The determination of Veterinary Antibiotics in live animals and animal products.

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Declaration

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I Abstract

"The development of antibiotic resistance in bacteria has been attributed to the use of antimicrobials in human medicine. The contributions of veterinary medicine and agriculture to antibiotic resistance are still being investigated."[1]

Hence, there is pressure on analytical scientists to detect and confirm the presence of antimicrobials in foods of animal origin. The aminoglycosides and macrolides are two families of antibiotics that are very similar in structure and have important applications in veterinary medicine. These antibiotics are widely used in the treatment of bacterial infections e.g. aminoglycosides for enteritis and mastisis and macrolides for enteric infections. They have also been used as feed additives for growth promoting. As a result, legislation has been laid down by the European commission in which member states must meet strict criteria for monitoring these residues[2].

This thesis was undertaken to develop a UPLC-MS/MS method for the simultaneous analysis of the aminoglycoside and macrolide antibiotics. This is the first time that a combination method for the two classes of compound was developed. A Waters Acquity/ Premier XE system with a dual ESI/APCI probe allowed for optimisation of detection for each component within a single run. For all target compounds the optimum MS ionisation mode and conditions were determined experimentally. The chromatographic conditions were investigated in order to improve separation, reduce analytical run times and meet validation requirements as per Commission Decision 2002/657/EC. While the scope of this thesis did not allow for the method to be expanded to cover the many sample matrices/ species that are required by the EU it is hoped that this will be investigated in the future. This thesis did however contain a comprehensive literature review of the current techniques employed to analyse for these residues both qualitatively and quantitatively. Sample preparation was researched and an extraction experiment was carried out comparing various solid phase extraction columns and discussed in detail in chapter 2. This experiment looked at various extraction methods to minimise matrix effects and optimise recoveries. Finally, the novel method developed and optimised for the analysis of the macrolide and aminoglycoside antibiotics using UPLC-MS/MS is presented. This will be applied to the analysis of real samples in the Central Meat Control laboratory which is a National Reference Laboratory (NRL) and expanded to cover the many species required for e.g. bovine, ovine and porcine tissues.

II Aknowledgements

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Finally, I would like to thank my family. My mum and dad, for too much to mention here, they always kept me going when I felt like I couldn't. To my husband David, for helping keep things in perspective and giving me emotional support. My sons Caden & Sam, for keeping me focused on the important things and my little dog Jack for making me get some fresh air now and again!.

III *List of Figures:*

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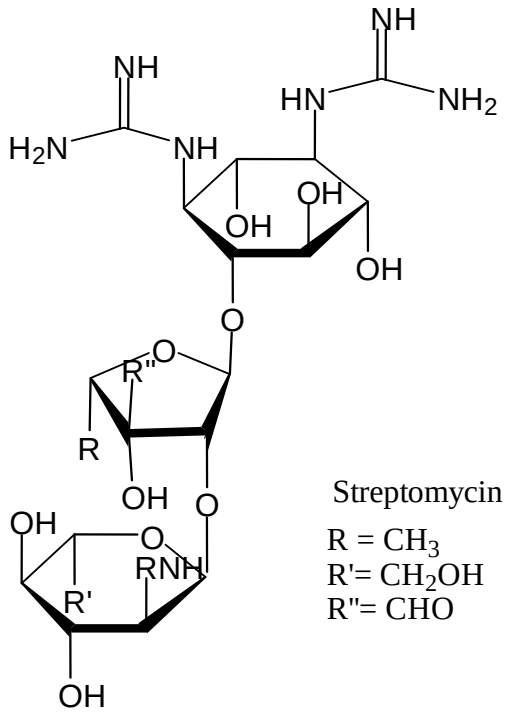
V ***Abbreviations***

ACN	Acetonitrile
AMG	Aminoglycoside
API	Atmospheric pressure ionisation
APCI	Atmospheric pressure chemical ionisation
ASE	Accelerated liquid extraction
CE	Capillary Electrophoresis
CMCL	Central Meat Control Laboratory
CVM	US Centre for Veterinary Medicine
2-D	Two-dimensional
DAD	Diode array detector
EIA	Enzyme Immunoassay
ELISA	Enzyme linked immunosorbent assay
ELSD	Evaporative light scattering detection
ESI	Electrospray ionisation
EU	European Union
EMIT	Enzyme multiplied immunoassay technique
FPT	Four-Plate Test
GC	Gas Chromatography
HFBA	Heptafluorobutyric acid
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IR	Infrared
LC	Liquid Chromatography
LIF	Laser induced fluoresence
LLE	Liquid-liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
MeOH	Methanol
MS	Mass Spectrometry
MSPD	Matrix solid phase dispersion
MRL	Maximum residues limit
MRM	Multiple reaction monitoring
PED	Pulsed electrochemical detection
PLE	Pressurised liquid extraction
NRL	National Reference Laboratory
RRS	Resonance Rayleigh scattering
SIM	Selected ion-monitoring
S/N	Signal to noise
SLM	Supported liquid membrane
SPE	Solid-phase extraction
SPR	Surface plasmon Resonance
TCA	Trichloroacetic acid
TIC	Total ion current
TLC	Thin Layer chromatography
TOF	Time of flight
UPLC	Ultra performance liquid chromatography
UV-VIS	Ultraviolet-Visible

Chapter 1: An Introduction to the Aminoglycoside and Macrolide antibiotics used in animal products.

1.1. Background

Aminoglycosides are a large class of antibiotics that are characterised by one or more amino sugars linked by glycosidic bonds to an aminocyclitol component. Aminoglycosides are classified according to the pattern of substitution of the cyclitol. The two most important subclasses are: 4, 5-disubstituted deoxystreptamine e.g. neomycin and 4, 6-disubstituted deoxystreptamine e.g. gentamicin, kanamycin[2]



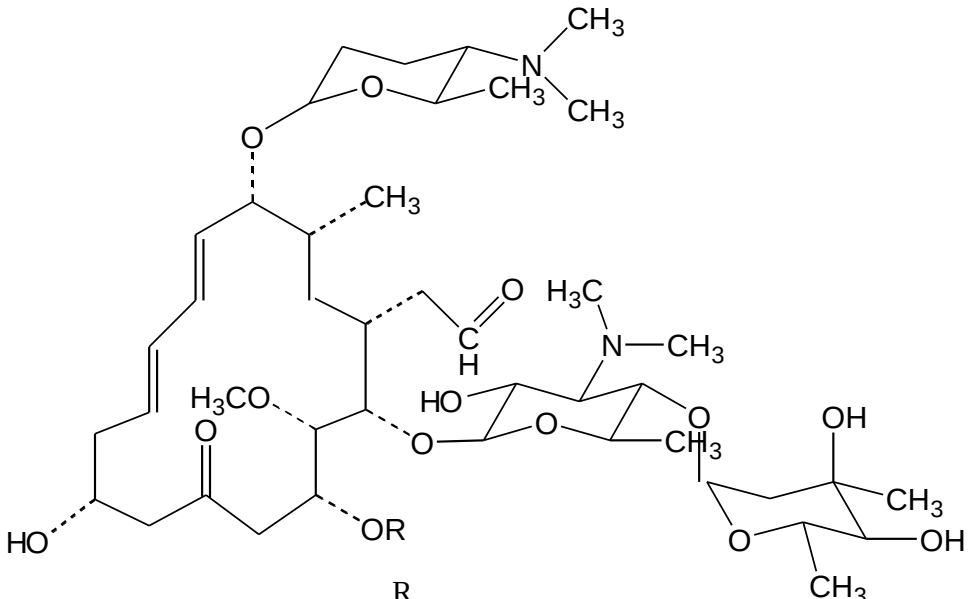
Streptomycin	Dihydrstreptomycin
R = CH_3	= CH_3
R'= CH_2OH	= CH_2OH
R''= CHO	= CH_2OH

Figure 1.1 Some Aminoglycoside Structures, Streptomycin (MW 581.57 $C_{21}H_{39}N_7O_{12}$) and Dihydrostreptomycin (MW 583.59 $C_{21}H_{41}N_7O_{12}$)

The aminoglycosides are polycationic compounds that contain an aminocyclitol and an amino sugar joined to a ribose unit. The aminoglycosides interfere with bacterial protein synthesis by binding irreversible to ribosome and could cause cell membrane damages. Bacterial resistance enzymes may inactivate them but bacteria could also display resistance through ribosomal modifications or by decreased uptake of antibiotic into the bacterial cell. Aminoglycosides are widely distributed in the body after injection and little is absorbed from the gastro-intestinal tract. They are excreted unchanged in the urine.

Streptomycin (Figure 1.1) is an aminoglycoside antibiotic produced by *Streptomyces griseus* and it is active against many gram-negative bacteria. Streptomycin is used in veterinary medicine. If this antibiotic is incorrectly used, residues may be found in large concentrations in foodstuffs from animal origin and represent a risk for the consumer and (or) a disruptive element for the manufacturing processes adopted by the food industry.

The macrolides are characterised by a macrocyclic lactone ring containing 14, 15 or 16 atoms with sugars linked via glycosidic bonds[3]. The macrolides with 16 atoms in the lactone ring represent the most commonly used macrolides in veterinary medicine and examples of these include tylosin (Figure 1.2) and spiramycin. Erythromycin (Figure 1.2) is another example of a macrolide antibiotic; it contains 14 atoms[3] and is produced by *Streptomyces erythrues.* It is active against gram-positive and some gram-negative bacteria[3]. Like the aminoglycosides the macrolide mode of action is protein synthesis inhibition, while the aminoglycosides bind to the 30S ribosomal subunit, the macrolides bind to the 50S ribosomal unit[4].



	R
Spiramycin I	H
Spiramycin II	$COCH_3$
Spiramycin III	$COCH_2CH_3$

Figure 1.2: A Macrolide Structure, Spiramycin (MW 843.0 $C_{43}H_{74}N_2O_{14}$)

The Aminoglycoside and macrolide groups of antibiotics are used in veterinary medicine and animal husbandry particularly for treatment of bacterial infections for e.g. mastitis, or for prophylaxis. They are banned for used as growth promoters in the E.U. [2] Legislation monitoring these residues in live animals and animal products are given in E.U. Council Directive 96/23/EC[5], S.I. 507/98 and E.U. Commission Decision 2002/657/EC[6].

In terms of their chemistries, the aminoglycosides are polar, resistant to acids, bases and heat and are not extensively bound to proteins[7]. Although some work has been carried out to date on this class of compound there is still huge potential for further research. The macrolides are more hydrophobic molecules and are unstable in acid[7]. The pKa values for the macrolides range from 7.4 for tylosin A to 8.8 for erythromycin[8]. They are soluble in methanol and range in molecular weight from 734 amu for erythromycin to 916 amu for tylosin.

1.2 Legislation

The increasing awareness of food safety by the consumer with respect to antimicrobial resistance has resulted in increasing pressure on laboratories responsible for food safety to monitor the use of these drugs and ensure the safety of food. There are increasingly resistant antibiotic strains of bacteria that are causing a threat to animal and human health. This interest is due primarily to the emergence and dissemination of multiple antibiotic resistant zoonotic bacterial pathogens[9].

As a result, to ensure that the aminoglycosides and macrolides are not used in non-approved situations and to control their use in meat producing animals, samples are taken at slaughterhouses and screened for the presence of residues. Analysis of positive screening tests for these residues in animal products must adhere to

legislation laid out in Council Directive 96/23/EC[5], S.I. 507/98 Commission Decision 2002/657/EC[6] whereby suitable confirmatory methods are based on chromatographic analysis using spectrometric detection[6].

Council regulation (EEC) 2377/90[10] of 26 June 1990 lays down the Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. See Table 1.1, 1.2 and 1.3 for details. Where a residue refers to: "***all pharmacologically active substances, whether active principles, excipients or degradation products, and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal product in question has been administered.***"

The definition of maximum residue limit according to this regulation is given as: "***the maximum concentration of residue resulting from the use of a veterinary medicinal product (expressed in mg/kg or µg/kg on a fresh weight basis) which may be accepted by the Community to be legally permitted or recognized as acceptable in or on a food."***

Confirmatory methods for these compounds must provide information on the chemical structure of the analyte. As a consequence methods that are based on chromatographic analysis only, without the use of spectrometric detection are unsuitable. However, if a single technique lacks the required specificity, a combination of techniques may be used. Table 1.4 details suitable confirmatory methods for the Aminoglycosides (Group B) compounds. A review paper by Rivier[11] details the criteria for the identification of compounds by LC/MS and LC/MS-MS and in particular the confirmation approach to comply with the European Union (EU) criteria for trace level organic analysis is presented.

Table 1.1 Pharmacologically active substances are divided into four Annex[10]

Annex I	Substances in respect of which maximum residue limits have been established.
Annex II	Substances for which it appears that it is not necessary for the protection of public health to establish maximum residue limits.
Annex III	Substances in respect of which provisional maximum residue limits have been established.
Annex IV	Where it appears that a maximum residue limit cannot be established in respect of a pharmacologically active substance used in veterinary medicinal products because residues of the substances concerned, at whatever limit, in foodstuffs of animal origin constitute a hazard to the health of the consumer

Table 1.2 Some aminoglycosides and macrolides with established MRL's (Annex I)[10]

Pharmacologically active substances	Marker Residue	Animal Species	MRL's	Target Tissues
Neomycin (including framycetin)	Neomycin B	All food producing species	500 µg/kg	Muscle
			500 µg/kg	Fat
			500 µg/kg	Liver
			5000 µg/kg	Kidney
			1500 µg/kg	Milk
			500µg/kg	Eggs
Kanamycin	Kanamycin A	All food producing species except fish	100 µg/kg	Muscle
			100 µg/kg	Fat
			600 µg/kg	Liver
			2500 µg/kg	Kidney
			150 µg/kg	Milk
Erythromycin	Erythromycin A	All food producing species	200 µg/kg	Muscle
			200 µg/kg	Fat
			200 µg/kg	Liver
			200 µg/kg	Kidney
			40 µg/kg	Milk
			150 µg/kg	Eggs
Gentamicin	Sum of Gentamicin C1, C1a, C2 and C2a	Bovine Porcine	50 µg/kg	Muscle
			50 µg/kg	Fat
			200 µg/kg	Liver
			750 µg/kg	Kidney
		Bovine	100 µg/kg	Milk

Table 1.2 Some aminoglycosides and macrolides with established MRL's (Annex I) Contd[10].

Pharmacologically active substances	Marker Residue	Animal Species	MRL's	Target Tissues
Streptomycin	Streptomycin	All ruminants	500 µg/kg	Muscle
			500 µg/kg	Fat
			500 µg/kg	Liver
			1000 µg/kg	Kidney
			200 µg/kg	Milk
Tylosin	Tylosin A	All food producing species	100 µg/kg	Muscle
			100 µg/kg	Fat
			100 µg/kg	Liver
			100 µg/kg	Kidney
			50 µg/kg	Milk
			200 µg/kg	Eggs

Table 1.3 Some aminoglycosides and macrolides with Provisional MRL's (Annex III)[10]

Pharmacologically active substances	Marker Residue	Animal Species	MRL's	Target Tissues
Aminosidine (paromomycin)	Aminosidine (paromomycin)	Bovine Porcine Rabbits Chicken	500 µg/kg	Muscle
			1500 µg/kg	Liver
			1500 µg/kg	Kidney

Table 1.4 Suitable confirmatory methods for organic residues or contaminants[6]

Measuring Technique	Substances Annex 1 96/23/EC	Limitations
LC or GC with mass-spectrometric detection	Groups A and B	Only if following either an on-line or an off-line chromatographic Separation Only if full scan techniques are used or using atleast3 (group B) or 4 (group A) identification points for techniques that do not record the full mass spectra.
LC or GC with IR spec detection	Groups A and B	Specific requirements for absorption in IR spectrometry have to be met.
LC-full-scan DAD	Group B	Specific requirements for absorption in UV spectrometry have to be met
LC –fluorescence	Group B	Only for molecules that exhibit native fluorescence and to molecules that exhibit fluorescence after either transformation or derivatisation
2-D TLC – full-scan UV/VIS	Group B	Two-dimensional HPTLC and co-chromatography are mandatory
GC-Electron capture detection	Group B	Only if two columns of different polarity are used
LC-immunogram	Group B	Only if at least two different chromatographic systems or a second, independent detection method are used
LC-UV/VIS (single wavelength)	Group B	Only if at least two different chromatographic systems or second, independent detection method are used.

1.3 Analytical Approaches to Macrolide and Aminoglycoside Determination

The aminoglycosides are a group of compounds that are polar, resistant to acids, bases and heat and are not extensively bound to proteins[7]. Although some work has been carried out to date on this class of compounds there is still huge potential for further research. The macrolides are more hydrophobic molecules and are unstable in acid[5]. In an excellent review paper by Stead[2] in 2000 the current methodologies for the analysis of aminoglycosides and macrolides are discussed with focus on both qualitative and quantitative methods. This literature review will focus especially on the current methodologies post 2000 and in particular for the analysis of aminoglycosides and macrolides used in veterinary medicine with reference to the European legislation Commission Decision 2002/657/EC[6].

A lot of the research (Table 1.5) focuses on bovine tissue and in many cases does not cover the diverse range of this antibiotic family. There are many factors which need to be addressed in order to develop a method which would be capable of analysing the range compounds to the required level e.g. pH, extraction methods and mobile phase. Over the next few chapters this will be explored in more detail.

Confirmatory methods must fall within strict criteria in order to comply with legislation as discussed in section 1.2. Detection of low levels of aminoglycoside residues in animal products by mass spectrometry is one of the more difficult analytical problems[12]. For example, for group B compounds there must be at least 3 identification points which means a parent mass and two daughter products are necessary. In a validated method by Heller[12] *et al*., the confirmation of gentamicin and neomycin by ESI/ ion trap tandem mass spectrometry at 30 pg/µl in milk was presented.

Posyniak[13] *et al.*, presents a HPLC method with fluorescence detection limits of quantitation for gentamicin and neomycin of 0.1 and 20 pg/µl respectively, in muscle, liver or kidney tissue. The determination of gentamicin in hospital wastewater by LC-MS/MS is described in a paper by Loffler[14] *et al.*, with limits of quantification of 0.2 pg/µl. Bruijnsvoort[15] *et al*., presents an LC/MS/MS method with a limit of quantitation for streptomycin of 2 pg/µl in honey and 10 pg/µl in milk and for dihydrostreptomycin these limits were a factor of 2 lower. A recent publication by Kaufmann[16] *et al*., described a method capable of reaching levels between 15 and 40 pg/µl for 11 aminoglycosides in a range of matrices including pork muscle and veal liver with run times of 15 minutes.

For the macrolides a number of sensitive methods have been reported. Detection limits of 0.4 ppb for erythromycin and 4ppb for tylosin in bovine meat by enzyme-linked immunosorbent assay (ELISA) are possible[17]. Tylosin and lincomycin residues in honey by LC-MS/MS gave detection limits of 5 and 2 ppb for lincomycin and tylosin, respectively[18]. Detection limits of 50 ppb for josamycin, kitamycin, microsamicin, spiramycin and tylosin were reported using HPLC[19]. A multi-residue method for seven macrolides including spiramycin, erythromycin and tylosin in poultry muscle by LC-MS achieved detection limits in the range 1-20 ppb[20].

