Analytical Strategies for Drug Residues in Various Matrices in a Regulatory Laboratory.

Geraldine Dowling
Dublin Institute of Technology

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Analytical Strategies for Drug Residues in Various Matrices in a Regulatory Laboratory

BY
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Thesis submitted in fulfilment of requirement leading to the award of the degree of Doctor of Philosophy on the Basis of Publications

School of Chemical and Pharmaceutical Sciences,
Dublin Institute of Technology, Kevin Street, Dublin 8.
November 2012

Supervisors: Dr Barry Foley

and

Dr Liam Regan
DECLARATION PAGE

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Candidate
ABSTRACT

Today, in modern farming practices veterinary drugs are given to food-producing animals. The generic term “veterinary drugs” covers a broad variety of classes of chemical compounds and the list of drugs is enormous and it is a significant analytical challenge in regulatory control to provide monitoring programmes. The major concerns in veterinary drug usage are the presence of harmful residues that may be introduced into the human food chain. The aim of this research is the development of analytical methods capable of screening and confirming increased numbers of these residues in target matrices by Gas Chromatography Tandem Mass Spectrometry (GC-MS/MS) and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The focus is on Non-Steroidal Anti Inflammatory Drugs (NSAIDs) residues in food of animal origin. The research resulted in the development and validation of methods for the analysis of NSAIDs in milk. The second topic under investigation was in the field of post-mortem forensic toxicology. Forensic toxicological activities are important in society and great efforts to implement rapid analytical procedures in a forensically credible manner is continuous across the globe. The main objectives of the research work in post-mortem forensic toxicology were to address the issues concerning the availability of rapid novel unequivocal confirmatory analytical methodologies for forensically important drugs. The aim of this work was to develop single fast, simple and reliable sample preparation procedures in blood and urine with detection by hybrid LC-MS as analytical strategies in a forensic laboratory for the determination of drugs of abuse in this study which were previously not available. All analytical methodologies developed in blood, milk and urine matrices were validated in accordance with EU legislation; Commission Decision
2002/657/EC. This validation legislation is concerned with the interpretation of results and the performance of analytical methods. Validation criteria were examined using protocols laid down in this legislation. These included specificity, accuracy, precision, repeatability, reproducibility, decision limits (CCα), detection capabilities (CCβ) and in addition measurement uncertainty (MU). The methods developed for the NSAIDs residues in milk had accuracies ranging from 73-109 % and the precision for all analytes ranged from 2-30%. The CCα and the CCβ values ranged from 0.46-0.79 ng mL⁻¹ and the MU ranged from 9-90%. The methods developed for the forensically important drugs in blood and urine had accuracies ranging from 80-113% and the precision for all analytes ranged from 4-22%. The CCα and the CCβ values ranged from 0.005-0.05 ug mL⁻¹ and the MU ranged from 13-47%. To ensure that methods were suitable for purpose the methods were evaluated in international proficiency testing schemes and results were satisfactory. In addition some of the methods were accredited in accordance with ISO17025 guidelines. A number of different analysts were trained on the new methodologies developed in this work thus providing further evidence of the development of rugged analytical methods and satisfactory results were obtained using quality control material. In addition the methods have replaced previous methods in The State Laboratory for the monitoring of NSAIDs and are used routinely at present in the Republic of Ireland in national monitoring programmes. The methods developed for blood and urine in the forensic toxicology section of this thesis have replaced previous methods in national forensic toxicology monitoring programmes in the past number of years at The State Laboratory and are used in toxicology testing for the law courts in forensic and Coroner’s cases in Ireland. The method in this thesis for forensic drugs in...
blood is submitted for accreditation in 2012 as satisfactory results were obtained using quality control and international proficiency test samples in the past number of years.
ACKNOWLEDGEMENTS

“The flower that blooms in adversity is the most rare and beautiful of all” – Mulan 1998

“The Emerald Isles is were I awakened and before me many were awake Boyle, Conway and Tyndall of Carlow their fragrance left behind for me to follow, Walton Kelvin and Joly then walked the path and joined others that had once stepped on green grass, Yeats, Shaw and Joyce then came along and made this land of St Patrick such a soul. I am but a simple soul but my dreams are high hidden in my soul. I wanted to reach high and conquer every mountain built high. I hoped and hoped and knew that blood, sweat and tears will bring what lay beyond. The hill was steep and pace at first slow but my belief was strong and the hill was climbed. I recalled the words of my mother” what doesn’t kill you will make you strong”. I feel proud of this land of mine as it has made my dreams come true. As I go forward I think of other sons whom have left these shores and left their spirit behind. I think of Kennedy, Swift and Wilde and know my work is not done until I have done my fair share of giving back to this dear dear land of mine” – Rosho 2012

I want to thank everyone who has offered their help through sharing their knowledge, understanding, advice, support and encouragement. I will never forget a second of this precious time and I am eternally grateful. I deeply appreciate it beyond words. I would like to particularly mention my supervisors Dr. Liam Regan and Dr. Barry Foley for believing in my idea to go forward with the PhD on the basis of publications. I would like to thank the State Laboratory in addition. I particularly would like to mention and thank Edward Malone for his kindness in answering my questions and giving help as required. I would like to thank John Power for his help on legislation. I would like to thank Mary G for her time to listen and I will always appreciate this. I would like to thank Michel Aliani for his kindness, support and funny dances!

I would like to thank Paddy, Mairead and Jean for their encouragement. I would like to thank gratefully Ramesh for the positivity and good wishes. I would like to thank Olivia and Mairead R for making me laugh and Myra and Johanna for their support. I would like to thank Maureen Ryan for her wisdom. I would most graciously like to thank my Mam and Dad for their support and acceptance of my unconventional ways of doing things. I would also like to thank Lisa, Emma and my brother Paul for being yourselves just as you are.

Geraldine Dowing 23rd November 2012
ABBREVIATIONS

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<th>Description</th>
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<tr>
<td>4-MAA</td>
<td>4-Methylaminoantipyrine</td>
</tr>
<tr>
<td>6-MAM</td>
<td>6-Monoacetylmorphine</td>
</tr>
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<td>AA</td>
<td>Arachidonic Acid</td>
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<td>ADHD</td>
<td>Attention Deficit Hyperactivity Disorder</td>
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<td>ADI</td>
<td>Acceptable Daily Intake</td>
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<td>AEME</td>
<td>Anhydroecgonine Methyl Ester</td>
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<td>AMP</td>
<td>Amphetamine</td>
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<tr>
<td>ANSES</td>
<td>National Agency for Food Environment and Occupational Health Safety</td>
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<td>AORC</td>
<td>Association of Official Racing Chemists</td>
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<tr>
<td>APCI</td>
<td>Atmospheric Pressure Chemical Ionisation</td>
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<td>API</td>
<td>Atmospheric Pressure Ionisation</td>
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<tr>
<td>APPI</td>
<td>Atmospheric Pressure Photo Ionisation</td>
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<tr>
<td>BENZOYL</td>
<td>Benzoylgonine</td>
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<td>BUPREN</td>
<td>Buprenorphine</td>
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<tr>
<td>BVL</td>
<td>Bundesamt für Verbraucherschutz und Lebensmittelsicherheit</td>
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<tr>
<td>BZP</td>
<td>N-Benzylpiperazine</td>
</tr>
<tr>
<td>CCα</td>
<td>Decision limit</td>
</tr>
<tr>
<td>CCβ</td>
<td>Detection capability</td>
</tr>
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<td>CD</td>
<td>Commission Decision</td>
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<td>CEDIA</td>
<td>Cloned Enzyme Donor Immunoassay</td>
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<td>Collision Energy Spread</td>
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<td>ChRM</td>
<td>Charge Residue Model</td>
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<td>Chemical Ionisation</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>COC</td>
<td>Cocaine</td>
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<td>COCA</td>
<td>Cocaethylene</td>
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<td>COD</td>
<td>Codeine</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>Carprofen</td>
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<td>CRL</td>
<td>Community Reference Laboratory</td>
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<td>CRM</td>
<td>Certified Reference Material</td>
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<td>DAPPI</td>
<td>Desorption Atmospheric Pressure Photo Ionisation</td>
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<td>DART</td>
<td>Direct Analysis in Real Time</td>
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<td>DCF</td>
<td>Diclofenac</td>
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<td>DESI</td>
<td>Desorption Electrospray Ionisation</td>
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<td>DHP</td>
<td>4,6-dimethyl-3-hydroxypyrimidine</td>
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<td>DIT</td>
<td>Dublin Institute of Technology</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>DNC</td>
<td>4,4'-dinitrocarbanilide</td>
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<td>Definitive Type 104</td>
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<td>EARS</td>
<td>European Antimicrobial Resistance Monitoring System</td>
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<td>EC</td>
<td>European Commission</td>
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<td>EDDP</td>
<td>2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine</td>
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<td>EI</td>
<td>Electron Ionisation</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assays</td>
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<td>EMCDDA</td>
<td>European Monitoring Centre for Drugs and Drug Addiction</td>
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<td>EMDP</td>
<td>2-ethyl-5-methyl-3,3-diphenylpyrroline</td>
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<tr>
<td>EMEA</td>
<td>The European Agency for the Evaluation of Veterinary Products</td>
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<tr>
<td>EMIT</td>
<td>Enzyme Multiplied Immunoassay Technique</td>
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<td>ENF</td>
<td>Enrofloxacin</td>
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<td>EPI</td>
<td>Enhanced Product Ion</td>
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<td>ESI</td>
<td>Electrospray Ionisation</td>
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<td>EU</td>
<td>European Union</td>
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<td>FAO</td>
<td>Food Agriculture Organisation</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FIRO</td>
<td>Firocoxib</td>
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<td>FLU-OH</td>
<td>Hydroxy Flunixin</td>
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<td>FPIA</td>
<td>Fluorescence -Polarisation Immunoassay</td>
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<td>FWHM</td>
<td>Formula Width Half Measurement</td>
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<td>GC</td>
<td>Gas Chromatography</td>
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<td>GC-MS</td>
<td>Gas Chromatography Mass Spectrometry</td>
</tr>
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<td>GC-MS-MS</td>
<td>Gas Chromatography Tandem Mass Spectrometry</td>
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<tr>
<td>GI</td>
<td>Gastrojntestine</td>
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<tr>
<td>HCX</td>
<td>Weak cation exchange sorbent</td>
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<tr>
<td>HFAA</td>
<td>HeptaFluorobutyric Acid Anhydride</td>
</tr>
<tr>
<td>HFG</td>
<td>Halofuginone</td>
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<td>HFPDOH</td>
<td>1,1,1-3,3,3-Hexafluoro-2-propanol</td>
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<tr>
<td>HLB</td>
<td>Hydrophilic/Lipophilic Balance</td>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>IBP</td>
<td>Ibuprofen</td>
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<tr>
<td>IDA</td>
<td>Information Dependent Acquisition</td>
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<tr>
<td>IEM</td>
<td>Ion Evaporation Mechanism</td>
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<td>IOC</td>
<td>International Olympic Committee</td>
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<tr>
<td>IP's</td>
<td>Identification Points</td>
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<tr>
<td>JVARM</td>
<td>Japanese Veterinary Anti-microbial Resistance Monitoring system</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>PHEN</td>
<td>Phenethylamine</td>
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<tr>
<td>PS-DVB</td>
<td>Polystyrene-divinylbenzene</td>
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<tr>
<td>PT</td>
<td>Proficiency Test</td>
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<tr>
<td>QqQ</td>
<td>Triplequadrupole</td>
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<tr>
<td>QTOF</td>
<td>Quadrupole Time of Flight</td>
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<td>QTRAP</td>
<td>Quadrupole Linear Ion Trap</td>
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<tr>
<td>r²</td>
<td>Regression coefficient</td>
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<td>RIA</td>
<td>Radio Immunoassay</td>
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<tr>
<td>RRLC</td>
<td>Rapid Resolution Liquid Chromatography</td>
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<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
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<tr>
<td>Rt</td>
<td>Retention Time</td>
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<tr>
<td>S/N</td>
<td>Signal to Noise Ratio</td>
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<td>SANCO</td>
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<td>SFE/ASE</td>
<td>Supercritical Fluid Extraction/Accelerated Solvent Extraction</td>
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<tr>
<td>SIM</td>
<td>Selected Ion Monitoring</td>
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<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
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<tr>
<td>SPME</td>
<td>Matrix Solid Phase Dispersion</td>
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<td>SPR</td>
<td>Surface Plasmon Resonance</td>
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<td>SRM</td>
<td>Single Reaction Monitoring</td>
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<td>Std</td>
<td>Standard</td>
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<td>SUXI</td>
<td>Suxibutazone</td>
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<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<td>TLF</td>
<td>Tolfenamic Acid</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of Flight</td>
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<tr>
<td>TR-FIA</td>
<td>Time resolved fluoroimmunoassay</td>
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<tr>
<td>TRL</td>
<td>Total Residue Level</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
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<tr>
<td>UN</td>
<td>United Nations</td>
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<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
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<td>USFDA</td>
<td>United States Food and Drug Administration</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VDP</td>
<td>Vedaprofen</td>
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<tr>
<td>WADA</td>
<td>World Anti-Doping Agency</td>
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2.3.4.2 Uses of piperazine type drugs

2.3.4.3 Risks to individuals ingesting piperazine type drugs

2.3.5 Ketamine

2.3.5.1 Pharmacokinetics and Metabolism

2.3.5.2 Uses of Ketamine

2.3.5.3 Risks to Individuals Injecting Ketamine

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2.5.2.1 Extraction Methods and Purification Strategies for amphetamines, cocaines, opioids and adulterants

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CHAPTER 1: INTRODUCTION
1.1 Aims, Objectives and Relevance of this Research Work

1.1.1 Introduction

This research was carried out in The State Laboratory, Ireland. The role of the State Laboratory is to provide expert scientific advice to government departments and other clients. The laboratory is well equipped with advanced instrumentation to meet the laboratories wide client base. A trend in the laboratory has been to introduce modern techniques to replace current ones in order to introduce better efficiency. The efficiencies gained can come in several different areas as the more modern technology may offer greater sensitivity, allow for the use of more simplified extraction procedures, or increases in sensitivity which may allow for detection of compounds not previously detectable, allow for wider ranges of substances to be analysed in a single detection protocol and in addition modern instruments will have computerised data systems which can be integrated with laboratory information management systems (LIMs) sample management technology. This can improve data transfer in order to speed up calculations and improve sample reporting efficiencies to improve turn around times for clients. The laboratory is also a National Reference Laboratory for Veterinary Drug Residues and gives advice to the Department of Agriculture and other national laboratories performing analysis on the National Veterinary Residue Control Plan. The research in this thesis was carried out on the topic of non-steroidal anti-inflammatory drugs (NSAIDs) as the State Laboratory is a National Reference Laboratory for these classes of veterinary drugs. Therefore the thesis covers the veterinary drug residues field as the first topic for investigation in order to improve monitoring at the laboratory for these drugs in Ireland.

As milk is a matrix that is directly consumed by the general public, careful consideration should be given in food safety to the possibility of veterinary drug residues.

The food safety issues concerning edible matrices and NSAIDs were studied.

It was the purpose of the work carried out in this thesis to develop analytical strategies for NSAIDs residues that were previously not available to meet legislative requirements.

The main objectives of the research work on veterinary drug residues described in four separate chapters in this thesis were to address food safety concerning NSAIDS and
edible matrices by developing new analytical strategies for the analysis of these substances that were previously not available to regulatory laboratories and included:

- The development of quantitative confirmatory Gas Chromatography Tandem Mass Spectrometry method that meets EU target limits set for the analysis of ibuprofen (IBP), ketoprofen (KPF), diclofenac (DCF) and phenylbutazone (PBZ) in milk.
- The development of a quantitative confirmatory method for the analysis of firocoxib (FIRO) in milk using Rapid Resolution Liquid Chromatography Tandem Mass Spectrometry.
- The development of a rapid quantitative confirmatory method for the determination of carprofen (CFF), diclofenac (DCF), mefenamic acid (MFN), niflumic (NIFLU), naproxen (NAP), oxyphenylbutazone (OXYPHEN), phenylbutazone (PBZ) and suxibuzone (SUXI) in milk using Rapid Resolution Liquid Chromatography Tandem Mass Spectrometry.
- The development of an analytical strategy for the determination of six NSAIDs in bovine plasma and an improved analytical strategy for the simultaneous determination of authorised and non-authorised residues of ten NSAIDs in milk by liquid chromatography tandem mass spectrometry.
- To adopt the criteria governing method validation for these substances according to Commission Decision 2002/657/EC.
- To apply these methodologies to routine analysis and implement into the National Reference Laboratory in Ireland for NSAIDs.

The thesis covers the field of post-mortem forensic toxicology as the second topic for investigation in order to improve monitoring at the laboratory for forensically important drugs. The State Laboratory provides a toxicological service to the Coroner's of Ireland. The work described in this thesis involved developing new methods in order to improve the toxicological service to the Coroner's of Ireland. Work was undertaken in order to
evaluate newer technology to improve overall analysis time in the laboratory and expand the classes of drugs for analysis. The second topic in this thesis covers the improvement of toxicological analyses in the post-mortem forensic toxicological field to give confirmatory results on the absence or presence of drugs and their metabolites. To help address the issues of obtaining rapid post-mortem forensic toxicology analytical results, the development of rapid analytical methods in blood and urine matrices in particular was focused on as the subject matter of this thesis targeting opioids, cocaines and amphetamine drugs. The availability of such analytical strategies would allow results to be given to Irish Court system, An Garda Siochana and families of the deceased more efficiently. Forensic toxicological activities are important in society and great efforts to implement rapid analytical procedures in a forensically credible manner is continuous across the globe. The main objectives of the research work on post-mortem forensic toxicology were to address the issues concerning the availability of rapid, novel, unequivocal confirmatory analytical methodologies for forensically important drugs. The aim of this work was to develop single fast, simple and reliable sample preparation procedures in blood and urine with detection by hybrid LC-MS as analytical strategies in a forensic laboratory for the determination of drugs of abuse which were previously not available. Areas focused on were the following:

- To adopt the criteria governing method validation for forensically important substances according to Commission Decision 2002/657/EC as an alternative validation protocol.
- To develop a hybrid liquid chromatography mass spectrometry strategy in a forensic laboratory for opioid, cocaine and amphetamine classes in human urine.
• To develop a new mixed mode solid phase extraction strategy for the determination of opioids, cocaines, amphetamines and adulterants in human blood.
• To apply these methodologies to routine analysis and implement into the National Confirmatory Laboratory in Ireland for Post-Mortem Forensic Toxicology in Ireland to replace traditionally used GC-MS techniques.
• The methods will be used routinely to establish the cause of death based on requests from the medical examiner, the coroner or the pathologists in Ireland.

1.2 Veterinary Drug Residue Issues in Foods for Human Consumption

1.2.1 Introduction

Today, in modern farming practices veterinary drugs are given to food-producing animals. The generic term “veterinary drugs” covers a broad variety of classes of chemical compounds and among these, for example include non-steroidal anti-inflammatory drugs (NSAIDs) quinolones, β-lactams, macrolides, lincosamides, sulphonamides, tetracyclines, antiparasitic agents such as anthelmintics or coccidiostats, β-agonists, nitrofurans and nitroimidazoles. However the list is enormous and it is a significant analytical challenge in regulatory control to provide monitoring programmes. The presence of pharmacologically active chemicals in a food-producing animal can give rise to the occurrence of residues in food. This is an important issue in the food safety area. The major concerns in veterinary drug usage are the presence of harmful residues that may be introduced into the human food chain. In addition ingestion of antibiotic drugs could cause residues that aid the development of antibiotic resistant pathogens. In the EU this food safety problem has been dealt with by providing legislation for veterinary drug residues in food. [Mitchell et al., 1998]. The inability to meet legislative requirements in relation to levels of residues in food could have severe results for the nation concerned. There is also on-going public interest in relation to drug residues in food worldwide due to more intense methods of animal husbandry. There is concern over the environmental impact of the agricultural practices and concern over animal welfare.
The global demand for animal products is projected to increase considerably due mainly to the increase in population. Predictions for the future growth of the population vary but a United Nations report in 2008 states that the world population will exceed 9 billion in 2050 [UN 2008]. In 2009 the Food and Agriculture Organisation (FAO) released a report entitled “The State of Food and Agriculture” which highlights that since the 1960’s consumption of milk per capita in the developing countries has almost doubled, meat consumption more than tripled and egg consumption increased by a factor of five [FAO 2009]. Trends showed that developing countries have responded to growing demand for livestock products by rapidly increasing production. Between 1961 and 2007, the greatest growth in meat production occurred in East and Southeast Asia, followed by Latin America and the Caribbean and most of the expansion in egg production was in East and Southeast Asia, while Southeast Asia dominated milk production [FAO 2009]. The increase in productivity has been as a result of a combination of factors such as advanced breeding and feeding technology, the use of hybridisation and artificial insemination accelerating the progress of genetic improvement, improvements in feed technology and improvements in animal health technology including the use of vaccines and antibiotics. The bulk of livestock produce is consumed generally within a country of production and does not enter international trade but for some countries livestock produce exports are important. Contamination of livestock products nationally and internationally from Asia and Latin America with veterinary drug residues can be the cause of international trade interventions leading to a loss of revenue for the exporting countries [Mitchell et al., 1998].

Veterinary therapeutic products are generally used in livestock production for one of three purposes [National Research Council 1999].

1. Therapeutic treatment of active infection.
2. Vaccination or prophylactic medication to prevent or to minimise infection.
3. Production enhancement-growth promotion and improvement of feed conversion efficiency with antimicrobial drugs and hormones.
1.2.2 Improper or illegal use of veterinary drugs

The incidence of residues in food, in violation of the levels set down in legislation, can occur in a variety of circumstances. These infringements result from either the inappropriate use of licensed products or the illegal use of unlicensed and prohibited substances [Kennedy et al., 2000]. A number of situations contribute to the improper use of licensed products, including, poor treatment records or failure to recognise treated animals and use of a drug other than as described on the product label. Residues occur chiefly by not observing sufficient withdrawal periods before slaughter of animals. As long as a licensed drug is used in accordance with its product licence and as long as the drug withdrawal periods are respected by farmers, drug residues should not occur in human food at concentrations exceeding the maximum residue limits (MRLs).

Deliberate use of prohibited drugs can occur for the following number of reasons which includes: the use of certain banned compounds that can be very efficient at controlling particular infections e.g. nitrofurans or banned compounds that can be very cheap and readily available e.g. phenylbutazone (PBZ) and the use of growth promoting hormones in order to enhance the weight of animals. Prohibited compounds such as PBZ which were once authorised but now banned. PBZ is widely used in the equine industry making it readily available [Dodman et al., 2010].

Cross contamination in feed mills is also another circumstance in which high levels of residues could potentially occur in food [Kennedy et al., 2000]. Trace quantities of medicated feed may be retained at various points along the production line, contaminating subsequent batches of feed as they are processed. The electrostatic properties, especially those of some drugs in powder form, exaggerate the problem, making it more complicated to purge the equipment between batches. Manufacturers have produced granular formulations with reduced electrostatic properties in reaction to this. The electrostatic properties of the drug nicarbazin (NCB) caused problems during the manufacture of NCB-free feeds, since NCB powder is strongly electrostatic and could not be removed from feed milling equipment. [Canavan et al., 2000]. NCB is a widely used coccidiostat drug, which is licensed as a feed additive for broiler chickens, but not for laying hens. There is a predicament in many countries world-wide with the incidence
of NCB residues in poultry tissues and eggs. Canavan et al., [2000] have reported a proportional relationship between the concentration of 4,4'-dinitrocarbanilide (DNC) and 4,6-dimethyl-3-hydroxypyrimidine (DHP) in eggs, the two marker residues for NCB, and the feed levels. Feed contaminated with NCB at concentrations higher than 2 mg kg$^{-1}$ gave rise to residue concentrations of DNC in eggs higher than 100 μg kg$^{-1}$. Yakkundi et al., [2002] designed an experiment to establish the relationship between halofuginone (HFG) in contaminated feed and HFG residues in eggs. HFG is used to treat coccidiosis also and has been registered as an anticoccidial feed additive for broiler hens and turkeys. In general the HFG concentration was much lower than those seen in similar studies on NCB. However comparison of the HFG concentrations measured in eggs and the MRL for HFG in bovine muscle suggested that feed contamination could give rise to potentially significant HFG residues in eggs. The study also showed that depletion of HFG from eggs was slower compared to NCB and lasalocid [Yakkundi et al., 2002].

1.2.3 Environmental contamination

Commercial fish farming could be another source of potential contamination where veterinary drugs or other contaminants, which have been given as feed additives, accumulate in sediments and may become ingested by marine species in the vicinity of farms [Kennedy et al., 2000]. The presence of drug residues may pose a health risk, if these wild species are harvested for human consumption. Murata et al., [2011] carried out a nationwide monitoring study of antibiotics in Japan. The antibiotics targeted in the study were sulphonamides, trimethoprim and macrolides. The results showed that the signals of the veterinary antibiotics were overwhelmed by those of human antibiotics in the lower reaches of most rivers. The concentrations were higher in urban rivers and were dominated by macrolides which were derived mainly from urban sewage although larger amounts of veterinary antibiotics are used in livestock. The authors postulate that lower concentrations of livestock antibiotics were found as livestock derived waste is unlikely to be readily discharged directly to surface waters. Nonetheless the threat posed by these residues is unknown. Other groups have found livestock waste caused antibiotic contamination of water supplies with veterinary drugs [Yang et al., 2003, Lissemore et al., 2006 and Wei et al., 2011].
1.2.4 Animal-to-animal transfer
Recycling of drugs due to ingestion of faeces and/or urine considerably contributes to the levels and persistence of residues in porcine tissues, poultry tissues and eggs [Kennedy et al., 2000, Cannavan et al., 2000]. Short-lived contact of unmedicated animals to the excretions of medicated animals in improperly cleaned accommodations during transport or in the slaughter house can result in high residues levels.
Cannavan et al., [2000] identified tenfold higher NCB values in the liver of broiler chickens treated with this drug and accommodated on flooring where litter can collect compared with those housed on wire flooring. Total exchange of the litter was the only way to stop the recycling by the birds.

1.3 The Risks to Human Health

1.3.1 Toxicity and allergenicity
A huge number of antibiotic and anthelmintic drugs administered in therapeutic and subtherapeutic forms to domesticated species are also approved for use in humans. Acute and chronic toxicity studies have been evaluated and shown these drugs to be reasonably safe [National Research Council, 1999]. The possibility of acute toxicity from veterinary drugs and their metabolites derived from animal tissues is exceptionally small. On the other hand the likelihood of chronic toxicity expressed in long-term, collective allergenic, mutagenic, teratogenic or carcinogenic effects is not easy to assess.
As a result veterinary drugs with a high probability to cause one or more of these toxic effects were banned by the EC for veterinary use in order to protect human health. Phenylbutazone (PBZ) is a NSAID that was authorised for administration in the United States for the treatment of rheumatoid arthritis and gout in 1952. Severe and often fatal adverse effects such as aplastic anemia and agranulocytosis appeared in the literature within a period of three years of its usage [Benjamin et al., 1981, Bottiger et al., 1973, Cameron et al., 1966, Chaplin 1986, Br Med J. 1952, Dunn et al., 1972, Etess et al., 1953, Hale et al., 1960, Leonard et al., 1953, Mauer et al., 1955, McCombs et al., 1958, Nelson et al., 1995, Ramsey et al., 1976, Steinberg et al., 1953]. Due to the bone marrow
toxicity of PBZ, the Food and Drug Administration (FDA) set no safe levels for PBZ in animals intended for food.

Chloramphenicol is another such compound which also causes toxic aplastic anaemia that does not correspond to dosage. Residues of tetracycline can reversibly slow down the growth of the skeleton and irreversibly tarnish the teeth of children younger than 8 years as tetracyclines are deposited in bones and teeth. Sulfonamides have caused increasing concern over their carcinogenic and mutagenic potential. Initially they had been used widely at sub-therapeutic and therapeutic concentrations in food-animal production but concerns have lead to decreased usage, longer withdrawal times and more intensive residue monitoring.

There is potential for toxicity from benzimidazole anthelmintic drugs [Delatour et al., 1986]. Certain benzimidazoles are mutagenic, however the effect is low in mammals even at high doses. A general property of benzimidazoles is teratogenicity and teratogenic metabolites have been identified and quantified in animal products such as milk, eggs and meat. For levamisole, the most significant of the adverse effects, which are rare, were agranulocytosis and neutropenia [Heitzman et al., 1998].

Therefore to protect human health, the EU has set up maximum residue limits (MRLs) for residues of licensed veterinary drugs in animal tissues entering the human food chain. The MRL may be defined as the maximum concentration of marker residue (e.g. parent compound, metabolites etc) resulting from the use of a veterinary drug, expressed as mg/kg, that is legally permitted or recognised as acceptable in or on a food [Mitchell et al., 1998]. The MRL is associated with the acceptable daily intake (ADI). This is an estimate of the amount of a substance in food and/or drinking water, expressed on a body weight basis that can be ingested daily over a lifetime without appreciable health risk to the consumer on the basis of all the known facts at the time of the evaluation. It is typically shown in milligrams of the chemical per kilogram of body weight. The ADI is calculated from the no-observable-effect level (NOEL) taking into account a safety factor (usually 100) [Grein et al., 2000]. The NOEL is the greatest concentration or amount of a substance, found by observation or experiment, which causes no detectable effect. The maximum acceptable total residue level (TRL) is calculated from the ADI considering the consumption pattern. The MRL is the detectable proportion of the marker residue that
corresponds to the TRL value. The MRL value refers to the permissible level for the marker residue.

Besides conventional toxicological effects, other issues such as the effects of drugs on the immune system and pharmacological effects together with specific effects of residues of veterinary antibiotics on the human gut flora, should be evaluated when considering safe residue levels [Boisseau et al., 1993].

Besides toxicological problems, hypersensitive reactions from β-lactam (e.g. penicillin) antibiotic residues in milk and meat [Mitchell et al., 1998, National Research Council, 1999] in sensitive individuals can occur.

Nonetheless, the risk to human health due to residues of veterinary drugs in foodstuffs must be taken in perspective. Microbial contamination of food is a major health problem worldwide. Infections of poultry products with Salmonella and Campylobacter are greater risks to public health than are residues of veterinary drugs in food. An emerging problem worldwide is the increasing prevalence of multi-drug resistant salmonella and resistance to clinically important antimicrobial agents such as fluoroquinolones and third generation cephalosporins [Brands et al., 2005, Chao et al., 2007, Chen et al., 2007, Gebreyes et al 2005].

The public risks of antibiotics and their metabolites are difficult to define, and the presence of high levels in food is illegal and subject to financial penalties in many countries.

1.3.2 Antibiotic resistance

Antibiotic resistance is a well documented major health threat around the world that has been given high priority by many health agencies. A particular example is the Definitive type 104 (DT104) which was first recognised in the UK in 1984 [Threlfall et al., 1996]. This phage has commonly exhibited resistance to five anti-microbial agents namely ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline [Gebreyes et al., 2004, Rayamajhi et al., 2008] Fluoroquinolones and third generation cephalosporins are recommended for use where microorganisms are resistant to these antibiotics [Hooper et al., 1991, Jacobson et al., 1989, Karczmarcz et al., 2010]. Recent research has identified that this phage and other stereotypes can acquire additional
resistance to fluoroquinolones and higher generation cephalosporins [Casin et al., 1999, 
Fey et al., 2000, Hsueh et al., 2004, Weill et al., 2006, Winokur et al., 2000].
The widespread use of anti-microbial agents in the treatment of humans and animals and 
also as growth promoting agents in livestock production has greatly promoted the 
appearance of anti-microbial resistant bacteria [Araque., 2009, Gonsia et al., 2011, Hur et 
al., 2011, Singh et al., 2010]. In addition, the potential exists for animal-to-human 
transfer of the resistance.
Animals fed with low (prophylactic) levels of antibiotics may give rise to bacteria with 
evolving resistance to these or other drugs. Humans may be exposed to these bacteria 
through consumption of food. In order to ensure cautious use of antimicrobials in 
livestock in the major production countries these nations have set up their own national 
monitoring system such as European Antimicrobial Resistance Monitoring system 
[EARS-Net 2010], National Antimicrobial Resistance Monitoring System in the United 
States [FDA 2010] and the Japanese Veterinary Anti-microbial Resistance Monitoring 
system [JVARM 2009].

1.3.3 Technological problems
The preliminary worry in relation to antimicrobial residues in food were not expressed by 
consumers but by dairy processors who identified that contaminated milk was inhibiting 
the starter cultures used in the manufacture of fermented milk commodities as well as 
interfering with the dye reduction tests used for milk quality [Mitchell et al., 1998]. 
Penicillin though not intrinsically toxic, can inhibit the fermentation process employed by 
the dairy industry if the concentrations exceed the MRL [Tamime et al., 1999]. Grunwald 
et al., [2003] showed that the presence of penicillin negatively affected yogurt 
production. The sensitivity of thermophilic and mesophilic starter cultures for 
manufacturing high-value fermented products such as cheese, yogurt, butter or raw 
sausages, requires biotechnologically safe raw supplies.
1.3.4 Effect of processing on residues

Most foods of animal origin are cooked (except milk and honey) before consumption but none of the required studies for licensed drugs evaluate the effect of processing on residues. Information about this influence is necessary to obtain more accurate estimates of consumer exposure to residues or possible breakdown products. Several drug residues during normal cooking and processing procedures were evaluated by Rose et al., [1997]. Residues of levamisole were identified as being stable to heating, but a fraction was lost from the meat into the juice. Studies also showed oxfendazole instability in boiling water was found after 3 hours and heating of samples with incurred residues of oxfendazole destroys the drug residues. Moats et al., [1999] reported that ordinary cooking procedures for meat, even to “well-done” must not be depended upon to break the more heat sensitive compounds such as penicillins and tetracyclines. Tarbin et al., [2005] evaluated the change in concentration of marker compound for the anti-coccidial drug nicarbazin, N,N′-bis(4-nitrophenyl)urea (dinitrocarbanilide) (DNC), in model oil, aqueous solutions and in chicken and egg. DNC residues in eggs were stable to microwave cooking and residues in chicken muscle were stable to stewing and microwave procedures. Other cooking procedures lead to a decreased amount of DNC and only minor amounts of residues leached into juices. Javadi et al., [2011] evaluated the effects of different cooking processes such as boiling, roasting and microwaving on enrofloxacin (ENF) residues in chicken muscle, liver and gizzard tissue from broiler chickens. The study showed that ENF was reduced after different cooking processes. In cooked meat and gizzard the most reduced levels of the residue were due to the boiling method. Cooked liver residue levels were the most depleted due to the roasting process. The highest residue levels remained after microwave cooking/heating. The relevance in food safety is unknown as the nature of the degradation products is uncertain generally. The identity of these degradation products should be established and their toxicity assessed. It would be useful if data generated from surveillance of raw tissue for dietary intake calculations and consumer exposure estimates be considered in the light of the effect of cooking on veterinary drug residues.
1.4 Legislative Aspects

1.4.1 Overview of EC Decisions, Council regulations and guidelines

The incidence of residues of veterinary drugs in food is an international dilemma. Food-producing animals and animal products are transported within EU countries and between EU countries and Third Countries. Much time and funds are spent by the EU to monitor residues and harmonise European legislation in the control of residues. The inspection of animals and of fresh meat for the presence of veterinary drug residues and specific contaminants was regulated by the European Commission by Council Directive 86/469/EEC [1986]. Current national surveillance schemes monitor residues under Council Directive 96/23/EC [1996] on measures to monitor certain substances and residues thereof in animal products and under Commission Decision 97/747/EC [1997] which lays down levels and frequencies of sampling in order to monitor some substances and residues thereof in certain animal products. The EU Decision details the number of samples to be analysed for each drug residue group. This directive gives an efficient and reliable approach for the monitoring and control of illegal substances or inaccurate use of authorised substances in animal products intended for human consumption within the EU. With the purpose of enabling this harmonised approach a number of particular requirements are noted in the text, these include:

1. Requirements related to the establishment and safeguarding of a network of EU and national (Member State) reference laboratories.

