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Development and Validation of Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Methods for the Analysis of Veterinary Drugs in Various Biological and Feed Matrices Utilizing Efficient Extraction Protocols

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Development and validation of liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the analysis of veterinary drugs in various biological and feed matrices utilizing efficient extraction protocols.

BY

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DECLARATION PAGE

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ABSTRACT

The transfer of farming practices from low intensity farming systems for livestock production to commercial enterprises which employ intensive practices has resulted in the use of veterinary drugs becoming a critical component of food production. Resulting residues of veterinary drugs occurring in food of animal origin may give rise to potential health risks to consumers. The aim of this research is the development of analytical methods capable of screening and confirming increased number of these residues in more target matrices by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Its focus is the analysis of nitroimidazole residues in food of animal origin and authorised and prohibited medicinal additives in animal feed.

This research resulted in the development and validation of methods for analysis of nitroimidazoles (NMZs) in plasma, eggs, milk and honey and prohibited and authorised medicinal additives in animal feed. The analytical technique used in all methods was the highly selective and sensitive LC-MS/MS. This technique allowed for multi-analyte methods to be developed for different matrices. NMZ residues examined were metronidazole, dimetridazole, ronidazole, ipronidazole, ternidazole, ornidazole, carnidazole and tinidazole along with three metabolite, hydroxy-metronidazole, 2-Hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI) and hydroxy-ipronidazole. Chloramphenicol was included with the analysis of NMZs in the matrices of milk and honey. Fourteen medicinal additives; metronidazole, dimetridazole, ronidazole, ipronidazole, clopidol, carbadox, sulfadiazine, sulfamethazine, dinitolimide, chloramphenicol, ethopabate, avilamycin, tylosin and virginiamycin were analysed for in animal feed. The final method developed allowed for coccidiostats; halofuginone, robenidine, nicarbazin, diclazuril, decoquinate,

semduramicin, lasalocid, salinomycin, monensin, narasin and maduramicin to be analysed for at levels related to unavoidable carryover in feed.

All veterinary residue methods used were validated in accordance with EU legislation; Commission Decision 2002/657/EC. This legislation is concerned with the performance of analytical methods and the interpretation of results. Validation criteria were examined using protocols set out in this legislation and these included specificity, accuracy, precision, repeatability, reproducibility, decision limits (CCα), detection capabilities (CCβ) along with measurement uncertainty (MU). In the four methods developed for the analysis of NMZ residues in plasma, egg, milk and honey the accuracy and precision for all analytes ranged from 87.2% to 108.9% and 3.7% to 11.3% respectively in all matrices. CCα and CCβ for all nitroimidazole residues ranged from 0.33 to 1.60 μg L^{-1} / μg kg^{-1} and 0.56 to 2.64 μg L^{-1} / μg kg^{-1} respectively with MUs ranging from 18 to 90% for all compounds in the various matrices. Chloramphenicol CCα and CCβ values were below its minimum required performance level (MRPL) of 0.3 μg L^{-1} / μg kg^{-1}.

At present there is no legislation in place describing validation approaches for methods used for the analysis of medicinal additives in animal feed. Therefore for the validation of the two feed methods developed as part of this research the veterinary residue legislation was used as a basis. In the case of the analysis of coccidiostats at unavoidable carry over levels; the method was validated entirely in accordance with veterinary residue legislation, Commission Decision 2002/657/EC. However the method for the analysis of 14 prohibited medicinal additives in feed was validated with some adaptations to this legislation. To ensure that the method was fit for purpose a wide variety of feed was used in validation and a wider concentration range was examined. Parameters of specificity, accuracy, precision,

repeatability, reproducibility were all examined and deemed to be acceptable along with measurement uncertainty.

ACKNOWLEDGEMENTS

ABBREVIATIONS

ACN	Acetonitrile
ADI	Average Daily Intake
AFRC	Ashtown Food Research Centre
AGP	Antibacterial Growth Promoters
ALARA	As Low As Reasonably Achievable
AMP	Amprolium
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photo ionization
ASE	Accelerated Solvent Extraction
AVIL	Avilamycin
BSA	Bistrimethylsilylacetamide
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
CAP	Chloramphenicol
CAR	Carbadox
CCα	Decision limit
CCβ	Detection capability
CD	Commission Decision
CLOP	Clopidol
CN	Cyano
CRL	Community Reference Laboratory
CRM	Certified Reference Material
CRZ	Carnidazole
CV	Coefficient of Variance
d-	Dueterated
DAD	Diode Array Detection
DC	Direct current
DEC	Decoquinate
DINIT	Dinitolimide
DIT	Dublin Institute of Technology
DMF	Dimethylformamide
DMZ	Dimetridazole
DNC	4,4'-dinitrocarbanilide
dSPE	dispersive Solid Phase Extraction
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EI	Electron Impact
ELISA	Enzyme linked immunoassay
EMEA	The European Agency for the Evaluation of Veterinary Products

ESI	Electrospray Ionisation
ETB	Ethopabate
EU	European Union
EURL	European Union Reference Laboratory
FAPAS	Food Analysis Performance Assessment Scheme
FF	Florfenicol
FFA	Florfenicol Amine
GC	Gas Chromatography
HAL	Halofuginone
HILIC	Hydrophilic Interaction Liquid Chromatography
HLB	Hydrophilic-lipophilic balance
HMMNI	2-Hydroxymethyl-1-methyl-5-nitroimidazole
HPLC	High performance liquid chromatography
HRMS	High Resolution Mass Spectometry
IARC	The International Agency for Research on Cancer
IP	Identification Points
IPZ	Ipronidazole
IPZ-OH	hydroxy-ipronidazole
IS	Internal Standard
ISO	International Organization for Standardization
LAS	Lasalocid
LC	Liquid Chromatography
LLE	Liquid Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
m/z	mass to charge ratio
MAD	Maduramicin
MALDI	Matrix Assisted Laser Desorption Ionisation
MCX	Mixed-mode cation exchange
MEOH	Methanol
MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid Phase Extraction
MNZ	Metronidazole
MNZ-OH	hydroxy-metronidazole
MON	Monensin
MRL	Maximum residue limit
MRM	Multiple Reaction Monitoring
MRPL	Minimum required performance level
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MSPD	Matrix solid phase dispersion
MU	Measurement Uncertainty
NaCl	Sodium chloride

NAR	Narasin
NCI	Negative Chemical Ionisation
NIC	Nicarbazin
NIG	Nigericin
NMZ	Nitroimidazole
NSAID	Non Steroidal Anti Inflammatory Drugs
OLA	Olaquindox
ORZ	Ornidazole
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzodioxins
PCDF	Polychlorinated dibenzofurans
PSA	Primary Secondary Amine
PT	Proficiency Test
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
r^2	Regression coefficient
RASFF	Rapid Alert System for Food and Feed
RC	Recommended Concentration
RF	Radio Frequency
RL	Recommended Level
RNZ	Ronidazole
ROB	Robenidine
Rpm	Revolutions per minute
RRt	Relative Retention Time
RSD	Relative standard deviation
Rt	Retention Time
S/N	signal to noise ratio
SAL	Salinomycin
SCX	Strong Cation Exchange
SDZ	Sulfadiazine
SEM	Semduramicin
SIM	Single Ion Monitoring
SIMBAG-FEED	Screening and Identification Methods for official control of Banned Antibiotics and Growth Promoters in Feedingstuffs
SMZ	Sulfamethazine
SPE	Solid Phase Extraction
SRM	Single Reaction Monitoring
Std	Standard
TAP	Thiamphenicol
TNZ	Tinidazole
TOF	Time of Flight

TRZ	Ternidazole
TYL	Tylosin
UHPLC	Ultra-high performance liquid chromatography
UPLC	Ultra performance liquid chromatograph
USA	United States of America
UV	Ultraviolet
VIR	Virginiamycin

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The production of food from animal origin in the form of meat, milk, eggs and honey is necessary in maintaining a sustainable food supply. Due to an increasing worldwide population there is a greater demand on food production but there is less space available to allow for it. In order to facilitate increased production, intensive farming practices have been implemented into the production of food from animal origins. To help aid these practices both authorised and prohibited veterinary drugs are either administered routinely as part of the feeding process or non-routinely in the form of injections, pour-ons or implants. This process of animal medication has become an integral part of animal husbandry. While this process leads to increased food production and less stock loss due to disease; it can result in the occurrence of veterinary residues in the food we eat. As a result of health risks, the issue of veterinary drug residues in foods of animal origin has become increasingly important throughout the European Union.

The main human health risks associated with the occurrence of veterinary residues in food are related firstly to the safety of food and secondly to the production of pathogens in humans with antibiotic immunity due to the continuous intake of residues from food. Therefore the European Union has acted in order to reduce the occurrence of residues in food by bringing into force a number of pieces of legislation such as Council Directives 96/22/EC, 96/23/EC and Commission Recommendation 2005/925/EC to set limits for and in some cases prohibit the use of veterinary drugs in food producing animals. These legislative acts have resulted in the need for countries to improve their farming practices by meeting set limits for veterinary residues by following withdrawal times and recommendations for veterinary products. Failure to meet requirements in relation to levels of residues in

food can have serious economic consequences for the country involved as heavy fines and exclusion from trading are associated with breaches. Despite this increase in legislation there is still growing public concern over drug residues in food due to the improper use of veterinary drugs in intense farming practices.

Due to an increasing population and a changing diet there has been an increase in the consumption of meat and dairy products, in particular poultry products [Abele *et al.*, 2004]. As a result, more intensive farming methods have evolved in order to meet this demand. Instead of mixed agricultural systems focusing on a number of areas on a small scale, current food production practices are commonly focused of one area of agriculture in the form of large commercial businesses with intensive farming systems in place for continuous production. This has resulted in a significant increase in the use of veterinary products such as coccidiostats and antibiotics to ensure stock is not lost to disease and growth promoting agents to ensure the largest profit can be made from the stock [Chevance *et al.*, 2009; Raloff, 2002; VMD, 2008]. Also in addition to this there is an increased percentage of food imported into Europe from third countries. Legislation in place in these countries may not be in line with European requirements which may result in the occurrence of residues in our food at possibly harmful levels.

Veterinary medicinal products used in animal production can be divided into a two main groups as set out in Council Directive 96/23/EC. These are Group A compounds which are unauthorized for use in food producing animals and Group B compounds which are authorized for use. Each group in divided into a number of classes of compounds and these classifications are seen in Table 1-1.

Table 1-1. Two Main Groups of Veterinary Medicinal Product

Group A Prohibited Substances	**Group B** Veterinary drugs and contaminants		
A1 STILBENES	B1 ANTIMICROBRIALS	B2a ANTHELMINTICS	B3a ORGANOCHLORINES including PCBs,
A2 THYROSTATS		B2b COCCIDIOSTATS	B3b ORGANOPHOSPHATES
A3 STEROIDS		B2c CARBAMATES/ PYRETHROIDS	B3c HEAVY METALS
A4 ZERANOL		B2d SEDATIVES	B3d MYCOTOXINS
A5 BETA-AGONIST		B2e Non Steroidal Anti-Inflammatory Drugs	B3e DYES
A6 (Nitroimidazoles, Chloramphenicol) Prohibited compounds listed in Table 2 of Regulation 37/2010/EC		B2f OTHERS e.g. corticoids	

The occurrences of residues in food that are in breach of levels stated in legislation can arise in a variety of different ways. These include either the improper use of licensed products or the illegal use of unlicensed and prohibited substances [Kennedy *et al.*, 2000]. In relation to licensed products, there are numerous factors that can result in residues being present in food above authorised levels; some of these are listed in Table 1-2. If the user of the authorized substance (Group B) adheres to the product license and provided that the drug withdrawal periods are respected, drug residues should not occur in food at concentrations greater than the maximum residue limits (MRLs). Prohibited veterinary products (Group A) are ones that are not permitted for use in the production of food from animal origin. Some of the main reasons for the occurrence of prohibited veterinary drug residues in the human food chain can be seen in Table 1-2.

Table 1-2. Reasons for drug residues from authorized and prohibited substances.

Authorized Substances	Prohibited Substances
1. Extended usage or excessive dosage of approved drugs	1. The use of growth promoting hormones in order to increase the weight of animals and reduce amount of feed needed by animals to gain weight [Debackere *et al.*, 1989]
2. Poor records of treatment leading to problems identifying in Treated-animal resulting in overdosing	2. The use of certain banned compounds that can be very effective at controlling particular infections e.g. nitrofurans [O'Keeffe *et al.*, 2004]
3. Use of incorrect veterinary drug for particular species.	3. The use of prohibited compounds that are very cheap and readily available.
4. Contaminated feed being used as withdrawal feed for target animals as a result of carry over issues in feed mills. [Commission Directive 2009/8/EC].	

Studies carried out on residues and metabolites of prohibited veterinary drugs (Group A) have highlighted the possibility of their harmful health effects. Reports published by both The European Agency for the Evaluation of Medicinal Products (EMEA) and The International Agency for Research on Cancer (IARC) have both suggested that nitroimidazoles and chloramphenicol are possible carcinogens and are therefore are classed as A6 compounds and are prohibited for use in food producing animals.

Although Group A substances are considered more of a human health risk than Group B substances; their use is not without there consequences. Apart from allergic reaction one of the main reasons for the control of some group B substance is the similarity between animal antibiotics and there human counterparts. It is feared that the continuous use of these compounds in animals may result in the increase of antibiotic immune bacteria that could transferred to humans. Immunity such as this has been seen for tetracycline [Smith *et al.*, 1957] and vancomycin in the late 1980s [Bates *et al.*, 1993].

With this ever increasing list of risks to human health, the European Commission has focused its attentions on consumer protection in relation to the use of veterinary drugs in animal husbandry. Legislation is continuously being updated and amended to assist with this. In order for each member state's regulatory authority to enforce this legislation and ensure ongoing consumer protection throughout Europe there is a need for robust and sensitive analytical methods for the detection of these residues to be developed.

1.2 Aims of the Study

The main aim of this research was to develop and validate reliable, robust and suitably sensitive analytical methods for the analysis of veterinary drugs and their residues in various biological and feed matrices. These methods were validated taking into consideration all relevant European legislation, ensuring that all criteria with regards to analytical methods, target analytes and suitable matrices were satisfied.

The initial part of the research dealt specifically with the analysis of 5-nitroimidazoles in biological matrices of food producing animals. Analysis of the suspected carcinogenic 5-nitroimidazole compounds is limited and this was the case in Ireland prior to this research as the analysis of these compounds was limited to two hydroxy-metabolites in a single matrix. In addition studies have shown that matrices such as muscle and liver don't allow for the best possible identification of abuse of these compounds. Prior to this research the analysis of these compounds often involved the examination of these unsuitable matrices.

The focus of the research then moved to the analysis of animal feed for the presence of medicinal feed additives. High levels of veterinary drug residues in food that can

pose a risk to human health may be the result of animals being fed contaminated feed. Therefore the development of analytical methods for the analysis of a wide variety of these additives in animal feeds is an important part of the research.

There were a number of specific aims within the research as a whole and these included:

I. To help improve the surveillance capabilities of The State Laboratory with regards to the analysis of nitroimidazoles by the development of a number of novel, rapid, confirmatory, multi residue methods using liquid chromatography tandem mass spectrometry in a variety of biological matrices.
    a) Nitroimidazoles in Plasma.
    b) Nitroimidazoles in Eggs.
    c) Nitroimidazoles and Chloramphenicol in Milk and Honey

II. In order to meet the requirements of EU legislation with regard to medicinal feed additives new liquid chromatography tandem mass spectrometry methods were developed. These included the analysis of:
    a) Prohibited Medicinal feed additives in pig and poultry feed
    b) Eleven Coccidiostats in animal feed at levels relating to unavoidable carry over from feed mills.

III. To determine the potential misuse of nitroimidazole compounds in the Irish Egg Industry by the analysis retail survey egg samples.

IV. Implementation of these methods into the National Reference Laboratory in Ireland designated for the control of the particular substance groups in question.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction

This chapter will give an overview of the main issues regarding veterinary drug analysis and the areas that should be taken into consideration when developing an analytical method. These issues include;

- Background information on nitroimidazoles, their metabolism, their stability and their carcinogenicity.
- Background information on chloramphenicol.
- Background information on medicinal feed additives and their use in feed.
- Review of the current legislation pertaining to veterinary drugs within the EU
- Review of the current legislation pertaining to medicinal feed additives and cross contamination issues.
- Review of validation protocols within the EU
- Review of extraction and purification procedures used in residue analysis for the compounds of interest, highlighting the relevant matrices.
- Review of LC-MS/MS and its uses in the field of veterinary residue analysis.

2.2 Overview of Veterinary Drugs Investigated

1.2.1. Nitroimidazoles

In the initial part of this research; the class of veterinary drugs of most interest was nitroimidazoles. This was due to the fact that although they are prohibited for the use in food producing animals, there remained a shortage of methods that could analyse for these carcinogenic compounds in a wide variety of matrices to ensure that a potential risk to human health was identified.

Nitroimidazoles are imidazole heterocycles with nitrogen groups incorporated in the structure. Examples of these compounds are metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (MNZ)], dimetridazole [1,2-dimethyl-5-nitroimidazole (DMZ)], ronidazole [1-methyl-2-[(carbamoyloxy)-methyl]-5-nitroimidazole (RNZ)], ipronidazole [2-isopropyl-1-methyl-5-nitroimidazole (IPZ)], carnidazole [1-(2-ethylcarbamothioic acid O-methyl ester)-2-methyl-5-nitroimidazole (CNZ)], ornidazole [1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole (ONZ)], ternidazole [1-propanol-2-Methyl-5-nitroimidazole (TRZ)] and tinidazole [1-(2-ethylsulfonylethyl)-2-methyl-5-nitroimidazole (TNZ)]. These examples are known as 5-nitroimidazoles as they contain a NO_2 group on the 5 ring position. This base structure is seen in Figure 2-1 along with the different compounds investigated as part of this research.

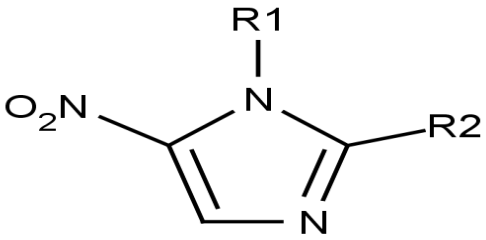
5-Nitroimidazoles are active against most obligate anaerobic bacteria and variety of protozoa where organism resistance is rare but have limited activity against aerobic bacteria [Bishop, 2005]. Nitroimidazole compounds that were licensed as veterinary medicines include metronidazole, dimetridazole and ronidazole. Metronidazole was the most commonly used 5-Nitroimidazole and can be used to treat a wide variety of infection in animals. It is used in the treatment of humans and animals for infections caused by *Trichomonas, Histomonas* and *Clostridium.* It can be used for treatment of dysentery in pigs and in combination with neomycin can treat *retentio secundinarum* in cows [EMEA Report, Metronidazole].

Dimetridazole is used mainly in the poultry industry in particular for the treatment of histomoniasis in turkeys and trichomoniasis in pigeons. It has also been used in the treatment of cattle for genital trichoniasis and pigs for haemorrhagic enteritis. [EMEA Report, Dimetridazole]. The final nitroimidazole that was licensed for use in

animal husbandry was ronidazole. This was used for the treatment of similar diseases such as histomoniasis in turkeys, genital trichoniasis in cows and haemorrhagic enteritis in pigs [EMEA Report, Ronidazole]. Articles published on nitroimidazoles have also shown that dimetridazole [Griffin, 1972] and ronidazole [Taylor, 1974] are effective in the treatment of swine dysentery. Another nitroimidazole, carnidazole, can also be used for the treatment and prophylaxis of trichomoniasis in pigeons [Bishop, 2005].

The antimicrobial effect of all 5-nitroimidazole derivatives is due to the same mode of action as each other i.e. the metabolic reduction of the nitro group by susceptible anaerobic microorganisms. The metabolic reduction of the nitro group is performed by microbial 'nitroreductases' [Voogd, 1981; Johnson, 1993]. This allows nitroimidazoles to be successful in the treatment of *Trichomonas, Histomonas* and *Clostridium*, as they interact with DNA destroying its ability to act as a template for DNA and RNA synthesis [Bishop, 2005].

Nitroimidazole compounds are believed to be carcinogenic and mutagenic to humans. The majority of studies have been carried out on metronidazole. The results of these studies show that it is believed to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals [IARC 1977, 1982, 1987]. Animal studies have shown that mice treated with metronidazole have shown increased incidence of lung tumors for both sexes and lymphomas in female mice [Rustia *et al.*, 1972]. Oral administration of the compound also caused mammary fibroadenomas and adenocarcinomas, and pituitary, testicular, and liver tumors in rats [IARC 1977, 1982, 1987]. There is however inadequate evidence for the carcinogenicity of metronidazole in humans [IARC 1982, 1987].



Nitroimidazole Base Structure

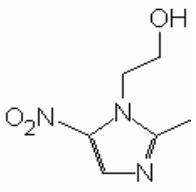
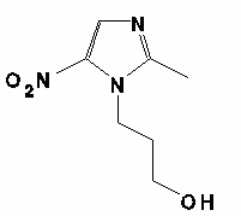
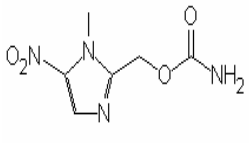
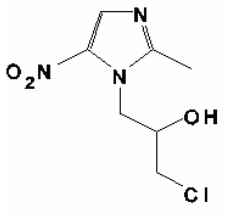
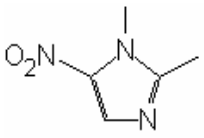
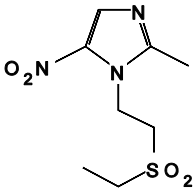
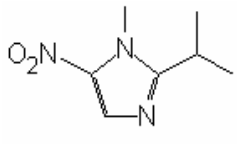
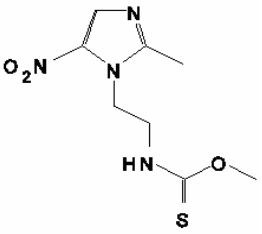
Metronidazole		**Ternidazole**	
Ronidazole		**Ornidazole**	
Dimetridazole		**Tinidazole**	
Ipronidazole		**Carnidazole**	

Figure 2-1: Basic 5-Nitroimidazole Structure and various different compounds examined in this research

The European Agency for the Evaluation of Veterinary Products (EMEA) has published summary reports on three nitroimidazole compounds; metronidazole

[EMEA Report, Metronidazole], dimetridazole [EMEA Report, Dimetridazole] and ronidazole [EMEA Report, Ronidazole]. While the reports suggest the studies carried out on humans are insufficient and inconclusive to prove or disprove carcinogenicity of these compounds, results in animal studies are conclusive [Rustia *et al.*, 1972; IARC 1977, 1982, 1987]. This, in their opinion, is enough to consider these compounds as carcinogenic and in the interest of human health they are prohibited for use in food producing animals. The legislation covering this is discussed fully in section 2.3.

A number of studies carried out on these compounds have shown that they are rapidly metabolised in bovine, porcine and avian species [MacDonald *et al.*, 1971; Craine *et al.*, 1974; Cala *et al.*, 1976; Rosenblum *et al.*, 1972]. The main metabolite of DMZ, IPZ and MNZ results from the oxidation of the side chain in the C-2 position of the imidazole ring to form hydroxy metabolites [MacDonald *et al.*, 1971; Craine *et al.*, 1974]. RNZ has a different degradation pathway but results in an identical metabolite to that of DMZ [Cala *et al.*, 1976; Rosenblum *et al.*, 1972]. The metabolite of DMZ and RNZ is HMMNI (2-hydroxymethyl-1-methyl-5-nitroimidazole), of MNZ is MNZ-OH (1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole) and of IPZ is IPZ-OH (1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole). There structures can be seen in figure 2-2. As these compounds still contain the imidazole ring their carcinogenicity cannot be overlooked [EMEA Report, Metronidazole]. Also these metabolites can be an indication of potential misuse of prohibited nitroimidazole compounds and therefore should also be analysed for.

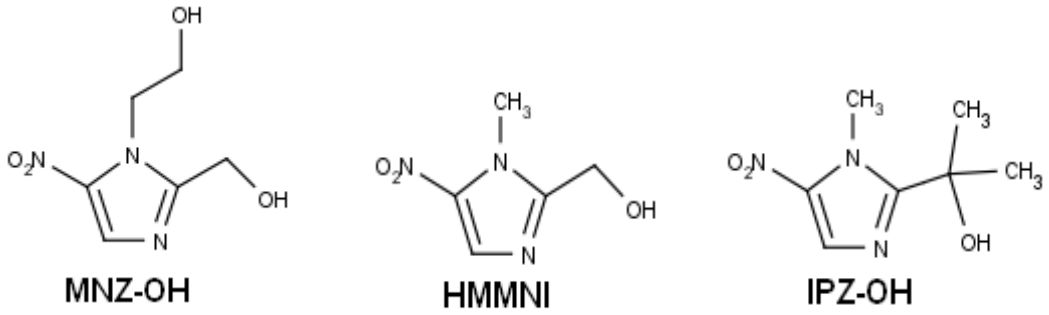


Figure 2-2. Structure of the hydroxy-metabolites MNZ-OH, HMMNI and IPZ-OH

The most recent study carried out on nitroimidazole metabolism by Polzer *et al.*, 2004 examined the amount of parent drug and the corresponding main hydroxy-metabolite in various matrices taken from treated turkeys and as a result a number of conclusions were made. The study demonstrated that the metabolites of DMZ and IPZ, HMMNI and IPZ-OH, should be chosen as target analytes to prove illegal treatment whereas to check for a treatment with RNZ or MNZ, the measurement of the parent drug is to be preferred. This is due to the fact that these are the most abundant residues left after administering nitroimidazoles. However the study also goes on to state that the ratio of parent drug to metabolite in the case of dimetridazole was found to vary with the length of the withdrawal time. Therefore it might be prudent to look for both the metabolite and parent residue for dimetridazole to ensure non compliant samples are identified. In addition to this without respective data on the behaviour of the other nitroimidazoles, a recommendation was made to monitor both, the parent drug and the respective metabolite, whenever possible in order to identify any possible abuse of these compounds. [Polzer *et al.*, 2004]

Methods used to analyse nitroimidazoles in food producing animals will be discussed fully in section 2.4.2. However studies on the stability and homogeneity of nitroimidazoles have been carried out by the European Union Reference Laboratory

(EURL) for nitroimidazoles in Berlin to identify suitable matrices and marker analytes for their analysis [Polzer *et al.*, 2004 and 2005]. These involved the analysis of turkeys administered nitroimidazoles in controlled experiments. In several animal studies turkeys were treated with different nitroimidazoles (DMZ, MNZ, RNZ, IPZ). Apart from the identification of target analytes as mentioned previously the main observations of their studies were; the repeatability of the analysis of muscle samples was unacceptable due to the inhomogeneity of the matrix and a rapid degradation of the analytes was observed during sampling and continues during storage in a non-frozen state [Polzer *et al.*, 2004 and 2005].

They showed clearly that in the stability studies they carried out, nitroimidazoles are not stable in muscle and liver and suggested that care must be taken to ensure an immediate and efficient cooling directly after sampling. The also showed that inhomogeneity of the analytes in muscle and liver and recommended to thoroughly homogenise sufficient quantities of these matrices in order to obtain representative sample material, e.g. by lyophilisation [Polzer *et al.*, 2004 and 2005].

Even with the implementation of these measures depletion studies of dimetridazole show there is a rapid disappearance of analyte in liver and muscle. In contrast they found that in plasma and eggs a longer detection period of the nitroimidazole residues is possible. They also found that these residues are more stable and there is no problem with inhomogeneity in the matrix of plasma and eggs. Furthermore, they feel that the matrix is readily available and is therefore very well suited for residue control purposes [Polzer *et al.*, 2004 and 2005]. Finally, the study determined that the same is true for retina. It stated that the highest concentrations of nitroimidazoles were measured in this matrix.

As these studies are carried out in turkeys the results could differ from poultry to larger animals such as pigs. That said these studies have suggested that, in order to accurately ensure that the misuse of nitroimidazoles is not taking place sampling, matrix and marker analyte selection is crucial. Taking this into consideration matrices that should be used for the analyses of nitroimidazoles are plasma, retina and eggs and once sampled should be cooled immediately [Polzer *et al.*, 2004 and 2005].

1.2.2. Chloramphenicol

Chloramphenicol (CAP) is a broad spectrum bacteriostatic antibacterial [Bishop, 2005] whose structure can be seen in figure 2-3. Like nitroimidazoles it is prohibited for the use in food producing animals. Much of the same European legislation governs both compounds. The analysis of CAP is often performed in single analyte methods (section 2.4.3) and it was felt that to combine these into one method would be beneficial.

Chloramphenicol is active against a wide range of illnesses and infections including rickettsial and chlamydophilial infections, numerous obligate anaerobes, gram positive aerobes, and non-enteric aerobes such as *Bordetella and Haemophilus* [Burnham *et al.*, 2000]. CAP is a simple lipid-soluble compound which readily crosses the cellular barrier. For this reason it is a very effective medicinal product as it diffuses throughout the body and reaches infection sites inaccessible to many other antibacterial drugs which include areas such as cerebrospinal fluid and the internal structures of the eye. The process by which CAP exerts its effect is by inhibiting bacterial protein synthesis. [Bishop, 2005].

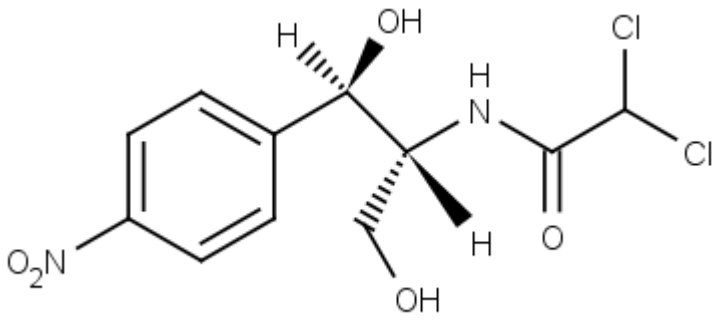


Figure 2-3. Structure of Chloramphenicol

Studies on the carcinogenicity of CAP in humans suggest that it is believed to be a human carcinogen. Case reports have shown leukemia to occur after medical treatment for chloramphenicol-induced aplastic anemia [IARC 1990]. Three case reports have also documented the occurrence of leukemia after chloramphenicol therapy in the absence of intervening aplastic anemia. In a case-control study in China, elevated risks of childhood leukemia were found, which increased significantly with the number of days chloramphenicol was taken [Shu *et al.* 1987, 1988]. Two case-control studies [Issaragrisil *et al.* 1997; Laporte *et al.* 1998] found high, but nonsignificant, increases in the risk of aplastic anemia associated with the use of chloramphenicol in the six months before onset of aplastic anemia. However, two case-control studies [Zheng *et al.*, 1993; Doody *et al.*, 1996] found no association between the use of chloramphenicol and the risk of adult leukemia, suggesting that children may be a particularly susceptible subgroup. One report by Zahm *et al.*, 1989 found an association between chloramphenicol use and increased risk of soft-tissue sarcoma. When considered together, the many case reports implicating chloramphenicol as a cause of aplastic anemia, the evidence of a link between aplastic anemia and leukemia, and the increased risk of leukemia found in

some case-control studies support the conclusion that chloramphenicol exposure is associated with an increased cancer risk in humans.

Another issue with CAP is that it is metabolized more slowly in babies and infants than in adults, which can result in gray baby syndrome. This syndrome is characterized by cardiovascular collapse in infants, apparently caused by accumulation of active, unconjugated chloramphenicol in the serum, resulting from low inactivation through glucuronide conjugation in the liver [Burnham *et al.*, 2000]. Taking this information into consideration, the European Union has prohibited the use of CAP in animals used in food production. The legislation governing this is discussed fully in section 2.3.

1.2.3. Veterinary Medicinal Additives

A major area of interest of this research was based on the development of methods for the analysis of medicinal additives in animal feed. New legislation governing the control of these additives is discussed in section 2.3.2. A wide range of compounds are detailed in this legislation which are either prohibited for use as a feed additive or are detailed specifically for certain species of animal. These medicinal additives include; compounds prohibited for use in food producing animals such as nitroimidazoles (section 2.2.1) and chloramphenicol (section 2.2.2); compounds specifically allowed only in medicated feed such as sulphonamides and compounds no longer allowed to be used as feed additives such as antibacterial growth promoters (AGPs) e.g. tylosin and virginiamycin. The other list of compounds that is governed by this new legislation are ionophore coccidiostats and histomonostats that are, as a result of this legislation, the only medicinal products that are allowed to be used as

feed additives but only for specific species. The two lists covered by the legislation can be seen in Table 2-3.

Previously there were a wide variety of medicinal products that were added to feed to help improve the general health of the livestock while also aiding in growth promotion of the animals. The majority of these additives are now prohibited by the new legislation. The history behind particular compounds investigated in this research is as follows. Carbadox (CAR) and olaquindox (OLA) have been approved for use as feed additives for pigs since 1974 and 1976 respectively. CAR and OLA are antimicrobial growth promotants (AGPs) used mainly in swine feed for growth promotion, to improve feed efficiency, increase the rate of weight gain, control swine dysentery and bacterial enteritis in young swine [Boison *et al.*, 2009]. These compounds have been expressly prohibited from inclusion in animal feedingstuffs in the EU since 1998 as they are believed to be carcinogenic and mutagenic [Regulation 2788/98/EC]. Other AGPs were also prohibited for use as feed additives around this time. These included spiramycin, virginiamycin and tylosin phosphate which were used for growth promotion as well as disease treatment and prophylaxis [Situ *et al.*, 2005] but were prohibited as they were believed to contribute to bacterial resistance in humans. [Regulation 2821/98/EC].

The concerns over antibiotic immunity and bacterial resistance are the major driving force behind prohibiting medicinal feed additives. The use of antimicrobial feed additives can result in the development of antibiotic immunity which could be transferred to humans. The first evidence of this occurring with antibiotics was tetracycline [Smith *et al.*, 1957] and later vancomycin in the late 1980s [Bates *et al.*, 1993] and most recently streptogramin resistances [Butaye *et al.*, 2001]. This new legislation prohibits the use of any medicinal feed additives except for the use of

coccidiostats and histomonostats [Recommendation 2005/925/EC]. It is envisaged that these will also be prohibited as feed additives before 2013 [Regulation 1831/2003/EC]. After this date, medical substances in animal feeds will be limited to therapeutic use by veterinary prescription [Castanon, 2007].

Until then eleven coccidiostats including monensin, narasin and nicarbazin are the only compounds permitted for use as feed additives. These compounds are only licensed for use in particular animals and therefore they are only allowed for the use as feed additives in feed intended for that specific target species [Recommendation 2005/925/EC]. Coccidiostats constitute the main choice to fight against coccidiosis. Coccidiosis is an infectious disease caused by several species of Eimeria and Isospora protozoa which results in significant loss of stock causing serious economical consequences in farming industry. In the European Union, coccidiostats are authorized mainly as feed additives, according to the Regulation 1831/2003/EC for the prevention and treatment of coccidiosis in rabbits and chickens. As a result of issues with carry over in feed mills the contamination of feed with coccidiostats needs to be monitored. Levels are set out in legislation for sensitive and less sensitive non-target animal species, withdrawal feed and non-target feed for ‘continuous food-producing animals’, such as dairy cows or laying fowl [Regulation 2009/8/EC] in order ensure animal and human welfare. This legislation is discussed fully in section 2.3.2.

2.3. Outline of European Legislation

2.3.1. Legislation regarding Substances that are prohibited for the use in Food Producing Animals

As a result of carcinogenic and other health risks some veterinary products previously used for treatment in animal husbandry, such as nitroimidazoles and chloramphenicol were prohibited firstly in Annex IV of Commission Regulation 2377/90 but are now included in table 2 of Council Regulation 37/2010/EC. Regulation 2377/90 was one of the first substantial pieces of legislation published by the European commission which tried to encompass the large amount of veterinary drugs in use and as a result try to limit the residues resulting from them. This was done by introducing maximum residue limits (MRLs) and this regulation defined what MRLs are and described the procedure for the establishment of MRLs for veterinary medicinal products in foodstuffs of animal origin. A definition of MRL is the maximum concentration of residue resulting from the use of a veterinary medicinal product (expressed in mg/kg on a fresh weight basis) which may be accepted by the European Community to be legally permitted or recognized as acceptable in or on a food. It is based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the acceptable daily intake (ADI), or on the basis of a temporary ADI that utilizes an additional safety factor. It also takes into account other relevant public health risks as well as food technology aspects. When establishing a MRL, consideration is also given to residues that occur in food of plant origin and/or come from the environment. Furthermore, the MRL may be reduced to be consistent with good practices in the use of veterinary drugs and to the extent that practical analytical methods are available. It also gave a definition of what constitutes residues of

veterinary medicinal products. They are 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.

Also included in this legislation were five annexes that cover the range of veterinary products in use and the allowed or prohibited residues resulting from that use. These annexes taken from Regulation 2377/90 are listed below.

Annex 1: List of pharmacologically active substances for which maximum residue levels have been fixed.

Annex 2: List of substances not subject to maximum residue levels.

Annex 3: List of pharmacologically active substances used in veterinary medicinal products for which maximum residue levels have provisionally been fixed.

Annex 4: Lists of pharmacologically active substances for which no maximum levels can be fixed.

Annex 5: Information and particulars to be included in an application for the establishment of a maximum residue limit for a pharmacologically active substance used in veterinary medicinal products.

The compounds listed in Annex 4 include dimetridazole, ronidazole, metronidazole and also chloramphenicol. As a result of their suspected negative human health effects, no MRL could be established and therefore they are prohibited for use in food producing animals. Other nitroimidazoles such as ipronidazole were never issued a veterinary licence for the use in food producing animals and therefore are also prohibited.

Regulation 2377/90 has recently been replaced by Council Regulation 470/2009/EC. This new regulation allowed for the use of a maximum residue limit established in

one species or foodstuff to be used for another species or another foodstuff. The new legislation also allowed for the provision of reference points of action for prohibited substances in order to harmonise trade between member states. These reference points of action would also take into account what concentrations of residue it is possible to measure in the laboratory. This regulation also refers to another new regulation that would replace Annexes 1-4 of 2377/90. This new regulation 37/2010; was brought in to simplify matters and only contains one Annex with two tables. The first table lists all the authorised substances in alphabetical order and the second table lists all the prohibited substances in alphabetical order.

As the compounds of interest in this study, nitroimidazoles and chloramphenicol, are in table 2 of regulation 37/2010 i.e. compounds that are prohibited substances, there is no maximum residue limit set for these and as such there is a zero tolerance policy towards their use within the EU. In practice however it is not possible to measure zero. In order to harmonise, to some extent, performance of laboratories from different Member States the Minimum Required Performance Level (MRPL) concept was devised, however legislative MRPLs have only been set for a small number of prohibited substances including, medroxyprogesterone acetate, chloramphenicol, malachite green and some nitrofuran metabolites (Table 2-1) [Commission Decision 2003/181/EC, Commission Decision 2004/25/EC]. These levels correspond to the average limits above which the detection of a substance or its residues can be construed as methodologically meaningful and therefore be used as the reference point for action to ensure a harmonised approach throughout the EU. Therefore all methods used to analyse for the compounds with MRPLs must be able to confirm there presence at this level. As chloramphenicol has an MRPL of 0.3μg/kg all methods developed for analysis of it in meat, eggs, urine, aquaculture products, milk

and honey must have the capability to confirm its presence at this level and therefore the method should be validated at this level.

Table 2-1: All Minimum required performance levels taken from legislation.

Substance and/or metabolite	Matrices	MRPL
Chloramphenicol	Meat, Eggs, Milk, Urine, Aquaculture products, Honey	0.3µg/kg
Medroxyprogesterone acetate	Pig kidney fat	1µg/kg
Nitrofuran metabolites: furazolidone, furaltadone, nitrofurantoin, nitrofurazone	Poultry meat, Aquaculture products	1µg/kg Total combined residue
Sum of malachite green and leucomalachite green	Meat of aquaculture products	2µg/kg

As there are very few legislative MRPLs, the European Union Reference Laboratories (EURL) which are in charge of the control veterinary residues in Europe went about producing a Guidance Document in 2007 [CRL Guidance 2007] that outlined recommended concentration/levels (RC/RL) that laboratories should aspire to measure for prohibited substances with no MRPL. The document also specified which matrices should be sampled and also what marker residue should be analysed for (parent drug or metabolite). The information given in this document in relation to nitroimidazoles can be seen in Table 2-2.

This document recommends that as a minimum, laboratories should analyse for DMZ, MNZ, RNZ, IPZ and their hydroxy metabolites HMMNI, MNZ-OH and IPZ-OH at a RL of 3 μg/kg in matrices such as plasma, retina and eggs. Therefore any methods that are developed for the analysis of NMZs must take these recommendations into consideration when selecting matrix, target analytes and levels at which to validate.

Table 2-2. EURL recommendations for nitroimidazoles adapted from CRL Guidance Document 2007.

Substances	Matrix	Recommended Concentaration *
Nitroimidazole: Dimetridazole, Ronidazole, Metronidazole hydroxy metabolites: MNZ-OH, HMMNI	Poultry: Plasma, Serum, Eggs Pigs (and others species): Plasma, Serum, Muscle	3ppb

* CCβ for screening methods or CCα for confirmatory methods should be lower than the value expressed in this column

2.3.2. Legislation Relating to Veterinary Products which are used as Feed Additives

In the European Union the use of feed additives is authorised according to Regulation No. 1831/2003 as long as various criteria are fulfilled including the need to provide suitable methods of analysis for official control of these compounds in feedingstuffs. Other requirements that need to be specified are the target animal, the level of active substance in feed and in some cases, as with coccidiostats, the withdrawal period before slaughter when the use of these substances are prohibited.

More recent legislation published in the EU, has gone about prohibiting the use of many veterinary products for use as feed additives. Commission Recommendation 2005/925/EC prohibits the use of many antibiotics, coccidiostats and antibacterial growth promoters as feed additives. This recommendation lists medicinal substances that should be monitored and the substances are divided into two groups; medicinal substances authorised as feed additives for certain animal species or categories e.g. ionophore coccidiostats and medicinal substances no longer authorised as feed additives e.g. carbadox, nitroimidazoles, tetracyclines. The two lists can be seen in Table 2-3.

Table 2-3: List of medicinal substances that should be monitored according to Commission Recommendation 2005/925/EC.

1. Medicinal substances authorised as feed additives for certain animal species or categories	2. Medicinal substances no longer authorised as feed additives
decoquinate (Deccox)	Amprolium
diclazuril (Clinacox 0,2 %)	amprolium/ethopabate
halofuginone hydrobromide (Stenorol)	Arprinocid
lasalocid A sodium (Avatec 15 %)	Avilamycin
maduramicin ammonium alpha (Cygro 1 %)	Avoparcin
monensin sodium (Elancoban G100, 100, G200, 200)	Carbadox
narasin (Monteban)	Dimetridazole
narasin — nicarbazin (Maxiban G160)	Dinitolmid
robenidine hydrochloride (Cycostat 66 G)	Flavophospholipol
salinomycin sodium (Sacox 120G, 120)	Ipronidazole
semduramicin sodium (Aviax 5 %)	Meticlorpindol
	meticlorpindol/methylbenzoquate
	Nicarbazin
	Nifursol
	Olaquindox
	Ronidazole
	Spiramycin
	Tetracyclines
	tylosin phosphate
	Virginiamycin
	zinc bacitracin
	other antimicrobial substances

This legislation went about harmonising the existing legislation on residues in animals and the existing legislation governing feedingstuffs. Compounds that are prohibited in food of animal origin are prohibited as feed additives e.g.

nitroimidazoles and compounds with MRLs in food of animal origin are only permitted in medicated feed e.g. tetracyclines. This has helped to reinforce the farm to plate philosophy i.e. ensure control of harmful residues through every stage of food production and as a result ensure consumer protection. Following the publication of this legislation no antibiotics other than coccidiostats and histomonostats can be marketed and used as feed additives within the European Union. Coccidiostats constitute the main choice to fight against coccidiosis. This is a major disease in poultry as well as in many other hosts [Matabudul *et al.*, 1999].

Following on from this legislation a problem was identified with carryover of coccidiostats from feed with additives added and non additive feed. This is due to the fact that feed business operators may produce within one establishment a broad range of feeds. Different types of products may have to be manufactured after each other in the same production line. It may happen that unavoidable traces of a product remain in the production line and end up in the beginning of the production of another feed product. This transfer from one production lot to another is called ‘carry-over’ or ‘cross-contamination’ and may occur for instance when coccidiostats or histomonostats are used as authorised feed additives. This may result in the contamination of feed produced subsequently by the presence of technically unavoidable traces of those substances in ‘non-target feed’, i.e. in feed for which the use of coccidiostats or histomonostats are not authorised, such as feed intended for animal species or categories not provided for in the additive authorisation. This unavoidable cross-contamination may occur at all stages of production and processing of feed but also during storage and transport of feed as cited in Regulation 2009/8/EC. This may lead to high concentrations of coccidiostats in non-target feed; which could pose a health risk to both the species itself and to humans.

As a result of concerns over the production of animal feed and in order to avoid carry-over, the Feed Hygiene Regulation 2005/183/EC was published. This lays down specific requirements for feed businesses using coccidiostats and histomonostats in the production of feed. In particular, the operators concerned have to take all appropriate measures concerning facilities and equipment, production, storage and transport in order to avoid any cross-contamination [Regulation 2005/183/EC].

Taking into account the application of good manufacturing practices set out in the feed hygiene regulation there are still levels of coccidiostat carryover that are considered unavoidable. The European Commission published legislation in the form of Regulation 2009/8/EC and this established maximum limits for unavoidable carry over of coccidiostats and histomonostats. Using the ALARA (As Low As Reasonably Achievable) principle the commission set maximum limits for unavoidable carry over. As a result and for the purpose of enabling the feed manufacturer to manage the unavoidable carry-over of coccidiostats, a carryover rate of approximately 3% of the authorised maximum content is considered acceptable with regard to feed for less sensitive non-target animal species, while a carry-over rate of approximately 1% of the authorised maximum content should be retained for feed intended for sensitive non-target animal species i.e. animals for which the additive might be harmful and ‘withdrawal feed’, i.e. feed used for the period before slaughter. The carry-over rate of 1% is also considered for allowed cross-contamination of other feed for target species to which no coccidiostats or histomonostats are added, and as regards non-target feed for ‘continuous food-producing animals’, such as dairy cows or laying hens, where there is evidence of transfer from feed to food of animal origin. The levels related to 1% and 3% carry

over of all the coccidiostats and histomonostats covered by this legislation are seen in Table 2-4.

Table 2-4. 1% and 3% carry over of all the coccidiostats as stated in legislation 2009/8/EC.

Compound	1% Carry Over (mg/kg)	3% Carry Over (mg/kg)
Halofuginone	0.03	0.09
Robenidine	0.70	2.10
Nicarbazin	0.50	1.50
Diclazuril	0.01	0.03
Decoquinate	0.40	1.20
Semduramicin	0.25	0.75
Lasalocid	1.25	3.75
Salinomycin	0.70	2.10
Monensin	1.25	3.75
Narasin	0.70	2.10
Maduramicin	0.05	0.15

2.3.3. Legislation describing procedures for the control of residues

Monitoring of veterinary residues in food of animal origin is carried out according to European legislation Council Directive 96/23/EC. This Directive lays down measures to monitor substances and groups of residues listed and defines the role of the national reference laboratories in monitoring. The main aims of this legislation is to give direction on the approach to be taken in monitoring illegal substances and incorrect use of authorised substances in food producing animals within the EU. As a result it is hoped that monitoring throughout the EU will be firstly effective but also consistent from one country to the next. In order to enable this harmonised approach this legislation lays down measures to monitor the substances and groups of residues. The legislation goes about setting up national reference laboratories in each member state that will be responsible for analysis of veterinary residues. Approaches to

sampling, frequency and numbers of samples taken and enforcement measures for non compliant results are all outlined in this legislation. This legislation also breaks up the different veterinary compounds into Group A and Group B. As mentioned previously Group A substances are those whose use are prohibited in food producing animals. The residues of compounds which were studied as part of this thesis i.e. nitroimidazoles and chloramphenicol are both categorised as A6 compounds in 96/23/EC.

2.3.4. Guidelines concerning the analytical performance and interpretation of results of residue methods.

As a result of legislation for the control of residues in food of animal origin, analytical methods for the analysis of the controlled substances needed to be developed. These methods needed to be sensitive, selective and fit for purpose. In order to ensure this the European Commission initiated the production of a legislation to ensure any method developed met certain quality criteria. This legislation lays down performance criteria for the analytical methods to be used for the analysis of certain substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC. The resulting piece of legislation is Commission Decision 2002/657/EC and it is concerned with the performance of analytical methods and the interpretation of results. The severe implications on countries where abuse of prohibited compounds (Group A) is identified resulted in the need for more rigorous criteria to be specified within 2002/657/EC for confirmation of the presence of these substances.

According to Commission Decision 2002/657/EC validation; in the case where no certified reference material is available; should be carried out as follows:

- Prepare a set of samples of identical matrices, fortified with the analyte to yield concentrations equivalent to 1, 1.5 and 2 times the MRPL/RL or 0.5, 1 and 1.5 times the MRL.
- At each level the analysis should be performed with at least six replicates.
- Analyse the samples and calculate the concentration present in each sample.
- Repeat these steps on at least two other occasions.
- Values for recovery, repeatability, within-laboratory reproducibility and the analytical limits of Decision Limit (CCα), Detection capability (CCβ) are calculated from the results obtained.

Technical guidelines and required performance criteria are detailed within Commission Decision 2002/657/EC and specific ones relevant to this research in terms of quantitative methods are as follows;

- Specificity: A method shall be able to distinguish between the analyte and the other substances under the experimental conditions. An estimate to which extent this is possible has to be provided. Strategies have to be employed in order to overcome any foreseeable interference with substances when the described measuring technique is used. These strategies include the use of internal standards such as homologues, analogues, metabolic products of the residue of interest used to correct for any interferences. It is of prime importance that interference, which might arise from matrix components, is also investigated.
- Confirmatory Requirements: For a analytical instrument to be deemed confirmatory, it must have a certain number of identification points while meeting criteria for relative ion intensities and relative retention times.
  - *Identification Points:* As the methods developed in this research concern the analysis of prohibited compounds i.e. NMZs and CAP the method

used for their analysis needs to achieve 4 identification points. The number of identification points gained by a particular method depends on the detector employed. Tandem mass spectrometry using a number of different triple quadrupole mass spectrometers operated in multi reaction monitoring mode was utilised as the determination step for all methods developed within the research carried out as part of this thesis. This technique is classified within 2002/657/EC as being low-resolution mass spectrometry (LR-MS), when operated in multi reaction monitoring mode; it gains one identification point for each precursor ion and 1.5 identification points for each product ion monitored. So if two product ions are monitored, which result from one precursor ion, a total of 4 identification points is achieved. List of MS techniques and the identification points associated as given in the directive are seen in Table 2-5.

Table 2-5. The relationship between a range of classes mass spectrometry detectors and identification points earned (CD 2002/657)

MS Technique	Identificaton points earned per ion
Low resolution mass spectrometry (LR)	1.0
LR-MS^n precusor ion	1.0
LR-MS^n transition products	1.5
HRMS	2.0
HR-MS^n precusor ion	2.0
HR-MS^n transition products	2.5

n: multiple mass spectrometers e.g tandem mass spectrometry (MS/MS)

- *Ion Ratio:* The relative intensities of the detected ions, expressed as a percentage of the intensity of the most intense ion, shall correspond to those of the calibration standard, either from calibration standard

solutions or from spiked samples, at comparable concentrations, measured under the same conditions, within the tolerances given in the legislation seen in Table 2-6.

Table 2-6. Maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques (CD 2002/657)

Relative Intensity (% of base peak)	EI- GC-MS (relative)	CI-GC-MS, $GCMS^n$, LC-MS, $LCMS^n$ (relative)
> 50%	± 10%	± 20%
> 20% to 50%	± 15%	± 25%
> 10% to 20%	± 20%	± 30%
≤ 10%	± 50%	± 50%

- *Relative Retention Times:* The ratio of the chromatographic retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, shall correspond to that of the calibration standards at a tolerance of ± 0.5 % for GC and ± 2.5 % for LC.