Table 1.5 Summary of Techniques for macrolide compounds

Reference	Compound analysed	Sample	Sample clean-up	Recovery (%)	Method	Range	Sensitivity
Granelli[21] *et al*., 2007	Tylosin & spiramycin	Muscle & kidney various species	Solvent extraction	80-86 from porcine muscle	LC-MS/MS	0 –4MRL	0.5 MRL
Xu[22] *et al*., 2007	Eight macrolides	Honey	SPE	60 – 130	LC-MS/MS	2-40 ppb	0.2 ppb
Civitareale[23] *et al*. 2004	Spiramycin, Tylosin	Animal feed	SPE –CN columns	99-74 spiramycin 81-53 tylosin	HPLC-UV/DAD	---	176 ppb Spiramycin 118 ppb Tylosin
Cherlet[24] *et al.* 2002	Tylosin	Porcine tissue	SCX- SPE	---	LC-MS/MS	50 – 200 ppb	0.2 – 0.8ppb
Codony[20] *et al*. 2002	Seven macrolides	Poultry muscle	Cation exchange SPE	56-93	LC-MS	Up to 1000 ppb	4 – 35 ppb
Berrada[25] *et al*., 2007	Seven macrolides	Bovine Liver and kidney	SPE	40 – 93	LC-DAD & LCMS	50-1000 µg/kg	CCα 60 – 1005 µg/kg
Gracia-Mayor[26]*et al*., 2006	Seven macrolides	Ovine milk	NaOH and ethyl acetate	55 –77	LC-UV/DAD	---	24 –72 ppb

Table 1.6 Summary of Techniques for aminoglycoside compounds

Reference	Compound analysed	Sample	Sample clean-up	Recovery (%)	Method	Range	Sensitivity
Bogialli[27] *et al*., 2005	Nine AMGs (incl. streptomycin & apramycin)	Bovine milk	Off-line hot water extraction	79 –92	LC-MS/MS	0.2 – 400 ppb	LOQ 2 ppb – 13 ppb
Bruijnsvoort[15] *et al*., 2004	Streptomycin & DHstrep	Bovine milk Honey	LLE SPE	81- 102	LC-MS/MS	50- 800 µg/kg (milk)	LOQ 1-10 µg/kg
Hornish[28] *et al*., 1998	Spectinomycin	Bovine kidney, liver, muscle and fat	SPE	80	LC-MS/MS	0.1 – 10 mg/g	LOQ 0.1 mg/g
Carson[29] *et al*., 1998	Spectinomycin	Bovine milk	Ion-pair SPE	69- 93	LC-MS/MS	0.1-5 mg/mL	LOQ 0.05- 0.1 mg/mL
Hammel[30] *et al*., 2008	3 AMGs, 7macrolides	Honey	Liquid -liquid extraction	28-214 amgs. 28–104 macrolides	LC-MS/MS	Matrix matched 6 point calibration	LOD 20 µg/kg
McLaughlin[31] *et al*., 1994	6 AMGs	Bovine Kidney	Matrix solid phase dispersion	---	LC-MS/MS	---	---

1.4 Macrolides and Aminoglycosides

The most commonly used aminoglycosides in veterinary medicine in Europe are gentamicin[32] along with neomycin, streptomycin and dihydrostreptomycin. The most commonly used macrolides are erythromycin and tylosin[32]. The use of aminoglycosides and macrolides for their growth promotion properties is banned in the EU. Therefore it is impossible to rule out the use of other members of this family e.g. spectinomycin and kanamycin which both have an established MRL.

Much of the research to date has focused on individual members of the family for e.g. a publication by Loffler[14] *et al.*, presents an analytical method for the determination of gentamicin in hospital wastewater by LC-MS/MS using kanamycin as an internal standard. In another method gentamicin and neomycin in animal tissue were investigated in terms of sample preparation[13].

Some authors have investigated the determination of a number of analytes, from the one family, in the same assay in bovine tissues by ion-pair LC-MS[31]. These analytes include spectinomycin, hygromycin B, streptomycin and dihydrostreptomycin This multi-residue method for the confirmation of aminoglycosides in bovine tissue used matrix solid-phase extraction (SPE) to isolate the aminoglycosides. It is clear that while researchers are developing useful methods for aminoglycosides and macrolides (Tables 1.5 and 1.6) there is a need for suitable multi-analyte confirmatory methods that would include the compounds with both established and provisional MRL values in the same assay.

Due to the safety issues surrounding these compounds, and the MRL's associated with them, there is huge pressure on the analytical assays to be extremely sensitive. The

trend has been to see more and more LC-MS methods being reported for this area of research. The review by Stead[2] investigated both qualitative and quantitative methodologies for the analysis of aminoglycosides. In that paper, the techniques most commonly used for the aminoglycosides were automated immunoassays for screening of aminoglycosides and HPLC with MS detection for quantitation. Limits of detection were of the order 50 ppb for streptomycin in milk by HPLC[33] and 70 ppb for spectinomycin in tissue extract by HPLC[34].

1.5 Aims and Objectives

This chapter has presented a thorough background into the chemistry of these two groups of antibiotics and the legislation covering their permitted use in animal products. Various methods in the literature have been tabulated to show the current research that has been carried out and summarises the extraction methods employed together with the individual anlaytes and the techniques employed together with their sensitivity. While it is clear from the above that some work on these compounds has been carried out, a suitable confirmatory method that would cover the compounds with established MRL's (Table 1.2) and provisional MRL's (Table 1.3) would be very useful. This forms the basis of this research. In summary, while it is apparent that low LOD values can be achieved, this can vary widely depending on the analyte being determined, the sample preparation, the technique used and the sample matrix. Another issue is that it can be difficult to reach the required sensitivity levels for all the analytes within one run.

References

1. Teuber M. (2001). *Current Opinion in Microbiology,* **4;** 493-499
2. Stead D. A. (2000). *Journal of Chromatography B,* **747;** 69-93
3. Kanfer I., Skinner M.F., Walker R.B. (1998). *Journal of Chromatography A,* **812**; 255-286
4. Tenover, F. C. (2006). *American Journal of Infection Control,* **34(5, Supplement 1);** S3-S10
5. Council Directive 96/23/EC, *Off. J. Europ. Comm.,* 1996; **L125,** 10.
6. Commission Decision 2002/657/EC; *Off. J. Europ. Comm.,* 2002; **L221,** 8.
7. Fedeniuk R.W., Shand P.J. (1998). *Journal of Chromatography A* , **812**; 3-15
8. Kolz, A.C., Ong S.K., Moorman, T. (2005). **60(2)**; 284-289
9. McDermott PF, Zhao S, Wagner DD, Simjee S, Walker RD, White DG. (2002). *Anim Biotechnol*. **13(1);** 71-84
10. Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official *Journal of the European Union* L 224, 18.8.1990, p. 1, as last amended by Commission Regulation (EC) No 1873/2003, *Official Journal of the European Union* L 275 149, 25.10.2003, p. 9
11. Rivier L. (2003). *Analytica Chimica Acta,* **492**; 69-82
12. Heller D.N., Clark S.B., Righter H.F. (2000). *Journal of Mass Spectrometry,* **35**; 39-49

13. Posyniak A, Zmudzki J, Niedzielska J. (2001). *Journal of Chromatography A,* **914;** 59-66

14. Loffler D., Ternes T. A. (2003). *Journal of Chromatography A,* **1000**; 583-588

15. Bruijnsvoort M., Ottink S.J.M., Jonker K.M., de Boer E. (2004). *Journal of Chromatography A,* **1058(1- 2)**; 137- 142

16. Kaufmann A., Maden K. (2005). *Journal of AOAC International*, **88(4);** 1118-1125

17. Draisci R., delli Quadri F., Achene L., Volpe G., Palleschi L., Palleschi G. (2001). *The Analyst,* **126**; 1942- 1946

18. Thompson T.S., Noot, D.K., Calvert J., Pernal S.F. (2005). *Rapid Communications in mass spectrometry,* **19(3);** 309-316

19. Horie M., Saito K, Ishii R., Yoshida T., Haramaki Y., Nakazawa H. (1998). *Journal of Chromatography A,* **812;** 295-302

20. Codony R., Compano R., Granados M., Garcio-Regueiro J.A., Prat M.D. (2002). *Journal of Chromatography A*, **959**; 131-141

21. Granelli, K., Branzell C. (2007). Rapid *Analytica Chimica Acta*, **586(1-2)**; 289-295

22. Xu, J.Z., Wu Z.X. (2007). *Fenxi Huaxue,* **35(2);** 166-170

23. Civitareale, C., Fiori M., Ballerini, A., Brambilla, G. (2004). *Journal of Pharmaceutical and Biomedical Analysis,* **36(2)**; 317-325

24. Cherlet, M., de Baere, S., Croubels, S., de Backer, P. (2002). *Analytica Chimica Acta,* **473(1-2)**; 167-175

25. Berrada, H., Borrull F., Font G., Moltó J.C., Marcé R.M. (2007). *Journal of Chromatography A*, **1157(1-2)**; 281-288

26. García-Mayor, M. A., Garcinuño R. M., Fernández-Hernando P., Durand-Alegría J.S. (2006). *Journal of Chromatography A*, **1122(1-2)**; 76-83

27. Bogialli S, Curini R, Di Corcia A, Laganà A, Mele M, Nazzari M. (2005). *Journal of Chromatography A*, **1067(1 -2)**; 93-100

28. Hornish R. E., Wiest J. R. (1998). *Journal of Chromatography A*, **812**; 123–133

29. Carson M. C., Heller D. N. (1998). *Journal of Chromatography B*, **718;** 95–102

30. Hammel, Y.-A., Mohamed R., Gremaud E., LeBreton M.H (2008). *Journal of Chromatography A*, **1177(1)**; 58-76

31. McLaughlin L.G.; Henion J.D.; Kijak P.J. (1994). *Biological Mass Spectrometry*, **23(7)**; 417-429

32. Diaz-Cruz, M. S., Barcelo, D. (2007). *TrAC Trends in Analytical Chemistry*, **26(6)**; 637-646

33. Edder, P., Cominoli A., Corvi, C. (1999). *Journal of Chromatography A*, **830(2)**; 345-351

34. Bergwerff A.A., Scherpenisse P., Haagsma N. (1998). *Analyst*, **123**; 2139 – 2144

Chapter 2: Sample Preparation used in the analysis of the Aminoglycoside and Macrolide antibiotics and presentation of an Experiment to extract Aminoglycoside and Macrolide antibiotics from Bovine Muscle.

2.1 Introduction

The analysis of the aminoglycosides and macrolides in animal tissues is made very challenging due to a number of important factors. The matrices involved are generally difficult by virtue of the fact that they are complex in many ways. Animal tissues like muscle and liver contain many possible interfering substances such as proteins and fats that need to be removed or reduced as much as possible without affecting the residues of interest, in this case the antibiotics. Another challenge, in sample preparation of these antibiotics, is that they can be present in very small quantities and there may be more than just one compound of interest present to be determined. These challenges are of utmost importance when developing a suitable extraction procedure.

2.2 Extraction and Clean-up Methods in the Literature

The target tissues specified by legislation that have to be monitored are such that extraction and clean up methods play a very important role in the overall analysis. Biological matrices like muscle and liver contain many possible interfering substances that need to be removed selectively.

The usual techniques employed for extraction and clean up of antibiotics from biological matrices include protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE)[1]. A good source of information on the methodologies for extraction and clean up of antibiotics in bio matrices can be found in "*Chemical Analysis for Antibiotics used in Agriculture"*[2]. An overview of different antibiotics and their clean-up and extraction from various matrices is given. Another reference for information on methodologies for extraction and clean up of antibiotics in food matrices has been published by Buldini[3] *et al.*, The determination of the macrolides from some matrices has

been reported to be possible with no sample clean-up. In one report, honey samples were diluted and injected directly into the LC-MS/MS system without additional steps such as solid-phase extraction or liquid-liquid extraction[4]. Normally, the issues of matrix interference and blocking of columns or injectors in systems necessitate some sample preparation prior to analysis.

2.2.1 Protein precipitation

Deproteinisation is a commonly method for the extraction/ clean-up step of sample preparation of antibiotics in bio matrices. It is used where removal of interferences is necessary whilst retaining good recoveries of the analytes of interest. An advantage of protein precipitation is that it is a relatively simple and inexpensive off-line procedure. An example of the advantages of deproteinisation was shown in Kowalski[5] *et al.,* where the determination of streptomycin in eggs was achieved using acetonitrile as the deproteinisation solution. A detection limit of 0.12 µg/g and recovery levels of ~72% have been reported[5]. This method demonstrated the effectiveness of deproteinisation using an organic solvent, as eggs are an especially difficult matrix to work with due to their proteinaceous nature. Acids such as trichloroacetic acid or perchloric acid[6,7] can also be used for protein precipitation prior to analysis of food samples.

2.2.2 Liquid-liquid extraction

Liquid-liquid extraction (LLE) has been exploited as an extraction procedure for aminoglycosides and macrolides from complex matrices. In a method published on determination of the aminoglycosides streptomycin and dihydrostreptomycin, milk samples were prepared using LLE[8]. The method was validated over a linear range from 50 to 800 µg kg^{-1}. The recoveries were found to be slightly low at 60% due to matrix

suppression. A number of papers have reported extraction with acetonitrile prior to clean-up of the extracts by LLE with hexane[9,10,11,12]. In some cases, this procedure was followed by solid-phase extraction.

Supported liquid membrane (SLM) extraction and/or enrichment is similar to liquid–liquid extraction and dialysis combined[13]. In SLM, an organic liquid is embedded in small pores of a polymer support and is held there by capillary forces. If the organic liquid is immiscible with the aqueous feed and strip streams, SLM can be used to separate the two aqueous phases. It may also contain an extractant, a diluent (which is generally an inert organic solvent to adjust viscosity) and sometimes also a modifier to avoid the formation of an emulsion[14]. One of the advantages of SLM is that the relatively small volume of organic components in the membrane and simultaneous extraction and re-extraction in one technological step results in high separation factors, easy scale-up, lower energy requirements and thus lower overall running costs[14].

The use of SLM has been reported for extraction of macrolides from kidney and liver tissue[15]. The macrolides were detected following extraction at concentration levels of 0.01, 0.03 and 0.08 µg kg^{-1} for tylosin tartrate, erythromycin and spiramycin, respectively. A 1-decanol/undecane (1:1) liquid membrane at pHs of 9 and 3 for donor and acceptor, respectively was utilised. SLM was also used to extract a mixture of aminoglycoside compounds - neomycin, gentamicin and streptomycin - from cow's milk and urine[16] using the same liquid membrane as above.

2.2.3 Solid Phase extraction

Solid phase extraction (SPE) involves liquid–solid partition, where the extracting phase is a solid sorbent. This technique and versions thereof, have been used extensively to extract and concentrate trace organic materials from food samples[17]. Wide choices of

sorbents are available which rely on different mechanisms for extraction/retention of analytes. While there are drawbacks associated with SPE such as the importance of packing uniformity to avoid poor efficiency, this technique can be used to extract veterinary residues from even the most challenging matrices such as shrimp[18].

The aminoglycosides are very hydrophilic compounds hence, prior to say LC analysis, a clean up with for e.g. SPE is necessary with complex matrices like tissues or honey[8]. SPE uses most of the phases and separation mechanisms that are available for HPLC. Medina *et al.,*[19] developed a method for the extraction of Hygromycin B from animal plasma and serum followed by detection by semi- quantitative TLC. The solid-phase extraction system used "clean screen DAU resin" which is co-polymeric bonded silica with hydrophobic and ionic functions. Various effects on the recovery of Hygromycin B were studied for e.g. the combined effects of amount of resin, sample volume and elution volume used. The acidified column packing was conditioned with 5% diethylamine-methanol followed by methanol and then de-ionised water. The pH was then adjusted to pH 6.0 with potassium phosphate buffer. The acidified samples were then loaded onto the columns followed by a rinse step with water and finally eluted with diethylamine-methanol.

Recently, Bruijnsvoort *et al.,*[8] found that a C_{18} packing in the SPE cartridge was preferable to a cation-exchange packing material. This method found the extraction of streptomycin and dihydrostreoptomycin from honey and milk with methanol from a C_{18} cartridge to be preferable. Variation was seen between different SPE brands with the Baker 200 mg C_{18} cartridge performing. Recoveries of >80% were achieved. Kaufmann *et al.,*[20] were able to extract 11 aminoglycosides from fish, pork and liver samples using a low-pH extraction with trichloroacetic acid followed by SPE. The cartridge material was a weak cation-exchanger. Babin *et al.,*[21]exploited on-line SPE for the extraction of three

aminoglycosides from veal tissues. This automated clean-up and analysis system enabled the analysis of 24 veal samples in half a day with recoveries of 51-76%.

Six macrolides were extracted from eggs, honey and milk using initial clean-up with acetonitrile or phosphate buffer (pH 8.0) followed by SPE[22]. Across all macrolides and all spiked concentration levels, recoveries were greater than 88%. Berrada *et a.,l*[23] used the same Oasis HLB cartridges, as employed by Wang[12], for extraction of seven macrolides from liver and kidney samples. Recoveries were > 67% for most of the antibiotics studied at the 200 µg kg^{-1} spiking level. Recoveries of 74-107% were obtained for six macrolides in animal feeds using the Oasis HLB cartridges again and an extra back extraction step[24]. Two macrolides were extracted using silica SPE cartridges but recovery was poor – estimated to be 40-55%[25]. Eight macrolides were extracted from honey by SPE with recoveries of 60-130%[26]. Carson reviewed the use of ion-pair SPE and discussed its potential application to multiclass multiresidue analysis[27].

2.2.4 Matrix Solid-phase Dispersion

Matrix solid-phase dispersion (MSPD) is a sample pre-treatment procedure that is increasingly used for extracting/purifying analytes from a variety of solid and semi-solid foodstuffs. MSPD is primarily used because of the possibility of performing extraction and clean-up in one step (illustrated in Figure 2.1), leading to a faster overall analysis time and lower consumption of solvents[28]. The aminoglycosides have been extracted using MSPD in the literature. Nine aminoglycosides were extracted from milk with heated water (70 ºC) followed by LC–MS/MS. After acidification and filtration, 0.2 mL of the aqueous extract was injected into the LC column. Recoveries ranged between 70 and 92%. The LOQ values for this method were between 2 and 13 g L^{-1}[,29]. An extraction method for the macrolides based on the MSPD technique with hot water as extractant

proved to be robust as matrix effects, even though present, did not significantly affect the accuracy of the method. After dispersing samples of milk and yogurt on sand, target compounds were eluted from the MSPD column by passing through it 5mL of water acidified with 30 mmol L^{-1} formic acid and heated at 70°C. After pH adjustment and filtration, a volume of 200 µl of the aqueous extract was directly injected onto the LC column. Hot water was found to be an efficient extracting medium, given absolute recoveries of the analytes from milk and yogurt were 68–86% and 82–96%, respectively[30].