2. Enforcement methods to be taken by Member States in the case of noncompliant results.
3. Designed sampling and analysis strategies, usually addressed as residue monitoring according to national control plans.

4. Requirements related to the authorisation of imports of food of animal origin from Third Countries that could contain residues.

Quality criteria for laboratory residue analyses are outlined in Commission Decision 93/256/EEC [1993] which states the methods to be used for detecting residues of substances having a hormonal or thyrostatic action. Reference methods for the list of National Reference Laboratories for detection of residues are outlined in Commission Decision 93/257/EEC [1993]. The two decisions are revised on a regular basis in order to take into account the current scientific information and the latest technological advances. A revised edition was submitted by the Directorate General for Agriculture as a draft Commission Decision SANCO/1805/2000 [2000] laying down performance criteria for the analytical methods to be used for certain substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC [1996]. This draft article was revised and enacted as Commission Decision 2002/657/EC [2002] implementing Council Directive 96/23/EC [1996] concerning the performance of analytical methods and the interpretation of results. The establishment of MRLs in the EU is governed by Council Regulation EEC/2377/90 [1990] amended by several EC Regulations. This regulation establishes details of compounds that have a fixed MRL (Annex I), that need no MRL (Annex II), or that have a provisional MRL (Annex III). Annex IV of this regulation is a list of compounds that are forbidden for use in livestock production. Annex V provides information and the data needed to determine the MRL values. Annex I of this regulation gives a list of substances that should be monitored in each EU member state. This catalogue of substances is broken into two separate sections: Category A substances and Category B substances. Category A substances are those whose use are partly or entirely illegal in food producing animals, whereas Category B substances are those products that are legal for use in food producing animals.
In addition to the protection of consumers from the potential risk of harmful residues, MRLs are also essential to smooth the progress of international trade. However, the introduction of the MRL has a severe impact on the availability of veterinary medicinal products. The data that must be generated is very complex requiring a huge number of experiments and the costs involved must be borne by the pharmaceutical company. This is because pharmaceutical companies that want to market their products must surrender full dossiers to regulatory authorities so MRLs can be set. These dossiers outline pharmacological and toxicological properties of their product. Most companies are wary of carrying out such trials because of the financial implications of preparing and carrying out such trials. Therefore compounds that may have been used in the past are now no longer available. This may be forcing the use of un-licensed products. Since some veterinary drugs are licensed for use in particular species only, farmers may use the products in an off label manner by treating a different species. The danger of the occurrence of residues in food when food producing animals are treated with un-licensed products or with products used in an off-label manner, is substantial. As the risks associated with this phenomenon potentially increase then consumer protection must be the highest priority. The European Commission issued a precautionary principle based on the Treaty of Amsterdam (Treaty of Amsterdam, 1997). A definition of this principle is stated by Fisher et al 2006 “Where, following an assessment of available scientific information, there are reasonable grounds for concern for the possibility of adverse effects but scientific uncertainty persists, provisional risk management measures based on a broad cost/benefit analysis whereby priority will be given to human health and the environment, necessary to ensure the chosen high level of protection in the Community and proportionate to this level of protection, may be adopted, pending further scientific information for a more comprehensive risk assessment, without having to wait until the reality and seriousness of those adverse effects become fully apparent”. In order for any regulatory agencies internationally to be in a situation to implement consumer protection a number of important factors need to be measured. A risk analysis needs to be carried out on the types and concentration of residues occurring, reliable data on the consumption of food types needs to be tabulated. In order to achieve reliable data and gain this
information rapid, sensitive and rugged analytical methodologies need to be developed and implemented in National Monitoring Plans in each nation.

1.5 Determination of Veterinary Drug Residues

1.5.1 Introduction

Analytical methods can be divided into screening, quantitative and confirmatory procedures. In the case of screening methodologies, they should ideally be rapid, simple to use and offer a positive or negative result for the test compound at a specified level. Screening tools include rapid test kits such as microbial receptor, receptor binding, radio or enzyme immunoassays used to monitor for veterinary residues. Screening methodologies can present some difficulties as they are not always sensitive at the required concentration (e.g. MRL), are often drug class and not compound specific and do not give quantitative information. Therefore supplementary analytical tests are required to determine if a sample is actually violative for an animal drug residue. Some tests, such as immunoassays, provide semi-quantitative results.

Quantitative methods are designed to separate, quantify and provide some qualitative information on the analyte of interest. Most gas and liquid chromatographic methods would fall into this category. Quantitative assays classify samples as positive or negative relative to specific drug concentration.

Confirmatory methods provide unambiguous identification of the drug residue being targeted. Due to its sensitivity and specificity, mass spectrometry is the preferred method for confirmation of drug residues.

1.5.2 Screening assays

A screening assay makes a division between compliant and suspect samples i.e. samples containing residues over an allowed limit. The most important requirement for a screening assay is that it yields very few false negatives, in essence the β-error (possibility of false negative result or a risk for the consumer) should be lower than 5%.
some false positives are acceptable as these will show a negative result when a confirmatory assay is performed.

The most basic methods used for detection of antimicrobial residues in food were based on the detection of growth inhibition of various sensitive bacterial strains [Mitchell et al., 1998]. The major disadvantages of these assays are that (i) they are not very definite for identification purposes, (ii) they are not quantitative and (iii) they have a limited detection level for many antimicrobials. Besides, they are lengthy requiring several hours before results are obtained. However they are cheap, easy to carry out, flexible for screening large numbers of samples and they have a reasonably broad antimicrobial detection spectrum.

The specificity of the immune system is demonstrated by its ability to distinguish subtle differences between antigens. Immunoassays exploit this and examples of such tests are enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA). Neogen Corporation developed an ELISA Kit for ibuprofen [Biocompare.com]. Saini et al., [2012] analysed diclofenac using ELISA in liver samples from livestock carcasses. FedMedco developed a MaxSignal Ketoprofen ELISA Kit for ketoprofen [FedMedco.com]. Jackman et al., [1996] described class specific enzyme immunoassays for the determination of most benzimidazole drugs. For thiabendazole a compound specific ELISA had to be developed. Wang et al., [2011] developed an ELISA for the determination of nitroimidazole in food products. Enzyme immunoassays are influential tools for evaluating food products for the occurrence of veterinary drug residues. The paybacks are high through-put of samples and low costs compared to instrumental methods, however commercial kits can be expensive. Van der Made et al., [2004] altered the sample preparation step of an enzyme immunoassay (RIDASCREEN™ Acetylgestagene EIA of R-Biopharm) to provide an easier and quicker assay for the detection of medroxyprogesterone acetate (MPA) in kidney fat. Another commercial kit was tested (MPA ELISA of Euro-Diagnostica B.V) in addition which showed poorer sensitivity for MPA. The results identified that screening using the former kit in combination with a simplified extraction procedure gave rise to a reliable screening method for the determination of MPA in kidney fat of pigs. With this test it was shown that 30 samples could be analysed per day by one analyst. Ferguson et al., [2005]
detected chloramphenicol and chloramphenicol glucuronide residues in poultry muscle, honey, prawn and milk using a surface plasmon resonance biosensor and Qflex kit chloramphenicol. Traynor et al., [2003] detected multi-β-agonist residues in liver matrix using surface plasma resonance biosensor. Peippo et al., [2004] described an easy and rapid time-resolved fluoro immunoassay (TR-FIA) for the screening of narasin in poultry plasma. Noot et al., [2004] described a surface plasmon resonance (SPR) based immunoassay using the Biacore Q™ SPR biosensor for multi-sulfonamide detection in porcine muscle. Following a simple preparation, results for 40 samples are available in just over five hours.

1.5.3 Confirmatory methods

1.5.3.1 Introduction
After samples have been analysed by a screening procedure and when certain samples screen positive further analysis by a confirmatory analytical method is required in order to be unambiguously declared positive. Chromatographic methodologies play an important role in the confirmatory tests for residues of veterinary drugs and contaminants. There are numerous types of chromatographic methods currently in use for residue analysis. These are categorised under thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). TLC has been used, generally for screening or qualitative assays only. Most veterinary drugs are non-volatile, polar, heat sensitive and or difficult to derivatise for GC. Therefore the most widely used analytical technique in residue analysis is HPLC. For confirmatory analysis chromatography with selective detectors is used commonly. Mass spectrometry (MS) is the favoured detection method for confirmation. Hyphenated techniques such as gas chromatography-tandem mass spectrometry (GC-MS-MS) and liquid
chromatography-tandem mass spectrometry (LC-MS-MS) are the most authoritative analytical tools in the analytical laboratory. On top of the general performance requirements, supplementary stipulations are essential for confirmatory methods. These comprise of the introduction of the concepts of identification points, defining criteria for relative ion intensities and the setting of limits for relative retention times. For a method to be deemed confirmatory, an exact number of identification points must be attained. For confirming the presence of compounds categorised within Group A; at least four identification points are required. For confirmation of substances within Group B; at least three identification points are required. The number of identification points obtained by a particular method depends on the determination technique employed. Tandem mass spectrometry using a number of different triple quadrupole mass spectrometers operated in multi reaction monitoring mode was used as the determination step for all veterinary drug residue methods developed within the research carried as part of this thesis. This technique is classified within 2002/657/EC [Commission Decision 2002] as being low-resolution mass spectrometry, 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 are earned.
1.6 Validation Of Analytical Methods For Use In Veterinary Drug Residue Determination

1.6.1 Introduction

Within the EU, each member state is developing their own surveillance methods for veterinary drug residues and these methodologies have to be validated to demonstrate their reliability. The validation parameters to be considered for these analytical strategies are described in: Commission Decision 2002/657/EC [2002] and they are: specificity, linearity, trueness, precision, recovery, analytical limits, stability and ruggedness. The criteria laid down in this decision were followed during the validation procedures carried out in this thesis. The legislation outlines how these parameters can be determined, however it does not make it compulsory for labs to use these approaches; as outlined 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”.

1.6.2 Validation parameters

1.6.2.1 Specificity

The specificity describes the ability of the method to measure the analyte of interest in the presence of other substances such as other analytes, metabolites and interferences in the sample matrix.

1.6.2.2 Linearity

The mathematical relationship between the response and the concentration of the analyte in the matrix must be established.

1.6.2.3 Trueness

This is a component of accuracy and is defined as the closeness of agreement between the mean value measured for an analyte from a large series of test results in a CRM and its
certified value, expressed as a percentage of its value. If no CRM is available, relevant parameters may be evaluated using fortified sample material.

1.6.2.4 Precision
The precision, another accuracy component, is the closeness of agreement between the results obtained by applying the experimental procedure several times under prescribed conditions and covers repeatability, within laboratory reproducibility and inter-laboratory reproducibility. The measure of precision is computed as standard deviation of the test result.

1.6.2.5 Recovery
Matrix effects in biological samples can cause reduced signal of analyte during the analytical procedure and can affect the recovery or extraction efficiency. Non matrix-related losses can occur due to the analytical procedure. If there is no CRM available to calculate trueness, the recovery has to be determined by experiments using fortified blank material. Whenever possible, incurred residue matrix material has to be used to optimise the method to recover as much incurred material as possible.

1.6.2.6 Analytical limits
The two analytical limits defined in Commission Decision 2002/657/EC [2002] are the decision limit (CCα) and the detection capability (CCβ). The decision limit is the value from which it can be concluded that a sample is truly violative with an error probability of α. The α-error, which is the probability that the tested sample is not truly violative, even though a violative measurement has been obtained (false positive decision) shall be 1% or 5% or lower for banned and MRL compounds, respectively. In the case of banned substances, CCα is the minimum concentration level at which a method can discriminate with a statistical certainty of 1-α whether the identified compound is present. For substances with an established MRL, CCα is the concentration above which it can be decided with a statistical certainty of 1-α that the identified compound content is truly greater than the MRL.
The detection capability is the smallest content of a substance that may be detected, identified and quantified in a sample with an error probability of $\beta$. The $\beta$-error, stated as the probability that the tested sample is truly violative, even though a non-violative measurement has been obtained (false negative decision), should be less than or equal to 5%. In the case of banned substances, $CC_\beta$ is the minimum concentration at which a method is able to truly detect residues in samples with a statistical certainty of $1-\beta$. For substances with an established MRL, $CC_\beta$ is the concentration level at which the method is able to distinguish MRL concentrations with a statistical certainty of $1-\beta$.

Substances for which no permitted limit has been established

![Graph showing detection capability](image)

- $CC_\alpha$: Response with a given $\alpha$-error and 50% $\beta$-error
- $CC_\beta$: Response with a given $\beta$-error and 50% $\alpha$-error
- $\overline{X}_B$: 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
- $\alpha$: Rate of false non-compliant results
- $\beta$: Rate of false compliant results
- $SB$: Standard error of the blank sample
- $SS$: Standard error of the contaminated sample

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1.6.2.7 Stability
The validation process should always include a study of the stability of the analyte or matrix constituents in the sample matrix as well as the stability of the standard analyte in solution. Whenever possible, incurred samples should be used to evaluate the stability in the matrix material.

1.6.2.8 Ruggedness
Ruggedness is tested by a separate systematic experimental strategy. Pre-investigation studies have to be carried out by selecting factors in the sample pre-treatment stage, clean-up stage, instrument stage and analysis, which may control the measured results obtained. Such factors may include the operator, the reagents, the instruments and the laboratory circumstances. The ruggedness of the method can be tested during determination of the within-laboratory reproducibility as part of the method validation.

1.6.3 Residue monitoring
Residue monitoring or surveillance strategies require the analysis of a huge number of samples. Conventionally screening techniques, such as inhibitory substance tests and immunoassays are used in residue laboratories. However multi-residue screening methods are more useful. Screening methods reduce the quantity of samples requiring more detailed analysis. Screening assays are an important part of the integrated approach to residue monitoring. It is necessary to combine different methods in an incorporated system in which a number of different tests are applied successively depending on the targets or objectives of the analysis. For regulatory purposes such a strategy should include at least two or more independent methods.
• Screening with a method optimised to prevent false negative results and with an acceptable number of false positive results at a low cost (e.g microbial growth inhibition tests).
• Intermediate tests to identify the residue category.
• Quantitative confirmation with an independent method optimised to prevent false positive results.

1.7 Post-mortem forensic toxicology drug monitoring

1.7.1 Introduction

The thorough investigation of the cause or sudden causes of death is a very significant civic responsibility. Establishing the cause of death is based on the medical examiner, the coroner or the pathologist arriving at an accurate conclusion which may depend on the combined effort of the pathologist and the toxicologist utilising sound analytical strategies. The cause of death in a poisoning cannot be proved beyond debate without toxicological analysis that establishes the presence of the toxicant in the tissues and body fluids. It is important to note that many drugs do not produce pathological lesions and therefore their presence in the body can only be demonstrated by chemical methods of isolation and identification. Frequently the issues surrounding the cause of death or criminal activity must be resolved by the judicial system. Forensic toxicology laboratories analyse specimens using a variety of analytical procedures. GC/MS and LC-MS are the most widely used methodologies for confirmation providing unequivocal identification. There is a need for rapid turnaround times, expansion of the number of chemicals measured in a specific test and to make methods, trained personnel and equipment available for instant response to toxicological emergencies.
1.8. Legislative Aspects

1.8.1 Overview

The possession of controlled substances is illegal. Drugs in Ireland are restricted by several different statutes of Irish Law; The Misuse of Drugs Act, 1977 is the main national legislation [1977] covering the forensically important drugs studied in this thesis and gives details of the list of controlled drugs. The Misuse of Drugs Act 1977 is divided into 43 sections. For example, in section 2 definition of a controlled drug is given. In this Act controlled drug means “any substance, product or preparation (other than a substance, product or preparation specified in an order under subsection (3) of this section which is for the time being in force) which is either specified in the Schedule to this Act or is for the time being declared pursuant to subsection (2) of this section to be a controlled drug for the purposes of this Act”. In sections 3 and 4, the Act describes the restrictions on possession of controlled drugs. In section 15 of the Act supplying of controlled drugs is detailed. In Section 17 unlawful cultivation of the opium poppy and plants of the genus Cannabis is described. In section 19 details are given in relation to occupier or owners of premises to be used for controlled substance cultivation, drug taking, manufacture or importation which is recognized as an offence. The Misuse of Drugs Act is a substantial document and has been amended regularly. In 1984 The Misuse of Drugs Act 1984 was brought into force in order to extend the law in relation to certain dangerous drugs [1984]. The Misuse of Drugs Act 1984 is divided into 16 sections. Section 2 gives a more comprehensive definition of “cannabis” and “opium poppy” plants. In section 10 the document details requirement for evidence by certificate as follows “the production of a certificate purporting to be signed by an officer of the
Forensic Science Laboratory of the Department of Justice and relating to an examination, inspection, test or analysis, as the case may be, specified in the certificate of a controlled drug or other substance, product or preparation so specified shall, until the contrary is proved, be evidence of any fact thereby certified without proof of any signature thereon or that any such signature is that of such an officer”. Generally the Misuse of Drugs Act 1977 and 1984 are quoted together when describing controlled substances. In 1987 the Misuse of Drugs Act, 1977 (Controlled Drugs) (Declaration) Order 1987 came into effect [1987]. The purpose of this order was to declare certain substances, products and preparations be controlled for the purposes of the Misuse of Drugs Act, 1977. This Declaration Order added Buprenorphine to the controlled drugs list. In 1993 the Misuse of Drugs Act, 1977 (Controlled Drugs) (Declaration) Order 1993 was published [1993]. Likewise the purpose was to declare certain substances, products and preparations be controlled for the purposes of the Misuse of Drugs Act, 1977. This Declaration Order added a number of other drugs e.g Ephedrine. In 1999 the Criminal Justice Act 1999 was brought into law [1999]. The Act contains 42 Sections. This legislation relates to the penalties for drug trafficking, providing evidence by certificate in relation to exhibits in court and in addition to other legal matters such as providing video evidence. In 2010 a huge departure was taken in Irish legislation with the publication of the Criminal Justice (Psychoactive Substances) Act 2010 which no longer followed the Misuse of Drugs Act format for legislation [2010]. This Act was divided into 26 sections. It is an act in order to prevent the misuse of dangerous or harmful psychoactive substances and provides for offences relating to the sale and importation, exportation or advertisement of these substances. In this Act a psychoactive substance
means “a substance, product, preparation, plant fungus or natural organism, which has when consumed by a person, the capacity to-(a) produce stimulation or depression of the central nervous system of the person, resulting in hallucinations or a significant disturbance in, or significant change to motor function, thinking, behaviour, perception, awareness or mood or, (b) cause a state of dependence including physical or psychological addiction. As a result of this legislation the Act brought into effect the removal of headshop drug products over the counter and shut down premises of this type. The Criminal Justice (Psychoactive Substances) Act 2010 listed substances such as synthetic cannibinoids, benzylpiperazine and piperazine derivatives, mephedrone and gamma butyrolactone (GBL) for example. The above legislation is utilised in cases where laboratories find substances that are illegal and thus severe penalties are enforced through the legal system based on the drug identified. In the post-mortem forensic toxicology area however there is a more of an emphasis on understanding the cause of a sudden or unexplained death. Although the Mis-Use of Drugs Act legislation described above may become relevant within this investigation if any of the controlled substances are detected. The principal legislation affecting the State Laboratory Post-Mortem Forensic Toxicology work carried out in this thesis is based on the Coroner’s Act 1962 [1962] and The Coroner’s (Amendment) Act 2005 in Ireland [2005]. The Human Toxicology section based in the State Laboratory, Ireland provides a toxicological service to the Coroner’s of Ireland. The post-mortem toxicology work in this thesis was carried out within this section at the State Laboratory. A Coroner in Ireland is an autonomous official with legal responsibility for the investigation of sudden
and unexplained deaths. The function of the Coroner is to enquire into the circumstances of unexpected, mysterious, violent and unusual deaths. This may require a post-mortem examination sometimes followed by an inquest. The post-mortem is carried out by a pathologist, who acts as the Coroner's agent for this purpose. The Coroner's examination initially is concerned with establishing whether or not the death was due to natural causes. If a death is due to natural causes then an inquest must be held by law. A post-mortem (or autopsy) is a procedure to establish the cause of death. If the cause of death cannot be determined beforehand, the Coroner will arrange for a post-mortem examination to be carried out. During this post-mortem examination samples such as blood and urine are sent for toxicological analysis. The State Laboratory performs toxicological analyses on these samples to give confirmatory results on the absence or presence of drugs and their metabolites. In addition chemicals such as ethanol, other volatile substances, carbon monoxide and other gases as well as toxic chemicals are monitored to identify if present in human fluids and tissues.

1.9 Determination of forensically important drug residues

The analysis of drug residues in the veterinary drug residue and post-mortem forensic toxicology fields is very similar. Analytical methodologies include screening, quantitative and confirmatory procedures also. In the case of screening methods, they should ideally be quick, simple to use and give a positive or negative result for the test compound at a specified level. Screening tools include rapid test kits such as microbial receptor, receptor binding, radio or enzyme immunoassays used to screen for drug
residues. Drawbacks of screening tests are mentioned below. Therefore additional analytical tests are needed to determine if a sample is actually positive for a drug residue. Quantitative methods are likewise designed to separate, quantify and provide some qualitative information on the analyte of interest. Most gas and liquid chromatographic methods would fall into this category. Quantitative assays classify samples as positive or negative relative to specific drug concentration.

1.9.1 Screening assays

In this thesis the requirements according to Commission Decision 2002/657/EC [2002] for veterinary drug residues were utilised as an alternative procedure in the post-mortem forensic toxicology field. The most important requirement for a screening assay is that it yields very few false negatives, in essence the β-error (possibility of false negative result) should be lower than 5%; some false positives are acceptable as these will show a negative result when a confirmatory assay is performed. Screening assays are developed to detect opioids, cocaines and amphetamine drugs or classes of drugs by utilising an immunologically based reaction coupled with a variety of detection techniques. Major advantages of these techniques include small sample volumes, inexpensive, high sample throughput, rapid turnaround times and long shelf lives. They are also popular due to their ease of use, potential for automation and their adaptability for use with blood and urine samples [Perrigo et al., 1995]. A disadvantage of immunoassay is their cross reactivity with substances that have similarities with the abused drug, yielding false positive results. Another disadvantage is that false negative results can occur if the
concentration of the drug is below the assay cut-off, if the urine sample is collected prior to the drug’s window of detection or if the sample has been diluted or adulterated [Eskridge et al., 1997, Lee et al., 2004, Ateshkadi et al., 1996]. There are a number of immunoassays available, for example the enzyme multiplied immunoassay technique (EMIT), the cloned enzyme donor immunoassay (CEDIA), fluorescence–polarisation immunoassay (FPIA), kinetic interaction of microparticles in solution (KIMs), the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA). In the analysis of amphetamine substances (brand name shown in brackets) such as amantadine/(Symmetrel) [Lee et al., 2004], benzathine salt forms [Berthier et al., 1995], brompheniramine, chloroquine/(Aralen), desipramine/(Norpramin) [Lee et al., 2004], doxepin/(Sinequan) [Merrigan et al., 1993] and levomethamphetamine/(Vick’s Nasal Inhaler) [Eskridge et al., 1997] to name just a few that have been illustrated in the literature as producing false positives. In the analysis of opioids substances doxylamine [Berthier et al., 1995], fluoroquinolones [Hauseman et al., 1983], gatifloxacin/(Tequin) [Baden et al., 2001], poppy seeds (foods) [Eskridge et al., 1997, Lee et al., 2004] and rifampin/(Rifadin) [Lee et al., 2004] have given false positive results. In the analysis of cocaines false positive results can occur if tolmetin /(Tolectin) [Young et al., 2004] or coca tea [Mayor et al., 2006] are present. In immunoassays substances causing false negative results for amphetamines can be aspirin based [Wagener et al., 1994, Linder et al., 1994]. Substances that give false negatives in analysis of opioids are salicylates [Merrigan et al., 1993, Wagener et al., 1994, Stout et al., 2004] and substances such as aspirin [Wagener et al., 1994, Linder et al., 1994]. Ascorbic acid, fluconazole/(Diflucan), mefenamic acid/(ponstel) [Young et al., 2004] can give false negative results for cocaine.
A study by Herring et al., [2001] gives comprehensive information on interferences with
drug screens in urine testing. Therefore due to cross-reactivity the immunoassay tests are
not specific enough to confirm the identity of the drugs and stand up in a court of law and
a second specific test is carried out by a confirmatory method for either blood or urine
matrices obtained from the deceased. Confirmatory methods provide unmistakable
identification of the drug residue in question. Due to its sensitivity and specificity, mass
spectrometry is the preferred method for confirmation of drug residues in the forensic
toxicology field.

1.9.2 Confirmatory methods

Samples that screen positive need to be further analysed by a confirmatory analytical
method in order to be unambiguously declared positive. The same chromatographic
techniques play an important role in the confirmatory tests for residues of veterinary
drugs and drug residues in the forensic toxicology area. There are a number of
chromatographic methods currently in use for residue analysis. These include thin layer
chromatography (TLC), gas chromatography (GC) and high performance liquid
chromatography (HPLC). TLC has found some use, generally for screening or qualitative
assays only. Some drugs are polar, non-volatile, heat sensitive and or difficult to
derivatise for GC. Therefore HPLC is the most commonly used analytical technique for
residue analysis. For confirmatory analysis chromatography with selective detectors is
used widely. Mass spectrometry (MS) is the preferred detection method for confirmation.
Hyphenated techniques such as gas chromatography-tandem mass spectrometry (GC-MS)
and liquid chromatography-mass spectrometry (LC-MS) are the most powerful analytical
tools in the analytical laboratory. The approach for validation and general method
performance parameter requirements are outlined in Commission Decision 2002/657/EC
[2002]. The requirements for confirmation relating to identification points, defining
criteria for relative ion intensities and setting limits for relative retention times were
utilised in the veterinary drug residue field according to Commission Decision
2002/657/EC. For a method to be deemed confirmatory, a specific number of
identification points must be attained. Group A substances are substances that are banned
for use as veterinary drug residues. Opioids, cocaines, amphetamines and adulterants
were treated as Group A substances in this thesis under veterinary drug residue legislative
requirements. For confirming the presence of compounds categorised within Group A; at
least four identification points are required. Tandem mass spectrometry using a number
of different triple quadrupole mass spectrometers operated in multi reaction monitoring
mode was used as the determination step for the veterinary drug residue methods
developed (NSAIDs in milk and plasma) within the research carried out as part of this
thesis. This technique is classified within Commission Decision 2002/657/EC [2002] as
being low-resolution mass spectrometry. 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 then a total of 4 identification points are earned. For confirming the
presence of opioids, cocaines and amphetamines hybrid mass spectrometry was used.
Hybrid mass spectrometry is a device that consists of two or more m/z separation
devices. The QTRAP is a hybrid triple quadrupole-linear ion trap operated in multiple
reaction monitoring mode and enhanced product ion mode as the determination step. So
4 identification points are obtained when two product ions are monitored as detailed above. In addition a full scan enhanced product ion spectrum is obtained for each drug thus giving a combination 4 identification and additional identification points for full scan spectra providing more data for confirmation. The QTRAP was used as the determination step for the opioids, cocaines, amphetamines and adulterants residues in blood and urine in post-mortem samples in the research carried out as part of this thesis in the post-mortem forensic toxicology field.

1.10 Validation Of Analytical Methods For Use In Post-Mortem Forensic Toxicology Residue Determinations

National regulatory laboratories throughout the world develop their own inspection methods for post-mortem forensic toxicology drug residues. These methods have to be validated to demonstrate they can be used in a court of law. The method performance parameters outlined in Commission Decision 2002/657EC [2002] in the veterinary drug residue field were adopted in post-mortem forensic toxicology studies in this work. Validation parameters to be evaluated for analytical methods are described in: Commission Decision 2002/657/EC [2002] and they are: specificity, linearity, trueness, precision, recovery, analytical limits, stability and ruggedness. The criteria laid down in this decision were followed during the validation procedures carried out in this thesis. The individual validation parameters are defined earlier in the veterinary drug residue section of the thesis.
Chapter 2: Literature Review
2.1 Introduction

This chapter will give an overview in relation to NSAIDs in veterinary drug residue analysis and also in relation to the forensically important drugs in post-mortem forensic toxicology being targeted by the State Laboratory for transfer to LC-MS technology. The chapter will also deal with issues in relation to analytical method development and legislation requirements for the drugs targeted. The chapter comprises of the following:

- Background information on NSAIDs, pharmacokinetics, metabolism, uses and risks to consumers
- Background information on forensically important drugs such as
  - Opioid drugs and their pharmacokinetics, metabolism, uses and risks to the consumers.
  - Cocaine and its pharmacokinetics, metabolism, uses and risks to the consumers.
  - Amphetamine drugs and their pharmacokinetics, metabolism and risks to the consumers.
  - Piperazine drugs and their pharmacokinetics, metabolism and risks to the consumers.
  - Ketamine and its pharmacokinetics, metabolism, uses and risks to the consumers.
  - Adulterant drugs
- Extraction and purification procedures
- Specific biological matrices available for testing
  - Veterinary drug residues monitoring
  - Forensic toxicology drug monitoring
- Overview of mass spectrometry

2.2 Overview of Veterinary Drugs Studied

2.2.1 Non-Steroidal Anti-Inflammatory Drugs

The chapters 3 to 6 of this thesis are based on work carried out on non-steroidal anti-inflammatory drugs (NSAIDs) as part of the veterinary drug residue section of the thesis
focusing on the detection of these substances in milk and plasma matrices. NSAIDs are defined as “compounds that are not steroidal and that suppress inflammation” [Booth et al., 2001]. NSAIDs compose an important class of drugs with therapeutic applications that have spanned several centuries in veterinary and human medicine. Historically, the discovery of certain plants and their extracts being applied for the relief of pain, fever and inflammation gave rise to the development of anti-inflammatory drugs. Salicylates were discovered in the 19th century to be the active components of Willow spp such as *Salix alba* in the treatment of fever, pain and inflammation [Rainsford, 2004]. Scientific advances in the 19th-20th century led to the development of the NSAIDs. The discovery of aspirin in 1946 (although this has been readdressed in article published by Sneader [2000]) followed subsequently by PBZ was the beginning of the NSAID revolution. PBZ was in the beginning employed as a combination with antipyrine in the assumption it would enhance the actions of the latter. In the 1950’s ibuprofen was the second drug to be available as an over the counter medication along with aspirin.

The general chemical classification of the NSAIDs gives rise to four classes. The four classes are Carboxylic acid, Carboxamides or Oxicams, Sulphonanilides and Diaryl-substituted Pyrazoles/Furanones [Rainsford 2007]. The list of NSAIDs is outlined in Figure 2-1.
Table 2-1. Chemical classification of the NSAIDs, taken from Rainsford [2004]

There are a number of diverse molecular structures and a more exhaustive list of NSAIDs outlined by Gupta [2007] and all of which share a common mechanism of action:

Salicylic acid derivatives (e.g. Acetylsalicylic acid, Diflunisal, Salicylates)

Oxicam Derivatives (e.g. Meloxicam, Piroxicam, Tenoxicam)

Fenamates/Anthranilic Acid Derivatives (e.g. Diclofenac, Aclofenac, Ibufenac, Melcogenamic Acid, Mefenamic Acid, Niflumic Acid, Tolfenamic Acid)
Propionic Acid Derivatives (e.g. Benoxaprofen, Carprofen, Ibuprofen, Fenbufen, Fenoprofen, Flurbiprofen, Ketoprofen, Suprofen, Tiaprofenic Acid)

P-Aminophenol derivatives (e.g. Acetaminophen)

Indolacetic acids (e.g. Etodolac, Indomethacin, Sulindac, Tolmetin, Zomepirac)

Aminonicotinic Acid Derivatives (e.g Flunixin meglumine)

Pyrazolone Derivatives (e.g. Dipyrone, Isopyrine, Phenylbutazone, Oxyphenylbutazone)

Quinolone Derivatives (e.g. Cinchopen)

The above is an incomplete list of all the NSAIDs available [Gupta 2007]. An example of the molecular structure of a representative of each of these listed above is detailed in Figure 2.1. The NSAIDs licensed for use in veterinary medicine in the EU for use in food producing animals is outlined in Table 2-2.

The mode of action of NSAIDs hinders one or more steps in the metabolism of arachidonic acid (AA). NSAIDs act chiefly to decrease the biosynthesis of prostaglandins (PG) by inhibiting cyclooxygenase (COX). The discovery of two isoforms of COX (COX-1 and COX-2) has advanced the knowledge of the mechanism of action and the possible side effects of NSAIDs. COX-1, expressed in almost all tissues of the body, catalyses the creation of constitutive PG which aids a variety of normal physiologic effects including GI mucosal protection, hemostasis and protection of the kidney from hypotensive insult. By difference, COX-2 is activated in inflamed or injured tissues and catalyzes the development of inducible PG, including PGE2, associated with intensifying the inflammatory reaction. COX-2 can also be implicated in thermoregulation and the pain reaction to injury. It is postulated that antipyretic, analgesic and anti-inflammatory actions of NSAIDs is due to the COX-2 inhibition. In addition simultaneous inhibition of
COX-1 may give rise to many of the undesirable effects of NSAIDs including gastric ulceration and renal toxicity [Merck Manual, Ninth Edition]. NSAIDs can vary in their capability to inhibit COX isoforms and any drug that is capable of inhibiting COX-2 at a lower concentration than that required to inhibit COX-1 could be classified by experts as a safer option. This has been the basis for the COX-2 selective NSAIDs and research has focused on development of these [Merck Manual, Ninth Edition].

