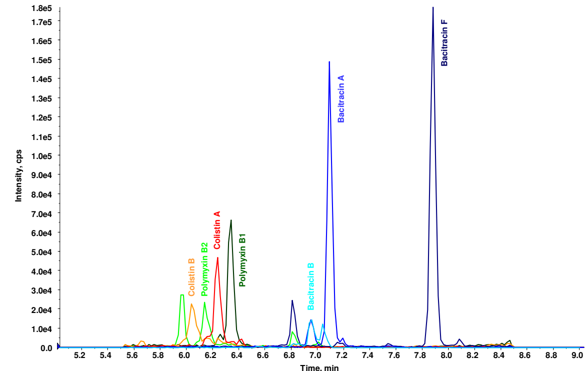

Figure 3-31. Spiked milk sample with 25 µg/kg (1/2 MRL for colistin).

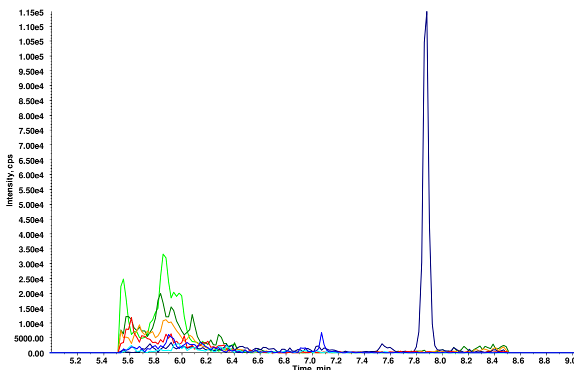

Figure 3-32. Blank meat sample spiked with 75 µg/kg of internal standard (bacitracin F).

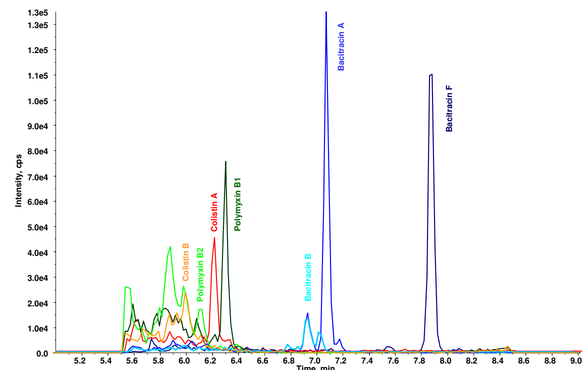

Figure 3-33. Spiked meat sample with 75 µg/kg (1/2 MRL).

Repeatability and within-laboratory reproducibility

Repeatability and within-laboratory reproducibility were assessed as described in 2.2.7. Table 3-15 and Table 3-16 list the determined values for milk and meat samples respectively. The bracketed values indicate the acceptable limit calculated according to Horwitz.

Repeatability is an indicator for the intra-day variation, when analysing the same sample a number of times by the same operator and instrument, while using the same chemicals (comparable conditions). Reproducibility on the other hand indicates how well the method can be reproduced under non-comparable conditions. For the determination of the reproducibility in this work the day of analysis and the operators were varied. Furthermore for meat two different homogenisation vessels were used and for milk 0.5 or 1 ml of HCl were added during sample preparation.

Generally both tables indicate that the repeatability is lower than the reproducibility, which is probably caused by the fact that there are more components of variance involved in the latter case, which generally is to be expected. All values for the analytes lie within the acceptable range, with the exception of polymyxin B_2 in meat samples. In comparison the analysis of meat samples is exposed to lower repeatability and reproducibility, when compared to milk samples. This could be caused by the complexity of the meat matrix.

In conclusion the method is able to give reproducible results over time, by varying variables such as operator and day.