❖ Calibration Curves: When calibration curves are used for quantification:

- at least five levels (including zero) should be used in the construction of the curve,
- the working range of the curve should be described,
- the mathematical formula of the curve and the goodness-of-fit of the data to the curve should be described,
- acceptability ranges for the parameters of the curve should be described.

❖ Recovery: During the analysis of samples the recovery shall be determined in each batch of samples, if a fixed recovery correction factor is used. If the recovery is within limits, the fixed correction factor may then be used. Otherwise

the recovery factor obtained for that specific batch shall be used; unless the specific recovery factor of the analyte in the sample is to be applied in which case the standard addition procedure or an internal standard shall be used for the quantitative determination of an analyte in a sample. % Recovery = 100 × measured content/fortification level

- Trueness/Accuracy: When no certified reference materials (CRMs) are available, it is acceptable that trueness of measurements is assessed through recovery of additions of known amounts of the analyte(s) to a blank matrix. The recovery can be determined as described above. Data for recovery are only acceptable when they fall within the ranges shown in Table 2-7.

Table 2-7. Minimum trueness of quantitative methods (CD 2002/657)

Mass Fraction	Range
≤ 1 µg/kg	-50% to 20%
> 1 µg/kg to 10µg/kg	-30% to 10%
≥10 µg/kg	-20% to 10%

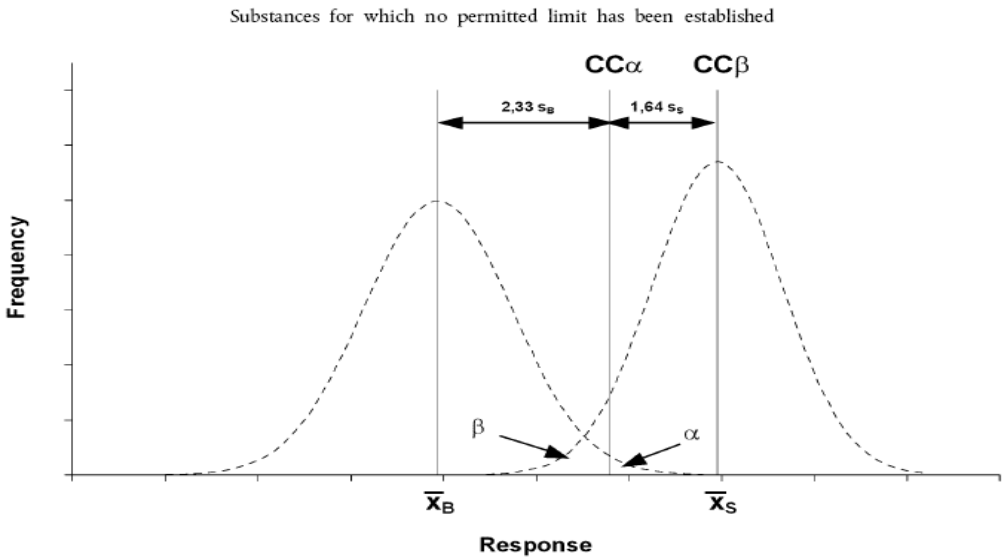
- Precision: The inter-laboratory coefficient of variation (CV) for the repeated analysis of a reference or fortified material, under reproducible conditions, shall not exceed the level calculated by the Horwitz Equation.
  - The equation is: $CV = 2^{(1 - 0.5 \log C)}$.
  - Where C is the mass fraction expressed as a power (exponent) of 10 (e.g. 1 mg/g = 10^{-3}).
  - Examples are shown in the Table 2-8.

Table 2-8. Examples for reproducibility CVs for quantitative methods at a range of analyte mass fractions. (CD 2002/657)

Mass Fraction	Reproducibility CV (%)
1 µg/kg	(*)
10 µg/kg	(*)
100 µg/kg	23
1000 µg/kg (1 mg/kg)	16

(*) For mass fractions lower that 100 µg/kg the application of the Horwitz equation gives unacceptably high values. Therefore, the CVs for concentrations lower than 100 µg/kg shall be as low as possible

- Analytical Limits: decision limit, CCα, and detection capability, CCβ, are also specified in this legislation and were intended in some way to take the place of performance characteristics; limit of detection (LOD) and limit of quantification (LOQ). CCα is defined as "the concentration at and above which it can be concluded with an error probability of α that a sample is non-compliant (contains the analyte)". CCβ is defined as "the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β". In β% of the cases, a non-compliant sample will be classified as compliant, and therefore reveals a false-negative result (Figure 2-4 gives a graphical illustration of these concepts).



$\overline{X}_B$	Mean Response of the blank sample
S_B	Standard deviation of the blank sample
$\overline{X}_S$	Mean Response of contaminated sample
S_S	Standard Deviation of the contaminated sample
α	Rate of false non-compliant results
β	Rate of false compliant results
CCα	Response with a given α -error and 50% β-error
CCβ	Response with a very small α-error and β-error

Figure 2-4: Adapted from 2002/657/EC illustrating CCα and CCβ

A variety of approaches on how these performance characteristics could be determined are also set out in this document. The approach used in this research is as follows;

- For the calculation of CCα blank material is used, which is fortified at and above the MRPL/RL in equidistant steps. The samples are analysed and after identification, a plot of the signal against the added concentration is made. The corresponding concentration at the y-

intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit. This is applicable to quantitative assays only (α = 1 %).

- For the calculation of CCβ, the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content at the decision limit equals the detection capability (β = 5 %).

The document describes approaches on how all these parameters can be determined, however it does not obligate laboratories to use these approaches; as stated in the document "Other approaches to demonstrate that the analytical method complies with performance criteria for the performance characteristics may be used, provided that they achieve the same level and quality of information".

2.4. Extraction and Purification Procedures

2.4.1. Introduction to Sample Preparation

Sample preparation is an essential stage in the analytical process. It takes place between sampling and measuring the prepared sample by means of an instrumental technique. It involves the extraction of the analyte of interest from either biological or feed matrix followed by the purification of this extract to help remove matrix components that may interfere with the instrument of detection. Although advancements in technology in the form of Ultra high performance liquid chromatography (UHPLC) and in tandem mass spectrometry (MS/MS) have reduced the need for labour intensive extraction protocols, sample preparation still plays a key role. However approaches and techniques used in sample extraction and purification have changed dramatically.

Historically analytical methods were performed on non specific instruments such as HPLC-UV. As a result methods used in the analysis of veterinary residues were often single analyte or at most single class methods which involved labour intensive extraction and purification protocols. These techniques often struggled to reach required sensitivity and therefore large sample sizes were necessary and large amounts of solvent were required for extraction. Quite often these methods included complex purification steps consisting of one or more solid phase extraction (SPE) steps (Figure 2-5), in order to make extracts suitable for non-specific detection techniques such as LC-UV [Sun *et al.*, 2007; Civitareale *et al.*, 2004; Dousa *et al.*, 2000].

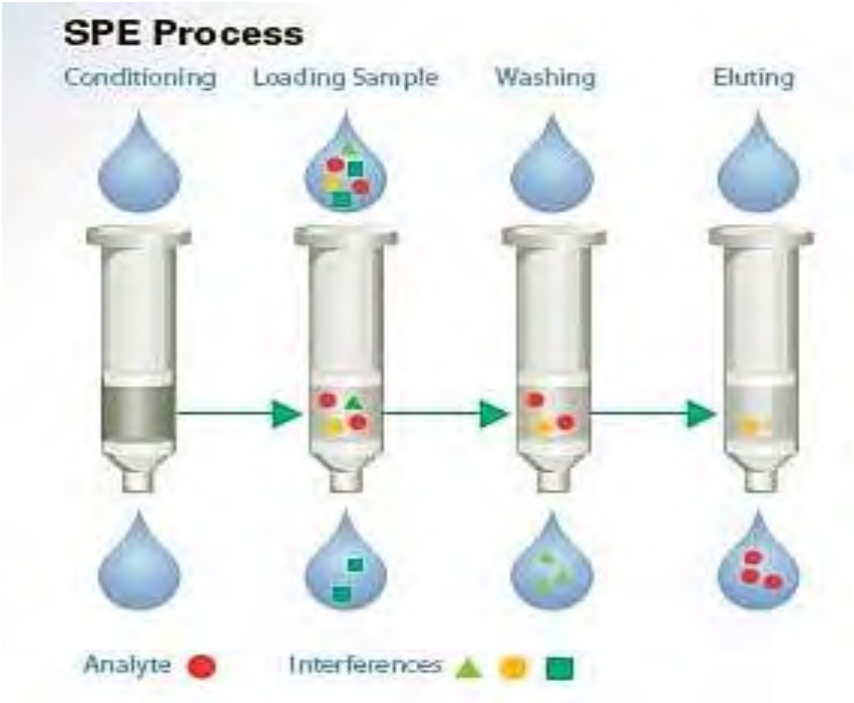


Figure 2-5. Schematic of Solid Phase Extraction operation

Recently the advancements in mass spectrometry have allowed for the development of methods for the analysis of potentially hundreds of compounds in a single experiment [Peters *et al.*, 2009; Kaufmann *et al.*, 2011]. These advancements have

resulted in a shift from complex labour intensive sample preparations to more generic extraction protocols and purification techniques being utilised. Resulting from this shift, a number of articles have been published involving generic sample preparation protocols which allow for a number of classes of drugs to be analysed in the same method [Boschera *et al.*, 2010; Stubbings *et al.*, 2009].

There are many benefits in using a generic extraction procedure for analysis of veterinary residues. There are significant economic benefits to this approach due to the fact that more analytes can be analysed in a single run with less solvent use and in a shorter time. Another benefit is that different classes of compounds previously analysed separately can now be analysed in a single run and as a result samples can be analysed for more residues in the same time. This also allows for more samples to be batched together allowing for reduced sample turnaround times. While the advantages of generic sample preparations are beneficial there are drawbacks to this approach when used with LC-MS in particular ion suppression which will be discussed in detail in section 2.5.4.

2.4.2. Extraction Methods and Purification Methodologies for Nitroimidazoles

Table 2-9: Overview of methods used for the analysis of nitroimidazoles as discussed in section 2.4.2

Reference	No. of compounds analysed	Matrix	Extraction Method	Measurement Technique	LOD (μg kg^{-1})
Egg Method					
Daeseleire 2000	3	Egg	Extraction with ACN; Evaporated and filtered	LC-MS/MS	0.50
Mohamed 2008	7	Egg	Extraction with ACN and NaCl; Clean-up on MIPs SPE	LC-MS/MS	1.00
Mottier 2006	7	Egg	Extraction with ACN and NaCl; Clean-up on Oasis HLB SPE	LC-MS/MS	0.60
Xia 2006	4	Egg, Muscle	Extraction with ACN and NaCl; Evaporated and filtered	LC-MS/MS	0.80
Tissue Methods					
Carretero 2008	1	Muscle	Extraction by ASE; Samples homogenised with EDTA washed sand and extracted with water at high temperature and pressure	LC-MS/MS	32.00
Clare Ho 2005	2	Muscle, Kidney, Liver	Extraction with Toluene mixed with Hexane; Clean-up on amine SPE cartridges	GC-MS/MS	1.90
Connolly 2007	5	Muscle	Extraction with Ethyl Acetate; Evaporated and re-constituted in HBS-EP	Optical Biosensor	2.00
Matusik 1992	4	Muscle	IPZ and IPZ-OH extracted with Benzene; Purified on Silica columns. DMZ and HMMNI extracted with acidic buffer and then extracted into Methylene Chloride	LC-MS/MS	10.00
Mottier 2006	7	Muscle, Fish	Extraction with Potassium Phosphate solution and Ethyl Acetate; Upper layer defatted with Hexane	LC-MS/MS	0.60
Polzer 2001	7	Muscle	Extraction by enzymatic hydrolysis; Clean-up on Kieselguhr SPE	GC-MS	2.80
Stubbings 2005	3	Muscle, Egg	Extraction with ACN; Extract dried with Sodium Sulphate; Acidified with Acetic Acid before clean-up on SCX SPE	HPLC-UV	5.00
Sun 2007	7	Muscle	Extraction with Ethyl Acetate; Clean-up on SCX SPE	HPLC-UV	0.80
Xia 2007	4	Liver	Extraction with Ethyl Acetate; Defatted with Hexane; Clean-up on Oasis MCX SPE	LC-MS/MS	0.50
Xia 2008	6	Muscle	Samples incubated overnight with HCl and 2-nitrobenzaldehyde; Neutralised with di-potassium hydrogen phosphate and NaOH; Clean-up on Oasis HLB SPE	LC-MS/MS	0.20
Xia 2009	7	Kidney	Extraction with Ethyl Acetate; Defatted with Hexane; Clean-up on MCX SPE	LC-MS/MS	0.50
Plasma Methods					
Aerts 1991	4	Plasma, Egg, Faeces	Extraction with Aqueous Buffer; Clean-up on Extrelute SPE followed by liquid-liquid partitioning with Isooctane	HPLC-UV	10.00
Fraselle 2007	7	Plasma	NaCl/Potassium Phosphate buffer and Protease solution added to samples; pH adjusted to 3 with HCl and hydrolised overnight; Defatted with Hexane and pH adjusted to 6 with NaOH; Clean-up on Chromobond XTR SPE	LC-MS/MS	1.25
Thompson 2009	7	Plasma, Kidney, Liver, Milk, Egg	Extraction with ACN followed by ultra-centrifugation	Optical Biosensor	3.00
Other Matrices Methods					
Capitan-Vallvey 2002	5	Water	Extraction with ACN; Clean-up on HLB SPE	LC-MS	0.20
Ding 2006	3	Royal Jelly	Samples dissolved in NaOH solution; Liquid-liquid extraction with Ethyl Acetate	LC-MS/MS	TBC
Stolker 2008	2	Milk	Extraction with ACN and dilution with water; Clean-up on Strata-X SPE	UPLC-TOF-MS	17.70
Zhou 2007	5	Honey	Extraction with Ethyl Acetate; Clean-up on amino SPE	HPLC-UV	TBC

The complexity of the matrices involved and also the high sensitivity that developed methods should be capable of achieving in order to identify treated animals are two main aspects that developed methods need to address. Nitroimidazole compounds were permitted for use in the European Union until the mid 1990s. As a result this research carried out on these compounds was limited until recently. Therefore methods for the analysis of these compounds are still relatively undeveloped in matrices recommended for analysis by EURL. Although in recent years more methods have been developed, these are limited in the analytes that they analyse for and in a lot of cases the sample matrix analysed is muscle and liver which as discussed previously is not suitable.

As discussed previously the selection of a suitable matrix for the analysis of these compounds is important to ensure abuse of these compounds is identified. Studies carried out by the EURL on the stability and homogeneity of nitroimidazoles in incurred muscle (i.e. in muscle of animals administered nitroimidazoles) [Polzer *et al.*, 2004; 2005] show that there is not a homogenous distribution of these analytes in turkey muscle and they also observed a rapid degradation in analyte concentration stored for prolonged periods above 4 °C. In contrast it was discovered that for plasma, retina and egg samples the analytes were stable during storage under the same conditions as the muscle samples which resulted in stable concentrations and allowed detection of these compounds for longer periods after medication had been halted. Therefore, it is advised that plasma, retina and eggs be used as target matrices for the residue control of nitroimidazoles [Polzer *et al.*, 2004; 2005]. The EURL has also put forward RL of 3 µg kg^{-1} (or ng mL^{-1}) for nitroimidazoles and therefore

developed methods used in their analysis must be sensitive enough for analysis, to at least this level.

Extraction methods largely depend on the matrix involved. Matrices differ greatly from one to the next. Although the extraction solvent may be the same the presence and absence of purification steps in the form of SPE is determined by the matrix in question. For this reason this section is broken up into extraction methods for specific matrices i.e. Egg, Tissue, and Plasma etc.

2.4.2.1.Egg Methods

There are a number of methods that exist for the determination of nitroimidazoles in the matrix of egg. The majority of these methods involve extraction with acetonitrile and the addition of NaCl. This is followed by either a purification step by SPE or by filtering before analysis. Mottier *et al.*, 2006 published a method for the detection of 4 nitroimidazoles and their three marker metabolites in eggs using detection by LC-MS/MS. Acetonitrile and NaCl were added to egg samples and the mixture was centrifuged. The resulting extracts were then purified on Oasis HLB SPE cartridges. The method was capable of detecting all analytes to a concentration of at least 0.6 µg kg^{-1}. Mohamed *et al.*, 2008 developed a method for the detection of 4 nitroimidazoles and their three marker metabolites in eggs using detection by LC-MS/MS. Acetonitrile was again used to extract the compounds of interest from egg, NaCl was then added to the resulting extract, the samples were centrifuged and the upper layer removed. In this method Molecular Imprinted (MIPs) SPE cartridges were then used for purification. The method was proficient in detecting each analyte to a concentration of at least 1 µg kg^{-1}. MIPs are cross-linked polymers with specific binding sites for a particular analyte. They possess recognition sites that, in terms of

size, shape and functionality, are complementary to the print molecule. MIPs provide good selectivity as separation materials, which is their most widely investigated use, their use as preconcentration and clean-up sorbents in MISPE (molecularly imprinted SPE) has recently been used [Mohamed *et al*., 2008]. One limitation of MIPs is that, depending on the synthetic procedure, there may be leaching of the template from the polymer, even after extensive washing, and this contaminates the sample. Since MIPs are made with large quantities of template, a small number of imprint molecules may remain in the resulting polymer and these may leak later during SPE, thus interfering with trace analysis.

The next two methods omit the use of an SPE step and replace it with a filtering step. Xia *et al.*, 2006 developed a method for the determination of 4 nitroimidazoles in egg, poultry muscle and porcine muscle. Samples were again extracted with acetonitrile and NaCl, samples were then concentrated by evaporation and filtered with detection by LC-MS/MS. The method had the capability to detect all analytes to 0.8 µg kg^{-1}. Daeseleire *et al.*, 2000 published work on a method for the determination of RNZ, DMZ and MNZ in egg with detection by LC-MS/MS. This time only acetonitrile was added to the egg samples to extract the analytes. The samples were then concentrated and filtered. In this case no SPE purification was employed. Compounds were detected to levels of 0.5 µg kg^{-1}.

From examination of these published methods it is clear that extraction with acetonitrile and the addition of NaCl is a popular and effective extraction technique for the analysis of nitroimidazoles in egg matrices. This is often followed by purification by SPE but methods have shown that this can be omitted but only for less analytes as SPE methods which analyse for seven residues. Our research will

attempt to adapt the rapid extraction protocols that omit SPE purification to allow for the analysis of a greater number of analytes than previously seen.

2.4.2.2.Tissue Methods (Liver, Kidney and Muscle)

Many methods exist for the determination of nitroimidazoles in tissue samples even though recent studies suggest that this may not be a suitable matrix for the detection of nitroimidazoles due to problems with stability. Xia *et al.*, 2007 developed a method for the detection of 3 nitroimidazoles and one metabolite in porcine liver with detection by LC-MS/MS. Liver samples were extracted with ethyl acetate, the extract was then evaporated to dryness, reconstituted in 0.1 M HCl and defatted with hexane. The extracts were further purified on Oasis MCX SPE cartridges. The method was capable of detecting each analyte at 0.5 µg kg^{-1}. Mottier *et al.*, 2006 reported a method for the detection of 7 nitroimidazoles in poultry muscle and fish using LC-MS/MS for determination. A potassium phosphate solution is added to the samples followed by ethyl acetate; this solution is then mixed and the upper organic layer removed. The extracts are then defatted with hexane; the method could detect each analyte to concentrations of 0.6 µg kg^{-1}. Matusik *et al.*, 1992 developed a method for the detection of 4 nitroimidazoles in turkey muscle with detection by LC-MS/MS. DMZ and its metabolite and IPZ and its metabolite are extracted using two different approaches. IPZ and IPZ-OH were extracted using benzene in the presence of borax with purification on silica columns. DMZ and HMMNI were extracted with an acidic buffer and then extracted into methylene chloride. Compounds could be determined to levels of 10 µg kg^{-1}. Clare Ho *et al.*, 2005 developed a method for the detection of 2 nitroimidazoles in poultry muscle and liver and porcine kidney and liver. Samples were extracted with toluene mixed with hexane and purified on amine

SPE cartridges. Residues are determined by GC-MS/MS, the method was capable of detecting each analyte to a concentration of 1.9 µg kg^{-1}. Polzer *et al.*, 2001 developed a method for the detection of 7 nitroimidazoles in poultry and porcine muscle using detection by GC-MS. Samples underwent an enzymatic hydrolysis followed by purification on kieselguhr SPE cartridges. The method was capable of detecting residues to a concentration of 2.8 µg kg^{-1}. Xia *et al.*, 2008 developed a multi-class, multi-residue method for the detection of 6 nitroimidazoles as well as a number of nitrofurans in porcine muscle. Hydrochloric acid and 2-nitrobenzaldehyde were added to the samples which were incubated overnight. Samples were then neutralized to pH 7 with di-potassium hydrogen phosphate and sodium hydroxide and purified on Oasis HLB SPE cartridges. The method was able to detect residues to a level of 0.2 µg kg^{-1} by LC-MS/MS. Carretero *et al.*, 2008 utilized accelerated solvent extraction (ASE) for extraction of RNZ from muscle samples. Samples were homogenized with EDTA washed sand and extracted with water at high temperature and pressure. The method was only able to detect residues to a level of 32 µg kg^{-1} by LC-MS/MS. Stubbings *et al.*, 2005 developed a screening method for the analysis of 3 nitroimidazoles in poultry muscle and egg with detection by HPLC-UV. Samples were extracted with acetonitrile then dried with sodium sulphate and acidified with glacial acetic acid before being purified on Bond Elut strong cation exchange SPE cartridges. The method was able to detect each analyte to a concentration of 5µg kg^{-1}. Sun *et al.*, 2007 reported a screening method for the detection of 7 nitroimidazoles in porcine and poultry muscle. Samples were extracted with ethyl acetate and purified using SCX SPE cartridges; determination was carried out by LC-UV. The developed method could detect each residue to a level of at least 0.8 µg kg^{-1}. Xia *et al.*, 2009 describes a method for the determination for 4 nitroimidazoles and 3 metabolites in

porcine kidney by LC-MS/MS. The compounds of interest were extracted from tissues with ethyl acetate. The crude extracts were subject to liquid–liquid partition with hexane followed by solid-phase extraction using mixed-mode strong cation-exchange column. The method could detect to levels of 0.5ug kg^{-1}. Connolly *et al.*, 2007 developed a screening method for the detection of 5 nitroimidazoles in poultry muscle with detection by optical biosensor. Samples were extracted with ethyl acetate, evaporated to dryness and resuspended in 0.5 ml HBS-EP (0.01 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) [pH 7.4], 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20). The method could detect each of the residues to at least 2 µg kg^{-1}.

Taking into consideration studies on the homogeneity and stability of nitroimidazole residues, it was decided that the extraction of muscle and liver samples do not ensure that the possible abuse of these compounds is detected. Therefore it was felt that their analysis should be from matrices such as plasma and eggs. Although the studies carried out pertain specifically to avian matrices it was felt that in order to ensure the methods developed were best suited for the routine analysis of nitroimidazoles in all species that plasma is chosen for analysis; along with eggs, milk and honey for specific species.

2.4.2.3.Plasma/Serum

Although plasma is one of the matrices recommended by the EURL for the analysis of nitroimidazoles there are only a few methods published for this matrix. Aerts *et al.*, 1991 developed a screening method for the detection of 4 nitroimidazoles in plasma, egg and faeces with LC-UV detection. The samples were extracted in an aqueous buffer and purified on Extrelut SPE cartridges followed by liquid-liquid

partitioning with isooctane. The method was able to detect all analytes to a level of 10 µg kg^{-1}.

Fraselle *et al.*, 2007 developed a method for the detection of 7 nitroimidazoles in porcine plasma by LC-MS/MS. A NaCl/Potassium phosphate buffer as well as a protease solution was added to the plasma. The pH was adjusted to 3 with HCl and the mixture was allowed to hydrolyze overnight. The mixture was then defatted with hexane adjusted to pH 6 with NaOH and purified on Chromabond XTR SPE cartridges. The method could detect each residue to at least 1.25 µg kg^{-1}. The method by Fraselle includes a hydrolysis step to free bound residues, there is some debate on whether this step is required for serum samples; Thompson *et al.*, 2009 does not include this step. Thompson *et al.*, 2009 developed a screening method for 4 nitroimidazoles and their 3 marker metabolites in serum, kidney, liver, milk and eggs. Acetonitrile was used for extraction followed by ultra centrifugation of the extract. Detection was carried out using an optical biosensor technique and the method was capable of detecting all residues to a level of 3 µg kg^{-1}.

It is quite clear from examination of published articles that despite reports recommending it, the analysis of nitroimidazoles in plasma is still not common practice. No rapid extraction methods exist for the confirmatory analysis of nitroimidazoles in plasma. By adapting the acetonitrile and NaCl method used in the used the extraction of nitroimidazole residues in eggs; it is felt that a rapid method could be developed to allow for an increased number of analytes to be analysed for.

2.4.2.4.Other

From investigation of literature only one method could be found that allows for the analysis of NMZs in honey. Zhou *et al.*, 2007, published a method for the analysis of

5 NMZs in honey by HPLC-UV. Samples were extracted with ethyl acetate and evaporated. The residue containing the NMZs was dissolved in ethyl acetate–hexane and subjected to solid-phase extraction cleanup by amino extraction columns. The eluent was evaporated, reconstituted and injected onto the column.

In relation to the analysis of nitroimidazoles in milk there are a limited number of published methods available [Ortelli *et al.*, 2009; Stolker *et al.*, 2008; Thompson *et al*., 2009]. These methods are all screening methods using either optical biosensor [Thompson *et al.*, 2009] or accurate mass instruments [Ortelli *et al.*, 2009; Stolker *et al.*, 2008]. Stolker *et al.*, 2008 developed a multi class, multi analyte method for the detection of various groups of veterinary residues in milk using UPLC-TOF-MS. IPZ and its hydroxy metabolite IPZ-OH were the only nitroimidazole compounds included. Milk was mixed with acetonitrile to precipitate proteins and the supernatant was diluted in water. This was then applied to Strata-X SPE columns and finally determined by MS. The method could reach 17.7 µg $kg^{-1}$for the detection of IPZ and 7.7 µg $kg^{-1}$for IPZ-OH. From investigation of literature there are no methods for the confirmatory analysis of NMZs in milk or honey at the levels desired.

Two other methods published for the analysis of nitroimidazoles are Ding *et al.*, 2006 who developed a method for the determination of 3 nitroimidazoles in royal jelly by LC-MS/MS. Samples were dissolved in sodium hydroxide solution to disassociate target analytes from the matrix. Liquid-liquid extraction methods using ethyl acetate as solvent were utilised to clean up the sample. The other was published by *Capitan-Vallvey et al.*, 2002 on the analysis of 5 NMZs in water by LC-MS. The extraction procedure was based on HLB (Hydrophilic-lipophilic balance) solid-phase extraction with acetonitrile followed by an evaporation step. The method was capable of identifying nitroimidazole residues at 0.2 µg L^{-1}.

It is quite clear that the matrices of milk and honey are not considered when methods for the analysis of nitroimidazoles are developed. This is most likely due to the fact that nitroimidazoles were previously not used routinely in honey and milk production practices. That being said, nitroimidazoles can be used to treat genital tricchoniasis in cattle and recently, reports from China suggest that the use of nitroimidazoles in beekeeping is being practiced [Zhou *et al.*, 2007]. Nitroimidazoles may be used to prevent and control Nosema apis in hives [Official Method (2003)]. Taking this into consideration the EURL for NMZs has suggested that honey be tested to ascertain any possible misuse of nitroimidazoles and if non-compliant results are found then this matrix should be included in monitoring plans. They also state that countries with high milk production should also analyse for these analytes in milk as their possible misuse in this matrix cannot be discounted. Therefore it was felt that the development of methods for the analysis of nitroimidazole residues in these two matrices should be developed. This would be performed by using previously developed methods for the other matrices as a starting point for the analysis of milk and honey.

2.4.3. Extraction Methods and Purification Methodologies for Chloramphenicol

Table 2-10: Overview of methods used for the analysis of chloramphenicol as discussed in section 2.4.3

Reference	No. of compounds analysed	Matrix	Extraction Method	Measurement Technique	LOD (µg kg^{-1})
Single Analyte Methods					
Forti 2005	1	Honey	Dissolution of samples in water before extraction with DCM and Acetone mixture; Evaporated and reconstituted in phosphate buffer; Clean-up on C18 SPE cartridges	LC-MS/MS	0.07
Huang 2006	1	Honey, Milk, Egg	Dissolution of samples in 20mM phosphate solution at pH 4.0; Samples passed through extraction tube containing a monolith microextraction polymer	LC-MS	0.02-0.04
Penney 2005	1	Milk, Eggs, Muscle, Liver, Kidney	Extraction with ACN and defatted with Hexane; Evaporated and reconstituted in mobile phase; Samples filtered prior to injection	LC-MS	0.20-0.60
Rejtharova 2009	1	Urine, Feed, Water, Milk, Honey	Extracion with ACN, Clean-up on MIP SPE cartridges	GC-MS-NCI	<0.30
Rocha-Siqueira 2005	1	Shrimp, Fish, Eggs; Poultry, Porcine and Bovine muscle	Extraction with phosphate solution followed by LLE with Ethyl Acetate	LC-MS/MS	0.10
Rodziewicz 2008	1	Milk Powder	Extraction with Ethyl Acetate and de-fatted with Hexane	LC-MS/MS	0.09
Ronning 2006	1	Meat, Seafood, Egg, Plasma, Honey, Milk, Urine	Extracion with ACN; Chloroform added to remove water; Evaproated and reconstitued in Methanol and water before injection	LC-MS/MS	0.02
Shen 2005	1	Seafood, Meat, Honey	Extraction with phosphoric buffer solution (pH = 6.88)/ Ethyl Acetate; Samples de-fatted with Hexane and purifed by SPE	ELISA, HPLC-UV, GC-ECD, GC-MS-EI-SIM, GC-MS-NCI-SIM	<0.30
Vinci 2005	1	?	Extraction with ACN and defatted with Hexane	LC-MS/MS	0.15
Vivekanandan 2005	1	Honey	Samples were diluted with water and purified using diatomaceous-based LLE cartridges	LC-MS/MS	0.05
Multi-Analyte Methods					
Hormazabal 2001	2	Meat, Milk	Extraction with ACN; water removed by $CHCl_3$; Clean-up on Bond Elut SPE cartridges	?	1.00
Shen 2009	4	Muscle, Liver	Extraction with Ethyl Acetate; Extracts frozen to remove lipids; Further purified by LLE with Hexane and SPE clean-up on HLB cartridges	GC-MS-NCI	0.10
Sheridan 2008	15	Honey	Extraction by Acid Hydrolysis; Clean-up on HLB SPE cartridges	LC-MS/MS	0.20
Wang 2007	3	Royal Jelly	Samples homogenised with water before extraction with Ethyl Acetate; Evaporated and reconstituted in phosphate buffer solution; Clean-up on C18 SPE cartridges	LC-MS/MS	0.10
Xie 2006	3	Feed	Extraction with Ethyl Acetate; Clean-up on C18 SPE cartridges	LC-MS/MS	0.10
Zhang 2008	4	Chicken Muscle	Extraction with Ethyl Acetate and defatted with Hexane; Clean-up on MCX SPE cartridges	LC-MS/MS	0.10

As discussed previously the use of CAP, due to health concerns, has been prohibited in food producing species. In order to ensure this is enforced effectively a legislative MRPL of 0.3 ng mL^{-1}/µg kg^{-1} has been issued for CAP which means all methods used in the analysis of this compound should be able to, at least, achieve this level. In

order to achieve enough sensitivity a common trend in analysis of CAP is the use of SPE as the sample purification technique. Common cartridge chemistries used in the analysis of CAP are Oasis HLB [Shen *et al.*, 2009], Oasis MCX [Zhang *et al.*, 2008] and the selective technique of Molecular Imprinted Polymers (MIPs) [Rejtharova *et al.*, 2009; Boyd *et al.*, 2007]. The need for very sensitive methods and increased sample purification in order to see down to the MRPL has resulted in CAP often being analysed in single analyte methods although some multi amphenicol methods do exist. An investigation into published literature found there are numerous methods available for the analysis of CAP in all matrices but found that some were quite labour intensive with use of SPE and resulted in the analysis of only one analyte [Rejtharova *et al.*, 2009; Ronning *et al.*, 2006], although there are some multi-amphenicol and multi-class methods available [Zhang *et al.*, 2008; Shen *et al.*, 2009]. Some of the more recently published methods are examined now under two headings single analyte and multi-analyte.

2.4.3.1. Single Analyte Chloramphenicol Methods

The majority of methods for the analysis of CAP are single analyte methods utilising SPE purification. Ronning *et al.*, 2006 developed a method that analysed for CAP residues in meat, seafood, egg, honey, milk, plasma and urine with liquid chromatography–tandem mass spectrometry. Meat, seafood, egg, honey and milk samples were extracted with acetonitrile. Chloroform was then added to remove water. After evaporation, the residues were reconstituted in methanol/water before injection. The urine and plasma were applied to a Chem Elut extraction cartridge, eluted with ethyl acetate, and hexane washed. These samples were also reconstituted in methanol/water after evaporation. CCα and CCβ for all matrices were 0.02 and

0.04 μg kg^{-1}. Rejtharova *et al.*, 2009 described a method for the analysis of CAP in urine, feed, water, milk and honey samples by GC-MS-NCI (Negative Chemical Ionization) using molecular imprinted polymer clean-up. CAP could be detected well below the MRPL in all matrices. Huang *et al.*, 2006 presented a single analyte method for the analysis of CAP in honey, milk, and eggs using polymer monolith micro-extraction followed by liquid chromatography-mass spectrometry determination. A poly(methacrylic acid-ethylene glycol dimethacrylate) monolithic capillary column was selected as the extraction medium. After dissolution in 20 mM phosphate solution at pH 4.0 and centrifugation, honey, eggs, or milk samples were directly passed through the extraction tube. The limits of detection for the method were 0.02 μg kg^{-1}, 0.04 μg L^{-1}, and 0.04 μg kg^{-1} in honey, milk, and eggs, respectively. Shen *et al.*, 2005 developed a method for the screening, determination and confirmation of chloramphenicol in seafood, meat and honey using ELISA, HPLC-UVD, GC-ECD, GC-MS-EI-SIM and GCMS-NCI-SIM methods. Extraction was with phosphoric buffer solution (pH = 6.88)/ethyl acetate, followed by defatting with hexane. For confirmation on GC-MS the samples underwent purification with SPE using LC-Si and LC-C18 cartridges. Forti *et al.*, 2005 described the detection and identification of CAP in honey. After a preliminary dissolution in water, samples were extracted with a mixture of dichloromethane/acetone and evaporated to dryness and reconstituted in a phosphate buffer solution. These were further cleaned up on an octadecyl (C18) SPE cartridge. CAP was determined by LC-MS/MS, using electrospray ionization in the negative ion mode with CCα of 0.07 μg kg^{-1} and CCβ of 0.10 μg kg^{-1}.

While the use of SPE is common there are published methods that omit this step. Vivekanandan *et al.*, 2005 published a method for the analysis of CAP in honey by

LC-MS/MS. Samples were diluted with water and were purified using diatomaceous-based supported liquid-liquid extraction cartridges. The LOD and LOQ of the method were 0.05 μg kg^{-1} and 0.1 μg kg^{-1} respectively. Rodziewicz *et al.*, 2008 published a method for the analysis CAP in milk powder by LC-MS/MS with negative electro-spray ionisation. Samples were extracted by using liquid-liquid extraction steps with ethyl acetate and lipids were removed using hexane. The CCα and CCβ of the method were 0.09 and 0.11 μg kg^{-1} respectively. Penney *et al.*, 2005 published a liquid chromatography/mass spectrometry (LC/MS) method for the determination of CAP residues in milk, eggs, chicken muscle and liver, and beef muscle and kidney. CAP is extracted from the samples with acetonitrile and defatted with numerous hexane washes. Samples are evaporated to dryness and reconstituted. They are then filtered before injection. The method detection limits of CAP ranged from 0.2 to 0.6 μg $kg^{-1}$for the various matrices. Rocha Siqueira et al., 2005 developed a LC-ESI-MS/MS method for determining chloramphenicol residues in fish, shrimp, poultry, eggs, bovine and swine samples. The samples were extracted with a phosphate extraction solution followed by liquid-liquid extraction with ethyl acetate. The LOQ of the method was 0.1 μg kg^{-1}. Finally a method was presented by Vinci *et al.*, 2005. CAP was extracted in acetonitrile and after liquid-liquid partitioning with n-hexane is identified and quantitatively determined by ion trap liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS) analysis in the negative ionisation mode. The CCα and CCβ of the method were 0.15 and 0.22 μg kg^{-1} respectively.

2.4.3.2.Multi-Analyte Methods including Chloramphenicol Analysis

While the majority of methods published on the analysis of CAP are single analyte methods there are a number of multi-amphenicol methods published. Xie *et al.*, 2006 published a method for the determination of chloramphenicol, thiamphenicol and florfenicol residues in original animal food by HPLC-ESI-MS/MS. The samples were extracted with basified ethyl acetate and cleaned up with C18 column. The detection limit of the method was 0.1 μg/kg. Shen *et al.*, 2009 developed a method capable of analysing for CAP, thiamphenicol (TAP), florfenicol (FF), and florfenicol amine (FFA) in poultry and porcine muscle and liver. Extraction was with ethyl acetate. The organic extracts were frozen to remove lipid and further purified by liquid-liquid extraction (LLE) with hexane and SPE using Oasis HLB cartridges. The target compounds were derivatized with BSTFA + 1% TMCS prior to GC-NCI/MS determination. A LOD of 0.1 μg/kg for CAP was obtained. Zhang *et al.*, 2008 published a LC-ESI-MS/MS method for the determination of CAP, TAP, FF and FFA in chicken muscle. Samples were extracted with basified ethyl acetate, defatted with hexane, and cleaned up on Oasis MCX cartridges. LOD was 0.1 μg/kg for CAP. Wang *et al.*, 2007 presented a method for simultaneous determination of residues of CAP, TAP and FF in royal jelly by using LC-MS/MS. After a preliminary homogenization of honey with water, samples were extracted with ethyl acetate, and evaporated to dryness, reconstituted in phosphate buffer solution followed by clean up on a C18 SPE cartridge. The method was capable of analysing for CAP at 0.1 μg kg^{-1}.

Apart from these multi-amphenicol methods there are a few multi-class methods which include the analysis of CAP. Sheridan *et al.*, 2008 developed a method for the analysis of 14 sulfonamide antibiotics and chloramphenicol in honey by liquid

chromatography-electrospray ionization-tandem mass spectrometry in negative mode for all 15 analytes. The method describes the use of an acid hydrolysis step to liberate the sugar-bound sulphonamides followed by a SPE method using Oasis HLB to remove potential interferences. The method had a limit of detection of 0.2 μg kg^{-1} for CAP. Hormazabal *et al.*, 2001 developed a method for determination of chloramphenicol and ketoprofen in meat and milk. The samples were extracted with acetonitrile, the organic layer was separated from water with $CHCl_3$, evaporated to dryness and then purified using Bond Elut SPE columns. The limit of detection for CAP was 1 μg L^{-1}.

Upon examining these published articles it was felt that incorporating the analysis of CAP with the analysis of nitroimidazoles would be beneficial. As CAP is often analysed on its own with the use of intensive extraction protocols the economic impact on the laboratory is significant. Therefore it's incorporation with nitroimidazole analysis greatly reduces labour hours, solvent usage and increases instrument capabilities, which greatly benefits the monitoring laboratory. Taking this into consideration methods were developed to allow for the analysis of both nitroimidazole and chloramphenicol residues in milk and honey.

2.4.4. Extraction Methods and Purification Methodologies for Veterinary Medicinal Additives in Animal Feed

An overall search of published literature on the analysis of veterinary products as feed additives shows that there are a limited number of methods available to analyse for them. In many cases there is only single analyte or at best single class methods available. Investigation into these methods shows that there is a wide variety of extraction and purification methods involved in their analysis. As the legislation is

divided into two lists, the published methods will be discussed under these two sets of compounds. The first will be methods for the analysis of prohibited medicinal additives and the second will be methods for the analysis of coccidiostats which are still permitted for use as feed additives.

2.4.4.1. Prohibited Medicinal Feed Additives

Table 2-11: Overview of methods used for the analysis of prohibited feed additives as discussed in section 2.4.4.1

Reference	Class of Compounds Analysed	Matrix	Extraction Method	Measurement Technique	LOD (mg kg^{-1})
Barbosa 2007; Vinas 2007; Wang 2006	Nitrofurans	Feed	Organic solvent extraction followed by SPE clean-up	LC-MS, HPLC-DAD	<0.01
Caballero 2002; Houglum 1997	Tetracyclines	Feed	Organic solvent extraction followed by SPE clean-up	HPLC-UV	<0.10
Capitan-Vallvey 2007	Nitromidizoles	Feed	Extraction with phosphate buffer solution (pH = 2); Clean-up on HLB SPE cartridges	LC-MS	0.05
Civitareale 2004	Antibacterial Growth Promoters	Feed	Extraction with methanol; Clean-up on CN SPE cartridges	HPLC-UV/DAD	<1.00
Dousa 2000; Tollomelli 1992; Ramos 1991	Nitromidizoles	Feed	Organic solvent extraction followed by SPE clean-up	HPLC-UV	1.00
Dusi 2000	Nicarbazine and Clopidol	Feed	Extraction with DMF; Clean-up on Alumina-basic SPE cartridge	HPLC-UV	1.00-2.50
Gramse 2004	Tylosin	Feed	Extraction with methanol; Clean-up on C18 SPE cartridges	HPLC-UV	0.22
Hajee 2001	Virginiamycin	Feed	Extraction with Ethyl Acetate; Clean-up on Sep-Pak Silica Gel and HLB SPE cartridges	LC-UV, LC-MS	2.70
Higgins 2002	Tylosin and Virginiamycin	Feed	Extraction by ASE using 65% aqueous acetone (pH = 2)	ELISA	10.00
Hormazabal 2002	AGPs and Ionophore Coccidiostats	Feed	Samples homogenised with methanol/acetone/THF; Samples mixed with water and centrifuged; Supernatant diluted and filtered through Spin-X microcentrifuge tube	LC-MS	1.50-2.50
Kesiunaite 2008	Antibacterial Growth Promoters	Feed	Extraction using matrix solid phase dispersion (MSPD)	HILIC-UHPLC-DAD	0.10
Qin 2005	Sulphonamides	Feed	Organic solvent extraction followed by SPE clean-up; Dilution before injection	LC-MS/MS	<0.02
Situ 2006	Antibacterial Growth Promoters	Feed	Analysis using Enzyme-linked Immuno-sorbant Assay (ELISA) Kit	ELISA	1.00-4.00
Squadrone 2008	Amprolium	Feed	Extraction with methanol/water (80:20 v/v); Extract filtered (filter paper 90g/m^2); Filtrate diluted with mobile phase before injection	LC-MS	0.20
Van Poucke 2003; 2005; 2006	Antibacterial Growth Promoters	Feed	Extraction with methanol/water (7:3 v/v); Extract diluted before clean-up on HLB SPE cartridges	LC-MS	<1.00
Vinas 2006	Chloramphenicol	Feed	Samples mixed with water; 2 LLE with Ethyl Acetate; Clean-up on SPE cartridge	LC-DAD	0.70
Wu 2009	Antibacterial Growth Promoters	Feed	Extraction with ACN/water (60:40 v/v); Clean-up on HLB SPE cartridges	LC-MS/MS	<0.02

As discussed previously NMZs and CAP are banned for use in food producing animals and therefore are also prohibited for use as feed additives. While there are numerous methods for the analysis of these compounds in biological matrices there are only a few methods developed which allow for their analysis in animal feed. The majority of methods that are used for the analysis of nitroimidazoles in feed are HPLC-UV methods with the use of SPE purification for the analysis of, at most, three analytes [Dousa, 2000; Tollomelli *et al.*, 1992; Ramos *et al.*, 1991]. Although one method is published which includes the analysis of six analytes by LC-MS, Capitan-Vallvey *et al.*, 2007 presented a method that allowed for the analysis of metronidazole, ronidazole, dimetridazole, secnidazole, tinidazole and ipronidazole in feedstuff. The 5-nitroimidazoles were extracted from animal feed with a pH 2 phosphate buffer solution followed by a SPE based on HLB cartridges. The method was applied successfully to determine 5-nitroimidazoles in feedstuff at level of 0.05 mg kg^{-1}. For analysis of CAP only two methods could be found in feed. One of these methods published by Vinas *et al.*, 2006 allows for the determination of CAP residues in animal feeds by liquid chromatography with photo-diode array detection. Feed was mixed with water and then underwent two liquid-liquid extractions (LLE) with ethyl acetate. Purification was performed using a Discovery DSC-18Lt SPE cartridge. LOD using the proposed procedure was 0.7 μg kg^{-1}.

Another set of antibiotic compounds prohibited for use in food producing animals are nitrofurans and in turn are banned for use as feed additives. From examination of literature there are three papers published for their analysis in feed. These methods analyse for a number of nitrofuran analytes by either LC-MS or HPLC-DAD with purification usually in the form of SPE [Barbosa *et al.*, 2007; Vinas *et al.*, 2007; Wang *et al.*, 2006].

Compounds such as sulphonamides and tetracyclines are now prohibited for use as feed additives although they are still permitted for use in medicated feed. While there are numerous methods for the analysis of both of these class of compounds in biological matrices [Shen *et al.*, 2010; Carretero *et al.*, 2008; Soto-Chinchilla *et al.*, 2007; Sergi *et al.*, 2007 Shao *et al.*, 2007] there are very few methods for their analysis in animal feed. Qin *et al.*, 2005 developed a method for the qualification and quantification of 10 sulfonamides in animal feedstuff by LC-MS/MS. Samples were solvent extracted, purified using SPE and diluted before injection. The LOQs for the 10 sulphonamides ranged from 0.5 - 2.0 μg/kg. In the case of tetracyclines there are no MS methods published that are capable of analysing animal feed although some chromatography methods are available [Caballero *et al.*, 2002; Houglum *et al.*, 1997].

The rest of the compounds in the prohibited feed additives list could be classified under the broad title of antibacterial growth promoters (AGPs). This list includes compounds such as tylosin, virginiamycin, avilamycin, spiramycin, zinc bacitracin, olaquindox, carbadox, aprinocid, methyclorpindol (Clopidol), dinitolimide amprolium and ethopabate. The analysis of some of these compounds in feed has in some cases not been published before whereas some analytes have been investigated as part of collaborative European wide studies.

A number of journal papers have been published as a result of the EC funded Feedstuffs-RADIUS project. This was a study in particular into the analysis of AGPs in animal feed. Situ *et al.*, 2006 published a method for the screening of zinc bacitracin, spiramycin, tylosin, virginiamycin and olaquindox in animal feedstuffs by the use of an enzyme-linked immuno-sorbent assay (ELISA) kit. The detection limits for the developed immunoassays were to detect concentrations of 4 mg kg^{-1}, or more,

of olaquindox and 1 mg kg^{-1} or more of the other compounds. A number of papers have been published by van Poucke *et al.*, 2003, 2005, 2006 on the area of AGPs analysis in animal feed by LC-MS. Some of these research papers were also funded by the Feedstuffs-RADIUS project. The three papers use the same method as a starting point with small alterations and are for the analysis of zinc bacitracin, spiramycin, tylosin, virginiamycin and olaquindox. Feed was extracted with 10 ml of methanol/water (7:3 v/v), and 3 ml of this extract was purified on an OASIS HLB column after dilution with 27 ml of water. The detection capability of the method for all compounds was <1 mg kg^{-1}.

Apart from van Pouke *et al.*, there are very few confirmatory methods for the analysis of AGPs in feed. Some of these methods are capable of seeing low levels of analyte but some are in the ppm range. Wu *et al.*, 2009 developed a method by LC-MS/MS for the simultaneous determination of carbadox, olaquindox, mequindox and quinocetone in swine feed. The analytes were extracted from the feed with acetonitrile/water (60:40, v/v), and then further purified by solid-phase extraction using Oasis HLB cartridges. The LOQs for the four compounds were <20 μg kg^{-1}. Squadrone *et al.*, 2008 published a LC-MS method which was able to detect amprolium in chicken feed. The samples were extracted with 100 mL methanol/water 80/20 (v/v) and blended for 60 min. An aliquot of these extracts was filtered on paper filter (90 g m^{-2}, 250 mm) and collected into a 50 mL flask. These filtrates were diluted 1:10 with initial mobile phase and filtered before injection. LOQ of the method was 0.2 mg kg^{-1}. Hormazabal *et al.*, 2002 developed a method for the determination of amprolium (AMP), ethopabate (ETB), lasalocid (LAS), monensin (MON), narasin (NAR) and salinomycin (SAL) in feed by LC-MS. Feed samples were homogenized with methanol-acetone-tetrahydrofuran. After addition of

water, the samples were mixed and centrifuged. The compact bottom layer was re-extracted with methanol-water. After centrifugation, the combined supernatants were diluted and filtered through a Spin-X micro-centrifuge tube. Three separate LC method were needed using three different columns in order to analyse for all analytes. The limits of detection were close to 2 mg kg^{-1} for AMP, LAS, MON, and NAR, 1.5 mg kg^{-1} for ETB, and 2.5 mg kg^{-1} for SAL in chicken feed. Hajee *et al.*, 2001 published a method for the detection of virginiamycin at sub-additive level in pig, calf, piglet, sow, poultry, cattle and laying hen feeds by LC-UV/LC-MS. Virginiamycin was extracted from animal feeds with ethyl acetate after wetting of the feed with water followed by clean-up on Sep-Pak silica gel and OASIS HLB cartridges. The LOQ of the method was 2.7 mg kg^{-1}.

The rest of the published methods dealing with the analysis of AGPs in animal feed are screening methods using chromatography or immunoassay techniques. Dusi *et al.*, 2000 described a method for the determination of nicarbazin and clopidol in poultry feeds by LC. Ground feed samples were extracted using aqueous dimethylformamide (DMF) after mixing with water. Co-extracted feed constituents were removed with a solid-phase extraction on alumina-basic columns and the eluates were directly analysed. LOD for nicarbazin and clopidol were 1 mg kg^{-1} and 2.5 mg kg^{-1} respectively. Kesiunaite *et al.*, 2008 developed a method involving matrix solid-phase dispersion (MSPD) extraction and hydrophilic interaction ultra-high-pressure liquid chromatography (HILIC-UHPLC) with photodiode array detection for the determination of carbadox and olaquindox in feed. Feed sample and 0.5 g of C18 sorbent were placed into an agate mortar and gently blended for about 2 min using a pestle and mortar to obtain a homogeneous mixture. The blend was then transferred into a 15 mL syringe with a frit on the bottom and a second frit was

placed over the dispersed sample with slight compression. The analytes were eluted from the cartridge with 10 mL of acetonitrile–methanol mixture (8:2, v/v) applying slight vacuum. This was then evaporated, reconstituted and filtered before injection. The method could analyse levels of 0.1 mg $kg^{-1}$for olaquindox and carbadox. Civitareale *et al.*, 2004 developed a method for the analysis of tylosin and spiramycin by HPLC-UV/DAD. After methanolic extraction, samples were cleaned up on SPE CN columns before analysis. Detection limits for the method were 176 and 118 μg kg^{-1} for spiramycin and tylosin respectively. Gramse *et al.*, 2004 published a method for the determination of tylosin in feeds. The method involves extraction of tylosin with methanol, concentration under a stream of nitrogen, and cleanup using C18 SPE cartridge followed by analysis using HPLC-UV. The limit of detection and quantitation of the method was 0.216 and 0.720 mg kg^{-1} respectively. Higgins *et al.*, 2002 developed a method for the screening of avoparcin, bacitracin zinc, spiramycin, tylosin and virginiamycin by immunoassay. Extraction of analytes from 5g portions of test samples was performed by use of a Dionex ASE200 accelerated solvent extraction system with use of 65% aqueous acetone (adjusted to pH 2 by addition of 12 ml of 1M-HCl per litre of solvent). All compounds could be screened at levels less than 10mg/kg.

It is quite clear from the published articles that there is no single method available to analyse for a broad range of these prohibited medicinal additives in animal feed. This is probably because legislation pertaining to these compounds in feed is relatively new and the range of compounds incorporated is quite diverse. Therefore it is anticipated the application of a generic extraction and purification protocol will allow an increased amount of analytes to be analysed in a single run, therefore increasing

the analytical capabilities of the laboratory and in doing so increase the likelihood of finding possible breaches of this legislation.