NSAID drugs studied in this thesis which are licensed for use in food producing animals were carprofen (CPF), diclofenac (DCF), firocoxib (FIRO), flunixin (FLU) and its metabolite hydroxy-flunixin (FLU-OH), meloxicam (MLX) and tolfenamic acid (TLF). Carprofen is administered orally or as an intravenous injection [EMEA/MRL/914/04-Final]. Diclofenac is administered intramuscularly [EMEA/CVMP/67421/2009]. Firocoxib is administered either orally or by intravenous route [EMEA/CVMP/383063-Final]. Flunixin is administered intravenously, orally and by intramuscular routes. In veterinary medicine flunixin is administered with meglumine as a solubiliser [EMEA/MRL/744/00-Final]. Tolfenamic acid can be administered orally or intramuscularly [EMEA/MRL/183/97-Final]. Meloxicam is administered subcutaneously or intravenously [EMEA/CVMP/152255/2006-Final]. Ketoprofen is administered by intravenous or intramuscular routes and it is of no risk [EMEA/MRL/020/95]. NSAIDs that are not licensed for use in food producing animals were also studied in this work and these included ibuprofen (IBP), mefenamic acid (MFN), niflumic acid (NIFLU), naproxen (NAP), PBZ, oxyphen (OXYPHEN) and suxibuzone (SUXI). In addition ketoprofen (KPF) was studied.
2.2.1.1 Pharmacokinetics/Metabolism of NSAIDs

The NSAIDs are weak organic acids that are well absorbed. It has been noted that food can affect the oral absorption of some NSAIDs e.g (flunixin meglumine and PBZ) [Merck Manual, Ninth Edition]. These drugs are available as parenteral formulations. Upon absorption NSAIDs are comprehensively bound (99 %) to plasma proteins with only a small percentage of unbound drug accessible and active in tissue. Most of the NSAID class are biotransformed in the liver to dormant metabolites and are excreted from the liver to inactive metabolites by the kidney via glomerular filtration and tubular secretion. The biotransformation and elimination half-lives vary significantly by species therefore it is difficult to extrapolate dosages from one species to another [Merck Manual, Ninth Edition].

2.2.1.2 Uses of NSAIDs

NSAIDs are extensively used as veterinary medicinal substances for therapeutic uses as the NSAIDs can relieve pain and inflammation without the immunosuppressive and metabolic side effects associated with corticosteroids. Generally NSAIDs give only indicative release from pain and inflammation and do not alter the course of pathologic damage. As analgesics, NSAIDs are less powerful than opioids so more functional for mild to moderate pain [Merck Manual, Ninth Edition]. NSAIDs are routinely used for the relief of pain from osteoarthritis, colic, navicular disease, laminitis, perioperative pain [Merck Manual, Ninth Edition], mastitis, metritis, agalactia [EMEA/MRL/744/00-Final] and respiratory disease [EMEA/MRL/183/97-Final]. They can also be used in
conjunction with other drugs [Adams 2001]. They are also utilised in combination with antibiotics for certain types of bovine respiratory disease [Lockwood et al 2003]. Meloxicam for instance can be used for the treatment of respiratory infections in combination with antibiotic therapy to reduce clinical symptoms. [EMEA/CVMP/152255/2006-Final]. NSAIDs are the second most prescribed class of drugs after microbials [Sundlof et al., 1995]. Dairy farmers and veterinarians are using NSAIDs in dairy animals more frequently [US Code 1988] and permitted limits are being violated more often [Smith et al., 2008]. Apart from these legal uses, NSAIDs may also be used illegally in order to develop quality characteristics of meat. NSAIDs can be administered close to slaughtering to thwart platelet formation thus inducing a faster exsanguination process. Meat therefore will have a paler colour which is highly attractive to some customers [Courtheyn et al., 2002]. In addition treatment with NSAIDs can also diminish lipogenesis which results in carcass’ containing less fatty tissue [BVL 2001]. Other illegal uses of NSAIDs are off-label application of these drugs to species in which they have not been licensed for treatment.

2.2.1.3 Risks to Consumers due to Animals Treated with NSAIDs

NSAIDs can cause adverse reactions some which can be life threatening. The most frequent is GI ulceration. When GI defensive mechanisms are missing this causes inhibition of constitutive PG that regulates blood flow to the gastric mucosa and stimulates bicarbonate and mucus defence. This in turn affects the alkaline shielding fence of the gut, thus allowing diffusion of gastric acid back into the mucosa, injuring
cells and blood vessels and promoting ulceration and gastritis [Merck Manual, Ninth Edition].

Food with residues of NSAIDs can have serious health effects for the consumer. Long-term exposure to NSAIDs has caused kidney tumors in mice and liver tumors in rats [Kari et al., 1995]. It has been reported in recent years also that the COX-2 inhibitor class of NSAIDs are implicated in cardiovascular damage in humans [Debabrata 2008; Staar et al., 2008]. Other potential side effects include gastrointestinal disorders, aplastic anemia and agranulocytosis [Goodman and Gilman 1990] and changes in renal function [Goodman and Gilman 1992]. These drugs can also be used in an off-label manner where they are utilised in species in which they are not licensed leading to residues which could potentially have ill health effects if consumed. Due to little data being available in relation to residues of these substances in unlicensed species these could lead to significant risk to the consumer.
Table 2-2: MRL listings for non-steroidal anti-inflammatory drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Marker Residue</th>
<th>Animal Species</th>
<th>MRL (µg/kg)</th>
<th>Target Tissue</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carprofen</td>
<td>Sum of CPF and CPF glucuronide</td>
<td>Bovine, equine</td>
<td>500</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
<td>1000</td>
<td>Fat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>allowed</td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>DCF</td>
<td>Bovine, porcine</td>
<td>5</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>Fat+skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
<td>0.1*</td>
<td>Milk</td>
</tr>
<tr>
<td>Firocoxib</td>
<td>FIRO</td>
<td>Equine</td>
<td>10</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>Fat</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>60</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>Kidney</td>
</tr>
<tr>
<td>Flunixin</td>
<td>As marker FLU-OH</td>
<td>Bovine</td>
<td>40</td>
<td>Milk</td>
</tr>
<tr>
<td>Flunixin</td>
<td>FLU</td>
<td>Bovine</td>
<td>20</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>Fat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>Kidney</td>
</tr>
<tr>
<td>Flunixin</td>
<td>FLU</td>
<td>Equine</td>
<td>10</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>15</td>
<td>Fat</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>60</td>
<td>Liver</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>10</td>
<td>Kidney</td>
</tr>
<tr>
<td>Flunixin</td>
<td>FLU</td>
<td>Porcine</td>
<td>50</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17</td>
<td>Skin+fat</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>200</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>Kidney</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>MLX</td>
<td>Equine, Porcine, Bovine</td>
<td>20</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
<td>15</td>
<td>Milk</td>
</tr>
<tr>
<td>Tolfenamic Acid</td>
<td>TLF</td>
<td>Bovine, porcine</td>
<td>50</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
<td>50</td>
<td>Milk</td>
</tr>
<tr>
<td>Vedaprofen</td>
<td>VEDA</td>
<td>Equine</td>
<td>50</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>Fat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000</td>
<td>Kidney</td>
</tr>
<tr>
<td>Metamizole</td>
<td>As marker 4-MAA</td>
<td>Bovine, porcine, equine</td>
<td>100</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>Fat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine</td>
<td>50</td>
<td>Milk</td>
</tr>
<tr>
<td>Acetylsalicylic acid,</td>
<td></td>
<td>Bovine, chicken</td>
<td>Not allowed</td>
<td>Milk</td>
</tr>
<tr>
<td>sodium acetylsalicylate,</td>
<td></td>
<td></td>
<td></td>
<td>Eggs</td>
</tr>
</tbody>
</table>
acetyl salicylic acid
DL-Lysine,
carbasalate calcium

*updated 21 July 2009*
Figure 2-1: Molecular structures of different types of NSAIDs: (i) Acetylsalicylic Acid, (ii) Meloxicam, (iii) Diclofenac, (iv) Ibuprofen, (v) Acetaminophen, (vi) Etodolac, (vii) Flunixin, (viii) Phenylbutazone and (xi) Cinchophen

2.3 Overview of Forensic Drugs Studied

Chapters 7 and 8 of this thesis cover the analysis of drugs of abuse in post mortem samples sent to the State Laboratory. The methods developed in this work have replaced the previous analytical strategies.

Drugs most generally targeted in post-mortem forensic toxicology are amphetamines, benzodiazepines, cannabis, cocaine and the opiates. In addition other illicit substances or almost any over-the-counter prescribed drug need to be evaluated. Possible poisons also need to be investigated. Chapters seven and eight in this thesis are based on work carried out on drugs of abuse focusing on opioid, cocaine and amphetamine drugs in human urine and blood matrices. The term “drugs of abuse” is often utilised to refer to long-established street drugs (e.g. cocaine, marijuana, heroin and amphetamines) although in Ireland these substances are more aptly called “controlled drugs”. Opioid drugs include naturally occurring opium poppy derivatives such as morphine and codeine and
semi-synthetic opiates like heroin and methadone. Opioids can produce intense euphoria and a sense of well being. Cocaine and amphetamines are stimulant drugs. Crack cocaine is a smokable form of cocaine which is formulated by chemically altering cocaine powder to form crystals and the term crack is utilised because it makes a crackling sound when burnt. Users of cocaine can feel more alert, confident and have an increased sex drive. Users of amphetamine drugs can have more energy, feel confident, exhilarated and require less sleep and food. The drugs of abuse can render users severely physically or psychologically addicted and lead to severe withdrawal symptoms and social problems.

Controlled drug testing involves screening by immunoassay and confirmation by chromatography (GC or LC) in an array of biological fluids. This thesis focuses on the development of analytical strategies for these substances in blood and urine in a regulatory laboratory.

2.3.1 Opioid Drugs

2.3.1.1 Types of Opioid Drugs

In 1803, a German chemist, Frederick Sertturner, purified opium and isolated morphine and in 1832, codeine was purified from opium [Fenton 2002]. Alexander Wood developed the hypodermic syringe in 1853. When morphine is injected it can give a much greater high resulting in more extensive abuse. Further chemical research on morphine led to the synthesis of diacetylmorphine or heroin in 1874 [Fenton 2002]. The pharmaceutical company Bayer marketed this compound in 1898 as a morphine substitute in order to help reduce addiction. Heroin however is even more addictive and has lead to an even greater worldwide problem with opiate addiction. Methadone was
developed in Germany in the second World War when Germany was unable to acquire opiates for medical uses. Its pharmacological activity is quite close to morphine. It is often used orally due to its reduced potency to suppress withdrawal symptoms and used widely in drug addiction programs e.g oral methadone substituted for injectable heroin. However methadone can produce marked sedative effects with repeated administration as a result of drug accumulation. Buprenorphine is a semisynthetic, highly lipophilic opioid derived from thebaine and possesses analgesic and opioid antagonist properties. [Baselt 2008;190]. It is 25 to 50 times more potent than morphine. In addition the drug can be administered as high dose tablets for the maintenance therapy of opiate addicts.

An opioid drug is a drug that binds to opioid receptors which are found mainly in the central and peripheral nervous system. The term opiate is often utilised as a synonym for opioid. Opiate should refer to the natural alkaloids found in the resin of the opium poppy (Papaver somniferum). Poppy seed, a common food ingredient contains morphine and may contribute to high oral morphine doses. It has been noted in the literature that the presence of thebaine, present in poppy seeds but not in illicit or pharmaceutical dosage forms could be used to identify dietary sources versus intentional drug use [Casella et al., 2006].

There are a number of classes of opioids and examples of molecular structure of each class are given below in Figure 2-2.

Opioids can be classified as follows:

(i) Natural Opiates (e.g. resin of opium poppy, morphine, codeine, thebaine, leaves of mitragyna speciosa, salvinorin A)
(ii) Semi-synthetic Opioids (e.g. Heroin, Hydromorphone, Hydrocodone, Buprenorphine)

(iii) Fully synthetic Opioids (e.g. Fentanyl, Methadone)

(iv) Endogeneous Opioid peptides (e.g. Endorphins, Dynorphins, Endomorphins)

2.3.1.2 Pharmacokinetics/Metabolism of Opioids

Morphine can be taken orally or by alternative routes. Morphine is N-demethylated to normorphine (5%) which is less active and normorpine is a urinary metabolite available in free (1%) and conjugated (4%) forms. However the majority of administered morphine is inactivated by conversion to morphine-3-glucuronide in urine [Baselt 2008:1057]. Free morphine, morphine-6-glucuronide, morphine-3-ethereal sulphate and morphine-3,6-diglucuronide are present in urine also. Morphine-6—glucuronide is a powerful analgesic but has less side effects compared to morphine and might be a useful alternative in post-operative pain relief [Binning et al., 2011]. Blood levels of morphine are less than those achieved by injection when morphine is orally taken. Low bioavailability of morphine is due to large quantities becoming metabolised while crossing the liver. If the drug is injected into a vein in the arm large amounts reach the nervous system. In addition opiates can be absorbed through smoke inhalation. Crossing the blood-brain barrier is difficult for morphine due to polarity of its two hydroxyl groups. Heroin is less polar thus crosses the blood brain barrier easily and is 2.5 times more potent than morphine [Fenton 2002]. Heroin and morphine are quickly metabolised by the liver. Heroin is rapidly converted to monoacetylmorphine and subsequently to morphine therefore no heroin will be found in the urine of a heroin user. However finding morphine in urine illustrates the
use of morphine or heroin. To distinguish if heroin or morphine, it is important to establish the presence of 6-monoacetylmorphine, the intermediary metabolite of heroin, in blood and urine which can only arise from heroin. Another issue is that codeine can be partly demethylated to morphine. A urinary morphine result could be due to codeine medication. If codeine medication was ingested then only small amounts of morphine would be present. It has also been reported that drinking 240 mL of *Papaveris fructis* herbal tea containing 10-32 mg L\(^{-1}\) produced urinary morphine concentrations of 1-7 mg L\(^{-1}\) [Thuyne et al., 2003]. Morphine can also be a metabolite of codeine, ethylmorphine, heroin and pholcodine [Baselt 2008:1057] which can complicate morphine analysis. The half-life of morphine is 1.3-6.7 hours [Baselt 2008:1057]. Codeine is transformed in humans via O-demethylation to morphine and via N-demethylation to norcodeine and all drugs are excreted as both free drugs and glucuronide conjugates [Bechtel et al., 1978].

After codeine intake the urinary codeine/morphine ratio (as total drug following hydrolysis) generally is greater than 1 in the first 24 hours and falls beneath 1 between 24-30 hours. After 30 hours only morphine can be detectable in urine by analytical methods. Often in forensic laboratories heroin usage can be inferred from detection of morphine alone. Urinary total codeine concentrations in 16 individuals who ingested poppy seed foods were generally less than 1 mg L\(^{-1}\) however total morphine levels were usually in the range 1-10 mg L\(^{-1}\) [Thevis et al., 2003]. In humans codeine is well absorbed following intramuscular or oral administration [Adler et al., 1955]. Vree et al., [1992] showed that an oral 30 mg dose achieved average plasma levels of 67 μg L\(^{-1}\) for codeine, 968 μg L\(^{-1}\) for codeine-6-glucuronide and 54 μg L\(^{-1}\) morphine-3-glucuronide with elimination half-lives in the study for these three species at 1.5, 2.8 and 1.7 hours.
respectively [Sasaki T.A. 2007]. Forensic toxicology widens net for drugs of abuse:

Codeine has a half-life of 1.2-3.9 hours [Baselt 2008:355]. Dihydrocodeine undergoes the same biotransformation steps in urine involving N- and O-dealkylation with glucuronide or sulphate conjugation at the 3- and 6- hydroxyl positions encountered. The plasma half-life for dihydrocodeine is 3.4-4.5 hours [Baselt 2008:465]. Heroin (diacetylmorphine, diamorphine) is rapidly deacetylated in whole blood to 6-acetylmorphine catalysed by blood esterases, and 6-acetylmorphine is further hydrolysed to morphine in the liver [Baselt 2008:730]. It was reported in the literature that 6-Acetylmorphine is present in urine in 64-73% of all heroin cases studied, averaging approximately 0.8 mg L\(^{-1}\) up to 10 mg L\(^{-1}\). [Fehn et al., 1985; Derks et al., 1986]. Papaverine metabolites are found in a high percentage of urine specimens from heroin users [Paterson et al., 2006] but also can be present in urine following poppy seed consumption [Trafkowski et al., 2006]. The plasma half-life of heroin is 2-6 min, the plasma half-life of 6-monoacetylmorphine is 6-25 min and the plasma half-life of morphine is 2-3 hours [Baselt 2008;730]. Methadone is metabolised in urine by mono- and di-N-demethylation, resulting in formation of 2-ethylidene-1,5-dimethyl-3,3- diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP) [Pohland et al., 1971]. The blood plasma half-life of methadone is 15-55 hours. [Baselt 2008 :941]. Buprenorphine is metabolised mainly by the N-dealkylation to form norbuprenorphine which is pharmacologically active and glucuronide conjugation of both buprenorphine and norbuprenorphine [Cone et al., 1985]. The half-life of buprenorphine in plasma is 2-4 hour (parenteral) and 18-49 hour (sublingual).
2.3.1.3 Uses of opioids

Opioids are a group of drugs that are used to treat moderate to severe pain after operations and in the treatment of cancer and rheumatoid arthritis. However, the drugs should be used cautiously. The drugs act on opioid receptors. Within the spinal cord, the brain, and other organs, opioid receptors exist. These bind endogenous and natural compounds known as beta endorphins or endorphin peptide transmitters. The endorphins bind to the opioid receptors, thus reducing the perception of pain. Drugs such as morphine or heroin increase the opioid receptor response by stimulating these receptors like that of the natural endorphin compounds. [Fenton 2002]. Codeine is not as potent an analgesic as morphine but is a very effective antitussive. Heroin has no accepted medical use. Methadone is used in narcotic maintenance programs. Buprenorphine can be administered as high dose tablets for the maintenance therapy of opiate addicts. Transdermal patches supplying buprenorphine have also become available for the treatment of moderate to severe chronic pain.

2.3.1.4 Risks to Individuals Ingesting Opioids

Addiction to these drugs develops due to opioid receptors in the brain’s limbic system (drug dependence region) binding to these drugs. There has been a cause for concern as the use of opioids has risen dramatically in control of pain and might contribute to the problem of opiate addiction [Okie 2010]. Opioids are generally used recreationally due to their ability to produce euphoria and addiction occurs as a result of physical/psychological dependence. It has been noted that psychological addiction to opioids is more likely if the drug is taken recreationally [Doyle et al., 2004]. Withdrawal
symptoms include sweating, nausea, depression, severe fatigue, pain and vomiting.

Eliminating the ingestion of opioids slowly over time (days/weeks) will reduce withdrawal symptoms. [Doyle et al., 2004]. Adverse effects due to morphine usage include constipation, pupillary constriction, nausea, vomiting, hypothermia, depression to name but a few. Adverse effects due to codeine in addition include miosis, respiratory depression. Adverse reactions to dihydrocodeine include dizziness, drowsiness, lightheadedness, nausea and constipation. The severity of withdrawal symptoms depends on the half-life of the opioid. Heroin and morphine withdrawal occur quickly but are more severe than methadone but methadone takes longer. Complications arising from heroin usage can result in liver disease [Edland 1972 ; Stimmel et al., 1972; Force et al., 1974], pulmonary hypertension [Kurtzman, 1970] and peripheral nerve lesions [Ricter et al., 1973]. The symptoms of opioid withdrawal can also be treated with other medications however they are not very effective. Ventricular arrhythmia has been reported in methadone maintenance patients receiving an average daily dose of 400 mg [Krantz et al., 2002].

![Molecular structures of different classes of opiates/opioids: (i) Morphine, (ii) Heroin and (iii) Methadone](image)

Figure 2-2: Molecular structures of different classes of opiates/opioids: (i) Morphine, (ii) Heroin and (iii) Methadone
2.3.2 Cocaine

Cocaine is a drug that is infamous for its potential for recreational abuse and is extremely popular. Cocaine is not a new drug on the market. Cocaine is a natural substance found in the leaves of the *Erythroxylum* coca plant. The plant can be found in Indonesia, Mexico, South America and the West Indies locally. It has been used for thousands of years by ancient tribes for ceremonial and religious reasons. Ancient rituals used a mixture of coca leaves and saliva as an anaesthetic for ritual trephinations [Altman et al., 1985; Gay et al., 1975] which involves removing a circular section of bone from the skull. When Spain invaded South America in 1492 taxes were placed on the coca leaf plant. In 1800’s the cocaine alkaloid was isolated in Germany. In 1884 it was first used as an anesthetic and subsequently became widely used as such during this time. In 1863 a new wine was marketed combining cocaine and alcohol which gave rise to a further compound called cocaethylene. The original 1886 recipe for Coca Cola contained cocaine. In 1879 cocaine was used to treat morphine addiction. In the early 1880’s Sigmund Freud experimented with cocaine and began to highly endorse its use. By 1906 Coca Cola began eliminating cocaine from the recipe.

In the US cocaine was sold over the counter until 1916. By the turn of the 20th century cocaine’s highly addictive qualities were well known. There are two chemical forms of cocaine, the free base and the hydrochloride salt. The free base refers to the drug not reacted with an acid and neutralised to make a hydrochloride salt. This free base is smokable. The powder form of cocaine is the hydrochloride salt and can be dissolved in water and taken intravenously or intranasal. It cannot be smoked because of the high melting and boiling points of cocaine hydrochloride.
2.3.2.1 Pharmacokinetics and Metabolism of Cocaine

The pharmacokinetics of cocaine is dependent on a number of factors such as route of administration, physical/chemical formulation, concurrent use with other drugs and genetics. The half-life of cocaine is from 0.7-1.5 hours and most of the drug is eliminated in a few hours [Klasco et al., 2008; Leikin et al., 2008; Baselt 2008:247-253; Hoffman et al., 2006; Kolbrich et al., 2006]. Cocaine undergoes metabolism through different enzyme pathways [Jeffcoat et al., 1989; Klasco et al., 2008; Leikin et al., 2008; Baselt 2008:247-253; Hoffman et al., 2006; Kolbrich et al., 2006; Huestis et al., 2007; Jatlow 1993]. Approximately half of the ingested cocaine is converted to the major metabolite benzoylecgonine hydrolysed by carboxylesterase in human liver and can be detected 1-4 hours after administration and lasts up to 144 hours [Klasco et al., 2008]. Plasma cholinesterases react with cocaine to form eegonine methyl ester. Benzoylecgonine and eegonine methyl ester are the two major inactive metabolites of cocaine. The half lives of benzoylecgonine and eegonine methyl ester are approximately 5-6 hours [Heustis et al., 2007]. Therefore benzoylecgonine has a longer half-life than cocaine [Capella-Piero et al., 2005]. Norcocaine is a metabolite formed by hepatic N-demethylation of cocaine. Cocaine users often ingest alcohol and cocaine simultaneously [Jatlow 1993] as this prolongs the high but results in cocaethylene formation which is a transesterification product. As in the case of cocaine, cocaethylene affects blood pressure, heart rate and promotes euphoria [MCClance et al., 1995]. The effects of cocaine and alcohol together could be additive and give rise to high mortality rates in individuals who use the drugs simultaneously. [MC Clance et al., 1995]. Cocaethylene has an elimination half-life of
150 minutes whereas with cocaine it is approximately 90 minutes [Boghdadi et al., 1997]. In addition the formation of cocaethylene leads to the reduced metabolism of cocaine and is frequently noted in patients with concurrent cocaine and ethanol use [Jatlow 1993]. When crack cocaine is smoked metabolites such as anhydroecgonine methyl ester (AEME), methylecgonine and carbomethoxycycloheptatriene derivatives [Klasco et al., 2008; Baselt 2008;247-253; Hoffman 2006; Kolbrich et al., 2006; Boghdadi et al., 1997; Cone et al., 1998] can be identified. The pyrolysis products of cocaine, anhydroecgonine and anhydroecgonine methyl ester can be used to identify “crack cocaine” smokers although it is worth noting that these products can also appear in GC-MS analysis as thermal degradation products of cocaethylene [Gonzalez et al., 1995].

2.3.2.2 Uses of Cocaine
Cocaine has a number of accepted medical uses. It can be utilised as a topical anaesthetic and peripheral vasoconstrictor, it has been employed in surgeries of the ear, eye, nose and mouth for decades. [Clauwaert et al.; Mueller et al., 2005]. Popularity of cocaine use in a clinical setting is due to its unique ability to induce local anesthesia and limit epistaxis. [Pithini et al.; Van Boexlaer et al., 2000].

2.3.2.3 Risks to Individuals Ingesting Cocaine
Cocaine shows central nervous system and cardiovascular toxicity. In the central nervous system cocaine affects the uptake of dopamine, norepinephrine and serotonin [Shanti et al., 2003; Hoffman et al., 2006]. When the serotonergic activity is increased then this can result in seizures and can be implicated in the addiction effects of cocaine [Lason et al.,
2001; O'Dell et al., 2000; Shanti et al., 2003; Knuepfer et al., 2003]. It is thought that the abundance of dopamine causes the majority of effects. Overuse of cocaine causes depletion of dopamine stores and can cause intense longing for the drug which is called "washed out" syndrome [Shanti et al., 2003; Hoffman et al., 2006]. Washed out syndrome causes difficulty with muscle movement, causes lethargy and anhedonia [Hoffman et al., 2006]. The drug also affects heat regulation and can cause hypothermia [Hoffman et al., 2006]. The cardiovascular effects of cocaine are as a result of its ability to block sodium channels and is a type I antidysrhythmic agent [Shanti et al., 2003; Hoffman et al., 2006]. The drug also affects hypertension as a result of its ability to cause vasoconstriction, cardiac ischemia, and end organ and tissue infarcts.

(i)  
(ii)

Figure 2-3: Molecular structures of (i) Cocaine and (ii) Benzoylecgonine

2.3.3 Amphetamines Drugs

Amphetamines have sympathomimetic activity (stimulate the sympathetic nervous system) together with strong central nervous system (CNS) stimulation. Amphetamines are a subgroup of the substituted phenethylamine class of compounds. There are a number of drugs in this category. Only an overview of the members studied in this thesis will be given. The members considered include amphetamine, methamphetamine,
pseudoephedrine, 3,4-methylenedioxyamphetamine (MDA) and 3,4-
methylenedioxyamphetamine (MDMA). Stimulants drugs can heighten mood,
increase alertness and decrease fatigue. Ingestion of amphetamine type drugs either alone
or simultaneously with other drugs such as cannabis and alcohol is widespread.
Amphetamine was first synthesised in Germany in 1887 but not until 1927 were its
physiological effects described. In 1927 the drug was investigated as an artificial
replacement for ephedrine. In 1933 the volatile base form of the drug was sold as an
inhaler or a decongestant. [Rasmussen 2006]. During World War II amphetamine was
utilised to combat fatigue. Amphetamine became widely abused recreationally and in
order to control weight gain. Methamphetamine was first synthesised in Japan in 1893
and was widely used in World War II by the German’s. Today homemade manufacture of
methamphetamine and concurrent abuse is widespread. Pseudoephedrine occurs naturally
as d-isomer in various Ephedra species in conjunction with l-ephedrine. The drug is
administered orally as a nasal decongestant and bronchodilator but can be found in non-
prescription cold remedies. The drug can be used as a starting material in manufacture of
methamphetamine. MDMA is a synthetic substance commonly known as ecstasy.

MDMA is generally taken in tablet form although if powdered could be snorted, injected
or inhaled. MDMA was first synthesised in 1914. In the late 1980’s MDMA use in the
rave culture became widespread. MDA is a psychotropic amphetamine which has similar
potency to p-methoxyamphetamine. It is taken orally and intravenously. MDA was first
synthesised in 1910. MDA was patented as a cough suppressant in 1958, as an ataractic
in 1960 and as anorectics in 1961 in the US. MDA is primarily a central stimulant and a
possible hallucinogen in large doses [Thiessen et al., 1973]. It is used illegally by
recreational drug users. Amphetamines acts on neurons in both the central nervous system and the peripheral nervous system that employ dopamine, norepinephrine and/or serotonin. The specific action of amphetamine is to enter vesicles that store neurotransmitters and produce leakage of the neurotransmitter into the synaptic junction. The nerve cell then transmits its message but in a way which is extreme and not related to standard physiological reaction.

2.3.3.1 Pharmacokinetics and Metabolism

The response to amphetamine drugs is dose dependent and also depends on the stereochemical nature of the drug. Amphetamine may be ingested or snorted and less often injected. The asymmetric α-carbon atom of amphetamine can give rise to two enantiomers. [EMCDDA Europe]. Amphetamine is available in d or l isomeric form with the d-isomer having 3-4 times more activity. The plasma half-life is 7-34 hours but is dependent on urinary pH [Baselt 2008:83-84]. An alkaline pH decreases elimination whereas an acidic pH increases it. Analysis of amphetamine in urine is complicated because it is a metabolite of methamphetamine and other drugs such as fenethylline, fenproporex [Baselt 2008:83-84]. A major metabolite is phenylacetone subsequently oxidised to benzoic acid and excreted as conjugates. A small amount is converted by oxidation to norephedrine and this and its parent are p-hydroxylated. [Baselt 2008:83-84].

Blood concentrations of d- and l- amphetamine developed steady-state peak plasma concentrations averaging 67 mg L⁻¹ for d-amphetamine and 22 mg L⁻¹ for l-amphetamine; elimination half-lives averaged 11-14 hours [Clausen et al., 2005]. Methamphetamine may be ingested, snorted and less often smoked or injected. It can exist as d- and l-
isomers. The d-isomer has stronger central stimulant action but less peripheral sympathomimetic activity than the l-isomer. D-Methamphetamine undergoes some N-demethylation to amphetamine, its major active metabolite. D-methamphetamine urine concentrations of 0.5-4 mg L\(^{-1}\) were observed in 24 hours after taking 10 mg dose. [Lebish et al., 1970]. In acid urine up to 76% parent drug is found and 7% amphetamine in 24 hours however in alkaline urine values of 2% and less than 0.1% (Beckett et al., 1965). 15% is excreted as free or conjugated p-hydroxy-methamphetamine whereas other minor metabolites are similar to those found after amphetamine administration [Caldwell et al., 1972; Shima et al., 2006]. A study by Heustis et al showed peak urine concentrations averaging 4 mg L\(^{-1}\) (range, 1.9-6.0) at 14 hours for methamphetamine and 0.74 mg L\(^{-1}\) (range, 0.27-1.6) at 17 hours for amphetamine after administration of 20 mg. [Heustis et al., 2007]. Urine half-lives were 23 hours for methamphetamine and 22 hours for amphetamine [Kim et al., 2004]. Blood concentrations when methamphetamine was administered orally at a dose of 0.125 mg kg\(^{-1}\) showed the plasma elimination half-life of 10 hours [Cook et al., 1992]. The half-life of d-methamphetamine was between 6-15 hours but is urine pH dependent [Baselt 2008: 947-949]. L-methamphetamine is believed to follow the same biotransformation pathway as the d-isomer but more slowly. L-methamphetamine is also a metabolite of selegiline making analysis difficult [Baselt 2008:950-951]. In urine l-amphetamine is metabolised slower and has an elimination half-life of 19 hours versus 13 hours for d-amphetamine [Wan et al., 1978;585]. The half-life is set at 13-15 hr [Baselt 2008:950-951]. Presence of only l-methamphetamine may be as a result of the use of Vick’s Inhaler. Presence of d-methamphetamine indicates prescription methamphetamine or the illicit drug prepared by reduction of l-ephedrine or
d-pseudoephedrine. Presence of racemate is due to illicit drug synthesis from phenyl-2-propanone [Fitzgerald et al., 1988; Cody et al., 1993; Hornbeck et al., 1993]. Blood elimination half-lives for the plasma species are approximately 10 and 33 hours respectively [Mendelson et al., 2006].

Methamphetamine has a higher potency than amphetamine. The half-life is approximately 9 hours [EMCDDA/Methamp]. The major metabolites are 4-hydroxymethamphetamine and amphetamine. Analysis of d-methamphetamine in urine is complicated as it can be a metabolite of other drugs such as benzphetamine and famprofazone [Baselt 2008:947-949]. Pseudoephedrine is metabolised to norpseudoephedrine but only in a minor way. In fact up to 88% of the dose is excreted unchanged in 36 hour urine with less than 1% present as norpseudoephedrine [Bye et al., 1975]. Blood concentration when given a dose of 180 mg gave plasma half-lives of 5.2-8.0 hours under normal conditions however if urine was kept acidic the half-lives ranged from 3-6.4 hours and when urine was basic was 9.2-16 hours (Kuntzman et al., 1971).

The half-life of pseudoephedrine is 3-16 hours [Kuntzman et al., 1971].

MDMA is metabolised by N-demethylation to MDA and by fission of the methylene bridge to form hydroxylated mono- and di- hydroxy derivatives. A study by Zhao et al., [2001] showed that 43 MDMA users had approximately 19 mg L⁻¹ MDA and 1.4 mg L⁻¹ MDA in the urine within 8 hours of drug administration. Most of the dose of MDMA excreted in the urine is unchanged. The half-life is 5-9 hours [Barnes et al., 2009]. Other metabolites include MDA and O-demethylated compounds.

The amount of MDA in blood or urine obtained from a person that has only ingested MDMA tends to be less than 10%. [Kolbrich et al., 2008, Barnes et al., 2009, Baselt
Human metabolism data of MDA in urine/blood is limited. Cimbura et al., [1072] states that in fatal cases up to 160 mg/L MDA were recorded as unchanged drug. Animal studies on MDA show metabolism is by O-dealkylation, deamination and conjugation [Midha et al., 1978].