Table 3-14. Validation results for repeatability and reproducibility of the method (milk samples)

Analyte	Repeatability [%] n=8 (maximum[1])	Reproducibility [%] n=6 (maximum[1])
Bacitracin A	7.4 (17)	11 (25)
Bacitracin B	5.7 (20)	20 (30)
Colistin A	3.7 (18)	15 (26)
Colistin B	4.9 (21)	15 (30)
Polymyxin B_1	7.3 (17)	16 (25)
Polymyxin B_2	9.9 (18)	18 (26)

[1] maximum repeatability / reproducibility according to Horwitz

Table 3-15. Validation results for repeatability and reproducibility of the method (meat samples)

Analyte	Repeatability [%] n=6 (maximum[1])	Reproducibility [%] n=6 (maximum[1])
Bacitracin A	6.6 (16)	13 (24)
Bacitracin B	11 (19)	16 (29)
Colistin A	12 (17)	9 (25)
Colistin B	9 (19)	11 (30)
Polymyxin B_1	9.6 (16)	15 (25)
Polymyxin B_2	20 (18)	53 (27)

[1] maximum repeatability / reproducibility according to Horwitz

In routine analysis quality control charts can then be used to determine if the analytical process is in a state of statistical control, especially for analytes which have low reproducibility like polymyxin B_2.

Recovery and trueness of quantitative method

According to the 2002/657/EC if no certified reference material is available to determine the trueness of the method, a spiked blank sample might be used instead. Table 3-17 and Table 3-16 show the corrected recovery values and trueness for milk and meat samples accordingly. These values were calculated as described in 2.2.7. The recovery values were obtained from the average of all the values used for method linearity, while the trueness was calculated based on the repeatability values. For trueness, values corrected with the mean recovery are only acceptable when they fall within the range of 80-110% (-20% to +10%). Judging from the Tables the method delivers "true" values for all the analytes, with the exception of colistin B in milk samples, which yields slightly higher values and should be further observed.

Table 3-16. Validation results for recovery and trueness of the method (milk samples)

Analyte	Recovery (corrected) [%]	Trueness [%]
Bacitracin A	103.1	106.4
Bacitracin B	104.0	104.4
Colistin A	111.0	101.8
Colistin B	109.7	113.9
Polymyxin B_1	106.0	105.4
Polymyxin B_2	113.6	103.1

Table 3-17. Validation results for recovery and trueness of the method (meat samples)

Analyte	Recovery (corrected) [%]	Trueness [%]
Bacitracin A	103.1	97.8
Bacitracin B	102.7	95.6
Colistin A	106.7	97.0
Colistin B	104.7	100.3
Polymyxin B_1	106.6	95.8
Polymyxin B_2	105.4	80.3

Limit of detection, limit of quantification, CCα and CCβ

CCα and CCβ were introduced as new parameter in order to ensure comparability and the performance of laboratories within Europe in regard to residue analysis in products of animal origin. In the past various parameters such as limit of detection,

limit of determination, limit of quantification, limit of identification and limit of detectability were employed and differently defined, which might hinder comparability.

LOD, LOQ, CCα, as well as CCβ were determined as described in 2.2.7. Results are shown in Table 3-18 and Table 3-19 for milk and meat samples respectively. LOQ values are, with the exception of polymyxin B_2 in meat, are all below ½ the MRL. Polymyxin B_2 as already visible Figure 3-33 interferes at low spiking levels with matrix compounds.

The decision limit (CCα) is the limit at and above which it can be concluded with an error probability of α (here 5%) that the analysed sample is non-compliant. This value is used to decide whether a sample is compliant or not. CCα is per definition above the defined limit value. Furthermore it already includes the measurement uncertainty in form of the within-laboratory reproducibility. The Tables indicate that is within an acceptable range for all analytes, when comparing it to the MRL.

The detection capability (CCβ) represents the smallest content of the substance that may be quantified in a sample with an error probability of β (here 5%). In the contrary to CCα it is not used to decide on the compliance of a sample. It is rather a parameter to assess the performance of an analytic method in regard to the probability of false negative results. It is associated with the CCα value, as well as the dispersion of the measured results. At CCα the rate of a false-negative result is 50%, while this declines to 5% at CCβ. As a function of the dispersion this rate declines with increasing concentrations. The Tables indicate that for milk samples, which have good repeatability and reproducibility as described above, CCβ values indicate good performance of the method. Analytes in meat samples on the other hand are exposed to higher dispersion values and therefore show higher CCβ values, especially for polymyxin.