2.4.4.2. Coccidiostats

Table 2-9: Overview of methods used for the analysis of coccidiostats in feed as discussed in section 2.4.4.2

Reference	No. of compounds analysed	Matrix	Extraction Method	Measurement Technique	LOD (mg kg^{-1})
Campbell 2006	3	Feed	Extraction with methanol and water (9:1 v/v); Samples shaken and diluted before injection	LC-UV	1.00
Delahaut 2010	11	Feed	Extraction with 10% Na2CO3solution and ACN; Samples agitated before centrifugation; Samples extracted a second time with ACN; Both organic extracts combined; An aliquot of extract evaportated and reconstituted before injection	LC-MS/MS	1% unavoidable carryover
Ebel 2004	2	Feed	Extraction with methanol; Samples filtered and de-fatted with hexane; Samples concentrated by evaporation before injection	LC-MS	<1.00
Focht 2008	1	Feed	Extraction with 0.5% HCl acidified methanol; Samples sonicated and shaken; Samples diluted amd filtered before injection	LC-FD	1.00
Hormazabal 2005	4	Feed	Samples homogenised with methanol/acetone/DHF; Samples mixed and centrifuged; After centrifugation supernatant was extracted with hexane; Evaporated to dryness and re-constituted in ACN and water; Filtered through Spin-X microcentrifuge tube before injection	LC-MS	0.20-0.60
Jong 2004 (a)	1	Feed	Extraction with ACN and methanol (1:1)	LC-UV	<20.00
Jong 2004 (b)	1	Feed	Extraction with methanol	LC-UV	2.00
Kot-Wasik 2005	1	Feed	Extraction with acidified methanol by ASE; Clean-up on Aluminium Oxide cartridges	HPLC-UV / LC-MS	0.02
Krabel 2000	1	Feed	Extraction with ACN and water (4:1 v/v); Filtered before injection	LC-UV	0.25
Mortier 2005 (a)	1	Feed	Extraction with methanol	LC-MS/MS	<0.01
Mortier 2005 (b)	6	Feed	Extraction with methanol	LC-MS/MS	<0.01
Sanchez 2008	1	Feed	Extraction with 1% Calcium Chloride in methanol solution using mechanical agitation; Samples centrifused and diluted; Filtered before injection	LC-FD	1.00
Thalmann 2004	1	Feed	Extraction with methanol and phosphate solution (9:1 v/v)	LC-UV	<20.00
Turnipseed 2001	4	Feed	Extraction with hexane and ethyl acetate; Clean-up on silica SPE cartridge	LC-MS	1.00-50.00
Vincent 2008	6	Feed	Extraction with methanol and water (9:1 v/v); Aliquot of extract cleaned-up on IST Isolute SPE cartridge before analysis	LC-MS/MS	0.01
Vincent 2011	6	Feed	Extraction with ACN; Samples sonicated and agitated before centrifugation; Quantification by Standard Addition	LC-MS/MS	1% unavoidable carryover
Wang 2000	2	Feed	Extraction with ACN; mixed for 1hr and then filtered; Clean-up on C18 SPE cartridge; Eluate diluted before analysis	MALDI-TOF-MS	2.40

The majority of methods published for the analysis of coccidiostats in animal feed are for the analysis of single analytes by LC-UV. These include a number of papers that validate LC methods as part of collaborative studies. These collaborative studies include the following methods. Sanchez *et al.*, 2008 developed a method for the

analysis of decoquinate in supplements, premixes, and complete animal feeds (i.e feed ready for use) at medicating and trace levels. Decoquinate was extracted from feed previously ground to homongeniety with 1% calcium chloride-methanol solution using mechanical agitation for 90 min. After centrifugation for 5 min and dilution of medicated levels (if necessary) into an acceptable analytical range. The diluted extracts are filtered and analyzed by reversed-phase LC with fluorescence detection and is capable of analysing to levels of 1 mg kg^{-1}. Focht, 2008 developed a LC method for the analysis of lasalocid in medicated premixes and complete animal feeds and at trace-level in feeds. The method employs a 0.5% HCl acidified methanol extraction followed by 20 min sonication in a water bath heated to 40°C. Samples are then shaken on a mechanical shaker for 1 h and stored overnight, followed by an additional 10 min shaking the following morning. Sample extracts are diluted if necessary with extractant, filtered, and injected onto an LC with fluorescence detection and is capable of analysing to levels of 1 mg kg^{-1}..

Other single analyte LC methods include two methods published by de Jong *et al.*, 2004 (a); Jong *et al.*, 2004 (b) describing the analysis of nicarbazin in broiler feeds and premixtures and maduramicin in feedingstuffs and premixtures at medicated levels. The extraction solvent was an acetonitrile-methanol (1:1) mixture for nicarbazin and was just methanol for maduramicin. Analysis was performed on LC-UV instrument and the LOD was <20 mg kg^{-1} for nicarbazin and 2 mg kg^{-1} for maduramicin. Kot-Wasik *et al.*, 2005 published an analytical procedure for the determination of robenidine in animal feeds. Robenidine was extracted from samples with acidified methanol using ASE. Extracts were dried and subjected to clean-up with aluminium oxide cartridges. Analysis was performed by HPLC coupled to DAD UV and MS. LOQ was determined to me 0.1 and 0.02 mg kg^{-1} for DAD-UV and MS

detection, respectively. Thalmann *et al.*, 2004 developed a reversed-phase LC method for the analysis of narasin in feedingstuffs and premixtures. The extraction solvent was methanol-K_2HPO_4 solution (9:1, v/v). Narasin was detected at 600 nm after post column derivatization with dimethylamino-benzaldehyde. The LOD was found to be <20 mg kg^{-1}. Krabel *et al.*, 2000 developed a method for the analysis of nicarbazin in animal feed. Feed is extracted with 200 ml acetonitrile/H_2O (4:1, v/v). An aliquot of the extract is filtered before analysis by LC-UV. The method has a LOD of 250 μg kg^{-1} and a LOQ of 500 μg kg^{-1}.

Some LC-UV methods are capable of analysing for more analytes. Campbell *et al.*, 2006 published a method for the analysis of monensin, narasin, and salinomycin in mineral premixes, supplements, and complete animal feeds at medicating and trace levels. The method uses methanol-water (9:1, v/v) extraction with mechanical shaking for 1 h, filtration, and dilution if necessary. Determination of the 3 ionophores is by reversed-phase LC using post-column derivatization with vanillin and detection at 520 nm. That said, the majority of multi analyte methods use LC-MS as the instrument of analysis. Hormazabal *et al.*, 2005 described a LC-MS method for the determination of lasalocid, monensin, narasin and salinomycin in feed. Samples were homogenized with methanol-acetone-tetrahydrofuran. The samples were mixed and centrifuged. After centrifugation, 100 μL supernatant was extracted with hexane, evaporated to dryness, diluted with acetonitrile-water, filtered through a Spin-X micro-centrifuge tube, and injected into the LC/MS. The LOQ of the method for all the analytes ranged from 0.2 to 0.6 mg kg^{-1}. Turnipseed *et al.*, 2001 published a LC-MS method for the analysis of monensin, lasalocid, salinomycin, and narasin. The drugs were extracted from the feed matrix using hexane-ethyl acetate and isolated using a silica solid-phase extraction cartridge.

These ionophores were confirmed in both medicated feeds and non medicated feeds fortified with these drugs at the 1-50 mg kg^{-1} level. Ebel *et al.*, 2004 published a method on the LC-MS analysis of monensin and lasalocid in feed samples. The samples were extracted with methanol. The extracts were filtered and then defatted with hexane. These were concentrated up by evaporation before injection. The method was capable of detecting compounds below 1 mg kg^{-1}. While Wang *et al.*, 2000 published a method for the analysis of salinomycin and narasin in poultry feed using MALDI-TOF MS. Ground feed samples were extracted with 10 ml acetonitrile for 1 h and the mixture was filtered. The filtrate was applied to a C18 SPE cartridge. The eluate was diluted before analysis. The LOD for both analytes was 2.4 mg kg^{-1}.

With the publication of European legislation Regulation 2009/8/EC laying down levels of unavoidable carry over for eleven coccidiostats in non target feed there is a need for confirmatory methods for the quantitation of these analytes at various levels in feed. LC-MS/MS offers a useful tool in the effort to ensure this legislation is enforced. Previous methods for the analysis of some of these compounds in feed show that LC-MS/MS is capable of confirming analytes at levels related to unavoidable carry over. Mortier *et al.*, 2005 (a) published a LC-MS/MS method for the detection of the coccidiostat diclazuril in poultry meat and feed. Feed samples are extracted with methanol. A portion of the extract is evaporated to dryness and then reconstituted in mobile phase. The samples are then filtered before injection onto the LC-MS/MS. Mortier *et al.*, 2005 (b) published another method using the same extraction protocol for the quantitative detection of the chemical coccidiostats halofuginone, robenidine, diclazuril, nicarbazin and dimetridazole and its main metabolite 2-hydroxydimetridazole in poultry eggs and feed. CCα and CCβ of the method were no higher than 10.7 and 14.5 μg kg^{-1} respectively for all analytes in

feed. While Vincent *et al.*, 2008 developed a LC-MS/MS method for the analysis of monensin, salinomycin, narasin, lasalocid, semduramicin and maduramicin in animal feed. Samples were extracted with 100 ml of a MeOH: H_2O mixture (90:10, v: v) for 60 min by agitation. 5ml of the extract was purified on IST Isolute cartridge before analysis. The LOD and LOQ of the method were different for various analyte/matrix combinations but were in all cases below 0.014 and 0.046 mg kg^{-1}.

More recently two papers have been published with the objective of analysing feed at levels relating to unavoidable carryover as stated in Regulation 2009/8/EC (Table 2.4). One of these methods published by Vincent *et al.*, 2011 was capabable of analysing for the six ionophore coccidiostats listed in the legislation. 5g of feed is extracted with 40ml of ACN. The mixture was then placed in an ultrasonic bath for 30 mins and this was followed by a head to head agitation for 60mins. The sample is centrifuged and seven 2ml aliquots are taken and used for analysis by utilising a standard addition approach. The method was validated and is capable of analysing the six compounds at levels relating to 1% and 3% unavoidable carry over values as stated in legislation.

The only method to include the analysis of feed for all 11 coccidiostats in a single method is one published by Delahaut *et al.*, 2010. Feed samples were extracted with a 10% Na_2CO_3 solution (w/v) and acetonitrile. The samples were shaken for 30 min on a mechanical agitator and then centrifuged. The supernatant was transferred into a tube and extraction with acetonitrile was repeated a second time and both organic extracts were combined. Finally, 1ml of acetonitrile extract was transferred into a tube and evaporated to dryness under nitrogen in a heated water bath. The sample was re-dissolved in 1ml acetonitrile/water mixture (80:20, v/v) before analysis by

LC-MS/MS. The method was validated using the levels permitted in feed of non target animals as stated in 2009/8/EC.

After investigation of the published literature it is apparent that there are very few methods available that can be applied in order to enforce Commission Regulation 2009/8/EC. To date only two methods have been published with the specific aim to analyse for these compounds at levels related to unavoidable carryover into non target feed. The similarities between the methods are that both use acetonitrile as the extraction solution because its polarity is the most suitable for the extraction of these compounds. It is also clear that dilution of sample extract rather that concentration results in improved repeatability of the methods due to the fact that the concentrations of the ionophore coccidiostats is above the analytical range of many mass spectrometers and also the matrix of feed is complex and dilution results in reduced interference from the matrix. Using these two papers as a starting point it is hoped that a method for the analysis of all eleven coccidiostat compounds can be developed for use in the analysis of the different feeds that are encountered in the laboratory on a routine basis.

2.5. LC-MS/MS

2.5.1. Introduction to Mass Spectrometry

After World War II, mass spectrometry began to have a broad application in chemistry and in particular organic chemistry. By the early 1950's there were a number of US companies building magnetic sector mass spectrometers. However it wasn't until the mid 1960's and the combination of gas chromatography with mass spectrometry did the use of mass spectrometry in the analysis of compounds become more prominent. The further development of GC-MS resulted in it becoming an

indispensable tool in a number of areas such as environmental, medical, food and flavour industries and also in forensics [Watson *et al*. 2009]. Its development continued from these early instruments into the extremely sensitive and selective instruments that are available today.

A definition of a mass spectrometer published by Price, 1991 is an instrument in which ions are analyzed according to their mass-to-charge ratio, and in which the number of ions is determined electrically. For the most part, there are four basic components that are standard in all mass spectrometers (Figure 2-6). These are; a sample inlet, an ionization source, a mass analyzer and an ion detector. Although there are many variation of mass spectrometers the process by which all sample molecules are analysed is similar regardless of instrument configuration. Sample molecules are introduced into the instrument through a sample inlet. Once inside the instrument, the sample molecules are converted to ions in the ionization source, before being electrostatically propelled into the mass analyzer. Ions are then separated according to their m/z within the mass analyzer. The detector converts the ion energy into electrical signals, which are then transmitted to a computer.

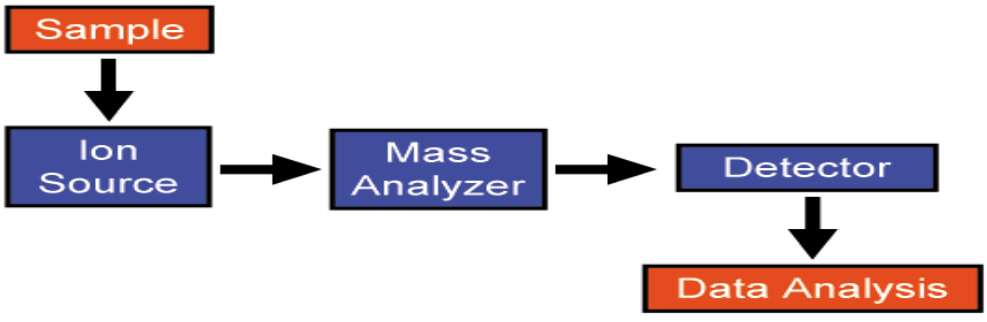


Figure 2-6: Basis components of a mass spectrometer

2.5.2. Instrument Selection LC-MS versus GC-MS

Upon commencing this research a decision needed to be made on what instrumental technique would result in improved methods for the analysis of pharmaceutical compounds in a variety of animal and feed matrices. According to CD 2002/657/EC there are only a number of instruments that can be used for the confirmatory analysis of veterinary residues in food matrices. The most commonly available of these is mass spectrometry. Therefore the majority of methods published in this area either use GC-MS^n or LC-MS^n for confirmatory analysis. Historically GC-MS was the most commonly used analytical instrument. This was as a result of GC-MS being a more mature technology, being less expensive and having an extensive list of established and approved operational protocols. However with improvements in the manufacture of LC-MS systems and increased research on there use, LC-MS is fast becoming the instrument of choice for analysis of veterinary residues in food matrices. The main reason for this is that liquid chromatography offers tremendous potential for analyzing non-volatile, polarized and ionized materials with reduced sample purification, extraction and more importantly there is no need for derivatisation.

For analysis by GC-MS, compounds need to be both volatile at the temperature needed for separation and also thermally stable. As a result, analysis by GC–MS, in many cases requires derivatization of the analytes in veterinary residue analysis. This is carried out by a number of processes such as silylation, acylation or oxime/silylation depending on the individual properties of the compounds to be analysed. However this can cause problems and this is seen in the case of nitroimidazoles where derivatisation results in the same trimethylsilylether product formed from the derivatisation of RNZ and HMMNI with BSA [Polzer *et al.*, 2001].

This is as a result of the breaking up of carbamoyloxymethyl group of RNZ during derivatisation. For this reason, a distinction between RNZ and HMMNI, which is the metabolite of DMZ and RNZ, cannot be made with the use of GC-MS. In addition to this a number of nitroimidazoles such as IPZ are not derivatisable with BSA [Polzer *et al.*, 2001].

The derivatisation process increases sample preparation times and in the case of nitroimidazoles does not allow for the analysis of all compounds. However in some cases GC-MS is a more specific and sensitive technique. This is due to the fact that gas chromatography and mass spectrometry are compatible because analytes need to be in the gas phase in order to be analysed by mass spectrometry. This gives GC-MS an advantage in terms of sensitivity over LC-MS in the analysis of some veterinary residues. The majority of the analyte which passes through the GC column will enter the mass spectrometer and be analysed. This is not the case with LC-MS as the combination of liquid chromatography and mass spectrometry is not as compatible as with GC which results in the large amount of analyte being lost when the sample is converted from liquid to gas phase ions. To allow for the use of this hyphenated technique the use of an interface is required. The primary purpose of this interface is the removal of the mobile phase and this results in the loss of analyte and a reduced amount ionised analyte reaching the mass analyzer. The types of interfaces and ionisation utilised in LC-MS are discussed further in the next section 2.5.3.

Taking this information into consideration, the most suitable instrument for use in multi residue methods is LC-MS. The reason for this is that unlike GC-MS it can be used for the analysis of all non volatile compounds without the need for derivatisation. This results in increased analytical capability as more analytes can be analysed for in single runs. Any problems with derivatisation are overcome by its

omission and as a result would allow all nitroimidazole residues to be analysed and distinguishable from each other. In the case of the feed methods it was felt that the only option for analysis was by LC-MS as the number and diversity of the compounds that needed to be incorporated into the method would not be achievable using GC-MS due to problems with derivatising all analytes with a single derivatising agent.

2.5.3. Liquid Chromatography to Mass Spectrometry: Interfaces and Ionisation Techniques

As mentioned previously the main obstacle in the development of the hyphenated technique LC-MS was the converting the analyte in the mobile phase to gas phase ions in order for them to be analysed by the MS. This resulted in the need for an interface linking the two techniques. This interface works at atmospheric pressure and allows for the liquid to be changed into gas phase and also ionises the analyte. This interface type is known as atmospheric pressure ionisation (API) interface. There are many different designs of this interface but an example of one can be seen in Figure 2-7.

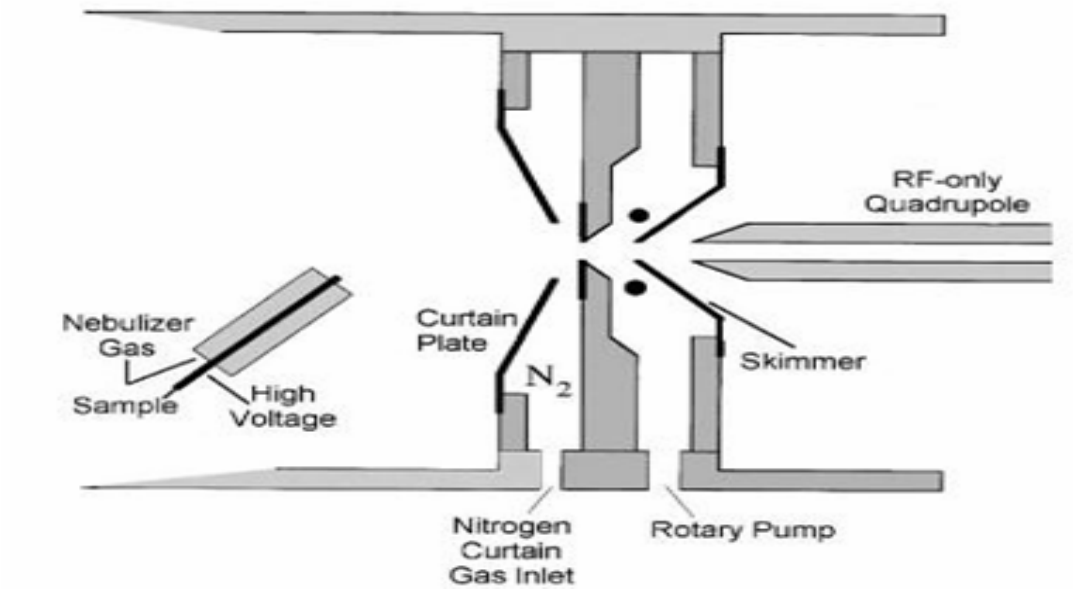


Figure 2-7. Diagram of API interface

Although ionisation is carried out at atmospheric pressure there are numerous different ionisation techniques that may be used with LC-MS. The most common ones used in veterinary residue analysis are electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI). From examination of Figure 2-8 it is clear that ESI works best over a broader range of different analytes. Compounds with higher polarity and molecular weights can only be analysed by ESI. Therefore it was decided that ESI was the best ionisation technique in this research as it was applicable to all the compounds that were to be analysed.

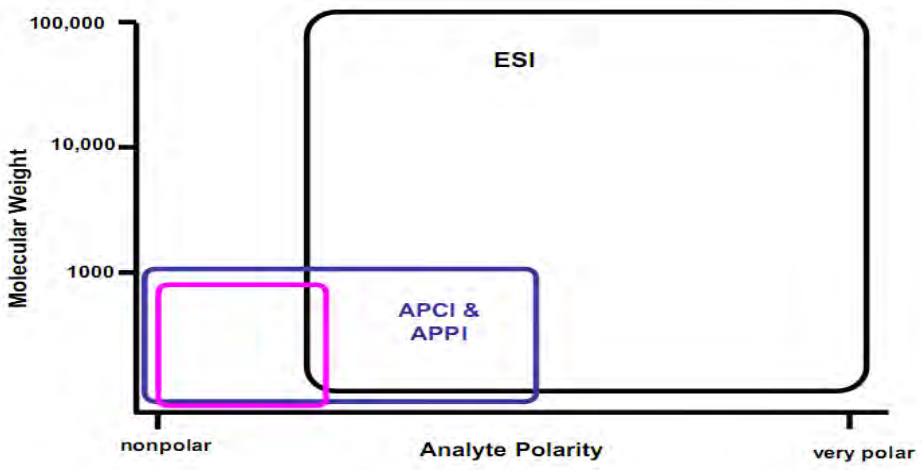
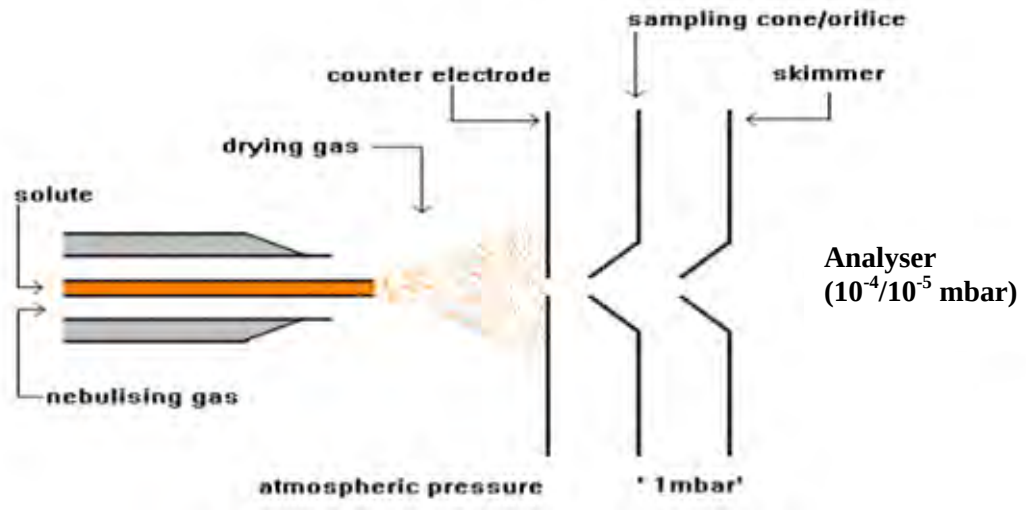


Figure 2-8. Portrayal of various ionization techniques such as ESI, APCI, and APPI as a function of compound polarity and molecular weight.

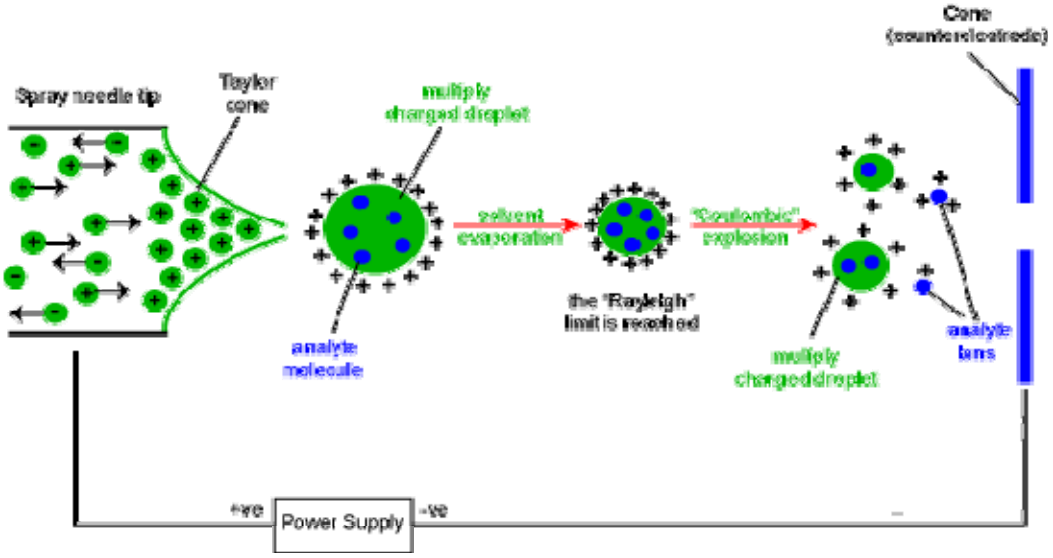
ESI is generally accomplished by forcing the LC mobile phase containing the analyte through a small capillary into an electric field of high positive or negative electrical potential typically of the order of 3-5 kV depending on whether positive ionization (higher voltages) or negative ionization (lower voltages) is required. (Figure 2-9).



(Source http://www.astbury.leeds.ac.uk/facil/MStut/mstutorial.htm)

Figure 2-9. Components of an ESI interface

When the solution reaches the end of the tube the strong electric field forces it to be nebulized into a spray of small highly charged droplets of solution in solvent vapour. Before entering the mass spectrometer the spray passes through a heated chamber, through which a flow of drying gas, typically air or nitrogen, is continually passed at high flow rates evaporating the solvent rapidly. Thus as the charged droplets get smaller, the electrical surface charge density increases until it reaches a point where the repulsive forces between charges of the same polarity at the surface of the droplet are greater than the cohesive forces of surface tension which hold the droplet together. This results in a "Coulombic explosion" (Figure 2-10), which produces a number of smaller droplets and this continues until charged analyte ions are formed which can be analysed by the mass spectrometer.



(Source http://www.bris.ac.uk/nerclsmsf/techniques/hplcms.html)

Figure 2-10. A simplified mechanism of ion formation in the electrospray ionization process.

2.5.4. LC-MS and Ion Suppression

While the advantages of LC-MS are in its capability to analyse for a very broad range of analytes with reduced sample purification and without the need for derivatisation, it is not without its own adverse aspects and limitations. Its main pitfall is a phenomenon known as ion suppression which occurs as a result of the presence of high concentrations of background matrix components. These components are primarily made up of endogenous substances for example organic or inorganic molecules present in the sample and that remain in the final extract; while other causes may be exogenous substances, i.e. molecules not present in the sample but may enter from various external sources during the sample preparation [Antignac *et al.*, 2005].

These matrix components can result in ion suppression by a number of mechanisms [Antignac *et al.*, 2005]. These mechanisms include;

- Decrease in evaporation efficiency due to the presence of matrix which results in increased viscosity and surface tension of droplets produced by ESI or APCI
- Co-precipitation of analytes with non volatile material such as macromolecules can also reduce their transfer into gas phase.
- Competition between analytes and interfering components for ionization
- Matrix can cause analytes to be basic in the gas phase and result in instability of analyte ions produced. This can result in neutralization processes which can affect analyte response.

These processes can all contribute to the occurrence of ion suppression which have consequences for results acquired in LC-MS. These consequences are;

- The detection capability is reduced due to the decrease of the analyte signal.
- The repeatability is also affected, because the degree of suppression may vary greatly from one sample to another.
- Ion ratio, linearity, and quantification, are also affected due to the variability of this unpredictable phenomenon.
- Ion suppression may lead to existing analytes to go undetected, to the underestimation of its real concentration or to the unsatisfactory results for identification criteria, with immediate consequences in terms of false negative (compliant) results.
- Finally if affecting the internal standard rather than the analyte, ion suppression may also lead sometimes to an overestimation of the analyte concentration with increased risk of false positive (non-compliant) results for maximum residue limit (MRL) compounds.

Therefore to avoid these consequences it is critical to always take ion suppression into consideration when developing a LC-MS method. There are a number of approaches that can be looked at in order to overcome these possible pitfalls [Antignac *et al.*, 2005]. These include;

- Modifying the mass spectrometric conditions if possible. This can be done by altering ionization technique as (ESI, APCI, APPI) as ion suppression may differ between different ionization techniques. Alternatively different ionization modes (positive or negative), or equipments with different source design can also reduce affect of ion suppression. Finding this type of solution is advantageous because it does not require any change in the rest of the developed analytical procedure (sample preparation and chromatographic condition)
- Another solution to overcome this problem is to use adequate internal standard, in order to balance the disturbance of the analyte signal by an equivalent disturbance on the internal standard. The best way to achieve this is to use a compound with a chemical structure and a retention time as close as possible to those of the analyte. For this purpose, C13-labelled or deuterated analogues of the analytes being tested significantly reduce signal variability observed for the analyte and consequently improve the repeatability of the measurement.
- Another way to reduce ion suppression is to adjust the LC conditions in order to shift the analyte of interest away from the matrix components.
- The previously described approaches only allow for the balancing of matrix effects or minimizing the consequences of ion suppression, but they do not eliminate it as the cause is not treated. The only way to definitively circumvent this problem remains to improve the sample preparation and purification, in order to limit the presence of interfering compounds in the final extract.

The conclusion of the article published by Antignac *et al.*, 2005 on ion suppression in LC-MS is that only a combination of sufficient sample purification and practical internal standard choice may ensure optimum performance in terms of repeatability and quantification. Therefore throughout this research ion suppression was always taken into consideration by the sourcing of suitable internal standards and by examining a variety of matrices in development and validation of methods to ensure that no effects were observed.

2.5.5. Types of Mass Analyzers

- Quadrupole: The quadrupole is the most widely used analyser due to its ease of use, mass range covered, good linearity for quantitative work, resolution and quality of mass spectra. The quadrupole is composed of two pairs of metallic rods. One set of rod is at a positive electrical potential, and the other one at a negative potential. A combination of DC and Rf (radio frequency) voltages is applied on each set. The positive pair of rods is acting as a high mass filter; the other pair is acting as a low mass filter. The resolution depends on the dc value in relationship to the Rf value. The quadrupoles are operated at constant resolution, which means that the Rf to DC ratio is maintained constant. For a given amplitude of the dc and Rf voltages, only the ions of a given m/z (mass to charge) ratio will resonate and have a stable trajectory to pass through the quadrupole and be detected. Other ions will be de-stabilized and hit the rods. As seen in figure 2-11.

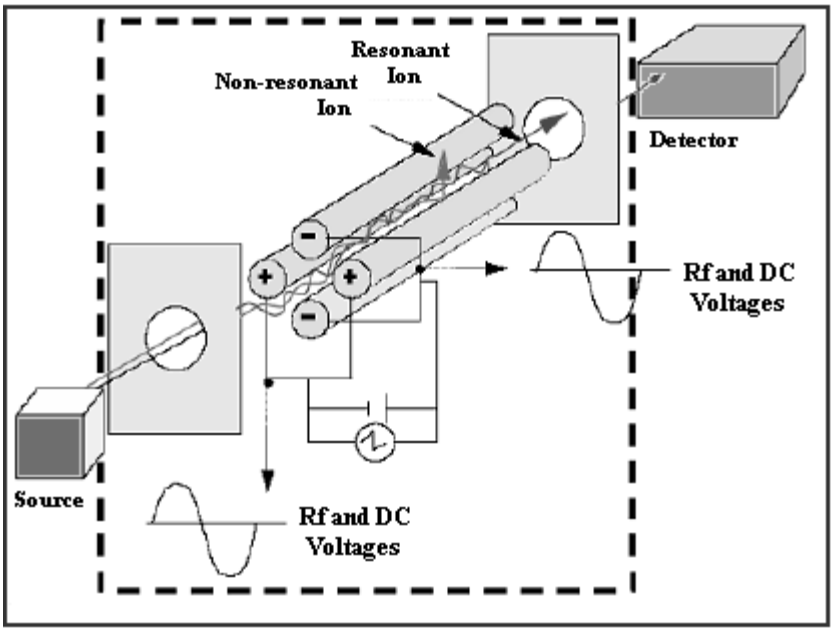


Figure 2-11. Quadropole Mass Analyzer

- Ion Trap: The principle of the trap is to store the ions in a device consisting of a ring electrode and two end cap electrodes. The ions are stabilized in the trap by applying a Rf voltage on the ring electrode. For maximum efficiency, the ions must be focussed near the centre where the trapping fields are closest to the ideal and the least distorted - maximizing resolution and sensitivity. This is achieved by introducing a damping gas (99.998% helium) that collisionally cools injected ions, damping down their oscillations until they stabilize. By ramping the Rf voltage, or by applying supplementary voltages on the end cap electrodes, or by combination of both, it is possible to: destabilise the ions, and eject them progressively from the trap or keep only one ion of a given m/z value in the trap, and then eject it to observe it specifically. The ion trap analyzer can be seen in figure 2-12.

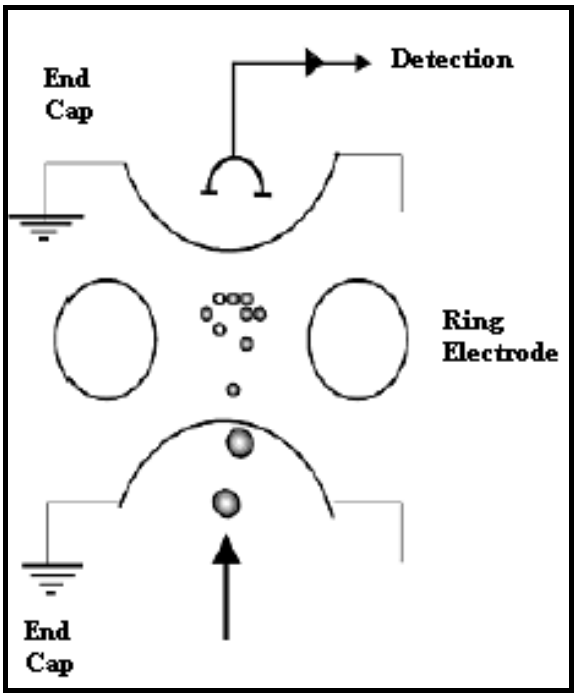


Figure 2-12. Ion Trap Mass Analyzer

- Time of Flight: In a Time–Of–Flight (TOF) mass spectrometer, ions formed in an ion source are extracted and accelerated to a high velocity by an electric field into an analyser consisting of a long straight 'drift tube'. The ions pass along the tube until they reach a detector. After the initial acceleration phase, the velocity reached by an ion is inversely proportional to its mass (strictly, inversely proportional to the square root of its m/z value). Since the distance from the ion origin to the detector is fixed, the time taken for an ion to traverse the analyser in a straight line is inversely proportional to its velocity and hence proportional to its mass (strictly, proportional to the square root of its m/z value). Thus, each m/z value has its characteristic time–of–flight from the source to the detector. In order to increase the resolution, the ion trajectory is bent by an electronic mirror, the reflectron. When going through the reflectron, the dispersion of ions of the same m/z value is minimized, leading to a great

improvement in resolution. The TOF instrument with and without the reflectron is seen in figure 2-13.

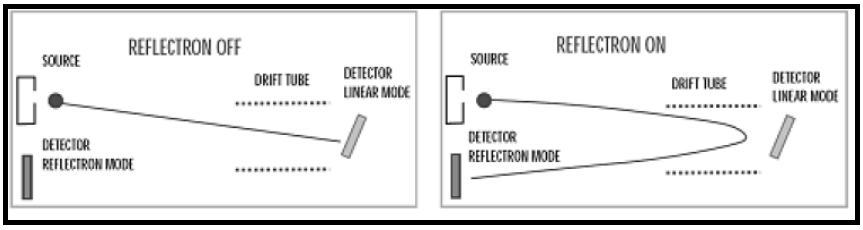


Figure 2-13. TOF Instrument with and without Reflectron .

2.5.6. Tandem Mass Spectrometry and Confirmatory Criteria

While the use of single mass analyzers is quite common, it is possible for ions to undergo separation by two different mass analysers in the same experiment. This is known as tandem mass spectrometry and it is a popular technique used in the analysis of veterinary residues in biological and feed matrices. This technique is concerned with the analysis of product ions formed from precursor ions as a result of their fragmentation due to collision induced dissociation. The most commonly used mode in tandem mass spectrometry for this purpose is "selected reaction monitoring" (SRM) usually carried out on a triple quadrupole instrument (Figure 2-14) [Le Bizec *et al.*, 2009]. As suggested previously quadrupoles have become the most widely used mass analysers in mass spectrometry [Dawson 1995]. In SRM mode, the molecular ion of the target compound is isolated in the first mass analyser, it subsequently undergoes fragmentation and only specific product ions are monitored in the second mass analyser. Le Bizec *et al.*, 2009 states that this technique offers many advantages for the analysis of trace levels of substances in complex matrices. The main advantage this technique gives is a significant decrease in noise observed in the signal of target analytes as a result of the small probability that interferences

from other compounds present in the final extract will have the same or very close molecular weights and product fragments as the analyte of interest.

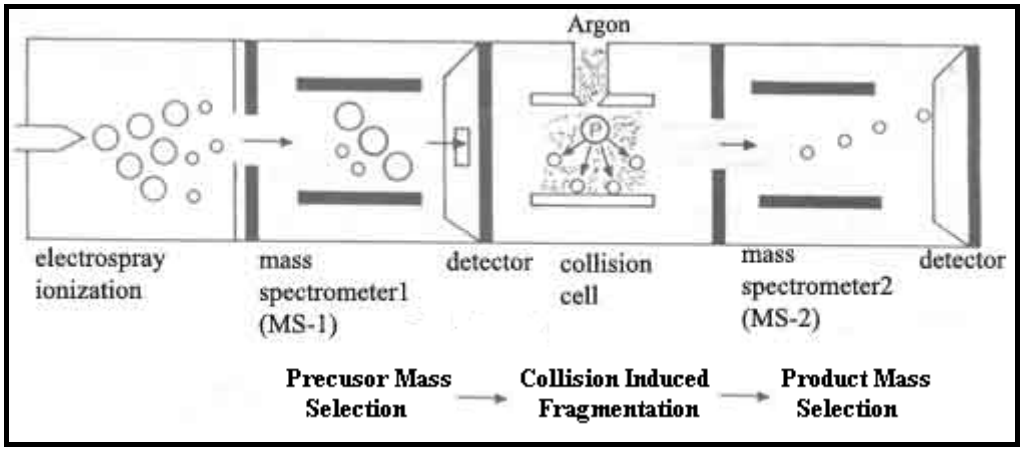


Figure 2-14. Diagram of triple quadrupole mass spectrometer (MS/MS)

The production of a precursor and two product ions in tandem mass spectrometry as discussed previously in section 2.3.4 yields four identification points (IPs). As a result this approach is classified as a confirmatory technique. The relative response of the two product ions (ion ratio) acts as a confirmatory criterion. Ion ratios for non compliant samples can be compared to the ion ratio of the analyte of interest which is usually determined from matrix matched calibration standards. This approach is similar to approaches for confirmation adopted by other regulatory bodies such as the Association of Official Racing Chemists (AORC), the US Food and Drug Administration (FDA), the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA) [Van Eenoo, 2004]. Triple quadrupole LC-MS/MS in SRM mode with 1 precursor and two product ions was the analytical technique utilised in all methods developed as part of this research.

Comment: - *Chapters 3-8 are reproductions of articles published as part of this research and are input into this thesis the same as they appear in the respective journals in which they are published or accepted for publication.*

CHAPTER 3: DEVELOPMENT AND VALIDATION OF A RAPID METHOD FOR THE DETERMINATION AND CONFIRMATION OF TEN NITROIMIDAZOLES IN ANIMAL PLASMA USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Published in *Journal of Chromatography B, Volume 877, Issues 14-15, 15 May 2009, Pages 1494-1500*

3.1 Abstract

A rapid LC-MS/MS method has been developed and validated for the simultaneous identification, confirmation and quantitation of ten nitroimidazoles in plasma. The method validated in accordance with Commission Decision (CD) 2002/657/EC and is capable of analysing for Metronidazole (MNZ), Dimetridazole (DMZ), Ronidazole (RNZ), Ipronidazole (IPZ) and their hydroxy metabolites MNZ-OH, HMMNI (Hydroxymethyl, Methyl Nitroimidazole), IPZ-OH. The method is also capable of analysing Carnidazole (CRZ), Ornidazole (ORZ) and Ternidazole (TRZ) which are rarely analysed by modern methods. MNZ, DMZ and RNZ have a Recommended Level (RL) of 3 ng mL^{-1} which this method is easily able to detect for all the nitroimidazole compounds. Plasma samples are extracted with acetonitrile, and NaCl is added to help remove matrix contaminants. The acetonitrile extract undergoes a liquid-liquid wash step with hexane; it is then evaporated and reconstituted in mobile phase. The reconstituted samples are analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The decision limits (CCα) range from 0.5-1.6 ng mL^{-1} and the detection capabilities (CCβ), range from 0.8-2.6 ng mL^{-1}. The results of the inter-assay study, which was performed by fortifying bovine plasma samples (n = 18) on three separate days, show the accuracy calculated for the various analytes range between 101-108%. The precision of the method, expressed as CV% values for the inter-assay variation of each analyte at the three levels of fortification (3, 4.5 and 6.0 ng mL^{-1}), ranged between 4.9-15.2%. A Day 4 analysis was carried out to examine species variances in animals such as avian, ovine, porcine and equine.

***Keywords*:** Nitroimidazoles; Method Validation; Liquid Chromatography- tandem Mass Spectrometry; Plasma

3.2 Introduction

Nitroimidazoles are imidazole heterocycles with a nitrogen group incorporated in the structure. Examples of these compounds are metronidazole (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, MNZ), dimetridazole (1, 2-dimethyl-5-nitroimidazole DMZ), ronidazole (1-methyl-2-[(carbamoyloxy) methyl]-5-nitroimidazole, RNZ), ipronidazole (2-isopropyl-1-methyl-5-nitroimidazole, IPZ), carnidazole (1-(2-ethylcarbamothioic acid O-methyl ester)-2-methyl-5-nitroimidazole, CNZ), ornidazole (1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole, ONZ) and ternidazole (2-Methyl-5-nitroimidazole-1-propanol; 3-(2-Methyl-5-nitroimidazol-1-yl)propan-1-ol, TRZ). These examples are known as 5-nitroimidazoles as they contain a NO_2 group on the 5^{th} position on its ring which is seen in Figure 3-1 and Figure 3-2.

These compounds are metabolised in bovine, porcine and avian species [Mottier *et al.*, 2006]. The main metabolite of DMZ, IPZ and MNZ results from the oxidation of the side chain in the C-2 position of the imidazole ring to form hydroxy metabolites. RNZ has a different degradation pathway but results in an identical metabolite to that of DMZ [Mottier *et al.*, 2006]. These metabolites are HMMNI (2-hydroxymethyl-1-methyl-5-nitroimidazole), MNZ-OH (1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole) and IPZ-OH (1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole). Structures of the nitroimidazole compounds and their metabolites are shown in Figure 3-1.

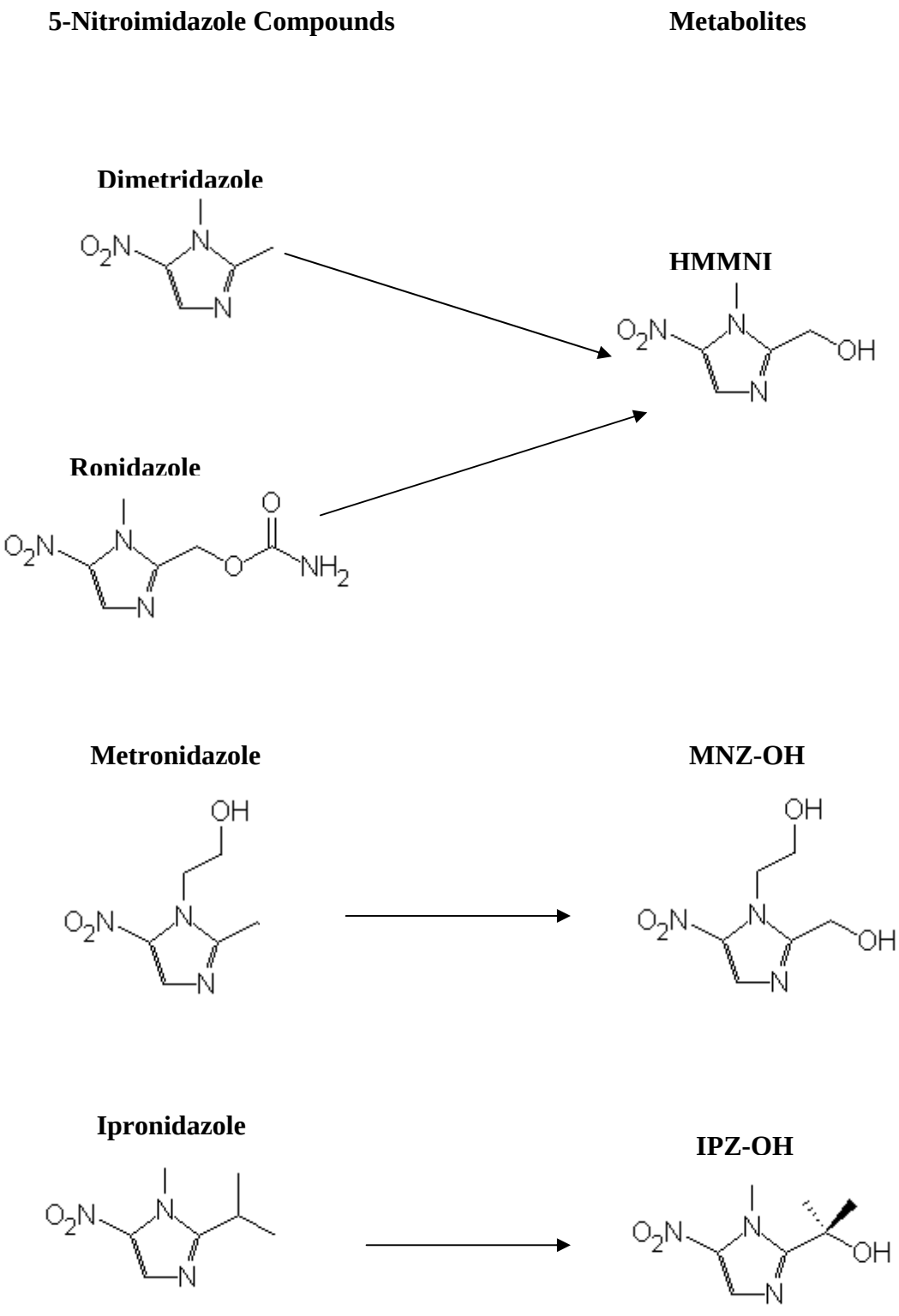
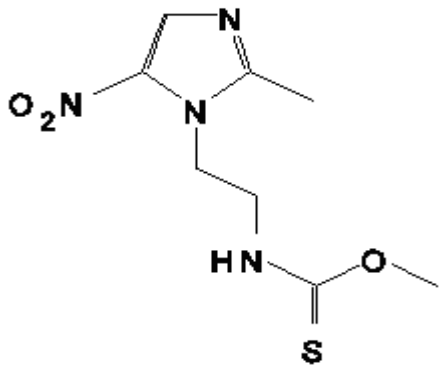
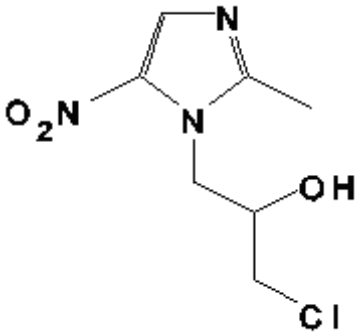


Figure 3-1: Chemical Structures of CRL Suggested Compounds and Metabolites

Carnidazole



Ornidazole



Ternidazole

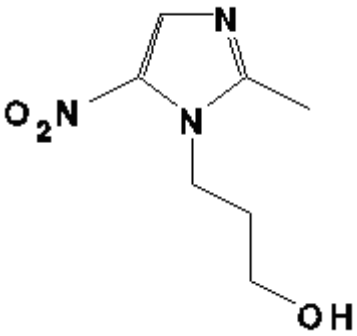


Figure 3-2: Chemical Structures of Three other 5-nitroimidazoles that the method can analyze for.

These compounds can be used for the prophylactic and therapeutic treatments of diseases such as histominiasis and coccidiosis in poultry, genital tricchoniasis in cattle and hemorrhagic enteritis in pigs. They are believed to be carcinogenic and mutagenic to humans and as a consequence were banned for the use in food producing animals within the European Union under Regulation 2377/90. They are also banned for use in the U.S.A and China [Xia *et al.*, 2007]. The analysis of these compounds is required under Council Directive 96/23/EC.

As a result of this ban, there is a need for rapid multi-residue analytical methods that have the capability of including a wide range of these analytes in order to ensure compliance with legislation. Previously the analysis of these compounds was carried out in liver and muscle [Xia *et al.*, 2007; Polzer *et al.*, 2001] but studies on the

stability and homogeneity of nitroimidazoles in incurred muscle [Polzer *et al.*, 2004; Polzer *et al.*, 2005] show that there is not a homogenous distribution of analyte in turkey muscle and also there is a rapid reduction in analyte concentration in muscle stored for prolonged periods above 4 °C. In contrast it was discovered that for plasma, retina and egg samples the analytes were stable during storage under the same conditions which resulted in constant concentrations and allowed detection of these compounds for longer periods after medication had been halted. Therefore, plasma, retina and eggs have been recommended as target matrices for the residue control of nitroimidazoles [Polzer *et al.*, 2004; Polzer *et al.*, 2005]. Current methods that are used for the analysis of these compounds are limited to the analysis of at most seven nitroimidazole compounds [Mottier *et al.*, 2006, Sun *et al.* 2007], but the majority analyse for fewer [Xia *et al.*, 2006; Polzer *et al.*, 2001; Fraselle *et al.*, 2007 Wang, 2001; Capitan-Vallvey *et al.*, 2002; Hurtaud-Pessel *et al.*, 2000; Ho *et al.*, 2005; Ding *et al.*, 2006; Mortier *et al.*, 2003; Thompson *et al.*, 2009]. These methods use varied extraction protocols including the use of acetonitrile [Mottier *et al.*, 2006] or ethyl acetate [Xia *et al.*, 2006; Sun *et al.* 2007] as extraction solvent. In some cases the samples were extracted using a buffer of $NaCl/KH_2PO_4$ with protease and adjusting the pH to 3 with 25% HCl [Fraselle *et al.*, 2007; Polzer *et al.*, 2001]. This was performed as it was believed that nitroimidazole compounds may be protein bound.

The majority of these current methods then employ a solid phase extraction step (SPE) in order to clean up their extract. [Mottier *et al.*, 2006; Xia *et al.*, 2007; Xia *et al.*, 2006; Polzer *et al.*, 2001; Polzer *et al.*, 2004; Polzer *et al.*, 2005; Fraselle *et al.*, 2007; Sun *et al.* 2007] and methods that do not employ SPE [Ding *et al.*, 2006; Mortier *et al.*, 2003] were not used to analyse plasma. Various techniques have been

used for the analysis of these compounds such as HPLC-UV [Sun *et al.* 2007] and GC-MS/MS [Polzer *et al.*, 2001; Ho *et al.*, 2005]. The use of GC-MS/MS limits the number of analytes that can be analysed due to problems arising from derivatisation, due to the fact that HMMNI and RNZ form the same derivatisation products [Xia *et al*., 2007]. Presently more methods are now being developed for this analysis by LC-MS/MS which allow for a greater number of analytes being analysed [Mottier *et al.*, 2006; Xia *et al.*, 2007; Xia *et al.*, 2006; Capitan-Vallvey et al., 2002]. This overcomes the problem of derivatisation and allows for quicker run times.