2.3.3.2 Uses of amphetamine type drugs

Amphetamine can be used in the treatment of attention deficit hyperactivity disorder (ADHD) or narcolepsy. Methamphetamine can be used according to the USFDA for the treatment of exogeneous obesity, ADHD, and off-label for treatment-resistant depression and narcolepsy [Mitler et al., 1993]. There are reports that amphetamines have been used as performance enhancers also. Since January 1st 2010, Pseudoephedrine has been banned due to its illegal use by athletes in order to enhance performance by World Anti-Doping Agency [WADA 2010]. Pseudoephedrine can be used as an oral or topical decongestant. It may also be useful as an anti-tussive drug [Minamizawa et al., 2006]. Some studies have shown that MDMA-assisted psychotherapy can be administered to post-traumatic stress disorder patients to reduce the severity of the condition [http://www.maps.org/research/mdma/]. (Accessed May 2012)

2.3.3.3 Risks to individuals Ingesting Amphetamines

In the central nervous system amphetamine acts as a stimulant and causes increased alertness, euphoria, wakefulness, lack of fatigue, decrease in appetite and increased motor and speech activity. As a result, amphetamine users can experience tremor, insomnia,
agitation, restlessness, sleep deprivation, fatigue and mental depression. [Fenton 2002].
Amphetamine is dangerous for people with a history of heart disease or hypertension or
people suffering with narrow-angle glaucoma and can induce mydriasis. In addition
amphetamine type drugs have been shown to transfer into breast milk [Skeiner et al.,
1984; Llett et al., 2007; Bartu et al., 2009]. Drug users can rapidly develop tolerance to
amphetamine drugs and require increasing amounts of the drug in order to obtain the
same effect [Merck Manual 2007]. Withdrawal can lead to depression, fatigue and
increased appetite, temporary stimulant psychosis such as paranoia, hallucinations,
delusions. Methamphetamine withdrawal can lead to depression, fatigue and increased
appetite. It passes into breast milk in addition. Methamphetamine drug users may lose
their teeth quickly and this is called “meth mouth”. Methamphetamine is highly
addictive. The mental depression associated with methamphetamine withdrawal lasts
longer and is more severe than that of cocaine withdrawal effects [Winslow et al., 2007].
Pseudoephedrine can be associated with the occurrence of stroke [Cantu et al., 2003]. It
may also cause palpitations, insomnia [http://home.intecom.com] and non-pigmenting
fixed drug eruption [Vidal et al., 1998]. It can also cause myocardial infarction [Biyik et
al., 2006]. MDMA effects such as grinding of teeth during sleep, lack of appetite, drug
mouth, concentration difficulties were common but occurred in vary amounts depending
on the gender [Liechti et al., 2001]. MDA causes tremor, agitation, tachycardia, rapid
breathing, hyperthermia, muscular rigidity convulsions and coma [Richards et al., 1971].
Figure 2-4: Molecular structures of (i) Amphetamine, (ii) Methamphetamine, (iii) Pseudoephedrine, (iv) MDMA, (v) MDA

2.3.4 Piperazine Type Drugs

Although the amphetamine drugs are extremely popular drug users searched for alternatives which produced similar effects that could be easily obtainable commercially and were not prohibited. Piperazine drugs became popular as a result. N-Benzylpiperazine is a piperazine derivative first synthesised in the 1940’s and initially underwent testing as an anthelminthic agent in livestock. Piperazines are synthetic substances and do not occur naturally. BZP has no stereoisomers. BZP has similar effects to amphetamines and it was reported that it should be controlled similarly [Campbell et al., 1973]. Amphetamine and Piperazine based stimulants are a cause for concern throughout the world today as both have been widely abused since the 1990’s. It has a potency approximately one-tenth that of d-amphetamine as a stimulant drug. N-Benzylpiperazine is generally purchased as hydrochloride salt capsules and tablets for oral administration.
The specific action of N-Benzylpiperazine is on the serotonin re-uptake transporter which increases the serotonin levels in extracellular fluids around the cell increasing activation of the serotonin receptors [Tekes et al., 1987; Lyon et al., 1986]. It has a lower potency effect on noradrenaline reuptake transporter and the dopamine reuptake transporter [Baumann et al., 2004].

2.3.4.1 Pharmacokinetics and Metabolism

N-Benzylpiperazine undergoes substantial biotransformation in rats and humans via ring hydroxylation, N-dealkylation, O-methylation and conjugation [Baselt 2008:155-156] and metabolites are found together with large amounts of unchanged drug in the urine. There are very few studies performed on this substance. One study was carried out by Antia et al., [2009] in which a 20 mg dose of N-Benzylpiperazine was administered orally and blood and urine samples were collected. The elimination half life was found to be 5.5 hours. Plasma major metabolites were BZP, 4-OH BZP and 3-OH BZP [Antia et al., 2009].

2.3.4.2 Uses of piperazine type drugs

N-Benzylpiperazine during the 1990’s was assessed for its anti-depressant properties in humans but never authorised for marketing and therefore has no real medical use.
2.3.4.3 Risks to individuals ingesting piperazine type drugs

After an average of 4.5 dosage units of the drug expected to contain 70-100 mg each, individuals showed symptoms of anxiety, agitation, sweating, hyperventilation, vomiting, dizziness, headache, confusion and collapse [Gee et al., 2005; Alansari et al., 2006]. The drug was found at levels of 1.7 mg L$^{-1}$ in blood simultaneously with methylenedioxymethamphetamine and methylenedioxyamphetamine and was implicated in the death of a young man in post-mortem blood [Wikstrom et al., 2004]. A single 100 mg oral dose of N-benzylpiperazine caused increased heart rate and systolic blood pressure rise [Bye et al., 1973].

(i)

![Molecular structure of (i) Benzylpiperazine](image)

Figure 2-5: Molecular structure of (i) Benzylpiperazine

2.3.5 Ketamine

Ketamine has been utilised in the United States as an anesthetic induction agent since 1972. It is structurally and pharmacologically related to phencyclidine which failed clinical trials due to its unpleasant side effects but subsequently became a popular street drug. Ketamine has some of the same hallucinogenic side effects and has gained abuse popularity as a result. The drug has been abused by the medical profession for its hallucinogenic effects [Ahmed et al., 1980]. Ketamine is a non competitive N-methyl-D-aspartate (NMDA) antagonist [Curran et al., 2001]. It binds to the NMDA receptor...
complex in the calcium channel. It inhibits a global number of excitatory amino acid
neuro transmitters [Cotman et al., 1987; Hampton et al., 1982] but in its action as a
NMDA agonist causes an increase in dopamine in the brain possibly causing schizotypal
and dissociative symptoms [Frances et al., 2011].

2.3.5.1 Pharmacokinetics and Metabolism
Ketamine is metabolised firstly by N-demethylation to norketamine which subsequently
undergoes dehydrogenation to dihydronorketamine and these two substances are
available at concentrations similar to ketamine in serum. In urine ketamine is eliminated
primarily as unchanged drug (2.3%), norketamine (1.6%), dihydronorketamine (16.2%)
and conjugates of hydroxylated derivatives of ketamine (80%). [Wieber et al., 1975]
Stenberg et al., [1981] has suggested that dihydronorketamine is an artefact of the
analytical procedure rather than a metabolite. In blood, men given an oral dose achieved
peak plasma concentrations averaging 0.08 mg L\(^{-1}\) at 0.5 hours for ketamine and 0.36 mg
L\(^{-1}\) at 0.8 hours for norketamine; the oral bioavailability of the drug averaged 20%
[Yanagihara et al., 2003]. Upon intravenous administration of 2.5 mg kg\(^{-1}\) to 5 patients an
average serum concentration of 1 mg/L was observed at 12 minutes after injection
decreasing to 0.5 mg L\(^{-1}\) by 30 minutes [Wieber et al., 1975]. The serum half-life of the
drug was estimated at 3-4 hours [Baselt:2008: 806].

2.3.5.2 Uses of Ketamine
Ketamine is used in human and animal medicine as an anaesthetic. There is research
being undertaken across the world evaluating the effectiveness of ketamine in the
treatment of pain, depression, alcoholism [Krystal et al., 2006] and heroin addiction [Jovaisa et al., 2006].

2.3.5.3 Risks to individuals ingesting Ketamine

Ketamine can cause hallucinations, delirium, irrational behaviour, nausea, vomiting, respiratory stimulation and depression, tachycardia, bradycardia, hypertension, seizures and cardiac arrhythmia [Baselt:2008:806] Ketamine may exhibit post-mortem redistribution as it was found that heart/femoral blood concentrations ratios averaged 1.6 (range 0.8-2.3) [Dalpe-Scott et al., 1995] and in another two cases averaged 3.3 (range 2.7-3.8) [Lalonde et al., 2004].

![Molecular structure of (i) Ketamine](image)

**Figure 2-6: Molecular structure of (i) Ketamine**

2.3.6 Adulterant Drugs

It is a common perception that illicit drugs contain substances other than the active ingredient and that adulteration is widespread. Illicit drug adulteration involves adding benign substances to bulk and dilute (sugars), substances that might enhance or mimic the effects of an illicit drug (such as procaine or lidocaine in cocaine which gives similar and stronger effects and the impression of high quality cocaine) or drugs that will help the administration of the illicit drug (such as caffeine in heroin which facilitates smoking or...
quinine in heroin which mimics the rush of heroin). Other agents in the production may be added accidentally such as a result of manufacturing, storage or production techniques.

A review carried out by Berridge [1978] showed that foreign substances were often added to opium pre and post importation [Berridge 1978]. A report on case studies by heroin users has stated that heroin has been cut “six or seven times” when it reaches the pusher [Richter et al., 1968]. More recently in 2010 a study entitled “A guide to adulterants and bulking agents and other contaminants found in illegal drugs” was published by public health bodies in the United Kingdom (UK) giving comprehensive details on the types of adulterants found [Cole et al., 2010]. Adulterants such as lidocaine and levamisole were included in the analysis of drugs in this thesis. The study in the UK in 2010 states that lidocaine and levamisole were usually found as adulterants in cocaine.

Lidocaine is an anesthetic which has stronger anaesthetic effects compared to cocaine and gives the impression of a higher quality cocaine. Levamisole is an anthelmintic medication (used for expelling worms) and it is unknown why it is added however it is hypothesised that it may give a more intense high.

![Molecular structure of (i) Lidocaine and (ii) Levamisole](image)

**Figure 2-7: Molecular structure of (i) Lidocaine and (ii) Levamisole**
2.4 Extraction and Purification Procedures

2.4.1 Introduction to Sample Preparation

In an analytical method the sample preparation stage is followed by a separation and detection stages. Generally the sample preparation stage can take the most time in an analytical method. In the field of residue analysis sample preparation is an extremely important stage. Selected compounds must be recovered in low concentrations from complicated matrices with a large number of potential interfering substances which can be present at high levels. The desired sample preparation procedure must ensure that the integrity and recovery of the selected analyte is not adversely affected prior to the separation and detection steps and that matrix components are minimised that may adversely affect the latter stages. In order to develop robust analytical procedures a proper sample preparation procedure is important. The principal objectives of sample preparation in residue analysis are preconcentration of analytes, dissolution of analytes in a particular solvent and separation of analytes from the largest number of interfering substances possible. Extraction techniques include liquid/liquid extraction, solid phase extraction (SPE), solid phase microextraction (SPME), matrix solid phase dispersion (MSPE), affinity techniques, column switching, supercritical fluid extraction/accelerated solvent extraction (SFE/ASE) only those utilised will be discussed.

2.4.1.1 Liquid/liquid extraction (LLE)

LLE is a traditional technique. The benefit of this technique is that through selective choices of solvent and pH manipulation, extremely clean extracts can be achieved with good recovery for the target analytes. Disposal of solvents can be a problem as solvent
volumes used can be large. A further drawback of the sample preparation technique is the difficulty in automation of this step. In a regulatory laboratory there is a need to develop a method for the analysis of a large number of samples in a single day and poor utilisation of this technique can limit sample throughput significantly.

2.4.1.2 Solid phase extraction (SPE)

SPE is a technique that has been used extensively in laboratories as a sample preparation technique for a number of analytical samples. The number of commercially available sorbent phases has expanded in the last number of years. Early problems relating to sorbent manufacture variability have been, in most cases, successfully eliminated. One of these disadvantages relates to the reverse phase silica sorbents and the occurrence of residual silanol groups which can lead to more than one retention mechanism being employed and this can cause variations in results between batches. Polymer based sorbents have been manufactured to overcome issues with residual silanol groups but in addition the polymer based sorbents i.e polystyrene-divinylbenzene (PS-DVB) copolymers allow a broader range of pH and stability for method development. Advantages including reduction of the presence of silanols mean one retention mechanism exists resulting in simpler protocols and PS-DVB allows a greater analyte retention compared with bonded silicas. Disadvantages of the polymer type sorbents include undesirable shrink/swell characteristics, hydrophobic PS-DVB samples require a condition step with a water wettable solvent. The overall advantages of SPE include preparation of a number of samples in parallel, low volumes of solvents and can be automated. Disadvantages of SPE include problems with maintaining a constant flow and in addition clogging of cartridges.
2.5 Specific Biological Matrices Available for Testing

2.5.1 Veterinary Drug Residue Monitoring

The rules regarding the sampling of matrices in veterinary drug residue monitoring are different depending on whether the substances analysed are non-authorised or authorised. Regulatory agencies involved in the detection of veterinary drug residues must take this into account when sampling. In the case of authorised veterinary drug substances, the EU legislation outlines the list of edible matrices which have MRLs established that require sampling to identify if MRL levels are being adhered to. Edible matrices sampled include skin, kidney, milk, liver, eggs, honey and muscle. For non-authorised veterinary drug substances matrices such as urine, plasma, hair, retina, liver and faeces can be taken. In the case of imported foods the availability of matrices for sampling is significantly reduced. In order to have retail import/export control and enhance consumer safety sampling can be restricted to edible matrices such as milk, muscle, honey, skin, fat and eggs to be tested. Milk is an important matrix therefore as it allows the detection of drugs in live animals prior to slaughter. Development of analytical strategies in milk is advantageous for residue control.

2.5.1.1 Extraction Methods and Purification Strategies for NSAIDs

NSAIDs are a class of anti-inflammatory drugs that are commonly used in veterinary medicine. Due to the potential for adverse effects to occur there is a need worldwide for regulatory agencies to identify residues in a variety of food animal and food animal products. NSAIDs have diverse structures and are not structurally related thus developing
analytical strategies in regulatory laboratories for these substances is complicated. Residues of these substances can be detected in slaughtered animals in kidney or liver matrices. The detection of these substances in live animals can be achieved in biological fluids such as plasma and in milk matrices. In 2009 the Community Reference Laboratory (CRL) for NSAIDs recommended that in order to achieve sufficient control of NSAIDs that the broadest number of residues should be monitored in analytical strategies. In general NSAIDs are extracted using an organic solvent with subsequent clean-up using liquid/liquid partitioning and/or SPE. In early days of NSAID method development there were a number of methods developed that monitored only single or a few NSAIDs. NSAIDs have been extracted with acetonitrile [Hardee et al., 1982; Neto et al., 1996; Fiori et al., 2004; Miksa et al., 2005, Jedziniak et al., 2007] from plasma and from milk [Daeseleire et al., 2003; Thompson et al., 2010]. NSAIDs have been extracted from plasma with ethyl acetate [Grippa et al., 2000] or by straight forward ultra centrifugation [De Veau et al., 1999]. NSAIDs have been extracted from urine with methanol [Stanley et al., 2007] or a mixture of dichloromethane:ethanol solution [Neto et al., 1996]. NSAIDs have been extracted from muscle using ethyl acetate:methanol and DL-dithio-threitol [Clarke et al 2002] and from kidney using water:ammonium hydroxide [Clarke et al., 2002]. NSAIDs have been extracted from bovine milk with a mixture of ethanol:ammonium hydroxide [Martin et al., 1983] or in presence of acetonitrile and sodium chloride [Jedziniak et al., 2009]. Dubreil et al., 2011 extracted NSAIDs from milk using methanol alone. After a hydrolysis step has been performed NSAIDs have been extracted from plasma using dichloromethane [Singh et al., 1991] or dichloromethane:n-hexane:diethylether [Hines et al., 2004]. Urine was extracted with chloroform [Igualada
et al., 2005], dichloromethane [Singh et al., 1991] and diethyl ether [Gonzalez et al.,
1996]. After hydrolysis or solvent extraction, solid phase extraction [SPE] is often used
in the purification of extracts containing NSAIDs. Bovine plasma has been shown to be
purified using C18 SPE for the determination of PBZ and DCF [Gowik et al., 1998], IBP,
KPF, DCF and PBZ [Vinci et al., 2006] and IBP, KPF and DCF [De Jong et al., 1989]
and NSAIDs from milk [Gallo et al., 2008; Gallo et al., 2010]. Plasma from pigs and
rabbits were purified using this approach to determine IBP, KPF, DCF and PBZ [Vinci et
al., 2006]. Equine plasma was purified using C18 for the determination of IBP, KPF, DCF
and PBZ simultaneously [Vinci et al., 2006] or PBZ alone [Taylor et al., 1995] or the C18
was substituted for an Oasis HLB™ cartridge [Quintana et al., 2004] for the
determination of PBZ. Bovine, equine and porcine muscle was purified using a Florisil
SPE cartridge. Bovine muscle extracts were applied to an Oasis HLB™ cartridge for the
removal of interferences in the determination of PBZ and KPF [Van Hoof et al., 2004].
Muscle samples were incubated with acetate buffer and B-glucuronidase followed by
extraction with acetonitrile and extracts passed through Sep Pak Alumina N cartridges
and the eluates were further purified using C18 SPE. [Jedziniak et al., 2010]. Chrusch et
al [2008] developed a method for NSAIDs in bovine muscle and kidney. Bovine kidney
extracts were applied to silica cartridges [Clarke et al., 2002] and bovine plasma extracts
were purified using affinity columns [Fiori et al., 2004] for the determination of PBZ.
Gallo et al., [2010] extracted 18 NSAIDs from milk using C18 SPE. Plasma of dogs and
horses have been diluted with water and samples were purified using Waters™ HLB for
the extraction of firocoxib using SPE [Kvaternick et al., 2007]. Urine and plasma from
dogs and horses was diluted with an aqueous solution of 5 % acetic acid for extraction of

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firocoxib using a Waters Oasis HLB™ 96-well solid phase extraction plate [Letendre et al., 2007].

Methods have been reported for the analysis of NSAIDs in plasma by LC-UV [De Veau et al., 1999; Kvaternick et al., 2007; Luo et al., 2004; Hardee et al., 1982; Neto et al., 1996; Grippa et al., 2000; Jedziniak et al., 2007; Singh et al., 1996; Gowik et al., 1998; Quintana et al., 2004; Fiori et al., 2004; Jedziniak et al., 2009], LC-fluorescence [Gallo et al., 2010], GC-MS [Neto et al., 1996; Singh et al., 1991; Hines et al., 2004; Gonzalez et al., 1996; Jaussaud et al., 1992], LC-MS [Luo et al., 2004; Miksa et al., 2005; Vinci et al., 2006; Quintana et al., 2004; You et al., 2008, Stolker et al., 2008, Chrusch et al., 2008; Thompson et al., 2010] and capillary electrophoresis [Gu et al., 1997]. The majority of methods that have been cited to date have been developed in equine plasma alone or in combination with other matrices with limits of detection ranging from 0.1 ng mL⁻¹ to 5 ng mL⁻¹ [Miksa et al., 2005; Luo et al., 2004; Hardee et al., 1982; Neto et al., 1996; Grippa et al., 2000; Singh et al., 1991; Hines et al., 2004; Gonzalez et al., 1996; Gowik et al., 1998; Vinci et al., 2006; Gu et al., 1997; You et al., 2008]. Other methods for the determination of NSAIDs in bovine plasma are available but with limits of detection ranging from 20 ng to 3.4 μg mL⁻¹ [De Veau et al., 1999, Miksa et al., 2005; Jedziniak et al., 2007; Gowik et al., 1998; Vinci et al., 2006; Quintana et al., 2004; Fiori et al., 2004]. Only two methods are available in equine plasma to date capable of meeting 5 ng mL⁻¹ level. A method by Luo et al [Luo et al., 2004] for a single residue had a limit of detection of 0.1 ng mL⁻¹ for FLU. A multi-residue method by Gonzalez et al [Gonzalez et al., 1996] had a limit of detection of 5 ng mL⁻¹ for IBP, FLU, DCF and TLF, but limits of detection of only 10-25 ng mL⁻¹ could be achieved for KPF, MFN and PBZ. Therefore
there are no methods available to date for KPF, MFN and PBZ in plasma that can meet the target level of 5 ng mL$^{-1}$. A disadvantage of the method developed by Gonzalez et al. [Gonzalez et al., 1996] is that the method monitors 3 ions and this is not a confirmatory method according to Commission Decision 2002/657/EC [European Commission Decision, 2002] and a second analytical technique is required. There are few analytical methods for the determination of authorised and non-authorised NSAIDs in milk and these usually analyse for only a few residues. Those that have been described use LC-UV [Martin et al., 1983; Gallo et al., 2008; Feely et al., 2002; Rubb et al., 1995; De Veau et al., 1996]. LC-MS [Gallo et al., 2008; Boner et al., 2003; Daeseleire et al., 2003; Malone et al., 2009. Dubreil et al., 2011] and GC-MS [Rubb et al., 1995]. A method by Gallo et al [Gallo et al., 2008] is capable of analysing 16 NSAIDs in milk using two separate analytical techniques and involves using a screening LC-DAD method with limits of detection (LOD) of between 2-15 ng mL$^{-1}$ and a runtime of 35 min with an equilibration time of 15 min per injection. Confirmation is achieved using an LC ESI-Iontrap -MS/MS method with an LOD of 5 ng mL$^{-1}$ except for flurbiprofen with a runtime of 40 min per injection. The LC-MS method does not meet the requirements for a confirmatory method according to Commission Decision 2002/657/EC and a third analytical technique is required. A method by Stolker et al. [2008] is capable of analysing 20 NSAIDs in milk using a quantitative screening method (UPLC-TOF-MS) with LOD's for specific NSAIDs such as NAP, PBZ and DCF at 12.5, 25 and 6.3 ng mL$^{-1}$ and a runtime of 8.5 min. The method cannot meet the 5 ng mL$^{-1}$ level set for NAP and PBZ or 0.1 ng mL$^{-1}$ level recently set for DCF; additionally, analysis by TOF-MS, medium to high resolution of approximately 10,000 FWHM is not included in
Commission Decision 2002/657/EC. Other methods for the determination of NSAIDs in milk have limits of detection of 20 ng mL$^{-1}$ for PBZ [Martin et al., 1983], 0.2 ng mL$^{-1}$ for FLU and FLU-OH [Bonner et al., 2003], 0.5 ug kg$^{-1}$ for FLU, FLU-OH and 1 ug kg$^{-1}$ for KPF [Daeseleire et al., 2003], 53.05, 15.82, 61.39, 45.04 ng mL$^{-1}$ for TLF, MLX, 4-MAA and FLU-OH [Malone et al., 2009], 0.46-2.86 ng mL$^{-1}$, 1 ng mL$^{-1}$ for FLU [Feely et al., 2002], 1.7 ng mL$^{-1}$ for FLU [Rubb et al., 1995] and the lowest fortification in matrix was 25 ng mL$^{-1}$ for PBZ [De Veau et al., 1996]. Dubreil et al., 2011 analysed 12 NSAIDs in milk and obtained limits of detection of between 0.69 and 27.54 for non-MRL substances which included PBZ, OBZ, FLU, NAP, MFN, KPF, VDP and decision limits for MRL substances were 0.1 for DCF, 15.37 for MLX, 45.08 for FLU-OH, and 62.96 µg kg$^{-1}$ for TLF [Dubreil et al., 2011]. There are limited methods available for the determination of NSAIDs in bovine plasma and milk matrices that meet EU legislative requirements and veterinary drug residue confirmatory criteria. It was the purpose of the work carried out in this thesis to develop analytical strategies for NSAIDs residues that were previously not available to meet these guidelines.

2.5.2 Forensic Toxicology Drug Monitoring

The use of drugs alone or in combination with other drugs such as cannabis or alcohol is widespread. The work described in this thesis involved developing new methods in order to improve the toxicological service to the Coroner's of Ireland. Work was undertaken in order to evaluate newer technology to improve overall analysis time in the laboratory and expand the classes of drugs for analysis. During the post-mortem examination samples of blood and urine are taken and sent to the laboratory for toxicological analysis. The
legislation that covers this work is based on the Coroner’s Act 1962 and The Coroner’s (Amendment) Act 2005. Drugs such as amphetamines, cocaines, opioids and adulterants were studied as they are commonly requested by the Coroners.

2.5.2.1 Extraction Methods and Purification Strategies for amphetamines, cocaines, opioids and adulterants

Therefore the availability of a rapid sample preparation procedure for urine was investigated. To date the simplest sample preparation procedures in urine in the literature for the determination of drugs included in this study such as AMP, MDA and MDMA [Andersson et al., 2008], MOR, M-3-G, M-6-G, COD, COD-6-glucuronide, ethyl morphine, M-6-G and 6-MAM [Gustavsson et al., 2007] and MOR, BENZOYL, 6-MAM, COC, COCA, METH and EDDP [Dams et al., 2003] were accomplished using direct injection. Alternatively KET has been filtered prior to direct injection [Chen et al., 2007] with subsequent LC-MS detection or KET and its metabolites in urine have undergone micro dialysis sampling coupled with online SPE [Chen et al., 2010]. Other simple sample preparation methodologies have utilised analyzing dried urine spot specimens on filter paper. The filter paper was soaked in water and the reconstituted urine analysed directly [Meany et al., 2009]. There is a scarcity of methods that provide rapid sample preparation procedures in urine that cover a wide range of drugs from different classes. There has been a move in recent years from Gas Chromatography Mass Spectrometry to Liquid Chromatography Mass Spectrometry. This thesis focuses on the application of LC-MS technology for the determination of drugs in this study. To date methods that have been cited in the literature for amphetamines, cocaines, opioids and
adulterants covered in this study have been analysed in urine by LC-Ion TRAP MS [Chen et al., 2007; Tsutsumi et al., 2005; Wu et al., 2005; Cheng et al., 2006; Katagi et al., 1996; Suni et al., 2011], LC Tandem MS [Andersson et al., 2008; Gustavsson et al., 2007; Dams et al., 2003; Cheze et al., 2007; Concheiro et al., 2007; Kuwayama et al., 2008; Gergov et al., 2009; Musshoff et al., 2004; Mueller et al., 2011; Nema et al., 2011; De Jager et al., 2011; Sasaki et al., 2009; French et al., 2009; Lamshoft et al., 2011; Menchijkanti et al., 2011; Noriaki et al., 2009; Mueller et al., 2005] and hybrid LC-MS [Mueller et al., 2005]. The LC-MS chromatography methods to date cover only a few analytes in the urine matrix usually with long chromatographic run-times.

Blood was the second matrix targeted in the Thesis. To date sample preparation procedures in whole blood for amphetamines, cocaines, opioids and adulterants in the literature for the determination of drugs included in this study was achieved by solvent extraction using methanol, liquid/liquid extraction [Juhascik et al., 2009; Gergov et al., 2009; Mueller et al., 2005; Gergov et al., 2003; Logan et al., 1987] or SPE [Juhascik et al., 2009; Mueller et al., 2005; Johansen et al., 2007; Bogusz et al., 1997; Dams et al., 2002; Decaestecker et al., 2003; Chen et al., 1993; Gerostamoulos et al., 1995]. A liquid/liquid extraction procedure for the determination of 122 drugs involved addition of sodium borate (pH 9.3) to blood and toluene:hexane:isoamyl alcohol (78:20:2, v/v/v). A back extraction was performed upon addition of sulphuric acid. Samples were mixed and centrifuged and the top layer aspirated to waste. Sodium hydroxide and n-butyl chloride was evaporated and extracts reconstituted in ethyl acetate [Juhascik et al., 2009]. The procedure was not suitable for morphine and benzoylecgonine. A liquid/liquid extraction procedure was developed by Gergov et al. [2009] for 25 opioid drugs in blood and urine.
Urine was initially hydrolysed using β-glucuronidase enzyme and sodium hydrogen phosphate buffer pH 9 was added to both blood and urine matrices. The samples were adjusted to pH 7 and extracted with butyl acetate. The method only covered a single class of drugs in either matrix. Mueller et al., [2005] extracted 301 forensically important drugs by performing a basic extraction by adding pH 11 buffer and extracting with butyl acetate. An acidic neutral extraction was performed by adding sodium chloride and phosphate buffer and phosphoric acid. Extraction was performed by addition of dichloromethane:2-propanol (95:5, v/v). Although a wide variety of drug classes were covered the method did not detect cocaethylene which is a forensically important substance. Gergov et al., [2003] added Tris (hydroxymethyl)-aminomethane buffer pH 11 and extracted with butyl acetate. Sodium chloride and phosphate buffer and phosphoric acid were extracted with dichloromethane:isopropanol (95:5 v/v). Logan et al., [1987] added pH 9 borate buffer to blood and subsequently added ethyl acetate. Drugs were re-extracted with sulphuric acid and samples centrifuged. The organic layer was discarded and aqueous layer saturated with ammonium carbonate and re-extracted with ethyl acetate.

SPE sorbent chemistries utilized were reverse phase [Decaestecker et al., 2003; Bogusz et al., 1997; Gerostamoulos et al., 1995], cation exchange [Johansen et al., 2007; Dams et al., 2002; Decaestecker et al., 2003], strong/weak mixed mode cation exchange sorbents [Dams et al., 2002; Decaestecker et al., 2003; Chen et al., 1993] and polymeric sorbents utilizing Oasis HLB [Decaestecker et al., 2003]. Chen et al [Chen et al., 1993] evaluated a number of sample pre-treatment methods for the extraction of morphine from blood prior to SPE. The pretreatments involved protein precipitation. The first involved
addition of zinc sulfate-methanol (70:30, v/v) and the supernatant was transferred into 0.1 M sodium acetate buffer. The second involved addition of acetonitrile and transfer of supernatant to tube with 0.1 M phosphate buffer. The third involved addition of methanol instead of acetonitrile. In the fourth method blood was vortexed upon addition of 0.1 M phosphate buffer at pH 6.0 or pH 3.3 and subsequently centrifuged. The fourth sample pre-treatment was found to be the most suitable and extracts were purified on BondElut Certify columns. Johansen et al., [Johansen 2007] pretreated blood by adding water and hydrochloric acid prior to loading onto SPEC MPI columns from Kingo Diag. Inc for the extraction of cocaine, ecgonine methyl ester, benzoylcegonine, norcocaine and ethylene cocaine. Bogusz et al., [1997] extracted morphine, M-3-G, M-6-G, codeine, codeine glucuronide, 6-monoacetylmorphine drugs using C18 SPE whereas Dams et al., [2002] extracted 17 opium alkaloids including drugs under investigation in this thesis such as buprenorphine, codeine, morphine, methadone, 6-MAM using Bond Elut CBA SPE columns [Dams et al., 2002]. Automated SPE was performed using the Zymark Rapid Trace SPE Workstation by Decaestecker et al., [2003]. The following sorbents were studied C2, C4, C18, C18 MF, PH, CN, Isolute HCX, HCX3, HCX5, Oasis HLB, MCX. Authors found that C8 was the best. Gerostamoulos et al., [1995] purified morphine and its metabolites from blood using C18 SPE.

To date drugs in our study have been analysed using LC coupled to different detectors including LC-diode array and fluorescence detectors [Dams et al., 2002], LC-electrochemical detector [Logan et al., 1987; Chen et al., 1993; Gerostamoulos et al., 1995], LC-MS [Gergov et al., 2009; Bogusz et al., 1997; Noriaki et al., 2009], LC-MS/MS [Shima et al., 2008; Gergov et al., 2003; Johansen et al., 2007, Decaestecker et
al., 2003; Jagerdeo et al., 2008; Sergi et al., 2010; Kjaerguard et al., 2010; Kriger et al., 2010; Oiestad et al., 2011; Scurati et al., 2009; Karinen et al., 2009; Gergov et al., 2009; Fernandez et al., 2009; Dress et al., 2009; Sadig et al., 2011] and hybrid LC-MS [Mueller et al., 2005]. There was a paucity of studies that used hybrid LC-MS technology in the literature as the majority of studies use LC-tandem MS. A method by Mueller et al [2005] analyses 301 drugs qualitatively in blood and urine by 3200 QTRAP hybrid LC-MS with a chromatographic runtime of 30 min. Blood was mentioned in the study but no validation results were evident and results were qualitative only. A disadvantage of the published methodology was that only one MRM transition is monitored and if a situation arises were the EPI scan does not trigger produce a satisfactory spectrum re-injection of the samples would be necessary. Three EPI scans in addition were utilised at three separate collision energies (CE) which increases the duty cycle of the study and substances such as COCA, BNZY, LEV and PHEN were not analysed in blood. The aim of this work was to develop single, fast, simple and reliable sample preparation procedures in blood and urine with detection by hybrid LC-MS as analytical strategies in a forensic laboratory for the determination of drugs of abuse which were previously not available.

2.6 Overview of Mass Spectrometry

2.6.1 Introduction to Mass Spectrometry

Mass Spectrometry is an analytical technique that measures the mass-to-charge ratio of charged particles or ions [Sparkman 2000]. Ions are generated by causing either loss or gain of a charge from a species that is neutral. The technique is utilised to establish the
mass of particles, to identify elemental composition of a sample or molecule, for identification of the chemical structure of a compound. In 1958, the combination of gas chromatography and mass spectrometry as the detector was achieved [Gohlke 1959].

John B. Fenn the 2002 Nobel Laureate in Chemistry for development of electrospray ionisation gave to date the most apt definition of mass spectrometry as

"the art of measuring atoms and molecules to determine their molecular weight. Such mass or weight information is sometimes sufficient, frequently necessary, and always useful in determining the identity of a species. To practice this art one puts charge on the molecules of interest, i.e., the analyte, then measures how the trajectories of the resulting ions respond in vacuum to various combinations of electric and magnetic fields. Clearly, the sine qua non of such a method is the conversion of neutral analyte molecules into ions. For small and simple species the ionization is readily carried by gas-phase encounters between the neutral molecules and electrons, photons, or other ions" [Fenn et al. 1990; Fenn 2002]

### 2.6.2 LC-MS versus GC-MS

The methodology used in the six chapters of this thesis focused on the use of gas chromatography and liquid chromatography mass spectrometry techniques as a means for the detection of trace residues in complicated matrices such as animal milk, plasma and human urine and blood. Chapter three utilised gas chromatography mass spectrometry and chapters four to eight focused on liquid chromatography mass spectrometry for detection of drugs. Gas chromatography mass spectrometry was utilised as the detection technique in the third chapter of this thesis focusing on non-steroidal anti-inflammatory...
drugs (NSAIDs). NSAIDs have been derivatised for GC-MS using the following derivatisation reagents; methelut (Neto et al., 2003), bis(tri-methylsilyl) trifluoroacetamide (Singh et al., 1991), n-trimethylsulfonium hydroxide (Hines et al., 2004) and N-methyl-N-(trimethylsilyl) fluoroacetamide and trimethylanilium hydroxide (Taylor et al., 1995), methyl iodide/anhydrous potassium carbonate (Gonzalez et al., 1996) and Trimethylsilyldiazomethane (Migowska et al., 2010). Derivatisation can be quite time consuming in order to improve peak shape, ionisation and/or volatility and may introduce a larger variability and decrease the precision of the methodology. The advantages of GC-MS include its low cost, superior resolution and low limits of detection and GC-MS detection is still a widely used analytical tool in laboratories. The availability of such methods is important to regulatory laboratories. Liquid chromatography mass spectrometry was utilised as the detection technique for NSAIDs, opioids, cocaines, amphetamines and adulterant drugs in the remaining five chapters of the thesis. The primary advantage of LC-MS is that it does not require derivatisation and that sample preparation can be more simplified. Sample preparation procedures such as SPE or liquid/liquid extraction can be performed but more simple procedures such as direct injection, dilution or protein precipitation can also be utilised. The reduction in the sample preparation stages saves both time and money and in addition the reduced number of steps can reduce the possibilities of errors and improve method precision. Therefore LC-MS was the most widely used technique in the thesis as more suitable to achieve the main aims in the thesis to develop rapid multi-residue strategies for regulatory laboratories. It should be noted that GC-MS is an established technique for the analysis
of complicated mixtures of compounds because of its combination of sensitivity and wide range of applicability and versatility and is widely used.