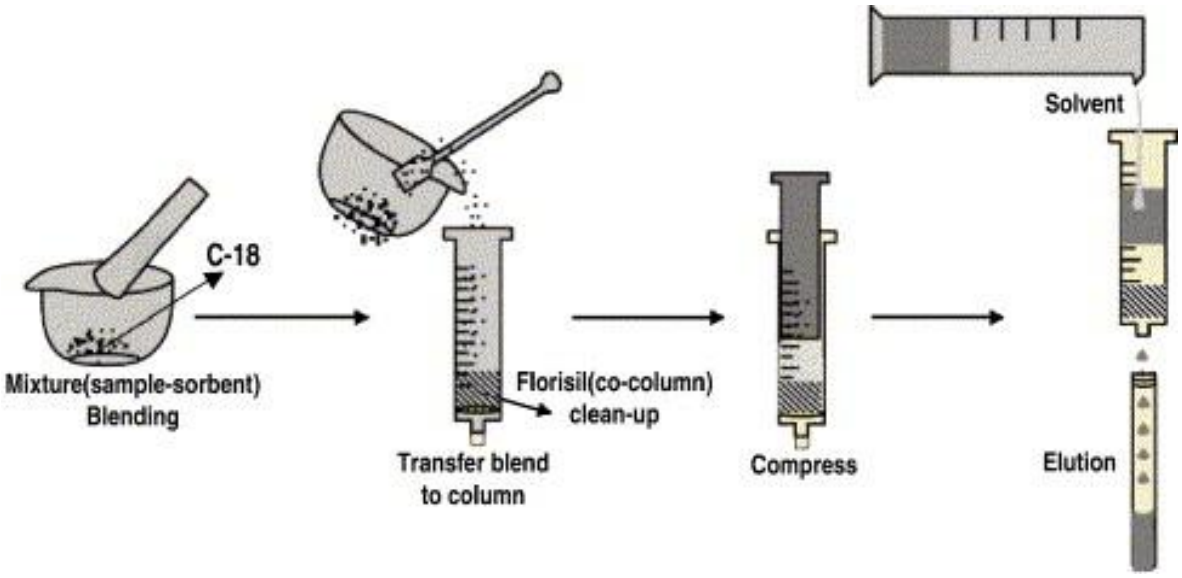


Figure 2.1 Schematic of a typical MSPD extraction procedure, Bogialli et al.,[30]

2.2.5 Pressurised liquid extraction

In the case of a number of complex sample matrices, pressurised liquid extraction (PLE) was employed[31, 32, 33] using an automated Dionex ASE 200 system. PLE is an accelerated liquid extraction (ASE) procedure, whereby increased temperature accelerates the extraction kinetics, and elevated pressure keeps the solvent below its boiling point. ASE is reported to use the same aqueous and organic solvents as traditional

extraction methods, and the method uses the solvents more efficiently. The extracts are completely transferred for further solid-phase extraction, typically using Oasis HLB sorbent or equivalent. The advantage of using PLE is the online capability and it was found to compare well against ultrasonic solvent extraction for extraction of macrolides[38]. In summary, there are many different ways to extract aminoglycosides and macrolides from food matrices. However, sometimes compromises are required. For example, for screening methods time and cost issues are more important than the removal of all matrix interferences so that a simple extraction system might be more suitable than a more complex extraction with higher recoveries. The number and type of analytes the method must selectively extract is also of consideration.

2.3 Introduction for Experiment to extract Aminoglycoside and Macrolide antibiotics from Bovine Muscle.

The Central Meat Control laboratory (CMCL) is the National Reference Laboratory (NRL) for these two groups. The laboratory is required to monitor these residues in accordance with the legislation, as discussed in Chapter 1, in a variety of animal tissues such as muscle and kidney. The sample preparation for these matrices is difficult as there are many possible interferences that need to be reduced/ removed prior to analysis, thus a good sample preparation technique that will remove interferences while retaining the analytes of interest is desirable.

2.4 Aim

The aim of this sample preparation experiment was to investigate various solid phase extraction cartridges in terms of their recovery and selectivity for a mixture of aminoglycoside and macrolide antibiotics.

2.5 Materials and Methods

2.5.1 Reagents

The following antibiotics, tobramycin (89549), apramycin sulfate (A2024), kanamycin sulfate (60616), dihydrostreptomycin sesquisulfate (37386), spectinomycin dihydrochloride pentahydrate (85555), streptomycin sulfate (2158X), spiramycin mixture of I, II and III (S-9132), tylosin tartrate (T-6134), roxithromycin (R4393), lincomycin (62143) and paromomycin sulfate (76261) were purchased from Sigma-Aldrich Corporation.

The following HPLC grade solvents, methanol, acetonitrile and ultra-pure water were purchased from Fisher Scientific. Negative (macrolide and aminoglycoside free) bovine muscle was sourced from Abbotstown farm and screened in-house using a microbiological 6-plate assay to ensure negativity, then homogenised and divided into 5g individual portions. Heptafluorobutyric acid (HFBA) was purchased from Sigma-Aldrich Corporation.

Various Waters Oasis® solid phase extraction cartridges used were WCX, WAX, MCX, MAX and HLB that were all purchaed from Waters Chromatography, Ireland. Varian bond Elut LRC-SCX 500mg part number 12113039, Phenomenex Strata X and Varian bond Elut C18 200mg/ 3mL part number 35402 were purchased from JVA Analytical.

2.5.2 Intrumentation

2.5.2.1 UPLC-MS/MS Instrumentation

The Ultra Performance Liquid Chromatography system (UPLC) was a Waters Acquity UPLC® system with a 2.1 x 50mm (1.7µm) C18 Acquity UPLC® BEH column,

Acquity Sample Organiser, In-line degasser AF, Column Manager and Heater/ Cooler, Binary Solvent Manager and Sample Manager. Data was processed using Waters Masslynx^{TM} software.

The mass spectrometer used was a Waters Quattro Premier^{TM} XE tandem quadrupole mass spectrometer utilising T-wave^{TM} collision cell, multi-mode ionisation and rapid polarity switching technologies.

2.5.2.2 Extraction Equipment

The extraction equipment used consisted of a REAX 2 overhead shaker together with a Heidolph test tube shaker, a Techne sample concentrator FSC 400D with Techne Dri-block heater DB-3 with 127mm needles and insert blocks were purchased from Lennox Chemicals Ltd.. The vacuum chamber was a VacElut 20 purchased from JVA Analytical and the Laboport vacuum/ pressure diaphram self drying pump was purchased from Carl Stuart Limited.

2.5.3 Preparation of Standards and Samples

2.5.3.1 Standard Solutions

Stock solutions (500µg/mL) of spiramycin, tobramycin, apramycin, kanamycin, dihydrostreptomycin, lincomycin, spectinomycin, tylosin, and roxithromycin were prepared in methanol. Stock solutions (500µg/mL) of streptomycin and paromomycin were prepared in ultra-pure water. Mixed standard solutions at 100ng/mL, 200ng/mL, 300ng/mL, 500ng/mL and 1000ng/mL were diluted from stocks in water. All standards were prepared in polypropylene volumetric units and stored in polypropylene amber storage jars at 4ºC.

2.5.3.2 Matrix Samples

2g muscle samples were homogenised, weighed and placed in 50mL polypropylene centrifuge tubes. Each tube was labelled with weight and date and stored at -20ºC until used. Matrix samples were spiked at appropriate levels using mixed standard solutions immediately prior to extraction.

Samples were then de-proteinised by adding 10mL of either 5% trichloroacetic acid or 5% ammonium hydroxide and placed on a shaker for 10 minutes. The samples were then centrifuged at 10,000rpm for 10 minutes and a 3mL aliquot was transferred to the solid phase extraction cartridge.

2.5.4 Assay Conditions

2.5.4.1 Extraction/ Clean-up Protocol

Cartridges were conditioned with 3mL methanol followed by an equilibration step with 3mL water. The 3mL sample was loaded onto the cartridge and the cartridge was washed with 3mL water. The analytes were then eluted in either 3mL methanol or 3mL of a 2% formic acid solution in methanol. This eluate was evaporated under nitrogen on a heating block set at ~45ºC and then re-constituted in 300µl water.

2.5.4.2 UPLC Method

Gradient elution was used with solvent A (ultra-pure water with 10mM HFBA) and solvent B (100% methanol or 100% acetonitrile, where indicated) as follows: T_0 90/10, $T_{0.5}$ 90/10, $T_{1.0}$ 50/50, $T_{2.5}$ 50/50 $T_{4.0}$ 40/60 $T_{5.0}$ 40/60, $T_{5.5}$ 90/10, $T_{6.0}$ 90/10. The system was conditioned with 15% solvent B for 1 hour prior to use on each day or until a delta pressure of <40psi was observed on the Solvent Manager. The mobile phase was degassed and filtered by passing through a 0.45µm pore size membrane filter (Milipore,

Milford, MA, USA) prior to use and further degassed with the in-line degasser. The flow rate was 0.450 mL min^{-1}. All injections were performed on column at temperature of 35ºC with injection volumes of 10 to 20µl as indicated.

2.5.4.3 MS Conditions

Tuning for all analytes was carried out for the mass spectrometer with the optimised conditions as follows: Capillary (kV) 3.5, Extractor (V) 4.00, RF lens (V) 0.4, Source Temperature (ºC) 120, Desolvation Temperature (ºC) 350, Cone Gas Flow (L/Hr) 200, Desolvation Gas Flow (L/Hr) 900, Ion Energy MS1 0.8 and Ion Energy MS2 1.0

Table 2.1: Parent and Daughter Ions with optimised collision and cone voltages

Analytes	**Parent Ion (M)+**	**Daughter Ions**	**Collision (eV)**	**Cone (V)**
Tylosin	916.5	174.2 & 101.4	40 & 45	50
Roxithromycin	837.4	158.4 & 679.5	30 & 20	30
Streptomycin	582.1	263.2 & 176.1	35 & 40	60
Dihydrostreptomycin	584.1	246.2 & 263.1	35 & 30	50
Paromomycin	616.2	163.2 & 203.2	40 & 40	45
Spiramycin	843.5	174.2 & 101.2	40 & 40	40
Spectinomycin	333.2	98.1 & 116.1	30 & 25	55
Lincomycin	407.2	126.4 & 359.2	30 & 20	40
Kanamycin	485.2	163.0 & 205.2	30 & 30	30
Apramycin	540.2	217.2 & 378.2	30 & 20	55
Tobramycin	468.2	163.2 & 205.3	25 & 25	30

2.6 Results and Discussion

The macrolides and aminoglycosides are basic analytes with known pkA's ranging from 5.4 for apramycin (one of four protons) to 9.5 for roxithromycin. During this experiment a number of different solid phase extraction cartridges were tested. A study using the waters Oasis® range of products (see Figure 2.2) was carried out to assess potential for extracting the analytes form bovine muscle samples.

The following cartridges were compared:

- HLB: Hydrophilic- lipophilic sorbent for all compounds.
- MCX: A mixed-mode Cation exchange and reverse-phase sorbent suitable for bases, high selectivity for basic compounds.
- MAX: A mixed-mode Anion exchange and reverse-phase sorbent suitable for acids, high selectivity for acidic compounds.
- WCX: A mixed-mode Cation exchange and reverse-phase sorbent with a high selectivity for strong bases.
- WAX: A mixed-mode Anion exchange and reverse-phase sorbent with a high selectivity for strong acids.

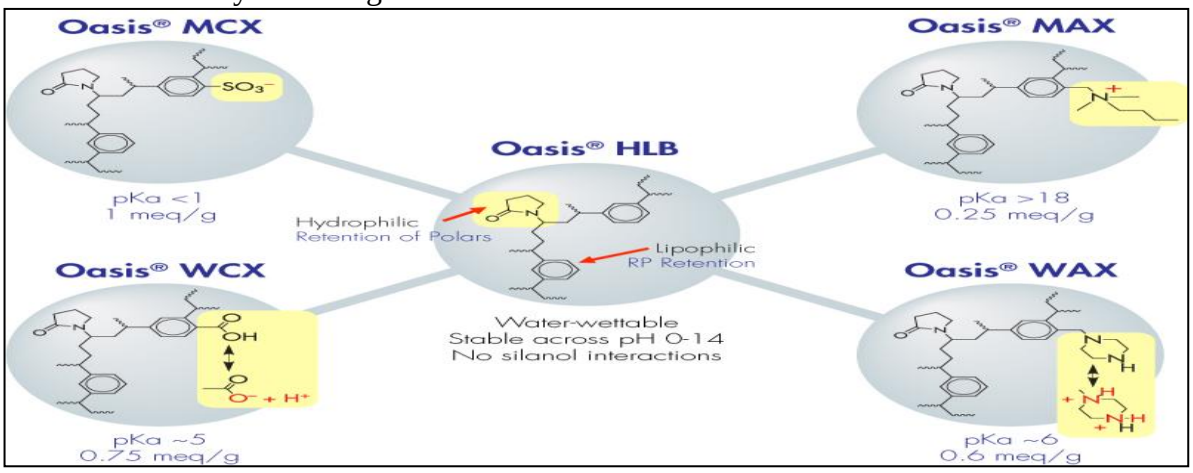


Figure 2.2: Waters Oasis® Solid-Phase Extraction Cartridges[34]

2.6.1 Waters Oasis® 2x4 method

This experiment used a mixture of standards that were prepared at the maximum residue levels and a number of 2g samples of muscle were spiked with this mixture. A weak acid, 10mL % trichloroacetic acid, and an alternative weak base, 5% ammonium hydroxide were then added to the samples to de-proteinise. Using the protocols set out in Figure 3.2 below, elutes 1 and 2 were collected and analysed by UPLC-MS/MS.

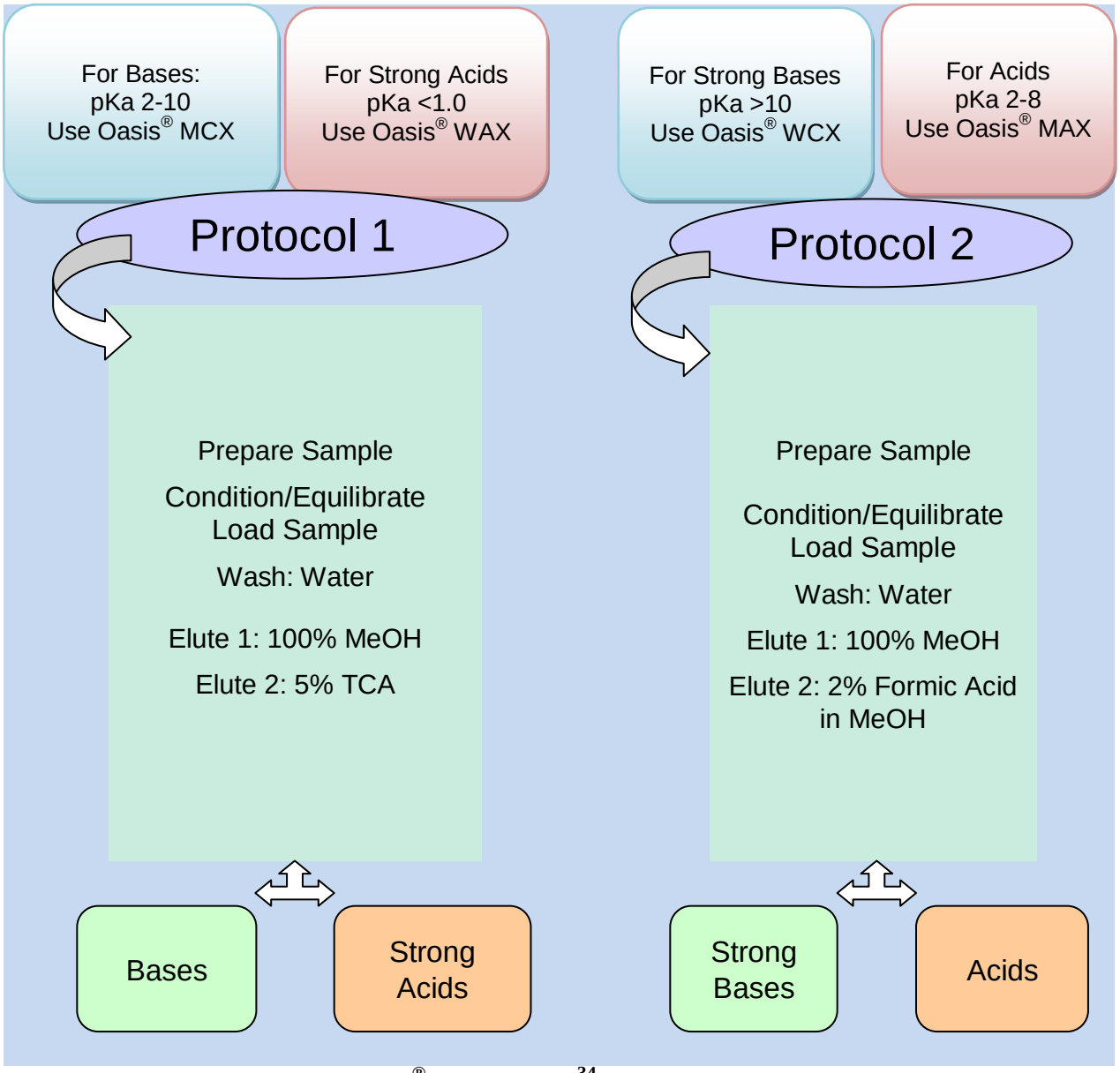


Figure 2.3: Adapted Waters Oasis® 2x4 Method[34]

The macrolides and aminoglycosides are basic analytes and as expected the best results were seen for the MCX and the WCX cartridges with good responses seen for seven of the 11 analytes in Elute 2 on the MCX cartridge. This elute was in 5% TCA and the de-proteinisation method used was 5% trichloroacetic acid (see Figure 2.3).