From examination of published literature no method was found that was capable of analysing the ten nitroimidazoles listed in this paper. The sample preparation described in this study is more efficient than previously published methods due to the absence of a SPE step. In previous studies a deconjugation step was utilised by addition of either a protease or an acid to deconjugate possible protein bound residues. In this study it was found that this step was not necessary by investigation of incurred samples of plasma received from the Community Reference Laboratory (CRL) in Berlin. A recently published method also omits this deproteination step [Thompson *et al.*, 2009].

A rapid, sensitive and specific multi-residue method for the detection and confirmation of a wide variety of nitroimidazoles in plasma has been developed and validated in accordance with CD 2002/657/EC. A recommended level (RL) for MNZ, DMZ and RNZ of 3 ng mL^{-1} has been proposed by the CRL hence this was used in validating these three compounds. This RL was also applied to the remaining seven compounds for which no RL has been proposed. During validation, all compounds were analysed in a single chromatographic run at 1, 1.5 and 2 times the RL (3 ng mL^{-1}) with six replicates at each level. Selectivity, linearity, recovery,

accuracy and precision were established and values for CCα and CCβ were determined.

3.3 Experimental

3.3.1 Materials and reagents

All analytical standards of nitroimidazoles, including deuterated substances were provided by the CRL (BVL, Berlin, Germany) except HMMNI, MNZ-OH, HMMNI-d_3, MNZ-OH-d_2, DMZ- d_3 and RNZ -d_3 (all from WITEGA Laboratorien Berlin, Germany), RNZ (Sigma, St. Louis, MO, USA) and DMZ (Fluka, St. Louis, MO, USA). Water LC-MS grade (Fluka, St. Louis, MO, USA). Methanol, Acetonitrile and Hexane HPLC Grade (Reagecon, Clare, Ireland). Sodium Chloride was AnalaR grade (VWR, Poole, England). Individual stock standards at 1mg mL^{-1} in ethanol were prepared and are stored at 4ºC for 1 year. Individual intermediate standard solutions (10 and 1µg mL^{-1}) in methanol were prepared and working standard solutions (mixture of (dueterated) nitroimidazoles) (200 and 500ng mL^{-1}) were prepared in methanol and are stored at 4ºC for 3 months.

3.3.2 *LC-MS/MS Instrumentation*

The LC-MS/MS system is a TSQ Quantum Ultra EMR coupled to a Finnigan Surveyor LC system (Thermo Fisher, Waltham, MA, USA). The instrument is controlled by Xcalibur software (Version 1.4)**.** Separation was achieved using a (100 × 2)mm, 3µm particle size, Luna C18 column (Part No. 00D-4251-B0) protected by a Security Guard guard cartridge system (20×2)mm, both supplied by Phenomenex (Torrance, California, USA). The oven temperature was set at 40ºC. The chromatographic separation performed on gradient mode using water acidified with

0.1% acetic acid (mobile phase A) and acetonitrile acidified with 0.1% acetic acid (mobile phase B), at a flow of 0.25mL min^{-1}. The initial conditions (0-4min) were 95% A. Then the conditions changed to 5% A (4-7min) and these were maintained until 9 min, the conditions returned to 95% A in 3min (9-12min), and were maintained until the end of the run at 20min. The ionisation used was positive electrospray ionisation (ESI) mode with a spray voltage of 4350V. The individual MRMs with their respective collision energies are listed in Table 3-1.

3.3.3 Plasma samples

Bovine, porcine, ovine, avian and equine plasma was obtained from local abattoirs and stored at –20 °C in polypropylene centrifuge tubes (50mL). Samples of this plasma were analysed and those found to contain no detectable residues of the analytes of interest were used as negative controls. Chromatograms of blank plasma are seen in Figure 3-3.

3.3.4 Sample extraction and clean-up

Bovine plasma (5mL) was pipetted into polypropylene centrifuge tubes (50mL). These were fortified with mixed internal standard (50 µL of 200 ng mL^{-1}) which corresponded to 2 ng mL^{-1}. Samples were fortified at 3, 4.5 and 6 ng mL^{-1} by adding mix working standard solution (30, 45 and 60 µL portions of 500 ng mL^{-1}) and these were vortexed (20secs). Acetonitrile (10mL) was added and vortexed. NaCl (2g) was added to this slurry which was then centrifuged (4500 rpm for 10min). The top organic layer from each sample was then transferred to polypropylene tubes (15mL) and evaporated (60ºC) to 6mL under nitrogen. Hexane (5mL) was added and the tubes were then vortexed (30secs). The hexane layers were discarded and the extracts

were evaporated to dryness under the same conditions. The extracts were reconstituted in Water: Acetonitrile (95:5, 200µL) and filtered through 0.2µm syringe filters. An aliquot (20µL) was injected onto the LC column. Chromatogram of a 2.5ng mL^{-1} fortified plasma sample is seen in Figure 3-4.

Table 3-1: Parent/Daughters fragmentations and corresponding collision energies.

Nitroimidazole Parent/Daughter Collision Energies			
Compound	**Parent Ion**	**Daughter Ions**	**Collision Energies**
DMZ	142.2	96.4	18
		81.4	28
MNZ	172.0	82.5	25
		128.2	15
RNZ	201.2	140.1	15
		55.7	20
		110.3	18
IPZ	170.0	124.3	18
		109.4	25
HMMNI	158.2	140.2	13
		55.7	18
		110.3	15
MNZ-OH	188.2	123.2	16
		126.2	17
IPZ-OH	186.0	168.1	14
		122.3	21
ORZ	220.0	128.2	17
		82.4	33
TRZ	186.0	128.2	17
		82.5	28
		111.3	25
CRZ	245.0	118.2	13
		75.3	33

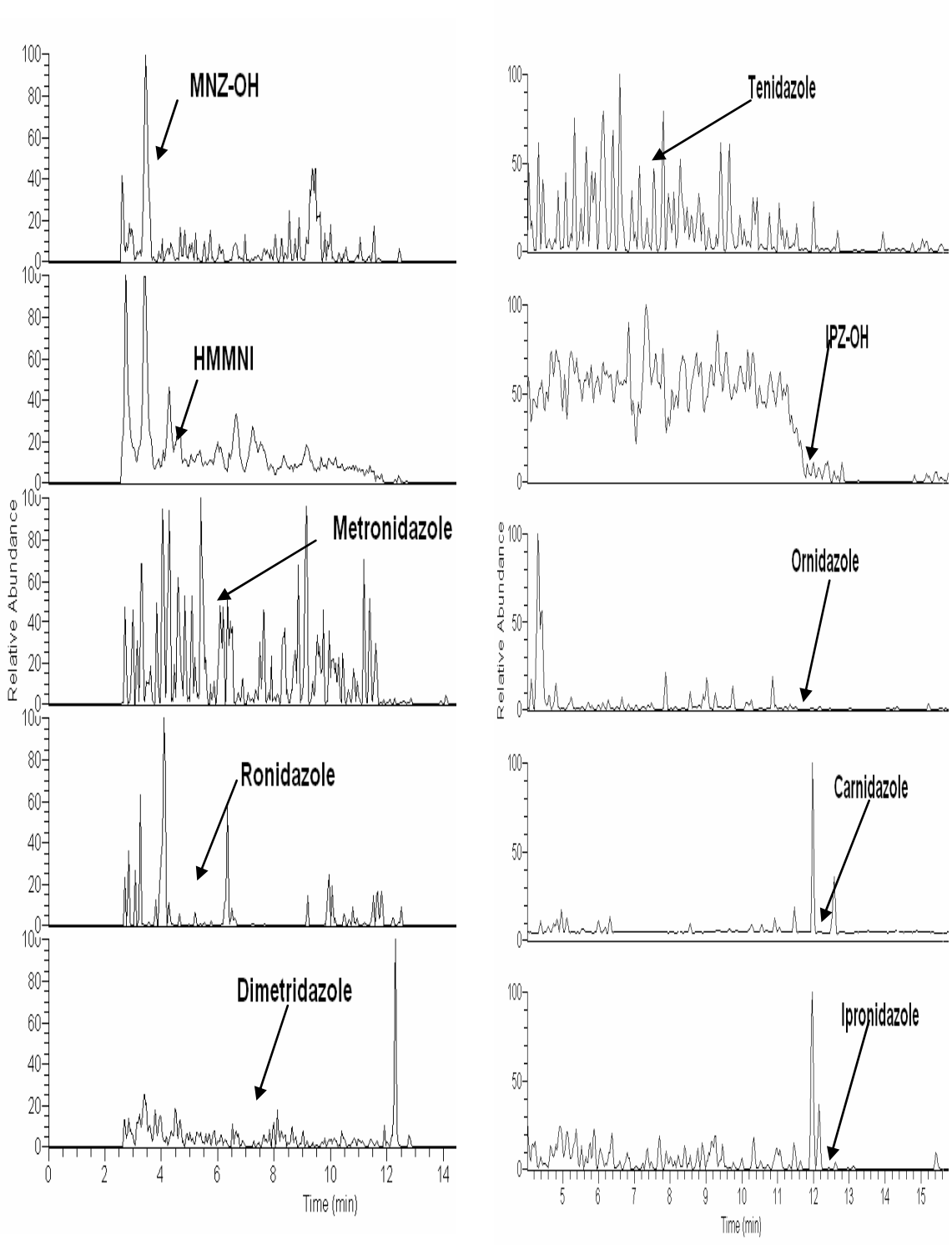


Figure 3-3: Chromatograms of Blank Bovine Plasma (the arrow points out the expected retention time of each compound).

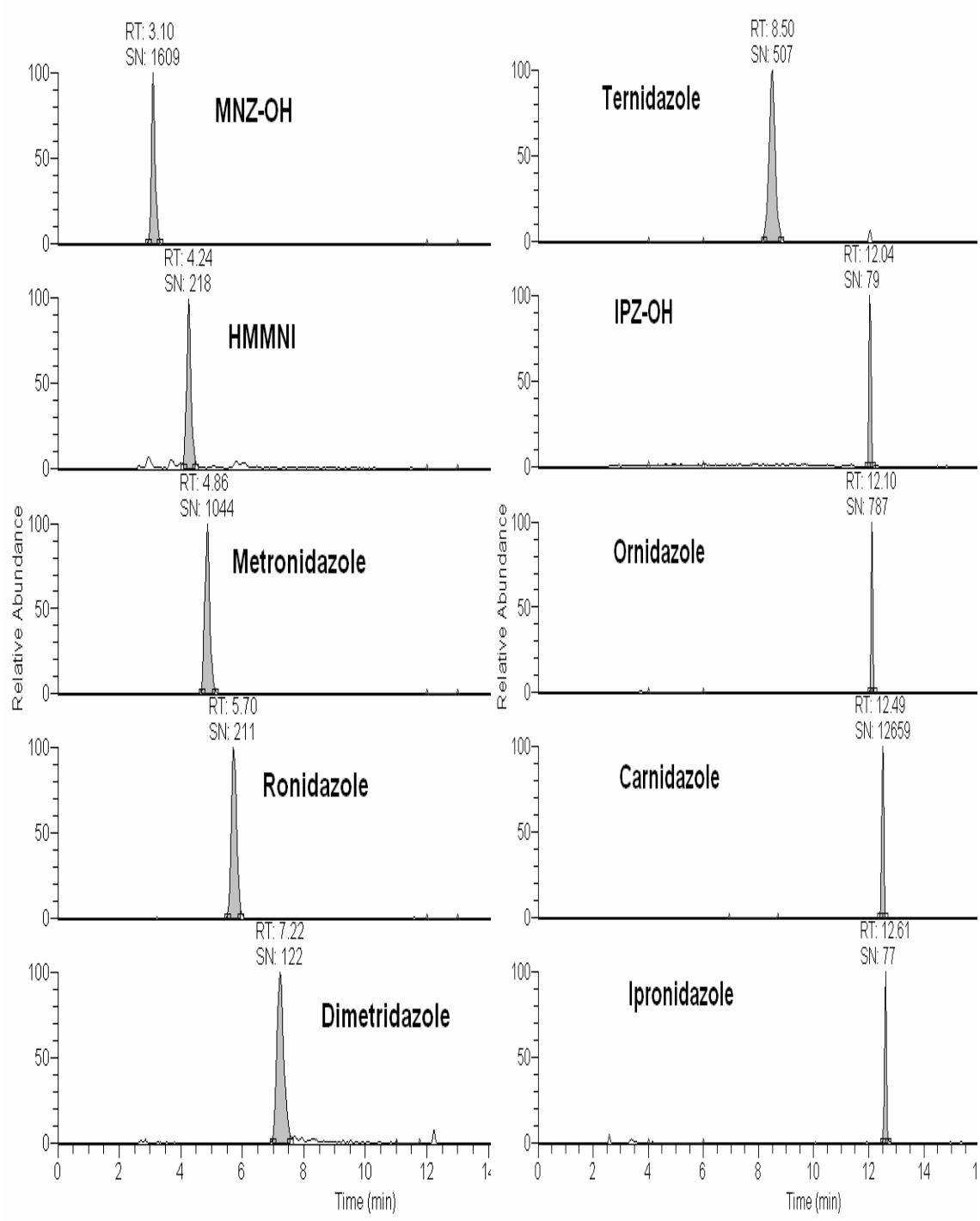


Figure 3-4: Chromatograms for the ten nitroimidazole compounds fortified at 2.5ug mL^{-1}

3.3.5 Matrix-Matched Calibration

Bovine matrix matched calibration curves were prepared and used for quantification. Control plasma was prepared as above (2.4). One control plasma sample was used for each calibration standard level. Plasma samples (5mL) were pipetted into

polypropylene tubes (50mL). Samples were fortified with mixed internal standard (50 μL of 200 ng mL^{-1}) at a level corresponding to 2 ng mL^{-1}. Samples were fortified at 0, 2.5, 5, 7.5, 10 and 20ng mL^{-1} by adding mixed working standard solution (0, 25, 50, 75, 100 and 200 μl aliquots of a 500 ng mL^{-1}).

Six deuterated internal standards are used; d3-DMZ, d3-RNZ, d3-HMMNI, d2-MNZ-OH, d3-IPZ and d3-IPZ-OH. For compounds with no deuterated form i.e. MNZ, TRZ, ORZ and CRZ, d3-HMMNI is used as internal standard.

3.3.6 Method validation

For estimation of accuracy, blank bovine plasma samples were fortified with each analyte at 3.0, 4.5 and 6.0ng mL-1. Six replicate test portions at each of the three fortification levels (18) were analysed on three separate days over a period of two weeks. To determine any matrix effects caused by biological variations arising from plasma from different species (bovine, ovine, porcine, avian and equine), a fourth day analysis was carried out. Two sets of ten plasma samples (two of each species) were analysed as in section 2.4. The first set was fortified with only internal standard, and the second set was fortified with both internal standard and with the analytes at a concentration equivalent to 4.5ng mL-1. The estimation of precision, intra-assay and inter-assay repeatability of the method were calculated along with CCα and CCβ. Absolute recovery was determined by analysing five replica plasma samples fortified at 3.0ng mL-1 prior to extraction and five replica plasma samples fortified at the equivalent concentration after extraction.

3.4 Results and Discussion

3.4.1 Preliminary experiments

The LC-MS/MS method was developed to provide confirmatory data for the analysis of 10 nitroimidazoles in plasma. The MS/MS fragmentation conditions were investigated and collision energies were optimised for each individual compound to give best response. For a method to be deemed confirmatory under CD 2002/657/EC it must yield 4 identification points. In this method a precursor ion (parent mass) and two daughter ions (corresponding to strong and weak ion) are monitored for each analyte (Table 3-1). This yields 4 identification points (1 for the parent ion and 1.5 for each daughter ion) hence it can be deemed a confirmatory method. When the MS/MS fragmentation conditions for HMMNI were optimised it was seen that the background noise was very high in its strong daughter ion. This was investigated further and it was found that the use of the laboratory's 18.2 MΩ water supply in the mobile phase was producing the high background. This problem was overcome by the use of LC-MS/MS grade water in the mobile phase.

From previous work carried out on these compounds it was seen that acetonitrile can be used as a suitable extraction solvent [Mottier *et al.*, 2006]. Various extraction procedures were examined. Double extractions are common practice in sample preparation but this in turn leads to an increased amount of solvent being used. Tests were carried out to see if this double extraction was necessary in the case of this procedure. Results were compared between a single and double extraction. These showed that recoveries were not significantly improved with the use of a double extraction therefore a single extraction of 10mL would suffice.

Past work carried out on nitroimidazoles showed that the addition of NaCl helped to remove impurities [Mottier *et al.*, 2006; Xia *et al.*, 2006]. In order to investigate at

which stage the NaCl should be added to yield the cleanest extract various experiments were performed. It was found that the addition of NaCl (2g) directly after addition of acetonitrile allowed for greater purification and produced a cleaner sample for analysis.

SPE is widely used as a sample clean-up method. Cartridges of extrelute NT20 [Polzer *et al.*, 2004], Oasis MCX [Xia *et al.*, 2007], SCX [Sun *et al.*, 2007] and HLB [Capitan-Vallvey *et al*., 2002], have been used in methods for the analysis of nitroimidazoles. With advances in LC-MSMS and its ability to perform Multi Reaction Monitoring (MRM) for compounds, which greatly reduces the effect of matrix interferences, the need for SPE is reduced. As a result it was found that addition of a quick liquid-liquid wash step was sufficient to remove an adequate amount of interferences and this in turn allows for quicker sample preparation times.

Hexane was used in previous work carried out on these compounds [Mottier *et al.*, 2006; Xia *et al.*, 2007] to help remove impurities, therefore the use of hexane as a liquid-liquid wash solvent was investigated in order to improve sample clean up. Hexane with varying amounts of ethyl acetate (0%, 2%, 5% and 10%) was used as the wash solvent. Ethyl acetate was added to help remove more polar impurities. Results concluded that the addition of this in any percentage gave poorer recoveries for some of the compounds due to the higher polarity of ethyl acetate. As a result hexane was chosen for the wash solvent. Samples were filtered before injection to remove any remaining impurities. Different types of syringe filters with varying pore sizes and various packing including PVDF, PTFE and Nylon filters were tested. Decreased analyte recovery was observed when using the PTFE and Nylon filters. Samples were cleaner when 0.25µm sized filters were used compared with 0.45µm. As a result 0.25µm PVDF syringe filters were chosen.

3.4.2 Validation study

As part of the validation study, various experimental parameters were determined. These included specificity, linearity, accuracy, absolute recovery, repeatability, CCα, CCβ and measurement of uncertainty. These parameters were determined in accordance with guidelines described in CD 2002/657/EC. In order to further demonstrate the applicability of the developed method analysis of incurred test material was also performed.

Table 3-2: Validation results of % CV, Accuracy, Absolute Recovery, CC*α*, CCβ, Measure of Uncertainty and Linearity.

Analytes	CV %	Accuracy %	Absolute Recovery %	CC*α* µg L^{-1}	CCβ µg L^{-1}	MU %	Linearity R^2
Metronidazole	4.9	106.6	50.4	0.52	0.89	50.4	>0.990
MNZ-OH	6.3	105.8	61.8	0.53	0.91	24.0	>0.990
Dimetridazole	6.1	101.8	61.4	0.58	0.99	22.8	>0.990
Ronidazole	5.6	101.5	67.5	0.60	1.02	23.5	>0.990
HMMNI	4.5	101.4	68.5	0.57	0.98	31.4	>0.990
Ipronidazole	15.1	103.5	71.8	1.49	2.54	54.4	>0.990
IPZ-OH	5.5	101.2	72.7	0.55	0.94	23.8	>0.990
Ternidazole	5.8	106.5	71.1	0.57	0.97	36.8	>0.990
Ornidazole	9.3	108.0	66.9	1.11	1.89	51.9	>0.990
Carnidazole	13.2	108.8	60.5	1.52	2.60	67.8	>0.990

3.4.2.1. Specificity

The technique of LC-MS/MS itself offers a high degree of selectivity and specificity due to its ability to operate in multi reaction monitoring mode (MRM), which greatly reduces the effect of matrix interferences. On each of the four occasions when

validation was performed no interfering peaks were observed at the retention time for any of the transitions. This allowed for clear identification and quantification of all analytes.

3.4.2.2. Linearity of the Response

Calibration curves were prepared by plotting the response factor (analyte peak area/internal standard peak area) as a function of analyte concentration (0 to 20 ng mL^{-1}). The regression coefficients (r^2) for all the calibration curves used in this study were ≥ 0.990. This showed that these analytes have a good fit to linearity within this range.

3.4.2.3. Absolute Recovery

Absolute recovery was calculated by determining concentration of samples fortified before extraction and dividing by the concentration of samples fortified after extraction and expressing this result as a percentage. Absolute recoveries were in the range of 50.4-72.7% (Table 3-2). These values fall within acceptable ranges.

3.4.2.4. Accuracy

CD 2002/657 states that accuracy should be between 70-110%. The mean corrected accuracy (n = 6) of the analytes were found by experiment to lie between 101% and 109 % for all analytes (Table 3-2) and therefore are acceptable.

3.4.2.5. Repeatability

Inter assay precision (CV %) should be as low as reasonably possible as the method works in the ng mL^{-1} range. Values for CV % in range of 4.5-15.1% were achieved for all compounds (Table 3-2). The main reason for the excellent precision

demonstrated here can be attributed to the availability of six deuterated analogues of the compounds being examined. Deuterated internal analogues were not available for metronidazole, ternidazole, carnidazole, ornidazole. For these analytes d_3-HMMNI was used as the corresponding internal standard and corrected well for any losses or matrix suppression.

3.4.2.6. CCα and CCβ

CCα is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. For prohibited substances an α value equal to 1 % is applied. CCβ is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of 1-β, where β = 5 %. CCα and CCβ were calculated using the calibration curve procedure according to ISO 11843. After identification, the signal is plotted against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the CCα. CCα values of for all 10 compounds are listed in Table 3-2 and are all below 1.52 ng mL^{-1}. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept + 3.97 times that standard error of the intercept). CCβ values for all 10 compounds are listed in Table 3-2 and are all below the RL of 3ng mL^{-1}.

3.4.2.7. Measurement of Uncertainty

The measurement of uncertainty was estimated by taking into account the within laboratory reproducibility over days 1, 2 and 3 as well as considering the repeatability on day 4 due to matrix effects caused by different species. These two

variability's were combined and multiplied by a coverage factor of three to give an overall figure for the MU. This approach of using the within laboratory reproducibility as a good estimator of measurement of uncertainty is taken from the SANCO/2004/2726rev4 document. It recommends using the within laboratory reproducibility and using a coverage factor of 2.33 to estimate expanded uncertainty, however as it was felt that not all the environmental and other factors that could be varied over the course of the validation were examined, hence a coverage factor of 2.33 may underestimate the true uncertainty of the method. So a value of 3 was chosen instead to give a more realistic value for the true uncertainty. Values for MU are seen in Table 3-2 and lie between 20 and 70%. High MU values are seen for MNZ, CNZ and ORZ as they have no deuterated analogue to use as an internal standard. These values are increased further due to a high value for reproducibility due to matrix as a result of a high biological variation between species (day 4). A high value is also observed for IPZ especially in day 1 due to insufficient data points across its short peak width. The dwell time for this compound was altered and results improved on day 2 and 3.

3.4.2.8. *Evaluation*

In order to evaluate this method two incurred samples were analysed. The samples were lyophilised plasma and were obtained from the CRL for nitroimidazoles in Berlin. One contained high levels of nitroimidazoles and the other contained lower levels. The results achieved from using the method are seen in Table 3-3 and when compared with the set values all but one lie well within the measurement uncertainty for each analyte. Exception is MNZ-OH in the V08G0247 sample where the result achieved was above the value stated by the CRL. Taken into consideration if the

CRL's measurement uncertainty was placed on their result the ranges of both labs would overlap.

Table 3-3: Results achieved using method, of two incurred plasma samples.

SAMPLE: *V08G0246*

Compound	Actual Amt (ng mL^{-1})	Calculated Amt (ng mL^{-1})	MU(%)	RANGE		
				MU of Cal Amt*	Upper	Lower
MNZ-OH	4.55	4.643	24.04	1.116	5.759	3.527
HMMNI	1.71	1.357	31.35	0.425	1.782	0.932
Metronidazole	1.48	1.198	50.42	0.604	1.802	0.594
Ronidazole	2.33	1.789	23.5	0.420	2.209	1.369
IPZ-OH	1.69	1.435	23.78	0.341	1.776	1.094

SAMPLE: *V08G0247*

Compound	Actual Amt (ng mL-1)	Calculated Amt (ng mL-1)	MU(%)	RANGE		
				MU of Cal Amt*	Upper	Lower
MNZ-OH	2.02	2.707	24.04	0.651	3.358	2.056
HMMNI	0.66	0.787	31.35	0.247	1.034	0.540
Metronidazole	0.6	0.672	50.42	0.339	1.011	0.333
Ronidazole	0.94	1.109	23.5	0.261	1.370	0.848
IPZ-OH	0.66	0.551	23.78	0.131	0.682	0.420

* MU calculated on result achieved by method.

3.5 Conclusions

A multi-residue confirmatory method has been developed that simultaneously identifies, confirms and quantifies ten nitroimidazole compounds in plasma by LC-MS/MS. It can be considered as a rapid method, as the only clean-up step required is

a hexane wash. The method also has a short chromatographic run time of only 20 minutes for each sample.

The method includes 10 nitroimidazole compounds including seven that are suggested by the CRL in Berlin to be analysed. In addition other nitroimidazoles that haven't been analysed previously such as Ornidazole and Carnidazole are included. The obtained data fulfils the requirements laid down in CD 2002/657/EC and allows the calculation of all relevant performance characteristics. This study shows that the developed method meets the required sensitivity of 3ng mL-1 which is the RL used for these compounds. The CCα and CCβ values determined for each analyte are lower than this level. The method performs very well in terms of accuracy and repeatability for each of the analytes due to the utilisation of six different deuterated internal standards. The values achieved for % accuracy, CV % and MU all fall within acceptable ranges. The applicability of the method for use on different types of plasma was demonstrated by the satisfactory results obtained from the Day 4 analysis of different species.

The reduced number of analytical steps within the method makes it very amenable for high through-put regulatory monitoring of these compounds. The objective of the work to develop a rapid confirmatory method capable of monitoring for these residues in plasma at ng mL^{-1} levels and validate according to the requirements in CD 2002/657/EC therefore has been achieved successfully.

3.6 Acknowledgements

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CHAPTER 4: RAPID CONFIRMATORY METHOD FOR THE DETERMINATION OF ELEVEN NITROIMIDAZOLES IN EGG USING LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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4.1 Abstract

A rapid confirmatory method has been developed and validated for the simultaneous identification, confirmation and quantitation of eleven nitroimidazoles in eggs by liquid chromatography tandem mass spectrometry (LC-MS/MS). The method is validated in accordance with Commission Decision 2002/657/EC and is capable of analysing Metronidazole (MNZ), Dimetridazole (DMZ), Ronidazole (RNZ), Ipronidazole (IPZ) and their hydroxy metabolites MNZ-OH, HMMNI (Hydroxymethyl, Methyl Nitroimidazole), IPZ-OH. The method is also capable of analysing Carnidazole (CRZ), Ornidazole (ORZ), Tinidazole (TNZ) and Ternidazole (TRZ). MNZ, DMZ and RNZ have been assigned a Recommended Level (RL) of 3 μg kg^{-1} by the Community Reference Lab (CRL) in Berlin. The developed method described in this study is easily able to detect all the nitroimidazole compounds investigated at this level and below. Egg samples are extracted with acetonitrile, and NaCl is added to help remove matrix contaminants. The acetonitrile extract undergoes a liquid-liquid wash step with hexane; it is then evaporated and reconstituted in mobile phase. The reconstituted samples are analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The decision limits (CCα) range from 0.33-1.26 μg kg^{-1} and the detection capabilities (CCβ), range from 0.56-2.15 μg kg^{-1}. The results of the inter-assay study, which was performed by fortifying hen egg samples (n = 18) on three separate days, show the accuracy calculated for the various analytes to range between 87.2-106.2%. The precision of the method, expressed as %CV values for the inter-assay variation of each analyte at the three levels of fortification (3, 4.5 and 6.0 μg kg^{-1}), ranged between 3.7-11.3%. A Day 4 analysis was carried out to examine species variances in eggs from different birds

such as duck and quail and investigating differences in various battery and free range hen eggs.

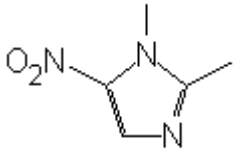
***Keywords*:** Nitroimidazoles; Method Validation; Liquid Chromatography- tandem Mass Spectrometry; Egg.

4.2 Introduction

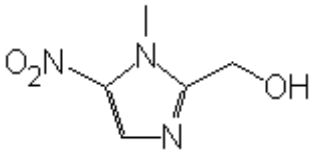
Nitroimidazoles are imidazole heterocycles with a nitrogen group incorporated in the structure. Examples of these compounds are metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ), ipronidazole (IPZ), carnidazole (CNZ), ornidazole (ONZ), tinidazole (TNZ) and ternidazole (TRZ). These examples are known as 5-nitroimidazoles as they contain a NO_2 group on the 5^{th} position on its ring which can be seen in figures 4-1.

Nitroimidazoles are extensively metabolised in avian, bovine and porcine species [Mottier *et al.,* 2006]. The main metabolites of DMZ, IPZ and MNZ result from the oxidation of the side chain in the C-2 position of the imidazole ring to form hydroxy metabolites. RNZ has a different degradation pathway but results in an identical metabolite to that of DMZ [Mottier *et al.,* 2006]. These metabolites are HMMNI, MNZ-OH and IPZ-OH. Structures of these metabolites are shown in figure 4-1.

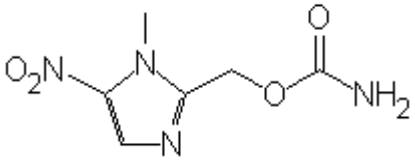
Dimetridazole
(1, 2-dimethyl-5-nitroimidazole)



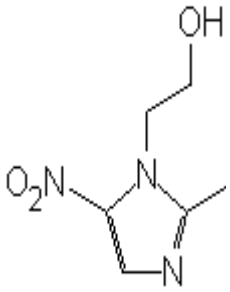
HMMNI
(2-hydroxymethyl-1-methyl-5-nitroimidazole)



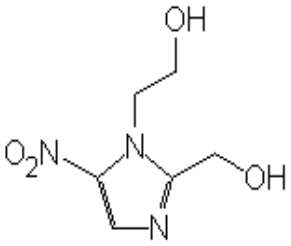
Ronidazole
(1-methyl-2-[(carbamoyloxy) methyl]-5-nitroimidazole)



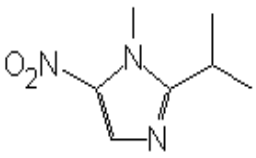
Metronidazole
(1-(2-hydroxyethyl)-2-methyl -5-nitroimidazole)



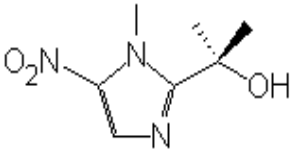
MNZ-OH
(1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole)



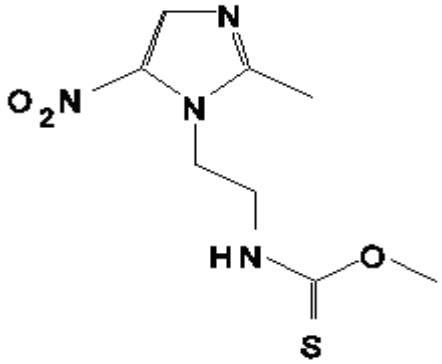
Ipronidazole
(2-isopropyl-1-methyl-5-nitroimidazole)



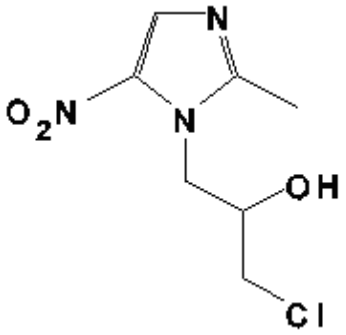
IPZ-OH
(1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole)



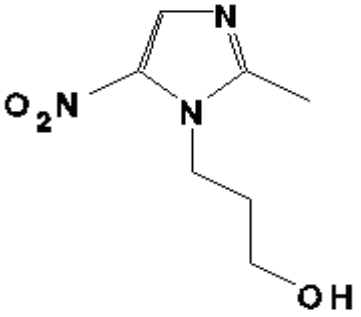
Carnidazole
(1-(2-ethylcarbamothioic acid O-methyl ester)
-2-methyl-5-nitroimidazole)



Ornidazole
(1-(3-chloro-2-hydroxypropyl)-
2-methyl-5-nitroimidazole)



Ternidazole
2-Methyl-5-nitroimidazole-1-
propanol; 3-(2-Methyl-5-



Tinidazole
1-(2-ethylsulfonylethyl)-2-
methyl-5-nitro-imidazole

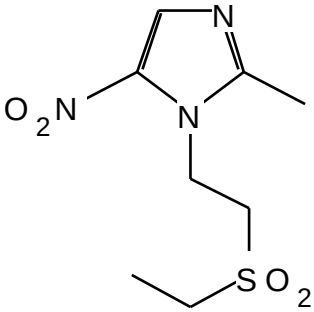


Figure 4-1: Chemical Structures of Nitroimidazole Compounds and Metabolites

These 5-nitroimidazoles are very effective for the prophylactic and therapeutic treatments of diseases such as histominiasis and coocidiosis in poultry, genital tricchoniasis in cattle and hemorrhagic enteritis in pigs. However these compounds are now suspected to be carcinogenic and mutagenic to humans and as a consequence were banned for the use in food producing animals within the European Union under Regulation 2377/90. They are also banned for use in the U.S.A and China [Xia *et al.*, 2007]. The analysis of these compounds is required under Council Directive 96/23/EC.

As the use of these substances are prohibited and food containing residues of these compounds may be dangerous to the consumer, there is a need for rapid multi residue analytical methods that have the capability of analysing a wide range of these analytes in order to ensure compliance with legislation. Previously, analysis of these compounds focused on liver and muscle [Xia *et al.*, 2007; Polzer *et al.*, 2001]. However studies on the stability and homogeneity of nitroimidazoles in incurred poultry muscle [Polzer *et al.*, 2004; Polzer *et al.*, 2005] show that there is not a homogenous distribution of the analyte(s) in turkey muscle and also there is a rapid degradation in analyte concentration in muscle stored for prolonged periods above 4 °C. In contrast it was demonstrated that for egg, plasma and retina samples; the analytes are stable during storage under the same conditions as the muscle samples and as a result concentrations are stable allowing detection of these compounds for longer periods after medication had ceased. Therefore, egg, plasma and retina have been recommended as target matrices for the residue control of nitroimidazoles [Polzer *et al.*, 2004; Polzer *et al.*, 2005]. Current methods used for the analysis of nitroimidazoles in any matrix are limited to the analysis of at most seven nitroimidazole compounds [Mottier *et al.*, 2006; Sun *et al.*, 2007], but the majority analyse for fewer [Xia *et al.*, 2006; Polzer *et al.*, 2001; Fraselle *et al.*, 2007 Wang, 2001; Capitan-Vallvey *et al.*, 2002; Hurtaud-Pessel *et al.*, 2000; Ho *et al.*, 2005; Ding *et al.*, 2006; Mortier *et al.*, 2003; Thompson *et al.*, 2009]. These methods use, varied extraction protocols including the use of acetonitrile [Mottier *et al.*, 2006] or ethyl acetate [Xia *et al.*, 2006; Sun *et al.* 2007]. In some cases the samples are extracted using a buffer of $NaCl/KH_2PO_4$ with protease and adjusting the pH to 3 with 25% HCl [Fraselle *et al.*, 2007; Polzer *et al.*, 2001]. This was performed as it was believed that nitroimidazole compounds may be protein bound.

The majority of these current methods then employ a solid phase extraction step (SPE) in order to purify the extract [Mottier *et al.*, 2006; Xia *et al.*, 2007; Xia *et al.*, 2006; Polzer *et al.*, 2001; Polzer *et al.*, 2004; Polzer *et al.*, 2005; Fraselle *et al.*, 2007; Sun *et al.* 2007] and of the methods that do not employ SPE [Ding *et al.*, 2006; Xia *et al.*, 2006] only one was used to analyse eggs [Xia *et al.*, 2006]. Various techniques have been used for the determination of these compounds such as HPLC-UV [Sun *et al.* 2007] and GC-MS/MS [Polzer *et al.*, 2001; Ho *et al.*, 2005]. The use of GC-MS/MS limits the number of analytes that can be analysed due to problems arising from derivatisation, such as the fact that HMMNI and RNZ form the same derivatization products [Xia *et al.*, 2007]. Presently more methods are being developed for this analysis by LC-MS/MS which allow for a greater number of analytes to be analysed [Mottier *et al.*, 2006; Xia *et al.*, 2007; Xia *et al.*, 2006; Capitan-Vallvey *et al.*, 2002]. This overcomes the problem of derivatisation and allows for quicker analysis times.

From the aforementioned papers it is clear that the analysis of egg as a target matrix for nitroimidazole [Polzer *et al.*, 2004; Polzer *et al.*, 2005] has been overlooked. From examining published literature, only three methods were found that were capable of analyzing nitroimidazoles in eggs. [Mottier *et al.*, 2006; Xia *et al.*, 2006; Mohamed *et al.*, 2008]. These methods are limited to at most the analysis of seven analytes. The method described by Xia *et al.* while rapid only analyses four compounds while the methods by Mottier *et al.*, 2006 and Mohamed *et al.*, 2008 analyse seven compounds but they incorporate time consuming SPE clean-ups in there methods.

From examination of published literature no method was found that was capable of the thorough analysis of eleven nitroimidazoles listed in this paper. The sample

preparation described in this study is more efficient than previously published methods due to the absence of a SPE step. In previous studies a deconjugation step was utilised by addition of either a protease or an acid to deconjugate possible protein bound residues. Newer methods [Thompson *et al.*, 2009] are now omitting this step and in this paper an incurred sample is tested to reinforce this theory.

A rapid, sensitive and specific multi-residue method for the detection and confirmation of a wide variety of nitroimidazoles in egg has been developed and validated in accordance with Commission Decision 2002/657/EC. A recommended level (RL) for MNZ, DMZ and RNZ of 3 µg kg^{-1} has been proposed by the CRL hence this was used in validating these three compounds. This RL was also applied to the remaining eight compounds for which no RL has been proposed. During validation, all compounds were analysed in a single chromatographic run at 1, 1.5 and 2 times the RL (3 µg kg^{-1}) with six replicates at each level over three separate days. The validation parameters selectivity, linearity, recovery, accuracy, precision, measurements of uncertainty as well as decision limits (CCα) capabilities (CCβ) have been established.

4.3 Experimental

4.3.1 Materials and reagents

MNZ, IPZ, CNZ, TNZ, TRZ, ORZ, IPZ-OH were provided by the CRL (BVL, Berlin, Germany). HMMNI, MNZ-OH, HMMNI-d_3, MNZ-OH-d_2, DMZ- d_3 and RNZ -d_3 (all from WITEGA Laboratorien Berlin), RNZ (Sigma) and DMZ (Reidel-de-Haen). Water is of LC-MS grade quality (Reidel-de-Haen). All other solvents were of LC quality and purchased from Reagecon (Clare, Ireland). Sodium Chloride was AnalaR grade and purchased from VWR (Poole, England). Individual stock

standards at 1mg mL^{-1} in ethanol were prepared and stored at 4ºC for 1 year. Individual intermediate standard solutions (10 and 1 µg mL^{-1}) in methanol were prepared and working standard solutions (mixture of nitroimidazoles) (500 ng mL^{-1}) were prepared in methanol and stored at 4ºC for 3 months. Deuterated standards were prepared similarly except mixed standard was 200 ng mL^{-1}.

4.3.2 LC-MSMS Instrumentation

The LC-MS/MS system is a TSQ Quantum Ultra EMR coupled to a Finnigan Surveyor LC system. The instrument is controlled by Xcalibur software (Version 1.4)**.** Separation was achieved using a (100 × 2) mm, 3 µm particle size, Luna C18 column (Part No. 00D-4251-B0) protected by a Security Guard guard cartridge system (20×2) mm, both supplied by Phenomenex. The oven temperature was set at 40 ºC. The chromatographic separation performed on gradient mode using water acidified with 0.1% acetic acid (mobile phase A) and acetonitrile acidified with 0.1% acetic acid (mobile phase B), at a flow of 0.25 mL min^{-1}. The initial conditions (0-4 min) were 95% A. Then the conditions changed to 5% A (4-7 min) and these were maintained until 9 min, the conditions returned to 95% A in 3 min (9-12 min), and were maintained until the end of the run at 20 min. The ionisation mode used was positive electrospray ionisation (ESI) mode with a spray voltage of 4350 V. The individual MRMs with there respective collision energies are listed in Table 4-1.

Table 4-1: MS/MS parent daughter fragmentation and Collision energies.

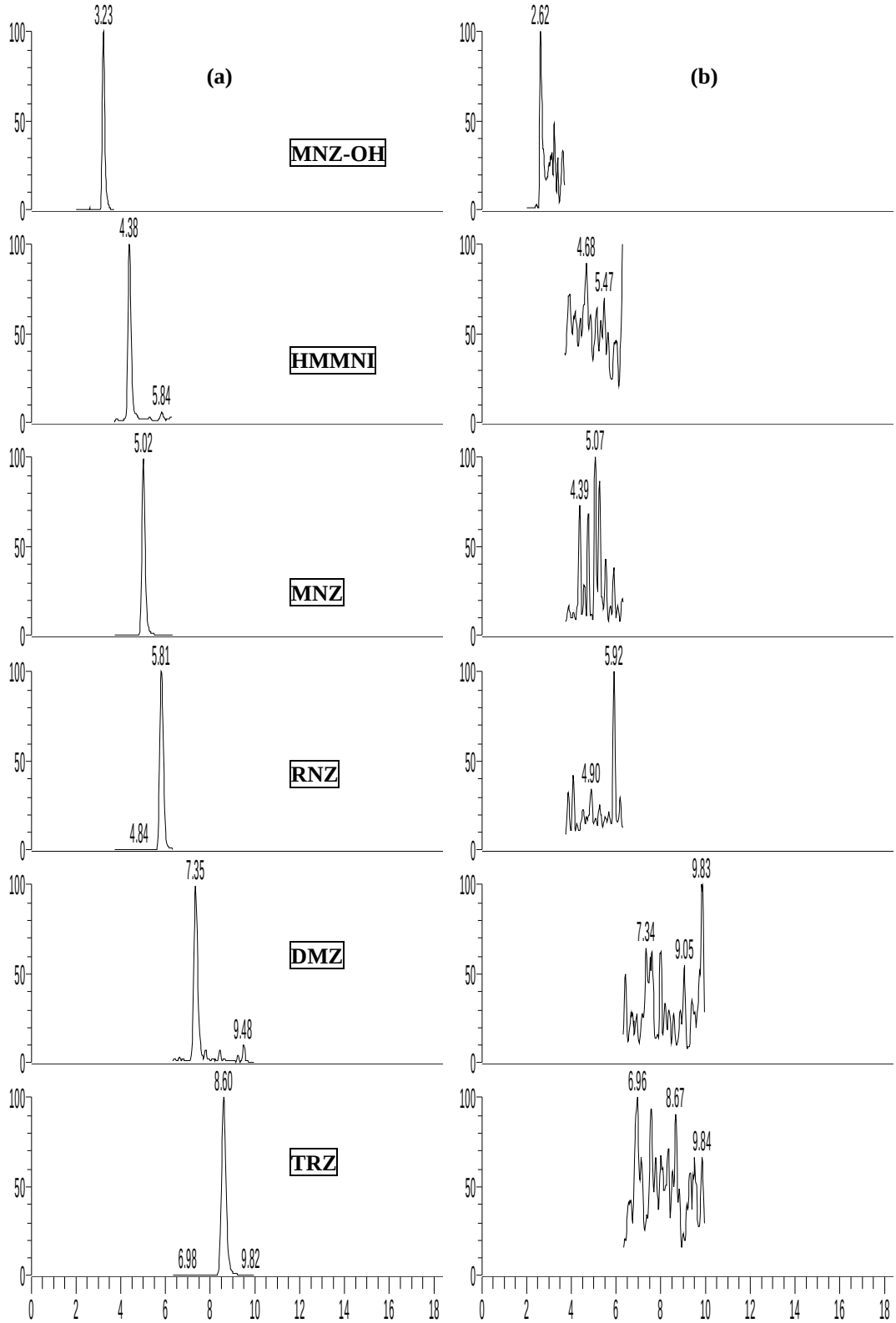
Nitroimidazole Parent/Daughter Collision Energies			
Compound	**Parent m/z**	**Daughters m/z**	**Collision Energies (eV)**
DMZ	142.2	96.4	18
		81.4	28
MNZ	172.0	82.5	25
		128.2	15
RNZ	201.2	140.1	15
		55.7	20
IPZ	170.0	124.3	18
		109.4	25
HMMNI	158.2	140.2	13
		55.7	18
MNZ-OH	188.2	123.2	16
		126.2	17
IPZ-OH	186.0	168.1	14
		122.3	21
ORZ	220.0	128.2	17
		82.4	33
TRZ	186.0	128.2	17
		82.5	28
CRZ	245.0	118.2	13
		75.3	33
TNZ	248.0	121.2	18
		202.0	15

4.3.3 Egg samples

Egg was obtained from local shops, homogenised and stored at –20 °C in polypropylene centrifuge tubes (50 mL). Samples of theses egg were analysed and those found to contain no detectable residues of the analytes of interest were used as blank egg. Chromatograms of blank egg are seen in figure 4-2.

4.3.4 Sample extraction and clean-up

Egg (3 g) is weighed into a polypropylene centrifuge tubes (50 mL). These are fortified with mixed internal standard (30 µL) which correspond to 2 µg kg^{-1}. Samples are fortified at levels corresponding to 3, 4.5 and 6 µg kg^{-1} by adding mix working standard containing each analyte (18, 27 and 36 µL) and these are vortexed (20 s). To this acetonitrile (6 mL) is added and the tubes are vortexed. NaCl (1.2 g) is added to this slurry which is then hand shaken and centrifuged (4350 x g for 10min). The top organic layer is then transferred to a polypropylene tube (15 mL). Hexane (3.5 mL) is added and this is vortexed (30 s). The hexane layer is then removed and the extracts are evaporated to dryness at 60ºC under a nitrogen stream. They are then reconstituted in 95:5 Water:ACN (200 µL). These are finally filtered through 0.2 µm PVDF syringe filters. An aliquot (20 µL) is injected onto the LC column. Chromatogram of a 2.5 µg kg^{-1} fortified egg sample is shown in figure 4-2.


(a)
(b)
MNZ-OH
HMMNI
MNZ
RNZ
DMZ
TRZ
3.23
2.62
4.38
5.84
4.68
5.47
5.02
5.07
4.39
5.81
4.84
5.92
4.90
7.35
9.48
9.83
7.34
9.05
8.60
6.98
9.82
6.96
8.67
9.84
100
50
0
0 2 4 6 8 10 12 14 16 18

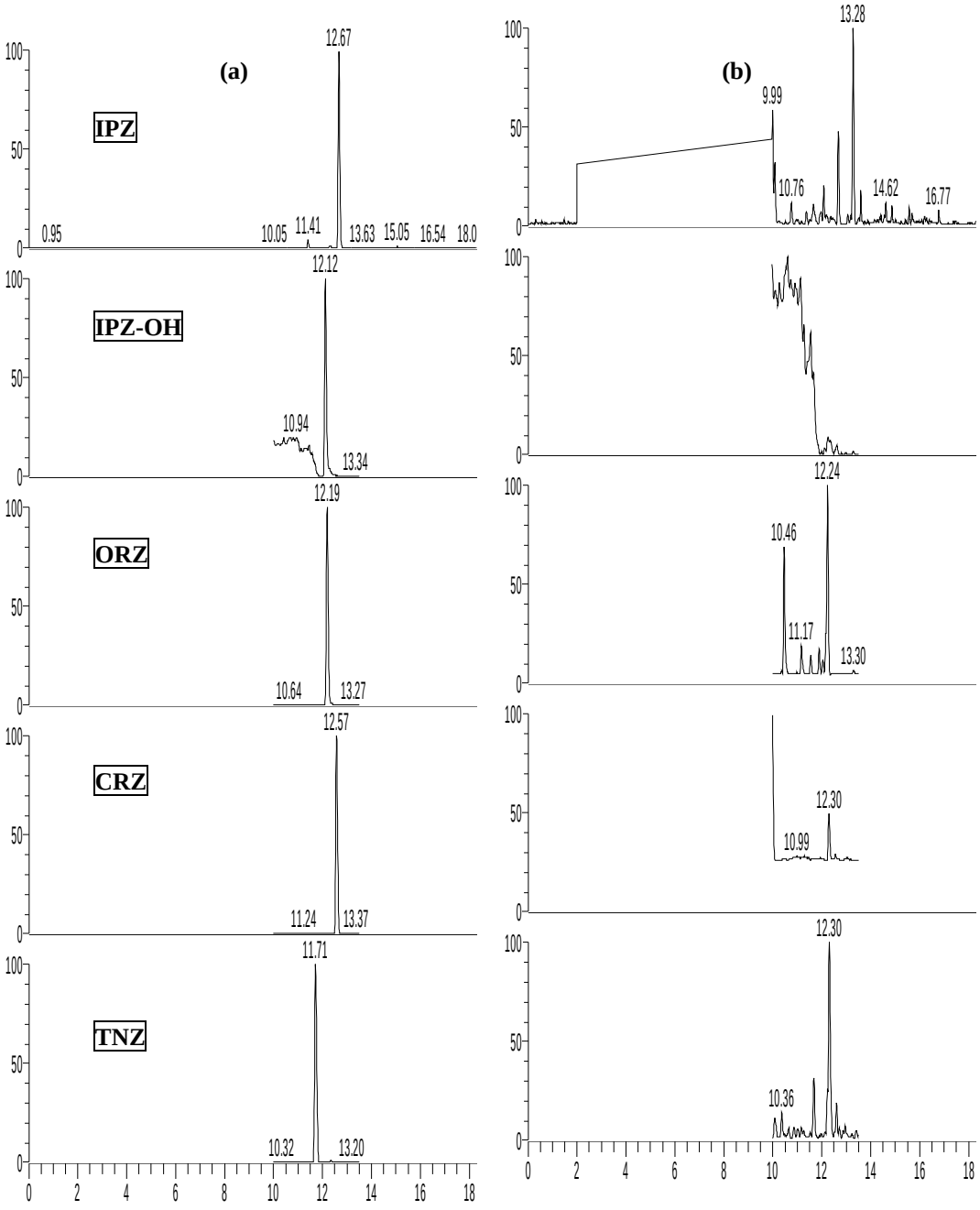


Figure 4-2: Chromatograms of all Nitroimidazole Compounds; (a) Fortified at 2.5µg kg-1 and (b) corresponding blank egg samples.

4.3.5 Matrix-Matched Calibration

Matrix matched calibration curves were prepared and used for quantification. Blank egg previously tested and shown to contain no residues was prepared as above. One blank egg sample was used for each calibration standard level. Samples were fortified at levels corresponding to 0, 2.5, 5, 7.5, 10 and 20µg kg^{-1} by adding mixed working standard solution (0, 15, 30, 45, 60 and 120 µl).

Calibration curves were prepared by plotting the response factor (peak area analyte/internal standard peak area) as a function of analyte concentration (0 to 20 µg kg^{-1}). Six deuterated internal standards are used; d3-DMZ, d3-RNZ, d3-HMMNI, d2-MNZ-OH, d3-IPZ and d3-IPZ-OH. For compounds with no deuterated form i.e. MNZ, TRZ, TNZ, ORZ and CRZ, d3-HMMNI is used as internal standard.

4.3.6 Method validation

For estimation of accuracy, blank hen egg samples were fortified with each analyte at 3.0, 4.5 and 6.0 µg kg^{-1}. Six replicate test portions, at each of the three fortification levels, were analysed. Analysis of the 18 test portions was carried out on three separate occasions. On a fourth occasion in order to determine any effects caused by different birds and egg types, 10 different blank samples from various birds were analysed, these samples consisted of five different sample types i.e. duck eggs, quail eggs, battery hen eggs, free range hen eggs and eggs containing omega three fatty acids and vitamin E. The samples were analysed in duplicate, the first were fortified with only internal standard, and the second set was fortified with both internal standard and with analytes at a concentration equivalent to 4.5 µg kg^{-1}. For the estimation of the precision of the method, intra and inter-assay repeatability was calculated.