![Mass spectrometer schematic](http://www.chem.mun.ca/courseinfo/c3500/Dawe/Lecture%20Notes.pdf)

**Figure 2-8 Schematic of Mass Spectrometer**

### 2.6.2.1 Overview of GC-MS

Gas chromatography- mass spectrometry is a hyphenated analytical technique arising from the combination of two separate analytical techniques. The gas chromatograph separates the constituents of a mixture such as volatile and semi-volatile compounds with great resolution in time and the mass spectrometer identifies each compound by providing structural information. The sample mixture is introduced into a mobile phase...
which is an inert gas such as helium. The mobile phase carries the sample mixture
through the stationary phase. The stationary phase is contained in a column. The column
is coated with a stationary phase that can selectively attract the constituents of the sample
mixture. Columns are available with a wide variety of stationary phases and dimensions
and can be manufactured with fused synthetic silica, glass or stainless steel. As the
constituents of the mobile phase are attracted to the stationary phase at different rates,
those that interact the least will elute from the column the most quickly. If the
stationary phase is altered then different mixtures of compounds can be separated due to
different interactions. The separation in GC can also be affected if the pressure of the
mobile phase or the temperature of the stationary phase is adjusted. The column in a GC
is held in an oven that is programmed to increase the temperature. As the temperature is
increased compounds that have low boiling points elute from column sooner compared to
substances that have higher boiling points. As the constituents of a mixture elute from a
column, they enter the mass spectrometer where they are bombarded with a stream of
electrons causing them to break apart. The fragments are charged ions with a certain
mass. The mass of the fragment divided by the charge is called the mass to charge ratio
(M/Z). Since most fragments have a charge of +1, the M/Z usually represents the
molecular weight of the fragment.

(Accessed May 2012)

Figure 2-9 Schematic of a GC-MS Instrument
Both techniques are highly compatible as in both techniques the sample is in the vapour phase and both techniques deal with approximately the same sample size and can be applied to a variety of analytical chemistry problems. Gas chromatographs operate at atmospheric pressure but the ion sources of mass spectrometers operate at low pressure and this can cause a considerable problem. Due to the incompatibility with pressure, suitable interfaces were designed in order to combine the two techniques. The interface allows the transference of all the column eluent at reduced pressure from GC into the mass spectrometer. The analyte must not decompose or condense in the interface before entering the mass spectrometer ion source. Also the gas load entering the ion source must be within the pumping capacity of the mass spectrometer. In the case of capillary columns the mass spectrometer can handle the entire column effluent however in the case of macrobore and packed columns the column effluent must be reduced. To reduce the column effluent flow splitting was used in the early days but is generally not as utilised as this reduces the sensitivity. These interfaces were no longer carrier gas splitters but carrier gas separators and they separated the carrier gas from organic target molecules and increased the concentration of the organic target molecules in the gas stream. The most valuable carrier gas separator that is commercially available is called the jet separator. The principle of the technology is that it takes advantage of the differences in diffusability between the carrier gas and the organic compound. Generally the carrier gas is a tiny molecule such as hydrogen or helium with a large diffusion coefficient whereas the target organic molecules have lower diffusion coefficients. While the device is functioning the GC effluent (contains the target molecules and the carrier gas) is sprayed through a tiny nozzle into a partially evacuated chamber (about $10^{-2}$ torr).
Due to helium’s diffusion coefficient, the helium is sprayed over a wide solid angle, whereas the heavier target molecules are sprayed over a much narrower angle and tend to go straight across the vacuum region. Upon collecting the middle section of the solid angle with a skimmer and passing it to the mass spectrometer, the higher molecular weight target organic molecules are separated from the carrier gas which is removed from the vacuum pump. Generally jet separators are made from glass by drawing down a glass capillary, sealing into the vacuum envelope and cutting out the middle spacing but it is imperative that the skimmer and the spray orifice are perfectly aligned. The higher flow rates used for packed columns work the best for these jet separators but there are certain disadvantages for consideration. Packed GC columns can be a source of small particles which can enter the jet separator and become stuck in the spray orifice. Subsequently this can block or drastically reduce the flow from the GC column into the mass spectrometer. The most common approach when utilising capillary GC columns is to pass all the carrier gas flow into the mass spectrometer if the flow is suitably small and the mass spectrometer pumping speed is sufficient to handle the gas flow. [Gas Chromatography Mass Spectrometry Ronald A Hites]

However it is custom now that GC-MS interfacing is performed by simply inserting the capillary column directly into the ion source. Other gases such as methane for chemical ionisation enter the ion source through a T joint around the capillary column. A separate line into the ion source is used for a thermocouple vacuum gauge tube to monitor source pressure. The mass spectrometer calibration standard is delivered into the ion source through another separate line into the ion source. The only removable fitting is at the junction of the GC column and the far end of the inlet tube and utilises Vespel ferrules.
Once GC columns are fitted with the ferrules it is recommended to cut off a few centimetres of the column to eliminate fine particles blocking the end of the column. The interface is held at temperatures between 250 to 280 °C.

The types of ionisation techniques used in GC-MS are electron ionisation and chemical ionisation. Electron ionisation is the most widely used technique when coupled to GC-MS. A heated filament emits electrons which are accelerated by a potential difference of generally 70 eV into the sample chamber. Ionisation of the sample is achieved by removal of an electron from the molecule thus generating a positively charged ion with one unpaired electron. The technique produces abundant fragment ions and library searchable spectra.

![Figure 2-10 Schematic of Ion Trap](http://www.hull.ac.uk/chemistry/massspec3/principles%20of%20ms.html#electronionisation)

(Accessed May 2012)

The second technique is called Chemical Ionisation which was developed from EI and similar except that a reagent gas is introduced into the chamber in excess of the sample. Negative CI uses methane gas in electron capture mode and Positive CI uses methane, isobutane or ammonia as reagent gas. The ionised reagent gas protonates the sample
molecules leaving a neutral reagent gas species. This is a softer ionisation technique than 
EI and is used to produce more abundant molecular ions. The spectra generated under CI 
conditions are not reproducible and therefore no CI libraries are commercially available. 
Electron ionisation was chosen as the ionisation technique for the work carried out in the 
following chapter of this thesis. Other authors have used electron ionisation in the 
literature for the determination of NSAIDs [Stanley et al., 1995]. 
In the technique of GC-MS virtually all compounds capable of passing through a GC 
column can be ionized. In LC-MS when a mass spectrometer is coupled to an LC system, 
compounds cannot be directly ionised and an interface must be utilised for the removal of 
the mobile phase.

2.6.2.2 Overview of LC-MS

Liquid chromatography-mass spectrometry (LC-MS) is a hyphenated analytical 
technique arising from the combination of two separate analytical techniques. The liquid 
chromatograph combines the physical separation capabilities of liquid chromatography 
and the mass spectrometer identifies each compound by providing structural information. 
A major difference between established high performance liquid chromatography 
(HPLC) and LC-MS is the scale. The size of the analytical columns used in LC-MS is 
generally much smaller and hence flow rates are reduced. If standard HPLC columns are 
used the flow is generally split. There are a number of different types of analysers that 
can be used in LC/MS such as single quadrupoles, triple quadrupoles, ion trap, time of 
flight (TOF), and quadrupole time of flight (QTOF) analysers. A difficulty for a long 
time was combining the liquid phase technique and the gas phase technique operated in a
vacuum. Interfaces were developed in order to deal with this difficulty. The use of atmospheric pressure ionisation (API) interfaces for coupling LC with quadrupole mass spectrometers overcame these difficulties and gave rise to analytical strategies with good selectivity, sensitivity and robustness [Niessen et al., 1992]. For API techniques the sample composition e.g the mobile phase, additives utilised, matrix constituents affect the ionisation and the subsequent analytical result.


**Figure 2-11 Schematic of a LC Instrument**

2.6.2.2.1 *API Interfaces and Electrospray Ionisation*

In the LC-MS systems, ionisation is achieved at atmospheric pressure (API). Horning and co-workers published the first bioanalytical applications of atmospheric pressure ionisation mass spectrometry (API-MS) [Horning et al., 1974].
A wide range of API source designs are available from the instrument vendors. These sources include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionisation (APPI), desorption electrospray ionisation (DESI) and direct analysis in real time (DART) when coupling to LC-MS. These sources ionise the sample at atmospheric pressure and the atmospheric pressure interface transfers ions into the high vacuum region of the mass analyser.

An API source according to Niessen [2003] consists of 5 separate components:

(i) The liquid introduction device.

(ii) The actual atmospheric-pressure ion-source region, where the ions are generated by ESI, APCI, or other means.

(iii) The ion-sampling aperture.

(iv) The atmospheric-pressure to high-vacuum interface: the transition region.

(v) The ion-optical system, where the ions generated in the source are analyte enriched and transported towards the high-vacuum mass analyser.

In this thesis the 5 papers illustrated in chapters four to eight utilised electrospray ionisation as the technique for ionisation. The use of electrospray has gained widespread acceptance in analytical chemistry due to its mild ionisation, high ion transmission, improved sensitivity at lower flow rates and its improved mass accuracy. The polar nature of NSAIDs studied in this research allows for the ESI source to be particularly suitable for their MS detection although some work has been reported using APCI sources in the literature [Abel-Hamid et al., 2001; Loffer et al., 2003]. NSAIDs are generally analysed in negative ion mode after deprotonation of the carboxylic acid functional group. In addition protonation of the same site can occur allowing detection in
positive mode [Ferrer et al., 2005]. LC-MS and LC-MS/MS have largely replaced GC-MS in the analysis of drugs of abuse. In the forensic toxicology part of this thesis the toxicologically relevant compounds studied have basic properties and positive mode was applied in their analysis by LC-MS.

The main advantage of LC-MS over GC-MS is that the LC-MS allows for the simultaneous analysis of both free and conjugated drugs of abuse in a single analysis without the need to carry out an intermediate hydrolysis step (e.g. GC analysis of cannabinoids). It also allows for the analysis of polar, low volatility and/or thermolabile drugs (e.g. cocaine, heroin) without the need to perform a derivatisation step (GC requirement). A number of papers have been published in the literature for the determination of drugs of abuse analysed in this work utilising electrospray ionisation in the positive mode [Clauwaert et al., 2000; Shima et al., 2006; Jeanville et al., 2000; Needham et al., 2000; Dams et al., 2003; Mueller et al., 2005; Maralikova et al., 2004] or APCI in the positive mode [Apollonio et al., 2006; Dams et al., 2003; Bogusz et al., 2000]. Other forensic toxicology studies utilised an atmospheric pressure photoionisation APPI source [Marchi et al., 2009] for the analysis of alprazolam and flunitrazepam metabolites in blood. Ambient mass spectrometry is a rapidly growing area in analytical chemistry and ambient ionisation methods such as desorption electrospray ionisation (DESI-MS) and desorption atmospheric pressure photoionisation mass spectrometry (DAPPI-MS) have also been utilised in the analysis of morphine and codeine [Suni et al., 2011].

Fenn et al. [1989] showed that multiply charged ions were obtained from proteins allowing their molecular weights to be determined [Fenn et al., 1989; Mann et al., 1989].
The electrospray interface is recommended for use with highly polar and ionized materials. It is a soft ionisation technique that results in little fragmentation.

As electrospray ionisation was utilised in all LC-MS studies in this research and the principle of this technique is outlined in this thesis.

In the literature numerous applications are reported ranging from the analysis of carbohydrates and nucleotides to small polar molecules utilizing ESI. The technique of ESI involves a number of steps including the formation of charged droplets, desolvation, ion generation, declustering and ion sampling [Mansalli et al., 2006]. A strong electric field is applied to create an excess of charge at the tip of the capillary which contains the analyte solution. The process of volatilising the samples is achieved due to setting a potential difference of 2-6 kV between the tip of the ES capillary and the counter electrode [Ross 2001; Smith et al., 1990]. Charged droplets emerge from the capillary as a spray and migrate at atmospheric pressure in an electrical gradient to the gas conductance limiting orifice or tube. The charged droplets emerging contain solvent, electrolyte cations, and analyte molecules. Solvent evaporation from the charged droplets gives rise to volume shrinkage thus increasing Coulombic repulsion. When a critical limit of repulsion (know as the Rayleigh stability limit) is exceeded, as a result of a small radius of droplets, asymmetric fission known as droplet-jet fission, generates daughter droplets and these droplets will be submitted to the same shrinkage and fission procedure. The overall timescale for the process of fission is a number of μs to ms [Smith 2006; Tang et al., 1993].

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Subsequently gas phase ions travel through various vacuum stages to the mass analyzer and finally the detector. ESI has been utilised with a variety of mass analysers each with their own advantages/disadvantages. A review of various mass analysers is given by Cole [1997]. The theory of electrospray has been outlined in a review by Manisali et al., [2006]. In ESI, a potential difference of a large magnitude is applied between an electrode shaped as a wire and a counter electrode and a strong electric field is produced at the tip. In practice in ESI the capillary containing the analyte solution has a high voltage applied. In the case of a positive potential being applied due to electric field gradient at the tip, charge separation in the solution occurs as anions migrate towards the meniscus of the droplet produced at the tip [Cole 2000]. Charged species are focused into the mass spectrometer using a series of counter electrodes with decreasing potential. The potential difference set depends on experimental parameters such as flow rate of solution, charge state of analyte, solvent composition, distance between tip and counter electrode. The Taylor cone was described after work completed by Taylor [1964] which detailed mathematically the formula behind the formation of the electrospray cone and hence this was referred to as a “Taylor cone” [Taylor 1964]. This is illustrated in Figure 2-13. As the electrostatic repulsion between the charged molecules at surface of the Taylor cone reaches the surface tension of the solution this is called meeting the Rayleigh Limit and charged droplet species are emitted from the tip. The principle of producing the Taylor cone is not understood. [Juraschek et al., 1998]. Spray modes can be affected by electrolyte concentration, capillary diameter and liquid flow rates if gradient elution is utilised. Valaskovic et al., [2004] overcame the problem with gradient elution by developing an orthogonal optoelectronic system with the ability to identify a large variety
of spray modes under different conditions. A small droplet released from the Taylor cone has less solvent therefore desolvation and ionization is more efficient. The size of the droplet ultimately depends on the flow rate and the capillary diameter. Smaller droplets undergo less evaporation-fission cycles to produce ions, the salt concentration in final offspring droplets can be less. Also this may lower the background noise in the mass spectrum. [Karas et al., 2000] but also smaller droplets that are not surface active will have a greater opportunity of transfer to gas phase instead of loss in larger parent droplets. Generally solvents used in ESI are acidified water and organic modifier. Organic solvents help lower the surface tension of liquid helping formation of gas phase ions. During evaporation of the solvent, the droplet shrinks and the electrostatic repulsion between the charges within the droplet becomes larger. As the Rayleigh limit is approached droplets begin to break away by ‘coulombic fission’ [Cole 2000]. Further evaporation of the droplets leads to a new fission series which is repeatable. Actual creation of gas phase ions from droplets is expected to be a combination of two mechanisms known as “ion evaporation mechanism [IEM] proposed by Iribarne et al., [1976] and the ‘charged residue model’ [ChRM] put forward by Dole et al., [Dole et al., 1968; Mack et al., 1969] and supported by Schmelzeisen-Redeker et al., [1989]. The differences between both mechanisms are in how the gas phase ions are generated. IEM proposes that if an electric field on a charged droplet is at a high level, single, solvated analyte molecules transporting some of the droplet charge are emitted with the gas phase as the energy of the ions close to the surface becomes high enough to allow evaporation to take place [Thompson et al., 1979]. The ChRM mechanism proposes that gas phase ions are formed when successive fissions lead to a charged droplet with a single analyte.
Some studies show that ChRM is the mechanism of choice in the case of producing charged globular protein in gas phase [Kebarle et al., 1999; Felitsyn et al., 2002; Gamero-Castano et al., 2000] even though it is not known which is the more exact. The mechanism of producing small analyte ions is not clear and can be achieved by more than one process [Cech et al., 2001]. The ESI source utilised in this work was supplied by Applied Biosystem (MDS Sciex). This ESI source technology involves a strategy for desolvation whereby heated auxiliary nitrogen is focused at an angle from the direction of the spray (TurboIonSpray™) [Li et al., 1996; Allanson et al., 1995]. In addition a counter current nitrogen gas flow (Curtain gas™) is emitted from the front of the gas conductance limiting orifice which further aids desolvation [Bruins 1997]. The curtain gas carries neutral molecules away from the sampling orifice by the nitrogen flow but charged molecules are focused through the gas flow under the effects of an electric field.

Source: Applera Corporation and MDS Inc Operator Training Manual

Figure 2-12: A schematic of an Turbo V™ source from Applied Biosystems
2.6.2.3 Mass Spectrometry and Confirmatory Criteria

Tandem mass spectrometry is a division of mass spectrometry which involves selection of a particular ion produced from a molecule by applying collision energies to form characteristic secondary fragment ions. (Futrell 2000). In a review by Le Bizec et al it was stated that “Among the different mass analysers applied for the target analysis, triple quadrupole (QqQ) is the most widely used for measuring and quantifying residues of veterinary drugs [Le Bizec et al., 2009]” The triple quadrupole (QqQ) consists of a Q1 quadrupole analyser for separation of the original precursor ion(s), an unscanned Q2 quadrupole that operates as a collision gas cell to fragment the ions sent to it by collision with a heavy gas molecule and a scanning Q3 quadrupole that can separate the fragments produced in the Q2 unit. There are four possible modes of operation of the two
analysers; Q1 scan/Q3 SIM, known as daughter mode or precursor scanning; Q1 SIM/Q3 scan, called parent mode or product scan; Q1 scan/Q3 scan, referred to as neutral loss scanning mode; and Q1 SIM/Q3 SIM, referred to as selected reaction monitoring (SRM) mode [McMaster 2005]. SIM refers to the operation of a mass spectrometer in which the intensities of several specific ion beams are recorded rather than the entire mass spectrum. SRM refers to data acquired from specific ions corresponding to m/z selected precursor ions recorded via two or more stages of mass spectrometry. In this research, triple quadrupole (QqQ) in SRM mode of operation was used in chapters three-six for the analysis of NSAIDs in the veterinary drug residue section of the thesis.

Figure 2-14 Schematic of a triple quadrupole mass spectrometer

Ion trap mass spectrometry is another analyser which has a wide number of uses in analytical chemistry. Compounds enter the ion trap from a pinhole orifice on the side of the ring electrode and are held in a three-dimensional spherical-segment stable orbit between the electrodes with a trapping voltage from the dc/RF source. They are emitted in increasing mass (m/z) by increasing the dc/RF voltage on the ring electrode. This
forces each fragment ion into an unstable orbit, causing it to escape through one of the seven holes in the exit electrode and into the dynode electron multiplier detector which distributes signal to the data system. An ionised molecular ion trapped between the ring electrodes and application of collision gas can lead to fragmentation to aid identification of target ion’s molecular structure [McMaster 2005]. A limited amount of sample can enter the ion trap without overloading and affecting performance. A newer technology ion trap called the linear ion trap was developed. This technology combines the separation capability of the quadrupole analyser with MS/MS capability of an ion trap. This type of analyser can be operated in normal scanning mode for separation and detection of mass ions, or the ends of electrodes can be turned on to retain a specific ion in the trap for collision with a damping gas and further fragmentation can be achieved by applying a resonance excitation voltage. The daughter ion fragments can be released to the ion detector sequentially by scanning the dc/RF voltage on the quadrupole rods while utilising a resonance ejection voltage on the trapping electrodes. The advantage of this type of analyser is the capacity is increased thus much greater sensitivity for analysing trace components in column effluent [McMaster 2005].

In this work hybrid LC-MS was used for the analysis of drugs of abuse (chapters 7 and 8). The mass analysers used in the hybrid LC-MS system were the combination of a triple quadrupole (QqQ) and linear ion trap (LIT) where Q3 in this technology can be utilised as a quadrupole or a linear ion trap with axial ion injection [Hager 2002]. The combination of both analysers in the same instrument allow running as a true QqQ triple quadrupole (very sensitive SRM) as well as the ability to provide sensitive full scan mass spectra which has the ability to perform MSn experiments thus allowing characterisation
of structures and ability to identify metabolites. This has been utilised in the analysis of drugs of abuse in the forensic toxicology section of this thesis utilising the 4000 hybrid linear ion trap-triple quadrupole mass spectrometer from Applied Biosystems. Triple quadrupole (QqQ) in SRM mode of operation was used for the forensic toxicology research and was previously described above. Enhanced product ion (EPI) mode was also utilised in the hybrid LC-MS for the forensic toxicology research where Q1 was used to filter the precursor ions. Q2 acted as a collision cell to generate fragments while Q3 was working in ion trap mode to scan product ions. This results in a triple quadrupole MS like fragmentation pattern but with higher sensitivity. In general operation as a triple quadrupole mass spectrometer is useful when high sensitivity and selectivity of SRM transitions is needed for example in quantitative analysis or targeted screening. In general, operation as a linear ion trap mass spectrometer is necessary when higher sensitivity in full scan experiments is required thus giving triple quadrupole MS like fragmentation patterns but with higher sensitivity. The QTRAP hybrid LC-MS system from Applied Biosystems instrument used in this research has a built in collision energy spread (CES) feature which allows for collection of data at different collision energies in one EPI spectrum. Commission Decision 2002/657/EC [Commission Decision 2002] outlines the use of identification points (IPs) in the veterinary drug residue field and the validation protocol has been utilised in the post-mortem forensic toxicology section of the thesis also as an alternative validation procedure. Commission Decision 2002/657/EC lays down performance criteria for analytical methods in the veterinary drug residue area. This decision in addition to general performance requirements delivered requirements for confirmation utilising identification points (IPs) and defined criteria for ion intensities.
The EU notion of IPs and tolerated ion intensities ratio for the confirmation of the identity of a compound is based on agreement obtained by members of the so-called EU working group of experts [Andre et al., 2001].

When SRM mode is utilised for samples and two precursor-product ion transitions are analysed an ion ratio can be obtained from the relative ratio of their response.

This ion ratio value obtained can be compared with that of the ion ratio for the known test compound (usually obtained by analysing material fortified with the test compound). This approach gives rise to four identification points (IPs) if a precursor ion and two transition product ions are monitored (Table below). A review by Stolker et al., [2005] gives a good overview of legislation and regulations in the veterinary drug residue field. Van Eenoo et al., [2004] summarises that there is no fundamental chemometric basis for the criteria utilised in the EU as regards the number of IPs and applied tolerances but it was interesting to note that they do not differ much from the criteria established by the Association of Official Racing Chemists (AORC), United States Food and Drug Administration (FDA), the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA) [Van Eenoo et al., 2004]. Stolker et al., [2005] states that the difference noted in the EU allows the use of a combination of different independent techniques to confirm the identity of a substance whereas the other bodies only allow the use of either GC-MS or LC-MS. The tables below outline the information contained in Commission Decision 2002/657/EC in relation to IP's and tolerances for ion intensities.
Table 2-3: Relationship between a range of classes of mass fragments and identification points earned $^b$

<table>
<thead>
<tr>
<th>MS technique $^a$</th>
<th>IP's gained per ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-resolution MS (LR MS)</td>
<td>1.0</td>
</tr>
<tr>
<td>LRMS$^a$ precursor ion</td>
<td>1.0</td>
</tr>
<tr>
<td>LRMS$^a$ transition product ions</td>
<td>1.5</td>
</tr>
<tr>
<td>High-resolution MS (HR MS)</td>
<td>2.0</td>
</tr>
<tr>
<td>HRMS$^a$ precursor ion</td>
<td>2.0</td>
</tr>
<tr>
<td>HRMS$^a$ transition product ions</td>
<td>2.5</td>
</tr>
</tbody>
</table>

$^a$ $n \geq 2 \hspace{1cm} ^b$ Source Table 5 2002/657/EC

Table 2-4: Examples of the number of IPs earned for a range of techniques and their combinations $^c$

<table>
<thead>
<tr>
<th>MS technique $^a$</th>
<th>Number of ions</th>
<th>IP's</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-MS (El or CI)</td>
<td>N</td>
<td>n</td>
</tr>
<tr>
<td>GC-MS (El and CI)</td>
<td>2 (EI) + 2 (CI)</td>
<td>4.0</td>
</tr>
<tr>
<td>GC-MS (El or Cl); 2 derivatives</td>
<td>2 (derivative A) + 2 (derivative B)</td>
<td>4.0</td>
</tr>
<tr>
<td>LC-MS</td>
<td>N</td>
<td>n</td>
</tr>
<tr>
<td>GC-MS/MS or LC-MS/MS</td>
<td>One precursor and two daughter products</td>
<td>4.0</td>
</tr>
<tr>
<td>GC-MS/MS or LC-MS/MS</td>
<td>Two precursors each with one daughter product</td>
<td>5.0</td>
</tr>
<tr>
<td>LC-MS$^T$</td>
<td>One precursor one daughter product and two daughter</td>
<td>5.5</td>
</tr>
<tr>
<td>HRMS</td>
<td>N</td>
<td>2n</td>
</tr>
<tr>
<td>Source Table 6  2002/657/EC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2-5: Maximum permitted tolerances for relative ion intensities\(^a\)

<table>
<thead>
<tr>
<th>Relative Intensity (% of base peak)</th>
<th>Relative Maximum Tolerance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EI-GC-MS</td>
</tr>
<tr>
<td></td>
<td>(relative)</td>
</tr>
<tr>
<td>&gt;50 %</td>
<td>±10 %</td>
</tr>
<tr>
<td>20-50 %</td>
<td>±15 %</td>
</tr>
<tr>
<td>10-20 %</td>
<td>±20 %</td>
</tr>
<tr>
<td>≤10 %</td>
<td>±50 %</td>
</tr>
</tbody>
</table>

\(^a\) Source Table 4  2002/657/EC
Chapter 3: Determination of ibuprofen, ketoprofen, diclofenac and phenylbutazone in bovine milk by gas chromatography tandem mass spectrometry

Published in Food Additives and Contaminants, Volume 25, No 12. December 2008, Pages 1497-1508
3.1 Abstract

A method has been developed to analyse for ibuprofen (IBP), ketoprofen (KPF), diclofenac (DCF) and phenylbutazone (PBZ) residues in bovine milk. Milk samples were extracted with acetonitrile and sample extracts were purified on Isolute™ C18 solid phase extraction cartridges. Aliquots were analysed by gas chromatography tandem mass spectrometry (GC-MS/MS). The method was validated in bovine milk, according to the criteria defined in Commission Decision 2002/657/EC. The decision limit (CCα) was 0.59, 2.69, 0.90 and 0.70 ng ml⁻¹, respectively, for IBP, KPF, DCF and PBZ and for the detection capability (CCβ) values of 1.01, 4.58, 1.54 and 1.19 ng ml⁻¹ respectively, were obtained. The measurement uncertainty of the method was 17.8, 80.9, 28.2 and 20.2 % for IBP, KPF, DCF and PBZ. Fortifying bovine milk samples (n = 18) in three separate assays, show the accuracy of the method to be between 104 and 112 %. The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5, 7.5 and 10 ng ml⁻¹) was less than 8 % for IBP, DCF and PBZ respectively. Poor precision was obtained for KPF with an RSD value of 28 %.

Keywords: Ibuprofen; Ketoprofen; Diclofenac; Phenylbutazone; Bovine Milk; Method Validation
3.2 Introduction

Phenylbutazone (PBZ), ibuprofen (IBP), ketoprofen (KPF) and diclofenac (DCF) are non-steroidal anti-inflammatory drugs (NSAIDs). The molecular structure of these compounds is shown in Fig. 3.1. NSAIDs are used widely in veterinary medicine in the treatment of food producing animals. Depending on their heterogeneous molecular structures the NSAIDs can be classified into four main sub-classes: (a) salicylic acid derivatives; (b) propionic acid derivatives; (c) pyrazole derivatives and (d) aniline derivatives including both anthranilic acid derivatives and nicotinic acid derivatives. PBZ is a pyrazole derivative, IBP and KPF are propionic acid derivatives and DCF is an aniline derivative. In the USA a survey was carried out involving 2000 veterinarians whose practices dealt 50% of the time with food producing animals [US Code of Federal Regulations 1988]. The survey showed that 93% of veterinarians use NSAIDs on a regular basis. Dairy practitioners reported more frequent use of NSAIDs than did beef practitioners. Overall, veterinarians indicated that NSAIDs were an important group of compounds which were routinely used for the treatment of food producing animals Kopcha et al., 1992. According to EU law, all substances for veterinary use need to be included in Annexes 1-3 of Regulation 2377/90 [European Commission 1990]. This regulation establishes lists of compounds that have a fixed MRL (Annex I), that need no MRL (Annex II) or that have a provisional MRL (Annex III). PBZ and IBP are compounds that have not been included in Annexes 1-3 and have no maximum residue limit (MRL) established. Substances that have no MRL established are prohibited for use in food producing animals. DCF is listed in Annex I and a provisional MRL has been set for different animal species and target matrices. DCF is however prohibited for use in
milk producing animals. KPF is listed in Annex II and is allowed for the application to species that produce milk but are not intended for human consumption. NSAIDs can cause adverse health effects in humans such as aplastic anaemia, gastrointestinal disorders and agranulocytosis [Insel 1990] and changes in renal function [Goodman et al., 1992]. Longterm exposure to PBZ has caused kidney tumors in mice and liver tumors in rats [Kari et al., 1995]. The widespread use of NSAIDs presents a potential risk to the consumer if food containing residues enter the food chain so there is a need for the development of methods to monitor compliance with legislation in the European Union in a variety of animal tissues and products. In the determination of NSAIDs in animal tissues PBZ has been shown to be extracted from equine plasma using acetonitrile [Hardee et al., 1982; Neto et al., 1996] or ethyl acetate [Grippa et al., 2000], from equine urine with methanol [Stanley et al., 2007] or a mixture of dichloromethane:ethanol solution [Neto et al., 1996]. PBZ has been shown to be extracted from bovine plasma using acetonitrile [Fiori et al. 2004; Miksa et al., 2005] or a straightforward ultracentrifugation procedure [De Veau 1999]. IBP, KPF and DCF has been shown to be extracted from bovine plasma using acetonitrile [Miksa et al., 2005]. PBZ have been shown to be extracted from ovine, equine, and porcine muscle using a mixture of ethyl acetate:methanol and DL-dithio-threitol [Clarke et al., 2002]. PBZ has been shown to be extracted from bovine kidney using a mixture of water: ammonium hydroxide [Clarke et al., 2002]. PBZ has been shown to be extracted from bovine milk with a mixture of ethanol:ammonium hydroxide [Martin et al., 1983]. KPF has been shown to be extracted from bovine milk using acetonitrile [Daeseleire et al., 2003]. The incorporation of a hydrolysis step at the beginning of the extraction procedure for the determination of
NSAIDs is also reported in the literature. This hydrolysis step allows the deactivation of
plasma bound proteins and the release of the protein bound NSAID residues. After
hydrolysis PBZ has been shown to be extracted from bovine plasma using
dichloromethane [Singh et aI., 1991] or a mixture of dichloromethane:n-hexane:diethyl
ether solution [Hines et aI., 2004], porcine, ovine, bovine and caprine urine were
extracted with chloroform [Igualada and Moragues 2005] and equine urine was extracted
with dichloromethane [Singh et aI., 1991] or diethyl ether [Gonzalez et aI., 1996] for the
determination of PBZ. After hydrolysis or solvent extraction, solid phase extraction
[SPE] is often used in the purification of extracts containing NSAIDs. Bovine plasma has
been shown to be purified using C 18 SPE for the determination ofPBZ and DCF [Gowik
et ai., 1998] IBP, KPF, DCF and PBZ [Vinci et aI., 2006] and IBP, KPF and DCF [De

long et al. 1989]. PIa ma from pigs and rabbits were purified using this approach to
determine IBP, KPF, DCF and PBZ [Vinci et aI., 2006]. Equine plasma was purified
Using C I8 for the determination of IBP, KPF, DCF and PBZ simultaneously [Vinci et aI.,
2006] or PBZ alone [Taylor and Westwood 1995] or the C I 8 was substituted for an Oasis

BLB ™ cartridge [Quintana et aI., 2004] for the determination ofPBZ. Bovine, equine
and porcine muscle wa purified using a Florisil SPE cartridge. Bovine muscle extracts
were applied to an Oasis HLB ™ cartridge for the removal of interferences in the
determination ofPBZ and KPF [Van Hoof et aI., 2004]. Bovine kidney extracts were
applied to ilica cartridge [ larke et aI. 2002] and bovine plasma extracts were purified
uSing affinity column [Fiori tal., 2004] for the determination ofPBZ. A few methods
have been reported for the analy i of

AIDs in animal tissues using LC-DV [Hardee et

al. 1982' ingh t ai. 1991' Taylor and We twood 1995; Gowik et aI., 1998; De Veau et

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al., 1999, Grippa et al., 2000; Quintana et al., 2004; Van Hoof et al., 2004; Vinci et al.,
2006] and GC-MS [Singh et al., 1991; Taylor and Westwood 1995, Gonzalez et al., 1996;
Neto et al., 1996; Hines et al., 2004]. There are a few methods that analyse for IBP, KPF,
DCF and PBZ in animal products such as milk and those are based on LC-UV [Martin et
al., 1983] for the determination of PBZ or LC-MS detection [Daeseleire et al., 2003] for
the determination of KPF and FLU. NSAIDs have been derivatised for GC-MS using the
following derivatisation reagents; Methelut [Neto et al., 1996], Bis(trimethylsilyl)
trifluoroacetamide [Singh et al., 1991], n-trimethylsulfonium hydroxide [Hines et al.,
2004] and N-methyl-N-(trimethylsilyl) fluoracetamidine and Trimethylanilinium
hydroxide [Taylor and Westwood 1995]. Gonzalez et al. [1996] monitored for IBP, KPF,
DCF and PBZ simultaneously in equine plasma and urine by GC-MS in SIM mode used
methyl iodide/anhydrous potassium carbonate as derivatisation agents. The method
developed in this study was based on a method developed by Vinci et al. [2006] but
adapted to include deuterated analogues of IBP, DCF and PBZ and the LC-MS detection
was replaced with GC-MS/MS coupled with a derivatisation step for detection of IBP,
KPF and DCF. The advantage of the additional derivatisation step is the ability to
produce two daughter ions for the determination of IBP whereas by LC-MS only
precursor (parent) and one daughter ion for these compounds could be obtained [Vinci et
al., 2006]. This method involves the addition of acetonitrile to bovine milk followed by
clean-up using Isolute™ C18 solid phase extraction (SPE) cartridges and analysis by GC-
MS/MS. To the best of our knowledge there are no methods published for the
determination of IBP, KPF, DCF and PBZ in bovine milk using GC-MS/MS in multiple
reaction monitoring mode.
3.3 Experimental

3.3.1 Materials and reagents

Water, ethanol, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid (37 %), n-hexane and iso-octane (HiPerSolv grade) were obtained from BDH (Merck, UK). Heptafluorobutyric Acid Anhydride was obtained from Pierce, (Pierce, USA). 1,1,1,3,3,3-Hexafluoro-2-propanol was obtained from Sigma (Sigma Aldrich, Ireland). PBZ was purchased from Sigma (Sigma Aldrich, Ireland). d$_{10}$-PBZ was obtained from Cambridge Isotope Labs (Cambridge Isotope Labs, USA). KPF was purchased from Fluka Riedel-de-Haen (Sigma Aldrich, Ireland). IBP and DCF were purchased from Sigma Aldrich (Sigma Aldrich, Ireland). d$_3$-IBP and d$_4$-DCF were obtained from CDN Isotopes (CDN Isotopes, Canada). Primary stock standard solutions of PBZ, KPF, IBP, DCF, d$_{10}$-PBZ, d$_3$-IBP and d$_4$-DCF (stable for 12 months) were prepared in ethanol at a concentration of 1 mg mL$^{-1}$. Intermediate single standards solutions of all analytes (stable...
for 6 months) were prepared in methanol at a concentration of 10 μg mL⁻¹. PBZ, KPF, IBP and DCF standard fortification solution (stable for 6 months) was prepared in methanol at a concentration of 500 ng mL⁻¹ from the 10 μg mL⁻¹ intermediate stock solution. A fortification solution of d₁₀-PBZ, d₃-IBP and d₄-DCF (stable for 6 months) was prepared in methanol at a concentration of 500 ng mL⁻¹ from the 10 μg mL⁻¹ intermediate stock solution. All standards were stored at 4 °C in the dark. Isolute™ endcapped C₁₈ solid phase extraction cartridges (6 mL, 1 g) were obtained from Biotage (Biotage, UK). Methanol:water (10:90, v/v) and 10 mM ascorbic acid were used as solid phase extraction wash solvents. N-hexane:diethyl ether (50:50,v/v) was used as the solid phase extraction elution solvent. Injection solvent was iso-octane.