Table 3-18. Validation results for LOD, LOQ, CCα and CCβ (milk samples)

Analyte	MRL [µg/kg]	LOD [µg/kg]	LOQ [µg/kg]	CCα [µg/kg]	CCβ [µg/kg]
Bacitracin A	100	11	32	116	121
Bacitracin B	100	5	16	108	115
Colistin A	50	6	17	58	67
Colistin B	50	2	5	56	63
Polymyxin B_1	50 (LPL)	8	23	57	71
Polymyxin B_2	50 (LPL)	7	22	58	71

Table 3-19. Validation results for LOD, LOQ, CCα and CCβ (meat samples)

Analyte	MRL [µg/kg]	LOD [µg/kg]	LOQ [µg/kg]	CCα [µg/kg]	CCβ [µg/kg]
Bacitracin A	150	10	30	170	190
Bacitracin B	150	6	19	167	183
Colistin A	150	16	47	174	201
Colistin B	150	10	30	157	165
Polymyxin B_1	150 (LPL)	33	99	166	221
Polymyxin B_2	150 (LPL)	23	69	168	208

In the case of bacitracin the MRLs laid down are expressed as the sum of bacitracin A, B and C. For this purpose guidelines for the implementation of decision 2002/657/EC have been issued by the Health and Consumer Protection Directorate of the EC (SANCO/2004/2726).

Similar LOD values for both bacitracin and colistin were reported by Sin et al. (2005) and Wan et al. (2006), which indicates that the method shows good performance. Values for polymyxin B were not found in literature since no publication included it as an analyte.

CHAPTER IV: CONCLUSION

4.1. Conclusion

The aim of this work was to develop a LC-MS/MS analytical method for the detection of polypeptide antibiotics bacitracin, colistin and polymyxin in food. First the mass-spectrometric measurement was developed. For this purpose, the substances of interest were measured with the MS/MS system while optimising ionisation conditions and formation of fragments. Triply charged ions showed higher intensities under the present conditions, compared to doubly charged ones. The composition of the employed reference standards was determined by mass spectrometric measurements. The successful detection of the analytes was followed by the establishment of a liquid chromatographic separation. Various tested reversed-phase C_{18} columns operated successfully, while a Thermo Scientific HyPurity column was chosen in the end. Chromatographic parameters were then optimised in order to obtain a sufficient, but also fast separation, which resulted in a gradient program with a total run time of 15 minutes. This instrumental part was followed by the development of an appropriate sample preparation protocol. Acidic solvent extraction combined with solid phase clean-up showed promising results. Each step of the sample preparation was optimised to obtain stable and high recovery values. Finally the overall method was successfully validated for milk and meat based on the European Commission regulation 2002/657/EC and showed good performance.

The here developed method will be shortly summarised in the following:

(1) Sample preparation: Animal muscles samples were weighted in representative portions of 1 g, while 2.5 g of milk were used. After the addition of 10 ml of MeOH/H_2O (1:1) extraction solution the samples were homogenised for 45 seconds in the case of solid samples, while milk was shaken for 5 minutes. Then 0.5 ml of 6 M HCl was added (1 ml in the case of milk) and another homogenisation took place. The mixture was centrifuged at 4600 rpm for 5 minutes. 5 ml of supernatant were taken and mixed with 15 ml of H_2O prior to clean-up and solid phase extraction. SPE columns were first conditioned with 3 ml MeOH and then 3 ml H_2O. The whole 20 ml water sample mixture was then loaded on to the column. This was followed by a washing step using 3 ml 10% MeOH solution. Elution took place with 2 ml 0.2/70/30

HFA/MeOH/H_2O. 500 µl of 10% ethylene glycol in ethanol was added as a keeper solution. Samples were concentrated through organic solvent evaporation using a gentle stream of nitrogen at 40°C and a block heater. The remaining leftover of the sample was filled up to 1 ml of final volume with 950 µl of 0.1/10/90 HFA/MeOH/H_2O. These extracts were then filled into vials for LC/MS/MS analysis.

(2) Chromatographic separation: Eluents for liquid chromatography were 0.1% HFA in water and 0.1% HFA in acetonitrile, while a gradient elution program as listed in Table 2-3 was used. The chromatographic lasted for 15 minutes under reversed-phase conditions. A HyPurity 150x3 mm, 5 µm (ThermoScientific) or similar column can be employed.

(3) Mass spectrometry: The mass spectrometric parameters for MRM measurement are listed in Table 2-4, together with the ionisation conditions as specified in Table 2-5.

In the future further steps could include:

- the evaluation of the method during ring trials with different laboratories,
- the addition of a more suitable internal standard such as deuterated bacitracin,
- and the validation of the method for further matrices like egg.