4.4 Results and Discussion

4.4.1 Preliminary experiments:-

The method was developed from an existing method used in the laboratory for the analysis of nitroimidazoles in plasma [Cronly *et al.*, 2009 (a)] to provide confirmatory data for the analysis of 11 nitroimidazoles in eggs. The MS/MS fragmentation conditions were investigated and collision energies were optimised for each individual compound to give best response. For a method to be deemed confirmatory under Commission Decision 2002/657/EC it must yield 4 identification points. In this method a precursor ion (parent mass) and two daughters (corresponding to strong and weak ion) are monitored for each analyte (Table 4-1). This yields 4 identification points (1 for the parent ion and 1.5 for each daughter ion) hence it can be deemed a confirmatory method. In addition to this relative retention times and ion ratios are tracked for each compound and ensured that they are within acceptable ranges stated in EC 2002/657.

When the compound HMMNI was optimised it was seen that the background noise was very high in its strong daughter ion when tuning its standard solution. This was investigated further and it was found that the use of the laboratory's 18.2 MΩ water supply in the mobile phase was producing the high background. This problem was overcome by the use of LC-MS grade water in the mobile phase.

From previous work carried out on these compounds it was seen that acetonitrile is an effective extraction solvent [Mottier *et al.*, 2006]. Various extraction procedures were examined. Double extractions are common practice in sample preparation but this in turn leads to an increased amount of solvent and time being used. Tests were carried out to see if this double extraction was necessary. Results were compared between a single and double extraction. Comparison of double and single extraction

recoveries only showed an improvement of between 5-7%. This combined with the fact that the lowest calibration level was easily distinguished above background noise allowed for a single extraction step of 6ml to be utilised.

Past work carried out on these compounds showed that the addition of NaCl helped to remove impurities. [Mottier *et al.*, 2006; Xia *et al.*, 2006]. The position at which NaCl addition gave the cleanest sample was ascertained by investigating whether addition with or after extraction solvent gave better results. The addition of NaCl (2 g) with extraction solvent allowed for greater impurities to be removed and produced a cleaner sample for analysis.

SPE is widely used as a sample clean-up method. Cartridges of extrelute NT20 [Polzer *et al.*, 2004], Oasis MCX [Xia *et al.*, 2007], SCX [Sun *et al.* 2007] and HLB [Capitan-Vallvey *et al.*, 2002], have been used in methods for the analysis of nitroimidazoles. Advances in LC-MSMS and its ability to operate in Multi Reaction Monitoring greatly reduce the effect of matrix interferences hence the need for SPE is reduced in some applications. As a result it was found that addition of a quick liquid-liquid wash step was sufficient to remove an adequate amount of interferences and this in turn allows for quicker sample-preparation times.

Hexane was employed as a wash solvent in prior work carried out on these compounds [Mottier *et al.*, 2006; Xia *et al.*, 2007] therefore the use of hexane as a liquid-liquid wash solvent was investigated. Hexane with varying amounts of ethyl acetate (0%, 2%, 5% and 10%) was used as the wash solvent. Ethyl acetate was added to help remove more polar impurities but on examination of samples purified with hexane which contained various amounts of ethyl acetate it was that ethyl acetate in any percentage gave poorer recoveries for some of the compounds due to its higher polarity. As a result hexane with no additive was chosen for the wash

solvent. Samples are filtered before injection to further remove impurities. Various types and sizes of syringe filters were investigated including PVDF, PTFE and Nylon filters, losses were noted for some of the analytes with both the PTFE and Nylon filters. Filtering the samples through 0.25 µm PVDF syringe filters gave the best results and these were chosen for the study.

4.4.2 Validation study

Validation of the method was carried out according to procedures described in Commission Decision 2002/657/EC covering specificity, calibration curve linearity, accuracy, absolute recovery, repeatability, decision limit (CCα), detection capability (CCβ) and measurement uncertainty. An evaluation of the method was also carried out by running an incurred sample from FAPAS. Ruggedness is demonstrated on an ongoing basis through the use of this method for the routine analysis of nitroimidazoles in eggs as part of the National Residue Control Plan in Ireland. Routine analysis has been carried out by various analysis and very similar results to those in validation have been observed. Nitroimidazole standards are stable over time. The peak areas of the analytes are monitored on an ongoing basis and the results achieved are consistent for standards stored 4 ºC.

Table 4-2: Results received from Validation; CV%, Accuracy, Absolute Recovery, CC*α*, CCβ, Measurement of Uncertainty and Linearity.

Analytes	CV %	Accuracy %	Absolute Recovery %	CC*α* µg kg-1	CCβ µg kg-1	MU %	Linearity R2
Dimetridazole	4.2	98.0	69	0.43	0.73	28	>0.990
Metronidazole	3.8	106.2	58	0.38	0.64	19	>0.990
Ronidazole	5.6	104.5	72	0.59	1.01	34	>0.990
Ipronidazole	4.7	100.3	70	0.53	0.90	27	>0.990
HMMNI	4.3	99.2	74	0.45	0.78	18	>0.990
MNZ-OH	3.7	102.5	67	0.33	0.56	22	>0.990
IPZ-OH	4.4	100.4	77	0.43	0.73	26	>0.990
Ornidazole	7.8	92.4	77	0.79	1.34	50	>0.990
Ternidazole	4.5	99.6	67	0.41	0.71	19	>0.990
Carnidazole	11.3	87.2	76	1.26	2.15	61	>0.990
Tinidazole	5.1	97.2	72	0.45	0.77	24	>0.990

4.4.2.1. Specificity

The technique of LC-MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, egg samples were fortified with the eleven analytes and the internal standards and non-fortified samples were also analysed. On each of Days 1, 2 and 3 egg samples were examined and on Day 4, 5 different types of egg samples were examined.

4.4.2.2. Linearity of the Response

The linearity of the chromatographic response was tested with matrix matched curves using 6 calibration points in the concentration range of 0 to 20 µg kg^{-1}. The regression coefficients (r^2) for all the calibration curves used in this study were ≥ 0.990.

4.4.2.3. Absolute Recovery

Absolute recovery was determined by analysing five replica egg samples fortified at 4.5 µg kg^{-1} before extraction and five replica egg samples fortified at 4.5 µg kg^{-1} after extraction and calculating the ratio as a % of one over the other. Results given in table 4-2 lie between 58-77%.

4.4.2.4. Accuracy

The accuracy of the method was determined using egg samples fortified at 3.0, 4.5 and 6.0 µg kg^{-1} for each analyte. Mean corrected accuracy (n = 6) of the analytes, determined in four separate assays shown in Table 4-2 was between 87.2% and 106.2% for the 11 analytes.

4.4.2.5. Repeatability

Quite low values for % CV (Table 4-2) were achieved for the majority of compounds, the main reason for this can be attributed to the availability of six deuterated analogues of the compounds being examined. For those compounds without a deuterated analogue namely metronidazole, ternidazole, carnidazole,

ornidazole and tinidazole; d_3-HMMNI is used and corrects quite well for any losses or matrix suppression of the other compounds.

4.4.2.6. CCα and CCβ

The decision limit (CCα) of the method was calculated according to the calibration curve procedure using the intercept (value of the signal, y, where the concentration, x is equal to zero) and 2.33 times the standard error of the intercept for a set of data with 6 replicates at 3 levels (3, 4.5 and 6 μg kg^{-1}). The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα. Blank egg tissue was fortified at 1, 1.5 and 2 times the RL of 3μg kg^{-1} for each analyte; 3μg kg^{-1} for each compound has been used for the method validation in this work as this is the RL suggested by the Community Reference Laboratory (CRL) in Berlin for some of these analytes. CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. CCα values of for all 11 compounds are listed in Table 4-2 and are all below 1.52 μg kg^{-1}. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept + 3.97 times that standard error of the intercept). CCβ values for all 11 compounds are listed in Table 4-2 and are all below the RL of 3 μg kg^{-1}.

4.4.2.7. Measurement Uncertainty

The measurement of uncertainty was estimated by taking into account the within laboratory reproducibility over days 1, 2 and 3 as well as considering the repeatability on day 4 due to matrix effects caused by different egg matrices These two variabilities were combined and multiplied by a coverage factor of three to

give an overall figure for the uncertainty of the measurement. This approach of using the within laboratory reproducibility as a good estimator of measurement of uncertainty is taken from the SANCO/2004/2726rev1 document (SANCO 2004/2726rev1 2004). It recommends using the within laboratory reproducibility and using a coverage factor of 2.33 to estimate expanded uncertainty, however as it was felt that not all the environmental and other factors that could be varied over the course of the validation were examined, hence a coverage factor of 2.33 may underestimate the true uncertainty of the method. So a value of 3 was chosen instead to give a more realistic value for the true uncertainty, this approach was acceptable to the ISO17025 (ISO/IEC 17025 2005) auditors as well. Values for MU are seen in Table 4-2 and lie between 18 and 61%. High MU values are seen for CNZ and ORZ as they have no deuterated analogue to use as an internal standard. These values are increased further due to a high value for reproducibility due to matrix as a result of a high biological variation between species (day 4 experiment).

4.4.2.8. Evaluation

Table 4-3: Results of FAPAS Proficiency Test

SAMPLE: *FAPAS PT 02120*							
Compound	Actual Amt (µg kg^{-1})	Calculated Amt (µg kg^{-1})	MU(%)	**RANGE** MU of Cal Amt	Upper	Lower	Z-Scores
MNZ	3.5	2.942	19.45	0.572	3.514	2.370	0.7
MNZ-OH	2.67	3.085	22.23	0.686	3.771	2.399	-0.7

In order to evaluate this method an incurred sample received as part of the Food Analysis Performance Assessment Scheme (FAPAS) was analysed. Figure 4-3 shows chromatograms with both the strong and weak ions for MNZ-OH and MNZ

which were found to be present in these samples. These samples were tested using the method described here and found to yield satisfactory results. Z-scores achieved for both compounds were less than 1. As well as good z-scores when the method measurement of uncertainty is applied, the assigned values fall within the possible range of concentrations given by this method. Also the method found no peaks of analytes that were not present in the sample. Results of this proficiency test are seen in table 4-3.

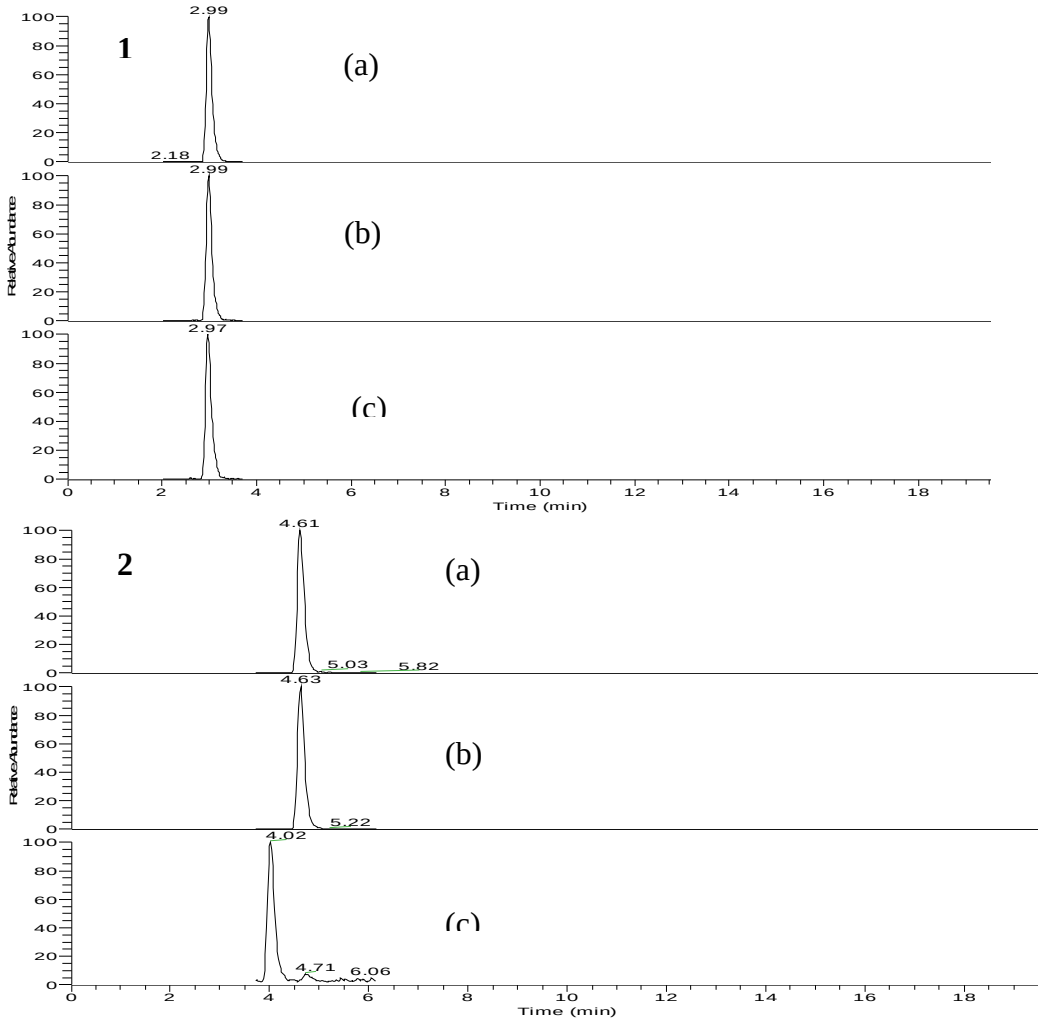


Figure 4-3: Chromatogram of FAPAS egg sample incurred with (1) MNZ-OH and (2) MNZ; (a) Strong Ion (b) Weak Ion (c) Internal Standard; (1) d2-MNZ-OH and (2) d3-HMMNI

4.5 Conclusions

The objective of this work was to develop a rapid confirmatory method capable of identifying, confirming and quantifying eleven nitroimidazole compounds in egg at μg kg^{-1} levels and to validate according to the requirements in Commission Decision 2002/657/EC and this was successfully completed.

The method can be considered as rapid, as it utilises only a hexane wash and omits the use of the time consuming SPE step, it also utilises chromatography which separates all analytes in a total run time of only 20 minutes. The method includes 11 nitroimidazole compounds including seven that are suggested by the CRL in Berlin to be analysed as well as other nitroimidazoles that are rarely if at all analysed such as tinidazole, ornidazole and carnidazole.

The obtained data fulfills the requirements laid down in Commission Decision 2002/657/EC and allows the calculation of all relevant performance characteristics. This study shows that the developed method meets the required sensitivity of 3 μg kg^{-1} which is the RL used for these compounds. The CCα and CCβ values determined for each analyte are lower than this level. The method performs very well in terms of accuracy and repeatability for each of the analytes due to the utilisation of six different deuterated internal standards. The values achieved for accuracy, %CV and measurement of uncertainty all fall within acceptable ranges. The applicability of the method for use on different types of eggs was demonstrated by the satisfactory results obtained from the Day 4 analysis of different species.The reduced number of analytical steps within the method makes it very amenable for high through-put regulatory monitoring of these compounds.

4.6 **Acknowledgements**

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CHAPTER 5: RETAIL SURVEY OF HEN AND DUCK EGGS AVAILABLE IN THE IRISH MARKET FOR 11 NITROIMIDAZOLE RESIDUES

Article in Press, Accepted Manuscript for publication in Food Additives and Contaminants Part B

5.1 Abstract

A liquid chromatography tandem mass spectrometry method recently developed, validated and accredited was used to screen for the presence of metronidazole, ronidazole dimetridazole ipronidazole, ternidazole, tinidazole, ornidazole carnidazole and three hydroxy metabolites hydroxy-metronidazole, HMMNI and hydroxy-ipronidazole in Irish retail egg samples . The method used had had decision limits (CCα) in the range 0.33-1.26 µg kg^{-1} and detection capabilities (CCβ), ranging from 0.56-2.15 µg kg^{-1} for all analytes. The internal standard corrected recovery calculated for the various analytes range between 87.2-106.2% while the coefficient of variance expressed as %CV range between 3.7-11.3%. The method was applied to 160 samples of caged, free range and organic hen and duck eggs available on the Irish Retail market as well as two incurred proficiency test egg samples. No nitroimidazole residues were detected in the survey samples above CCα and the results achieved for the two proficiency test samples were acceptable when compared with the assigned values.

Keywords: Nitroimidazoles; eggs; retail survey; LC-MS/MS

5.2 Introduction

Nitroimidazoles (NMZs) are imidazole heterocycles with a nitrogen group incorporated in the structure. Examples of these compounds are metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ), ipronidazole (IPZ), carnidazole (CNZ), ornidazole (ONZ), ternidazole (TRZ) and tinidazole (TNZ). These examples are known as 5-nitroimidazoles as they contain a NO_2 group on the 5 ring position. The structures of these compounds can be seen in figure 5-1. 5-NMZs are active against most obligate anaerobic bacteria and a variety of protozoa; as a result they

are very effective in the prophylactic and therapeutic treatment of histominiasis and coccidiosis in poultry [Bishop, 2005].

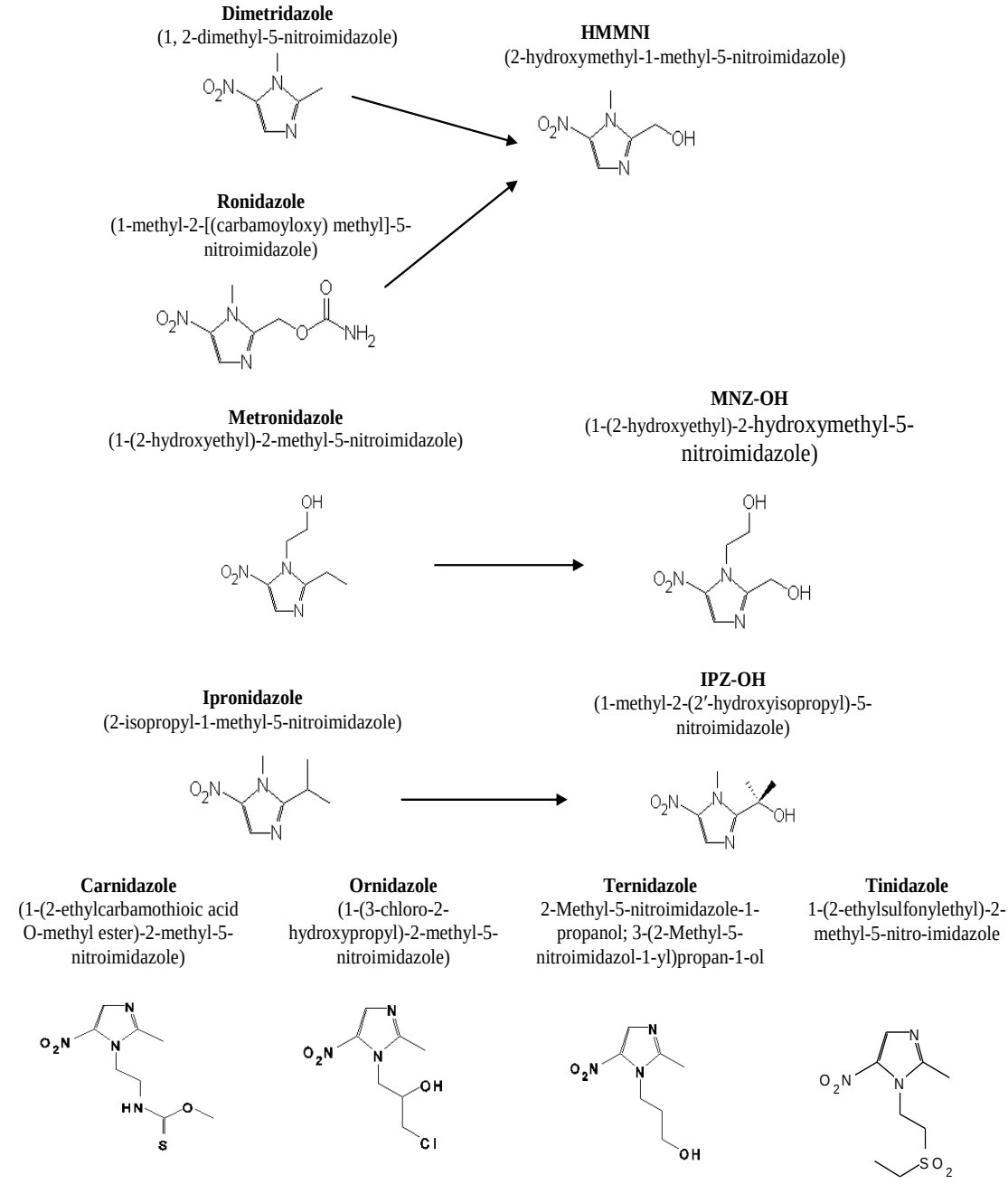

Figure 5-1: Structures of eleven nitroimidazole residues that were analysed for as part of the survey.

Toxicological studies carried out on these compounds suggest that they are possibly carcinogenic and mutagenic in humans. The European Agency for the Evaluation of Veterinary Products (EMEA) have published summary reports on three nitroimidazole compounds; metronidazole [EMEA Report, Metronidazole], dimetridazole [EMEA Report, Dimetridazole] and ronidazole [EMEA Report, Ronidazole]. While the reports suggest the studies carried out on humans are insufficient and inconclusive to prove or disprove carcinogenicity of these compounds, results in animal studies are conclusive [IARC, 1987]. This in their opinion is enough to consider these compounds as carcinogenic and in the interest of human health they are prohibited for use in food producing animals.

Due to these possible health risks associated with their use, nitroimidazole compounds previously used for treatment in animal husbandry, were prohibited originally by there inclusion in Annex IV of Commission Regulation 2377/90 but now by inclusion table 2 of Commision Regulation 37/2010. Compounds in this table are ones for which no Maximum Residue Level (MRL) can be fixed and therefore are prohibited for use in food producing animals. However because of concerns over these compounds no other 5-NMZ such as IPZ and TRZ has been issued a license for use in food producing animals and are therefore also prohibited.

NMZ compounds are rapidly metabolised in bovine, porcine and avian species. The main metabolite of DMZ, IPZ and MNZ results from the oxidation of the side chain in the C-2 position of the imidazole ring to form hydroxy metabolites [EMEA Report, Metronidazole]. RNZ has a different degradation pathway but results in an identical metabolite to that of DMZ. These metabolites are 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI), hydroxy-metronidazole (MNZ-OH) and hydroxy-ipronidazole (IPZ-OH). The structures of these compounds are seen in

figure 5-1. As these compounds still contain the imidazole ring their carcinogenicity cannot be overlooked. Also these metabolites can be an indication of potential misuse of prohibited nitroimidazoles compounds and therefore should also be examined. From studies carried out by Polzer *et al*., 2004 on the measurements of the parent drug and the corresponding main hydroxy-metabolite in various incurred materials it can be concluded that HMMNI should be chosen as target analyte to prove a treatment with DMZ. The metabolite IPZ-OH is recommended to detect an illegal medication with IPZ. To check for a treatment with RNZ or MNZ, the measurement of the parent drug is to be preferred. The studies also go on to state that the ratio of parent drug to metabolite was found to vary with the length of the withdrawal time in the case of a treatment with DMZ and respective data on the behaviour of the other nitroimidazoles are not available to date, it is recommended to monitor both, the parent drug and the respective metabolite, whenever possible in order to get more reliable results [Polzer *et al*., 2003].

The European Union Reference Laboratories (EURLs) published a Guidance Document in 2007 (CRL Guidance 2007) that outlined recommended concentration/levels (RC/RL) that laboratories should aspire to measure for prohibited substances with no legislative minimum required performance level (MRPL). The document also specified which matrices should be sampled and also what marker residue should be analysed (parent drug or metabolite). The information given in this document in relation to nitroimidazoles can be seen in table 5-1. Matrices that should be used for the analysis of nitroimidazoles are plasma, retina and eggs (Polzer at al., 2003). A level of 3 μg kg^{-1} has been assigned as the RL for NMZs and all methods used in the analysis of NMZ residues should be capable to detect to at least this level.

Table 5-1: EURL recommendations for nitroimidazoles adapted from CRL Guidance Document 2007.

Substances	Matrix	Recommended Concentaration *
Nitroimidazole: Dimetridazole, Ronidazole, Metronidazole hydroxy metabolites: MNZ-OH, HMMNI	Poultry: Plasma, Serum, Eggs Pigs (and others species): Plasma, Serum, Muscle	3ppb

* CCbeta for screening methods or CCalpha for confirmatory methods should be lower than the value expressed in this column

Although these compounds have been prohibited for use in food producing animals since the mid 1990s their analysis in eggs has not always been carried out. This was the case in Ireland where their analysis in eggs was not carried out prior to 2007. From examination of non compliant findings in Europe, reported in the European Food Safety Authority (EFSA) annual report and on the Rapid Alert System for Food and Feed (RASFF) the abuse of these prohibited compounds cannot be overlooked. The 2009 report from EFSA states that there were 6 non compliant findings of nitroimidazole residues in 2007 and 5 in 2008. All positive findings were in poultry meat and egg products for metronidazole and ronidazole in two member states [EFSA Report, 2009]. From examination of the RASFF reports two positive results for poultry meat products also contained metronidazole and ronidazole. Hence taking into consideration these non compliant findings and the lack of analysis carried out on these compounds in eggs in Ireland prior to 2007, it was felt that an examination

of eggs available in the Irish retail market for the presence of NMZ residues would be beneficial. It would firstly help ensure that the ban on these compounds is being observed and secondly it would give confidence to the consumer that the eggs that reach the table are free from these harmful residues.

To the best of our knowledge, no data on the presence of NMZ residues in Irish Eggs has previously been published. Therefore the aim of this study was to examine occurrences of NMZ residues in eggs sold on the Irish retail market. The analysis of 160 egg samples for eleven NMZ compounds was carried out with an in house validated and ISO 17025 accredited LC-MS/MS method (Cronly *et al*., 2009 (b)).

5.3 Materials and methods

5.3.1 Sample collection and preparation

Table 5-2: Information on samples taken as part of the survey

		Hen Eggs	Duck Eggs
Total Numbers		148	12
Purchased in	***Leinster***	36	3
	Munster	62	5
	Connaght	30	2
	Ulster	20	2
Farming Practices	***Free Range***	118	12
	Organic	14	0
	Caged	11	0
	Barn	5	0

This survey was carried out in conjunction with Ashtown Food Research Centre (AFRC) and the collection of eggs was performed by them. Hen and duck eggs were purchased from farmer markets, small shops, convenience stores and large chain

supermarkets all over Ireland. To ensure representative samples 12 eggs were used for each one. Whole egg samples were homogenised, transferred into polypropylene tubes (50ml) and stored at -20ºC until transfer to our laboratory. These samples were delivered by courier in a frozen state and stored at -20ºC until analysis. A table of samples analysed and information related to them can be seen in table 5-2.

5.3.2 Chemicals and materials

CNZ, TNZ, TRZ, ORZ were provided by the EURL (BVL, Berlin, Germany). IPZ-OH, MNZ, IPZ, HMMNI, MNZ-OH, HMMNI-d3, MNZ-OH-d2, DMZ- d3 and RNZ -d3 (all from WITEGA Laboratorien Berlin), RNZ and DMZ (Sigma, St. Louis, MO). Water is of LC-MS grade quality (Fluka). All other solvents were of LC quality and purchased from Reagecon (Clare, Ireland). Sodium Chloride was AnalaR grade and purchased from VWR (Poole, England). Individual stock standards at 1mg mL^{-1} in ethanol were prepared and stored at 4ºC for 1 year. Individual intermediate standard solutions (10 and 1 µg mL^{-1}) in methanol were prepared and working standard solutions (mixture of nitroimidazoles) (300 ng mL^{-1}) were prepared in methanol and stored at 4ºC for 3 months. Deuterated standards were prepared similarly except mixed standard was 300 ng mL^{-1}.

5.3.3 Sample extraction

Extraction method is one taken from Cronly *et al.*, 2009 (b). In brief; egg samples (3 g) are weighed into a polypropylene centrifuge tubes (50 mL). These are fortified with mixed internal standard. A calibration curve is run with each batch by fortifying blank egg at levels corresponding to 0, 2.5, 5, 7.5, 10 and 20 µg kg^{-1} by adding mix working standard containing each analyte (0, 25, 50, 75, 100 and 200µL) and these

are vortexed (20 s). To these samples acetonitrile (6 mL) is added and the tubes are vortexed. NaCl (1.2 g) is added and shaken vigorously by hand before centrifuging (4350 x g for 10min). The top organic layer is then transferred to a polypropylene tube (15 mL). Hexane (3.5 mL) is added and this is vortexed (30 s). The hexane layer is then removed and the extracts are evaporated to dryness at 50ºC under a nitrogen stream. They are then reconstituted in 95:5 Water: ACN (200 µL). Before injection the samples are filtered through 0.2 µm PVDF syringe filters. An aliquot (20 µL) is injected onto the LC column. A chromatogram of a 2.5 µg kg^{-1} fortified egg sample is presented in figure 5-2.

5.3.4 Liquid chromatography tandem mass spectrometry conditions

Two LC-MS/MS systems were used in the analysis of these samples, first was a TSQ Quantum Ultra EMR coupled to a Finnigan Surveyor LC system and controlled by Xcalibur software (Version 1.4) and the second one an AB Sciex Triple Quad 5500 couple to Schimadzu UFLC XR LC system which is controlled by Analyst Software 1.5. Separation was achieved using C18 column with an oven temperature was set at 40 ºC. The chromatographic separation performed on gradient mode using water acidified with 0.1% acetic acid (mobile phase A) and acetonitrile acidified with 0.1% acetic acid (mobile phase B). As the two LC are different one being HPLC and the other UPLC the gradients and runs time are different. The gradients and mass spectrometer parameters for the respective instruments can be seen in table 5-3. The ionisation mode used was positive electrospray ionisation (ESI) mode and the individual MRMs with there respective collision energies were optimised for both instruments and are listed in table 5-4.

Table 5-3: LC and MS/MS parameters for Instrument 1 (Thermo TSQ Quantum) and Instrument 2 (AB Sciex 5500) used in analytical method.

Instrument 1				**Instrument 2**			
HPLC Gradient				**UHPLC Gradient**			
Time (min)	%A	%B	Flow mL min^{-1}	Time (min)	%A	%B	Flow mL min^{-1}
0	95	5	0.25	0	95	5	0.55
4	95	5	0.25	1.5	95	5	0.55
7	5	95	0.25	3	5	95	0.55
9	5	95	0.25	5	5	95	0.55
12	95	5	0.25	6	95	5	0.55
20	95	5	0.25	9	95	5	0.55
TSQ Quantum Mass Spectrometer Parameters				**AB Sciex 5500 Spectrometer Parameters**			
Ionisation Mode:	ESI (Positive Mode)			Ionisation Mode:	ESI (Positive Mode)		
Spray Voltage:	4350V			Spray Voltage:	4500V		
Capiliary Temperature:	325ºC			Source Temperature:	650ºC		
Source CID:	0			CAD Gas:	8		
Collision Pressure:	1.5 Torr			Entrance Potential:	10V		
Tube Lens Offset:	75			Curtain Gas Pressure:	45psi		
Quad MS/MS Bias:	-1			Ion Source Gas1 Pressure:	50psi		
Sheath Gas Pressure:	65			Ion Source Gas2 Pressure:	60psi		
Auxilary Gas Pressure:	35			Resolution Q1:	unit		
Ion Sweep Gas Pressure:	0			Resolution Q3:	unit		

Table 5-4: Information on precursor ion, product ion, collision energies and retention times for each of the eleven nitroimidazole residues on both instruments.

Compound	Precursor (M/z)	Product (M/z)	Collision Energy (eV)		Retention Time (min)	
			Instrument 2	Instrument 1	Instrument 2	Instrument 1
MNZ-OH	188	123	17	16	1.34	3.23
		126	23	17		
HMMNI	158	110	20	18	1.77	4.38
		140	17	13		
MNZ	172	82	33	25	2.11	5.02
		128	19	15		
RNZ	201	140	17	15	2.38	5.81
		55	29	20		
DMZ	142	96	21	18	3.05	7.35
		81	31	28		
TRZ	186	128	19	17	3.69	8.6
		82	37	28		
TNZ	248	121	23	18	3.97	11.71
		82	47	15		
IPZ-OH	186	168	19	14	4.09	12.12
		122	27	21		
ORZ	220	128	23	17	4.11	12.19
		82	39	33		
CRZ	245	118	21	13	4.27	12.57
		75	43	33		
IPZ	170	124	25	18	4.32	12.67
		109	33	25		

5.3.5 Validation studies

The validation studies of this method can be seen in Cronly *et al*. 2009(b). The method was validated in accordance with Commission Decision 2002/657. The validation results associated with this method can be seen in table 5-5. To ensure the ongoing competency of the method proficiency test samples were analysed using the method. The results achieved from this scheme must meet requirements set out by both the organiser of the scheme in this case Food Analysis Performance Assessment Scheme (FAPAS) as well as in house laboratory requirements for performance in proficiency schemes.

Table 5-5: Validation results of %CV, accuracy, absolute recovery, CC*α*, CCβ, MU and linearity.

Analytes	CV %	Accuracy %	Absolute Recovery %	CC*α* µg kg^{-1}	CCβ µg kg^{-1}	MU %	Linearity R^2
Dimetridazole	4.2	98.0	69	0.43	0.73	28	>0.990
Metronidazole	3.8	106.2	58	0.38	0.64	19	>0.990
Ronidazole	5.6	104.5	72	0.59	1.01	34	>0.990
Ipronidazole	4.7	100.3	70	0.53	0.90	27	>0.990
HMMNI	4.3	99.2	74	0.45	0.78	18	>0.990
MNZ-OH	3.7	102.5	67	0.33	0.56	22	>0.990
IPZ-OH	4.4	100.4	77	0.43	0.73	26	>0.990
Ornidazole	7.8	92.4	77	0.79	1.34	50	>0.990
Ternidazole	4.5	99.6	67	0.41	0.71	19	>0.990
Carnidazole	11.3	87.2	76	1.26	2.15	61	>0.990
Tinidazole	5.1	97.2	72	0.45	0.77	24	>0.990

5.4 Results and discussions

The LC–MS/MS method published in Cronly *et al*., 2009(b) was used to provide confirmatory data for the analysis of 11 nitroimidazole compounds in hen and duck eggs. The MS/MS fragmentation conditions were optimised in the method for each individual compound to give best response. For a method to be deemed confirmatory under Commission Decision 2002/657/EC it must yield 4 identification points. In this method a precursor ion (parent mass) and two product ions (corresponding to strong and weak ion) are monitored for each analyte (Table 5-4). This yields 4 identification points (1 for the precursor ion and 1.5 for each product ion) hence it can be deemed a confirmatory method. In addition to this relative retention times and ion ratios are monitored for each compound and evaluated to ensure that they are within acceptable ranges as stated in EC 2002/657. The developed extraction method assisted with the reduction of matrix associated ion suppression but did not eliminate it completely; this possible problem was overcome by the use matrix matched calibration curves and deuterated internal standards. The method was fully validated in accordance with Commission Decision 2002/657. The validation results associated with this method can be seen in table 5. The method has also received ISO 17025 accreditation.

Table 2 lists the breakdown of samples analysed as part of this survey. The majority incorporated in this survey were hen eggs as they are the most widely available on the Irish market. The survey also analysed a number of duck egg samples. The samples are broken into these two categories and information associated to province of purchase and farming type is given. The samples were batched in numbers of ten to fifteen each month and analysed using the accredited method. They were analysed of the AB Sciex 5500 LC-MS/MS or Thermo Quantum LC-MS/MS. With each batch a calibration curve was run and certain parameters were examined to ensure the

extractions and instrumental analyses were successful. The 2.5 µg kg^{-1} matrix extracted standard was run at the start and the Signal to Noise (S:N) for the weakest transition for each analyte needed to be greater than 25. A chromatogram for each compound at the lowest calibration level 2.5 µg kg^{-1} with associated S:N can be seen in figure 5-2.

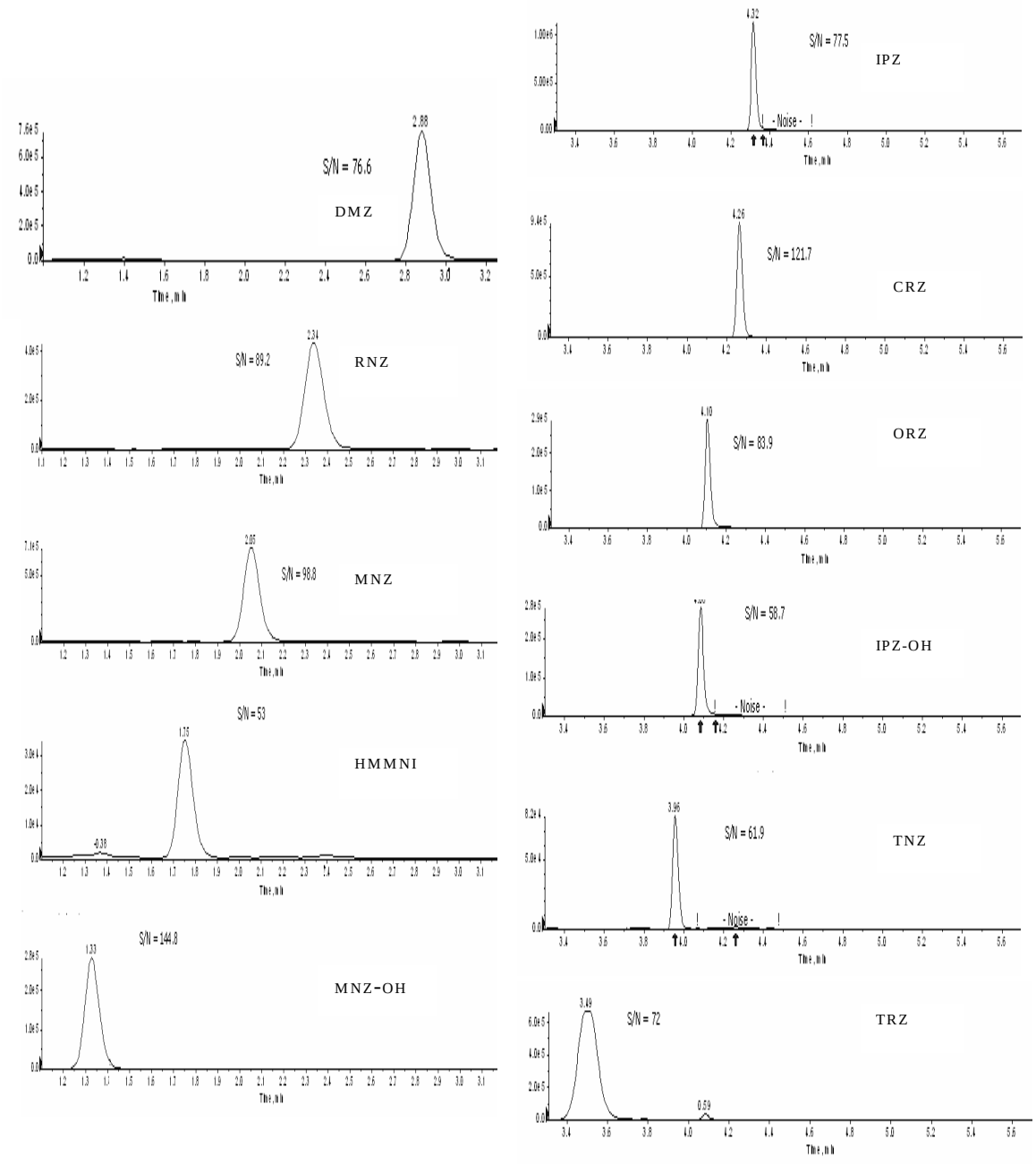

Figure 5-2: Chromatogram showing signal to noise of the eleven nitroimidazole residues in 2.5 µg kg-1 fortified blank egg sample relating to the lowest calibration.

The calibration curve had to have a correlation coefficient of greater than 0.99 and an intercept smaller than ± 0.3. Finally the internal standard in each sample had to have a S:N greater than 25 to ensure that the extraction had worked for each sample. Example of internal standards in samples with their associated S:N values can be seen in figure 5-3. If these parameters were not met the sample would be repeated.

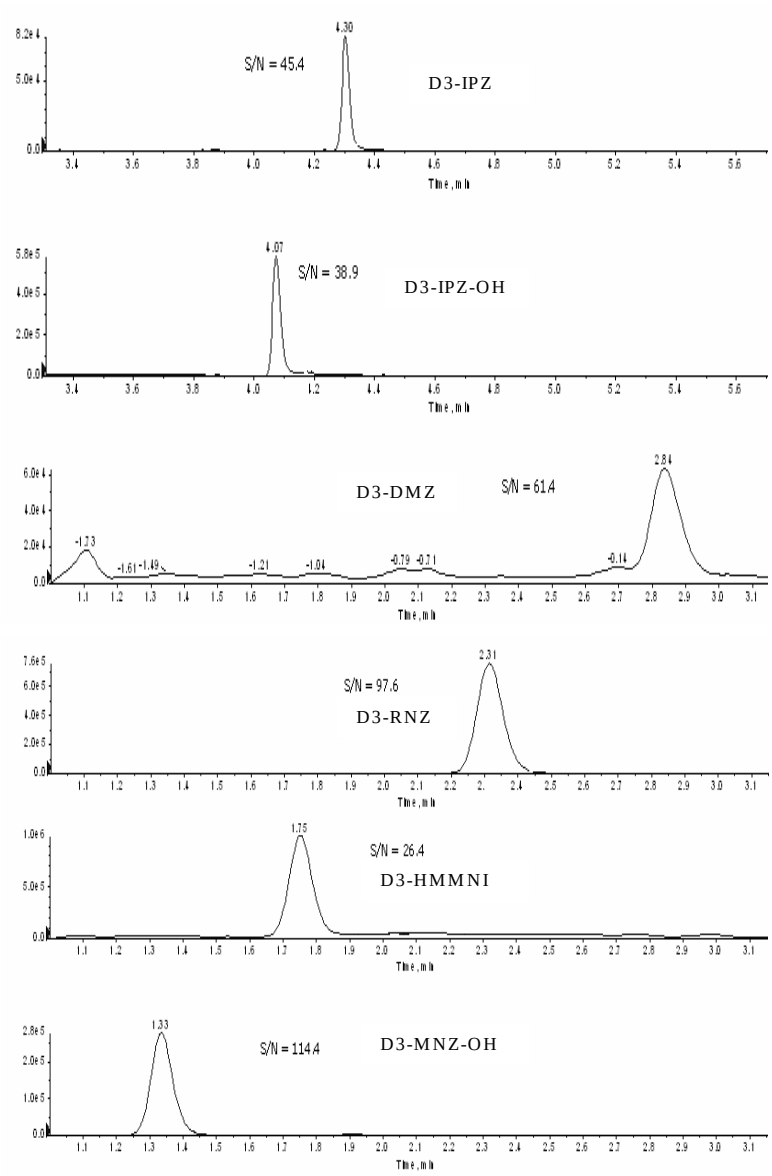

Figure 5-3: Chromatogram showing the signal to noise of the six internal standards in a survey sample.

In addition to these batch suitability tests, the method was tested on an ongoing basis by its use to analyse two proficiency test (PT) samples provided by FAPAS. These were run in the same way as the survey samples and the results achieved were compared to the assigned values. The chromatograms relating to the strong and weak ions and internal standard for the positive PT samples can be seen in figure 5-4. The results achieved gave very acceptable Z scores of less than +/- 2 and were well within the measurement uncertainty MU of the method.

The samples analysed as part of the survey were collected all over the country over a two year period in 2009 and 2010. The samples were sourced from different retail outlets and markets from every province in Ireland to ensure that the egg samples analysed were representative of eggs that are available to the Irish consumer. From examination of the results of all 160 hen and duck egg samples no non compliant findings were observed for any of the 11 nitroimidazole residues investigated.

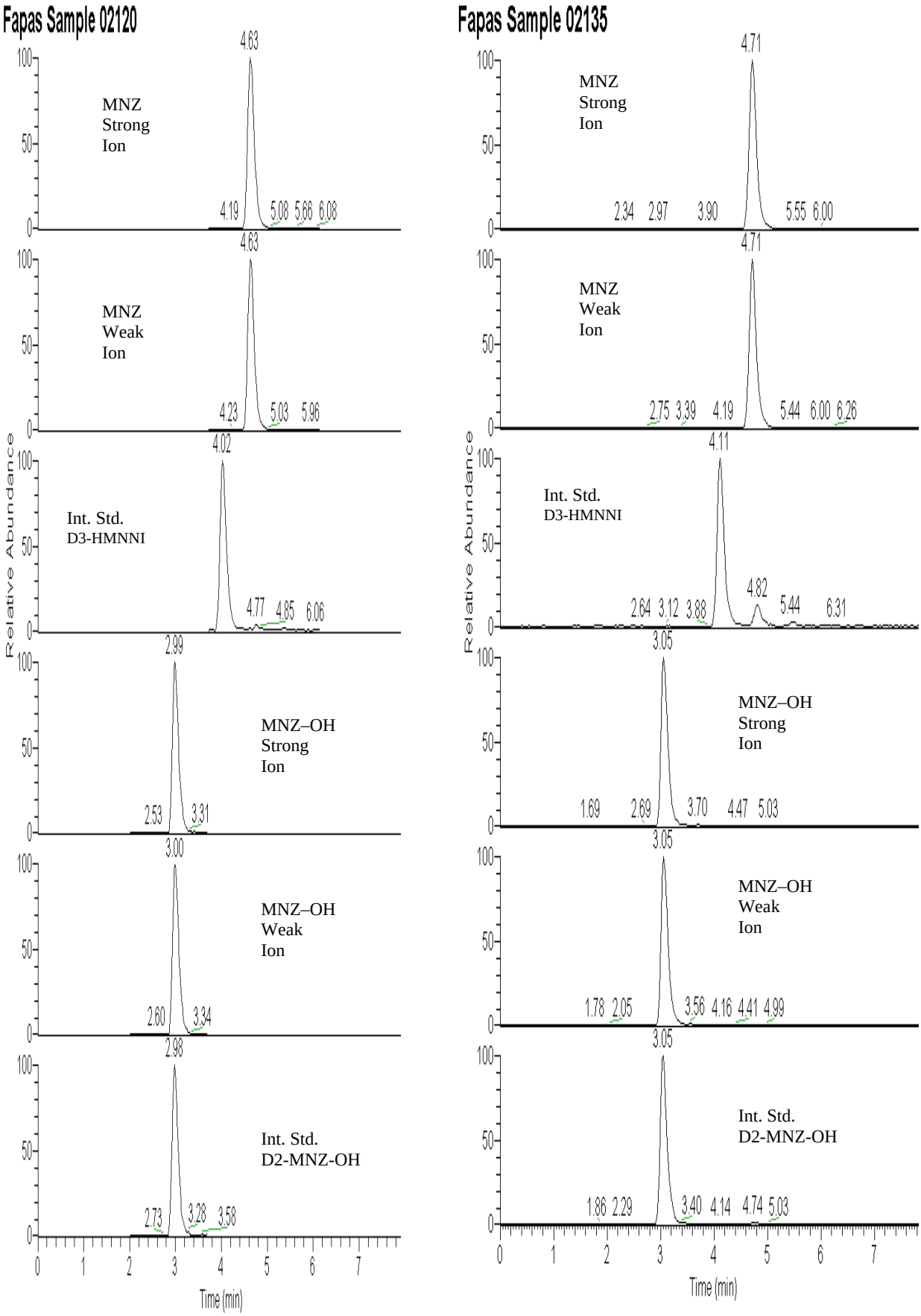

Figure 5-4: Chromatograms of strong and weak ions and internal standards relating to non compliant results associated with FAPAS proficiency test samples

5.5 Conclusions

The results indicate a zero incidence of illegal or accidental use of nitroimidazoles in Irish poultry egg industry. The method used in the analysis of 160 hen and duck egg samples was one published by Cronly *et al*., 2009(b) and was validated in accordance with Commission Decision 2002/657/EC and has achieved ISO 17025 accreditation. Each batch analysed had to meet certain requirements for acceptance and in the case of all samples this was achieved. The method was further tested by its use in the analysis of PT samples and results achieved demonstrated the method was capable of the confirmation and quantitation of nitroimidazole residues in egg samples. Results achieved had Z scores less 2 and were within MU of the method. Confirmatory criteria of Ion ratios and RRT were within criteria set out in European legislation.

When taking into consideration non compliant findings in eggs in Europe and the lack of analysis carried out on these compounds in eggs in Ireland prior to 2007, it was felt that an evaluation of eggs available in the Irish retail market for the presence of NMZ residues would be beneficial. Upon carrying out this survey which resulted in complaint findings for all samples analysed it is felt that this demonstrates that the ban on these compounds is being observed and secondly it gives confidence to the consumer that the eggs that reach the table are free from these harmful residues. Taking this into consideration it is felt that the monitoring of these compounds in eggs which is being carried out as part of the national monitoring plan is sufficient to ensure the continued enforcement of these prohibited compounds in eggs. In addition to this the results achieved for this study will be added to other research which is ongoing in AFRC in the hope of producing a dietary exposure assessment of food on the Irish market.

CHAPTER 6: RAPID MULTI-CLASS MULTI-RESIDUE METHOD FOR THE CONFIRMATION OF CHLORAMPHENICOL AND ELEVEN NITROIMIDAZOLES IN MILK AND HONEY BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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6.1. Abstract

A confirmatory method was developed to allow for the analysis of eleven nitroimidazoles and also chloramphenicol in milk and honey samples. These compounds are classified as A6 compounds in Annex IV of Council Regulation 2377/90 (European Commission 1990) and therefore prohibited for use in animal husbandry. Milk samples were extracted by acetonitrile with the addition of NaCl; honey samples were first dissolved in water before a similar extraction. Honey extracts underwent a hexane wash to remove impurities. Both milk and honey extracts were evaporated to dryness and reconstituted in initial mobile phase. These were then injected onto a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system and analysed in less than 9min. The MS/MS was operated in multiple reaction monitoring (MRM) mode with positive and negative electrospray ionization. The method was validated in accordance with Commission Decision 2002/657/EC and is capable of analysing metronidazole, dimetridazole, ronidazole, ipronidazole and there hydroxy metabolites hydroxymetronidazole, 2-hydroxymethyl-1-methyl-5-nitroimidazole, and hydroxyipronidazole. The method can also analyse for carnidazole, ornidazole, ternidazole, tinidazole, and chloramphenicol. A recommended level of 3 $\mu gL^{-1}/\mu gkg^{-1}$ for methods for metronidazole, dimetridazole, and ronidazole has been recommended by the Community Reference Laboratory (CRL) responsible for this substance group, and this method can easily detect all nitroimidazoles at this level. A minimum required performance level of 0.3 $\mu gL^{-1}/\mu gkg^{-1}$is in place for chloramphenicol which the method can also easily detect. For nitroimidazoles, the decision limits (CCα) and detection capabilities (CCβ) ranged from 0.41 to 1.55 μgL^{-1} and from 0.70 to 2.64

μgL^{-1}, respectively, in milk; and from 0.38 to 1.16 μgkg^{-1} and from 0.66 to 1.98 μgkg^{-1}, respectively, in honey. For chloramphenicol, the values are 0.07 and 0.11 μgL^{-1} in milk and 0.08 and 0.13 μgkg^{-1} in honey. Validation criteria of accuracy, precision, repeatability, and reproducibility along with measurement uncertainty were calculated for all analytes in both matrices.

Keywords: Nitroimidazoles; chloramphenicol; milk; honey; lc-ms/ms; validation

6.2. Introduction

6.2.1. Nitroimidzoles

5-Nitroimidazoles are primarily used for the prophylactic and therapeutic treatments of diseases such as histominiasis and coocidiosis in poultry, hemorrhagic enteritis in pigs and genital trichomoniasis in cattle. Recently, reports from China suggest that the use of nitroimidazoles in beekeeping is being practiced [Zhou *et al.*, 2007]. Nitroimidazoles may be used to prevent and control Nosema apis in hives. Nosema Apis is a microsporidian pathogen that is commonly found in Apis mellifera throughout the beekeeping world [Official Method (2003)].