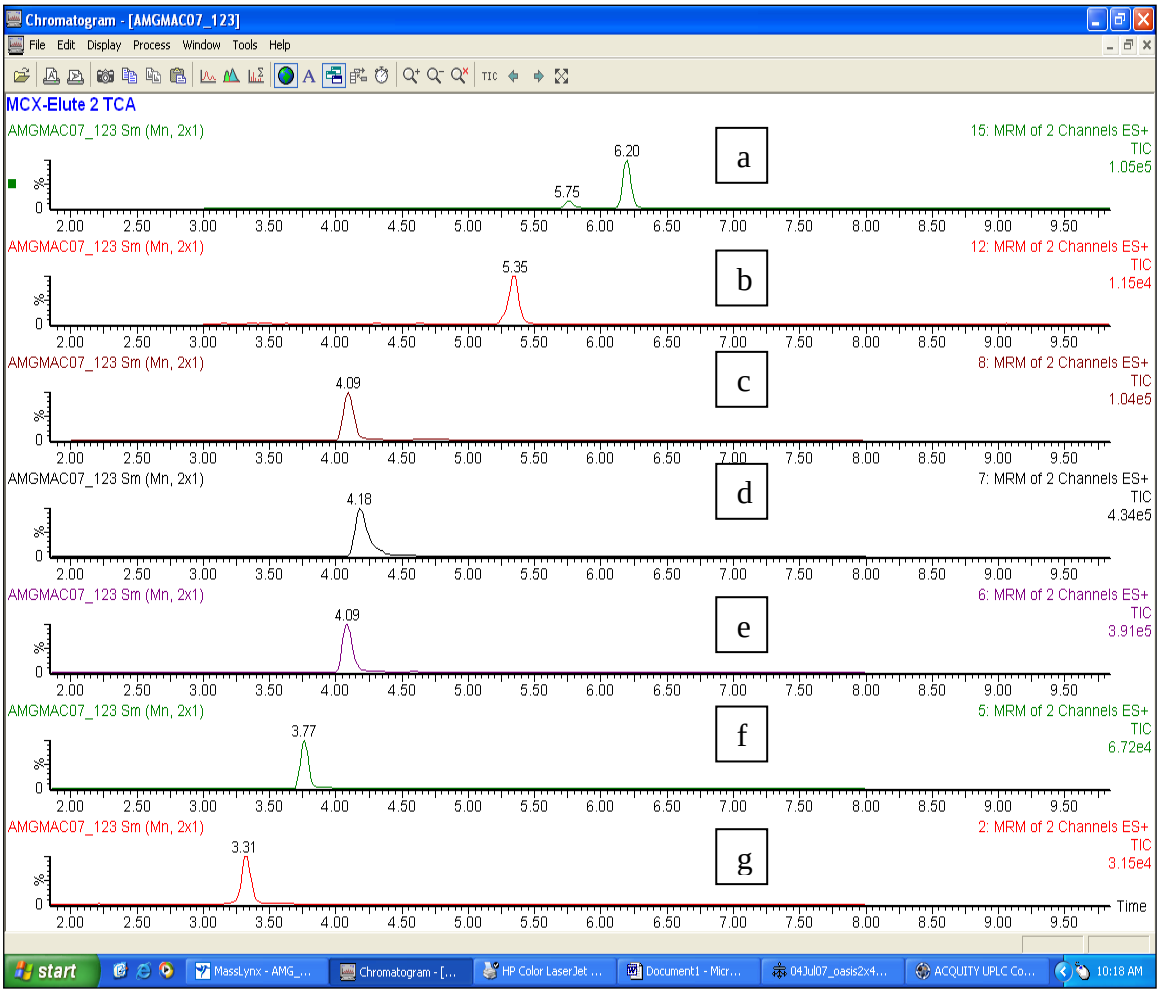


Figure 2.4: TIC data for elution of 7 aminoglycosides and macrolides. X axis time, y axis response. UPLC conditions: Solvent A 10mM HFBA, Solvent B Acetonitrile. Gradient, Initial 90% A, 4.5min 50% A, 8.5min 30% A, 9.5min 90% A, 10.5min 90% A. Flow rate: 0.3ml/min. Peaks a to g, roxithromycin, tilmicosin, paromomycin, tobramycin, apramycin, kanamycin and lincomycin.

2.6.2 Waters HLB Cartridge and Phenomenex Strata X

The waters HLB 6cc 200mg Cartridges and the Phenomenex Strata X 6cc 200mg Cartridges were also compared. They were chosen for their ability to extract across a wide pKa range. Two extraction solutions were chosen, 50:50 MeOH:water and 50:50 ACN:water. A number of 2 gram samples were taken and spiked at the MRL level for 11 analytes of various aminoglycosides and macrolides and the extraction method below was performed.

Waters HLB and Strata X extraction method

- Condition with 5mL MeOH and equilibrate with 5mL H2O
- Load 5mL sample. *Collect*
- Wash with 5mL 10% MeOH. *Collect*
- Elute with 5mL 100% MeOH. *Collect*
- Evaporate to dryness under nitrogen and re-constitute in 1mL H2O

Results proved disappointing with various problems encountered such as pressure problems on system and column, the sample cone in the mass spectrometer tended to get dirty very quickly which meant that it needed cleaning regularly. This is a problem with muscle samples and their clean up as discussed in section 2.2. There was also a difficulty resolving eluting compounds of similar molecular weights and a difficulty in finding an extraction method to reduce matrix effects while retaining extraction of all the compounds. The HLB results were better in terms of the number of analytes recovered (Figure 2.5). Comparing the two extraction solvents the 50:50 acetonitrile:water mix gave higher recoveries e.g. lincomycin when compared with a standard directly analysed gave a recovery of ~20% in the acetonitrile mix compared to only about 5% in the methanol

mix. The overall results for the study however were poor with few compounds extracting with satisfactory recoveries.

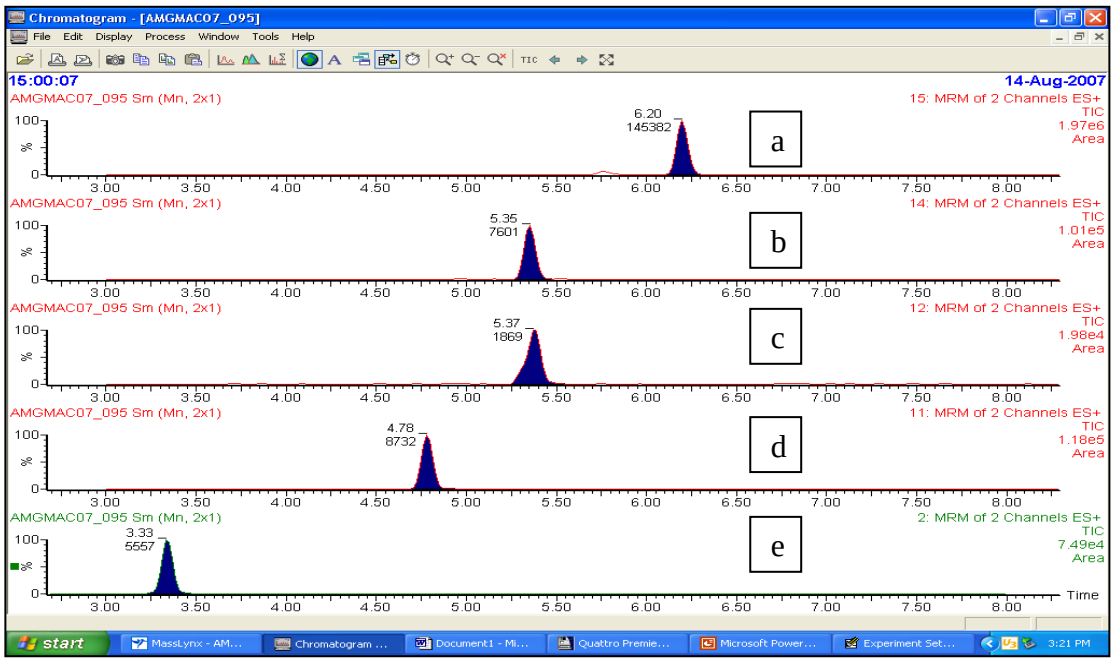


Figure 2.5: TIC data for elution of 5 aminoglycosides and macrolides. X axis time, y axis response. UPLC conditions: Solvent A 10mM HFBA, Solvent B Acetonitrile. Gradient, Initial 90% A, 4.5min 50% A, 8.5min 30% A, 9.5min 90% A, 10.5min 90% A. Flow rate: 0.3ml/min. HLB Elute at MRL level. Peaks a to e: Roxithromycin, tylosin, tilmicosin, spiramycin and lincomycin.

2.7 Conclusion

There are a number of ways to extract these compounds from complex matrices like animal tissue. Protein precipitation is relatively cheap and fast enabling clean up to of samples where speed is critical for e.g. in keeping turn-around times down. Liquid-liquid extraction and supported liquid membrane are useful for extracting mixtures of the aminoglucosides and macrolides at low levels however the recoveries were poor for analysis in milk. Overall either of these techniques would have the advantage of being easy to scale up and cheap to run. For an extraction experiment to look at a mixture of

aminoglycosides and macrolides in tissue samples the technique that offers the most options is SPE. In terms of choice of sorbent, the ability to concentrate trace materials from complex matrices, automation and good recoveries, SPE is the most versatile.

A significant amount of work was carried out in order to develop and optimise the extraction of these two groups of antibiotics from very difficult matrices such as bovine muscle. The current techniques in the literature were thoroughly researched to look at the various options prior to carrying out many trials with various solid-phase extraction cartridges. The sample preparation for these analytes is a critical step for any method that intends to confirm their quantity and presence and thus a number of different solid-phase extraction techniques have been presented here which could be investigated further to look at optimising their use for these groups.

The sample preparation technique using the Oasis® HLB cartridge resulted in a greater number of analytes recovered (Figure 2.5) when compared to the Phenomenex Strata X cartridge. For the Oasis® 2x4 method the MCX cartridge resulted in the greatest number of analytes eluting. This was expected as the MCX cartridge is recommended when trying to extract bases like the aminoglycosides and macrolides.

Comparing extraction solvents, for the Oasis® HLB cartridge, the 50:50 acetonitrile:water mix gave higher recoveries e.g. lincomycin when compared with a standard directly analysed gave a recovery of ~20% in the acetonitrile mix compared to only about 5% in the methanol mix.

References

1. Hernandez M., Borrull F., Calull M. (2003). *Trends in Analytical Chemistry,* **22**; 7-8
2. Hisao Oka, Hiroyuki Nakazawa, Ken-ichi Harada, and James D. MacNeil, Editors**,** Chemical Analysis of Antibiotics Used in Agriculture Copyright © (1995),AOAC INTERNATIONAL.,452 Pages.

3. Buldini P.L., Ricci L., Sharma J.L. (2002). *Journal of Chromatography A*, **975(1);** 47-70
4. Thompson T.S., Noot, D.K., Calvert J., Pernal S.F. (2005). *Rapid Communications in mass spectrometry*,**19(3);** 309-316
5. Kowalski, P., Oledzka, I., Okoniewski, P., Switala, M., Lamparczyk, H. (1999). *Chromatographia* , **50 (1-2);** 101-104
6. Xie, W.; Ding, H.-Y.; Xi, J.-Y.; Qian, Y.; Huang, L.-F. (2007). *Se Pu*, **25 (3);** 404-407
7. Viñas P., Balsalobre N., Hernández-Córdoba M. (2007). *Talanta,* **72(2);** 808-812
8. Bruijnsvoort M., Ottink S.J.M., Jonker K.M., de Boer E. (2004). *Journal of Chromatography A*, **1058** (**1- 2)**; 137- 142
9. Yue, Z.F, Chen, X.X., Xie, L.Q., Ji, C.N., Hua, H.H. (2007). *Fenxi Huaxue*, **35 (9)**; 1290-1294
10. Takegami, H.; Horie, M.; Nakazawa, H. (2006). *Bunseki Kagaku*, **55 (9)**; 651-659
11. Wang, J., Leung, D., Lenz, S. P. (2006). *Journal of Agricultural and Food Chemistry*, **54 (8)**; 2873-2880
12. Wang J., Leung D., Butterworth F. (2005). *Journal of Agricultural and Food Chemistry*, **53(6)**; 1857-1865
13. Torto N., Mmualefe L.C., Mwatseteza J.F., Nkoane B., Chimuka L., Nindi M.M., Ogunfowokan A.O. (2007). *Journal of Chromatography A*, **1153(1-2)**; 1-13
14. Kocherginsky N.M., Yang Q., Seelam L. (2007). *Separation and Purification Technology*, **53(2)**; 171-177
15. Msagati T.A.M., Nindi M.M. (2004). *Microchim. Acta*, **148(3-4);** 199-214
16. Msagati T.A.M., Nindi M.M. (2005). *Bull. Chem. Soc. Jpn*., **78;** 2135

17. Ridgway K., Lalljie S. P. D., Smith R.M. (2007). *Journal of Chromatography A*, **1153(1-2);** 36-53

18. Li H., P. Kijak J., Turnipseed S.B., Cui W. (2006). *Journal of Chromatography B*, **836(1-2)**; 22-38

19. Medina M.B., Unruh J.J. (1995). *Journal of Chromatography B*, **663**; 127-135

20. Kaufmann A., Maden K. (2005). *Journal of AOAC International*, **88** (**4);** 1118-1125

21. Babin Y., Fortier S. (2007). *Journal of AOAC International*, **90(5)**; 1418-1426

22. Wang J., Leung D. (2007). *Rapid Communications in Mass Spectrometry*, **21(19);** 3213-3222 a

23. Berrada, H., Borrull F., Font G., Moltó J.C., Marcé R.M. (2007). *Journal of Chromatography A*, **1157(1-2)**; 281-288

24. González de la Huebra M.J., Vincent U., von Holst C., (2007). *Journal of Pharmaceutical and Biomedical Analysis*, **43(5)**; 1628-1637

25. Heller D. N., Nochetto C. B., (2004). *Journal of Agricultural and Food Chemistry*, **52(23);** 6848-6856

26. Xu, J.Z., Wu Z.X. (2007). *Fenxi Huaxue*, **35(2);** 166-170

27. Carson M. C. (2000). Journal *of Chromatography A*, **885(1-2)**; 343-350

28. Bogialli S., Di Corcia A. (2007). *Journal of Biochemical and Biophysical Methods*, **70(2);** 163-179 a

29. Bogialli S, Curini R, Di Corcia A, Laganà A, Mele M, Nazzari M. (2005). *Journal of Chromatography A*, **1067(1 -2)**; 93-100

30. Bogialli S., di Corcia A., Laana A., Mastrantoni V., Sergi M., (2007). *Rapid Communications in Mass Spectrometry*, **21(2)**; 237-246 b

31. Gobel A., Thomsen A., McArdell C.S., Alder A.C., Giger W., Theis N., Loffler D., Ternes T.A. (2005). *Jourrnal of Chromatography A*, **1085;** 179–189

32. Schlusener M.P., Spiteller M., Bester K (2003). *Journal of Chromatography A*, **1003;** 21–28

33. Jacobsen A.M., Halling-Sorenses B., Ingerslev F., Hansen S.H. (2004). *Journal of chrormatography A*, **1038**; 157–170

34. Waters Corporation 2007. *Purity by SPE: Cleaner, Simpler and Faster SPE-LC/MS Analysis.* Waters Corporation.

Chapter 3: Methods of analysis for the Aminoglycoside and Macrolide antibiotics.

3.1 Introduction

The presence of aminoglycoside and macrolide residues in animal products must be monitored according to legislation set out in chapter 1. Due to the safety issues surrounding these compounds, and the MRL's associated with them, there is huge pressure on the analytical assays to be extremely sensitive. The trend has been to see more and more LC-MS methods being reported for this area of research. There are however many methods, both chemical and biological, used to determine the presence (screening/ qualitative assays) and amount (quantitative assays) of these residues in animal tissues, and these will be discussed here.

3.2 Screening Methods-Chemical

3.2.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is one of the most popular and widely used separation techniques due to a number of factors including ease of use, wide applications, good sensitivity, speed and low cost[1]. Chromatography is a method of separating a mixture into its various components. The mobile phase carries the sample through the fixed stationary phase and based on the heterogeneous equilibrium between these two phases the components of the sample are separated. The stationary phase can be either a solid or liquid and the mobile phase a gas or a liquid. Chromatography is often defined by these as liquid-solid, liquid-liquid, gas-solid or gas-liquid[1].

The measurement parameter in any form of chromatography is the distribution coefficient (k) of a substance between the two phases. k is dependent on the temperature and concentration of the solute (Touchstone 1983).

Equation 3.1:

$$\text{DistributionCoefficient}(k) = \frac{\text{Amount of Solute per Unit of Stationary Phase}}{\text{Amount of Solute per Unit of Mobile Phase}}$$

In a review by Stead[2], the use of TLC as a qualitative method for the aminoglycosides has been well documented. The separation of aminoglycosides by normal phase and reverse phase TLC is presented in a paper by Bhushan *et al.*,[3] where streptomycin, kanamycin, gentamicin and tobramycin were determined with detection limits of 0.4 – 0.6 μg possible. The macrolides have also been assayed by TLC[4].

3.2.2 Capillary Electrophoresis

Capillary electrophoresis (CE) describes a family of techniques used to separate a variety of compounds. These analyses, all driven by an electric field, are performed in narrow tubes and can result in the rapid separation of many hundreds of different compounds. The versatility and number of ways that CE can be used means that almost all molecules and even whole organisms can be separated using this powerful method.

There are a number of different ways of performing CE separations. This makes the technique especially useful when optimised for a separation of interest in e.g. ensuring purity during manufacture, or diagnosing illness in a hospital. Separations driven by electrophoresis also have a novel separating mechanism. This makes them useful in situations where other liquid phase separation techniques are limited or impractical. Some of the advantages of capillary electrophoresis include very high efficiencies (meaning hundreds of components can be separated at the same time), small sample size, automation, and it is quantitative. However, one of the drawbacks associated

with the use of CE for determining trace levels of residues is that it is sometimes not sensitive enough due to the lower sample injection volumes required and short optical path-length for on-capillary detection[5].

The aminoglycosides are difficult to analyse by capillary electrophoresis due to the problems in detecting these compounds using spectrophotometric means as many of these compounds lack chromophores[6]. Hence, initial work saw the aminoglycosides first detected by CE using indirect UV detection with imidazole as the background electrolyte under low pH and reversed polarity conditions[7]. The problems encountered were mainly in the difficulty separating closely related compounds. Subsequent work by Flurer *et al.*,[6] showed that borate buffers could be used to allow direct detection of the aminoglycosides by UV detection at 195 nm. Based on the formation of negatively charged complexes between the hydroxyl moieties minor differences were emphasized between compounds and separation of twelve aminoglycosides was possible.

Post 2000, work on capillary electrophoresis continued and in a study by Yang *et al.*,[8] the separation of five aminoglycoside antibiotics used in veterinary medicine with electrochemical detection is demonstrated. In this study a copper micro particle-modified carbon fiber micro disk array electrode was fabricated. This array showed catalytic activity for the aminoglycosides using a separation voltage of 6.2 kV and electrophoresis medium of 125 mM NaOH resulting in the separation of the 5 antibiotics within 20 min. With linear calibration curves over two orders of magnitude of concentration and detection limits of 2µM for all compounds except lincomycin in pharmaceutical injectables and further human urine. This investigation showed that with further research it would be feasible to determine these antibiotics in other matrices.

Capillary zone electrophoresis was successfully applied to separate eight related substances of kanamycin and several minor unknowns from the main component[9]. The standard curves were linear over the concentration range of 0.007–1.01 mg/mL for the main component and 0.003–0.1 mg/mL for the related substances. The limit of quantitation was 0.14% (m/m) for the related substances and impurities (*S*/*N*=10). A review of the use of CE for the aminoglycosides to 2002 found that the choice of detector had a great influence on the separations with laser-induced fluorescence (LIF) showing the best sensitivity with improvements of up to three orders of magnitude[10]. CE-LIF was used by Serrano *et al.,*[11] to determine four aminoglycosides in milk. Following derivatisation the separation took 20 min and the antibiotics were readily detected at 0.5–1.5 μg kg^{-1} levels. A general review of CE methods for antibiotics in a variety of matrices including food has been reported[5].