Furthermore a possible integration of the method into a multi method for the parallel detection of further antibiotics such as spiramycin, tylosin and virginiamycin could be considered. Currently in residue analysis there is an emerging trend in the development of methods that are capable of monitoring a wide variety of compounds of similar, but also different chemical classes, in a single run (Turnipseed *et al.*, 2008). Some methods which include the analysis of zinc bacitracin in feed are already reported (Boscher *et al.*, 2010, De Alwis and Heller, 2010, Gibson *et al.*, 2012, van Poucke *et al.*, 2003). Furthermore a multi-residue method for confirmatory determination of antibiotics in milk was presented by Samanidou and Nisyriou (2008).

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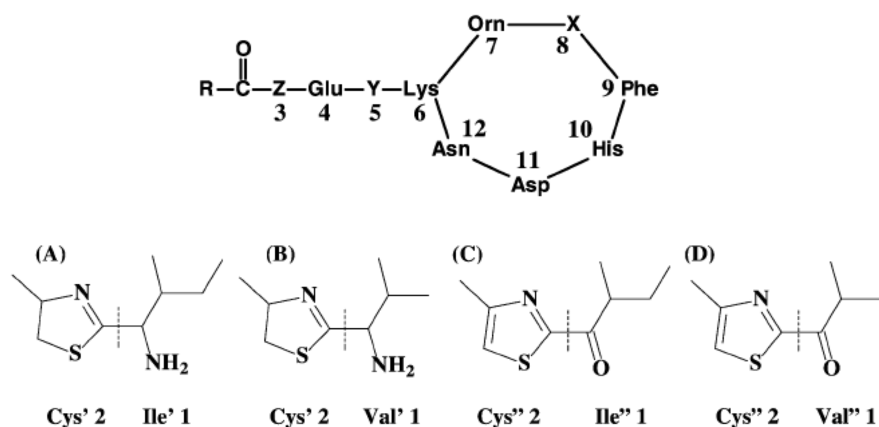
APPENDICES

Appendix A Structural information

A.1 Bacitracin

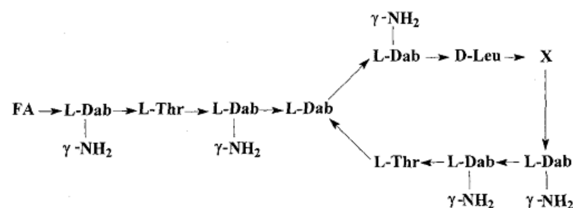


Measured monoisotopic mass	X (8)	Y (5)	Z (3)	R	Peak	Sequenced bacitracins	Ikai *et al* Bacitracin	Siegel *et al* Bacitracin	Morris Bacitracin
1421.7	Ile	Ile	Leu	(A)	14	A	A	A	A
1407.7	Ile	Ile	Leu	(B)	10	B_1	B_1	B_{1b}	B_1
1407.7	Val	Ile	Leu	(A)	11	B_2	B_2	B_{1a}	B_2
1407.7	Ile	Val	Leu	(A)	12	B_3	B_3	B_2	B_3
1393.7	Val	Ile	Leu	(B)	6	C_1	D_1	C_{1c}	C_1
1393.7	Ile	Val	Leu	(B)	7	C_2	D_2	C_{1b}	C_2
1393.7	Val	Val	Leu	(A)	8	C_3	D_3	C_{1a}	C_3
1379.7	Val	Val	Leu	(B)	4	D	E	D_{1a}	D
	Val	Val	Val	(A)				D_{2a}	
1418.7	Ile	Ile	Leu	(C)	22	F	F		
	Ile	Ile	Leu	(D)			H_1		
1404.7	Val	Ile	Leu	(C)	19	H_2	H_2		
1404.7	Ile	Val	Leu	(C)	21	H_3	H_3		
1404.7	Ile	Ile	Val	(C)	18	H_4			
	Val	Ile	Leu	(D)			I_1		
	Ile	Val	Leu	(D)			I_2		
	Val	Val	Leu	(C)			I_3		
1-epimers of bacitracin									
1421.7	Ile	Ile	Leu	(A)*	13	1-epi-A		A_{2a}	
1407.7	Ile	Ile	Leu	(B)*	9	1-epi-B_1			
	Val	Ile	Leu	(A)*		1-epi-B_2			
1393.7	Val	Ile	Leu	(B)*	5	1-epi-C_1			