Nitroimidazoles(NMZs) are believed to be carcinogenic and mutagenic to humans and as a consequence are placed in the group A6 (prohibited) substances and their use in food producing animals within the European Union is not permitted under Regulation 2377/90. They are also prohibited for use in the U.S.A and China. The analysis of these compounds is required under Council Directive 96/23/EC. The CRL has also established a recommended level (RL) of 3 μgL^{-1}/μgkg^{-1} for these compounds. There is a need for rapid multi residue analytical methods that have the capability to include a wide rage of these analytes in all matrices where abuse may be

found. The structure of these 5-nitroimidazoles and their metabolites are seen in table 6-1.

The Community Reference Laboratory (CRL) in Berlin has carried out homogeneity and stability studies on NMZs in various matrices [Polzer *et al.*, 2004, Polzer *et al.*, 2005]. These studies showed that there is not a homogenous distribution of these analytes in turkey muscle and they also observed a rapid degradation in analyte concentration stored for prolonged periods above 4 °C. In contrast it was discovered that for plasma, retina and egg samples; the analytes were stable during storage under the same conditions which resulted in stable concentrations and allowed detection of these compounds for longer periods after medication had been halted. Therefore, it is advised that plasma, retina and eggs be used as target matrices for the residue control of NMZs [Polzer *et al.*, 2004, Polzer *et al.*, 2005].

As a result of these published studies there has been an increase in the methods being developed for the analysis of these compounds in matrices such as egg [Mottier *et al*. 2006, Mohammed *et al.*, 2008, Xia *et al.*, 2006, Daeseleire *et al.*, 2000, Cronly *et al.*, 2009(b)] and plasma [Aerts *et al.*, 1991, Fraselle *et al.*, 2007, Thompson *et al.*, 2009, Cronly *et al.*, 2009(a)]. The majority of methods published for the analysis of eggs involve extraction with acetonitrile and the addition of NaCl. Purification methodologies of these extracts varied. Solid Phase Extraction (SPE) such as Hydrophilic Lypophilic Balance (HLB) catridges [Mottier *et al.*, 2006] or Molecular imprinted (MIPs) SPE cartridges [Mohamed *et al.*, 2008] were used. In some methods the samples were just filtered before injection and satisfactory results were still achieved [Xia *et al.*, 2006, Daeseleire *et al.*, 2000, Cronly *et al.*, 2009(a), (b)]. Methods for the analysis of these compounds in plasma are varied in their extraction protocols. Extraction solutions such as aqueous buffer [Aerts *et al.*, 1991],

NaCl/Potassium phosphate buffer (Fraselle *et al.*, 2007) and acetonitrile [Thompson *et al.*, 2009, Cronly *et al.*, 2009 (b)] have been used in the extraction of NMZs from plasma. SPE catridges of Extrelut [Aerts *et al.*, 1991] and Chromabond kieselguhr [Fraselle *et al.*, 2007] have been used in the sample purification in this matrix. Cronly *et al.*, 2009 (a) omits the use of SPE and clean up is achieved by purifying samples with the use of a hexane wash step and filtering before injection.

While suitable methods now exist for monitoring these analytes, no suitable methods in matrices such as milk and honey exist for the comprehensive confirmatory analysis of NMZs. The CRL for NMZs has suggested that honey be tested to ascertain any possible misuse and if non-compliant results are found then this matrix should be included in monitoring plans. They also state that countries with high milk production should also analyse for these analytes in milk as their possible misuse in this matrix cannot be discounted. From investigation of literature only two method could be found that allows for the analysis of NMZs in honey. Zhou *et al.*, 2007 published a method for the analysis of 5 NMZs in honey by HPLC-UV. Samples were extracted with ethyl acetate and evaporated. The residue containing the NMZs was dissolved in ethyl acetate–hexane and subjected to solid-phase extraction cleanup by amino extraction columns. The eluent was evaporated, reconstituted and injected onto the column. The second by Mol et al., 2008, was a multi-class multiresidue method for the analysis of veterinary residues in honey. While the method was confirmatory for eight nitroimidazole compounds it could only analyse to 10 $\mu g\ kg^{-1}$ which is three times higher the RL for these compounds.

In relation to the analysis of nitroimidazoles in milk there are a limited number of published methods available [Ortelli *et al.*, 2009; Stolker *et al.*, 2008, Thompson *et al.*, 2009]. These methods are all screening methods using either optical biosensor

[Thompson *et al.*, 2009] or time of flight mass spectrometers [Ortelli *et al.*, 2009; Stolker *et al.*, 2008]. From investigation of literature there are no methods for the confirmatory analysis of NMZs in milk at the levels desired.

6.2.2. Chloramphenicol

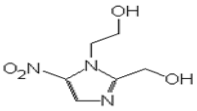
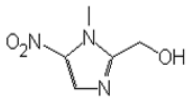
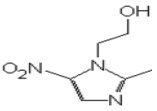
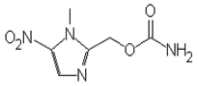
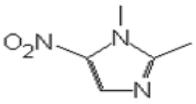
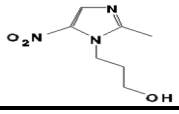
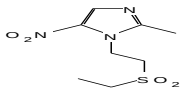
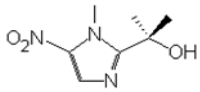
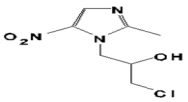
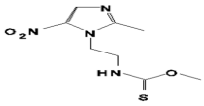
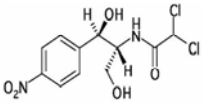
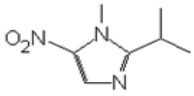
Chloramphenicol (CAP) is a broad spectrum bacteriostatic antibiotic that exerts its effect by inhibiting bacterial protein synthesis. The structure of CAP is seen in table 6-1. Research carried out on CAP has shown that it has adverse toxicological affects in human such as aplastic anaemia or grey-syndrome. Due to these health concerns it has been classified as an A6 compound in annex IV of council regulation 2377/90 which means it is prohibited for use in food producing species. CAP has been issued a minimum required performance limit (MRPL) of 0.3 ng mL^{-1} which means all methods should be able to at least see to this level. As a result of this low level; CAP is often analysed in single analyte methods [Rejtharova *et al.*, 2009; Ronning *et al.*, 2006] although some multi amphenicol methods do exist [Zhang *et al.*, 2008; Shen *et al.*, 2009]. In addition to this there are two other multiclass methods which include the analysis of CAP [Lopez et al., 2008 and Sheridan et al., 2008.] These analyse for CAP with compounds that are not prohibited for use such as sulfonamides and tetracyclines.

A common trend in analysis of CAP in any matrix is the use of an SPE clean-up. Common cartridge chemistries used in the analysis of CAP are Oasis HLB [Shen *et al.*, 2009], Mixed Cation Exchange (MCX) [Zhang *et al.*, 2008] and the selective technique of Molecular Imprinted Polymers (MIPs) [Rejtharova *et al.*, 2009; Boyd *et al.*, 2007] An investigation into the methods used to analyse for CAP found that it is often analysed singly in several matrices including milk and honey. Ronning *et al.*,

2006 developed a method that anaylse for CAP residues in meat, seafood, egg, honey, milk, plasma and urine with liquid chromatography–tandem mass spectrometry. Samples were extracted with acetonitrile and chloroform was added to remove water. Extracts were then evaporated to dryness, reconstituted and filtered before injection. Rejtharova *et al.*, 2009 described a method for the analysis of CAP in urine, feed water, milk and honey samples by GC-MS-NCI using molecular imprinted polymer clean-up.

On examining published literature it is quite clear that the confirmatory analysis of NMZs in milk and honey has not been examined to date. No confirmatory methods could be found for the analysis of these analytes in the matrices of honey and milk. With growing concerns from China about the use of NMZs in honey the CRL having recommended that this matrix be examined. The CRL have also recommended that countries with high milk production should analyse for these compounds in milk. Therefore there is a need for a comprehensive method to allow for the confirmatory analysis of both these matrices. In addition to this, while the analysis of CAP in all matrices is being performed it is often performed using single analyte methods which contain time consuming SPE clean-up steps. From literature investigation no method was found that was capable of analysing for CAP and the eleven NMZs listed in this paper. The sample preparation described in this study is less time consuming than previously published methods. The milk method has a sample size of 1ml which allows for fast extraction times. The majority of methods for the analysis of CAP incorporate the use of an SPE clean up. The method presented here omits this step and still achieves satisfactory results.

Table 6-1: Structures, Retention time (Rt), Precursor and Product ions and typical ion ratios for all 12 analytes

Compound	Structure	Rt	Precursor(M/z)	Product (M/z)	Collision Energy	Typical Ion Ratio
MNZ-OH 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole		1.34	188	123	17	0.71
				126	23	
HMMNI 2-hydroxymethyl-1-methyl-5-nitroimidazole		1.77	158	110	20	0.73
				140	17	
Metronidazole (MNZ) 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole		2.11	172	82	33	0.70
				128	19	
Ronidazole (RNZ) 1-methyl-2-[(carbamoyloxy) methyl]-5-nitroimidazole		2.38	201	140	17	0.33
				55	29	
Dimetridazole (DMZ) 1, 2-dimethyl-5-nitroimidazole		3.05	142	96	21	0.30
				81	31	
Ternidazole (TRZ) 2-Methyl-5-nitroimidazole-1-propanol; 3-(2-Methyl-5-nitroimidazol-1-yl)propan-1-ol		3.69	186	128	19	0.46
				82	37	
Tinidazole (TNZ) 1-(2-ethylsulfonylethyl)-2-methyl-5-nitroimidazole		3.97	248	121	23	0.19
				82	47	
IPZ-OH 1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole		4.09	186	168	19	0.50
				122	27	
Ornidazole (ORZ) 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole		4.11	220	128	23	0.58
				82	39	
Carnidazole (CNZ) 1-(2-ethylcarbamothioic acid O-methyl ester)-2-methyl-5-nitroimidazole		4.27	245	118	21	0.17
				75	43	
Chloramphenicol (CAP) 2,2-dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide		4.25	321	257	-16	0.90
				152	-12	
Ipronidazole (IPZ) 2-isopropyl-1-methyl-5-nitroimidazole		4.32	170	124	25	0.65
				109	33	

Materials and Methods

6.3.1. Materials and Reagents

CAP and CAP-d5 were purchased from Sigma (St. Louis, MO) and Dr. Ehrenstorfer GmbH(Germany) respectively. CNZ, TNZ, TRZ, ORZ were provided by the CRL (BVL, Berlin, Germany). RNZ and DMZ were purchased from Sigma (St. Louis, MO) and MNZ, IPZ, IPZ-OH, HMMNI, MNZ-OH, HMMNI-d3, MNZ-OH-d2, DMZ-d3, RNZ-d3 were purchased from WITEGA Laboratorien (Berlin, Germany). Water is of LC-MS grade (Fluka). All other solvents were of LC grade and purchased from Reagecon (Clare, Ireland). Sodium chloride was AnalaR grade and purchased from VWR (Poole, England). Individual stock standards of each analyte at 1mg ml^{-1} in ethanol were prepared and stored at 4˚C for 1 year. Individual intermediate standard solutions (10,000 and 100 ng ml^{-1}) in methanol were prepared. Two working standards solutions (mixture of analytes) were prepared in methanol containing all NMZs at levels of 300ng ml^{-1} and 100ng ml^{-1} respectively (for honey) and CAP at levels of 30ng ml^{-1} and 10ng ml^{-1} respectively (for milk). Deuterated standards were prepared similarly except the mixed standard contained 200ng ml^{-1} deuterated NMZs and 20ng ml^{-1} deuterated CAP.

6.3.2. LC-MS/MS Instrumentation

The LC-MS/MS system is an AB Sciex Triple Quad 5500 couple to Shimadzu UFLC XR LC system. The instrument is controlled by Analyst Software 1.5 and operated in positive and negative electrospray ionisation (ESI +/-).

Separation was achieved using a 100x2mm, 1.8 micron particle size Zorbax Eclipse Plus C18 column supplied by Agilent Technologies (Santa Clara, CA). The column

oven temperature was set at 45 ºC. The chromatographic separation was achieved using gradient mode consisting of water acidified with 0.1% acetic acid (mobile phase A) and acetonitrile acidified with 0.1% acetic acid at flow rate 0.5ml min^{-1}. The gradient is as follows; 95% A for the first 1.5 min. Then this changes to 5% A from 1.5-3.0 min and maintained for 2.0 min. The conditions then return to the initial 95% A in 1.0 min (5-6min) and remain the same till the end of the run of 9.0 min. A divert valve is utilised to help remove any matrix impurities from entering the MS/MS. The LC flow is diverted for the first minute and the last three minutes of the method. The ionisation mode used was positive electrospray ionisation for the NMZs and negative electrospray ionisation for CAP. The MS/MS method was segmented in order to obtain enough data points on each peak. The first three minutes is run in positive mode and from then on it is run with positive and negative switching. A source temperature of 650ºC with a spray voltage of 4500V was used to produce parent to product ions. The individual precursor and products ions for each analyte with their respective collision energies are listed in table 6-1.

6.3.3. Milk and Honey Samples

Milk and honey were obtained and stored at -20 ºC. Portions of these samples were analysed and those found to contain no detectable residues of the analytes of interest were used as blanks for the validation study. Chromatograms of blank milk and honey samples can be seen in figure 6-1 and 6-2. For the day four validation studies of variability due to matrix variances a wide range of milk and honey samples were obtained. Milk samples comprised of high fat, low fat, organic milk and also milk with added extra calcium, folic acid and vitamins A, B, D and E, a sample of goat's milk was also examined. Honey samples comprised of the following types

Kapetanios Pure Greek; Rowse Australian Eucalyptus; Capilano Australian Organic Blended; Tropical Forest Ltd. Ethiopian Forest; Marks and Spencer's New Zealand Clover; Famille Michaud-Lavender Honey, Provence, France; New Zealand Manuka Honey, Irish Honey ; Irish Honeycomb, Dublin; De Rit Blended Flower, Holland.

6.3.4. Methods

6.3.4.1. Milk Extraction

Milk (1mL) was pipetted into polypropylene centrifuge tubes (15mL). These were fortified with mixed internal standard (30 µL) which corresponded to 6 ng mL^{-1} of deuterated NMZs and 0.6 ng mL^{-1} deuterated CAP. Acetonitrile (2mL) was added and vortexed. NaCl (0.5g) was added to this slurry which was shaken (30 secs) and then centrifuged (4350 x g for 10min). The top organic layer from each sample was then transferred to amber vials (5mL) and evaporated (50ºC) to dryness under a stream of nitrogen. The extracts were reconstituted in Water: Acetonitrile (95:5, 200µL) and filtered through 0.2µm PVDF syringe filters. An aliquot (10µL) was injected onto the LC column.

6.3.4.2. Honey Extraction

Honey (3 g) was weighed into polypropylene centrifuge tubes (50 mL). These were then placed in an oven at 50 ºC for 30 min to soften. The samples were then fortified with mixed internal standard (50 µL) which correspond to 2 µg kg^{-1} of deuterated NMZs and 0.2 µg kg^{-1} deuterated CAP. Water (5ml) was then added to each sample and these are then placed back in the oven for a further 10 min. The samples were then thoroughly vortexed until the honey was fully dissolved in the water. To this acetonitrile (10 mL) was added and the tubes were vortexed (20secs). NaCl (2 g) was

added to this slurry which was then shaken (30secs) and centrifuged (4350 x g for 10min). The top organic layers were then transferred to polypropylene tubes (15 mL) and evaporated (50ºC) to 6mL under nitrogen. Hexane (5 mL) was added and this was vortexed (30secs). The hexane layer was then discarded and the extracts were evaporated to dryness at 50ºC under a nitrogen stream. They were then reconstituted in Water:ACN (200 µL of 95:5 and filtered through 0.2 µm PVDF syringe filters. An aliquot (10 µL) was injected onto the LC column.

6.3.5. Matrix Matched Calibration Curves

Quantitation was carried out using matrix-matched calibration curves. Blank honey and milk samples were used. These samples were fortified with mixed working standard and submitted to the full extraction procedure of the method. A matrix matched calibration curve is performed with every batch. Six samples are fortified with internal standard and mixed working standard for a calibration range of 0 to 20 ng mL^{-1} (µg kg^{-1} Honey) for the NMZs and a range of 0-2 ng mL^{-1} (µg kg^{-1} Honey) for CAP. Calibration curves were prepared by plotting the response factor (the ratio of peak area analyte over peak area of internal standard) against analyte concentration. Seven deuterated internal standards are used; d3- DMZ, d3-RNZ, d3-HMMNI, d2-MNZ-OH, d3-IPZ, d3-IPZ-OH and d5-CAP. For those compounds with no deuterated analogues; MNZ, TRZ, TNZ, ORZ and CRZ, d3-HMMNI is used as an internal standard. For each analyte calibration curves were linear in the given range with a correlation coefficient of at least 0.99.

6.3.6. Method Validation

The LC–MS/MS method was validated according to 2002/657/EC guidelines. The same validation protocol was used for both the honey and milk matrix. LC–MS/MS identification criteria were verified throughout the validation study by monitoring relative retention times, ion detection (signal-to-noise ratio (S/N)) and relative ion intensities. LC-MS/MS identification criteria as set out in the legislation were verified throughout the validation of the method.

Several method validation parameters were determined including linearity, specificity, recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (decision limit CCα and detection capability CCβ). Specificity was determined by analysing 10 different blank milk and honey samples sourced from different suppliers. No interfering peaks were observed at the retention time for any of the transitions. This allows for clear identification and quantification of all analytes. To investigate the linearity of the method, matrix-matched calibration curves were prepared and run with each of the validation batches to give 6 point calibration curves ranging from 0 to 20μg L^{-1} / μg kg^{-1} for the NMZs and 0 to 2μg L^{-1} / μg kg^{-1} for CAP.

Since no certified reference materials were available for the analytes and matrices of interest, the recovery from fortified negative samples was measured as an alternative to trueness. The recovery and precision were determined through the analysis of negative milk and honey samples fortified in six replicates at 1, 1.5 and 2 times the RL and the MRPL of 3 and 0.3 μg L^{-1} / μg kg^{-1} for NMZs and CAP respectively. Six replicate test portions at each of the three fortification levels (n = 18) were analysed on three separate days over a period of two weeks. Samples were fortified with

NMZs at 3, 4.5 and 6 μg L^{-1} / μg $kg^{-1}$and with CAP at 0.3, 0.45 and 0.6 μg L^{-1} / μg kg^{-1} by adding mix working standard solution (30, 45 and 60 μL) and analysed. To determine any matrix effects caused by biological variations arising from various milk and honey samples a fourth day analysis was carried out. For each matrix two sets of ten different samples were analysed. The first set was fortified with only internal standard, and the second set was fortified with both internal standard and with the analytes at a concentration equivalent to 4.5μg L^{-1} / μg $kg^{-1}$of NMZs and 0.45μg L^{-1} / μg kg^{-1} of CAP. From these four separate validation days an estimation of recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (decision limit CCα, and detection capability CCβ) were determined.

6.4. Results and Discussion

6.4.1. Method Optimisation

The method was developed to provide confirmatory data for the analysis of CAP and 11 NMZs in milk and honey. The method was developed from an existing method used in the lab to analyse for NMZs in plasma and eggs [Cronly *et al.*, 2009(a), (b)]. The LC-MS/MS instrument used for this method was different than the one used previously so fragmentation conditions were investigated and collision energies were optimised for each individual compound to give best response. For a method to be deemed confirmatory under Commission Decision 2002/657/EC it must yield 4 identification points. In this method a precursor ion (parent mass) and two product ions (corresponding to quantifier and qualifier ion) are monitored for each analyte (Table 6-1). This yields 4 identification points (1 for the precursor ion and 1.5 for

each product ion) hence it can be deemed a confirmatory method. In addition to this relative retention times and ion ratios are tracked for each compound and ensured that they are within acceptable ranges stated in EC 2002/657. The LC gradient was also optimised in order to have a quick run time but also have enough data points for each peak. For a method to achieve reliable quantitation each analyte peak should have at least 10-12 data points. As this method involved positive and negative ionisation switching the MS/MS method had to be segmented. This along with altering the LC gradient allowed for the analysis of all 12 analytes in a complete run time of less than 9 minutes.

In developing the milk method; the sample size was reduced to as low as possible to allow for a more efficient extraction method with reduced extraction costs as 1ml of milk was extracted with 2ml of acetonitrile with the addition of 0.5g of NaCl. The extract was then evaporated to dryness and reconstituted in initial mobile phase. The samples were filtered and run on the LC-MS/MS. The instrument was sensitive enough to see all the analytes at low concentrations with a sample size of only 1ml.

The matrix of honey is more complex and not much work had been carried out previously on it. The majority of methods use a SPE clean-up and this was overcome by adapting the previous methods used for aqueous based matrices (eggs and plasma). The honey sample was first diluted in water before extraction with acetonitrile and the addition of NaCl. Initial studies saw that the honey was quite difficult to dissolve in the water so the honey was first softened in an oven before the addition of water and this allowed the honey to dissolve fully. It was also seen that when acetonitrile was added to this solution that two layers formed after shaking. It was decided to take top organic layer and investigate if the analytes had been extracted into this layer. It was clear upon evaporation of this layer that some honey

had been taken into the layer and therefore could not be reconstituted. This did not occur when NaCl (2g) was added to the mixture and shaken. The extract was hexane washed and evaporated to dryness. The samples were reconstituted and filtered before been run on the LC-MS/MS. This is a much less involved extraction than used previously with these analytes in honey but results achieved were still satisfactory.

6.4.2. Validation

Validation is carried out in accordance with the procedures outlined in Commission Decision 2002/657/EC covering specificity, calibration curve linearity, accuracy, precision, decision limits (CCα), decision capability (CCβ) and measurement uncertainty. Results are seen in table 6-2 for these criteria. The ruggedness of the method is demonstrated on an ongoing basis through the use of it to analyse National Residue Control Plan milk and honey samples in Ireland. The criteria of relative retention times (RRT) and ion ratios were monitored for all analytes in the four validations days. The values identified for these were all within European requirements. The RRT tolerance of 2.5% was adhered to when standards were compared to samples in the validation runs. Two transition ions were monitored for each of the twelve analytes. The most intense was used for quantitation. All ion ratios of samples were within tolerances as set out by European criteria when compared with standards used during validation.

Table 6-2: Validation Results for Milk and Honey; Coefficient of Variance (%CV), Accuracy, Decision Limits (CCα) and Detection Capabilities (CCβ), Measurement Uncertainty (MU) and Correlation Coefficients (R^2).

Analytes	CV%		Accuracy %		CCα µg L^{-1} / µg kg^{-1}		CCβ µg L^{-1} / µg kg^{-1}		MU %		R^2 Value
	Milk	Honey	Milk	Honey	Milk	Honey	Milk	Honey	Milk	Honey	
MNZ-OH	4.5	4.1	92.0	103.8	0.43	0.39	0.73	0.67	27	25	>0.99
HMMNI	4.1	4.6	94.5	104.2	0.42	0.42	0.71	0.71	27	29	>0.99
MNZ	7.8	6.7	92.6	108.9	0.65	0.72	1.10	1.22	30	51	>0.99
RNZ	4.3	3.5	94.3	102.4	0.41	0.38	0.70	0.66	23	24	>0.99
DMZ	9.2	9.4	96.2	94.7	1.08	0.73	1.83	1.24	40	42	>0.99
TRZ	5.4	9.0	93.9	102.0	0.53	0.78	0.91	1.34	23	56	>0.99
TNZ	15.3	12.4	90.5	104.4	1.55	1.16	2.64	1.98	61	64	>0.99
IPZ-OH	4.8	6.2	94.2	100.8	0.46	0.64	0.78	1.10	20	39	>0.99
ORZ	11.4	11.3	90.8	100.7	1.08	1.00	1.85	1.71	69	81	>0.99
CRZ	10.8	10.7	91.7	101.0	1.23	0.95	2.09	1.62	40	90	>0.99
CAP	7.5	8.0	95.1	100.7	0.07	0.08	0.11	0.13	32	31	>0.99
IPZ	4.7	4.3	95	97.8	0.52	0.40	0.88	0.68	25	35	>0.99

6.4.2.1. Specificity

The technique of LC-MS/MS itself offers a great deal of specificity and selectivity. To establish the specificity and selectivity of the method blank milk and honey samples and samples fortified with all 12 analytes were analysed over the 3 validation days. On the fourth day 10 different types of milk and honey samples were also analysed. Blank samples showed no interfering peaks in the area of interest for any of the analytes. Chromatograms of blank milk and honey and milk and honey fortified at the RL and MRPL for each of the analytes are seen in figure 6-1 and 6-2.

6.4.2.2. Linearity

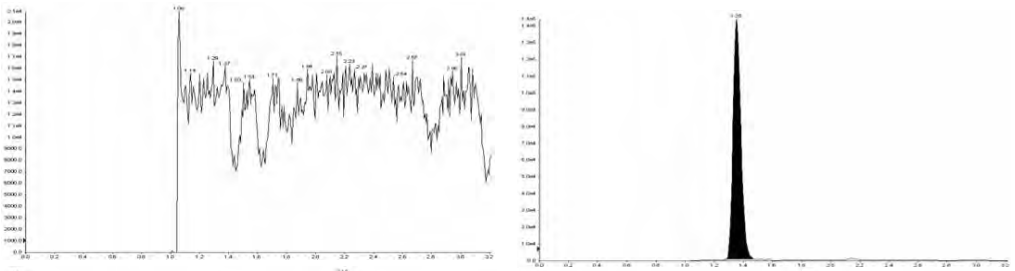
The linearity of the chromatographic response was tested with matrix matched calibration curves using six calibration points in the range of 0-20 μg L^{-1} / μg kg^{-1} NMZs and 0-2.0 μg L^{-1} / μg $kg^{-1}$for CAP. The regression coefficients for all the analytes on each of the validation days in both matrices were greater than 0.99.

6.4.2.3. Accuracy/Trueness

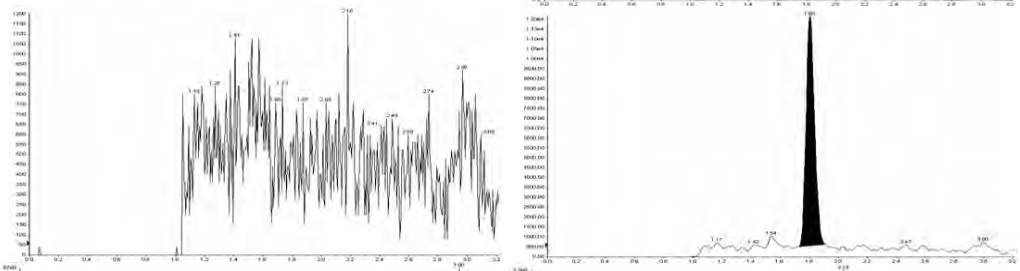
The accuracy (trueness) of the method was determined by fortifying 6 replicate milk and honey samples at 1, 1.5 and 2 times the analytes respective RL or MRPL on three separate days. Mean corrected recovery (n=6) of the analytes, determined in the three separate validation batches are shown in table 6-2 range between 90.8 and 108.9% for the twelve analytes in both matrices. No absolute recovery was determined as the use of internal standards means that each sample is individually corrected for.

Compound ***A*** ***B***

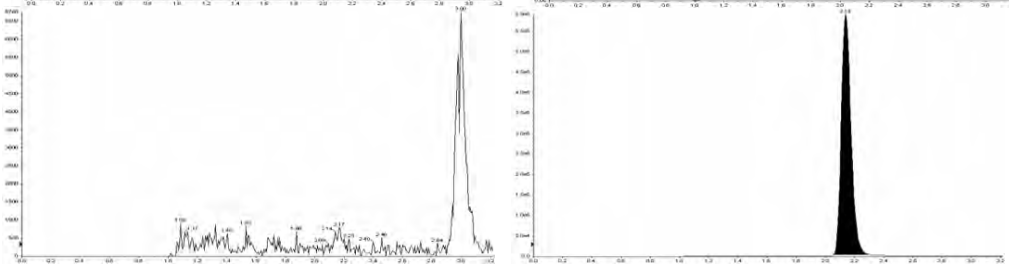
MNZ-OH



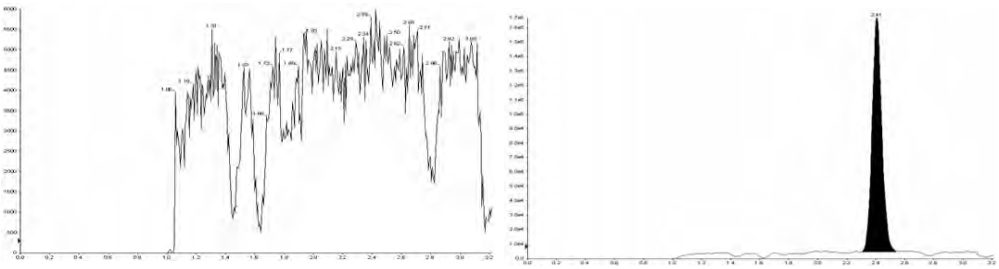
HMMNI



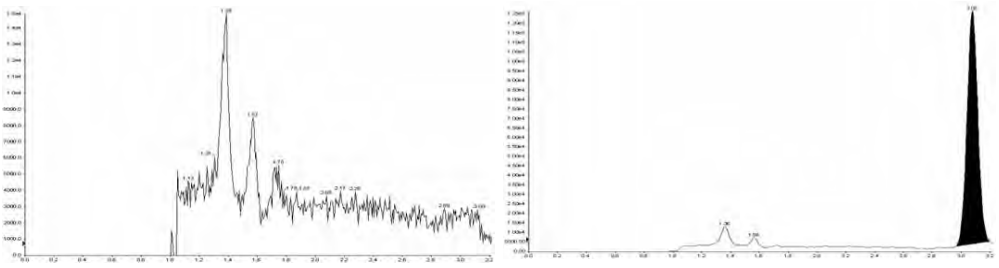
MNZ



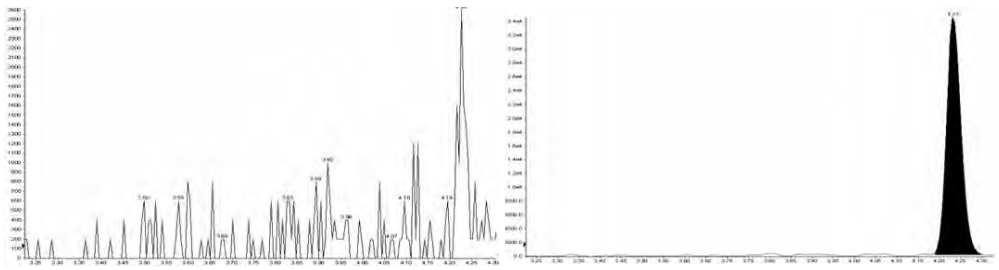
RNZ



DMZ



CAP



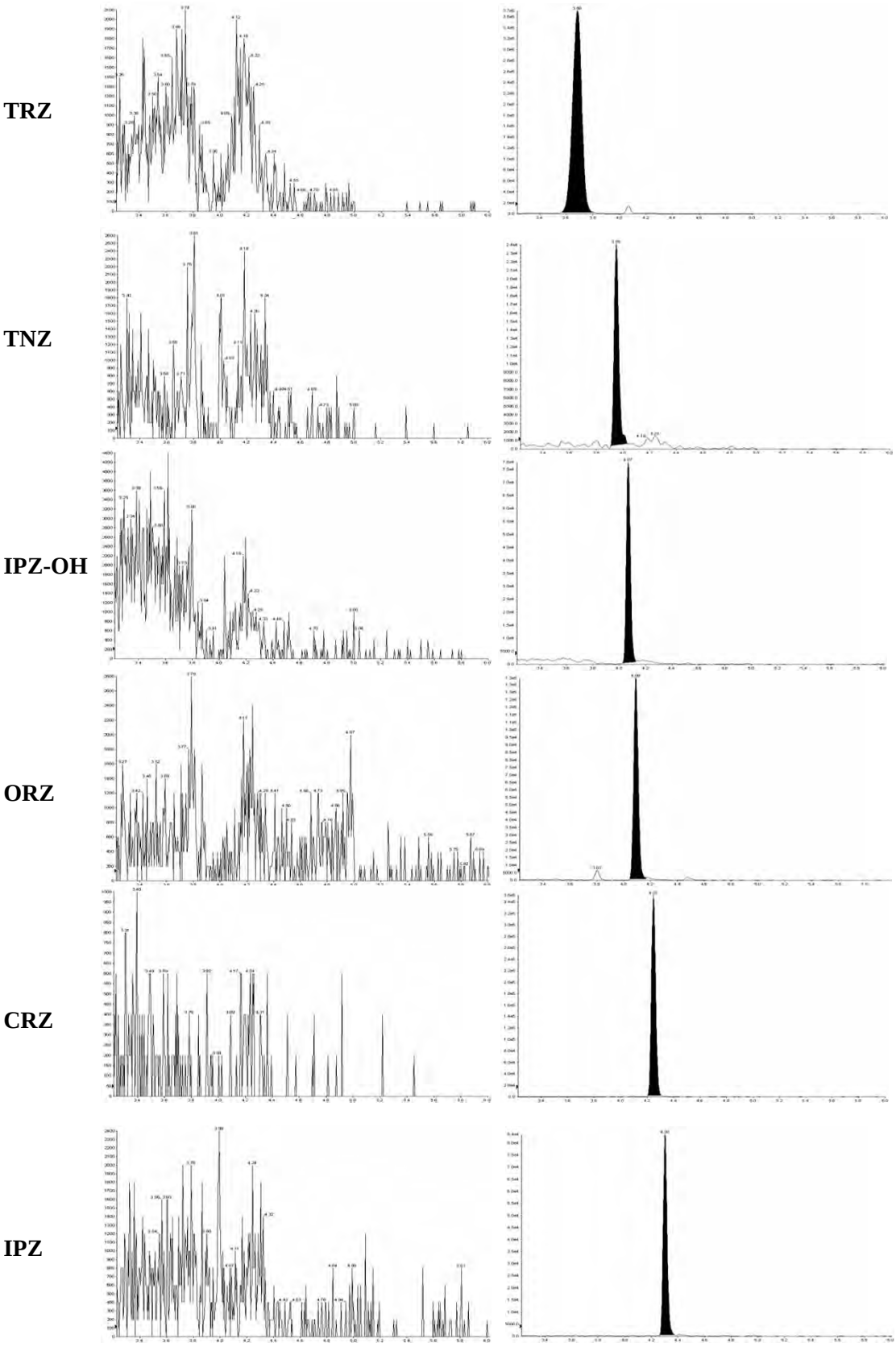
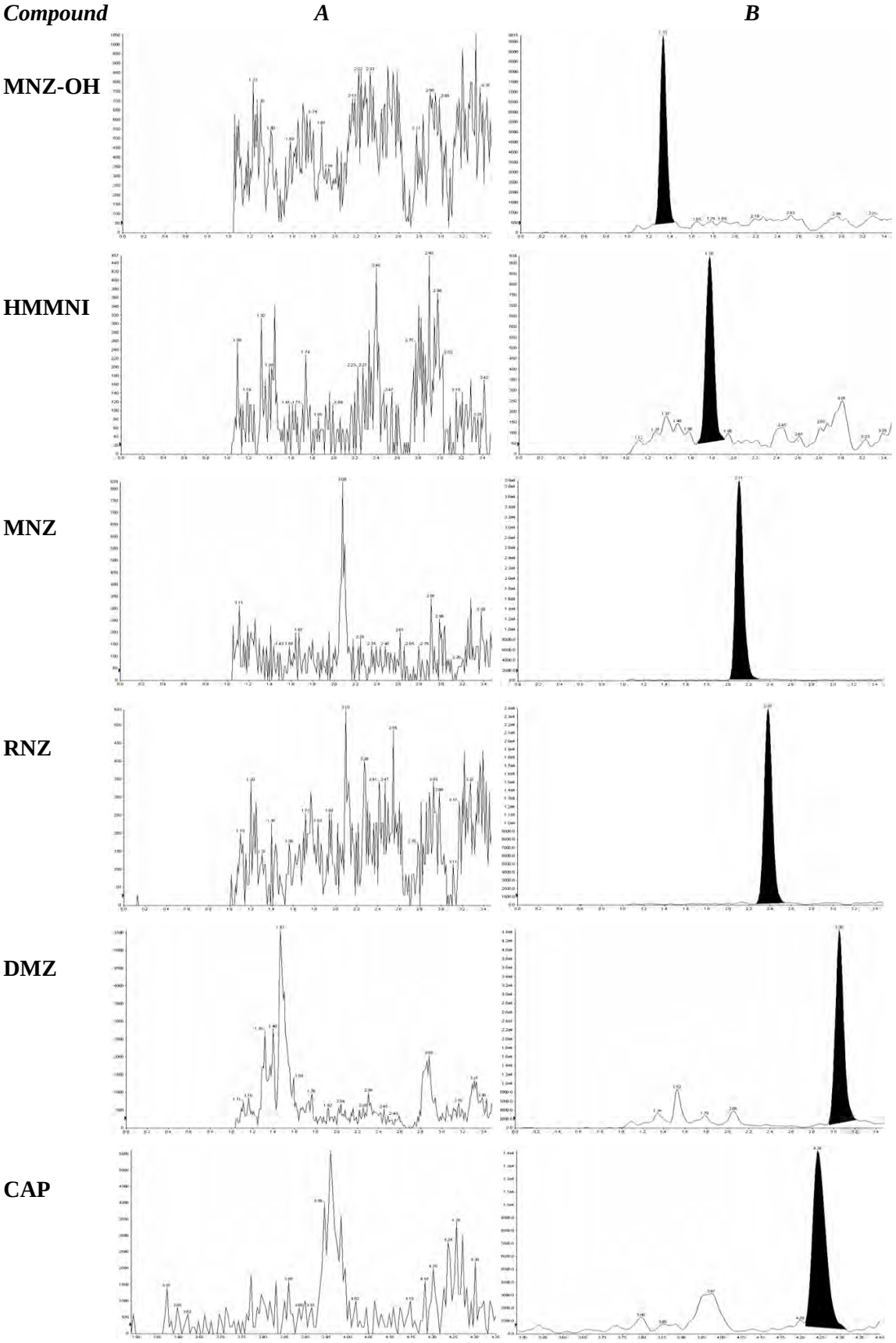


Figure 6-1. Chromatogram of blank milk (A) and milk (1ml) fortified at 2.5μg L^{-1} for NMZs and 0.25μg L^{-1} for CAP (B).


Compound
A
B
MNZ-OH
HMMNI
MNZ
RNZ
DMZ
CAP

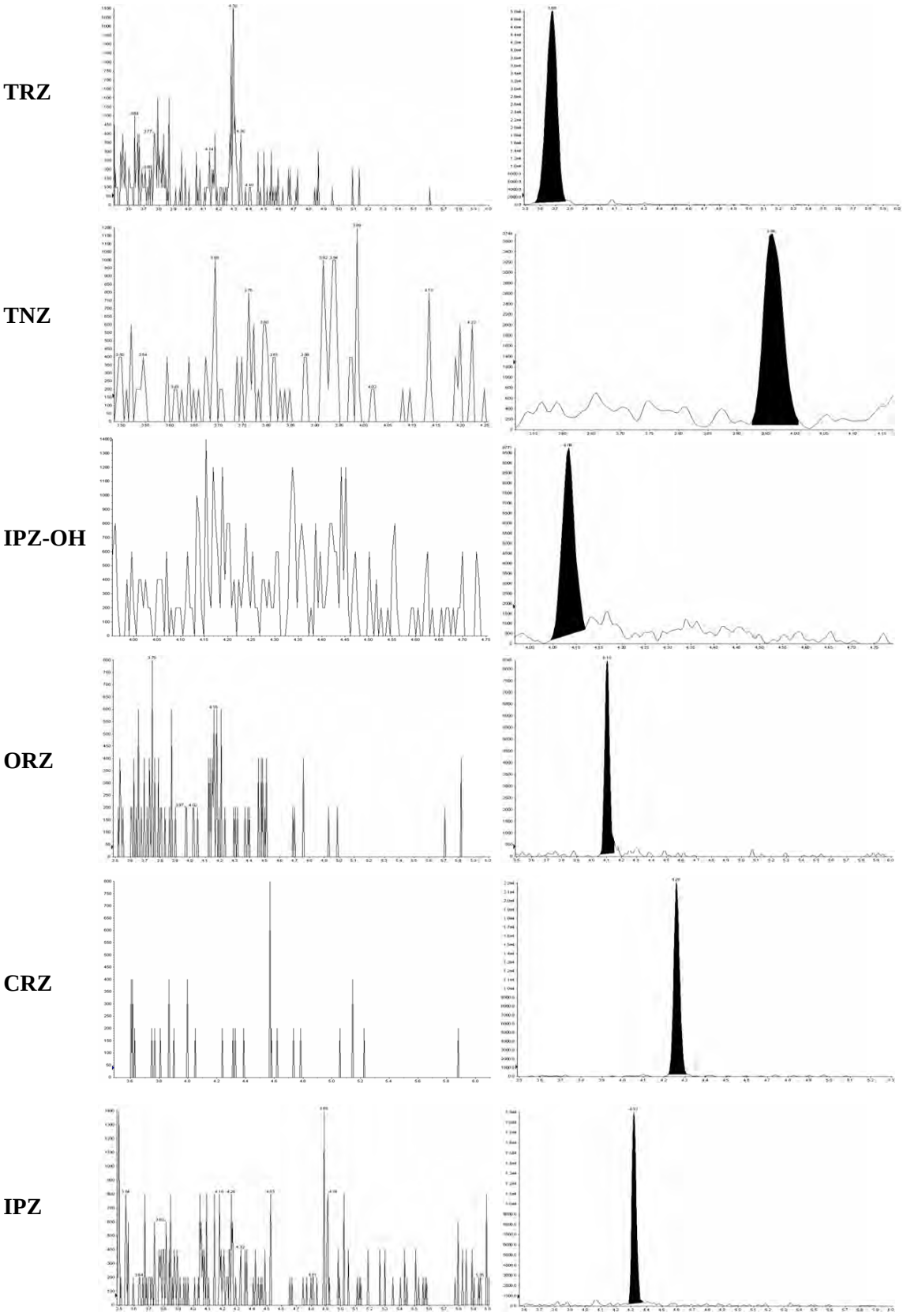


Figure 6-2: Chromatogram of blank honey (A) and honey (3g) fortified at 2.5μg kg^{-1} for NMZs and 0.25 μg $kg^{-1}$for CAP (B)

6.4.2.4. Precision

Satisfactory values for inter-assay precision expressed as %CV values for the within lab reproducibility (table 6-2) were achieved for all analytes in both matrices. According to Commission Decision 2002/657/EC this coefficient of variance for the repeated analysis of fortified material under reproducible conditions shall not exceed the level calculated by the Horwitz equation. For a concentration of 100 μg L^{-1} / μg kg^{-1} this equation gives a value of 23%. However when concentrations go under this value the equation gives unacceptably high results. Therefore its stated in Commission Decision 2002/657/EC that %CV should be kept as low as possible. Results achieved range from 3.5 to 15.3% for all analytes in both matrices. These acceptable results can be attributed to the availability of 7 deuterated analogues to use as internal standards. HMMNI-d3 was used as an internal standard and worked well for compounds with no deuterated internal standards such as CNZ, ORZ, TRZ and TNZ.

6.4.2.5. CCα and CCβ

CCα is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. For prohibited substances an α value equal to 1 % is applied. CCβ is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of 1-β, where β = 5 %. CCα and CCβ were calculated using the calibration curve procedure in accordance with ISO 11843. After identification, the signal is plotted against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept

equals the CCα. CCα values of for the 12 compounds in honey and milk are listed in Table 6-2 and are all below 1.55 μg L^{-1} / μg $kg^{-1}$for the NMZs and below 0.08 μg L^{-1} / μg kg^{-1} for CAP. CCβ is the concentration corresponding to the signal at CCα + 1.64 times the standard error of the intercept (i.e. the intercept + 3.97 times the standard error of the intercept). CCβ values for all compounds in honey and milk are listed in table 6-2 and are all below the RL of 3ng $mL^{-1}$and MRPL of 0.3 μg kg^{-1} for NMZs and CAP respectively.

6.4.2.6. Measurement Uncertainty

The measurement uncertainty (MU) was estimated by taking into account the within laboratory reproducibility over days 1, 2 and 3 as well as considering the repeatability on day 4 due to matrix effects caused by various honey and milk samples. These two variability's were combined and multiplied by a coverage factor of three to give an overall figure for the MU. This approach of using the within laboratory reproducibility as a good estimator of measurement of uncertainty is taken from the SANCO/2004/2726rev4 document. It recommends using the within laboratory reproducibility and using a coverage factor of 2.33 to estimate expanded uncertainty, however it was felt that not all the environmental factors that could be varied over the course of the validation were examined. Therefore a coverage factor of 2.33 may underestimate the true uncertainty of the method and instead a value of 3 was chosen to give a more realistic value for the true uncertainty. Values for MU are seen in Table 6-2 and lie between 23 and 69% for all compounds in milk and between 24 and 90% for all analytes in honey.

Higher MUs are seen in milk for some compounds with no deuterated analogues to use as internal standards which is expected. Honey results in general display higher MUs. High MU estimates are again seen for some of the compounds with no deuterated analogues to use as internal standards in particular ORZ (81%) and CRZ (90%). Their MU estimates in honey are the highest due to large values for reproducibility due to matrix as a result of a variation between matrices used in the day 4 experiment and the lack of suitable internal standards.

6.5. Conclusions

The objective of this work was to develop a rapid multi-class multi-residue confirmatory method capable of identifying, confirming and quantifying eleven NMZ compounds and CAP in milk and honey at μg L^{-1} and μg kg^{-1} levels and to validate according to the requirements in Commission Decision 2002/657/EC. This was successfully completed.

The method can be considered as rapid, as it utilises an efficient extraction protocol without the use of SPE. It also utilises chromatography which separates all analytes in a total run time of only 9 minutes. The method includes the confirmatory analysis of CAP and 11 NMZs in milk and honey which has not been seen before.

The obtained data fulfils the requirements laid down in Commission Decision 2002/657/EC and allows the calculation of all relevant performance characteristics. This study shows that the developed method meets the required sensitivities of 3 μg L^{-1} / μg kg^{-1} for NMZs and 0.3μg L^{-1} / μg $kg^{-1}$for CAP which are the RL and MRPL used for these compounds. The CCα and CCβ values determined for each analyte are lower than this level. The method performs very well in terms of accuracy and

repeatability for each of the analytes due to the utilisation of seven different deuterated internal standards. The values achieved for accuracy, %CV and measurement of uncertainty all fall within acceptable ranges. The applicability of the method for use on various types of milk and honey samples was demonstrated by the satisfactory results obtained from the Day 4 analysis of different species. The reduced number of analytical steps within the method makes it very amenable for high through-put regulatory monitoring of these compounds.

From examination of published literature, no method was found that was capable of the sensitive confirmatory analysis of CAP and eleven NMZs in milk and honey. Methods published on these matrices analysed at most seven analytes and in the case of CAP often analysed as a single analyte method. The method developed in this study allows for improvement on any existing method as it allows for the analysis of an increased number of analytes in matrices that have been previously overlooked. It also allows for reduced sample preparation times as SPE clean-up has been omitted. In the case of the extraction protocol for milk time and solvent usage is greatly reduced compared to other published methods as a result of reduced sample size of 1mL.

CHAPTER 7: DEVELOPMENT AND VALIDATION OF A RAPID MULTI-CLASS METHOD FOR THE CONFIRMATION OF FOURTEEN PROHIBITED MEDICINAL ADDITIVES IN PIG AND POULTRY COMPOUND FEED BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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7.1 Abstract

A confirmatory method has been developed to allow for the analysis of fourteen prohibited medicinal additives in pig and poultry compound feed. These compounds are prohibited for use as feed additives although some are still authorised for use in medicated feed. Feed samples are extracted by acetonitrile with addition of sodium sulphate. The extracts undergo a hexane wash to aid with sample purification. The extracts are then evaporated to dryness and reconstituted in initial mobile phase. The samples undergo an ultracentrifugation step prior to injection onto the LC-MS/MS system and are analysed in a run time of 26 minutes. The LC-MS/MS system is run in MRM mode with both positive and negative electrospray ionisation. The method was validated over three days and is capable of quantitatively analysing for metronidazole, dimetridazole, ronidazole, ipronidazole, chloramphenicol, sulfadiazine, sulfamethazine, dinitolimide, ethopabate, carbadox and clopidol. The method is also capable of qualitatively analysing for tylosin, virginiamycin and avilamycin. A level of 100 μg kg^{-1} was used for validation purposes and the method is capable of analysing to this level for all the compounds. Validation criteria of trueness, precision, repeatability and reproducibility along with measurement uncertainty are calculated for all analytes.

Keywords: Antibiotics; pig and poultry compound feed; lc-ms/ms; validation**.**

7.2 Introduction

The use of many antibiotics, coccidiostats and antibacterial growth promoters as feed additives has been prohibited in Europe since 2006 by Commission Recommendation 2005/925/EC. This recommendation lists medicinal substances that should be monitored and the substances are divided into two groups; medicinal substances authorised as feed additives for certain animal species or categories and medicinal substances no longer authorised as feed additives. This paper focuses on the analysis of the second group of medicinal substances specifically antibacterial growth promoters (AGPs) which are no longer authorised as feed additives; this group consists of various different types of compounds. Nitroimidazoles and chloramphenicol are banned for use in food producing animals. Other AGPs which include virginiamycin and tylosin are prohibited for use as feed additives. Finally some compounds such as sulfonamides are only permitted for use in medicated feed. The structures for all fourteen analytes are presented in Tables 7-1a and 7-1b.

Table 7-1a. Molecular Structures, Retention time (Rt), Precursor and Product ions and typical ion ratios for all 11 analytes

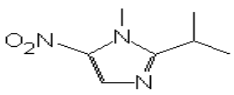
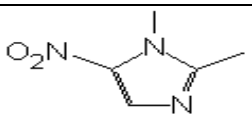
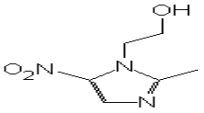
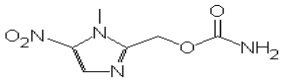
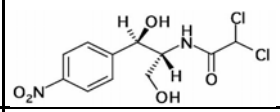
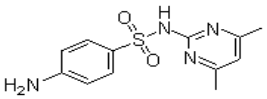
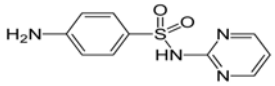
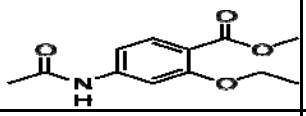
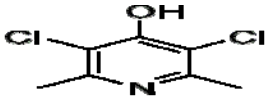
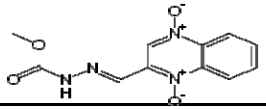
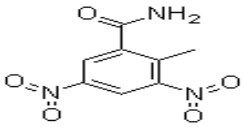
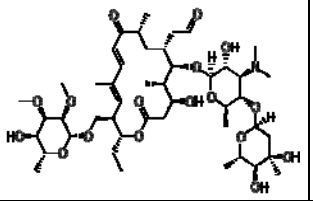
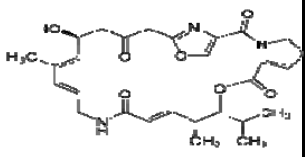
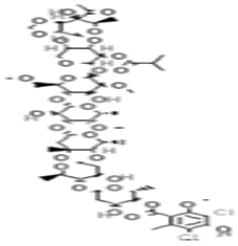
Compound	Molecular Structure	Rt	Precursor Ion (M/z)	Product Ions	Collision Energy
Ipronidazole (IPZ) 2-isopropyl-1-methyl-5-nitroimidazole		9.90	170	124	18
				109	25
Dimetridazole (DMZ) 1, 2-dimethyl-5-nitroimidazole		2.83	142	96	18
				81	28
Metronidazole (MNZ) 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole		1.95	172	82	25
				128	15
Ronidazole (RNZ) 1-methyl-2-[(carbamoyloxy) methyl]-5-nitroimidazole		2.21	201	140	15
				110	18
Chloramphenicol (CAP) 2,2-dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide		13.46	321	257	18
				152	12
Sulfadiazine (SDZ) 4-amino-N-pyrimidin- 2-yl-benzenesulfonamide		2.39	251	110	23
				156	17
Sulfamethazine (SMZ) 2-(p-Aminobenzenesulfonamido)-4,6-dimethylpyrimidine		4.29	279	186	17
				156	19
Ethopabate (EPB) methyl 4-(acetylamino)-2-ethoxybenzoate		14.16	238	136	32
				206	13
Clopidol (CLOP) 3,5-Dichloro-2,6-dimethyl-pyridin-4-ol		2.00	192	128	24
				101	26
Carbadox (CAR) methyl (2E)-2-[(1,4-dioxidoquinoxalin-2-yl) methylene]hydrazinecarboxylate		2.65	263	175	19
				130	22
Dinitolmide (DINIT) 2-Methyl-3,5-dinitrobenzamide		7.68	224	151	18
				181	12

Table 7-1b: Molecular Structures, Retention time (Rt), Precursor and Product ions and collision energies for all 3 analytes.