3.3.2 GC conditions

The GC system consisted of a Varian CP-3800 Gas Chromatograph coupled to a Varian 1200 L Quadrupole MS and a Varian 8400 Autosampler (Varian, CA, USA). The carrier gas was helium at flow of 1.5 mL min⁻¹. IBP, KPF, DCF and PBZ were chromatographed on a column with film thickness of 0.25 μm, diameter 0.25 mm, length 30 m packed with SE-54/CP Sil 8 type material (Restek, Buchs, UK). The injector temperature was maintained at 275 °C and it was a constant temperature splitless programmable temperature vaporising (PTV) injection. The injection volume was 2 μl. The oven temperature was set at 100 °C for 1 min and ramped to 300 °C in increments of 10 °C per minute. The temperature is held at 300 °C is held for 1 min. The runtime is 22 minutes. Data acquisition and integration were performed using Varian MS Workstation (version
6.5) chromatographic management software (Varian, CA, USA). The described GC system was shown to be suitable for the analysis of IBP, KPF, DCF and PBZ (Figures 3-2 to 3-9).

3.3.3 MS/MS parameters

The analysis was performed using EI positive MS/MS with multiple reaction monitoring (MRM) mode. Two transitions per compound were used and the collision voltages were optimised as shown (Table 1). The MS/MS detector conditions were as follows: electron energy, -70 eV; detector voltage, 1950 volts; detector setting, fixed voltage; collision cell pressure, 1.5 mTorr; ion source temperature, 250 °C; transfer line temperature, 290 °C; and the collision gas was argon.

3.3.4 Milk samples

Milk obtained for use as negative controls was separated into 50 mL aliquots and stored at -20 °C. The milk was obtained from local supermarkets and was analysed in previous batches. The milk was found to contain no detectable residues of IBP, KPF, DCF and PBZ and was used as negative controls.

3.3.5 Sample extraction and clean-up

Milk samples (5 ml) were aliquoted into 50 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 15 ng mL⁻¹ by adding a 150 μL portion of a 500 ng mL⁻¹ mix solution of d₃-IBP, d₄-DCF and d₁₀-PBZ. Samples were fortified at levels corresponding to 5, 7.5 and 10 ng mL⁻¹ by adding 50, 75 and 100 μL
portions of a 500 ng mL\textsuperscript{-1} solution of IBP, KPF, DCF and PBZ. After fortification, samples were held for 15 min prior to extraction. Acetonitrile (5 mL) was added and the samples were vortexed (30 sec), centrifuged (3500 rpm, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The sample pellet is re-extracted with 5 mL of acetonitrile and the supernatants were combined. A total of 10 mM ascorbic acid (20 mL) and 1 M hydrochloric acid (0.2 mL) were added to the extracts and the pH of the samples were checked to ensure they were at pH 3 before proceeding to the solid phase extraction (SPE) stage. The sample extracts were purified by SPE using C\textsubscript{18} SPE cartridges. Sample extracts were loaded onto the cartridges (preconditioned with n-hexane:diethyl ether (50:50, v/v) (3 mL), methanol (3 mL) and water (5 mL). The samples were loaded onto cartridges under gravity. The cartridges were washed with 10 mM ascorbic acid (3 mL) and methanol:water (10:90, v/v) (3 mL). The cartridges were dried under vacuum (30 min). The cartridges were eluted with n-hexane:diethyl ether (50:50, v/v) (3 mL). The eluates were reduced to dryness under nitrogen without heat before re-dissolving in 50 \mu L of heptfluorobutyric acid anhydride (HFAA) and 25 \mu L 1,1,1-3,3,3-Hexafluoro-2-propanol (HFPOH) and vortexed (1 min). Samples are capped and placed in an oven at (60 °C, 45 min). Samples were allowed to come to room temperature and the derivatisation reagent is evaporated under a gentle stream of nitrogen with no heat. Samples were reconstituted in 50 \mu L of iso-octane. An aliquot (2 \mu L) is injected onto the GC column.
Table 3.1 MS/MS parameters for determination of IBP, KPF, DCF and PBZ

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition</th>
<th>Scan time (s)</th>
<th>Collision (V)</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBP</td>
<td>356 &gt; 161</td>
<td>0.20</td>
<td>-18</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>356 &gt; 252</td>
<td>0.20</td>
<td>-18</td>
<td>6.0</td>
</tr>
<tr>
<td>d&lt;sub&gt;1&lt;/sub&gt;-IBP</td>
<td>359 &gt; 316</td>
<td>0.20</td>
<td>-18</td>
<td>6.0</td>
</tr>
<tr>
<td>KPF</td>
<td>404 &gt; 327</td>
<td>0.12</td>
<td>-22</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>404 &gt; 181</td>
<td>0.12</td>
<td>-15</td>
<td>11.9</td>
</tr>
<tr>
<td>DCF</td>
<td>214 &gt; 151</td>
<td>0.12</td>
<td>-25</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>214 &gt; 179</td>
<td>0.12</td>
<td>-20</td>
<td>14.5</td>
</tr>
<tr>
<td>d&lt;sub&gt;4&lt;/sub&gt;-DCF</td>
<td>218 &gt; 183</td>
<td>0.12</td>
<td>-20</td>
<td>14.4</td>
</tr>
<tr>
<td>PBZ</td>
<td>308 &gt; 183</td>
<td>0.20</td>
<td>-21</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>308 &gt; 252</td>
<td>0.20</td>
<td>-13</td>
<td>16.1</td>
</tr>
<tr>
<td>d&lt;sub&gt;10&lt;/sub&gt;-PBZ</td>
<td>318 &gt; 193</td>
<td>0.20</td>
<td>-21</td>
<td>16.1</td>
</tr>
</tbody>
</table>
Figure 3-2 Chromatogram of Negative Control Milk fortified at 15 ng.mL⁻¹ with internal standard d₃-IBP
Figure 3-3 Chromatogram of Negative Control Milk fortified with 2 ng.ml\(^{-1}\) of IBP and at 15 ng.ml\(^{-1}\) with internal standard d\(^3\)-IBP
Figure 3-4 Chromatogram of Negative Control Milk fortified at 15 ng.mL$^{-1}$ with internal standard d$_3$-IBP
Figure 3-5 Chromatogram of Negative Control Milk fortified with 2 ng.ml\(^{-1}\) of KPF and at 15 ng.ml\(^{-1}\) with internal standard d\(_3\)-IBP
Figure 3-6 Chromatogram of Negative Control Milk fortified at 15 ng.ml\(^{-1}\) with internal standard \(d_4\)-DCF
Figure 3-7 Chromatogram of Negative Control Milk fortified with 2 ng.ml\(^{-1}\) of DCF and at 15 ng.ml\(^{-1}\) with internal standard \(d_4\)-DCF
Figure 3-8 Chromatogram of Negative Control Milk fortified at 15 ng.ml\(^{-1}\) with internal standard \(d_{10}\)-PBZ
Figure 3-9 Chromatogram of Negative Control Milk fortified with 2 ng.ml\(^{-1}\) of PBZ and at 15 ng.ml\(^{-1}\) with internal standard d\(_{10}\)-PBZ
3.3.6 Matrix-Matched Calibration

Matrix matched calibration curves were prepared and used for quantification. Control milk previously tested and shown to contain no residues was prepared as above (3.3.4). One control milk sample was used for each calibration standard level. Milk samples (5 ml) were aliquoted into 50 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 15 ng mL\(^{-1}\) by adding a 150 µL portion of a 500 ng mL\(^{-1}\) mix solution of \(d_3\)-IBP, \(d_4\)-DCF and \(d_{10}\)-PBZ. Samples were fortified at levels corresponding to 0, 2, 5, 7.5, 10 and 20 ng mL\(^{-1}\) by adding 0, 20, 50, 75, 100 and 200 µL portions of a 500 ng mL\(^{-1}\) standard solution of IBP, KPF, DCF and PBZ. After fortification, samples were held for 15 min prior to the extraction procedure as described above (3.3.5). Calibration curves were prepared by plotting the response factor (peak area analyte/peak area internal standard) as a function of analyte concentration (0 to 20 ng mL\(^{-1}\)) to quantify samples.

3.3.7 Method validation

For estimation of accuracy, blank milk samples were fortified with IBP, KPF, DCF and PBZ at 5, 7.5 and 10 ng mL\(^{-1}\). 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. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility was calculated. The decision limit (CC\(\alpha\)) 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 six replicates at 3 levels. The detection capability (CC\(\alpha\)) was calculated by adding 1.64 times the standard error to the CC\(\beta\).
3.4. Results and Discussion

3.4.1 Preliminary experiments

The GC-MS/MS method was developed to provide confirmatory data for the analysis of bovine milk for IBP, KPF, DCF and PBZ. The MS/MS fragmentation conditions were investigated and collision energies were optimised. For a method to be deemed confirmatory four identification points are required. These identification points can be obtained by monitoring one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion).

IBP, KPF and DCF are derivatised with a mixture of HFAA and HFPOH. The reagents form a derivative with the hydroxyl group of the carboxylic acid on IBP, KPF and DCF. PBZ does not have any functional groups that are easily derivatised. The ions monitored for each analyte generally result from cleavage of the aliphatic chains containing the hexafluoropropyl moiety.

IBP, KPF, DCF and PBZ are chromatographed on a Restex-1 MS column with retention times of 6.1, 11.9, 14.4 and 16.4 min respectively. \( \text{d}_3 \)-IBP is used as I.S for IBP and KPF. \( \text{d}_4 \)-DCF and \( \text{d}_{10} \)-PBZ were used as I.S for DCF and PBZ.

3.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [European Commission 2002] covering specificity, calibration curve
linearity, recovery (accuracy), precision, decision limit (CCα) and detection capability (CCβ).

### 3.4.2.1 Specificity

The technique of GC-MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, a variety of milk samples were fortified with the IBP, KPF, DCF and PBZ and d₃-IBP, d₄-DCF and d₁₀-PBZ and non-fortified samples were also analysed. Additionally samples were fortified with 2.0 ng ml⁻¹ of other NSAIDs which included vedaprofen (VDF), tolfenamic acid (TOLF), mefenamic acid (MEF), flunixin (FLU), oxyphenylbutazone (OXYPHEN) and suxibutazone (SUXI). No interferences were observed in the retention window of IBP, KPF, DCF and PBZ in chromatograms when fortified with these substances.

### 3.4.2.2 Linearity of the response

The linearity of the chromatographic response was tested with matrix matched curves using six calibration points in the concentration range of 0 to 20 ng mL⁻¹. The regression coefficients (r²) for all the calibration curves used in this study were ≥ 0.99.

### 3.4.2.3 Accuracy

The accuracy of the method was determined using bovine milk samples fortified at 5.0, 7.5 and 10.0 ng ml⁻¹. Mean corrected recovery (n = 18) of the analyte, determined in three separate assays (Table 3.2) was between 104 and 112 % for IBP, KPF, DCF and PBZ.
3.4.2.4 Precision

The usefulness of suitable deuterated standards is demonstrated in the excellent repeatability and within-laboratory reproducibility obtained for IBP, DCF and PBZ (Table 3.2). No deuterated analogue is available for KPF so poorer precision was obtained for this analyte. The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5, 7.5 and 10 ng ml⁻¹), for IBP, DCF and PBZ was less than 8%. The RSD values were less than 28% for KPF. Even by applying correction by means of the I.S of the other analytes to KPF a much higher RSD was achieved and this indicates the necessity for the incorporation of a structurally identical isotopically-labelled I.S of KPF in the method. Due to the low precision obtained for KPF without a suitable I.S, the method developed was suitable for qualitative confirmation only.

| Table 3.2 Intra- and inter-assay variation for accuracy of IBP, KPF, DCF and PBZ from bovine milk |

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Percentage RSD</th>
<th>Percentage RSD within-laboratory reproducibility</th>
<th>Percentage corrected recovery</th>
<th>MU (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBP</td>
<td>4.4</td>
<td>5.9</td>
<td>104.1</td>
<td>17.8</td>
</tr>
<tr>
<td>KPF</td>
<td>23.7</td>
<td>28.8</td>
<td>108.3</td>
<td>80.9</td>
</tr>
<tr>
<td>DCF</td>
<td>7.9</td>
<td>9.9</td>
<td>111.6</td>
<td>28.2</td>
</tr>
<tr>
<td>PBZ</td>
<td>4.7</td>
<td>6.7</td>
<td>105.3</td>
<td>20.2</td>
</tr>
</tbody>
</table>

3.4.2.5 CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL.
substances an \( \alpha \) equal to 1% is applied. The detection capability (CC\( \beta \)) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of 1-\( \beta \), were \( \beta = 5\% \). CC\( \alpha \) and CC\( \beta \) were calculated using the intercept (value of the signal, \( y \), were the concentration, \( x \) is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (5.0, 7.5 and 10.0 ng ml\(^{-1} \)). Blank milk was fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng ml\(^{-1} \) set for IBP, KPF, DCF and PBZ. CC\( \alpha \) is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. CC\( \alpha \) values of 0.59, 2.69, 0.90 and 0.70 ng ml\(^{-1} \) were achieved for IBP, KPF, DCF and PBZ. CC\( \beta \) is the concentration corresponding to the signal at CC\( \alpha \) + 1.64 times the standard error of the intercept (i.e the intercept + 3.97 times that standard error of the intercept). CC\( \beta \) values of 1.01, 4.58, 1.54 and 1.19 ng ml\(^{-1} \) were achieved for IBP, KPF, DCF and PBZ.

Table 3.3 Calculated CC\( \alpha \) and CC\( \beta \) values based on data from Assay 1, 2 and 3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>CC( \alpha ) (ng ml(^{-1} ))</th>
<th>CC( \beta ) (ng ml(^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBP</td>
<td>0.59</td>
<td>1.01</td>
</tr>
<tr>
<td>KPF</td>
<td>2.69</td>
<td>4.58</td>
</tr>
<tr>
<td>DCF</td>
<td>0.90</td>
<td>1.54</td>
</tr>
<tr>
<td>PBZ</td>
<td>0.70</td>
<td>1.19</td>
</tr>
</tbody>
</table>

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3.4.2.6 Measurement Uncertainty

According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [SANCO 2004]. For the calculation of the extended uncertainty a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CCα, corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different milk sourced from different animals it was decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of the method was estimated at 17.8, 80.9, 28.2 and 20.2%. This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

3.4.2.7 Evaluation

The confirmatory method developed in this study has been used to confirm the presence of IBP, KPF, DCF and PBZ in bovine milk in the National Monitoring Plan in Ireland in 2006, 2007 and 2008. In routine monitoring for these substances at our laboratory, it was possible to detect the precursor ion and two daughter ions (at 2 ng ml⁻¹) in multiple reaction monitoring mode. Furthermore the relative retention time versus internal standard requirement and the product ion ratio requirement was also met. The method has been carried out by different analysts over a period of three years under varying environmental conditions and the method was shown to be robust. The method was
accredited by the Irish National Accreditation Board in 2006 according to ISO17025 standard used by testing and calibration laboratories.

3.5 Conclusions

A relatively fast, simple and selective GC-MS/MS method for the detection of IBP, KPF, DCF and PBZ in bovine milk has been developed. There is no published confirmatory method for the simultaneous determination of IBP, KPF, DCF and PBZ in bovine milk by GC-MS/MS that is validated according to Commission Decision 2002/657/EC [Commission Decision 2002]. Recently it was proposed by Community Reference Laboratories (CRLs) in Europe that laboratories should be capable of monitoring for KPF, DCF and PBZ in bovine milk at a level of 5 ng ml\(^{-1}\) and IBP at a level of 10 ng ml\(^{-1}\) in EU member states [SANCO 2007]. This study shows that the required sensitivity was achieved for all compounds that easily meet the proposed levels. The method performs very well in terms of accuracy and within-laboratory reproducibility. For KPF improvements can be made if a suitable deuterated analogue becomes available.

The objective of the work to develop, validate and accreditate a method for these residues in bovine milk at low ng ml\(^{-1}\) levels and validate according to the requirements in Commission Decision 2002/657/EC therefore has been achieved successfully.

3.6 Acknowledgements

The authors would like to thank staff at The State Laboratory, Ireland and The Istituto Zooprofilatico Sperimentale del Mezzogiorno, Italy for their practical assistance.
Chapter 4: Confirmatory analysis of firocoxib in bovine milk by rapid resolution liquid chromatography tandem mass spectrometry

Published in *Journal of Chromatography B*, 877, 2009, Pages 541-546
4.1 Abstract

A rapid method has been developed to analyse for firocoxib (FIRO) residue in bovine milk. Milk samples were extracted with acetonitrile and sample extracts were purified on Evolute™ ABN solid phase extraction cartridges. Aliquots were analysed by rapid resolution liquid chromatography tandem mass spectrometry (RRLC-MS/MS). The method was validated in bovine milk, according to the criteria defined in Commission Decision 2002/657/EC. The decision limit (CCα) was 1.18 ng/mL and for the detection capability a (CCβ) value of 2.02 ng/mL was obtained. The measurement uncertainty of the method was 27%. Fortifying bovine milk samples (n = 18) in three separate assays, show the accuracy of the method to be between 96 and 105%. The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5, 7.5 and 10 ng/mL) was less than 11% respectively.

Keywords: Firocoxib; Bovine Milk; Method Validation; Decision Limit; Detection Capability; Mass Spectrometry
4.2 Introduction

Firocoxib (3-cyclopropymethoxy-5,5-dimethyl-4-[4-(methyl sulfonyl) phenyl]-2-(5H)-furanone), (FIRO) is a non-steroidal anti-inflammatory drug (NSAID). The molecular structure of this compound is shown in Fig. 4.1.

![Figure 4.1 Structure of Firocoxib](image)

NSAIDs are used widely in veterinary medicine in the treatment of food producing animals. FIRO gives therapeutic efficacy due to inhibition of prostaglandin synthesis via selective binding to the type II cyclooxygenase (COX-2) isoenzyme [McCann et al., 2004, McCann et al., 2005]. A survey involving 2000 veterinarians reported that 93 % of veterinarians use NSAIDs in food producing animals and dairy practitioners reported the most frequent use [US Code 1988]. Overall NSAIDs are an important group of compounds which are routinely used for the treatment of food producing animals [Kopcha et al., 1992]. A survey in 1995 reported that NSAIDs were the second most prescribed class of drugs after microbial for dairy practitioners [Sundlof et al., 1995]. In 2008 a study reported the increased incidence of residue violations for NSAIDs in cattle [Smith et al., 2008] in the past 10 years. According to EU law, all substances for veterinary use need to be included in Annexes 1-3 of Regulation 2377/90 [European
Commission 1990]. This regulation establishes lists of compounds that have a fixed MRL (Annex I), that need no MRL (Annex II) or that have a provisional MRL (Annex III).

FIRO is a compound that has been included in Annex I and has a maximum residue limit (MRL) established only in equine tissues. Substances that have no MRL established are prohibited for use in food producing animals. FIRO has no MRL established in bovine species. It is anticipated that due to the large increase in NSAID use in recent years that this substance may be used to treat food producing animals other than equines. Off label application of veterinary drug compounds to cows that produce milk for human consumption in the Republic of Ireland and the European Union is illegal. In Ireland in 2007, Ivermectin, a veterinary drug which is licensed in liver, kidney and fat of all mammalian food producing species but not authorised in animals that produce milk for human consumption was found in milk by the National Reference Laboratory for Avermectins in Ireland. The finding of this substance in milk is illegal within the EU.

FIRO is a newly licensed NSAID in horses [EMEA 38346206] and has become available on the market under the trade name Previcox since 2007 [EMEA 082] and Equioxx since 2008 [EMEA V-142]. FIRO cannot be used in mares in which milk is intended for human consumption. FIRO has been shown to be comparable in efficacy to meloxicam and carprofen [EMEA 082] and also been shown to be comparable in efficacy to phenylbutazone [Doucet et al., 2008]. In the case of carprofen and meloxicam these substances are licensed for use in horses and cattle, therefore it cannot be excluded that FIRO would not be used in cattle. As in the case of ivermectin, there is a need to anticipate the requirements of the future where risks could occur due to the administration of FIRO to milk producing species. Therefore the development of an
analytical method at the National Reference Laboratory for NSAIDs in Ireland was undertaken to provide an analytical tool to monitor for this substance. Long-term exposure to NSAIDs has caused kidney tumors in mice and liver tumors in rats [Kari et al., 1995].

It has been reported in recent years that the COX-II inhibitor class of NSAIDs of which FIRO is a member has been implicated in cardiovascular harm in humans [Van Staa et al. 2008, Debabrata et al., 2008]. FIRO shows the same undesirable side effects [EMEA 082] as other NSAIDs (diarrhea, mouth lesions and lethargy) therefore monitoring of its illegal use in milk producing animals is important for consumer protection. There are very limited methods for the determination of FIRO in food producing animals and no methods for the determination of this substance in animal products.

Plasma of dogs and horses have been diluted with water and samples were purified using Waters™ HLB solid phase extraction cartridges and analysed by LC-UV [Kvaternick et al., 2007]. Urine and plasma from dogs and horses was diluted with an aqueous solution of 5% acetic acid and passed through a Waters Oasis HLB™ 96-well solid phase extraction plate and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) [Letendre et al., 2007].

This method involves the addition of acetonitrile to bovine milk followed by clean-up using Evolute™ ABN solid phase extraction (SPE) cartridges and analysis by RRLC-MS/MS. To the best of our knowledge there are no methods published for the determination of FIRO in bovine milk. The objective of this study was to develop and validate a rugged, sensitive, selective and efficient method for the analysis of FIRO in bovine milk for implementation into the National Programme in the Republic of Ireland.
4.3 Experimental

4.3.1. Materials and methods

Water, ethanol, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid (37%), n-hexane and iso-octane (HiPerSolv grade) were obtained from BDH (Merck, UK). FIRO was given as a gift from Merial (Saint-Vulbas, France). Primary stock standard solution of FIRO (stable for 12 months was prepared in ethanol at a concentration of 1 mg/mL. Intermediate single standard solution of FIRO (stable for 6 months as standard injected throughout 6 months period gave similar results) was prepared in methanol at a concentration of 10 μg/mL. FIRO standard fortification solution (stable for 6 months as standard injected throughout 6 month period gave similar results) was prepared in methanol at a concentration of 500 ng/mL from the 10 μg/mL intermediate stock solution. All standards were stored at 4 °C in the dark. Isolute™ Evolute ABN 50 μm solid phase extraction cartridges (10 mL, 100 mg) were obtained from Biotage (Biotage, UK). Methanol:water (10:90, v/v) and 10 mM ascorbic acid were used as solid phase extraction wash solvents. N-hexane:diethyl ether (50:50,v/v) was used as the solid phase extraction elution solvent. Injection solvent was water:acetonitrile (90:10, v/v).
4.3.2 LC conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). FIRO was chromatographed on a 1.8 μm Agilent Eclipse Plus C₁₈ column (2.1 x 50 mm) (Agilent, Ireland) and the column temperature was maintained at 55 °C. A gradient was applied with water and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B). The flow rate throughout the chromatographic analysis was 0.75 mL/min and the following gradient was applied: 0 min, 90% A; 0.4 min, 90% A; 1.0 min, 85% A; 3.1 min, 80% B; and 4.7 min, 90% A. The column was regenerated for 1.8 min before injection (Table 4.1). The total run time was 6.5 minutes. The injection volume was 15 μL. The mass spectrometer used was a QTRAP 4000 with a TurboIonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.4.2 of Analyst software. The described LC-MS/MS system was shown to be suitable for the analysis of FIRO (Figure 4.2-4.3).
Figure 4.2 Chromatogram of negative control bovine milk (A) and negative control bovine milk fortified with 2 ng/mL of FIRO (B)
Figure 4-3. Chromatogram of negative control bovine milk (A) and negative control bovine milk fortified with 2 ng/mL of FIRO
4.3.3 MS/MS parameters

The analysis was performed using positive ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. Two transitions were used and the collision voltages were optimised as shown (Table 4.2). Each transition was performed with a 13 ms dwell time and a pause time of 3 ms. The MS/MS detector conditions were as follows: Ion mode electrospray positive; curtain gas 45 psi; ion spray voltage 4400 V; temperature 650 °C; ion source gas 1 70 psi; ions source gas 2 70 psi; Interface heater on; entrance potential 10 V; Resolution Q1 unit; Resolution Q3 unit; CAD gas =high.

Table 4.1 LC gradient profile for determination of FIRO.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Component A (%)</th>
<th>Component B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>0.4</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>3.1</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>4.1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>4.7</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>6.5</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Component A: Component A: water containing 0.001 M acetic acid + acetonitrile (90 + 10, v/v)
Component B: Acetonitrile

Table 4.2 MS/MS parameters for the determination of FIRO.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition</th>
<th>Declustering potential [V]</th>
<th>Collision [V]</th>
<th>CKP [V]</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIRO</td>
<td>337.2&gt;213.2 (strong)</td>
<td>71.24</td>
<td>13</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>337.2&gt;237.0 (weak)</td>
<td>71.24</td>
<td>23</td>
<td>16</td>
</tr>
</tbody>
</table>
4.3.4 Milk samples

Untreated milk from 8 individual cows was obtained by veterinary inspectors and milk (5 different brands of whole milk) obtained from a local supermarket were used as negative controls. The milk was analysed separately and no detectable residues of FIRO were found. Milk samples previously analysed were pooled together and separated into 50 mL aliquots and stored at –20 °C and used as negative controls in the experiments.

4.3.5 Sample extraction and clean-up

Milk samples (5 ml) were aliquoted into 50 ml polypropylene tubes. Samples were fortified at levels corresponding to 5, 7.5 and 10 ng/mL by adding 50, 75 and 100 μL portions of a 500 ng/mL solution of FIRO. After fortification, samples were held for 15 min prior to extraction. Acetonitrile (5 mL) was added and the samples were vortexed (30 sec), centrifuged (3500 rpm, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The sample pellet is re-extracted with 5 mL of acetonitrile and the supernatants are combined. 10 mM ascorbic acid (20 mL) and 1 M hydrochloric acid (0.2 mL) were added to the extracts and the pH of the samples were checked to ensure they were at pH 3 before proceeding to the solid phase extraction (SPE) stage. The sample extracts were purified by SPE using Evolute ABN™ SPE cartridges. Sample extracts were loaded onto the cartridges (preconditioned with methanol (3 mL) and ascorbic acid (3 mL)). The samples were loaded onto cartridges under gravity. The cartridges were washed with methanol:water (10:90, v/v) (2 mL). The cartridges were dried under
vacuum (15 min). The cartridges were eluted with n-hexane:diethyl ether (50:50, v/v) (2 \times 2 \text{ mL}). The eluates were reduced to dryness under nitrogen without heat before redissolving in 150 \text{ µL} water:acetonitrile (90:10, v/v) and vortexed (1 min). An aliquot (15 \text{ µL}) is injected on the LC column.

4.3.6 Matrix matched calibration

Matrix matched calibration curves were prepared and used for quantification. Control milk previously tested and shown to contain no residues was prepared as above (4.3.4). One control milk sample was used for each calibration standard level. Milk samples (5 ml) were aliquoted into 50 ml polypropylene tubes. Samples were fortified at levels corresponding to 0, 2, 5, 7.5, 10 and 20 ng/mL by adding 0, 20, 50, 75, 100 and 200 \text{ µL} portions of a 500 ng/mL standard solution of FIRO. After fortification, samples were held for 15 min prior to the extraction procedure as described above (4.3.5). Calibration curves were prepared by plotting the peak area as a function of analyte concentration (0 to 20 ng/mL) to quantify samples.

4.3.7 Method validation

For estimation of accuracy, blank milk samples were fortified with FIRO at 5, 7.5 and 10 ng/mL. 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. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility was calculated. 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. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα.

4.4 Results and Discussion

4.4.1 Preliminary experiments

The LC-MS/MS method was developed to provide confirmatory data for the analysis of bovine milk for FIRO. The MS/MS fragmentation conditions were investigated and collision energies were optimised. For a method to be deemed confirmatory four identification points are required. These identification points can be obtained by monitoring one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion).

FIRO was chromatographed on a 1.8 µm Agilent Eclipse Plus C18 column with retention time of 2.57 min.

4.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [Commission Decision 2002] covering specificity, calibration curve linearity, recovery (accuracy), precision, decision limit (CCα) and detection capability (CCβ).
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, a variety of milk samples were fortified with the FIRO and non-fortified samples were also analysed. No interfering peaks were observed at the retention time for FIRO (Figure 4.2-4.3). Additionally samples were fortified with 2.0 ng/mL of other NSAIDs which included flunixin (FLU), carprofen (CPF), meloxicam (MLX), oxyphenylbutazone (OXYPHEN) and diclofenac (DCF). No interferences were observed in the retention window of FIRO in chromatograms when fortified with these substances.

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 ng/mL. The regression coefficients ($r^2$) for all the calibration curves used in this study were ≥ 0.99.

4.4.2.3 Accuracy

The accuracy of the method was determined using bovine milk samples fortified at 5.0, 7.5 and 10.0 ng/mL. Mean corrected recovery ($n = 18$) of the analyte, determined in three separate assays (Table 3) was between 96 and 105%.

4.4.2.4 Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5, 7.5 and 10 ng/mL) was less than 11% (Table 4.3).
Table 4.3 Intra- and inter-assay variation for recovery of FIRO from milk

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fortification level (ng/mL⁻¹)</th>
<th>Recovery (%)</th>
<th>Within Run CV (%)</th>
<th>Between Run CV (%)</th>
<th>Total CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIRO</td>
<td>5</td>
<td>104.4</td>
<td>2.0</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>98.3</td>
<td>5.8</td>
<td>7.5</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>105.2</td>
<td>6.6</td>
<td>5.2</td>
<td>10.4</td>
</tr>
<tr>
<td>Combined variance</td>
<td>5, 7.5, 10</td>
<td></td>
<td></td>
<td></td>
<td>9.1</td>
</tr>
</tbody>
</table>

4.4.2.5 *CCα and CCβ*

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1 % is applied. The detection capability (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, were β = 5 %. CCα and CCβ were calculated using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (5.0, 7.5 and 10.0 ng/mL). Blank milk was fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng/mL set for FIRO. CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. CCα value of 1.18 ng/mL was achieved for FIRO. 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 value of 2.02 ng/mL was achieved for FIRO.
4.4.2.6 Measurement Uncertainty

According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [SANCO 2004]. For the calculation of the extended uncertainty a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the $CC_\alpha$, corresponding to a confidence level of 99 %. As the only source of variation during the validation was the different days and different milk sourced from different animals it was decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of the method was estimated at 27 %. This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

4.5 Conclusions

A relatively fast, simple, sensitive and selective RRLC-MS/MS method for the detection of FIRO in bovine milk has been developed. There is no published confirmatory method for the determination of FIRO in bovine milk that is validated according to Commission Decision 2002/657/EC [Commission Decision 2002]. This is the first time that milk extracts have been purified using Evolute™ ABN solid phase extraction cartridges for the determination of FIRO and the first time that FIRO has been analysed using RRLC-MS/MS. The method performs very well in terms of accuracy and within-laboratory reproducibility. In routine monitoring for this substance at our laboratory in 2008 it was possible to detect the precursor ion and two daughter ions (at 2 ng/mL) in multiple
reaction monitoring mode in the lowest standard in the matrix matched curve. Furthermore the product ion ratio requirement was also met. The method meets the requirements for a confirmatory method according to 2002/657/EC. The method has been carried out by different analysts under varying environmental conditions and the method was shown to be robust.

The objective of the work to develop and validate a method for this residue in bovine milk and incorporate into the National Monitoring Programme in Ireland at low ng/mL levels and validate according to the requirements in Commission Decision 2002/657/EC therefore has been achieved successfully.

4.6 ACKNOWLEDGEMENTS

The authors would like to thank staff at The State Laboratory, Ireland for their practical assistance and Merial Pharmaceuticals for the gift of the Firocoxib analytical standard.
Chapter 5: Rapid confirmatory analysis of non-steroidal anti-inflammatory drugs in bovine milk by rapid resolution liquid chromatography tandem mass spectrometry

Published in Journal of Chromatography A, 1216, 2009, Pages 8117-8131
5.1 Abstract

A rapid method has been developed to analyse carprofen (CPF), diclofenac (DCF), mefenamic acid (MFN), niflumic acid (NIFLU), naproxen (NAP), oxyphenylbutazone (OXYPHEN), phenylbutazone (PBZ) and suxibuzone (SUXI) residues in bovine milk. Milk samples are extracted with acetonitrile and sample extracts were purified on Evolute™ ABN solid phase extraction cartridges. Aliquots were analysed by rapid resolution liquid chromatography tandem mass spectrometry (RRLC-MS/MS) with a runtime of 6.5 min. The method was validated in bovine milk, according to the criteria defined in Commission Decision 2002/657/EC. CCα values of 0.46, 1.08, 0.92, 1.26, 1.29, 2.12, 0.55 and 2.86 ng mL⁻¹ were determined for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI respectively. CCβ values of 0.79, 1.85, 1.56, 2.15, 2.19, 3.62, 0.94 and 4.87 ng mL⁻¹ were determined for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI respectively. The measurement uncertainty of the method was estimated at 9, 28, 28, 45, 46, 45, 10 and 39 % for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI. Fortifying bovine milk samples (n = 18) in three separate assays, show the accuracy of the method to be between 82 and 108 %. The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5, 7.5 and 10 ng mL⁻¹) was less than 16 % respectively. The advantage of the method is that low ng mL⁻¹ levels can be detected and quantitatively confirmed rapidly in milk and that 3 batches of samples can be analysed within a single day using RRLC-MS/MS with a runtime of 6.5 minutes.
5.2 Introduction

Carprofen (CPF), diclofenac (DCF), mefenamic acid (MFN), niflumic acid (NIFLU), naproxen (NAP), oxyphenylbutazone (OXYPHEN), phenylbutazone (PBZ) and suxibuzone (SUXI) are non-steroidal anti-inflammatory drugs (NSAIDs). The molecular structure of these compounds is shown in Fig. 5-1.