(A)* position 1 D-Ile instead of L-Ile
(B)* position 1 D-Val instead of L-Val

Figure A-1. Structure of Bacitracin (adapted from Govaerts *et al.*, 2003b)

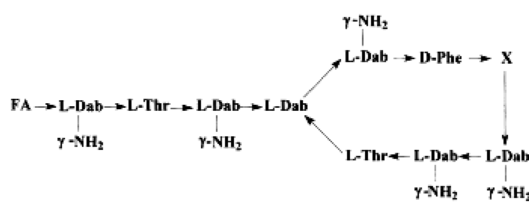
A.2 Colistin and Polymyxin



Polymyxin	X	FA
E_1*	L-leucine	6-MOA
Ile-E_1*	L-isoleucine	6-MOA
Nva-E_1	L-norvaline	6-MOA
Val-E_1	L-valine	6-MOA
E_2	L-leucine	6-MHA
Ile-E_2	L-isoleucine	6-MHA
Nva-E_2	L-norvaline	6-MHA
Val-E_2*	L-valine	6-MHA
E_3	L-leucine	OA
E_4	L-leucine	HA
E_7**	L-leucine	7-MOA
Ile-E_8**	L-isoleucine	7-MNA

* Isolated compounds
** New compounds

Figure A-2. Structure of Colistin (adapted from Orwa *et al.*, 2001a); Dab, diaminobutyric acid; FA, fatty acid; 6-MOA, 6-methyloctanoic acid; 6-MHA, 6-methylheptanoic acid; OA, octanoic acid; HA, heptanoic acid; 7-MOA, 7-methyloctanoic acid; 7-MNA, 7-methylnonaoic acid.



Polymyxin	X	FA	Calculated mol. wt.	MS +ve mode singly charged	MS +ve mode doubly charged
B_1	L-leucine	6-MOA	1202	1203.6	602.7
Ile-B_1	L-isoleucine	6-MOA	1202	1203.5	602.6
B_2	L-leucine	6-MHA	1188	1189.6	595.5
B_3	L-leucine	OA	1188	1189.7	595.6
B_4	L-leucine	HA	1174	1175.6	588.6
B_5	L-leucine	NA*	1202	1203.7	602.6
B_6	L-leucine	3-OH-6-MOA*	1218	1219.6	610.6

*New

Figure A-3. Structure of Polymyxins (adapted from Orwa *et al.*, 2001b); Dab, diaminobutyric acid; FA, fatty acid; 3-OH-6-MOA, 3-hydoxy-6-methyloctanoic acid; 6-MOA, 6-methylocta-noic acid; 6-MHA, 6-methylheptanoic acid; OA, octanoic acid; NA, nonanoic acid; HA, heptanoic acid.

Appendix B Mass spectrometry

B.1 Tuning results (double-charged ions)

B.1.1 Bacitracin

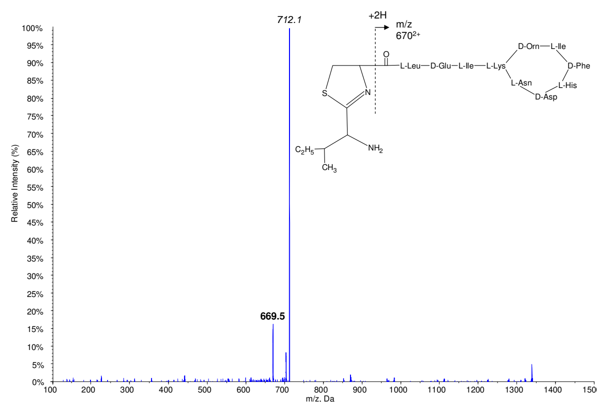


Figure B-4. Product ion spectrum of bacitracin A; double-charge precursor ion with m/z 712.1; product ion is 669.5.

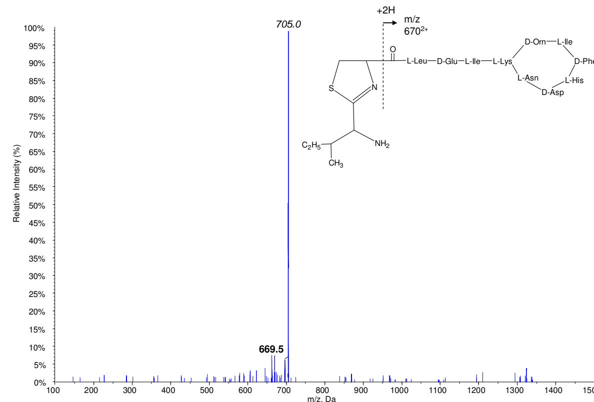


Figure B-5. Product ion spectrum of bacitracin B; double-charge precursor ion with m/z 705.0; product ion is 669.5.