Compound	Molecular Structure	Rt	Precursor Ion (M/z)	Product Ions (M/z)	Collision Energy
Tylosin (TYL)		14.03	917	772	29
				174	37
Viginiamycin (VIR)		16.12	526	355	20
				508	15
Avilamycin (AVIL)		14.89	791	373	45
				391	48

Nitroimidazoles and chloramphenicol are classified as prohibited substances in table 2 of Commission Regulation 2010/37/EC and therefore prohibited for the use in animal husbandry. As a result these should not be found in animal feeds. While there are single class methods for the analysis of some of compounds [Vincent *et al.*, 2008; Galarini *et al.*, 2009; Pecorelli *et al.*, 2003; Barbosa *et al.*, 2007; van Holthoon *et al.*, 2010] there are very few published methods for nitroimidazoles and chloramphenicol in animal feed. Capitan-Vallvey *et al.*, 2007 describes a method for the analysis of nitroimidazoles in feed by LC-MS and Vinas *et al.*, 2006 describes a method for chloramphenicol in feed by LC-photo diode array detector.

The use of 5 AGPs including tylosin and virginiamycin were prohibited for this use in Council Regulation 2821/98. As a result there are some published methods for the

analysis of these compounds. Van Poucke *et al.* described a method for the analysis of tylosin and virginiamycin in feed by LC-MS/MS [Van Poucke *et al.*, 2003 Van Poucke *et al.*, 2005] and Civitareale *et al.*, 2004 describes a method for the analysis of tylosin by LC-UV/DAD. Other medicinal additives listed in 2005/925/EC also have LC methods for their analysis such as clopidol/nicarbazin [Dusi *et al.*, 2000], amprolium/ethopabate [Tan *et al.*, 1996] and carbadox [Kesiunaite *et al.*, 2008; Hutchinson *et al.*, 2005] while for compounds such as dinitrolimide no published methods exist for their analysis. The majority of methods published for the list of compounds specified in 2005/925/EC allow for the analysis of these compounds at levels relating to therapeutic level or in the mg kg^{-1} range while only a few allow for the analysis in the µg kg-1 range. Also, from examination of literature the majority of methods are single or dual analyte methods while very few are capable of analysing for a particular class of compounds.

From a review of the literature it would seem there is a lack of published methods available that would help with the enforcement of Commission Recommendation 2005/925/EC. In addition to this, methods available are for single analytes/classes at mg kg-1 range; often utilising large sample sizes which in turn need large amounts of solvent for extraction which can prove expensive and time consuming. Reports from the Screening and Identification Methods for official control of Banned Antibiotics and Growth promoters in Feedingstuffs study (SIMBAG-FEED study) suggested that methods be able to identify compounds to at least 5 times lower than the lowest contents formerly described in the Directive 70/524/CEE. In many cases this was around the 1ppm range [de Jong, 2005]. To aid compliance with Commission Recommendation 2005/925/EC there is a need for an efficient sensitive multi-class method to analyse for as many of the analytes listed in this recommendation as

possible. To this end; this paper describes the analysis of 14 of these prohibited medicinal additives at 100 µg kg^{-1} levels in pig and poultry compound feed by LC-MS/MS utilising a small sample size of 2 g and an efficient sample extraction procedure.

7.3 Materials and Methods

7.3.1 Chemicals and Reagents

Dimetridazole (DMZ), ronidazole (RNZ), chloramphenicol (CAP), sulfadiazine (SDZ), sulfamethazine (SMZ), dinitolimide (DINIT), ethopabate (ETB), carbadox (CAR), clopidol (CLOP) and sulfaphenazole (SPZ) were purchased from Sigma (St. Louis, MO, USA), metronidazole (MNZ), ipronidazole(IPZ), d3-IPZ, d3-DMZ, d3-RNZ were purchased from WITEGA Laboratorien (Berlin, Germany), d5-chloramphenicol were purchased from Dr Ehrenstorfer (Augsborg, Germany) and tylosin, virginiamycin and avilamycin were received from RIKILT (Wageningen, The Netherlands). Water was of LC-MS grade from Fluka (Germany). All other solvents were of LC grade and purchased from Reagecon (Clare, Ireland). Anhydrous Sodium sulphate was AnalaR grade and purchased from Acros (Geel, Belgium). Individual stock standards of each analyte ranging between 0.25-1.00 mg ml^{-1} in ethanol were prepared and stored at 4˚C. A working standard solution (mixture of analytes) (10 ug mL^{-1}) was prepared in acetonitrile and stored at 4º. Internal standards were prepared similarly.

7.3.2 Instrumentation

The LC-MS/MS system was a TSQ Quantum Ultra EMR coupled to a Finnigan Surveyor LC system. The instrument was controlled by Xcalibur software (Version

1.5). Separation was achieved using a (100 × 2)mm, 3µm particle size, Luna C18 column (Part No. 00D-4251-B0) protected by a Security Guard guard cartridge system (20×2)mm, both supplied by Phenomenex. The oven temperature was set at 40ºC. The chromatographic separation was performed in gradient mode using water acidified with 0.2% acetic acid (mobile phase A) and acetonitrile acidified with 0.2% acetic acid (mobile phase B), at a flow rate of 0.25mL min^{-1}. The initial conditions from 0-6min were 85% A. This was changed to 50% A over 2 minutes from 6-8min and was maintained until 10 min. The conditions were changed again to 10% A over 2 minutes from 10-12 min and these were maintained until 15.20 min. Finally the conditions returned to 85% A over 2.8 minutes from 15.20-18min and were maintained until the end of the run at 26min. Electrospray ionisation (ESI) was used in the MS with both positive and negative ionisation mode, with a spray voltage of 4350V and a cone temperature of 325 ºC. The individual precursor and products ions for each analyte with their respective collision energies are listed in Tables 7-1a and 7-1b.

7.3.3 Pig and Poultry Compound Feed Samples

Different varieties of pig and poultry compound feed were sourced from various feed mills. These were milled upon receipt to 1mm using a Retsch SM 100 mill and stored in amber jars at 4ºC. Portions of these samples were analysed and those found to contain no detectable residues of the analytes of interest except for residues of sulfadiazine were used as blanks for the validation study. To ensure true robustness of the method a high number of different feed samples were used in validation. These included 18 different pig feeds and 18 different poultry feeds. Chromatograms of blank feed can be seen in Figure 7-1b.

7.3.4 Extraction

Feed (2 g) was weighed into polypropylene centrifuge tubes (50 mL). The sample was fortified with mixed internal standard (50 µL) which corresponds to a concentration of 250 µg kg^{-1} of internal standard in the feed material. To this acetonitrile (12 mL) was added and the tubes were vortexed (20 secs). Anhydrous sodium sulphate (3.5 g) was added to this slurry which was shaken (30 mins) and centrifuged (5100rpm for 20 min). The organic layer was transferred to a clean polypropylene tube (15 mL) and evaporated at 50ºC to 6 mL under nitrogen. Hexane (5 mL) was added and the tubes contents were vortexed (30 secs) and centrifuged (3750 rpm for 20 min). The hexane layer was discarded and the extracts were evaporated to dryness at 50ºC under a nitrogen stream. The extract was reconstituted in water: acetonitrile (85:15, 800 µL) and vortexed thoroughly for 45 secs. The sample underwent an ultra-centrifugation step at 13750 rpm for 30 mins. This centrifugation step separated the sample into two distinct layers. 200 µL of the clear lower layer (containing the analytes) was transferred into an LC-MS vial. An aliquot (20 µL) was injected onto the LC column.

7.3.5 Matrix Extracted Calibration Curves

Quantitation was carried out using matrix extracted calibration curves. Blank pig and poultry feed samples were used. These samples were fortified with mixed working standard and submitted to the full extraction procedure. Matrix extracted calibration curves were performed with every batch. Six different feed samples are fortified with internal standard and mixed working standard yielding a calibration range of 0 to 1000 µg kg^{-1} for all the 11 quantitation analytes. Calibration curves were prepared by plotting the response factor (the ratio of peak area analyte over peak area of internal

standard) against analyte concentration. Five internal standards were used; d3- DMZ, d3-RNZ, d3-IPZ, d5-CAP and Sulfaphenazole. For those compounds for which no suitable deuterated internal standard could be acquired; MNZ, CLOP, DIN, ETB and CAR, d3-DMZ was used as an internal standard. For each analyte; calibration curves were linear in the given range with a correlation coefficient of at least 0.98. In the case of the 3 qualitative analytes, TYL, VIR and OLA no suitable internal standard could be found. This resulted in poor linearity as matrix effects could not be corrected for in a repeatable manner. For these analytes six different feed samples were fortified; one at 0 μg kg^{-1} and five at the 100 μg kg^{-1}. d3-DMZ was used as an internal standard for these in order to compensate for any extraction errors.

7.3.6 Method Validation

LC–MS/MS identification criteria were verified throughout the validation study by monitoring relative retention times and relative ion intensities. LC-MS/MS identification criteria as set out in the Commission Decision 2002/657 were verified throughout the validation of the method.

Several method validation parameters were determined including linearity, specificity, trueness, precision (repeatability and within-laboratory reproducibility). Specificity was determined by analysing a number of different blank animal feed samples sourced from different mills. To investigate the linearity of the method, matrix-extracted calibration curves were prepared and run with each of the validation batches to give 6 point calibration curves in the range of 0 to 1000 μg kg^{-1} for all eleven quantitation analytes. To ensure linearity across the range of different feed samples that could be encountered for these species a different type of feed alternating between pig and poultry was used for each calibration point.

Since no certified reference materials were available for the analytes and matrices of interest, the trueness from fortified negative samples was measured as an alternative to trueness. The trueness and precision of the method were determined through the analysis of negative pig and poultry compound feed fortified in six replicates at 100 µg kg^{-1}, 500 µg kg^{-1} and 1000 µg kg^{-1} with the eleven quantifiable analytes for a total of 18 samples. This was repeated on three separate days. For the three qualititative analytes all 18 samples were fortified at 100 µg kg^{-1}. The type of feed was varied for each of the six replicates in ordered to ensure that the method was fully fit for purpose. From these three separate validation days an estimation of trueness, precision (repeatability and within-laboratory reproducibility) and LC-MS/MS confirmatory criteria were all evaluated.

7.4 Results and Discussion

7.4.1 LC-MS/MS Optimisation

The LC-MS/MS method was developed to provide confirmatory data for the analysis of 14 antibiotics in pig and poultry compound feed. The MS/MS fragmentation conditions were investigated and collision energies were optimised for each individual compound to give best response. For a method to be deemed confirmatory under Commission Decision 2002/657/EC it must yield 4 identification points. In this method a precursor ion (parent mass) and two product ions (corresponding to strong and weak ion) were monitored for each analyte (Tables 7-1a and 7-1b). This yielded 4 identification points (1 for the precursor ion and 1.5 for each product ion) hence it can be deemed a confirmatory method. In addition to this relative retention times and ion ratios were monitored for each compound and evaluated to ensure that they are within acceptable ranges as stated in CD 2002/657/EC. As this method

involved positive and negative ionisation switching the MS/MS method had to be segmented. The LC gradient was optimised in order to have as an efficient run time as possible in order to allow successful segmentation of the MS/MS method. Only when the positive and negative ionisation switching was isolated to one segment was there enough data points for each peak. Lowering scan time and dwell time of the instrument was not sufficient to achieve this. For a method to achieve reliable quantitation each analyte peak should have at least 10-12 data points. The LC gradient along with segmentation permitted for the analysis of all 14 analytes in a complete run time of 26 minutes with each peak having a minimum of 12 data points.

7.4.2 Sample Extraction Development

The development of the extraction method faced two major obstacles; one the need to extract a wide variety of analytes with a single extraction and the other the need to purify the sample sufficiently without losing the analytes in question. A variety of extraction solutions including water, acetonitrile and methanol and various mixtures of the three were tested. Immediately it was visibly evident that methanol and water extracted far more matrix contaminants than acetonitrile and this resulted in lower recoveries for the analytes using these extraction solvents. It was also observed that acetonitrile consistently extracted the broad range of analytes therefore acetonitrile was chosen as the extraction solvent. The next stage was to sufficiently clean up the acetonitrile extract in order to determine down to the levels of interest. The use of anhydrous sodium sulphate in sample clean up when extracting these analytes has been previously seen. [Stubbings *et al.*, 2009]. Hence the use of anhydrous sodium sulphate was investigated followed by addition of a hexane wash step. This

purification procedure sufficiently removed background interferences resulting in the fact that a SPE clean-up step was not needed. The purification was completed when the reconstituted extract underwent an ultracentrifugation step. This removed further interferences and also allowed all analytes to be determined at levels in the μg kg^{-1} range.

7.4.3 Internal Standard Selection

While the extraction method allowed all analytes to be seen in the μg kg^{-1} range the variability in sample recovery was noticeable from feed sample to feed sample. To overcome this problem the sourcing of suitable internal standards was pursued. In some cases deuterated analogues were available for the analytes such as d3-DMZ, d3-IPZ, d3-RNZ and d5-CAP. These corrected well for all variabilities encountered in extraction. Sulfaphenazole is a sulphonamide and it has been used as an internal standard for sulfonamides in previous work [McDonald *et al.*, 2009]. This was used for SDZ and SMZ compounds and corrected sufficiently for them. Erythromycin was tried for use with VIR, TYL and AVIL but did not correct consistently well for them. As a last attempt the internal standards used for other compounds were used for the remaining compounds without internal standards. It was observed that d3-DMZ extracted consistently and as a result could be used as an internal standard for CLOP, CAR, DIN and ETH. This allowed for eleven compounds to be analysed quantitatively. For the remaining three compounds VIR, TYL and AVIL no suitable internal standard could be identified. Therefore the method could only be used as a qualitative extraction method for these compounds.

7.4.4 Validation Approach Selection

As of yet no official EU validation protocol exists for the analysis of veterinary drugs in animal feed. Therefore a validation protocol was designed in order to best show that the method was fit for purpose. It was seen in development the variability due to the matrix feed is significant. In order to prove that the method would extract all analytes in a wide range of pig and poultry compound feed it was decided that feed samples would be varied as much as possible. For each of the calibration curve points a different feed would be used on each validation day to ensure linearity held through for all feeds. Eighteen samples were analysed on each day of the three validation days containing six different types of animal. A level of 100µg kg^{-1} was chosen as a reporting level and this is significantly lower for the majority of the analytes presented in this paper than observed in previous methods. SIMBAG study suggested levels around 1000 µg kg^{-1} but it was felt that as these compounds are banned they should not be present at any level. These compounds are prohibited for use as feed additives and therefore these compounds should not be present at any level and therefore the ALARA (as low as reasonably achievable) principle was adopted. Work carried out prior to validation indicated that a level of 100µg kg-1 was achievable. This was chosen as it was felt that the method could be used to determine this level on a routine basis for all analytes. For the three qualitative analytes TYL, VIR, OLA it was decided that all eighteen samples on the three different days would be fortified at the reporting level of 100 µg kg^{-1}. The measurement of uncertainty for each analyte would be calculated and added onto the 100 µg kg^{-1}level and give us a value above which would result in a positive. For the eleven quantitative analytes a different approach was taken. The eighteen samples on

the three days would be made up of six replicates of 100, 500 and 1000µg kg^{-1}. This was done in order to validate the method over the complete calibration range for which positive results might be obtained. Specificity, trueness, precision (repeatability and within-laboratory reproducibility); along with confirmatory criteria laid out Commission Decision 2002/657 were determined during validation.

7.4.5 Specificity

The technique of LC-MS/MS itself offers a great deal of specificity and selectivity. To establish the specificity and selectivity of the method 18 blank pig and poultry compound feed samples and samples fortified with all 14 analytes were analysed over the 3 validation days. All blank samples showed no interfering peaks in the area of interest for any of the analytes except for sulfadiazine. This is as a result of low levels of sulfadiazine found in the majority of feed samples available. Sulfadiazine is still permitted to be used in medicated feed and this might possibly be the reason for low levels been found in the feed. In order to correct for this, the feed samples were analysed prior to validation and the response observed for SDZ was subtracted from the results achieved during the validation procedure. Although this corrected the results somewhat, the variability in the background sulfadiazine resulted in worse validation results for this compound than the others. Chromatograms of blank feed and feed fortified at 100 µg kg^{-1} for each of the fourteen analytes are seen in Figure 7-1a and 7-1b.

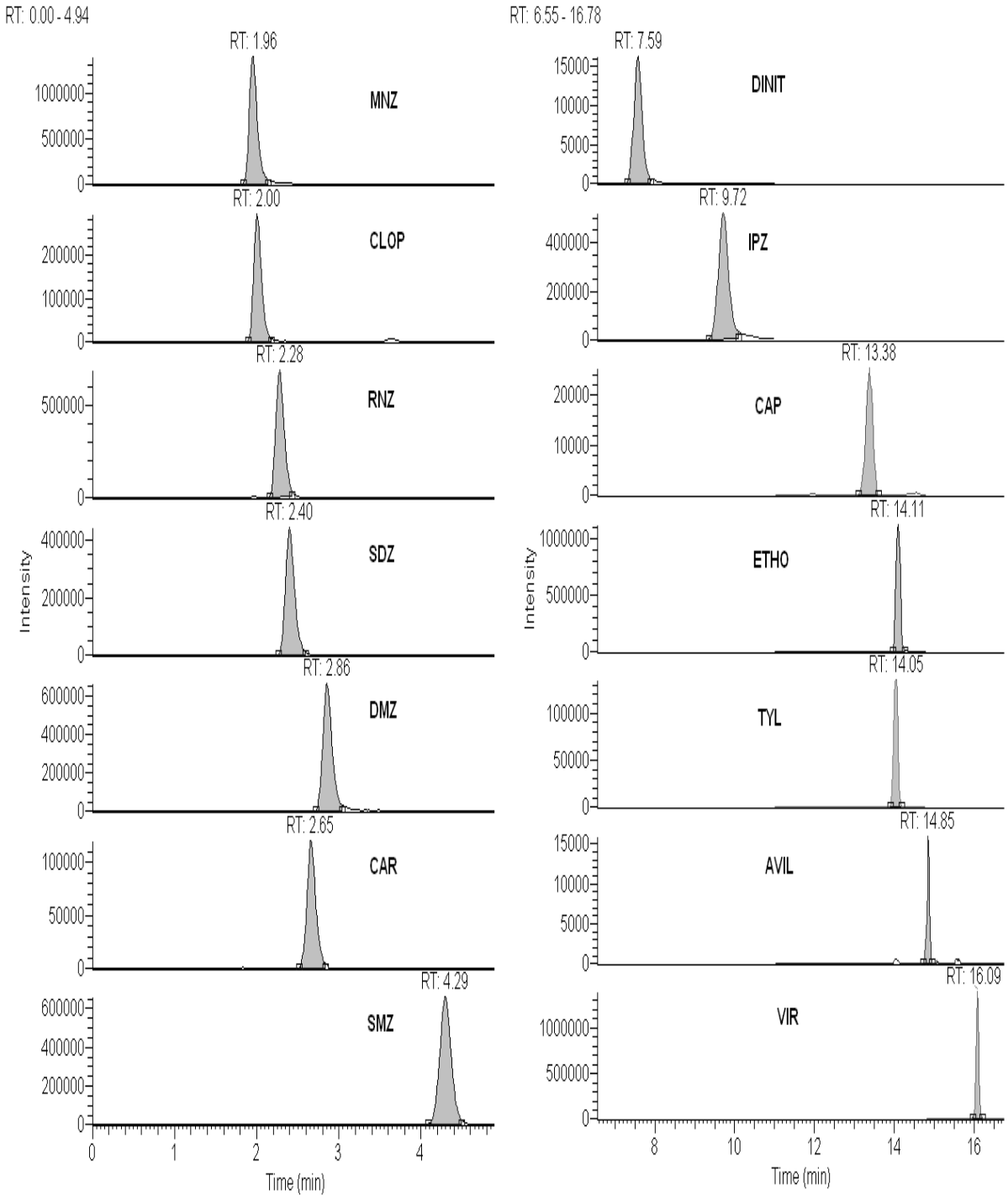


Figure 7-1a: Chromatograms of Feed Fortified at a level equal to 100 μg kg^{-1} for all 14 compounds.

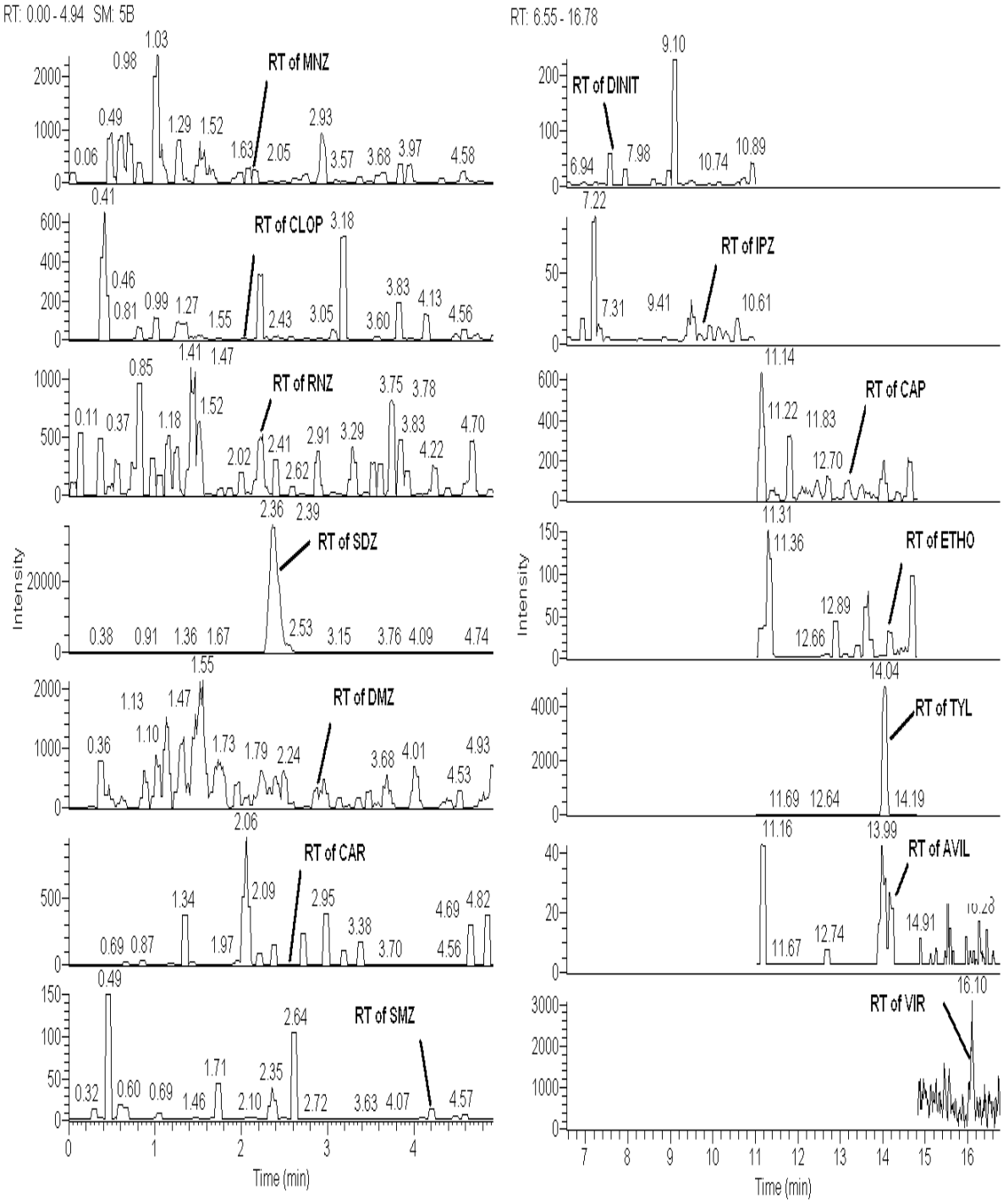


Figure 7-1b: Chromatograms of Blank Feed

7.4.6 Linearity of Response

The linearity of the chromatographic response was tested with matrix extracted calibration curves using six calibration points in the range of 0-1000µg kg^{-1} for all eleven quantitative analytes on each of the validation days. The regression

coefficients for all the analytes on each of the validation days in were greater than 0.98.

Table 7-2: Validation results for Accuracy, Repeatability, Reproducibility and Measurement Uncertainty (MU) and Confirmatory data of typical ion ratios and relative retention times(RRT) for all 14 analytes.

Analyte	Internal Standard	Accuracy (%)	Repeat %RSD	Reprod %RSD	MU (%)	Typical RRT	Typical Ion Ratio	Cut-Off Level µg/kg
DMZ	DMZ-d3	98.9	4.5	8.9	27	1.0100	0.2344	100
RNZ	RNZ-d3	99.1	6.3	9.0	27	1.0053	0.0395	100
MNZ	DMZ-d3	102.5	5.8	9.3	28	0.6911	0.2964	100
IPZ	IPZ-d3	99.4	4.3	7.2	24	1.0164	0.8382	100
SDZ	SPZ	101.4	23.3	28.0	84	0.1666	0.4667	100
SMZ	SPZ	101.4	16.8	20.6	55	0.2987	0.2815	100
CAR	DMZ-d3	99.9	12.6	13.9	42	0.9466	0.1610	100
CAP	CAP-d5	101.2	11.4	12.0	36	1.0082	0.8108	100
CLOP	DMZ-d3	103.3	10.8	16.0	48	0.7125	0.3653	100
DINIT	DMZ-d3	96.3	7.7	14.8	44	2.7345	0.1880	100
ETB	DMZ-d3	99.4	9.1	16.3	49	5.0406	0.5094	100
TYL	DMZ-d3	95.6	16.8	21.8	69	5.0000	0.7275	169
VIR	DMZ-d3	100.0	22.7	22.9	65	5.7381	0.3777	165
AVIL	DMZ-d3	89.2	21.1	22.0	66	5.2961	0.4851	166

7.4.7 Ion Ratios

Two transition ions were monitored for each of the fourteen analytes. The most intense was used for quantitation. Ion ratios were calculated for all analytes. The ion ratio is a ratio of ion responses. The ratios of weak ion responses/strong ion responses are presented in Table 7-2. All ion ratios of samples were within tolerances as set out by European criteria when compared with standards used during validation. Control charts were used to ensure all ion ratios were acceptable. The example of metronidazole is seen in Figure 7-2.

7.4.8 Relative Retention Times (RRT)

RRTs were calculated for all fourteen analytes in this method by calculating the ratio of the retention time of the analyte over the retention time of its corresponding internal standards. The RRTs tolerance for LC-MS/MS of 2.5% was adhered to when standards were compared to samples in the validation runs. Control charts were again used to ensure all ion ratios were acceptable. The example of metronidazole is seen in Figure 7-3. The typical RRT for all the analytes are shown in Table 7-2.

Sample	Metronidazole				
	Strong Ion Peak Area	Weak Ion Peak Area	Ion Ratio	20%+	20%-
Std 100 μg kg^{-1}	15194729	4752084	0.3127	0.3556	0.2371
Std 250 μg kg^{-1}	42333522	12107683	0.2860	0.3556	0.2371
Std 500 μg kg^{-1}	72310544	20962631	0.2899	0.3556	0.2371
Std 750 μg kg^{-1}	111573188	33615028	0.3013	0.3556	0.2371
Std 1000 μg kg^{-1}	141124965	41197657	0.2919	0.3556	0.2371
Level 1 A	15930883	4802808	0.3015	0.3556	0.2371
Level 1 B	13779107	4100715	0.2976	0.3556	0.2371
Level 1 C	15060999	4687121	0.3112	0.3556	0.2371
Level 1 D	14015787	4045653	0.2886	0.3556	0.2371
Level 1 E	14936729	4494700	0.3009	0.3556	0.2371
Level 1 F	15109412	4330588	0.2866	0.3556	0.2371
Level 2 A	82300381	23812667	0.2893	0.3556	0.2371
Level 2 B	55668164	16886535	0.3033	0.3556	0.2371
Level 2 C	98201444	27970852	0.2848	0.3556	0.2371
Level 2 D	86217956	24778305	0.2874	0.3556	0.2371
Level 2 E	88044794	25699990	0.2919	0.3556	0.2371
Level 2 F	88971771	27257657	0.3064	0.3556	0.2371
Level 3 A	183983033	50847934	0.2764	0.3556	0.2371
Level 3 B	157024199	45227252	0.2880	0.3556	0.2371
Level 3 C	170214626	50069929	0.2942	0.3556	0.2371
Level 3 D	170597905	48706960	0.2855	0.3556	0.2371
Level 3 E	177451858	50805992	0.2863	0.3556	0.2371
Level 3 F	163816078	47947886	0.2927	0.3556	0.2371

Average Std Ion Ratio: 0.2964
Average + 20% : 0.3556
Average – 20% : 0.2371

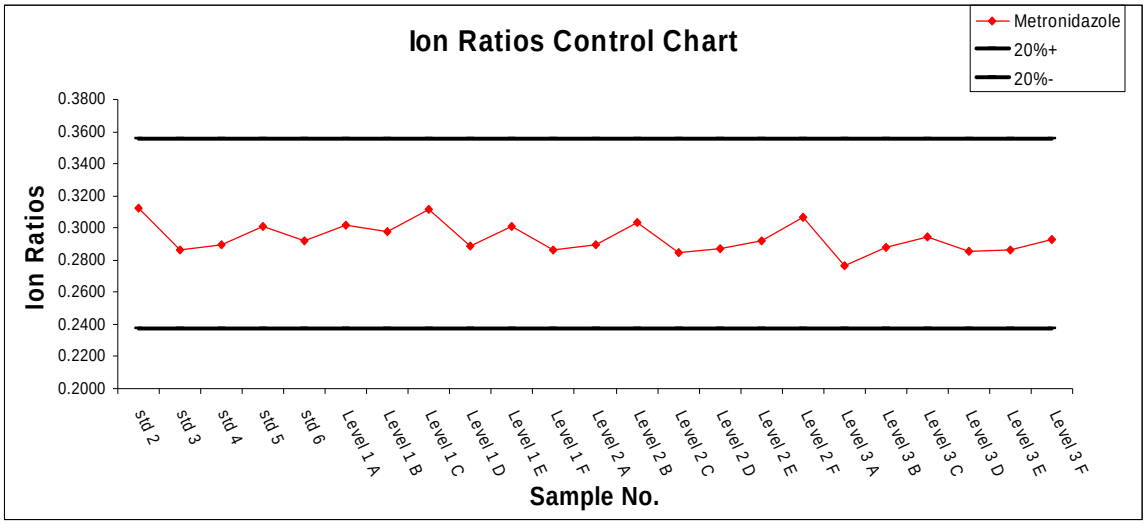


Figure 7-2: Control Chart for Ion Ratio of Metronidazole

	Sample	Metronidazole				
		Retention Time	Retention Time of Internal Standard	Relative Retention Time (RRT)	2.5%+	2.5%-
Std2	Std 100	1.94	2.82	0.6879	0.7084	0.6738
Std3	Std 250	1.95	2.80	0.6964	0.7084	0.6738
Std4	Std 500	1.94	2.82	0.6879	0.7084	0.6738
Std5	Std 750	1.94	2.80	0.6929	0.7084	0.6738
Std6	Std 1000	1.94	2.81	0.6904	0.7084	0.6738
	Level 1 A	1.95	2.82	0.6915	0.7084	0.6738
	Level 1 B	1.94	2.82	0.6879	0.7084	0.6738
	Level 1 C	1.94	2.80	0.6929	0.7084	0.6738
	Level 1 D	1.93	2.80	0.6893	0.7084	0.6738
	Level 1 E	1.95	2.80	0.6964	0.7084	0.6738
	Level 1 F	1.94	2.82	0.6879	0.7084	0.6738
	Level 2 A	1.94	2.82	0.6879	0.7084	0.6738
	Level 2 B	1.94	2.82	0.6879	0.7084	0.6738
	Level 2 C	1.94	2.82	0.6879	0.7084	0.6738
	Level 2 D	1.94	2.82	0.6879	0.7084	0.6738
	Level 2 E	1.94	2.82	0.6879	0.7084	0.6738
	Level 2 F	1.94	2.82	0.6879	0.7084	0.6738
	Level 3 A	1.96	2.82	0.6950	0.7084	0.6738
	Level 3 B	1.94	2.80	0.6929	0.7084	0.6738
	Level 3 C	1.94	2.80	0.6929	0.7084	0.6738
	Level 3 D	1.94	2.80	0.6929	0.7084	0.6738
	Level 3 E	1.95	2.82	0.6915	0.7084	0.6738
	Level 3 F	1.95	2.82	0.6915	0.7084	0.6738

Average RRT: 0.6911

Average + 2.5%: 0.7081
Average – 2.5%: 0.6738

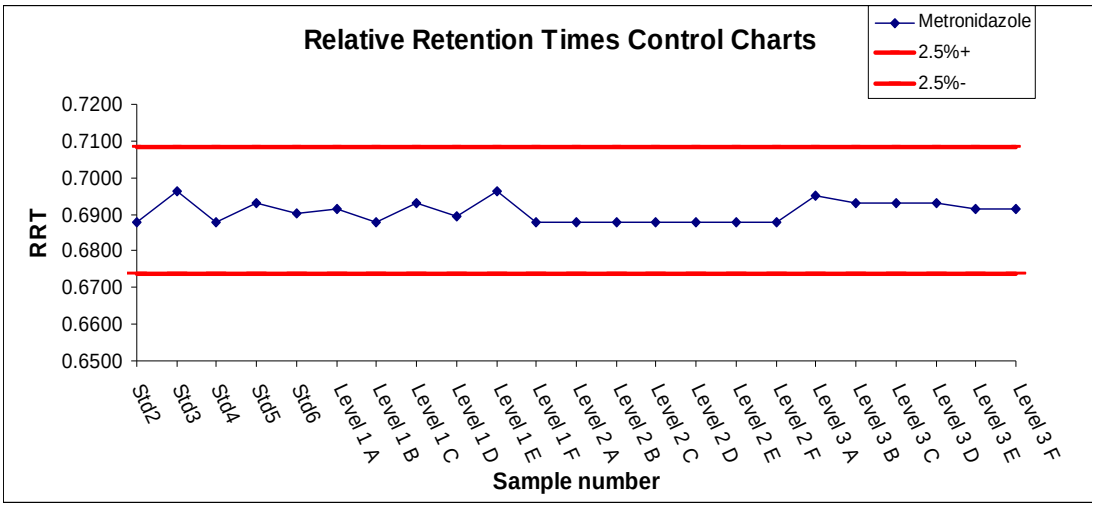


Figure 7-3: RRT Control Chart for Metronidazole

7.4.9 Trueness

The trueness of the method was determined by fortifying 18 replicate feed samples on three separate days. For the eleven quantitative analytes six replicates were fortified at 100, 500, 1000μg kg^{-1} while the three qualitative analytes were all spiked at 100μg kg^{-1} for the 18 replicates. Mean corrected trueness (n=6) of the analytes, determined in the three separate validation batches, are shown in Table 7-2 ranging between 89.2 and 103.3 for the fourteen analytes in pig and poultry feed. No recovery was determined as the use of internal standards means that each sample is individually corrected for.

7.4.10 Precision (Repeatability and within-lab Reproducibility)

Repeatability (within-day) and within-laboratory reproducibility (different days and operators) were determined by calculating relative standard deviations (RSD, %) for the repeated measurements. Overall repeatability (RSD, %) and within-laboratory reproducibility (RSD, %) ranged from 4.3 to 23.3% and from 7.2 to 28.0%, respectively, for all analytes (Table 7-2).

The usefulness of suitable deuterated internal standards is demonstrated in the acceptable results for repeatability and within-laboratory reproducibility obtained for DMZ, RNZ, IPZ and CAP. Although deuterated analogues could not be obtained by our laboratory for use as internal standards for over half of the analytes investigated, acceptable repeatability and within-laboratory reproducibility is obtained by using the d3-DMZ for MNZ, CLOP, DINIT, ETH, CAR and using sulfaphenazole for SMZ. Less favorable is the situation for SDZ. Rather high RSD values were obtained for both the repeatability and within-laboratory reproducibility of SDZ (between 20 and 28%), even when applying correction by means of an internal standard

(sulfaphenazole). This is as a result of the variability for the feed sample due to the low levels of SDZ present in the feed.

7.4.11 Measurement of Uncertainty

The measurement uncertainty (MU) was estimated by taking into account the within laboratory reproducibility over days 1, 2 and 3. This value was multiplied by a coverage factor of three to give an overall figure for the MU. This approach of using the within laboratory reproducibility as a good estimator of measurement of uncertainty is taken from the SANCO/2004/2726rev4 document. It recommends using the within laboratory reproducibility and using a coverage factor of 2.33 to estimate expanded uncertainty, however it was felt that not all the environmental factors that could be varied over the course of the validation were examined. Therefore a coverage factor of 2.33 may underestimate the true uncertainty of the method and instead a value of 3 was chosen to give a more realistic value for the true uncertainty. Values for MU are seen in Table 7-2 and lie between 24 and 84% for all the analytes.

Higher MUs are seen in some compounds with no deuterated analogues for use as internal standards which is expected. In particular the MU for SDZ (84%) is the highest observed for any of the analytes investigated due to problems with low levels of SDZ observed in the majority of feed used. This resulted in greater variability in results achieved for SDZ and in turn increased its MU.

7.5 Conclusions

The objective of this work was to develop a rapid multi-class confirmatory method capable of analysing for fourteen prohibited medicinal additives in pig and poultry

compound feed at 100 μg kg^{-1} and to validate in such a way as to best show the method as fit for purpose. This was successfully completed to allow for the quantification of 10 analytes and qualitative analysis of 4 analytes.

The method can be considered as rapid, as it utilises an efficient extraction protocol without the use of large sample sizes, extraction volumes and SPE. It also utilises chromatography which separates all analytes in a total run time of only 26 minutes. The method permits the analysis of 14 medicinal additives in pig and poultry compound feed which has not been seen in literature before.

The obtained confirmatory criteria of ion ratios and relative retention times fulfill the requirements laid down in Commission Decision 2002/657/EC. The calculation of all relevant performance characteristics was performed during validation. This study shows that the developed method meets the desired sensitivity of 100 μg kg^{-1} for all the compounds. The method performs satisfactorily in terms of trueness and repeatability for each of the analytes investigated with the exception of sulfadiazine due to the utilisation of five different internal standards. The values achieved for trueness, %RSD and measurement of uncertainty all fall within acceptable ranges except for sulfadiazine. The applicability of the method for use on various types of pig and poultry compound feed was demonstrated by the satisfactory results obtained from the validation. The validation data shows that the method allows for the quantitation of 10 analytes and the qualitative analysis of 3 analytes. While sulfadiazine was validated in order to be quantified the validation results achieved were not acceptable. This is as a result of varying background sulfadiazine in the feeds that were used in validation. That said, the reduced number of analytical steps within the method makes it very amenable for high through-put regulatory

monitoring of these compounds and enforcing Commission Recommendation 2005/925/EC.

The method developed in this study is an improvement on existing methods as it allows for the analysis of an increased number of analytes in this matrix. It also allows for reduced sample preparation times and solvent usage than other published methods.

7.6 Acknowledgements

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CHAPTER 8: DETERMINATION OF ELEVEN COCCIDIOSTATS IN ANIMAL FEED BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY AT CROSS CONTAMINATION LEVELS

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8.1 Abstract

A confirmatory multi-residue method has been developed to allow for the detection, confirmation and quantification of eleven coccidiostats in animal feed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method can be used to determine halofuginone, robenidine, nicarbazin, diclazuril, decoquinate, semduramicin, lasalocid, monensin, salinomycin, narasin, maduramicin at levels relating to unavoidable carry over as stated in Regulation 2009/8/EC. Feed samples are extracted with water and acetonitrile with the addition of anhydrous magnesium sulphate and sodium chloride. The extract then undergoes a freezing out step before being diluted and injected onto the LC-MS/MS system. The LC-MS/MS system is run in MRM mode with both positive and negative electrospray ionisation and can confirm all eleven analytes in a run time of 19 minutes. The sensitivity of the method allows quantification and confirmation for all coccidiostats at a 0.5% carry over level. The method was validated over three days in accordance with of European legislation; Commission Decision 2002/657/EC. Validation criteria of accuracy, precision, decision limit (CCα), and detection capability (CCβ) along with measurement uncertainty are calculated for all analytes. The method was then successfully used to analyse a number of feed samples that contained various coccidiostat substances.

Keywords: Coccidiostats; Regulation 2009/8/EC; LC-MS/MS; Animal Feed; Validation.

8.2 Introduction

The use of many antibiotics, coccidiostats and antibacterial growth promoters as feed additives has been prohibited in Europe since 2006 by Commission Recommendation 2005/925/EC. This recommendation lists medicinal substances that should be monitored and the substances are divided into two groups; medicinal substances authorised as feed additives for certain animal species or categories and medicinal substances no longer authorised as feed additives. Following this ban of antibiotics such as tylosin, virginiamycin and spiramycin as feed additives no antibiotics other than coccidiostats and histomonostats can be marketed and used as feed additives within the European Union. Coccidiostats constitute the main choice to fight against coccidiosis. Coccidiosis is a parasitic disease of the intestinal tract caused by unicellular organisms. The disease is highly contagious and spreads from one animal to another by contact with infected faeces. Coccidiosis is a major disease in poultry as well as in many other hosts. [Matabudul *et al.*, 1999]

During the production of feed containing coccidiostats as feed additives, unavoidable carry-over of the coccidiostats from target to non target feed occur from the use of the same production lines. This may lead to high concentrations of coccidiostats in non-target feed; which could harm certain species and also accumulation of coccidiostats may be a risk to human health. As a result of these concerns and in order to avoid coccidiostat carry-over, Regulation 2005/183/EC sets specific requirements for companies using coccidiostats in the production of feed, pertaining particularly to facilities and equipment, production, storage and transport, to avoid any cross-contamination. In addition to this EU legislation was published in the form of Regulation 2009/8/EC and this established maximum limits for unavoidable carry over of coccidiostats and histomonostats. Taking into account the application of good

manufacturing practices, carry-over rates of approximately 1 and 3% of the authorised maximum content should be considered for sensitive and less sensitive non-target animal species, respectively. A carry-over rate of 1% should also be considered for feed used during the period before slaughter, for other target species feed to which no coccidiostats are added, and for non-target feed for 'continuous food producing animals' (dairy cows or laying hens). The structures and the 1% and 3% carry over levels of all the coccidiostats covered by this legislation are seen in table 8-1.

As a result of this legislation there is a need for reliable multi-residue methods to help enforce it. From examination of literature it was observed that there is a limited amount of methods available to carry out this work. There are number of methods for the analysis of these substances in various biological matrices such as liver, muscle and eggs [Dubois *et al.*, 2004; Olejnik *et al.*, 2009; Mortier *et al.*, 2005(a)]. Methods for the analysis of feed for some of these compounds are varied; from the analysis of one analyte [de la Huebra *et al.*, 2010; Mortier *et al.*, 2005(b); Kot-Wasik *et al.*, 2005] up to the analysis of several [Vincent *et al.*, 2008; Mortier *et al.*, 2005(a)]. A method by Vincent *et al.*, 2008 allowed for the analysis of the six ionophore coccidiostats by LC-MS/MS. Purification was by solid phase extraction and quantification was by matrix matched standards or by standard addition. A method published by Mortier *et al.*, 2005(a) allowed for the analysis of four substances of interest; HAL, ROB, DNC and DIC in feed by LC-MS/MS. Samples were extracted with methanol and concentrated up before filtering through a 0.22μm filter. Quantification was performed with the use of internal standards and calibration curves. To the laboratory's knowledge only one method has been published in literature to allow for the analysis of all eleven coccidiostats in feed. Delahaut *et al.*,

2010 published a method for the analysis of eleven coccidiostats in feed by LC-MS/MS. Samples were extracted with 10% Na_2CO_3 and double extraction acetonitrile. No purification was performed and quantitation was performed by the use of internal standards and quadratic regression model [Delahaut *et al.*, 2010].

From previous work carried out on the analysis of feed in our laboratory it is clear that feed is a very complex matrix [Cronly *et al.*, 2010 (b)]. Each feed sample can almost be unique at time therefore developing a method to allow for its analysis can prove difficult. None of the methods available in literature could be applied successfully to allow for the analysis of all eleven coccidiostats in the feed that is encountered in our laboratory. As a consequence a method had to be optimised in our laboratory that would allow for the extraction of all eleven analytes but would also overcome the issue of feed variability that would be encountered on a routine basis. To this end; this paper describes the analysis of eleven coccidiostats in pig and poultry feed at unavoidable carry-over levels described in Regulation 2009/8/EC by LC-MS/MS utilising an efficient extraction protocol.

8.3 Materials and Methods

8.3.1 Chemicals and Reagents

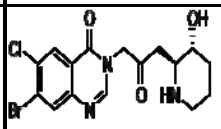
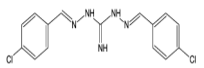
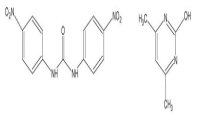
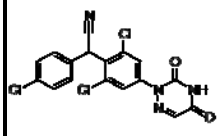
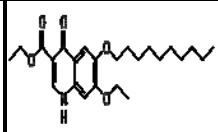
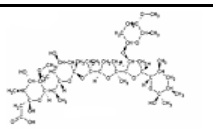
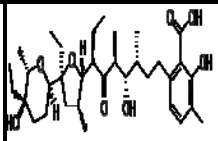
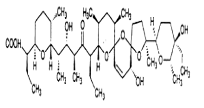
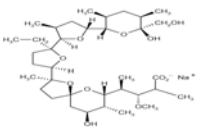
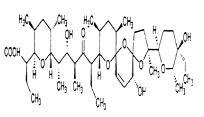
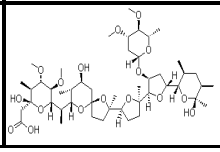
Robenidine (ROB), nicarbazin (NIC), diclazuril (DIC), decoquinate (DEC), lasalocid (LAS), monensin (MON), salinomycin (SAL), narasin (NAR), maduramicin (MAD), nigericin (NIG), d8-Robenidine (d8-ROB), d8-4,4'-dinitrocarbanilide (d8-DNC) and d5-decoquinate (d5-DEC) were all purchased from Sigma (St. Louis, MO, USA). Halofuginone (HAL) was purchased WITEGA Laboratorien (Berlin, Germany) , bis-diclazuril (bis-DIC) from Janssen (Beerse, Belgium) and semduramicin (SEM) from Phibro (Pittsburgh, PA, USA). Water is of LC-MS grade from Fluka (Berlin,

Germany). All other solvents are of LC grade and purchased from Reagecon (Clare, Ireland). Anhydrous magnesium sulphate is AnalaR grade and purchased from Sigma (St. Louis, MO, USA). Individual stock standards of each analyte ranging between 0.50-1.00 mg ml^{-1} were prepared in methanol for all compounds except for halofuginone (water) and nicarbazin (dimethylsulfoxide) and stored at 4˚C. Working standard solution (mixture of analytes) was prepared in acetonitrile by diluting stock standard into a range that equated to the carryover levels in 2.5g of feed and stored at 4ºC for. Internal standards were prepared similarly.

8.3.2 Instrumentation

The LC-MS/MS system is a TSQ Quantum Ultra EMR coupled to a Finnigan Surveyor LC system. The instrument is controlled by Xcalibur software (Version 1.5). Separation was achieved using a (100 x 3)mm, 3.5µm particle size, Symmetry C8 column protected by a Security Guard guard cartridge system (20 x 2)mm, supplied by Waters and Phenomenex respectively. The oven temperature was set at 40ºC. The chromatographic separation was performed in gradient mode using water acidified with 0.1% formic acid (mobile phase A) and acetonitrile acidified with 0.1% formic acid (mobile phase B), at a flow rate of 0.6mL min^{-1}. The initial conditions (from 0-1min) were 100% A. Then the conditions were changed to 2% A over 4 minutes from 1-5min and this were maintained until 11.50 min. Finally the conditions returned to 85% A over 0.5 minutes from 11.5-12min, and were maintained until the end of the run at 19min. Electrospray ionisation (ESI) was used in the MS with both positive and negative ionisation utilised with a spray voltage of 4500V and a cone temperature of 350 ºC. The individual precursor and products ions for each analyte with their respective collision energies are listed in Table 8-1.

Table 8-1: Molecular Structures, 1% and 3% Carry-over, Electrospray Ionisation (ESI) Retention time(Rt), Precursor and Product ions for all 11 analytes.

Compound	Structure	1% Carry Over (mg/kg)	3% Carry Over (mg/kg)	Rt	ESI	Precursor (M/z)	Product (M/z)	Collision Energy
Halofuginone		0.030	0.090	5.48	positive	416	100.4*	21
							120.0	29
Robenidine		0.700	2.100	6.02	positive	333.9	138.0	31
							155.0*	18
Nicarbazin		0.500	1.500	7.30	negative	301	107.2	38
							136.9*	18
Diclazuril		0.001	0.003	7.56	negative	405	333.9	20
						407	335.9*	22
Decoquinate		0.400	1.200	8.21	positive	418	203.9*	42
							372.1	29
Semduramicin		0.250	0.750	9.08	positive	895.4	833.3*	39
							851.3	41
Lasalocid		1.250	3.750	9.38	positive	613.3	377.2*	32
							577.2	32
Salinomycin		0.700	2.100	9.69	positive	773.5	431.0*	50
							531.4	45
Monensin		1.250	3.750	9.76	positive	693.4	461.5	55
							675.3*	41
Narasin		0.700	2.100	10.08	positive	787.4	431.4*	50
							531.4	45
Maduramicin		0.050	0.150	10.30	positive	935.9	719.5	65
							877.7*	35

8.3.3 Pig and Poultry Compound Feed Samples

A variety of pig and poultry compound feed samples were sourced from various feed mills. These were milled upon receipt to 1mm using a Retsch SM 100 mill and stored in amber jars at 4ºC. Portions of these samples were analysed and those found to contain no detectable residues of the analytes of interest were used as blanks for the validation study. To ensure robustness of the method a high number of different feed samples were used in validation. These included 12 different pig feeds and 12 different poultry feeds. Chromatograms of blank feed can be seen in Figure 8-1a.

8.3.4 Extraction

Feed (2.5 g) was weighed into polypropylene centrifuge tubes (50 mL). The sample was fortified with mixed internal standard (25 µL). To this deionised water (12 mL) was added and the tubes were shaken (15 min). To this acetonitrile (25mL) was added and the tubes were shaken (15min). Anhydrous $MgSO_4$ (4.0 g) and NaCl (2g) was added to this slurry which was then shaken (15 min) and centrifuged (5100rpm for 20 min). The organic layer was then transferred to a clean polypropylene tube (50 mL) and placed in a -80 ºC freezer for 15mins to allow for freezing out of matrix components. The samples were then removed from the freezer. 10mL of the extract is then transferred to a clean polypropylene tube (15mL) and centrifuged at 4500rpm for 10min. The extract is then run twice. The first was a straight extract and the second was the extract diluted 15 times in mobile phase B. An aliquot (20 µL) of each is injected onto the LC-MS/MS.

8.3.5 Matrix Extracted Calibration Curves

Quantitation was carried out using matrix extracted calibration curves. Blank pig and poultry feed samples were used. These samples were fortified with mixed working standard and submitted to the full extraction procedure. A matrix extracted calibration curve was performed with every batch. Feed samples were fortified with internal standard and mixed working standard yielding a calibration range relating 0 to 4% carryover of each of the eleven analytes. Calibration curves were prepared by plotting the response factor (the ratio of peak area analyte over peak area of internal standard) against analyte concentration for the four compounds with internal standard. Four internal standards were used; d8- DNC, d8-ROB, d5-DEC and bis-DIC. For compounds for which no suitable internal standard could be acquired; HAL and the six ionophore coccidiostats SEM, LAS, SAL, MON, NAR and MAD; calibration curves were prepared by plotting the peak area of the analyte against analyte concentration.