**Structure a: Carprofen**

![Structure a: Carprofen](image)

**Structure b: Diclofenac**

![Structure b: Diclofenac](image)
Structure c: Mefenamic Acid

Structure d: Naproxen

Structure e: Niflumic Acid
Structure f: Oxyphenylbutazone

Structure g: Phenylbutazone
Fig. 5-1 Structures of the NSAIDs

Depending on their heterogeneous molecular structures, NSAIDs can be classified into six main subclasses, sharing common pharmacological activity: (a) salicylic acid derivatives; (b) propionic acid derivatives; (c) pyrazole derivatives; (d) aniline derivatives; (e) oxicam derivatives and (f) COX II inhibitors. According to EU law, all substances for veterinary use need to be included in Annexes 1-3 of Regulation 2377/90 [European Commission 1990]. This regulation establishes lists of compounds that have a fixed maximum residue limit, MRL (Annex I), that need no MRL (Annex II) or that have a provisional MRL (III). In milk CPF has been included in Annex II of the regulation only for bovine milk [European Commission 2005] and there is no MRL level for this but it was still included in this study. DCF is not authorised for use in animals that produce milk for human consumption [European Commission 2004]. No MRL is established for
MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI and are considered as prohibited substances. The European Council recommend a stringent control of NSAIDs in food producing animals [SANCO 2000] because of the potential teratogenic and carcinogenic effects due to the considerable use of these drugs. NSAIDs can cause other adverse health effects in humans such as aplastic anaemia, gastrointestinal disorders and agranulocytosis [Insel 1990] and changes in renal function [Goodman and Gilman 1992]. Longterm exposure to PBZ has caused kidney tumors in mice and liver tumors in rats [Kari et al., 1995]. NSAIDs are widely used in veterinary medicine in the treatment of food producing animals. In 2008, a study reported the increased incidence of residue violations for NSAIDs in cattle [Smith et al., 2008] in the past 10 years. A survey in 1995 reported that NSAIDs were the second most prescribed class of drugs after microbials for dairy practitioners [Sundlof et al., 1995]. A survey involving 2000 veterinarians reported that 93% of veterinarians use NSAIDs in food producing animals and dairy practitioners reported the most frequent use [US Code 1988]. NSAIDs are an important group of compounds which are routinely used for the treatment of food producing animals [Kopcha et al., 1992]. The widespread use of NSAIDs presents a potential risk to the consumer if food containing residues enter the food chain so there is a need for the development of methods to monitor compliance with legislation in the EU in a variety of animal tissues and products. Milk is an important matrix for residue control. In Ireland milk is one of the target matrices chosen to identify the misuse of NSAIDs in animal production. There are few analytical methods for the determination of NSAIDs in milk and those that have been described use high-performance liquid chromatography (HPLC) [De Graves et al., 1993, De Graves et al., 1996, De Veau et al., 1998, Musser et al., 1998,
De Veau 1996, Martin et al., 1983, Gallo et al., 2008], Liquid chromatography/mass spectrometry [Gallo et al., 2008, Feely et al., 2002, Boner et al., 2003, Daeseleire et al., 2003, Stolker et al., 2008, Malone et al., 2009] and gas chromatography/mass spectrometry [Rubb et al., 1995, Dowling et al., 2008]. Most of the detection methods focused on just one or a few NSAID substances. However some multi-residue methods exist. A method developed by Gallo et al [18] is capable of analysing for 16 NSAIDs in milk using two separate analytical techniques and involves a screening LC-DAD method with limits of detection between 2 and 15 ng mL\(^{-1}\) and a runtime of 35 min with an equilibration time of 15 min per injection followed by confirmation using a LC ESI-Iontrap- MS/MS method with an LOD of 5 ng mL\(^{-1}\) except for flurbiprofen and a runtime of 40 min per injection. The LC-MS method does not meet the requirements for a confirmatory method according to Commission Decision 2002/657/EC [Commission Decision 2002/657/EC] and a third analytical technique is required. A method by Stolker et al. [Stolker et al., 2008] is capable of analysing 20 NSAIDs in milk using a quantitative screening method (UPLC-TOF-MS) with LOD’s for specific NSAIDs such as NAP, PBZ and DCF at 12.5, 25 and 6.3 ng mL\(^{-1}\) with a runtime of 8.5 min. The method cannot meet the minimum level for analysis of NSAIDs in milk set at 5 ng mL\(^{-1}\) for these substances and furthermore the analysis by TOF-MS, medium to high resolution of approximately 10,000 FWHM is not included in Commission Decision 2002/657/EC [Commission Decision 2002]. Therefore a second analytical technique is required. Other LC-MS/MS methods available analyse between 1 and 4 NSAIDs residues in milk [Feely et al., 2002, Boner et al., 2003, Daeseleire et al., 2003, Malone et al., 2009] with runtimes of 17, 20 and 20 and 33 min with detection limits ranging from 0 to 5 ng mL\(^{-1}\) [Feely et al., 2002, 2003, 2008].
Although some NSAIDs are licensed for use in milk, this study in milk concentrated on the detection of those NSAIDs that are prohibited for use (except carprofen) as there is a lack of available analytical methods. In Ireland in 2007, ivermectin, a veterinary drug which is licensed in liver, kidney and fat of all mammalian food producing species but not authorised in animals that produce milk for human consumption was found in milk by the National Reference Laboratory for Avermectins in Ireland. The presence of this substance in milk is illegal. The development of a rapid analytical method at the National Reference Laboratory for NSAIDs in Ireland was undertaken to provide an analytical tool to monitor for non-authorised substances in bovine milk. The NSAIDs selected in this study were chosen to allow a method to be developed for the analysis of a wide variety of non-authorised drugs from different NSAID sub-classes (b-d). The newly developed water wettable polymer based Evolute™ ABN (acidic basic neutral) solid phase extraction (SPE) sorbent from Biotage was selected due to its drying properties and the potential for monitoring structurally diverse substances with a single cartridge chemistry. The new technology of Rapid Resolution Liquid Chromatography (RRLC, trade name given by Agilent Technologies) was selected as can obtain rapid analytical results, speed up method development times and utilise less solvent. This is the first time that milk extracts have been purified for the determination of the tested NSAIDs residues using Evolute™ ABN solid phase extraction (SPE) cartridges and the first time that the NSAIDs chosen have been analysed using RRLC-MS/MS to the best of our knowledge at levels below 5 ng mL⁻¹ for all substances tested. The advantages of the developed
method are that it is a rapid quantitative confirmatory method that meets the requirements according to Commission Decision 2002/657/EC [Commission Decision 2002] for a broad number of NSAIDs using a single analytical technique. Also the developed method is the most sensitive available to the best of our knowledge and the theoretical values calculated for the CCα according to 11843 calibration curve procedure were shown experimentally to be valid by spiking milk at levels below the CCα values calculated to illustrate the sensitivity [CRL 2007]. Furthermore the method represents savings in time and consumables (solvents) over existing methodologies. The objective of this work was to develop a method that is capable of the determination of NSAIDs residues in bovine milk below 5 ng mL⁻¹, a level set in accordance to requirements of the Community Reference Laboratories in the EU [SANCO 2004] and to validate the method according to the guidelines of 2002/657/EC [Commission Decision 2002, CRL 2007]. In this paper a fast, simple and reliable RRLC-MS/MS method is described for a wide range of NSAIDS in milk. The proposed method does not cover the glucuronides.

5.3 Experimental

5.3.1 Materials and reagents

Water, ethanol, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid (37%), n-hexane and iso-octane (HiPerSolv grade) were obtained from BDH (Merck, UK). CPF, DCF, MFN, NIFLU, NAP and PBZ were purchased from Sigma (Sigma-Aldrich, Ireland). OXYPHEN and SUXI were obtained as a gift from Istituto Zooprofilatico Sperimentale del Mezzogiorno (Naples, Italy). d₁₀-PBZ was obtained from Cambridge Isotope Labs (Cambridge Isotope Labs, USA). d₃-CPF and d₄-DCF were obtained from
CDN Isotopes (CDN Isotopes, Canada). \(d_3\)-TLF was obtained as a gift from Agri-Food & Biosciences Institute (Belfast, UK). Primary stock standard solutions (stable for 12 months) were prepared in ethanol at a concentration of 1 mg mL\(^{-1}\). Intermediate single standard solutions (stable for 6 months) were prepared in methanol at a concentration of 10 \(\mu\)g mL\(^{-1}\). CPF, DCF, MFN, NIFLU, NAP, OXYPHEN PBZ and SUXI standard fortification solution (stable for 6 months) was prepared in methanol at a concentration of 500 ng mL\(^{-1}\) from the 10 \(\mu\)g mL\(^{-1}\) intermediate stock solution. Internal standard fortification solution of \(d_3\)-CPF, \(d_4\)-DCF, \(d_3\)-TLF and \(d_{10}\)-PBZ was prepared at a concentration of 500 ng mL\(^{-1}\). All standards were stored at 4 °C in the dark. Evolute™ ABN 50 \(\mu\)m solid phase extraction cartridges (10 mL, 100 mg) were obtained from Biotage (Biotage, UK). Methanol:water (10:90, v/v) and 10 mM ascorbic acid (used as a stabiliser) were used as solid phase extraction wash solvents. N-hexane:diethyl ether (50:50, v/v) was used as the solid phase extraction elution solvent. Injection solvent was water:acetonitrile (90:10, v/v).

5.3.2 LC conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). The NSAIDs were chromatographed on a 1.8 \(\mu\)m Agilent Eclipse Plus C\(_{18}\) column (2.1 x 50 mm) (Agilent, Ireland) and the column temperature was maintained at 55 °C. A gradient was applied with water and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B) (Table 5.1). The total run time was 6.5 minutes. The injection volume was 15 \(\mu\)L. The mass spectrometer used was a QTRAP 4000 with a
TurboIonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.4.2 of Analyst software. The described LC-MS/MS system was shown to be suitable for the analysis of NSAIDs (Figure 5.2-5.11).

5.3.3 MS/MS parameters

The analysis was performed using negative ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. Two transitions were used and the collision energy was optimised as shown (Table 2). Each transition was performed with a 13 mSec dwell time and a pause time of 3 mSec. The MS/MS detector conditions were as follows: ion mode electrospray negative; curtain gas 45 psi; ion spray voltage 4400 V; temperature 650 °C; ion source gas 1 70 psi; ion source gas 2 70 psi; interface heater on; entrance potential 10 V; resolution Q1 unit; resolution Q2 unit; collision -activated dissociation CAD gas =high

Table 5.1 LC gradient profile for determination of CPF, DCF, MFN, PBZ, NAP, NIFLU, OXYPHEN and SUXI

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Component A (%)</th>
<th>Component B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
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<tr>
<td>0.4</td>
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<tr>
<td>4.7</td>
<td>20</td>
<td>60</td>
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<tr>
<td>5.5</td>
<td>10</td>
<td>90</td>
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Component A: water containing 0.001 M acetic acid + acetonitrile (90 + 10, v/v)
Component B: Acetonitrile
Table 5.2: MS/MS parameters for determination of CPF, DCF, MFN, PBZ, NAP, NIFLU, OXYPHEN, and SUXI

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Transition</th>
<th>Declustering potential (V)</th>
<th>Collision energy (eV)</th>
<th>Collision cell exit potential (V)</th>
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</thead>
<tbody>
<tr>
<td>CPF</td>
<td>3.2</td>
<td>271.9&gt;277.9 (strong)</td>
<td>-60</td>
<td>-11</td>
<td>-17</td>
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<tr>
<td></td>
<td></td>
<td>271.9&gt;225.8 (weak)</td>
<td>-60</td>
<td>-11</td>
<td>-17</td>
</tr>
<tr>
<td>DCF</td>
<td>3.3</td>
<td>294.0&gt;250.0 (strong)</td>
<td>-70</td>
<td>-10</td>
<td>-15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>294.0&gt;165.0 (weak)</td>
<td>-70</td>
<td>-10</td>
<td>-15</td>
</tr>
<tr>
<td>MFN</td>
<td>3.5</td>
<td>230.8&gt;185.0 (strong)</td>
<td>-60</td>
<td>-11</td>
<td>-17</td>
</tr>
<tr>
<td>PBZ</td>
<td>3.5</td>
<td>305.0&gt;250.0 (strong)</td>
<td>-60</td>
<td>-11</td>
<td>-17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>305.0&gt;131.0 (weak)</td>
<td>-70</td>
<td>-16</td>
<td>-16</td>
</tr>
<tr>
<td>OXYPHEN</td>
<td>4.2</td>
<td>222.0&gt;295.0 (strong)</td>
<td>-94</td>
<td>-7</td>
<td>-9</td>
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<tr>
<td></td>
<td></td>
<td>222.0&gt;133.0 (strong)</td>
<td>-94</td>
<td>-7</td>
<td>-9</td>
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<tr>
<td>SUXI</td>
<td>3.3</td>
<td>437.0&gt;301.1 (strong)</td>
<td>-45</td>
<td>-7</td>
<td>-7</td>
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<tr>
<td></td>
<td></td>
<td>437.0&gt;130.8 (weak)</td>
<td>-45</td>
<td>-7</td>
<td>-7</td>
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</tbody>
</table>

continued

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Transition</th>
<th>Declustering potential (V)</th>
<th>Collision energy (eV)</th>
<th>Collision cell exit potential (V)</th>
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<td>NIFLU</td>
<td>3.4</td>
<td>200.0&gt;237.0 (strong)</td>
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<td>-13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200.0&gt;176.0 (weak)</td>
<td>-85</td>
<td>-13</td>
<td>-13</td>
</tr>
<tr>
<td>NAP</td>
<td>2.0</td>
<td>230.0&gt;185.0 (strong)</td>
<td>-45.5</td>
<td>-10</td>
<td>-10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250.0&gt;170.0 (strong)</td>
<td>-45.5</td>
<td>-10</td>
<td>-10</td>
</tr>
<tr>
<td>d₃-PBZ</td>
<td>3.5</td>
<td>316.0&gt;310</td>
<td>-70</td>
<td>-16</td>
<td>-16</td>
</tr>
<tr>
<td>d₄-CFF</td>
<td>3.2</td>
<td>274.0&gt;230.0</td>
<td>-69</td>
<td>-16</td>
<td>-16</td>
</tr>
<tr>
<td>d₄-DCF</td>
<td>3.3</td>
<td>258.0&gt;254</td>
<td>-70</td>
<td>-15</td>
<td>-15</td>
</tr>
<tr>
<td>d₅-TLF</td>
<td>3.6</td>
<td>364&gt;220</td>
<td>-60</td>
<td>-15</td>
<td>-5</td>
</tr>
</tbody>
</table>

Note: Matrix matched curves were used for quantification of all compounds.

d₃-CPF was used as internal standard (I.S) for CPF, d₄-DCF was used as internal standard I.S for DCF, d₁₀-PBZ was used as I.S for PBZ, d₅- TLF was used as I.S for MFN. No I.S was used for remaining analytes.
5.3.4 Milk samples

Milk obtained for use as negative controls were separated into 50 mL aliquots and stored at -20 °C. The milk was analysed in previous batches and milk found to contain no detectable residues of NSAIDs were used as negative controls.

5.3.5 Sample extraction and clean-up

Milk samples (5 ml) were aliquoted into 50 ml polypropylene tubes. The milk aliquots (5 mL) were fortified with internal standard at levels corresponding to 15 ng mL⁻¹ by adding a 150 µL portion of a 500 ng mL⁻¹ mix solution of d3-CPF, d4-DCF, d3-TLF and d10-PBZ. Samples were fortified at levels corresponding to 5, 7.5 and 10 ng mL⁻¹ by adding 50, 75 and 100 µL portions of a 500 ng mL⁻¹ solution of CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI. After fortification, samples were held for 15 min prior to extraction. Acetonitrile (5 mL) was added and the samples were vortexed (30 sec), centrifuged (3568 x g, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The sample pellet is re-extracted with 5 mL of acetonitrile and the supernatants are combined. 10 mM ascorbic acid (20 mL) and 1 M hydrochloric acid (0.2 mL) were added to the extracts and the pH of the samples were checked to ensure they were at pH 3 before proceeding to the solid phase extraction (SPE) stage. The sample extracts were purified by SPE using Evolute™ ABN SPE cartridges. Sample extracts were loaded onto the cartridges (preconditioned with methanol (3 mL) and ascorbic acid (3 mL). The samples were loaded onto cartridges under gravity. The cartridges were washed with methanol:water (10:90, v/v) (2 mL). The cartridges were dried under vacuum (15 min). The cartridges were eluted with n-hexane:diethyl ether (50:50, v/v) (2
× 2 mL). The eluates were reduced to dryness under nitrogen without heat before re-dissolving in 150 μL water:acetonitrile (90:10, v/v) and vortexed (1 min). An aliquot (15 μL) is injected on the LC column.

5.3.6 Matrix matched calibration

Matrix matched calibration curves were prepared and used for quantification. Control milk previously tested and shown to contain no residues was prepared as above (5.3.4). One control milk sample was used for each calibration standard level. Milk samples (5 ml) were aliquoted into 50 ml polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 15 ng mL⁻¹ by adding a 150 μL portion of a 500 ng mL⁻¹ mix solution of d₃-CPF, d₄-DCF, d₃-TLF and d₁₀-PBZ. Samples were fortified at levels corresponding to 0, 2, 5, 7.5, 10 and 20 ng mL⁻¹ by adding 0, 20, 50, 75, 100 and 200 μL portions of a 500 ng mL⁻¹ standard solution of CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ, and SUXI. After fortification, samples were held for 15 min prior to the extraction procedure as described above (5.3.5). The concentration of the NSAIDs (ng mL⁻¹) was determined from the matrix matched calibration curves. The calibration curves were calculated by linear regression, plotting the response factor (peak area analyte/internal standard peak area of CPF, DCF, MFN, PBZ) or peak areas (NIFLU, NAP, OXYPHEN and SUXI) of the strong transition as a function of analyte concentration. The equation of the line $y=mx+c$ is used to obtain the concentration of the sample response factor or peak area (y).
5.3.7 Method validation

For estimation of accuracy, blank milk samples were fortified with CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI at 5, 7.5 and 10 ng mL\(^{-1}\). 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. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility was calculated. The decision limit (CC\(\alpha\)) of the method was calculated according to the ISO 11843 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. The detection capability (CC\(\beta\)) was calculated by adding 1.64 times the standard error to the CC\(\alpha\). Milk samples were fortified at theoretical CC\(\alpha\) values calculated according to ISO 11843 calibration curve procedure to ensure the calculated levels were realistic using the developed methodology.

5.4 Results and Discussion

5.4.1 Preliminary experiments

The RRLC-MS/MS method was developed to provide confirmatory data for the analysis of CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI. The ionisation of all NSAIDs were studied in negative and positive mode. Most NSAIDs can be detected by ESI-MS both in the negative mode and the positive mode, showing different ionisation efficiencies. The optimum conditions (declustering potential, collision energy, collision cell exit potential) were determined for each drug and the best diagnostic ions for MS/MS
analysis were obtained and can be seen in Table 5.2. Negative ion mode was chosen as the required sensitivity was satisfactory for all compounds and less baseline noise was obtained. For a method to be deemed confirmatory 4 identification points must be obtained. This is achieved by monitoring one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion) which is a suitable confirmatory method in accordance with 2002/657/EC [Commission Decision 2002]. Chromatographic tests were carried out using a 1.8 μm Agilent Eclipse Plus C_{18} column (3.0× 50 mm). All analytes were eluted with a flow rate of 750 μL min⁻¹ and a run time of 6.5 min per injection with good peak shape when using a mobile phase of water and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B). As a result a batch of 30 samples can be analysed using the developed RRLC-MS/MS method in less than 4 hours allowing the running of up to 3 batches of extracted samples within a single day. The primary advantage of this developed RRLC-MS/MS method is the speed of analysis and to the best of our knowledge this is the most rapid multi-residue detection technique for the determination of NSAIDs published in the literature. The second advantage of the developed RRLC-MS/MS method in this study is that the quantitation and confirmation can be carried out using a single technique as opposed to other multi-residue methods which require longer runtimes and two separate analytical techniques [Gallo et al., 2008, Malone et al., 2009] and this method meets the requirements of 2002/657/EC as a confirmatory method. The developed methodology has been applied for the analysis of other NSAIDs not analysed in this study and satisfactory results were obtained [Dowling et al., 2009, Dowling et al., 2008].
Carprofen (strong)

$d_3$-Carprofen

Fig. 5-2A. Chromatogram of Negative Control Milk fortified at 15 ng.ml$^{-1}$ with internal standard $d_3$-CPF
Fig. 5-2B. Chromatogram of Negative Control Milk fortified at 15 ng.ml\(^{-1}\) with internal standard \(d_3\)-CPF and Negative Control Milk fortified at 2 ng.ml\(^{-1}\) with CPF
Fig. 5-3A. Chromatogram of Negative Control Milk fortified at 15 ng.mL⁻¹ with internal standard d₄-DCF.
Fig. 5-3B. Chromatogram of Negative Control Milk fortified at 15 ng.ml\(^{-1}\) with internal standard \(d_4\)-DCF and Negative Control Milk fortified at 2 ng.ml\(^{-1}\) with DCF
Fig. 5-4A. Chromatogram of Negative Control Milk

Fig. 5-4B. Chromatogram of Negative Control Milk fortified with 2ng mL\(^{-1}\) MFN
Phenylbutazone (strong)

d_{10}-Phenylbutazone

Fig. 5-5A. Chromatogram of Negative Control Milk fortified at 15 ng.ml\textsuperscript{-1} with internal standard d_{10}-PBZ
Fig. 5-5B. Chromatogram of Negative Control Milk fortified at 15 ng.ml$^{-1}$ with internal standard d$_{10}$-PBZ and Negative Control Milk fortified at 2 ng.ml$^{-1}$ with PBZ.
Fig. 5-6A. Chromatogram of Negative Control Milk

Fig. 5-6B. Chromatogram of Negative Control Milk fortified with 2ng mL⁻¹ OXYPHEN
Fig. 5-7A. Chromatogram of Negative Control Milk

Fig. 5-7B. Chromatogram of Negative Control Milk fortified with 2 ng mL⁻¹ SUXI
Fig. 5-8A. Chromatogram of Negative Control Milk

Fig. 5-8B. Chromatogram of Negative Control Milk fortified with 2ng mL⁻¹ NAP
**Fig. 5-9A.** Chromatogram of Negative Control Milk

**Fig. 5-9B.** Chromatogram of Negative Control Milk fortified with 2 ng mL$^{-1}$ NIFLU

Niflumic Acid (strong)
Fig. 5-10A. Chromatogram of Incurred Milk containing CPF
Fig. 5-11A. Chromatogram of Incurred Milk containing DCF
Fig. 5-12A. Chromatogram of Incurred Milk containing PBZ

5.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [Commission Decision 2002] covering specificity, calibration curve linearity, accuracy, precision, decision limit (CCα) and detection capability (CCβ).
5.4.2.1 Specificity

The technique of RRLC-MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, milk samples were fortified with CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ, and SUXI and non-fortified samples were also analysed. No interfering peaks were observed at the retention time for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI. Additionally samples were fortified with licensed NSAIDs for use in milk producing animals which included flunixin (FLU), meloxicam (MLX), tolfenamic acid (TLF) and vedaprofen (VDP). No interferences were observed in the retention window of CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ, and SUXI in chromatograms when fortified with these substances.

5.4.2.2 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 ng mL\(^{-1}\). The regression coefficients \((r^2)\) for all the calibration curves used in this study were \(\geq 0.99\).

5.4.2.3 Accuracy

The accuracy \((n=18)\) of the method was determined using bovine milk samples fortified at 5.0, 7.5 and 10.0 ng mL\(^{-1}\) for CPF, DCF, MFN and PBZ in three separate assays was 82-108\%.
5.4.2.4 Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (5.0, 7.5 and 10.0 ng mL\(^{-1}\)) was less than 16% (Table 5.3). Commission Decision 2002/657/EC states that the precision for quantitative methods for mass fractions lower than 100 ng mL\(^{-1}\) the application of the Horwitz Equation gives unacceptable high values. Therefore, the RSD values for concentrations lower than 100 ng mL\(^{-1}\) shall be as low as possible.

Table 5.3 Intra- and inter-assay variation for accuracy of CPF, DCF, MFN, PBZ, NAP, NIFLU, OXYPHEN and SUXI

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fortification level (ng mL(^{-1}))</th>
<th>Accuracy (%)</th>
<th>Within run CV (%)</th>
<th>Between run CV (%)</th>
<th>Total CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPF</td>
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<td>2.6</td>
<td>1.7</td>
<td>3.1</td>
</tr>
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<td>103</td>
<td>2.7</td>
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<td></td>
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<tr>
<td></td>
<td>Combined variance</td>
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<td>5.8</td>
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<td>13.0</td>
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<td>6.4</td>
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<td>11.7</td>
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<td>14.7</td>
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<td>14.7</td>
</tr>
<tr>
<td></td>
<td>Combined variance</td>
<td>5, 7.5, 10</td>
<td></td>
<td></td>
<td>13.0</td>
</tr>
</tbody>
</table>

180
5.4.2.5 $CC\alpha$ and $CC\beta$

The decision limit ($CC\alpha$) is defined as the limit above which it can be concluded with an error probability of $\alpha$, that a sample contains the analyte. In general, for non-MRL substances an $\alpha$ equal to 1% is applied. The detection capability ($CC\beta$) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of $1-\beta$, where $\beta = 5\%$. $CC\alpha$ and $CC\beta$ were calculated using the intercept (value of the signal, $y$, were the concentration, $x$ is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (5.0, 7.5 and 10.0 ng ml$^{-1}$). Blank milk was fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng ml$^{-1}$ set for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI. $CC\alpha$ is the concentration corresponding to the intercept $+ 2.33$ times the standard error of the intercept. $CC\alpha$ values of 0.46, 1.08, 0.92, 1.26, 1.29, 2.12, 0.55 and 2.86 ng ml$^{-1}$ were determined for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI respectively. According to SANCO/2004/2726 rev 1 [SANCO 2004] it is recognised in some instances where ISO 11843 has been used the extrapolated theoretical values for the decision limit ($CC\alpha$) may be too low to be confirmed experimentally. Milk samples were subsequently fortified at a level of 0.4 ng ml$^{-1}$ for CPF, DCF, MFN, NIFLU, NAP and PBZ and at a level of 2 ng ml$^{-1}$ for OXYPHEN and SUXI to establish that the theoretical decision limits ($CC\alpha$'s) calculated according to ISO11843 calibration curve procedure were valid.

$CC\beta$ is the concentration corresponding to the signal at $CC\alpha$ $+ 1.64$ times the standard error of the intercept (i.e the intercept $+ 3.97$ times that standard error of the intercept).
CCβ values of 0.79, 1.85, 1.56, 2.15, 2.19, 3.62, 0.94 and 4.87 ng mL⁻¹ were determined for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI respectively.

**Table 5.4 Calculated CCα and CCβ values based on data from Assay 1, 2 and 3**

<table>
<thead>
<tr>
<th>Substance</th>
<th>CCα (ng mL⁻¹)</th>
<th>CCβ (ng mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPF</td>
<td>0.46</td>
<td>0.79</td>
</tr>
<tr>
<td>DCF</td>
<td>1.08</td>
<td>1.85</td>
</tr>
<tr>
<td>MFN</td>
<td>0.52</td>
<td>1.56</td>
</tr>
<tr>
<td>PBZ</td>
<td>0.55</td>
<td>0.84</td>
</tr>
<tr>
<td>NAP</td>
<td>1.26</td>
<td>2.10</td>
</tr>
<tr>
<td>NIFLU</td>
<td>2.12</td>
<td>3.63</td>
</tr>
<tr>
<td>OXYPHEN</td>
<td>2.86</td>
<td>4.87</td>
</tr>
<tr>
<td>SUXI</td>
<td>2.46</td>
<td>4.57</td>
</tr>
</tbody>
</table>

### 5.4.2.6 Measurement Uncertainty

According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [SANCO 2004]. For the calculation of the extended uncertainty a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CCα, corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different milk sourced from different animals it was decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of the method was estimated at 9, 28, 28, 45, 46, 45, 10, and 39 % for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI. This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.
5.4.2.7 Evaluation

The method developed in this study has been used to evaluate the presence of CPF, DCF, MFN, NIFLU, NAP OXYPHEN, PBZ and SUXI in bovine milk. In monitoring for these substances at our laboratory it was possible to detect the precursor ion and two daughter ions (at 2 ng mL\(^{-1}\), reporting level) in multiple reaction monitoring mode for CPF, DCF, MFN, NIFLU, OXYPHEN, PBZ and SUXI. Furthermore the product ion ratio requirement was also met. The method has been carried out by different analysts under varying environmental conditions and the method was shown to be robust. To demonstrate the applicability of the method milk samples taken from animals treated with CPF, DCF and PBZ from the Community Reference Laboratory in Berlin were tested. These samples had values ranging from about 4-13 ng mL\(^{-1}\). The samples were analysed by the method developed in this study and all samples were found to be non-compliant as they contained levels above the calculated \(CC\alpha\) and also met the confirmatory criteria for both ion ratio and relative retention time. This study shows that suitable sensitivity was obtained and that the method performs very well in terms of accuracy and within-laboratory reproducibility. The developed method was evaluated by comparison of results when the method was performed by different analysts under different environmental conditions, using different batches of reagents and solid phase extraction cartridges. The results (unpublished) were highly acceptable providing evidence of the development of a rugged analytical method in this study.
5.5 Conclusions

A fast, simple, sensitive and selective RRLC-MS/MS method for the determination of CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI in bovine milk has been developed. There is no published method available to the best of our knowledge for the simultaneous determination of CPF, DCF, KPF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI in bovine milk that purifies sample extracts using Evolute TM ABN solid phase extraction cartridges and analyses extracts by RRLC-MS/MS with a runtime of 6.5 min. This is the first time that a rapid multi-residue methodology for the above NSAIDs has been validated according to Commission Decision 2002/657/EC [Commission Decision 2002] and the measurement uncertainty of the methodology has been described. The RRLC-MS/MS provides quantitative confirmatory data for the analysis of bovine milk for CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI using a single technique and as a result a batch of 30 samples can be analysed using the developed RRLC-MS/MS method in less than 4 h allowing the running of up to 3 batches of extracted samples in a single day. The rapid sample procedure and the reduced run-time per analyses of the RRLC method make it very amenable for high throughput regulatory monitoring. The primary advantage of the developed RRLC-MS/MS is the speed of the analysis and to the best of our knowledge this is the most rapid multi-residue technique available for the determination of NSAIDs available to date in which quantitation and confirmation can be carried out using a single technique that meets the requirements according to Commission Decision 2002/657/EC. Other multi-methods require longer runtimes and do not meet the requirements according to Commission Decision 2002/657/EC for a quantitative confirmatory method. The method developed allows for
the analysis of a wide variety of non authorised drugs from different NSAID sub-classes such as CPF, NAP (arylpropionic acid derivatives), SUXI, PBZ and OXYPHEN (pyrazolidinedione derivatives), DCF, MFN (anthranilic derivatives) and NIFLU (nicotinic acid derivatives). Recently it was proposed by Community Reference Laboratories (CRLs) in Europe that laboratories should be capable of monitoring for NSAIDs at a level of 5 ng mL\(^{-1}\) in EU member states where no MRL exists in milk and this study shows that these limits can be reached using the developed methodology [Commission 2002]. The second advantage of the developed RRLC-MS/MS method in this study is that the values determined for the decision limit (CC\(\alpha\)) in milk by RRLC-MS/MS are lower than those recorded for these substances recorded in the literature to date to the best of our knowledge and furthermore the calculated values according to the ISO11843 calibration curve procedure were verified by spiking milk samples at levels between 0.4- 2 ng mL\(^{-1}\) to illustrate the sensitivity in the method. A third advantage is that the NSAID RRLC-MS/MS detection method can be used with other sample preparation procedures as it is an efficient detection technique for the determination of NSAIDs providing the sample extraction and clean-up is sufficient.

The objective of the work to validate a method for these residues in bovine milk at low ng mL\(^{-1}\) levels and validate according to the requirements in Commission Decision 2002/657/EC therefore has also been achieved successfully. The present study provides an analytical tool to anticipate the requirements of the future where risks could occur due to the administration of these NSAIDs in milk.

5.6 Acknowledgements

The authors would like to thank staff at The State Laboratory, Ireland.
Chapter 6: Analytical strategy for the determination of non-steroidal anti-inflammatory drugs in plasma and improved analytical strategy for the determination of authorised and non-authorised non-steroidal anti-inflammatory drugs in milk by liquid chromatography tandem mass spectrometry

Published in Food Additives and Contaminants, Vol 27, No 7, 2010, Pages 962-982
6.1 Abstract

A sensitive and selective method for the determination of six non-steroidal anti-inflammatory drugs (NSAIDs) in bovine plasma was developed. An improved method for the determination of authorised and non-authorised residues of ten non-steroidal anti-inflammatory drugs in milk was developed. Analytes were separated and acquired by high performance liquid chromatography coupled with an electrospray ionisation tandem mass spectrometer (ESI-MS/MS). Target compounds were acidified in plasma and plasma and milk samples were extracted with acetonitrile and both extracts were purified on an improved solid phase extraction procedure utilising Evolute™ ABN solid phase extraction cartridges. The accuracy of the methods for milk and plasma was between 73 and 109 %. The precision of the method for authorised and non-authorised NSAIDs in milk and plasma expressed as % RSD, for the within-laboratory reproducibility was less than 16 %. The % RSD for authorised NSAIDs at their associated MRL(s) was less than 10 % for meloxicam, flunixin and tolfenamic acid and was less than 25% for hydroxy flunixin. The methods were validated according to Commission Decision 2002/657/EC.

Keywords: Non-steroidal Anti-Inflammatory Drugs; Plasma; Milk; Liquid Chromatography Tandem Mass Spectrometry; Method Validation
6.2 Introduction

Carprofen (CPF), diclofenac (DCF), ibuprofen (IBP), ketoprofen (KPF), mefenamic acid (MFN), phenylbutazone (PBZ), flunixin (FLU), hydroxy-flunixin (FLU-OH), tolfenamic acid (TLF) and meloxicam (MLX) are non-steroidal anti-inflammatory drugs (NSAIDs) and their structures are illustrated in Figure 6.1.