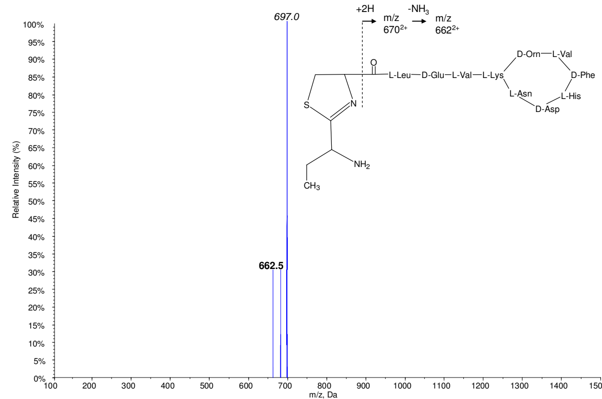
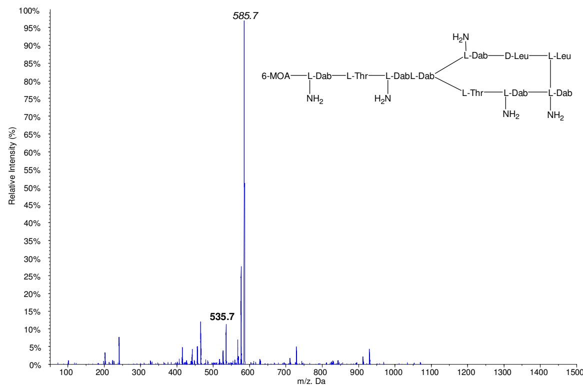


Figure B-6. Product ion spectrum of bacitracin C; double-charge precursor ion with m/z 697.0; product ion is 662.5.

B.1.2 Colistin



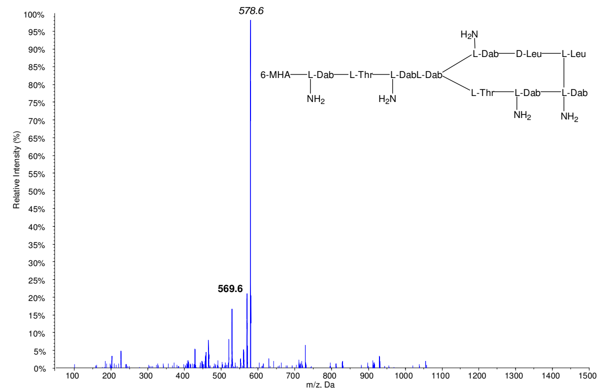
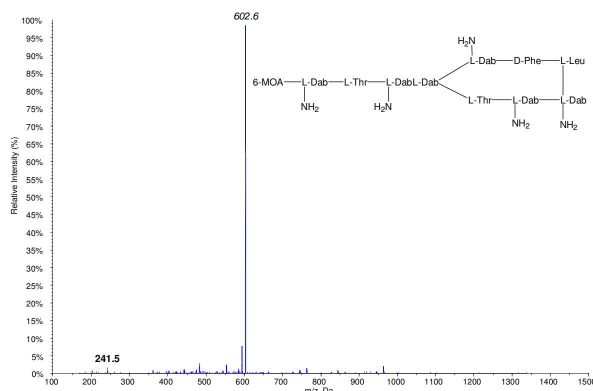


Figure B-7. Product ion spectrum of colistin A; double-charge precursor ion with m/z 585.7; product ion is 535.7.

Figure B-8. Product ion spectrum of colistin B; double-charge precursor ion with m/z 578.6; product ion is 569.6.