3.2.3 Optical Biosensor

Biosensor systems using the surface plasmon resonance (SPR) detection principle are recent and provide rapid and reliable results with minimal sample preparation. The detection of streptomycin and dihydrostreptomycin residues in milk, honey and meat samples using this technique has been reported[12]. The study compared a commercially available biosensor kit with a commercially available enzyme immunoassay kit (EIA) and a confirmatory HPLC method. The results demonstrated that the biosensor technology compared favourably with the immunoassay and HPLC methods. Antibody specificity for streptomycin and dihydrostreptomycin was good with < 0.1% cross-reaction with other aminoglycosides for e.g. neomycin, gentamicin, kanamycin or other antimicrobials for e.g. penicillin G and chlortetracycline. The LOD values were 15, 30,

50 and 70 µg kg^{-1} for honey, milk, kidney and muscle respectively. Recoveries ranged from 77% to 110% using the biosensor kit. One false positive result for kidney was found but no false negatives were found (which is more important in the case of screening tests). The Biacore 3000, an optical biosensor with four flow channels was used for the detection of five aminoglycosides in reconstituted skimmed milk, in combination with a mixture of four specific antibodies. The limits of detection were between 15 and 60 µg kg^{-1}, which were well below the MRLs, and the total run time between samples was 7 min[13]. Biosensors have the advantages of simple, fast, sensitive and cost-effective detection[14] thus making them ideal for use in the screening of residues in food.

A cell-based microbial biosensor for macrolides utilised a luminescence biosensor based on the coupling of structural luciferase genes of *Vibrio fischeri* to the regulatory control mechanism of a bacterial erythromycin resistance operon[15]. This system was tested on its ability to isolate and characterise picromycin from a Streptomyces species.

3.2.4 Ultraviolet and Visible Spectra

Visible and ultra violet spectra are associated with the transitions between electronic energy levels; these transitions are mostly between a bonding or lone-pair orbital and an anti-bonding or unfilled non-bonding orbital. When the electrons in the p- and d- orbitals are excited which occurs above 200 nm, the spectra are steady and informative[16]. In order for a compound to perform with this method it must contain a chromophore, this is the part of the compound which contains the electrons responsible for the absorption. Generally a conjugated chromophore perform best for e.g. when conjugation between two isolated double bonds occurs the energy level of the highest occupied orbital is raised and the lowest unoccupied anti-bonding orbital lowered

resulting in a strong and easily detected maximum. Aminoglycosides lack chromophores and are therefore not amenable to direct UV or fluorescence detection however they may be derivatised after separation with UV absorbent or fluorescent agents thus allowing analysis with more commonly available spectrometric detectors[2].

3.2.5 Resonance Rayleigh scattering

Resonance Rayleigh scattering (RRS) is a new analytical method developed in recent years that can be used as an alternative to UV-Vis or microbiological assays for screening of aminoglycosides. It is based on the aggregation of a conjugated structure in biological macromolecules or the ion-association complexes that are formed by the reaction between electrostatic and hydrophobic interaction in small molecules[17].

A study by Liu *et al.,*[18] compared RRS with time consuming microbiological assays and lower sensitivity UV-Vis for the aminoglycosides. Results indicated that when Evans blue dye and some individual aminoglycosides; kanamycin, gentamicin, tobramycin and neomycin react together, an ion-association complex is formed which enhances the individual spectrums and a new RRS spectrum is observed. The linear range reported was 0.01 – 6.0 ug/mL with a detection limit of 5.2 ng/mL for kanamycin and 0.02 – 6.0 ug/mL for the other compounds with detection limits for neomycin, tobramycin and gentamicin of 5.5, 6.2 and 6.9 ng/mL respectively[18].

This phenomenon has also been reported for pontamine sky blue dye with aminoglycosides[19]. While RRS of aminoglycosides has not been used for food samples, it has been used in serum and therefore may be applicable to food matrices[18, 19].

3.3 Screening Methods-Biological

3.3.1 Enzyme Immunoassay

There are two classifications for the enzyme immunoassays based on the presence of the enzyme labelled antigen. The heterogeneous assay where the enzyme-labelled antigen or antibody is separated from the antibody-antigen complex prior to measurement of enzyme activity in both e.g. enzyme linked immunoassay (ELISA). The homogenous assay where the enzyme-labelled antigen or antibody is measured in the presence of the antibody-antigen complex e.g. enzyme multiplied immunoassay technique (EMIT).

A rapid and sensitive screening ELISA for gentamicin in swine tissues was developed and reported an analysis time less than 45 min, excluding coating and blocking, with negligible cross-reactivity with other aminoglycosides[20]. LOD values ranged from 2.7–6.2 µg kg^{-1} in the different tissues and recoveries were between 90 and 101% in muscle, 77 and 84% in liver and 65 and 75% in kidney[20]. A report by Haasnoot *et al.*,[21] described the detection of gentamicin, neomycin, streptomycin and dihydrostreptomycin using three ELISA assays for applications in milk and kidney samples. The detection limits were 0.7-5.1 µg L^{-1} and the recoveries were 47-78% for milk and 70-96% for kidney. Real samples were taken and analysed from the kidneys of healthy pigs (n=124) and milk (n = 776). The aminoglycoside residues found were all below the established MRLs.

An electrochemical ELISA for the detection of two macrolides (erythromycin and tylosin) in bovine muscle has been reported[22]. The detection limit of the assay was 0.4 µg L^{-1} for erythromycin and 4.0 µg L^{-1} for tylosin. Results were confirmed by LC-MS/MS.

3.3.2 Microbiological Assay

An advantage of the microbiological tests is that they are inexpensive, easy to perform on a large scale and they possess a wide, non-specific spectrum in sensitivity[23], however, a comparative study carried out by Sachetelli *et al.*,[24] on tobramycin standards/ samples by ELISA, HPLC and microbiological assay found that the M-agar assay resulted in an over-estimation of the actual quantity in comparison with the other procedures. The aminoglycosides are commonly screened by the four-plate test (FPT) in the EU. There are many drawbacks with the four-plate test such as the fact that it takes at least six hours before the results are known[13].

3.4 Quantitative Methods of Analysis

3.4.1 Introduction

Chromatography can be defined as the separation of molecules by differential migration, i.e. separation is achieved on the basis of different speeds of transportation for different molecules[25]. The most common way to classify the different chromatographic techniques is by the nature of the phase involved for e.g. a gas in the case of gas chromatography or a liquid in the case of liquid chromatography. For the aminoglycosides and macrolides, the focus of this review will be on liquid chromatography.

Separation is achieved on a chromatographic column where there are two chromatographic phases: the mobile phase and the stationary phase. Depending on the polarity of the sample the chromatography is described as normal-phase or reversed-phase. With the addition of pairing agents it is referred to as ion-exchange or ion-pair chromatography. In normal phase chromatography, the sample is soluble in non-polar

solvent such as n-hexane as mobile phase and the stationary phase is more polar for e.g. In reverse phase chromatography, the sample is polar and therefore the mobile phase is polar and the stationary phase less polar for e.g. C18. Further sub-division by detector type will also be discussed with the main focus on methods that use mass spectrometric detection.

In the case of the aminoglycosides and macrolides, group B compounds, where quantitative analysis at the MRL and lower is required, mass spectral detection can be employed. When a confirmatory assay for antibiotic residues in food is required, the method must provide information on the chemical structure of the analyte. A paper by Rivier describes the criteria for the identification of compounds by LC-MS and LC-MS/MS in order to comply with the EU criteria for trace level organic analysis[26].

Mass spectrometry is the detection method of choice for the aminoglycosides due to the lack of chromophores and fluorophores in the molecule[27]. The mass spectrometer is designed to perform three basic functions: vaporise volatile compounds, to produce ions or neutrals from the vaporised gas-phase compounds and to separate/ detect these ions on a mass-to-charge ratio (*m/ze*). Generally the charge *z* is one because usually the ions are singly charged. The charge of one electron is a constant and therefore *m/z* gives the mass of the ion. When an array of ions is separated and recorded this is known as the mass spectrum with the most abundant ion, the molecular ion equal to the molecular weight[16].

There are many types of mass spectrometers available which can perform MS analysis for e.g. magnetic sector instruments which are used when ions of one mass unit are to be separated, or time-of-flight instruments where ions are separated over a given distance where the larger the mass the lower the velocity and hence the longer the time of flight over the given distance[16]. For MS–MS (MRM) methods, two types of mass

spectrometers are available: triple quadrupole and ion trap mass spectrometer or a hybrid of both. Triple quadrupole instruments produce ions with collision induced fragmentation for MS–MS (e.g. daughter ions for MRM) while, ion trap mass spectrometer can produce MS^n fragment ion (e.g. grand daughter ions). In the case of group B compounds, where quantitative analysis about the MRL is required, quadrupole or ion trap instruments can be employed. De Wash *et al*., discussed the advantages and the disadvantages of these two types of instruments by considering the analysis of sedative residues and the comparison of the lower LOD versus linearity.

Direct mass spectral analysis of the aminoglycosides is difficult due to their thermal lability. It is possible to use thermospray ionisation to volatise and ionise the compounds from chromatographic eluents however it is difficult to get to low detection limits[29] A summary of some of the most relevant LC-based analytical methods, many with mass spectral detection, published for the aminoglycosides and macrolides can be seen in Tables 1.5 & 1.6.

3.4.2 Aminoglycosides

There are a large number of HPLC methods available in the literature for the aminoglycosides and macrolides. Analysis of the aminoglycosides by HPLC has been dealt with in a number of review papers most recently by Stead[2]. Therefore in this review the focus will be given to post 2000 published methods.

Many authors have overcome the problem of a lack of UV chromophore or fluorophore for the aminoglycosides by using derivatising agents for detection by fluorescence[30, 31, 32, 33] or UV absorbance[34]. Derivatisation steps however render the analytical process more time consuming and may even introduce impurities. Another

problem associated with derivatisation is the possibility of the derivatives themselves degrading within a few hours after formation. Limits of detection using LC-fluorescence methods can be low for aminoglycosides in foods e.g. 7.5-15 μg kg^{-1} for streptomycin and dihydrostreptomycin in honey, milk, eggs and liver[30] and 15 μg L^{-1} for gentamicin in milk[32]. Indirect UV or fluorescence methods have also been employed for determining the aminoglycosides, though not in foods[35, 11].

Instead of an optical technique, evaporative light scattering detection (ELSD) can be employed. ELSD offers sensitive, universal detection of any sample less volatile than the mobile phase it is in, and both chromophores and non-chromophores can be detected[36]. A HPLC method combined with ELSD capable of analysing four aminoglycosides including amikacin, neomycin, streptomycin and tobramycin has been described[37]. In this publication, the response for all four antibiotics was much improved when detected by ELSD as opposed to UV @ 220nm. Enhancement techniques for ELSD method development are available[36]. Since the chromatographic requirements are similar, methods developed with ELSD are easily transferable to MS[38]. Rapid and simple methods for the separation and quantitation of gentamicin and neomycin by HPLC coupled with ELSD have been developed for pharmaceutical preparations[39, 40].

Manyanga *et al.,* compared a number of LC methods for the analysis of gentamicin and found, on the basis of selectivity, sensitivity and ease of use, that LC-ELSD or LC with pulsed electrochemical detection (PED) were best[41]. It was also shown that method transfer between PED and ELSD is not straightforward. LC methods combined with electrochemical detection have been reported for other aminoglycosides[42]. Cai *et al.,* employed pulsed amperometric detection after ion-exchange chromatography

for determination of three aminoglycosides in milk. Detection limits between 5 and 47 μg L^{-1} were obtained[43].

Mass spectrometry is the detection method of choice for the aminoglycosides due to the lack of chromophores and fluorophores in the molecule. It offers the advantages of sensitivity and confirmation of identity. However, direct mass spectral determination of the aminoglycosides can be difficult due to their thermal lability. The ionisation mode of choice for the production of the ions for residue determination is atmospheric pressure ionisation (API). This technique, coupled to high-performance liquid chromatography and tandem mass spectrometry (LC–MS/MS) has heralded a new era in qualitative and quantitative determination of veterinary drug residues[44]. API techniques include both electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) and enable the determination of compounds with a range of molecular masses as well as non-volatile substances without a need to derivatise. This technique, based on triple quadrupole mass spectrometer and ion trap technologies has become more accessible and affordable to veterinary residue control laboratories[44]. The first mass spectral method which met the U.S. Centre for Veterinary Medicine (CVM) criteria for regulatory compliance was a multi-residue confirmation of six aminoglycoside antibiotics in bovine kidney using ion spray combined with tandem MS detection[45]. The method yielded limits of detection of 25 μg kg^{-1} for gentamicin and neomycin in kidney.

A sensitive method for the determination of streptomycin and dihydrostreptomycin in milk and honey was developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS)[46]. The method was optimised in regard to sensitivity and chromatographic efficiency, and validated by a procedure consistent with EU directive 2002/657[47]. The mass spectrometer conditions were optimised while

infusing a 0.2 mg L^{-1} aqueous solution of the analytes, acidified with 0.1% formic acid. Streptomycin and dihydrostreptomycin generated a similar mass spectrum. The fragments m/z 263, 246, 221, 176 and 407 were found to be the most abundant transitions of the respective protonated molecular ions (m/z 582.1 for streptomcyin and m/z 584.2 for dihydrostreptomycin) to m/z 263 used for screening and quantification, while the ratios with the product ion m/z 246 were used for confirmation of the identity. The LOQ of streptomycin was 2 µg kg^{-1} in honey and 10 µg kg^{-1} in milk and the values for dihydrostreptomycin were a factor of two lower again[46].

An LC-MS procedure for determining nine widely used aminoglycoside antibiotics in bovine milk was developed with LOQ values between 2 µg L^{-1} (apramycin) and 13 µg L^{-1} (streptomycin)[48]. Extraction was carried out using matrix solid-phase dispersion (MSPD) followed by a gradient LC system using increasing methanol concentration. Heptafluorobutyric acid was included in the mobile phase as an ion pair agent. Detection was carried out in multi reaction-monitoring (MRM) mode and quantitation performed by selecting at least two fragmentation reactions for each analyte. Table 3.1 shows the mass spectral conditions and individual limits of quantitation. Babin *et al.,* reported an even more sensitive LC-MS/MS method for the determination of aminoglycosides in food where the LOD values were between 0.1 and 0.4 µg kg^{-1} in various tissue samples[49].

Table 3.1: Time-scheduled MRM conditions for detecting aminoglycoside antibiotics[48]

Compound	MRM transition (m/z)	Cone voltage (V)	Collision energy (eV)	LOQ µg kg^{-1}
Spectinomycin	351 > 315, 333	32	20	5
Dihydrostreptomycin	**293** > 176, 409	20	12	3
Streptomycin	**308** > 176, 263	20	15	13
Aminosidine	**309** > 161, 455	15	12	–
Apramycin	**271** > 163, 217	15	12	2
Gentamicin C1a	**226** > 129, 322	10	6	5
Gentamicin C2, C2a	**233** > 126,143, 322	12	6	7
Gentamicin C1	**240** > 139,157, 322	15	10	6
Neomycin B	**308** > 161, 455	15	10	4

3.4.3 Macrolides

The macrolides do contain chromophores and hence quantitative, direct UV determination is possible. The determination of seven macrolides in sheep's milk has been described using LC–DAD[50]. Erythromycin and roxythromycin were quantified at 210 nm, josamycin and spiramycin at 231 nm, and tylosin at 287 nm. LODs ranged from 24 to 72 µg kg^{-1}. Another study using LC-DAD was shown to be capable of determining seven macrolides in animal liver and kidney samples[51]. The analytes were separated using a gradient elution system with an aqueous phosphate/phosphoric acid buffer (pH 3.5) for mobile phase A and acetonitrile for mobile phase B. Validation was carried out according to the European Commission Decision 657/2002[47].

When the results were compared to those obtained by LC-MS detection in selected-ion monitoring (SIM) mode, the LC-DAD method was found to be robust, selective and stable. The LC-DAD method was found to be sensitive enough for detecting macrolides in liver samples with LOD values at or close to the MRLs but the LOD values were ten times lower using LC-MS (15-50 µg kg^{-1}). The method was applied to rabbit liver samples (see Figure 3.1). An LC-UV method for determination of spiramycin and tylosin in feedstuffs yielded detection limits of 176 and 118 µg kg^{-1} respectively[52].

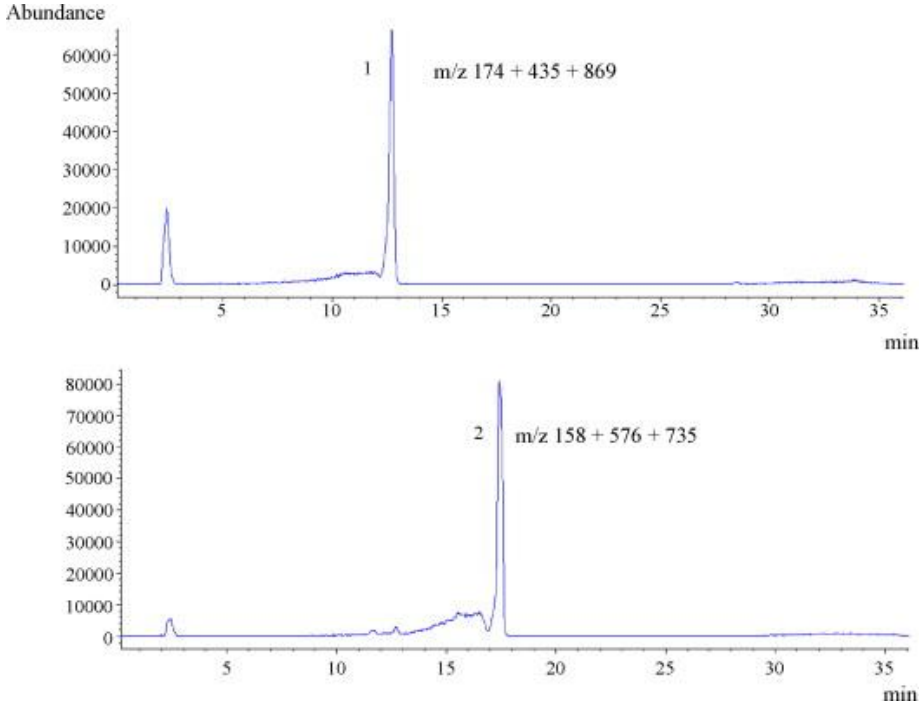


Figure 3.1: SIM chromatograms corresponding to the extract of rabbit liver sample where tilmicosin (1) was found at 250 µg $kg^{-1}$and erythromycin (2) at 168 µg kg^{-1} (51) (Berrada 2007)

LC-MS using electrospray ionisation has been used to successfully determine seven macrolides in chicken muscle[53]. The protonated molecular ion was used for quantitation purposes under selected ion monitoring (SIM) mode. Detection limits ranged from 1-20 µg L^{-1}.