8.3.6 Method Validation

The LC–MS/MS method was validated according to Commission Decision 2002/657/EC guidelines. LC–MS/MS identification criteria were verified through-out the validation study by monitoring relative retention times, ion recognition (signal-to-noise ratio (S/N)) and relative ion intensities. LC-MS/MS identification criteria as set out in the legislation were verified throughout the validation of the method.

Several method validation parameters were determined including linearity, specificity, recovery, precision and analytical limits (decision limit CCα, and detection capability CCβ). Specificity was determined by analysing 28 different

blank pig and poultry samples sourced from different mills. To investigate the linearity of the method, matrix-matched calibration curves were prepared and run with each of the validation batches to give 6 point calibration curves ranging from 0 to 4% carryover for each of the analytes.

Since no certified reference materials were available for all the analytes and matrices of interest, the recovery from fortified blank samples was measured as an alternative to trueness. The accuracy and precision were determined through the analysis of wide variety of blank pig and poultry feed samples fortified in seven replicates relating to 0.5%, 1% and 3% carryover of each of the analytes. Seven replicate test portions at each of the three fortification levels (n = 21) were analysed on three separate days over a period of two weeks. Samples were fortified by adding mix working standard solution (12.5, 25 and 75 μL) and analysed. From these three separate validation days the estimation of recovery, precision and analytical limits (decision limit CCα, and detection capability CCβ) were determined along with the methods measurement uncertainty.

8.4 Results and Discussion

8.4.1 LC-MS/MS Optimisation

The LC-MS/MS method was developed to provide confirmatory data for the analysis of 11 coccidiostats in pig and poultry compound feed. The MS/MS fragmentation conditions were investigated and collision energies were optimised for each individual compound to give best response. For a method to be deemed confirmatory under Commission Decision 2002/657/EC it must yield 4 identification points. In this method a precursor ion (parent mass) and two product ions (corresponding to strong and weak ion) are monitored for each analyte (Table 8-1). This yields 4

identification points (1 for the parent ion and 1.5 for each daughter ion) hence it can be deemed a confirmatory method. In addition to this relative retention times and ion ratios are monitored for each compound and evaluated to ensure that they are within acceptable ranges as stated in EC 2002/657.

Previous work carried out on coccidiostats in various matrices used a range of different columns and gradients. A popular choice with many methods is C18 with acidified water and acetonitrile gradient [Mortier *et al.*, 2005 (a); Dubois *et al.*, 2004; Delahaut *et al.*, 2010]. Other methods used phenylhexyl [Olejnik *et al.*, 2009] and C8 [Dubreil-Chéneau *et al.*, 2009] columns with three mobile phases. During the development of this method it was found that a combination of C8 column with a mobile phase of water and acetonitrile acidified with 0.1% formic acid gave the best retention, peak shape and separation for all compounds in an acceptable run time. As this method involved positive and negative ionisation switching, the MS/MS method had to be segmented. The LC gradient was optimised in order to have the negative ionisation in one segment. Only when the positive and negative ionisations were isolated to separate segments was there enough data points for each peak. For a method to achieve reliable quantitation each analyte peak should have at least 12 -15 data points. The LC Gradient along with segmentation allowed for the analysis of all 11 analytes in a complete run time of 19 minutes with each peak having a minimum of at least 12 data points.

8.4.2 Extraction Optimisation

The aim of the development work was to develop a quick efficient extraction protocol for the analysis of eleven coccidiostats in a wide variety of feed. From investigation of literature it was found that acetonitrile is commonly used as an

extraction solvent for these compounds in various matrices [Olejnik *et al.*, 2009; Dubois *et al.*, 2004; Delahaut *et al.*, 2005]. From initial tests it was clear that although acetonitrile extracted all the analytes of interest it also extracted a lot of matrix interferences as feed is a very complex matrix. Previous research carried out in our lab has demonstrated difficulties with this matrix [Cronly *et al.*, 2010 (b)]. Feed is not a standardised formula and is a lot more variable than other biological matrices such as plasma and milk. Even one batch of feed to the next can vary and therefore each feed can be considered a unique sample.

Taking this into consideration there is a need for a suitable sample clean up procedure. A number of procedures were investigated; the extraction of analytes by water and acetonitrile with addition of NaCl [Cronly *et al.*, 2009 (a)], extraction by acetonitrile with the addition of anhydrous sodium sulphate [Cronly *et al.*, 2010(b)] and the extraction by water and acetonitrile with addition of NaCl and magnesium sulphate [Walorczyk, 2008] in order to reduce the amount of interferences extracted from the matrix. While all these helped to improve the recovery of the analytes and the ion suppression in the LC-MS/MS the addition of NaCl and magnesium sulphate with an extraction of water and acetonitrile gave the best results.

To help improve the method further the addition of a dispersive SPE step was added. This improved the method repeatability but on investigation of literature it was found that this step could be replaced by a freezing out step which can remove many of the same matrix components as the dispersive SPE [Walorczyk, 2008; Mastovska *et al.*, 2010]. This step was performed by placing the extract into a -80ºC freezer for fifteen minutes. Upon removal from the freezer a visible amount of particles had fallen to the bottom of the extract. The addition of this step improved repeatability in different feeds.

The next step in the development was the selection of suitable internal standards. From investigation of literature it was observed that some deuterated internal standards were available [Dubreil-Chéneau *et al.*, 2009]. Deuterated standards available were ROB-d8, DNC-d8 and DEC-d5. Other internal standards used were bis-DIC for diclazuril and nigericin for the ionophore coccidiostats [Mortier *et al.*, 2005; Vincent *et al.*, 2008]. These were obtained and used as internal standards for the various analytes. It was seen that while the deuterated standards and bis-DIC corrected satisfactorily for their respective analogues, nigericin did not correct the ionophore coccidiostats adequately. It was observed that in calibration curves of the six ionophore coccidiostats the curve started levelling off near the top. Recent work published [Delahaut *et al.*, 2010] suggested the use of quadratic instead of linear calibration curves. While in single feeds this gave coefficient of determination (R^2) values of 0.99, the use of this curve for calculating fortified samples gave very variable results in various different types of feed. Therefore in order to use this approach the selection of feed for matrix matched curves had to be very similar to the feed being analysed. As feed can vary greatly it was felt that this approach would not be fit for purpose in our laboratory where a wide range of feed samples would be received for analysis.

In order to overcome this problem it was decided that diluting the extract would allow for less matrix interference while still having enough analyte signal. Various dilutions were tested and it was found that a 1 in 15 dilution gave linear curves in a single feed with R^2 of 0.99 using nigericin as an internal standard for the ionophore coccidiostats. When this was used for calculating the levels in fortified feed samples it was seen that while in some feeds, results were satisfactory, there were cases in which the results were not acceptable in relation to accuracy. Upon further

investigation it was seen that this was due to the unsuitability of nigericin as an internal standard for these compounds in feed. Results showed that when peak areas for the analyte increased, the results for nigericin decreased and vice versa. This resulted in larger errors in the results for these feeds. It was seen that repeatability and accuracy were greatly improved when no internal standard was used for these compounds. The only drawback to this was that each extract would have to be run twice, once as a straight extract and once diluted 15 times. This was as a result of the levels and low responses for DIC and HAL in the diluted samples.

In order to ensure that this procedure would hold true in the wide variety of feed samples likely to be encountered in the laboratory on a routine basis a validation procedure was developed in accordance with Commission Decision 2002/657. In each validation a single feed would be used for the six point matrix extracted calibration curve and seven different feeds would be used for the replicates at the three validation levels. This would allow the validation to fully cover the various feeds likely to be encountered.

8.4.3 Validation

Validation was carried out in accordance with the procedures outlined in Commission Decision 2002/657/EC covering specificity, calibration curve linearity, accuracy, precision, decision limits (CCα), decision capability (CCβ) and measurement uncertainty. Results are seen in table 8-2 for these criteria. The criteria of relative retention times and ion ratios were monitored for all analytes in the three validations days. The values identified for these were all within European requirements.

Table 8-2: Validation results for accuracy, precision (%CV), decision limit (CCα), detection capability (CCβ) and measurement uncertainty (MU) and confirmatory data of typical ion ratios and relative retention times (RRT) for all 11 analytes.

Analyte	Internal Standard	Accuracy (%)	CV (%)	CCα (mg/kg)		CCβ (mg/kg)		Typical RRT	Typical Ion Ratio	MU (%)
				1%	3%	1%	3%			
HAL	None	88.8	8.8	0.029	0.092	0.033	0.109	0.912	0.334	25
ROB	ROB-d8	95.0	6.1	0.786	2.370	0.871	2.639	1.000	0.863	19
DNC	DNC-d8	97.3	4.4	0.532	1.669	0.564	1.837	1.000	0.069	12
DIC	bis-DIC	96.3	8.0	0.011	0.035	0.013	0.040	0.990	0.849	17
DEC	DEC-d5	95.0	7.0	0.439	1.333	0.478	1.466	1.000	0.589	15
SEM	None	99.9	7.6	0.294	0.863	0.338	0.976	1.511	0.129	21
LAS	None	98.1	8.6	1.570	4.524	1.890	5.298	1.561	0.455	31
SAL	None	92.3	9.1	0.821	2.418	0.942	2.737	1.612	0.548	22
MON	None	96.6	6.3	1.388	4.178	1.527	4.606	1.624	0.354	21
NAR	None	101.7	7.0	0.775	2.380	0.850	2.660	1.677	0.463	19
MAD	None	104.5	8.4	0.057	0.168	0.064	0.187	1.714	0.037	24

8.4.3.1 Specificity

The technique of LC-MS/MS itself offers a great deal of specificity and selectivity. To establish the specificity and selectivity of the method 24 blank pig and poultry compound feed samples and samples fortified with all 11 analytes were analysed over the 3 validation days. All blank samples showed no interfering peaks in the area of interest for any of the analytes. Chromatograms of blank feed and feed fortified at 0.5% carryover of each analyte are seen in Figure 8-1a and 8-1b.

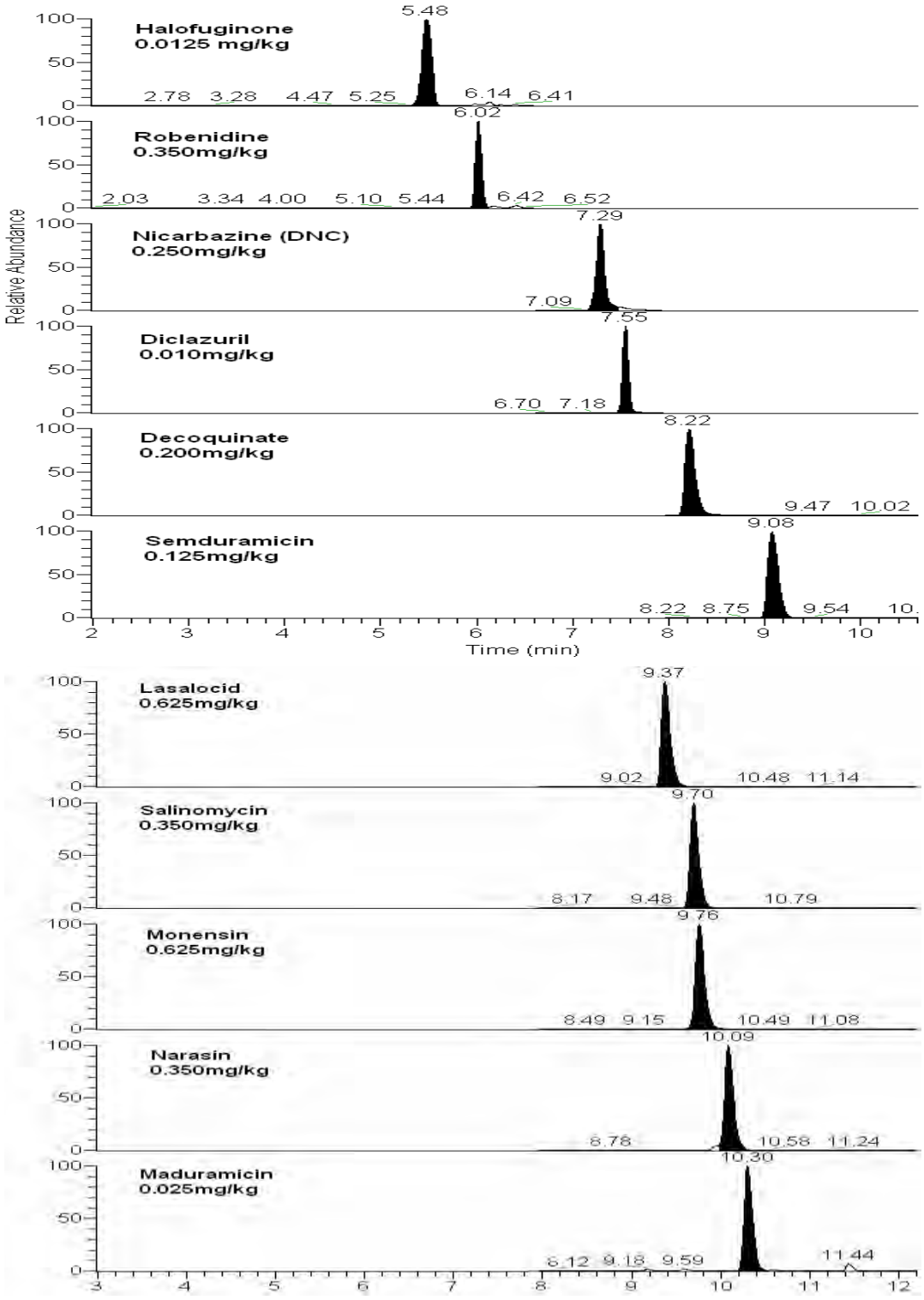


Figure 8-1a: Chromatogram of feed fortified at 0.5% Carry-Over

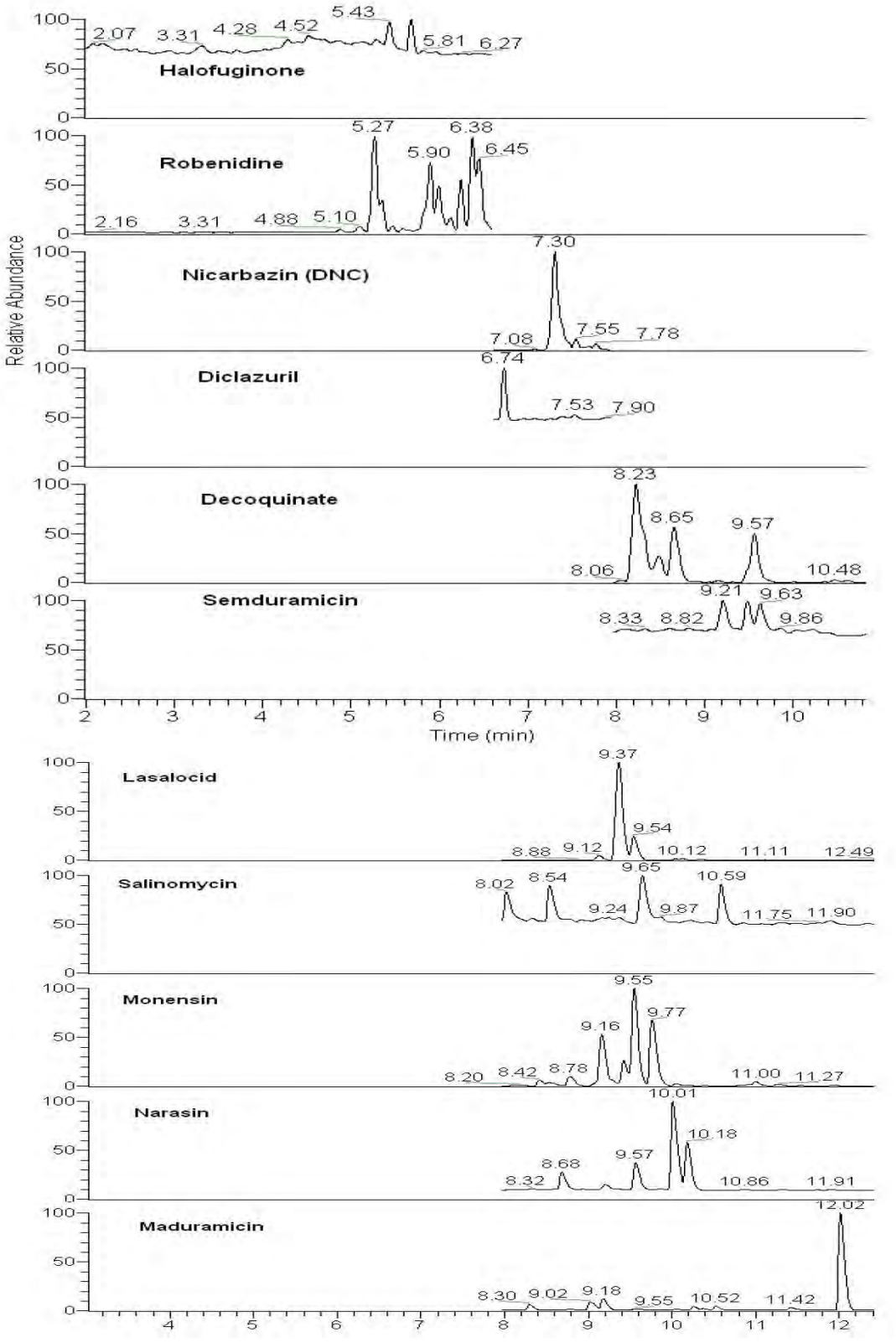


Figure 8-1b: Chromatogram of blank feed

8.4.3.2 Linearity of Response

The linearity of the chromatographic response was tested with matrix extracted calibration curves using six calibration points in the range of 0-4% carryover for all eleven analytes on each of the validation days. For each analyte; calibration curves were linear in the given range with a coefficient of determination (R^2) of at least 0.99 for all compounds except for LAS and MAD which were at least 0.98.

8.4.3.3 Ion Ratios

Two transition ions were monitored for each of the fourteen analytes. The most intense was used for quantitation. Ion ratios were calculated for all analytes by calculating the ratio of the strong ion over the weak ion. All ion ratios of samples were within tolerances as set out by European criteria when compared with standards used during validation. Control charts were used to ensure all ion ratios were acceptable. The typical ion ratios for all the analytes are shown in Table 8-2.

8.4.3.4 Relative Retention Times (RRT)

RRTs were calculated for all analytes in this method by calculating the ratio of the retention time of the analyte over the retention time of its corresponding internal standards. For the seven compounds that do not use an internal standard, ROB-d8 was used for their RRT calculations. The RRT tolerance for LC-MS/MS of 2.5% was adhered to when standards were compared to samples in the validation runs. Control charts were again used to ensure all ratios were acceptable. The typical RRT for all the analytes are shown in Table 8-2.

8.4.3.5 Accuracy/Trueness

The accuracy (trueness) of the method was determined by fortifying 21 replicate feed samples on three separate days. For the eleven coccidiostat analytes seven replicates were fortified at 0.5%, 1% and 3% carryover. Mean corrected accuracy (n=7) of the analytes, determined in the three separate validation batches are shown in Table 8-2 range between 88.8% and 104.5% for the eleven analytes in pig and poultry feed. These are within acceptable limits set out in European legislation.

8.4.3.6 Precision

Satisfactory values for inter-assay precision expressed as %CV values for the within lab reproducibility (table 8-2) were achieved for all analytes. According to Commission Decision 2002/657/EC this coefficient of variance for the repeated analysis of fortified material under reproducible conditions shall not exceed the level calculated by the Horwitz equation. For a concentration of 1 mg kg $^{-1}$ this equation gives a value of 16%. Results achieved range from 4.4 to 9.1% for all analytes and this is less the desired 16%. These acceptable results can be attributed to the availability of 4 suitable internal standards. For compounds without suitable internal standards the optimisation of the extraction protocol can be attributed to the acceptable values.

8.4.3.7 CCα and CCβ

In the case of substances with an established permitted limit, the decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant and the detection capability (CCβ) is the concentration at which the method is able to detect permitted limit concentrations

with a statistical certainty of 1 – β. CCα and CCβ values were determined in accordance with sections 3.1.2.5 and 3.1.2.6 of commission decision 2002/657/EC by fortifying seven samples at the 1% and 3% allowed carry over levels on three different days (n=21) and calculating standard deviations. The concentrations at the 1% and 3% plus 1.64 times the calculated standard deviation was used to yield CCα, CCβ was determined by addition of another factor of 1.64 times the standard deviation. Values of CCα and CCβ for each of the eleven analytes for the 1% and 3% carryover are shown in Table 8-2.

8.4.3.8 Measurement Uncertainty

The measurement uncertainty (MU) was estimated by taking into account the within laboratory reproducibility over days 1, 2 and 3. This value was multiplied by a coverage factor of two to give an overall figure for the MU. This approach of using the within laboratory reproducibility as a good estimator of measurement of uncertainty is taken from the SANCO/2004/2726rev4 document. It recommends using the within laboratory reproducibility and using a coverage factor of 1.64 to estimate expanded uncertainty for permitted substances, however it was felt that not all the environmental factors that could be varied over the course of the validation were examined. Therefore a coverage factor of 1.64 may underestimate the true uncertainty of the method and instead a value of 2 was chosen to give a more realistic value for the expanded uncertainty. Values for MU are seen in Table 8-2 and lie between 12 and 31% for all the analytes.

8.4.3.9 Method Evaluation

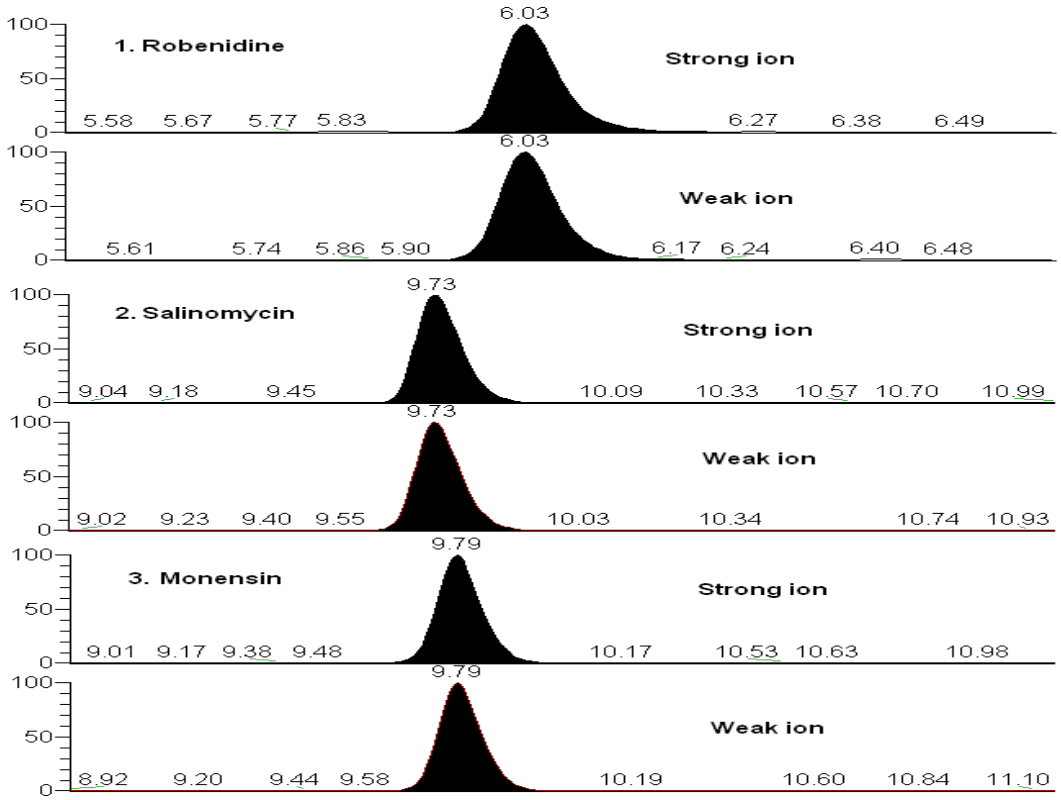


Figure 8-2: Chromatogram containing strong and weak ions of non compliant samples

In order to evaluate this method non compliant feed samples that were received from Rikilt (Netherlands) at levels relating to carry over issues were analysed. Figure 8-2 shows chromatograms with both the strong and weak ions for robenidine, salinomycin and monensin which were found to be present in these samples. These samples were tested using the method described here and found to yield satisfactory results. When the methods measurement uncertainty is applied, the assigned values fall within the possible range of concentrations given by this method. Also the method found no peaks of analytes that were not present in the sample. Results of all non compliant samples tested are seen in table 8-3.

Table 8-3: Results of non compliant sample received from Rikilt

Feed Sample No.	Compound	MU (%)	RESULTS			Actual (mg kg $^{-1}$)
			Calculated (mg kg $^{-1}$)	Result + MU (mg kg $^{-1}$)	Result -MU (mg kg $^{-1}$)	
12	ROB	19	3.24	3.86	2.62	2.9
7	SAL	22	2.24	2.73	1.75	2.3
14	MON	21	1.61	1.95	1.27	1.3

8.5 Conclusions

The objective of this work was to develop a rapid confirmatory method capable of identifying, confirming and quantifying eleven coccidiostats at levels relating to unavoidable carry-over in a variety of feed samples that the laboratory might encounter on a routine basis and to validate in accordance with the requirements set out in Commission Decision 2002/657/EC. This was successfully completed.

The method can be considered as rapid, as it utilises an efficient extraction protocol without the use of large sample sizes, extraction volumes and SPE. It also utilises chromatography which separates all analytes in a total run time of 19 minutes. The method includes the analysis of 11 coccidiostats in a wide variety pig and poultry compound feed.

The obtained confirmatory criteria of ion ratios and relative retention times fulfill the requirements laid down in Commission Decision 2002/657/EC. The calculation of all relevant performance characteristics was performed during validation. This study shows that the developed method meets the desired sensitivity of 0.5% carry-over for all the compounds. The method performs satisfactorily in terms of accuracy and precision (%CV) for each of the analytes investigated and all fall within acceptable

ranges. The analytical limits in terms of decision limit (CCα), and detection capability (CCβ) of the method were calculated for all eleven coccidiostats. Therefore applicability of the method for use on various types of pig and poultry compound feed was demonstrated by the satisfactory results obtained from the validation. The validation data shows that the method allows for the quantitation of 11 analytes. The method was further evaluated by using it to analyse for these compounds in non compliant samples. The reduced number of analytical steps within the method makes it very amenable for high through-put regulatory monitoring of these compounds and enforcing Commission Directive 2009/8/EC.

CHAPTER 9: CONCLUSIONS AND FUTURE WORK

9.1 Conclusions

The main areas on which this research focused was the development of analytical methodologies for the detection of nitroimidazole residues in various biological matrices. These included plasma, eggs, milk and honey [Cronly *et al.*, 2009(a); 2009(b); 2010(a)]. By using generic extraction protocols the work also demonstrated that different classes of drugs could be analysed together such as chloramphenicol and nitroimidazoles. The developed method was then used to carry out a survey for the presence of nitroimidazole residues in Irish retail egg samples [Cronly *et al.*, 2011(a)]. The second area of research focused on the analysis of medicinal additives in animal feed. New legislation has been introduced in this area by the EU which prohibits the use of wide number of these compounds [Commission Recommendation 2005/925/EC]. As a result there is a requirement for multi-class methods that can analyse for a wide variety of these additives in feed. The remaining authorised additives are only permitted for use in target species. Levels for allowed unavoidable carryover of these additives in non target feed were set by the EU [Commission Directive 2009/8/EC] and hence a need existed for an analytical method capable of measuring all these substances at these carry over levels. Therefore as part of this research two methods were developed for the analysis of animal feed, firstly for the presence of 14 prohibited medicinal additives [Cronly *et al.*, 2010(b)] and secondly for the coccidiostats at levels relating to unavoidable carry over [Cronly *et al.*, 2011(a)]. The conclusions resulting from these two areas of research are discussed fully in the following two sections.

9.1.1 Analytical methodologies for detection of nitroimidazoles and chloramphenicol residues in biological matrices

When initiated the main focus of this research was on the development of multi-residue analytical methods for the analysis of nitroimidazole residues in plasma and eggs by LC-MS/MS. There were a number of reasons for this;

- Nitroimidazole compounds are believed to be carcinogenic and mutagenic to humans.
- They are prohibited for use in food producing animals by their inclusion in Table 2 of Council Directive 37/2010/EC.
- Matrices of plasma and egg are recommended as target matrices for these compounds by the EURL.
- Finally their monitoring as part of the national residue control plan in Ireland was limited to the analysis of two residues in one matrix.
- LC-MS/MS offered a selective and sensitive confirmatory technique that could overcome problems associated with derivatisation of these analytes for analysis by GC-MS/MS.

From examination of literature it was apparent that there was a shift from traditional labour intensive extraction and purification techniques such as SPE to more efficient generic extraction protocols which resulted in significant economic and time benefits.

The approach to method development taken as part of this research was to try to incorporate as many nitroimidazole analytes as possible and develop a rapid and efficient method. Initially a method published by Xia *et al.*, 2006 for the analysis of nitroimidazoles in muscle and eggs was used as the basis for the developed

extraction protocol. Xia *et al.,* 2006 method was limited to the analysis of four NMZ residues so needed to be adapted to incorporate increased number of analytes. This paper described the use of a fast LLE using acetonitrile. The miscible aqueous and organic phases were separated by the addition of salt. The salt used was NaCl and this resulted in the phases separating into two layers. The NMZ residues were extracted into the acetonitrile phase which resulted in a cleaner extract.

Using this protocol as a basis for method development; methods were developed for the analysis of NMZ residues in plasma and eggs. This was achieved by making the following adaptations to the original method;

- The samples were extracted with a single extraction rather than a double extraction.
- The samples were purified by the addition of a hexane wash step which removed any further non polar impurities
- Two centrifuge steps were incorporated, one to ensure complete phase separation and another to remove any particulates prior to evaporation.
- The sample was evaporated to dryness and reconstituted in a small volume to increase sensitivity.
- Finally after testing numerous filters, samples were filtered through 0.25 µm PVDF filters which resulted in a clean extract for injection.

These adaptations allowed for rapid, multi-residue, confirmatory methods to be developed that simultaneously identifies, confirms and quantifies ten NMZ residues in plasma [Cronly *et al.,* 2009(a)] and eleven NMZ residues in eggs [Cronly *et al.,* 2009(b)] by LC-MS/MS.

The developed methods were an improvement on existing methodologies and the benefits of the analytical methods are as follows;

- An increased number of analytes are incorporated both in plasma and egg than previously published in the literature. The method is capable of analysing for the seven NMZ residues that are recommended for analysis by the EURL as well as other nitroimidazoles that haven't been analysed previously such as ornidazole and carnidazole
- The method can be considered as rapid, as it utilises an efficient extraction protocol with purification by hexane wash and filtering.
- The method is validated and the obtained data fulfils the requirements laid down in CD 2002/657/EC and allows the calculation of accuracy, repeatability, reproducibility and MU. These parameters fall within acceptable ranges for each analytes.
- The required sensitivity of the method is demonstrated by the values for CCα and CCβ. These values are lower than the RL for NMZ residues of 3 μg kg^{-1}/ng mL^{-1}.
- The developed methods were capable of analysing for plasma and eggs from a variety of species. These include bovine, avian, porcine, ovine and equine plasma and caged, free range and organic hen eggs along with duck and quail eggs.
- Finally both methods performed satisfactorily in evaluation tests carried out on incurred plasma and egg samples where results achieved were within MUs when compared to assigned values.

Upon completion of validation of the developed method for the analysis of nitroimidazoles in eggs, a survey of retail egg samples commenced [Cronly *et al.*, 2011(b)]. The two main reasons for undertaking this survey were that non compliant findings of NMZ residues in eggs had been recorded in Europe [EFSA Report, 2009] and also there was no analysis carried out on these compounds in eggs in Ireland prior to 2007. Therefore it was felt that an evaluation of eggs available in the Irish retail market for the presence of NMZ residues would be beneficial. The survey was carried out throughout the course of the research with retail samples taken every month over two years. Upon completion of this survey; 160 hen and duck egg samples were analysed and no non-compliant samples were identified. It is felt that this demonstrates that the ban on these compounds is being observed and it gives confidence to the consumer that the eggs that reach the table are free from these harmful residues. It is felt that the continued monitoring of these compounds in eggs which is being carried out as part of the national monitoring plan is sufficient to ensure the continued enforcement of these prohibited compounds in eggs.

Carrying on from the development of the methods for plasma and eggs other matrices were examined. In order to ensure continued consumer protection the EURLs are, on an ongoing basis, suggesting that a wider variety of residues and matrices be examined. Two such matrices suggested by the EURL for nitroimidazoles were milk and honey. Honey bees and dairy cows were originally not target species for nitroimidazole drugs but abuse in them now cannot be overlooked. The EURL suggest that countries with high milk production should test for NMZ residues in this matrix. Reports from China about the potential use of nitroimidazoles in beekeeping suggest that honey should also be investigated [Zhou *et al.*, 2007]. Examination of literature found that there was a lack of methods for these matrices with regards to the

analysis of nitoimidazoles with only one confirmatory method for the analysis of NMZ residues in milk and none in honey. Therefore it was felt that the previously developed method could be adapted to include the analysis of milk and honey.

Due to the generic nature of the extraction procedure it was envisaged that it may be possible to include other classes of compounds. From examination of literature and legislation it was felt that it would be beneficial to include chloramphenicol. The main reasons for this were that it is also listed in Table 2 of Commission 37/2010/EC (prohibited substances), it is commonly analysed in single analyte labour intensive methods which are time consuming and target matrices for this compound include milk and honey. Therefore it was decided to adapt the method for the analysis of 11 NMZ residues and CAP in milk and honey by LC-MS/MS.

The main benefits of this method over existing published methods are as follows;

- The method includes the confirmatory analysis of CAP and 11 NMZs in milk and honey which has not been seen before.
- The method can be considered as rapid, as it utilises an efficient extraction protocol without the use of SPE and utilises rapid chromatography with a total run time of only 9 minutes.
- Methods previously published on these matrices analysed at most seven NMZ analytes and in the case of CAP it was often analysed as a single analyte method.
- This study shows that as the developed method uses a newer sensitive instrument that even with reduced sample sizes required sensitivities of 3 $\mu g\ L^{-1}$ / $\mu g\ kg^{-1}$ for NMZs and 0.3$\mu g\ L^{-1}$ / $\mu g\ kg^{-1}$for CAP which are the RL and MRPL used for these compounds are easily achieved.

- The method performs very well in terms of accuracy and repeatability for each of the analytes due to the utilisation of seven different deuterated internal standards.
- In the case of the extraction protocol for milk; time and solvent usage is greatly reduced compared to other published methods as a result of reduced sample size of 1mL.

This concluded the research on the analysis of nitroimidazole residues in biological matrices. The main aim of this research was to help improve the surveillance capabilities of The State Laboratory with regards to the analysis of nitroimidazoles. To this end, a number of novel, rapid, confirmatory, multi residue methods using liquid chromatography tandem mass spectrometry in a variety of biological matrices were successfully developed. Other aims including determining the potential misuse of nitroimidazole compounds in eggs in the Irish poultry industry by the analysis of retail survey samples and the implementation of these methods in the National Reference Laboratory designated for nitroimidazoles in Ireland were also successfully completed.

9.1.2 Analytical methodologies for the detection of medicinal additives in animal feed.

The second phase of the research was concerned with the development of multi-class analytical methods for the analysis of medicinal additives in animal feed. This included methods for the analysis of 14 prohibited medicinal additives and the analysis of 11 coccidiostats at levels relating to unavoidable carryover. The reasons for researching this area are;

- Contaminated feed has the potential to result in possibly harmful levels of residues in any animal fed such feed.
- Recent legislation; Commission Recommendation 2005/925/EC; has prohibited all previously used feed additives except for one class of compounds coccidiostats and histomonostats.
- Further legislation, Commission Directive 2009/8/EC established maximum limits for unavoidable carry over of coccidiostats and histomonostats into sensitive and less sensitive non target species.
- Previously used single analyte HPLC methods used for the analysis of additives at medicated level are not sensitive or selective enough to enforce levels set out in the new legislation.
- No multi-class multi-residue analytical methods were available for analysis of these compounds and prior to this legislation, LC-MS/MS was not utilised in this area.

It was felt that the best analytical tool for analysing these various feed additives and enforcing new legislation was LC-MS/MS. However the use of LC-MS/MS in medicinal additive analysis in animal feed is not routinely used and therefore this research focused on the development and implementation of LC-MS/MS methods for this purpose. Upon carrying out literature review it was decided that two methods would be developed; one for the analysis of as many prohibited medicinal feed additives as possible and one for the analysis of all allowed coccidiostat additives at levels set out in legislation.

The first of these methods was developed to allow for the analysis of 14 prohibited medicinal additives in pig and poultry compound feed. The method was one of the

first multi-class feed additive methods to be published and it had significant benefits over previously published articles. These included;

- It is a rapid multi-class confirmatory method that allowed for the quantitative analysis of 10 analytes and qualitative analysis of 4 analytes in pig and poultry compound feed which had not been seen in literature before.
- The method was capable of analysing all these compounds to a level of at least 100 µg kg^{-1}.
- The method developed in this study is an improvement on existing methods as it allows for the analysis of an increased number of analytes in this matrix with reduced sample preparation times and solvent usage.
- The applicability of the method for use on the various different types of pig and poultry compound feed was demonstrated by the satisfactory results obtained from the validation.
- The values achieved for accuracy, repeatability, reproducibility and MU all fall within acceptable ranges.
- The obtained confirmatory criteria of ion ratios and relative retention times for all analytes fulfil the requirements laid down in Commission Decision 2002/657/EC.

The development of this method had great economical benefits as well as improved analytical capabilities for monitoring laboratories. Previous methods were often single analyte methods that needed large samples sizes and large volumes of extraction solvents for analysis. As well as this a number of analytes such as dinitolimide did not have methods for their analysis. This method allowed for all

samples to be analysed for 14 analytes in a single run with less sample and solvent required.

The final LC-MS/MS method to be developed as part of this research was a method for the analysis of all eleven authorised coccidiostat feed additives at levels related to unavoidable carry over in non target feed. Methods routinely used in monitoring laboratories and many of the published methods in lliterature could only analyse for a select number of these compounds. The allowed levels in non target feed for these compounds ranged from the 5 mg kg^{-1} down to 10 µg kg^{-1} and some existing methods had difficulty reaching the required sensitivity. The method developed as part of this research could identify, confirm and quantify eleven coccidiostats at levels relating to unavoidable carry-over in a variety of feed samples that the laboratory might encounter on a routine basis and was validated in accordance with the requirements set out in Commission Decision 2002/657/EC. The benefits of this method are as follows;

- The method can be considered as rapid, as it utilises an efficient extraction protocol without the use of large sample sizes, extraction volumes or SPE. It also utilises chromatography which allows for the analysis of all analytes in a total run time of 19 minutes.
- This study shows that the developed method meets the desired sensitivity of 0.5% carry-over of the medicated level for all the compounds.
- The method performs satisfactorily in terms of accuracy and precision (%CV) for each of the analytes investigated and all fall within acceptable ranges. Levels for CCα and CCβ were calculated for all compounds.

- The method was further evaluated by using it to analyse for these compounds in non compliant samples. Results achieved were within MU for all analytes when compared to the assigned values.
- The reduced number of analytical steps within the method makes it very amenable to high throughput regulatory monitoring of these compounds and as a result enforcing Commission Directive 2009/8/EC.

The primary aim of this research; meeting the requirements of new EU legislation with regard to medicinal feed additives was achieved by developing two new liquid chromatography tandem mass spectrometry methods for the analysis of prohibited medicinal feed additives in pig and poultry feed and eleven coccidiostats at unavoidable carry over levels in animal feed. Both methods used efficient extraction protocols with reduced usage of extraction solvents and decrease in sample preparation time which will result in economical benefits for monitoring laboratories. This work will contribute greatly to the research that is ongoing in this area as it has shown improvements on many existing methods published in literature.

9.2 Future Work

9.2.1 Generic extractions for multi-class methods.

This research has demonstrated that in some cases the use of highly selective and sensitive mass spectrometry techniques reduces the need for complex extraction protocols utilising labour intensive purification steps. Leading on from this research it is envisaged that future work in this area will be focused on the use of generic extraction protocols for the analysis of multiple residues from multiple classes of compounds in multiple matrices in a single method. An example of this is the extraction method utilised in this study for nitroimidazoles. This was used for the

analysis of nitroimidazoles and chloramphenicol in milk and honey. From examination of literature similar extraction protocols with acetonitrile and phase separation with NaCl has been used for the analysis of non steroidal anti inflammatory drugs (NSAIDs) and corticosteroids in milk [Malone *et al*., 2009; Malone *et al*., 2010]. Taken this into consideration this extraction procedure has the capabilities of analysing for at least four classes of veterinary residues that routinely monitored for as part of a European wide monitoring program.

Although there has been some work in the development of generic extraction methods for the analysis of veterinary residues in biological matrices it is still somewhat in it's infancy. However the use of such extraction protocols has, for some time now, been utilised in the analysis of pesticides. The most commonly used extraction method utilised in this area is known as QuEChERS which stands for Quick, Easy, Cheap, Effective, Rugged, and Safe. There is a wide range of these type of methods used in pesticide residue analysis but they have the same core elements involved in their extraction with some minor adaptations for specific analytes. Anastassiadiades *et al*., 2003 and Lehotay *et al*., 2005 were some of the first to publish methods utilising this type of generic method in pesticide analysis. Samples are extracted with acidified acetonitrile. LLE is induced by addition of $MgSO_4$ and NaCl to remove water. The sample then undergoes purification with what is known as dispersive solid phase extraction (d-SPE) by the addition of primary secondary amine (PSA), C18 sorbents and $MgSO_4$. The extract is centrifuged before injection and initially allowed for the analysis of 80 different pesticides in a single extraction but has been further developed to allow up to 229 pesticides to be incorporated [Lehotay *et al*., 2005].

There are a number of benefits to this type of approach in pesticide analysis which might be beneficial in veterinary residue analysis [Lehotay *et al*., 2005]. These are;

- High recoveries (>85%) are achieved for a wide range of polarity and volatility.
- Very accurate results are achieved because an IS is used to correct for commodity to commodity water content differences and volume fluctuations.
- High sample through-put of 10-20 preweighed samples in 30-40min.
- A single person can perform the extraction without much training or technical skill.
- Despite ease of method the procedure is still quite rugged.

This type of generic extraction if adapted for the analysis of veterinary residues could greatly improve the analytical capabilities of monitoring laboratories. To date there has been only one published method for the analysis of veterinary residues attempting to use QuEChERS protocol. Stubbings *et al*., 2009 published results of their research for the analysis of 11 different classes of verteinary residues in animal tissue. The published method was capable of screening for near 50 different residues but was limited by there use of LC-MS/MS system. They needed to run samples several times in order to achieve a complete screen.

This is where problems with the use of LC-MS/MS in this type of multi-class multi-analyte work arise. This analytical instrument while it gives unequivocal confirmation it can only analyse for a finite amount of analytes. The number of analytes it can analyse for is governed by a number of factors such as scanning speed, dwell time, need for pos/neg switching as well as column separation in order to allow for segmentation of the MS/MS program. Therefore while a large number of analytes could be determined using this instrument it might be a limiting factor. With the advent of UHPLC-MS/MS more analytes can be incorporated due to increased separation of analyte in shorter run times. However it is felt that future work in this

area will lead to use of these type of generic extraction procedures in combination with liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). The benefits of this instrument technique are discussed fully in the next section.

9.2.2 Screening for veterinary residues by High Resolution Mass Spectrometry (HRMS).

Many analytical methods currently employed in monitoring laboratories throughout Europe for the analysis of veterinary residues are performed using LC-MS/MS. The major drawback of this technique is that it only offers targeted analysis i.e. only compounds that the instrument is tuned may be detected. However, with the advent of HRMS combined with recent advances in extraction procedures, the possibility exists to monitor for potentially 100's of harmful substances in one assay. Hence there will be increased confidence that the food that reaches the consumer will be free from harmful residues. From examination of published literature it has become apparent that the capabilities of HRMS may be applied to the area of veterinary residue analysis and offers many exciting opportunities [Kaufmann *et al*., 2011; Peters *et al*., 2009]. The relatively few articles published to date in this area have shown the impressive capabilities and possibilities of this technique.

To date this instrument is not considered a confirmatory instrument but never the less its screening capability is vast and when applied to veterinary residues analysis has the ability to improve analytical capability immensely. It is felt that future work in this area will look at the possible combination of generic extraction procedures and HRMS for the analysis of large numbers of residues in a single run. Two common instruments that work on the basis of HRMS are Orbitrap and Time of Flight

instruments. These instruments work on the basis of accurate mass calculations with high resolution that allow for molecular weight determination to 4 decimal places. This allows for compounds to be distinguished from each other. These instruments can provide full scan targeted/untargeted screening and this approach offers a number of advantages:

- Using these instruments in untargeted full scan with a generic extraction protocol allows samples of edible matrices (milk, eggs, meat etc.) containing possibly dangerous levels of veterinary residues to be identified that may be otherwise declared compliant.
- It also allows for the re-interrogation of the data at a future date to examine for the presence of possible residues which laboratories may not have been aware of at the time of analysis.
- The chemical formula of unknown residues can be determined which may allow for identification of previously unknown compounds that are potentially being abused.

It is anticipated that with decreasing cost of these instruments over time they will be introduced more for performing targeted and untargeted screening assays of veterinary residues in monitoring laboratories. It is believed the combination of this powerful screening tool with generic extraction protocols such as QuEChERS will lead to an improved monitoring program throughout EU member states for veterinary residues.

9.2.3 Development of a "risk based" approach to the monitoring of veterinary residues.

The overall goal for this research was to improve the veterinary residue surveillance capabilities of monitoring laboratories by the development of novel, efficient multi-residue analytical methods. As previously stated the use of veterinary drugs is becoming a critical component of food production which has resulted in an increased demand for veterinary residue surveillance systems. The current approach adopted throughout the EU member states involves the analysis of a percentage of animals slaughtered in the previous year as directed by Council Directive 96/23/EC. An example of this approach is given below for bovine species taken from the legislation;

"The minimum number of animals to be controlled each year for all kinds of residues and substances must at least equal 0.4 % of bovine animals slaughtered the previous year, with the following breakdown:

Group A: 0.25 % divided as follows:

- one half of the samples are to be taken from live animals on the holding;
- one half of the samples are to be taken at the slaughterhouse.

Each sub-group in Group A must be checked each year using a minimum of 5 % of the total number of samples to be collected for Group A. The balance must be allocated according to the experience and background information of the Member State.

Group B: 0.15 %

30 % of the samples must be checked for Group B 1 substances.

30 % of the samples must be checked for Group B 2 substances.

10 % of the samples must be checked for Group B 3 substances.

The balance must be allocated according to the situation of the Member State species for each class of compound every year."

Key concerns over this type of approach are the economic viability of such surveillance programmes when the prevalence of a residue approaches zero.

As human and financial resources available to support government monitoring of veterinary residues are becoming more limited in many countries world-wide a search to find a more efficient monitoring protocol is being considered. Hence the concept of "risk based" monitoring programs has become an area of interest in veterinary residue analysis. The main tenet of this approach focuses on the analysis of compounds in particular species that pose a higher risk to human health. It is felt, issues that present higher risks merit higher priority for residue surveillance resources as investments will yield higher benefit-cost ratios.

As part of this research a survey of retail egg samples available on the Irish market for the presence of nitroimidazole residues was performed. The results of this found no noncompliant results in 160 samples. Therefore the assumption could be made that the risk posed by nitroimidazole residues in eggs is relatively small. However more factors need to be taken into consideration when developing risk based assessments. This is where problems have arisen in implementing a risk based approach to residue analysis. It is difficult to clarify the meaning of risk. Factors such as toxicity of residues, overall occurrence of residues, species in which residues typically occur, the intake of residues into humans from food and the deterioration of residues after traditional cooking processes need to be taken into consideration when developing risk based monitoring programs.

As a result of this, a lot of future work should go into assessing the risk associated with these factors. Further toxicological studies should be carried out to assess the

risk of residues to humans. Surveys like the one carried out in this research for nitroimidazole residues in eggs, in addition to current monitoring plans, should be carried out throughout Europe in a wide range of matrices to assess the occurrences of different residues and the species they typically occur in. This information along with values of Average Daily Intake (ADI) of certain foods for humans should be used in determining the risk associated with certain residues. This associated risk will then be used to determine what residues in what species should be given higher priority when drafting residue plans.

9.2.4 Future work on contaminants in animal feed

Following an investigation into the Irish Dioxin crisis resulting from contaminated feed fed to pigs in 2008 it was noted that there is too much emphasis placed on the analysis of the quality of feed rather than on the monitoring for the presence of undesirable substances in the feed. Therefore it was suggested that more samples should be taken for the analysis of undesirables in animal feed. Hence as part of this research, methods for the analysis of medicinal feed additives were developed. These included detecting medicinal substances in feed that are no longer authorized as feed additives as listed in Annex II of Commission Recommendation 2005/925/EC and monitoring of unavoidable carryover of coccidiostats in non-targeted feed as per Commission Directive 2009/8/EC.

Future work in this area will continue and should focus on the development of multi-analyte methods for the analysis of contaminants in animal feed such as mycotoxins and dioxins. Another area that should possibly be examined is the setting of unavoidable carryover levels for medicines such as sulfadiazine and chlortetracycline

in non medicated feed similar to what is set in legislation for cocciodisats in non target feed. This work would be beneficial for a number of reasons;

- *Analytical methods for analysis of mycotoxins in animal feed:* Fungal growths and moulds on crops and animal feed can produce various mycotoxins. These mycotoxins can pose a threat to animal health, consumer safety and food processing activities if they occur at unacceptable limits. There are a number of different parent classes of mycotoxins such as fusarium, aspergillus and penicillium mycotoxins all with numerous sub components. Commission Regulation (EC) No. 1881/2006 sets out maximum levels for contaminants in foodstuffs. Levels are set for a number of mycotoxins including aflatoxins, ochratoxin and deoxynivalenol. With the advent of newer, more selective and sensitive analytical techniques emphasis should be place on the development confirmatory multi analytes method for the analysis of a wide range of these analytes in animal feedingstuffs in order to ensure these harmful contaminants are below permitted levels.
- *Analytical methods for analysis of dioxins in animal feed:* The general term "dioxin" collectively refers to a class of structurally and chemically related compounds known as halogenated aromatic hydrocarbons. They include poly-chlorinated dibenzo-p-dioxins (PCDD or Dioxin's), polychlorinated dibenzofurans (PCDF or Furans) and the "dioxin-like" Biphenyls (PCBs). Dioxins and planar PCBs are carcinogenic and may have adverse effects on reproduction. Dioxins and planar PCBs are fat soluble chemicals and exposure of humans to these contaminants is largely from fat-containing foods of animal origin. Many of the dioxin scares and crises are the result of animals fed dioxin contaminated feed. This was the case in France in 1998 where dioxin-

contaminated citrus pulp from Brazil was used in feed for dairy animals and resulted in contaminated milk, and also in Belgium in 1999 where gross contamination of waste edible oil with machine oil, resulted in contaminated animal feed and contaminated food products such as poultry, eggs, red meat and milk. More recently, there have also been two dioxin crises in Ireland (2008) and Germany (2011). In Ireland, animal feed was contaminated with dioxins as a result of an improper fuel being used in a direct drying burner system used to dry animal feed which resulted in dioxin contaminated pork products on the international market. Also in Germany the substitution of dioxin-contaminated industrial fats for vegetable fats in animal feed resulted in the contamination in possibly a 1000 pig and poultry farms. As a result of these contaminations and the potential major health risks associated with these compounds increased focus should be put on the analysis of these compounds in animal feed and the development of multi-class method for their analysis.

- *Setting of unavoidable carryover levels of medicines in non medicated feed:*

  During this research, work was carried out on the analysis of medicinal additives no longer authorised as feed additives. While some of these compounds such as nitroimidazoles are no longer licensed for use in animals some are still permitted to be used as medicines. Examples of these compounds are sulfadiazine, tylosin and chlortetracycline are still used in medicated feed. Part of the validation process involved sourcing blank feed material for use as negative controls. However this proved quite challenging as it became apparent that low levels of these medicines were present in a number of non medicated feed samples possibly due to their carryover in feed mills. Currently the EU has a zero tolerance policy to the presence of these substances in non medicated

feed. However this is not practical and a similar approach of setting maximum unavoidable carryover levels as used with coccidiostats in non target feed should be considered.

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