B.1.3 Polymyxin



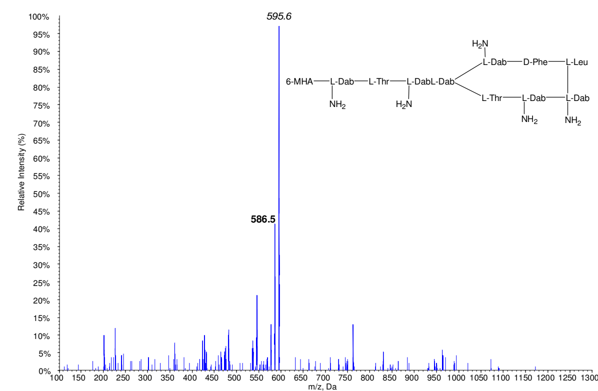


Figure B-9. Product ion spectrum of polymyxin B_1; double-charge precursor ion with m/z 602.6; product ion is 241.5.

Figure B-10. Product ion spectrum of polymyxin B_2; double-charge precursor ion with m/z 595.6; product ion is 586.5.

B.2 MRM transitions

Table B-1. All mass spectrometric parameters of the reference substances

Substance	Ion	Precursor	MRM ion	DP [V]	EP [V]	CE [V]	CXP [V]
Bacitracin A	$[M+2H]^{2+}$	712.5	670	110	12	32	10
			704.1	110	12	33	10
			870.4	110	12	36	13
			1339	110	12	35	12
Bacitracin A	$[M+3H]^{3+}$	475.3	86.1	70	12	60	10
			356.2	70	12	26	10
			470	70	12	20	10
			447.2	70	12	21	10
			661.7	70	12	23	10
			670.1	70	12	20	10
			869.4	70	12	23	10
Bacitracin B	$[M+2H]^{2+}$	705	669.5	100	12	34	10
			696.8	100	12	34	10
Bacitracin B	$[M+3H]^{3+}$	470.3	662.6	70	12	20	10
			669.6	70	12	20	10
			869.5	70	12	23	10
Bacitracin C	$[M+2H]^{2+}$	697.9	662.5	140	11	32	10
			688.8	140	11	30	10
Bacitracin C	$[M+3H]^{3+}$	465.4	227	70	12	31	10
			662.4	70	12	19	10
Bacitracin F	$[M+2H]^{2+}$	710.6	869.5	100	11	36	10
			309.2	100	11	43	10
			280.9	100	11	53	10
			702.1	100	11	32	10
Bacitracin F	$[M+3H]^{3+}$	475	670	85	11	20	10
			556.7	85	11	20	10
			869.5	85	11	23	10
Colistin A	$[M+2H]^{2+}$	585.5	465.6	130	12	27	10
			535.5	130	12	27	10
			541.5	130	12	32	10
			576.8	130	12	26	12
Colistin A	$[M+3H]^{3+}$	390.7	241.3	70	13	20	10

			384.9	70	13	17	11
			456.7	70	13	17	10
			465.6	70	13	21	10
Colistin B	$[M+2H]^{2+}$	578.6	227.4	115	12	28	10
			528.8	115	12	33	10
			569.8	115	12	25	10
			728.6	115	12	28	10
Colistin A	$[M+3H]^{3+}$	386.1	101	75	12	26	10
			227.3	75	12	21	10
			374.5	75	12	19	10
			380.2	75	12	17	10
			465.6	75	12	17	10
Polymyxin B_1	$[M+2H]^{2+}$	602.6	241.5	120	11	32	10
			593.8	120	11	27	10
			482.4	120	11	27	10
			552.8	120	11	27	10
Polymyxin B_1	$[M+3H]^{3+}$	402.1	101.2	75	12	26	10
			241.2	75	12	29	10
			390.5	75	11	20	10
			396.1	75	11	18	12
			482.6	75	12	17	10
Polymyxin B_2	$[M+2H]^{2+}$	595.6	227.1	100	11	32	10
			545.6	100	11	27	10
			586.5	100	11	24	10
			762.4	100	11	24	10
Polymyxin B_2	$[M+3H]^{3+}$	397.2	385.6	70	10	20	10
			391.8	70	10	18	10
			482.5	70	10	18	10

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EDUCATION AND QUALIFICATIONS

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Oct 2011 – Sept 2012	*Cranfield University, Bedfordshire, United Kingdom* **MSc Analytical Biotechnology (Double Degree)** Thesis: Development of a reagentless aptasensor for the detection of kanamycin A (taking place at University of Linköping, Sweden)
Feb – Jul 2011	*University of Copenhagen, Faculty of Life Sciences, Denmark* **Exchange semester**
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