Table 3.2: Typical ions detected for macrolide antibiotics using LC–ESI-MS[56]

Compound	M_w	Base peak ions (amu)	Other main ion Masses (amu)
Erythromycin	733.9	734.5 $(M+H)^+$	716.4, 576.3
Oleandomycin	688.9	688.4 $(M+H)^+$	670.4, 544.3
Kitasamycin	771.9	772.5 $(M+H)^+$	702.5, 558.3
Josamycin	828.0	828.5 $(M+H)^+$	860.4, 786.4
Mirosamicin	727.9	728.4 $(M+H)^+$	554.3
Spiramycin	843.1	422.3 $(M+2H)^{2+}$	843.5, 699.5, 540.3
Neospiramycin	698.8	350.2 $(M+2H)^{2+}$	721.5, 699.5, 540.3
Tilmicosin	869.2	435.3 $(M+2H)^{2+}$	869.5, 695.5
Tylosin	916.1	916.5 $(M+2H)^{2+}$	742.3, 582.3

A confirmatory method for three macrolides using micro-LC-MS/MS in bovine tissues was published in 2001[54]. This method used an atmospheric pressure source with an ion spray interface to detect molecular ions $[M+2H]^{2+}$ at m/z 435 for tilmicosin, and $[M+H]^+$ ions at m/z 734 for erythromycin and 918 for tylosin. Two diagnostic daughter ions for each compound were studied to fulfil the confirmation requirements. LOQ values in kidney, liver and muscle ranged from 20-150 µg kg^{-1}. An LC-tandem mass spectrometric method for the determination of tylosin in honey yielded an LOD and LOQ of < 3 µg kg^{-1} and < 5 µg kg^{-1} respectively[55].

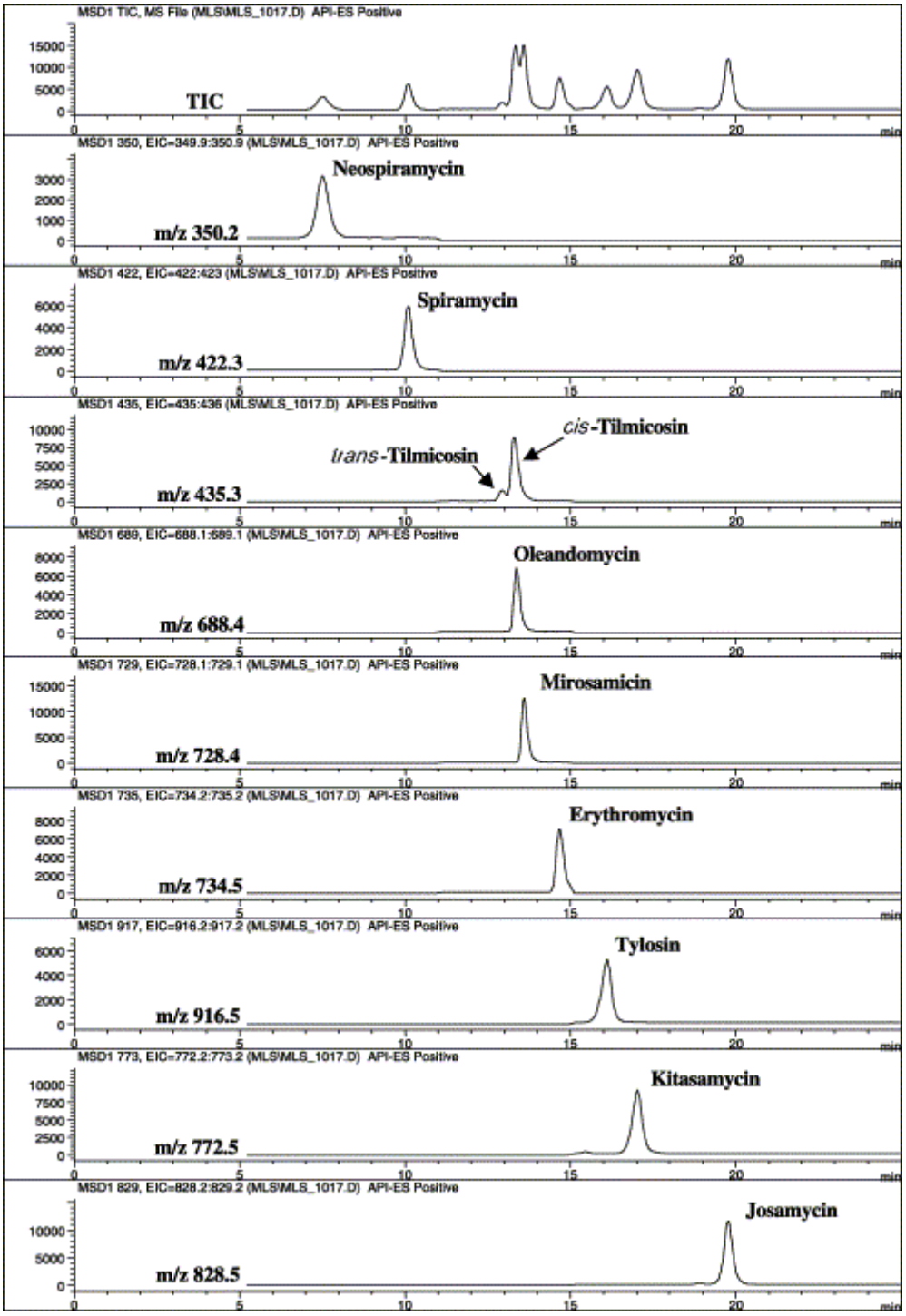


Figure 3.2: Typical LC chromatograms of a standard mixture 0.05 µg mL (Horie et al., 2003) [56]

The assay, developed for the control of unauthorised use of antibiotics in bee-keeping, was validated according to the guidelines laid down by Commission Decision

2002/657/EC[47]. Another LC–MS method for determination of eight macrolides in meat and fish samples resulted in LOQ values of 10 g kg^{-1} (Table 3.2) [56]. A total ion current (TIC) trace and extracted ion chromatograms for these antibiotics are shown in Figure 3.2.

In recent years, sensitivity has improved using LC-MS techniques with detection limits less than 1 µg kg^{-1} being reported for some macrolides in food matrices[57, 58, 59]. Building on analytical methods reported previously by the author, Wang and Leung compared two LC-MS assays for the determination of six macrolides in eggs, milk and honey[58]. The first technique was UPLC-QTof MS with an electrospray interface, which allowed unambiguous confirmation of positive findings and identification of degradation products but was not as sensitive as LC-MS/MS. The second technique was a triple quadrupole LC-MS/MS, which gave better repeatability and lower LOD concentrations of 0.01-0.5 µg kg^{-1}.

Summary

This chapter describes the various analytical methods for the determination of aminoglycosides and macrolides in food matrices focusing mainly on methods published in the past decade. Extraction of these two classes of antibiotics from food has also been explored. This is a very important area for the monitoring of veterinary residues in agriculture as there are so many different compounds and matrices required to be monitored under the legislation and their concentration levels are getting lower all the time. Despite the activity in this area of research, there still exist many gaps for certain matrices and species that residue laboratories are required to monitor in their national residue plans. With this in mind, multi-residue ‘catch-all’ methods or even combination methods for both aminoglycosides and macrolides using definitive techniques such as

LC-MS are highly appealing in terms of fulfilling the legislation requirements as well as their high throughput and sensitivity.

References

1. Touchstone J.C., Dobbins M.F., "*Practice of Thin Layer Chromatography"* 2nd Ed., **1983**, John Wiley and Sons Inc.
2. Stead D. A. (2000). Current *Journal of Chromatography B*, **747;** 69-93
3. Bhushan R., Arora M. (2001). *Journal of Planar Chromatography*,**14**; 35-438
4. Kanfer I., Skinner M.F., Walker R.B. (1998). *Journal of Chromatography A,* **812**; 255-286
5. Hernandez M., Borrull F., Calull M. (2003). *Trends in Analytical Chemistry,* **22**; 7-8
6. *Flurer C.L. (1995). Journal of Pharmaceutical and Biomendical Analysis, **13**; 809-816*
7. Ackermans, M.T., Everaerts, F.M., Beckers J.L. (1992). *Journal of Chromatography*, **606**; 229
8. Yang W.C., Yu A.M., Chen H.Y (2001). *Journal of Chromatography A*, **905**; 309-318
9. Kaale E., van Schepdael A., Roets E., Hoogmartens J. (2001). *Journal of Chromatography A*, **924**; 451-458
10. Kaale E., van Schepdael A., Roets E., Hoogmartens J. (2002). *American Laboratory*, **34**; 21, 22, 24, 26
11. Serrano J.M., Silva M. (2006). *Journal of Chromatography B*, **843(1);** 20-24

12. Ferguson, J.P**.**, Baxter, G.A., McEvoy, J.D.G., Stead, S., Rawlings, E. and Sharman, M. (2002). *Analyst*, **127;** 951-956.

13. Haasnoot W., Cazemier G., Koets M., van Amerongen, A., (2003). *Analytica Chimica Acta*, **488(1)**; 53-60

14. Tschmelak J., Proll G., Gauglitz G. (2005). *Talanta*, **65(2);** 313-323

15. Moehrle V., Stadler M., Eberz G. (2007). *Analytical and Bioanalytical Chemistry*, **388(5-6)**; 1117-1125

16. Williams D.H., Fleming I., *"Spectroscopic methods in organic chemistry"* 4th edition revised, **1989**, McGraw-Hill.

17. Pasternack R. F., Collings P. J. (1995). *Science*, **269;** 935

18. Liu, S.P, Hu X.L., Luo, H.Q. (2003). *Analytical Sciences*, **17**; 927-932

19. Hu X.L., Liu **S.P.,** (2003). Rayleigh *Analytical and Bioanalytical Chemistry*, **376 (1);** 42-48

20. Chen Y., Shang Y., Li X., Wu X., Xiao X. (2008). *Food Chemistry*, **108(1)**; 304-309

21. Haasnoot W., Stouten P., Cazemier G., Lommen A., Nouws J.F.M., Keukens H.J., (1999). *The Analyst*, **124 (3)**; 301-305

22. Draisci R., delli Quadri F., Achene L., Volpe G., Palleschi L., Palleschi G. (2001). *The Analyst*, **126**; 1942- 1946 (a)

23. Nouws J., van Egmond H., Smulders I., Loeffen G., Schouten J., Stegeman H. (1999). *International Dairy Journal*, **9(2)**; 85-90

24. Sachetelli S., Beaulac C., Lagace J. (1998). *Biochimica et Biophysica Acta;* **1379;** 35-41

25. Schoenmakers P.J., "*Optimisation of Chromatographic Selectivity- a guide to method development"* 1st Ed., **1986**, Elsevier Press.

26. Rivier L. (2003). *Analytica Chimica Acta,* **492**; 69-82

27. Loffler D., Ternes T. A. (2003). *Journal of Chromatography A,* **1000**; 583-588

28. De Wash, K., Alam, Z., Benoot, J., Van Hoof, N., Poelmans, S., De Brabander, H., in: Poster Presented at the Fourth International Symposium on Hormone and Veterinary Drug Residue Analysis, Antwerpen, June 2002

29. Getek T.A., Vestal M.L., Alexamder T.G. (1991). *Journal of Chromatography A,* **554;** 191-203

30. Viñas P., Balsalobre N., Hernández-Córdoba M. (2007). *Talanta,* **72 (2);** 808-812

31. Hornish R. E., Wiest J. R. (1998). *Journal of Chromatography A,* **812**; 123–133

32. Kijak P. J., Jackson J., Shaikh B. (1997). *Journal of Chromatography B: Biomedical Sciences and Applications,* **691(2)**; 377-382

33. Posyniak A, Zmudzki J, Niedzielska J. (2001). *Journal of Chromatography A,* **914;** 59-66

34. Jiang H., Liu S.P.**,** Hu X.L.**,** Liu Z.F.**,** Qin Z.H.**,** Zhan H.L. (2003). *Fenxi huàxué,* **31(9);** 1053-1057

35. Yang M, Tomellini S.A. (2001). *Journal of Chromatography A,* **939**; 59-67

36. Megoulas N.C., Koupparis **M.A.** (2005). Twenty years of evaporative light scattering detection. *Critical Reviews in Analytical Chemistry,* **35(4)**; 301-316

37. Manis L.E., Wilcox M.J. (2001). Reversed-phase HPLC Analysis of Aminoglycoside Antibiotics using Evaporative light Scattering Detection.. *Alltech Associates Inc.*

38. Chitneni S.K., Govaerts C., Adams E., van Schepdael A., Hoogmartens J. (2004). *Journal of Chromatography A*, **1056(1-2)**; 111-120

39. Clarot I., Chaimbault P., Hasdenteufel F., Netter P., Nicolas A. (2004). *Journal of Chromatography A*, **1031(1)**; 281-287

40. Clarot I., Regazzeti A**.,** Auzeil N., Laadani F., Citton M., Netter P., Nicolas A. (2005). *Journal of Chromatography A*, **1087(1-2)**; 236-244

41. Manyanga V., Grishina O., Yun Z., Hoogmartens J., Adams E. (2007). *Journal of Pharmaceutical and Biomedical Analysis*, **45(2)**; 257-262

42. Wang J., Wang D.D., Ni K.Y., Hu X.J. (2007). *Journal of Liquid Chromatography & Related Technologies*, **30(5-8);** 1001-1013 b

43. Cai Y.E., Cai Y.Q., Mou S.F., Lu Y.Q. (2006). *Fenxi shiyanshi*, **25(8)** ; 7-9

44. Balizs G., Hewitt A. (2003). *Analytica Chimica Acta,* **492 (1-2);** 105-131

45. McLaughlin L.G.; Henion J.D.; Kijak P.J. (1994). *Biological Mass Spectrometry,* **23(7)**; 417-429

46. Bruijnsvoort M., Ottink S.J.M., Jonker K.M., de Boer E. (2004). *Journal of Chromatography A*, **1058 (1- 2)**; 137- 142

47. Commission Decision 2002/657/EC; Performance of Analytical Methods. *Off. J. Europ. Comm.*, 2002; **L221,** 8.

48. Bogialli S, Curini R, Di Corcia A, Laganà A, Mele M, Nazzari M. (2005). *Journal of Chromatography A*, **1067(1 -2)**; 93-100

49. Babin Y., Fortier S. (2007). *Journal of AOAC International*, **90(5)**; 1418-1426

50. García-Mayor, M. A., Garcinuño R. M., Fernández-Hernando P., Durand-Alegría J.S. (2006). *Journal of Chromatography A,* **1122(1-2)**; 76-83

51. Berrada, H., Borrull F., Font G., Moltó J.C., Marcé R.M. (2007). *Journal of Chromatography A*, **1157(1-2)**; 281-288

52. Civitareale, C., Fiori M., Ballerini, A., Brambilla, G. (2004). *Journal of Pharmaceutical and Biomedical Analysis,* **36(2)**; 317-325

53. Codony R., Compano R., Granados M., Garcio-Regueiro J.A., Prat M.D. (2002). *Journal of Chromatography A,* **959**; 131-141

54. Draisci R., Palleschi L., Ferretti L., Achene L., Cecilia A. (2001). *Journal of Chromatography A*, **926**; 97-104 (b)

55. Benetti C., Dainese N., Biancotto G., Piro R., Muntnelli F. (2004). *Analytica Chimica Acta*, **520(1-2);** 87-92

56. Horie M., Tekegami K.T., Nakazwa H. (2003). *Analytica Chimica Acta*, **492, 1-2;** 187-197

57. Yue, Z.F, Chen, X.X., Xie, L.Q., Ji, C.N., Hua, H.H. (2007). *Fenxi Huaxue*, **35 (9)**; 1290-1294

58. Wang J., Leung D. (2007). *Rapid Communications in Mass Spectrometry,* **21(19);** 3213-3222 a

59. Xu, J.Z., Wu Z.X. (2007). *Fenxi Huaxue,* **35(2);** 166-170

Chapter 4: Development and optimisation of an analytical assay for the macrolide and aminoglycoside classes of antibiotics using UPLC-MS/MS

4.1 Introduction

This study on a number of antibiotics from the macrolide and aminoglycoside families was carried out using ultra-performance liquid chromatography (UPLC) with a triple quadrupole tandem mass spectrometer as a detector. The optimisation of the mass spectrometer, dwell times, cone voltages, flow rates of gas is discussed in detail and the chromatographic conditions that were explored discussed.

This study outlines the extraction and optimisation of a UPLC method, and the development of a mass spectroscopy method for a number of antibiotics from the macrolide and aminoglycoside family of antibiotics in bovine muscle. These antibiotics are approved for use within strict guidelines set down in Irish legislation and hence screening of animal products such as meat, milk, and eggs to ensure that the legislation is adhered to be very important. Analysis of positive screening tests for aminoglycosides and macrolide residues in animal products must adhere to Commission Decision 2002/657/EC[1] where suitable confirmatory methods are based on chromatographic analysis using spectrometric detection. The maximum residue limits (MRLs) allowed is very low and hence it can be challenging to achieve the sensitivity required in an assay that can determine a number of compounds simultaneously. These analytes are polar, resistant to acids, bases and heat and are not extensively bound to proteins[2]. They are found to adsorb to glass.

A number of analytes from the macrolide and aminoglycoside groups were chosen, the individual standards were tuned in the mass spectrometer and then a mixture of the analytes was injected onto the analytical column using a gradient elution system for separation. A major advantage of UPLC technology over conventional HPLC is the use of sub 2µm particles which improve resolution, allow for shorter run times thereby

reducing solvent use, costs and waste[3].

The objective of this study is to present a method that allows for the quantitation of a number of different compounds from the aminoglycoside and macrolide groups together in a mixture in an animal tissue. The development of this method and the various parameters for optimisation are also presented and discussed.

4.2. Experimental

4.2.1 Materials and Reagents

The following antibiotics were purchased from Sigma-Aldrich Corporation, tobramycin (89549), apramycin sulfate (A2024), kanamycin sulfate (60616), dihydrostreptomycin sesquisulfate (37386), spectinomycin dihydrochloride pentahydrate (85555), streptomycin sulfate (2158X), spiramycin mixture of I, II and III (S-9132), tylosin tartrate (T-6134), roxithromycin (R4393), lincomycin (62143) and paromomycin sulfate (76261).

The following HPLC grade solvents were purchased from Fisher Scientific, methanol, acetonitrile and ultra-pure water. Negative (macrolide and aminoglycoside free) bovine muscle was sourced from abbotstown farm and screened in-house using the 6-plate microbiological screen to ensure negativity, then homogenised and divided into 5g individual portions. Heptafluorobutyric acid (HFBA) was purchased from Sigma-Aldrich Corporation.

Various Waters Oasis® solid phase extraction cartridges used were WCX, WAX, MCX and MAX that were all purchased from Waters Chromatography, Ireland. Varian solid phase extraction cartridges used were the bond Elut LRC-SCX 500mg part number 12113039 and Varian bond Elut C18 200mg/ 3mL part number 35402 and were

purchased from JVA Analytical.

4.2.2 UPLC and Mass spectrometer

The Ultra performance liquid chromatography system (UPLC) was a Waters Acquity UPLC® system with a 2.1 x 50mm (1.7µm) C18 Acquity UPLC® BEH column, Acquity Sample Organiser, In-line degasser AF, Column Manager and Heater/ Cooler, Binary Solvent Manager and Sample Manager. Data was processed using Waters Masslynx™ software. The mass spectrometer used was a Waters Quattro Premier™ XE tandem quadrupole mass spectrometer utilising T-wave™ collision cell, multi-mode ionisation and rapid polarity switching technologies.

4.2.3 Extraction

The extraction equipment used consisted of a REAX 2 overhead shaker together with a Heidolph test tube shaker, a Techne sample concentrator FSC 400D with Techne Dri-block heater DB-3 with 127mm needles and insert blocks were purchased from Lennox Chemicals Ltd. The vacuum chamber was a VacElut 20 purchased from JVA Analytical and the Laboport vacuum/ pressure diaphram self drying pump was purchased from Carl Stuart Limited.

4.2.4 Preparation of Standards and Samples

4.2.4.1 Standard Solutions

Stock solutions (500µg/mL) of spiramycin, tobramycin, apramycin, kanamycin, dihydrostreptomycin, lincomycin, spectinomycin, tylosin, and roxithromycin were prepared in methanol. Stock solutions (500µg/mL) of streptomycin and paromomycin were prepared in ultra-pure water. Mixed standard solutions at 100ng/mL, 200ng/mL, 300ng/mL, 500ng/mL and 1000ng/mL were diluted from stocks in water. All standards

were prepared in polypropylene volumetric units and stored in polypropylene amber storage jars at 4ºC.

4.2.4.2 Matrix Samples

Homogenised muscle samples (2g) were weighed and placed in 50mL polypropylene centrifuge tubes. Each tube was labelled with weight and date and stored at -20ºC until used. Matrix samples were spiked at appropriate levels using mixed standard solutions immediately prior to extraction. Samples were then de-proteinised by adding 10mL of either 5% trichloroacetic acid or 5% ammonium hydroxide and placed on a shaker for 10 minutes. The samples were then centrifuged at 10,000rpm for 10 minutes and a 3mL aliquot was transferred to the solid phase extraction cartridge.

4.2.5 Assay Conditions

4.2.5.1 Extraction/ Clean-up Protocol

Cartridges were conditioned with 3mL methanol followed by an equilibration step with 3mL water. The 3mL sample was loaded onto the cartridge and the cartridge was washed with 3mL water. The analytes were then eluted in either 3mL methanol or 3mL of a 2% formic acid solution in methanol. This eluate was evaporated under nitrogen on a heating block set at ~45ºC and then re-constituted in 300µl water.

4.2.5.2 UPLC Method

Gradient elution was used with solvent A (ultra-pure water with 10mM HFBA) and solvent B (100% methanol or 100% acetonitrile, where indicated) as follows: T_0 90/10, $T_{0.5}$ 90/10, $T_{1.0}$ 50/50, $T_{2.5}$ 50/50 $T_{4.0}$ 40/60 $T_{5.0}$ 40/60, $T_{5.5}$ 90/10, $T_{6.0}$ 90/10. The system was conditioned with 15% solvent B for 1 hour prior to use on each day or until a delta pressure of <40psi was observed on the Solvent Manager. The mobile phase was degassed and filtered by passing through a 0.45µm pore size membrane filter (Milipore,

Milford, MA, USA) prior to use and further degassed with the in-line degasser. The flow rate was 0.450 mL min^{-1}. All injections were performed on column at temperature of 35ºC with injection volumes of 10 to 20µl as indicated.

4.2.5.3 MS Conditions

Tuning for all analytes was carried out for the mass spectrometer with the optimised conditions as follows: Capillary (kV) 3.5, Extractor (V) 4.00, RF lens (V) 0.4, Source Temperature (ºC) 120, Desolvation Temperature (ºC) 350, Cone Gas Flow (L/Hr) 200, Desolvation Gas Flow (L/Hr) 900, Ion Energy MS1 0.8 and Ion Energy MS2 1.0

Table 4.1: Parent and Daughter Ions with optimised collision and cone voltages

Analytes	Parent Ion (M)+	Daughter Ions	Collision (eV)	Cone (V)
Tylosin	916.5	174.2 & 101.4	40 & 45	50
Roxithromycin	837.4	158.4 & 679.5	30 & 20	30
Streptomycin	582.1	263.2 & 176.1	35 & 40	60
Dihydrostreptomycin	584.1	246.2 & 263.1	35 & 30	50
Paromomycin	616.2	163.2 & 203.2	40 & 40	45
Spiramycin	843.5	174.2 & 101.2	40 & 40	40
Spectinomycin	333.2	98.1 & 116.1	30 & 25	55
Lincomycin	407.2	126.4 & 359.2	30 & 20	40
Kanamycin	485.2	163.0 & 205.2	30 & 30	30
Apramycin	540.2	217.2 & 378.2	30 & 20	55
Tobramycin	468.2	163.2 & 205.3	25 & 25	30

4.3. Results and Discussion

4.3.1 Development of Extraction Protocol

As discussed in section 2.3, the macrolides and aminoglycosides are basic analytes with known pkA's ranging from 5.4 for apramycin (one of four protons) to 9.5 for roxithromycin. A number of different solid phase extraction cartridges were tested. The following Waters cartridges were compared:

- HLB: Hydrophilic- lipophilic sorbent for all compounds.
- MCX: A mixed-mode Cation exchange and reverse-phase sorbent suitable for bases, high selectivity for basic compounds.
- MAX: A mixed-mode Anion exchange and reverse-phase sorbent suitable for acids, high selectivity for acidic compounds.
- WCX: A mixed-mode Cation exchange and reverse-phase sorbent with a high selectivity for strong bases.
- WAX: A mixed-mode Anion exchange and reverse-phase sorbent with a high selectivity for strong acids.

4.3.2 Development of UPLC Method

4.3.2.1 Mobile Phase Composition

During the course of the laboratory work for this study, the instrument chosen was a Waters Acquity UPLC coupled with a Waters Premier XE mass spectrometer. This equipment was designed to allow for fast sensitive analysis of samples thus proving an excellent choice for method development especially in terms of the sheer number of anlaytes proposed in this study.

Initially an isocratic mobile phase of mobile phase A ultra-pure water (0.2% acetic) with mobile phase B acetonitrile (0.2% acetic) was chosen to look at some individual analytes. Tylosin at a level of 20ng on column was run through the Acquity column at a number of different conditions from 85% A down to 30% A. Retention was found to improve at the lower aqueous level as these conditions allowed for better interaction between analyte and the non-polar stationary phase. Retention of streptomycin and tilmicosin was also observed using this mobile phase.

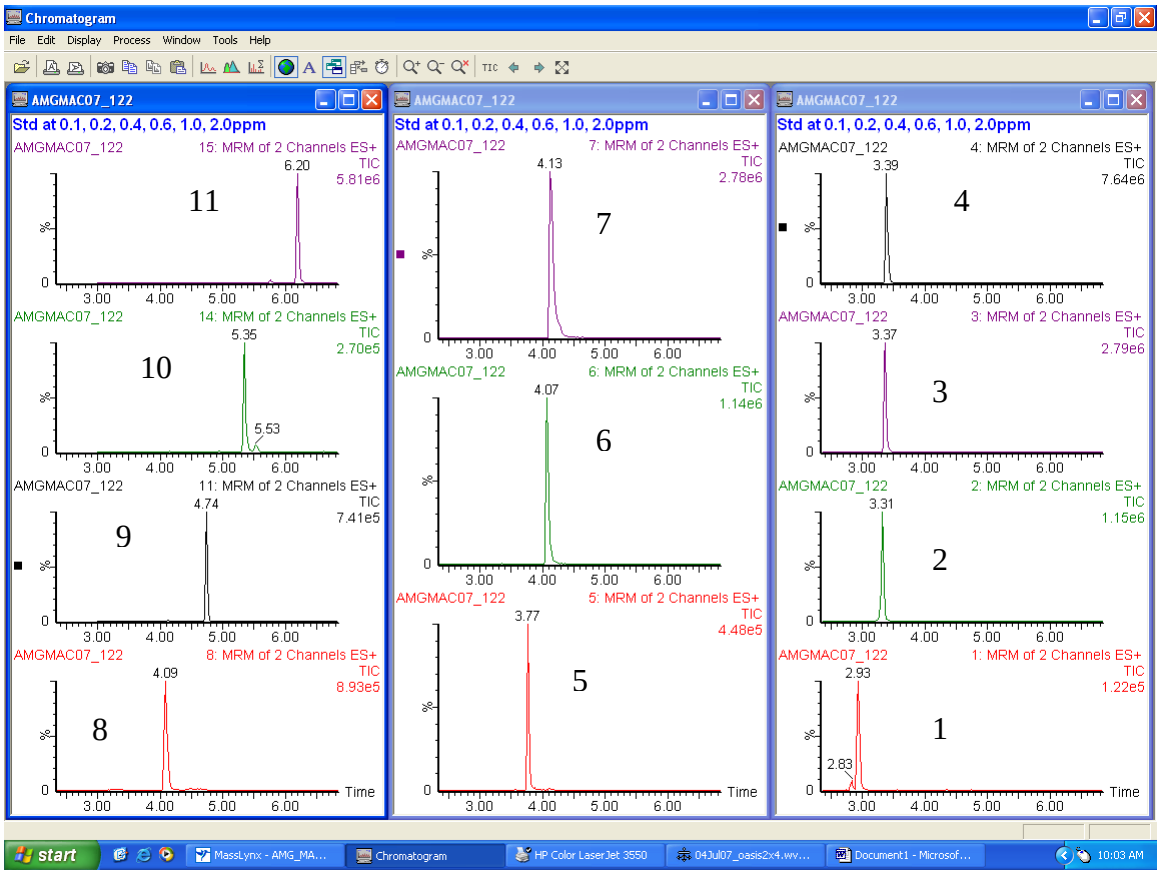


Figure 4.1: Standard mix of aminoglycoside and macrolides at their MRL levels, 50ppb-1000ppb. Peaks 1 to 11: Spectinomycin, lincomycin, streptomycin, dihydrostreptomycin, kanamycin, apramycin, tobramycin, paromomycin, spiramycin, tylosin and roxithromycin.

The aminoglycosides and macrolides eluted in general according to their polarities with spectinomycin of highest polarity eluting first and roxithromycin eluting last (Figure 4.1) as expected using reverse phase chromatography with the following UPLC Conditions: Flow Rate: 0.3mL/min, Solvent A: 10mM HFBA, Solvent B: Acetonitrile, Gradient: Initial 90%A, 4.5min 50%, 8.5min 30%, 9.5min 90%, 10.5min 90%.

Gentamicin was a more difficult analyte to work with as it was less polar than the others. No retention of gentamicin was seen using the same mobile phase conditions above. Methanol was substituted for acetonitrile as mobile phase B to see if the slightly more polar methanol would improve the result for this analyte. However no retention was achieved.

4.3.2.2 Use of Ion-pair reagent

A literature review for these analytes provided a solution for the problem with gentamicin in the form of the use of an ion-pairing agent. The effects of increasing amounts of pentafluoroporpionc acid (PFPA) from 0.2% to 0.4% were found to cause more retention of the aminoglycosdies on a reversed phase column[4] (Manis 2001). The retention of the aminoglycosides on reversed-phase columns increased with ion-paring chain length for e.g. HFBA > PFPA[5] (Heller 2000). Therefore, a mobile phase containing 10mM HFBA in ultra-pre water was chosen as mobile phase A and 100% methanol for mobile phase B in a ratio of (50:50) resulted in a broad peak at ~2 minutes observed for gentamicin.

In the case of tilmicosin, which is more polar to gentamicin, a (50:50) ratio resulted in less retention as the peak eluted very quickly in the mobile phase, changing to a less polar mobile phase ratio of (30:70) improved retention (Figure 4.2). The peak

quality characteristics (Table 4.2) were also improved with the kurtosis value closer to a more normal peak shape of 0 for peak A compared to peak B.

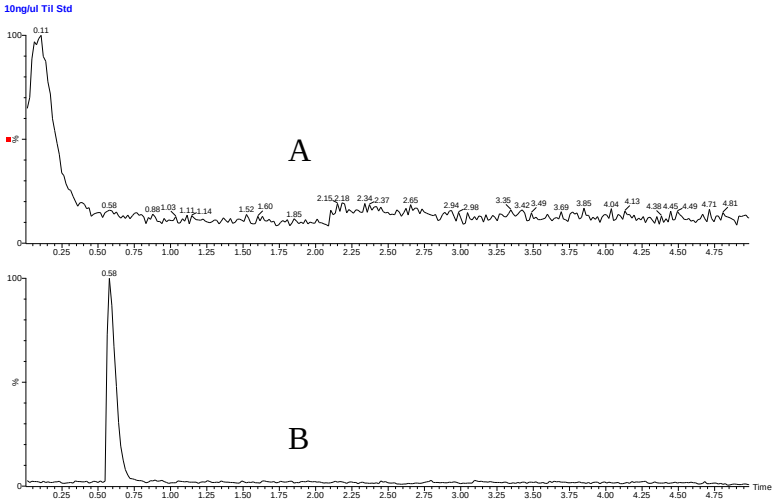


Figure 4.2: Tilmicosin standard @ 10ng/µl, Mobile phase 10mM HFBA/ methanol Chromatogram A (50:50) and chromatogram B (30:70)

The Waters Acquity UPLC® ,columns are designed to combine faster seperations with high resolution. These columns are capable of running under high pressure conditions of up to 15000 psi (1000 bar) and are available in many different configurations and chemistries.

Table 4.2: Peak quality factors for Tilmicosin standard @ 10ng/µl

Chromatogram	Skewness	Kurtosis	Signal/ Noise
Chromatogram A	0.128	-1.077	10
Chromatogram B	0.229	0.430	239

Mobile phases: chromatogram A 10mM HFBA/ methanol (50:50) and chromatogram B (30:70)

Once all the individual analytes had been assessed individually a mixture of all 8 analytes in 30% methanol was prepared at 10 ng/µl and 5 ng/µl respectively. A gradient mixture of the mobile phases was employed to look at separating the analytes. Although there is some variation in the polarities of the analytes the known pka's lie in a close range from 7.1 for tylosin to 9.5 for roxithromycin[6] (Merck Index, 13th edition) so the peaks eluted in quite a close group between 1.97 min and 5.09 min for 100 ng on column.

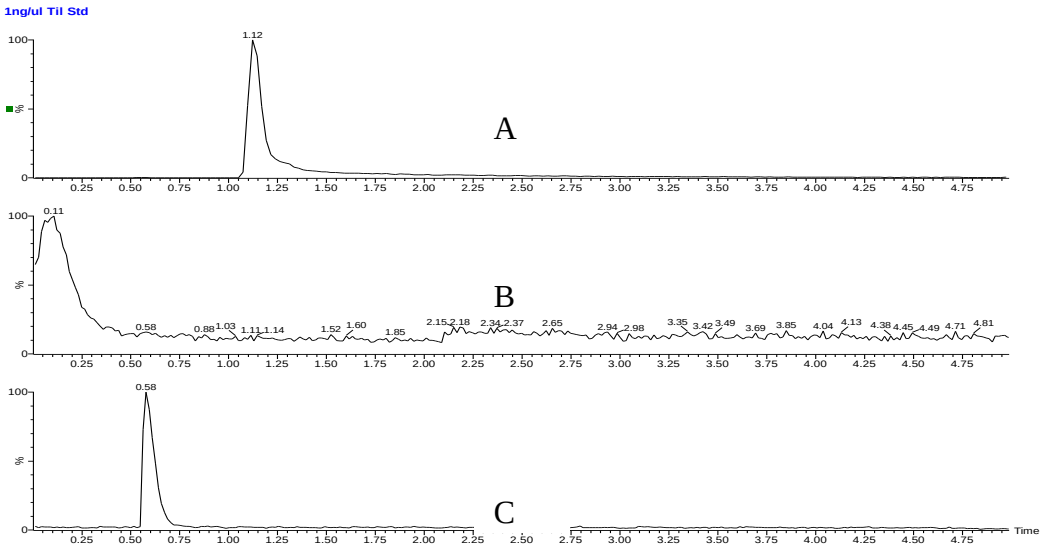


Figure 4.3: Illustration of peak tailing for tilmicosin. Chromatogram A shows tilmicosin eluting in ultra-pure water (0.2% acetic: acetonitrile (0.2% acetic) (30:70), B shows elution in 10 mM HFBA: methanol (50:50) and C 10 mM HFBA: methanol (30:70).

Peak shape was found to be affected by changes in the mobile phase polarity and the addition of an ion-pairing agent (Figure 4.3). The tailing for tilmicosin was improved with the addition of the ion-pairing agent HFBA compared with acetic acid (Figure 4.3, chromatograms A and C) and further when the mobile phase went from 50% aqueous

(Figure 4.3, chromatograms B) to 30% aqueous (Figure 4.4, chromatograms B). The peak quality criteria also reflect this (Table 4.3) with the kurtosis and skewness closest to the expected normal distribution of 0 for chromatogram C.

Table 4.3: Peak quality factors Tilmicosin standard at 10ng/µl.

Chromatogram	Skewness	Kurtosis	Signal/ Noise
Chromatogram A	1.585	2.071	586
Chromatogram B	0.128	-1.077	10
Chromatogram C	0.229	0.430	239

Chromatogram A ultra-pure water (0.2% acetic: acetonitrile (0.2% acetic) (30:70), B 10 mM HFBA: methanol (50:50) and C 10 mM HFBA: methanol (30:70).

4.3.2.3 Introducing Matrix and Injecting mixed standards in matrix

As each analyte has an individual MRL, a mixture of the analytes were prepared to reflect the levels of interest from 50 µg/L to 250 µg/L separation of all 7 analytes was possible (Figure 4.4).

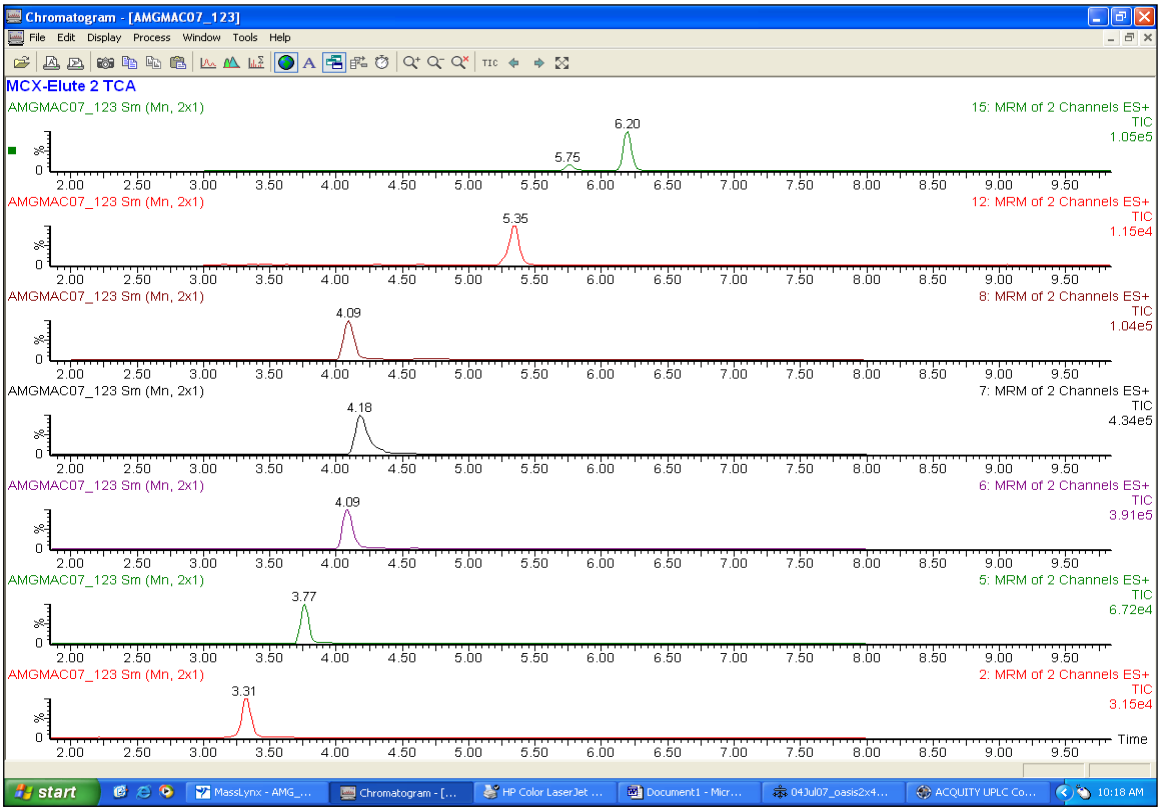


Figure 4.4: Extracted bovine muscle sample. 8 analytes extracted from bovine muscle at 50 ppb to 250 ppb, from bottom to top, lincomycin, dihydrostreptomycin, streptomycin, spiramycin, tylosin, erythromycin and roxithromycin.

4.3.3 Development of MS Conditions

4.3.3.1 MS tuning and optimisation for parent and daughter ions

Mass spectrometry is the detection method of choice for the aminoglycosides due to a lack of chromophores and fluorophores in the molecule ruling out the use of UV or fluorescence detection without the use of derivatisation agents as demonstrated by many authors[7, 8, 9, 10] or UV absorbance[11]. The macrolides on the other hand do contain chromophores and fluoresce but since the aim of this study was to determine the aminoglycosides and macrolides as one group mass spectrometry was the detection method of choice. Mass spectral data offers the advantages of sensitivity and

confirmation of identity, however, direct mass spectral determination of the aminoglycosides can be difficult due to their thermal lability[12].

A sample solution of each individual analyte in methanol was infused directly into the MS through the capillary at a flow rate of 40µl/min. Mobile phase was mixed with analyte at a rate of 0.450 mL min^{-1} through the T-piece. The mass range of interest i.e. the mass of the parent ion (M+) was focused on and the various settings for the instrument such as cone voltage, capillary voltage, gas flows and temperatures, optimised to get the best response for each parent ion. An example of the tuning spectrum for tylosin with mass 917 can be seen in Figure 4.5.

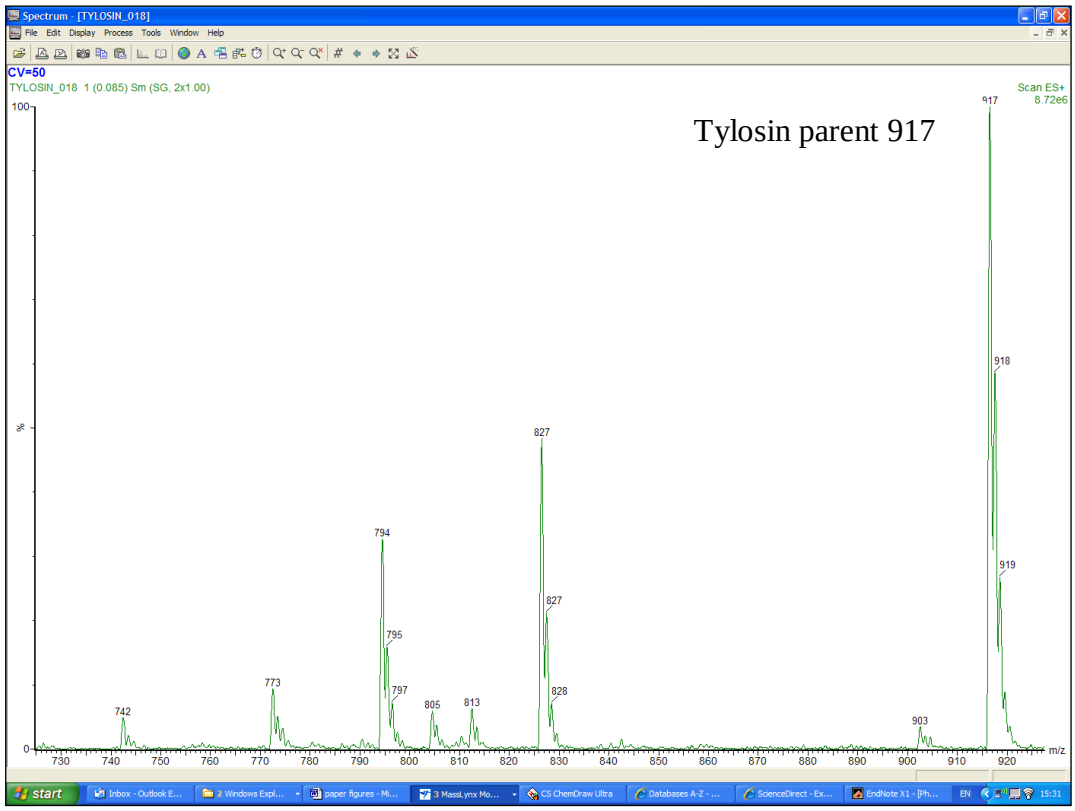


Figure 4.5: Mass spectrum of parent ion for tylosin [M^{+1}] 917.

The ionisation of these analytes can be difficult due to their polarity and the ratio for the signal to concentration can be very small in comparison to other analytes[13]. The aminoglycoside and macrolide analytes showed similar fragmentation patterns and the fragment of highest intensity used for quantification. Figure 4.6 shows the daughter ions produced when tylosin is fragmented.

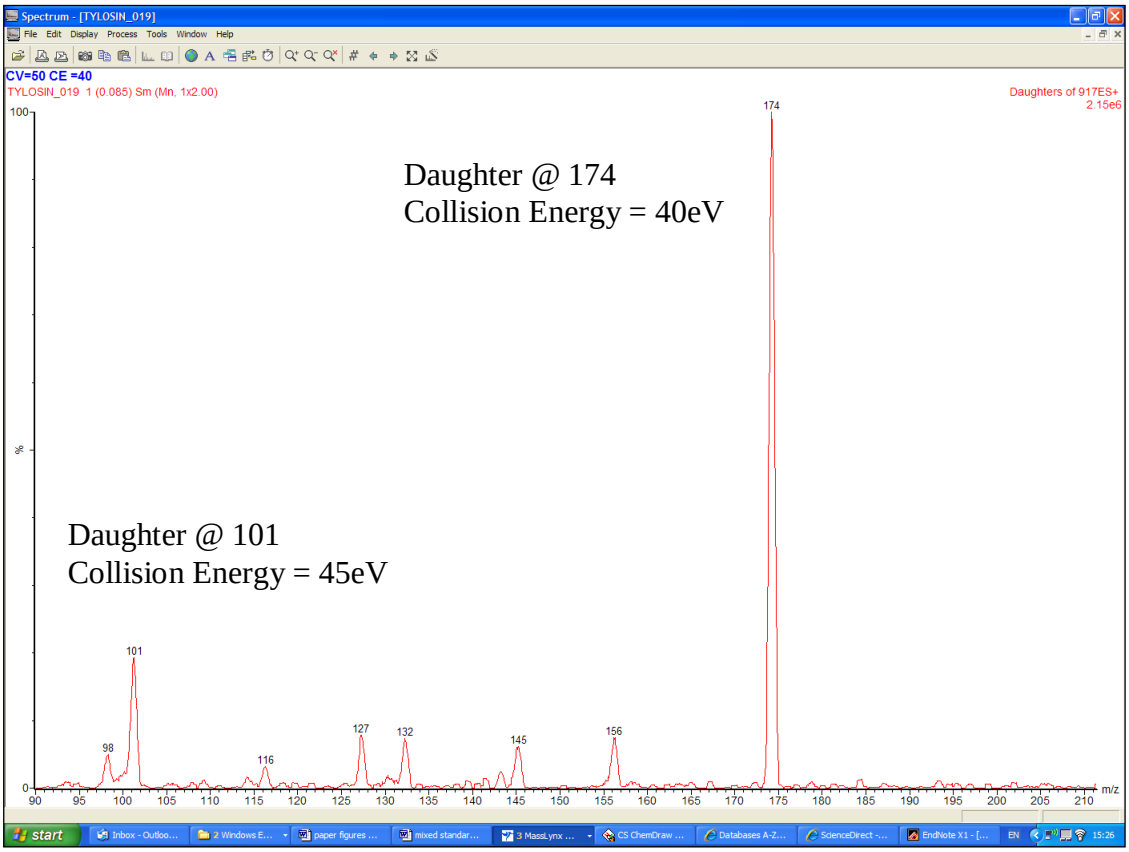


Figure 4.6: Mass Spectrum for daughter ions of Tylosin [M^{+1}] 917

A Multiple Reaction Monitoring (MRM) file was built for each analyte using the optimised conditions. Figure 4.7 shows the total ion chromatogram (TIC) and two MRM files for a tylosin standard at 1ng/µl. This process was carried out for each analyte with each parent to daughter ion transition occurring under specific conditions (Table 4.1).

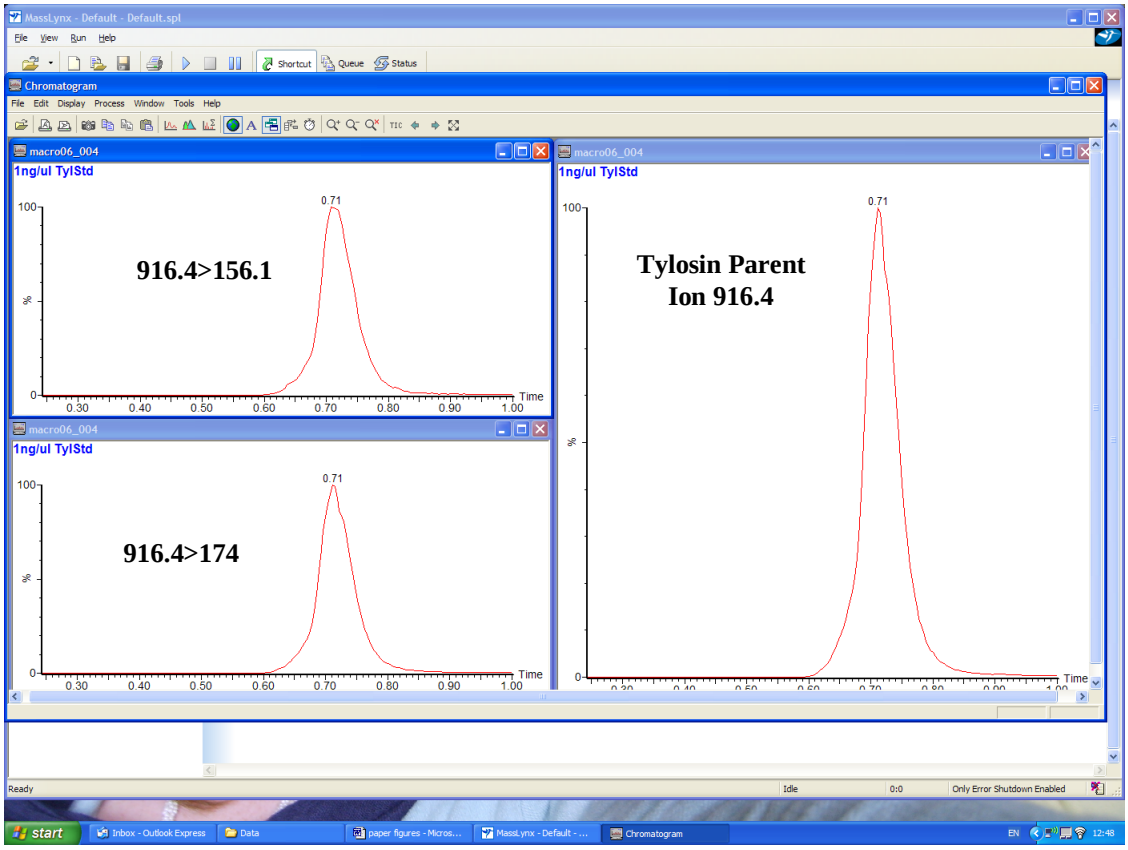


Figure 4.7: Total ion chromatogram TIC for Tylosin and daughter ion chromatrograms. Note: 1ng/µl in 30% methanol, infused into MS through syringe.

4.3.3.2 Effects of dwell time, cone voltage and injection volumes

Dwell times, inter-channel delays and inter scan times on the peak shapes were studied. In the case of gentamicin the dwell time was changed from 0.01 to 0.5 to see if the peak shape could be improved. A very broad peak was observed for gentamicin as this analyte consists of a number of different components, which are difficult to separate. Changes to the dwell time affect the number of scans seen across the peaks and due to the fact that the genatmicin peak was very broad, lowering and raising this function did not have an apparent effect on the peak shape. A bigger effect of changes to dwell time would be seen in very narrow peak with less than 15 scans across. Again for the inter

scan and inter channel functions, changing from 0.1 to 0.05 did not appear to have much effect on the peak shape of gentamicin.

Summary

This method looked at a number of antibiotics from the macrolide and aminoglycoside families using ultra-performance liquid chromatography (UPLC) coupled with a triple quadrupole tandem mass spectrometer as a detector. The optimisation of the mass spectrometer, dwell times, cone voltages, flow rates of gas etc. was discussed in detail and the chromatographic conditions that were explored discussed.

This method outlines the extraction and optimisation of a UPLC method, and the development of a mass spectroscopy method for a number of antibiotics from the macrolide and aminoglycoside family of antibiotics in bovine muscle. This method provides a fast and economical way for a laboratory such as this, the National Reference Laboratory, to carry out its functions to comply with the legislation for monitoring these two groups of antibiotics in animal tissues. Many possibilities exist for developing this research to look at other groups and/ or matrices. Much knowledge has been acquired in the technique of UPLC-MS/MS in terms of optimising the mass spectrometer instrument coditions and the UPLC chromatographic conditions.

References

1. Commission Decision 2002/657/EC; Performance of Analytical Methods. *Off. J. Europ. Comm.*, 2002; **L221,** 8.
2. *Fedeniuk R.W., Shand P.J. (1998). Journal of Chromatography A , 812; 3-15*

3. Al-Dirbashi O.Y., Aboul-Enein H.Y., Jacob M., Al- Qahtani K., Rashed M.S. (2006). *Anal Bioanal Chem*, 385(8); 1439-43

4. Manis L.E., Wilcox M.J. 2001, Alltech Associates Inc

5. Heller D.N., Clark S.B., Righter H.F. (2000). *Journal of Mass Spectrometry,* **35**; 39-49

6. O'Neil, M.J., (Ed.). (2001). The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals (13th ed.).

7. Kijak P. J., Jackson J., Shaikh B. (1997). *Journal of Chromatography B: Biomedical Sciences and Applications,* **691(2)**; 377-382

8. Hornish R. E., Wiest J. R. (1998). *Journal of Chromatography A,* **812**; 123–133

9. Posyniak A, Zmudzki J, Niedzielska J. (2001). *Journal of Chromatography A,* **914;** 59-66

10. Viñas P., Balsalobre N., Hernández-Córdoba M. (2007). *Talanta***, 72 (2);** 808-812

11. Jiang H., Liu S.P.**,** Hu X.L.**,** Liu Z.F.**,** Qin Z.H.**,** Zhan H.L. (2003). *Fenxi huàxué*, **31(9);** 1053-1057

12. Balizs G., Hewitt A. (2003). *Analytica Chimica Acta,* **492 (1-2);** 105-131

13. Oertel R., Neumeister V., Kirch W. (2004). *Journal of Chromatography A,* **1058 (1-2);** 197-201

Chapter 5: Overall Conclusions

5.1. Conclusion

This study focused on two groups of antibiotics the aminoglycosides and the macrolides. A thorough background was given detailing the legislation covering these groups and the chemical properties of these compounds in chapter 1. These compounds are required to be monitored in accordance with EU legislation by the National Reference Laboratory in Ireland, the Central Meat Control Laboratory (CMCL).

These antibiotics are widely used in the treatment of bacterial infections e.g. enteric infections. They have also been used as feed additives for growth promotion. Legislation monitoring these residues in live animals and animal products are given in Council Directive 96/23/EC3, S.I. 507/98 and Commission Decision 2002/657/EC4.

In order to comply with the legislation it is important for the laboratory to continually develop and keep up to date with scientific research. This project is part of improving the expertise within the laboratory and bringing on board new methods using state of the art instrumentation such as the Waters Acquity UPLC system.

The aim of this project was ultimately to develop and method that would analyse the two groups within one analytical run. The method presented in chapter 4 outlines the extraction and optimisation of a UPLC method, and the development of a mass spectroscopy method for a number of antibiotics from the macrolide and aminoglycoside family of antibiotics in bovine muscle. This method is of huge benefit to the work of the laboratory in terms of enabling compliance with the legislation governing National Reference laboratories and also in terms of developing our expertise in the field of analytical science and keeping up to date with the latest techniques available. While this method did not fulfil the “catch all” ideal for both groups it did however provide a

starting point where a large amount of data was analysed and can be further developed to look at separate methods for these groups perhaps.

Finally, the novel method will be applied to the analysis of real samples in the Central Meat Control laboratory which is a National Reference Laboratory (NRL) for e.g. bovine, ovine and porcine tissues.

Appendix I

List of Publications & Poster Presentations

Publications

- McGlinchey T.A.; Rafter P.A.; Regan F.; McMahon G.P. (2008). A review of analytical methods for the determination of aminoglycoside and macrolide residues in food matrices. *Analytica Chimica Acta* **2008**, 624, 1, 1-15

Poster Presentations

- 4th International Symposium on Recent Advances in Food Analysis (RAFA 2009)
- Strategies for Antibiotics- Group B1 Confirmatory methods, AFSSA Fougeres, France, Community Reference Laboratory (CRL), 11th & 12th September 2006
- Analytical Research Forum 2006 (ARF06), Royal Society of Chemistry/Tyndall Institute, University College Cork, 17-19 July 2006.
- 4th Biennial Conference on Analytical Sciences in Ireland, Dublin Institute of Technology, Kevin Street, 11th & 12th April 2006.

Conferences & Training Courses Attended

- Workshop on Multi-antimicrobial screening by LCMSMS, AFSSA Fougeres, Community Reference Laboratory (CRL), 10th & 11th October 2007.
- 5th International Symposium on Hormone and Veterinary Drug Residue Analysis, Antwerp, Belgium, 16th & 19th May 2006.
- 2nd International Symposium on Recent Advances in Food Analysis, International Association of Environmental Analytical Chemistry (IAEAC), Institute of Chemical Technology, Prague, Czech Republic, 2 – 4th November 2005.