Structure a: Carprofen

Structure b: Diclofenac
Structure c: Mefenamic Acid

\[
\begin{align*}
\text{Mefenamic acid} \\
\end{align*}
\]

Structure d: Ibuprofen

\[
\begin{align*}
\text{Ibuprofen} \\
\end{align*}
\]

Structure e: Ketoprofen

\[
\begin{align*}
\text{Ketoprofen} \\
\end{align*}
\]
Structure f: Phenylbutazone

\[
\begin{align*}
\text{Phenylbutazone} \\
\end{align*}
\]

Structure g: Flunixin

\[
\begin{align*}
\text{Flunixin} \\
\end{align*}
\]

Structure h: Hydroxy-Flunixin

\[
\begin{align*}
\text{Hydroxy-Flunixin} \\
\end{align*}
\]
Over the past number of years, residues of NSAIDs in food are a cause for concern. Studies have shown that the second most prescribed class of drugs after microbials is NSAIDs [Sundlof et al., 1995]. Dairy farmers and veterinarians are using NSAIDs in dairy animals more frequently [US Code, 1988] and studies have shown that their
Increased use [Kopcha et al., 1992] poses a threat to human health as permitted residue levels are being violated [Smith et al., 2008]. In 2007 the EC Rapid Alert System for Food and Feed reported alert notifications in relation to horse meat for these substances. The European Council recommend rigorous control of NSAIDs in food producing animals [SANCO 2000] because of the health effects in humans such as aplastic anaemia, gastrointestinal disorders, agranulocytosis [Insel 1990] and changes in renal function [Goodman et al. 1992]. Long term exposure to PBZ has caused kidney tumors in mice and liver tumors in rats [Kari et al., 1995]. In recent years the COX-II inhibitor class of NSAIDs has been implicated in cardiovascular harm in humans [Debabrata et al., 2008, Van Staa et al., 2008]. According to EU law, all drugs for veterinary use need to be included in Annexes 1-3 of Regulation 2377/90 [Commission Decision 1990]. This regulation establishes lists of compounds that have a fixed maximum residue limit, MRL (Annex I), that need no MRL (Annex II) or that have a provisional MRL (III). There are no MRL’s set in plasma as is not an edible matrix. The recommended minimum concentration for NSAIDs in plasma is set at 5 ng mL \(^{-1}\) [SANCO 2007]. In milk FLU, FLU-OH, TLF and MLX are included in Annex I. CPF has been included in Annex II of the regulation only for bovine milk [European Commission 2005]. DCF was not authorised for use in animals that produce milk for human consumption [European Commission 2004] until recently [EMEA]. KPF is listed in Annex II of the regulation. PBZ, MFN and IBP are considered as prohibited substances and are not included in Annexes 1-3 and have no maximum residue limit (MRL) established however the minimum recommended concentration for analysis of NSAIDs with no MRL set in milk is 5 ng mL \(^{-1}\). The widespread use of NSAIDs presents a prospective risk to the consumer.
if food containing residues enter the food chain. In Ireland plasma and milk are some of the target matrices chosen to identify the misuse of NSAIDs in animal production. The advantages of using plasma as a target matrix in regulatory control are that it is an easy matrix to handle for analysis and PBZ residues can be found in this matrix for a long time (personal communication with the CRL). Therefore the analytical method developed in this study in plasma concentrated on the analysis of six NSAIDs in bovine species.

Methods have been reported for the analysis of NSAIDs in plasma by LC-UV [De Veau, 1999; Kvaternick et al., 2007; Luo et al., 2004; Hardee et al., 1982; Neto et al., 1996; Grippa et al., 2000; Jedziniak et al., 2007; Singh et al. 1991; Gowik et al. 1998; Quintana et al., 2004; Fiori et al., 2004], GC-MS [Neto et al., 1996; Singh et al., 1991; Hines et al., 2004; Gonzalez et al., 1996; Jausaud et al., 1992], LC-MS [Luo et al., 2004; Miksa et al., 2005; Vinci et al., 2006; Quintana et al., 2004; You et al., 2008] and capillary electrophoresis [Gu et al., 1997]. The majority of methods that have been cited to date have been developed in equine plasma alone or in combination with other matrices with limits of detection ranging from 0.1 ng ml⁻¹ to 5 mg ml⁻¹ [Miksa et al., 2005; Luo et al., 2004; Hardee et al., 1982; Neto et al., 1996; Grippa et al., 2000; Singh et al., 1991; Hines et al., 2004; Gonzalez et al., 1996; Gowik et al., 1998; Vinci et al., 2006; Gu et al., 1997; You et al., 2008]. Other methods exist for the determination of NSAIDs in bovine plasma are available but with limits of detection ranging from 20 ng to 3.4 µg ml⁻¹ [De Veau et al., 1999, Miksa et al., 2005; Jedziniak et al., 2007; Gowik et al., 1998; Vinci et al., 2006; Quintana et al., 2004; Fiori et al., 2004]. Only two methods are available in equine plasma to date capable of meeting this requirement. A method by Luo et al [2004] for a single residue had a limit of detection of 0.1 ng mL⁻¹ for FLU. A multi-residue method
by Gonzalez et al [1996] had a limit of detection of 5 ng mL$^{-1}$ for IBP, FLU, DCF and TLF, but limits of detection of only 10-25 ng mL$^{-1}$ could be achieved for KPF, MFN and PBZ. Therefore no methods available to date for KPF, MFN and PBZ in plasma that can meet the target level of 5 ng mL$^{-1}$. A disadvantage of the method developed by Gonzalez et al [1996] is that the method monitors 3 ions and this is not a confirmatory method according to Commission Decision 2002/657/EC [Commission Decision. 2002] and a second analytical technique is required. Overall there is a paucity of methods in the literature that are available for the analysis of NSAIDs in bovine plasma and of those available, the methods are not sensitive enough to meet the minimum required concentration of analysis set at 5 ng mL$^{-1}$. Milk is the second target matrix analysed in this study and is important in food safety because sampling can often be restricted to sampling of meat, milk, eggs and honey, as in the case of retail import/exports. Milk also allows the detection of drugs in live animals prior to slaughter. There are few analytical methods for the determination of authorised and non-authorised NSAIDs in milk and usually analyse for only a few residues. Those that have been described use LC-UV [Martin et al., 1983; Gallo et al., 2008; Feely et al., 2002; Rubb et al., 1995; De Veau et al. 1996], LC-MS [Gallo et al. 2008; Boner et al. 2003; Daeseleire et al. 2003; Malone et al. 2009; Dowling et al. 2009] and GC-MS [Dowling et al. 2008; Rubb et al., 1995]. A method by Gallo et al [Gallo et al., 2008] is capable of analysing 16 NSAIDs in milk using two separate analytical techniques and involves using a screening LC-DAD method with limits of detection (LOD) of between 2-15 ng mL$^{-1}$ and a runtime of 35 min with an equilibration time of 15 min per injection. Confirmation is achieved using an LC ESI-Iontrap -MS/MS method with an LOD of 5 ng mL$^{-1}$ except for flurbiprofen with a
The LC-MS method does not meet the requirements for a confirmatory method according to Commission Decision 2002/657/EC and a third analytical technique is required. A method by Stolker et al [Stolker et al., 2008] is capable of analysing 20 NSAIDs in milk using a quantitative screening method (UPLC-TOF-MS) with LOD's for specific NSAIDs such as NAP, PBZ and DCF at 12.5, 25 and 6.3 ng mL⁻¹ and a runtime of 8.5 min. The method cannot meet the 5 ng mL⁻¹ level set for NAP and PBZ or 0.1 ng mL⁻¹ level recently set for DCF; additionally, analysis by TOF-MS, medium to high resolution of approximately 10,000 FWHM is not included in Commission Decision 2002/657/EC.

Other methods for the determination of NSAIDs in milk have limits of detection of 20 ng/mL for PBZ [Martin et al., 1983], 0.2 ng mL⁻¹ for FLU and FLU-OH [Boner et al., 2003], 0.5 µg kg⁻¹ for FLU, FLU-OH and 1 µg kg⁻¹ for KPF [Daeseleire et al., 2003], 53.05, 15.82, 61.39, 45.04 ng mL⁻¹ for TLF, MLX, 4-MAA and FLU-OH [Malone et al., 2009], 0.46-2.86 ng mL⁻¹ for CPF, DCF, MFN, niflumic acid (NIFLU), naproxen (NAP), oxyphenylbutazone (OXYPHEN), PBZ and suxibuzone (SUXI) [Dowling et al., 2009], 0.59, 2.09, 0.90 and 0.70 ng mL⁻¹ for IBP, KPF, DCF and PBZ [Dowling et al., 2008], 1 ng mL⁻¹ for FLU [Feely et al., 2002], 1.7 ng mL⁻¹ for FLU [Rubb et al., 1995] the lowest fortification in matrix was 25 ng mL⁻¹ for PBZ [De Veau et al., 1996]. The objective of this study was to develop an analytical strategy for the determination of NSAIDs in bovine plasma and for authorised and non-authorised simultaneously in milk that meet the EU target levels set and validate according to Commission Decision 2002/657/EC.
In this study an improved purification procedure was developed using Evolute ABN™ solid phase extraction cartridges for the analysis of a wider range of NSAIDs including authorised and non-authorised NSAIDs in bovine milk. The developed procedure was suitable for the purification of six NSAIDs in bovine plasma. An improved liquid chromatography tandem mass spectrometry detection technique was developed to analyse ten NSAIDs simultaneously with a run-time of 15 min. The methods in each matrix were comprehensively validated according to Commission Decision 2002/657/EC. The methods were implemented into the National Monitoring Programme in Ireland for veterinary drugs residues and accredited according to ISO 17025 Standard. The proposed method in milk does not cover the glucuronides. This is the first time that suitably sensitive methods for the analysis of NSAIDs in plasma and for the analysis of the selected range of authorised and non-authorised NSAIDs using Evolute ABN™ solid phase extraction cartridges simultaneously in bovine milk are available.

6.3 Experimental

6.3.1 Materials and reagents

Water, ethanol, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid (37%), n-hexane and iso-octane (HiPerSolv grade) were obtained from BDH (Merck, UK). CPF, DCF, IBP, MFN, FLU, KPF, MLX, TLF and PBZ were purchased from Sigma (Sigma Aldrich, Ireland). FLU-OH was obtained as a gift from The Community Reference Laboratory for NSAIDs in the EU in Germany. \( \text{d}_{10}-\text{PBZ} \) was obtained from Cambridge Isotope Labs (Cambridge Isotope Labs, USA). \( \text{d}_3-\text{MLX}, \text{d}_3-\text{IBP} \) and \( \text{d}_4-\text{DCF} \) were obtained from CDN Isotopes (CDN Isotopes, Canada). \( \text{d}_3-\text{FLU} \) was obtained from
Witega (Witega, Germany). \(d_4\)-TLF was obtained as a gift from Stormont, (Stormont, UK). Primary stock standard solutions (stable for 12 months) were prepared in ethanol at a concentration of 1 mg mL\(^{-1}\). Intermediate single standard solutions (stable for 6 months) were prepared in methanol at a concentration of 10 \(\mu\)g mL\(^{-1}\). CPF, DCF, IBP, MFN, FLU, FLU-OH, KPF, TLF, MLX and PBZ standard fortification solution for plasma (stable for 6 months) was prepared in methanol at a concentration of 500 ng mL\(^{-1}\) from the 10 \(\mu\)g mL\(^{-1}\) intermediate stock solution. Internal standard fortification solution for milk or plasma containing \(d_3\)-MLX, \(d_4\)-DCF, \(d_3\)-IBP, \(d_3\)-FLU, \(d_4\)-TLF and \(d_{10}\)-PBZ was prepared at a concentration of 1.25 \(\mu\)g mL\(^{-1}\). CPF, DCF, IBP, MFN, KPF and PBZ standard fortification solution for milk (NMRL) was prepared in methanol at a concentration of 500 ng mL\(^{-1}\) from the 10 \(\mu\)g mL\(^{-1}\) intermediate stock solution. MLX, FLU, FLU-OH and TLF standard fortification solution for milk (MRL) was prepared in methanol at a concentration of 1.5, 4, 4 and 5 \(\mu\)g mL\(^{-1}\). All standards were stored at 4 °C in the dark.

Isolute™ Evolute ABN 50 \(\mu\)m solid phase extraction cartridges (10 mL, 100 mg) were obtained from Biotage (Biotage, UK). Methanol:water (10:90, v/v) and 10 mM ascorbic acid were used as solid phase extraction wash solvents. N-hexane:diethyl ether:acetonitrile:methanol (45:45:7:3, v/v) was used as the solid phase extraction elution solvent. Injection solvent was water:acetonitrile (90:10, v/v).

### 6.3.2 LC conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). The NSAIDs were chromatographed on a 1.8 \(\mu\)m Agilent Eclipse Plus...
C_{18} column (3.0 x 50 mm) (Agilent, Ireland) and the column temperature was maintained at 55 °C. A gradient was applied with water containing 0.001 M acetic acid and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B) (Table 6.1). The total run time was 15 minutes. The injection volume was 15 µL. The mass spectrometer used was a QTRAP 4000 with a TurboIonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.5 of Analyst software. The described LC-MS/MS system was shown to be suitable for the analysis of NSAIDs in plasma (Figure 6.2-6.7) and milk (Figure 6.2-6.11).

6.3.3 MS/MS parameters

The analysis was performed using negative ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. The collision voltages were optimised as shown (Table 6.2). Each transition was performed with a 13 msec dwell time and a pause time of 3 msec. The MS/MS detector conditions were as follows: Ion mode electrospray negative; curtain gas 45 psi; ion spray voltage 4400 V; temperature 650 °C; ion source gas one 70 psi; ions source gas two 70 psi; Interface heater on; entrance potential 10 V; Resolution Q1 unit; Resolution Q2 unit; CAD gas =high
Table 6.1: LC gradient profile for determination of CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX

<table>
<thead>
<tr>
<th>Time</th>
<th>Component A (%)</th>
<th>Component B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(min)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>0.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>1.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>3.5</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>7.5</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>9.5</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>11.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>15.0</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Component A: water containing 0.001 M acetic acid + acetonitrile (90 + 10, v/v)

Component B: Acetonitrile

Table 6.2: MS/MS parameters for determination of CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition</th>
<th>Declustering potential (V)</th>
<th>Collision energy (eV)</th>
<th>Collision cell exit potential (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPF</td>
<td>271.8 &gt; 223 (strong)</td>
<td>-60</td>
<td>-16</td>
<td>-13</td>
</tr>
<tr>
<td>DCF</td>
<td>294.0 &gt; 250 (strong)</td>
<td>-50</td>
<td>-19</td>
<td>-15</td>
</tr>
<tr>
<td>IBP</td>
<td>264.0 &gt; 214(weak)</td>
<td>-10</td>
<td>-14</td>
<td>-9</td>
</tr>
<tr>
<td>PBZ</td>
<td>306.9 &gt; 276 (strong)</td>
<td>-70</td>
<td>23</td>
<td>-12</td>
</tr>
<tr>
<td>FLU</td>
<td>294.9 &gt; 250.2 (strong)</td>
<td>-80</td>
<td>-18</td>
<td>-15</td>
</tr>
<tr>
<td>FLU-OH</td>
<td>294.9 &gt; 191.0 (strong)</td>
<td>-80</td>
<td>-25</td>
<td>-17</td>
</tr>
<tr>
<td>MFN</td>
<td>306.9 &gt; 266.0 (strong)</td>
<td>-65</td>
<td>-41</td>
<td>-9</td>
</tr>
<tr>
<td>MLX</td>
<td>349.9 &gt; 285.9 (strong)</td>
<td>-65</td>
<td>-54</td>
<td>-11</td>
</tr>
<tr>
<td>KPF</td>
<td>252.8 &gt; 205.0 (strong)</td>
<td>-60</td>
<td>-18</td>
<td>-12</td>
</tr>
<tr>
<td>TLF</td>
<td>259.8 &gt; 215.5 (strong)</td>
<td>-60</td>
<td>-18</td>
<td>-12</td>
</tr>
<tr>
<td>d3-DCF</td>
<td>298.0 &gt; 231.1 (strong)</td>
<td>-70</td>
<td>-16</td>
<td>-9</td>
</tr>
<tr>
<td>d3-IBP</td>
<td>208.0 &gt; 164.0 (strong)</td>
<td>-70</td>
<td>-16</td>
<td>-9</td>
</tr>
<tr>
<td>d3-PBZ</td>
<td>316.9 &gt; 289.0 (strong)</td>
<td>-70</td>
<td>-16</td>
<td>-9</td>
</tr>
<tr>
<td>d3-FLU</td>
<td>298.0 &gt; 254.0 (strong)</td>
<td>-70</td>
<td>-16</td>
<td>-9</td>
</tr>
<tr>
<td>d3-MLX</td>
<td>353.0 &gt; 280.0 (strong)</td>
<td>-70</td>
<td>-16</td>
<td>-9</td>
</tr>
<tr>
<td>d3-TLF</td>
<td>384.0 &gt; 323.0 (strong)</td>
<td>-70</td>
<td>-16</td>
<td>-9</td>
</tr>
</tbody>
</table>

Note: Matrix matched curves were used for quantification of all compounds.

d3-IBP was used as internal standard (IS) for CPF, IBP and KPF, d3-DCF was used as IS for DCF, d10-PBZ was used as IS for PBZ, d3-FLU was used as IS for FLU and FLU-OH, TLF and MLX.
6.3.4 Plasma/milk samples

Plasma/milk obtained for use as negative controls was separated into 50 mL aliquots and stored at -20 °C. The plasma/milk was analysed in previous batches and plasma/milk found to contain no detectable residues of NSAIDs were used as negative controls.

6.3.5 Sample extraction and clean-up

6.3.5.1 Plasma Extraction

Plasma samples (5 mL) were aliquoted into 50 mL polypropylene tubes. The plasma aliquots (5 mL) were fortified with internal standard at levels corresponding to 15 ng mL⁻¹ by adding a 60 µL portion of a 1.25 µg mL⁻¹ mix solution of d₃-MLX, d₄-DCF, d₃-IBP, d₃-FLU, d₄-TLF and d₁₀-PBZ. Samples were fortified at levels corresponding to 5, 7.5 and 10 ng mL⁻¹ by adding 50, 75 and 100 µL portions of a 500 ng mL⁻¹ solution of CPF, DCF, IBP, MFN, KPF and PBZ. After fortification, samples were held for 15 min prior to extraction. Hydrochloric acid (500 µL, 1 M) was added to the plasma samples and they were left to stand at room temperature (10 min). Acetonitrile (5 mL) was added and the samples were vortexed (30 sec), centrifuged (4500 rpm, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. 10 mM ascorbic acid (15 mL) was added and the samples were vortexed (30 sec) and the pH of the samples were
checked to ensure they were at pH 3 before proceeding to the solid phase extraction (SPE) stage.

6.3.5.2 Milk Extraction

Milk samples (5 mL) were aliquoted into 50 mL polypropylene tubes. The milk aliquots (5 mL) were fortified with internal standard at levels corresponding to 15 ng mL\(^{-1}\) by adding a 60 \(\mu\)L portion of a 1.25 ug mL\(^{-1}\) mix solution of \(d_3\)-MLX, \(d_4\)-DCF, \(d_3\)-IBP, \(d_3\)-FLU, \(d_4\)-TLF and \(d_{10}\)-PBZ. Samples were fortified at levels corresponding to 5, 7.5 and 10 ng mL\(^{-1}\) by adding 50, 75 and 100 \(\mu\)L portions of a 500 ng mL\(^{-1}\) solution of CPF, DCF, IBP, MFN, KPF and PBZ (NMRL solution) and at 7.5, 15 and 22.5 ng mL\(^{-1}\) with MLX, at 20, 40 and 60 ng mL\(^{-1}\) with FLU and FLU-OH and at 25, 50 and 75 ng mL\(^{-1}\) with TLF by fortifying with 25, 50 and 75 \(\mu\)L portions of a 1.5, 4, 4 and 5 ug mL\(^{-1}\) of MLX, FLU, FLU-OH and TLF (MRL solution). After fortification, samples were held for 15 min prior to extraction. Acetonitrile (5 mL) was added and the samples were vortexed (30 sec), centrifuged (3568 x g, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The sample pellet is re-extracted with 5 mL of acetonitrile and the supernatants are combined. 10 mM ascorbic acid (20 mL) and 1 M hydrochloric acid (0.2 mL) were added to the extracts and the pH of the samples were checked to ensure they were at pH 3 before proceeding to the solid phase extraction (SPE) stage.
6.3.5.3 Solid phase extraction

The sample extracts were purified by SPE using Evolute ABN™ SPE cartridges. Sample extracts were loaded onto the cartridges (preconditioned with 3 mL of n-hexane:diethyl ether (50:50, v/v) 3 mL of methanol and 5 mL of ascorbic acid. The samples were loaded onto cartridges under gravity. The cartridges were washed with 3 mL of methanol:water (10:90, v/v). The cartridges were dried under vacuum (15 min). The cartridges were eluted with 2 × 1.5 mL of n-hexane:diethyl ether: acetonitrile: methanol (45:45:7:3, v/v). The eluates were reduced to dryness under nitrogen without heat before re-dissolving in 150 μL of water: acetonitrile (90:10, v/v) and vortexed (1 min). An aliquot (15 μL) was injected on the LC column.

6.3.5.4 Matrix-Matched Calibration

Matrix matched calibration curves were prepared and used for quantification. Control plasma/milk previously tested and shown to contain no residues was prepared as above (6.3.4). One control plasma sample and one control milk was used for each calibration standard level. Plasma samples (5 mL) or milk samples (5 mL) were aliquoted into 50 mL polypropylene tubes. Individual plasma or milk samples were fortified with internal standard at levels corresponding to 15 ng mL⁻¹ by adding a 60 μL portion of a 1.25 μg mL⁻¹ mix solution of d₃-MLX, d₄-DCF, d₃-IBP, d₃-FLU, d₄-TLF and d₁₀-PBZ. Plasma samples were fortified at levels corresponding to 0, 5, 7.5, 10 and 20 ng mL⁻¹ by adding 0, 50, 75, 100 and 200 μL portions of a 500 ng mL⁻¹ standard solution of CPF, DCF, IB, KPF, MFN and PBZ. After fortification, plasma samples were held for 15 min prior to
the extraction procedure as described above (6.3.5). Milk samples were fortified at levels corresponding to 0, 5, 7.5, 10 and 20 ng mL\(^{-1}\) by adding 0, 50, 75, 100 and 200 \(\mu L\) portions of a 500 ng mL\(^{-1}\) standard solution of CPF, DCF, IBP, MFN, KPF and PBZ (NMRL solution) and at levels corresponding to 0, 7.5, 15, 22.5, 30, 60 ng mL\(^{-1}\) of MLX, 0, 20, 40, 60, 80 and 160 ng mL\(^{-1}\) FLU and FLU-OH and 0, 25, 50, 75, 100 and 200 ng mL\(^{-1}\) of TLF by adding 0, 25, 50, 75, 100 and 200 \(\mu L\) portions of a 1.5, 4, 4, 5 \(\mu g\) mL\(^{-1}\) standard solution of MLX, FLU, FLU-OH and TLF (MRL solution). After fortification, milk samples were held for 15 min prior to the extraction procedure as described above (6.3.5). Calibration curves of plasma or milk were prepared by plotting the response factor as a function of analyte concentration (0 to 20 ng mL\(^{-1}\)) to quantify samples.

6.3.6 Method validation

For estimation of accuracy, blank plasma samples were fortified with CPF, DCF, IBP, MFN, KPF and PBZ at 5, 7.5 and 10 ng mL\(^{-1}\). For estimation of accuracy, blank milk samples were fortified with CPF, DCF, IBP, MFN, KPF and PBZ at 5, 7.5 and 10 ng mL\(^{-1}\) and at 7.5, 15 and 22.5 ng mL\(^{-1}\) with MLX, at 20, 40 and 60 ng mL\(^{-1}\) with FLU and FLU-OH and at 25, 50 and 75 ng mL\(^{-1}\) with TLF. 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 for each matrix. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility was calculated. For unauthorised substances the decision limit (CC\(\alpha\)) 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. The detection capability (CC\( \beta \)) was calculated by adding 1.64 times the standard error to the CC\( \alpha \). For authorised substances the decision limit (CC\( \alpha \)) of the method was calculated according to the ISO 11843 calibration curve procedure by plotting the corresponding concentration at the permitted limit plus 1.64 times the standard deviation of the within laboratory reproducibility for a set of data with six replicates at three levels. The detection capability (CC\( \beta \)) was calculated by adding 1.64 times the standard deviation of the within laboratory reproducibility to the CC\( \alpha \).

6.4 Results and Discussion

6.4.1 Development/optimisation experiments

The ionization of all NSAIDs was studied in negative and positive mode. Most NSAIDs can be detected by ESI-MS both in negative and positive mode showing different ionization efficiencies. The optimum parameters (polarity mode, decustering potential, collision energy, collision cell exit potential) were determined for each drug and the best diagnostic ions for MS/MS analysis are shown in Table 6.2. Negative ion mode was chosen as the required sensitivity was obtained for all compounds and less baseline noise was obtained. The MS/MS method was developed to monitor one precursor ion (parent mass) and two daughters (corresponding to strong and weak ion), which is a suitable confirmatory method yielding four identification points in accordance with 2002/657/EC. Only one daughter ion could be obtained for KPF and IBP, but two daughter ions could
be obtained for all other compounds investigated in the study. A previous method developed at the authors laboratory utilising GC-MS/MS was capable of obtaining 2 daughter ions for these substances after derivatisation [Dowling et al., 2008]. The LC method developed in this study was based on a method developed at the author’s laboratory for the determination of eight banned non-steroidal anti-inflammatory drugs but was not suitable for incorporation of the new range of analytes in this study. [Dowling et al., 2009]. Chromatographic tests were carried out to evaluate the suitability of the 1.8 μm Agilent Eclipse Plus C₁₈ column (3.0 × 50 mm) and the LC mobile phase utilised in this study when additional NSAIDs were added. The tests showed that the internal standards of DCF and FLU overlapped in each internal standard transition when the analytes were eluted with a flow rate of 750 μL min⁻¹ and a run time of 6.5 min per injection. A study was performed using the same composition of mobile phase A and B. The times in the gradient were adjusted, the flow rate was reduced and the chromatographic runtime was extended to separate the internal standard of DCF and FLU. This resulted in the analytes being eluted with good peak shape when using a mobile phase of water containing 0.001 M acetic acid and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B) with a flow rate of 0.5 μL.min⁻¹ and a runtime of 15 min. The internal standards d₄-DCF and d₃-FLU were completely separated under these conditions. As a result a batch of 30 samples can be analysed using the developed LC-MS/MS method for 10 NSAID residues in 7.5 hours allowing the running of up to 3 batches of extracted samples within a 24 hour period. The extraction of the NSAIDs from plasma was based on methods developed by Gowik et al. (Gowik et al., 1998) and by Vinci et al (Vinci et al., 2006) but modified with the addition of
acetonitrile. The extraction of NSAIDs in milk was based on a method previously
developed at the author’s laboratory [Dowling et al., 2009]. The extraction procedures
were found to be satisfactory in the extraction of the NSAIDs from milk and plasma in
this study. The purification of NSAIDs from the plasma and milk extracts was
investigated initially using a solid phase extraction procedure previously developed at the
authors laboratory using Evolute ABN™ cartridges but the original procedure was not
satisfactory for the additional range of new analytes in this study. The NSAID FLU-OH
was poorly recovered when the method previously developed at our laboratory was
utilised. Elution studies were performed to ascertain where losses were occurring. The
cartridges were eluted with different compositions and volumes of solvents including, 3
mL diethyl ether:hexane:acetonitrile (45:45:10, v/v/v), 3 mL diethyl ether:hexane,
acetonitrile:methanol (45:45:5:5, v/v/v/v), 1.5 mL diethyl ether:hexane (80:20, v/v-
elution 1) and 1.5 mL acetonitrile:methanol (90:10, v/v-elution 2), 1.5 mL
acetonitrile:methanol 90:10, v/v-elution 1) and 1.5 mL diethyl ether:hexane 50:50, v/v-
elution 2), 3 mL methanol and 3 mL diethyl ether:hexane:acetonitrile:methanol
(37.5:37.5:20:5 v/v/v/v). The results showed that elution of the cartridge with a solvent
1.5 mL) gave the best results for all the analytes tested in this study. This is the first time,
to the best of our knowledge, that CPF, DCF, IBP, KPF, MFN and PBZ residues have
been purified from bovine plasma using Evolute ABN™ solid-phase extraction
cartridges. The methodology is capable of meeting the 5 ng mL⁻¹ set for NSAIDs in
plasma. Moreover the values determined for the decision capability (CCa) in this study
were lower than those recorded for these substances in the literature in plasma by LC-
MS/MS to date. This is the first time that FLU, TLF, FLU-OH, IBP, KPF and MLX have been purified from milk using Evolute ABN™ solid phase extraction cartridges. The method meets the target level of 5 ng mL\(^{-1}\) for IBP and KPF in milk for the first time.

There are no analytical methods that monitor for authorised and non-authorised NSAIDs in milk as well as FLU and FLU-OH simultaneously in milk that meet the stringent validation requirements according to Commission Decision 2002/657/EC. The primary advantage of the developed analytical strategy in this study is that quantitation and confirmation can be carried out using a single analytical technique according to Commission Decision 2002/657/EC (Commission Decision 2002), except for IBP and KPF. Confirmation of these residues using a second analytical technique is described elsewhere (Dowling et al., 2008).

After validation of this method, the EU changed the legislation for DCF and an MRL of 0.1 ng mL\(^{-1}\) was set in milk. Preliminary spiking studies at the new MRL for DCF were carried out using the developed analytical strategy in this study. Results showed that this analytical strategy was sensitive enough to detect DCF at this level. The same extract was also analysed on an Applied Biosystems 5500 triple quadrupole mass spectrometer and the response using this system gave a better signal-to-noise ratio compared to the 4000 QTRAP. This analytical strategy with detection using the 4000 QTRAP or 5500 Applied Biosystems LC-MS/MS technology shows, for the first time during initial studies, that the new MRL set for DCF in milk at 0.1 ng mL\(^{-1}\) could be achieved.
Fig. 6-2. Chromatogram of negative control milk (A) and plasma (C) fortified at 15 ng mL\(^{-1}\) with internal standard d\(_3\)-IBP and fortified with 5 ng mL\(^{-1}\) of CPF in milk (B) and plasma (D)
Fig. 6-3. Chromatogram of negative control milk (A) and plasma (C) fortified at 15 ng mL\(^{-1}\) with internal standard \(\text{d}_4\)-DCF and fortified at 5 ng mL\(^{-1}\) with DCF in milk (B) and plasma (D)
Fig. 6-4. Chromatogram of negative control milk (A) and plasma (C) fortified at 15 ng mL\(^{-1}\) with internal standard d\(_3\)-IBU and fortified at 5 ng mL\(^{-1}\) with IBP in milk (B) and plasma (D).
Fig. 6-5. Chromatogram of negative control milk (A) and plasma (C) fortified at 15 ng mL\(^{-1}\) with internal standard d\(_3\)-IBU and fortified at 5 ng mL\(^{-1}\) with KPF in milk (B) and plasma (D).
Fig. 6-6. Chromatogram of negative control milk (A) and plasma (C) fortified at 15 ng mL$^{-1}$ with internal standard d$_4$-TLF and fortified at 5 ng mL$^{-1}$ with MFN in milk (B) and plasma (D).
Fig. 6-7. Chromatogram of negative control milk (A) and plasma (C) fortified at 15 ng mL$^{-1}$ with internal standard $d_{10}$-PBZ and fortified at 5 ng mL$^{-1}$ with PBZ in milk (B) and plasma (D)
Fig. 6-8. Chromatogram of Negative Control milk (A) fortified at 15 ng mL⁻¹ with internal standard d₃-MLX and fortified at 7.5 ng mL⁻¹ with MLX (B)

Fig. 6-9. Chromatogram of negative control milk (A) fortified at 15 ng mL⁻¹ with internal standard d₃-FLU and fortified at 20 ng mL⁻¹ with FLU (B)
Fig. 6-10. Chromatogram of negative control milk (A) fortified at 15 ng mL$^{-1}$ with internal standard d$_3$-FLU and fortified at 20 ng mL$^{-1}$ with FLU-OH (B)

Fig. 6-11A. Chromatogram of negative control milk (A) fortified at 15 ng mL$^{-1}$ with internal standard d$_4$-TLF and fortified at 25 ng mL$^{-1}$ with TLF (B)
6.4.2.2 Method Validation

Validation of the method in plasma and milk was according to procedures described in Commission Decision 2002/657/EC [Commission Decision 2002] covering specificity, calibration curve linearity, recovery (accuracy), precision, decision limit (CCα) and detection capability (CCβ).

6.4.2.1 Specificity

The technique of LC-MS/MS itself offers a high degree of selectivity and specificity. To establish the selectivity/specificity of the method, a variety of plasma and milk samples were fortified with analytes and internal standards and non-fortified samples were also analysed. No interfering peaks were observed at the retention time of the analytes. To further test specificity in plasma and milk, samples were also fortified with 5.0 ng mL\(^{-1}\) of naproxen (NAP), niflumic acid (NIFLU), oxyphenylbutazone (OXYPHEN) and suxibuzone (SUXI). No interfering peaks were observed at the retention window of the analytes.

6.4.2.2 Linearity

The linearity of the chromatographic response in plasma was tested with matrix matched curves using 5 calibration points in the concentration range of 0 to 20 ng mL\(^{-1}\) when fortified with CPF, DCF, IBP, KPF, MFN and PBZ. The linearity of the chromatographic response in milk was tested with matrix matched curves using 6 calibration points in the concentration range of 0 to 20 ng mL\(^{-1}\) when fortified with CPF, DCF, IBP, KPF, MFN and PBZ (NMRL substances). For MRL substances the linearity of the chromatographic response in milk was tested with matrix matched curves using 5
calibration points in the concentration range of 0 to 30 ng mL\(^{-1}\) for MLX, 0 to 160 ng mL\(^{-1}\) for FLU and FLU-OH and 0 to 100 ng mL\(^{-1}\) for TLF. Overall the regression coefficients \((r^2)\) were ≥ 0.98 except for FLU-OH.

6.4.2.3 Accuracy

The accuracy was determined using bovine plasma fortified at 5.0, 7.5 and 10.0 ng mL\(^{-1}\) with CPF, DCF, IBP, KPF, MFN and PBZ. Mean corrected recoveries \((n = 18)\) determined in three separate assays in plasma (Table 6.3) were between 99 and 109 %. The accuracy was determined using bovine milk fortified at 5.0, 7.5 and 10.0 ng mL\(^{-1}\) with CPF, DCF, IBP, KPF, MFN and PBZ and the mean corrected recoveries \((n = 18)\) determined in three separate assays in milk (Table 6.4) were between 74 and 109 %. The accuracy was determined using bovine milk fortified at MRL levels of 7.5, 15 and 22.5 ng mL\(^{-1}\) for MLX, at 20, 40 and 60 ng mL\(^{-1}\) with FLU and FLU-OH and fortified at 25, 50 and 75 ng mL\(^{-1}\) with TLF and the mean corrected recovery \((n = 18)\) of the analytes determined in three separate assays in milk (Table 6.4) were between 73 and 102 %.

6.4.2.4 Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility of CPF, DCF, IBP, MFN and PBZ in plasma was less than 16 % (Table 6.3). No deuterated analogue was available for CPF, MFN, KPF and FLU-OH in our laboratory at the time of carrying out this work. d\(_3\)-IBP was used as I.S for CPF and KPF, d\(_3\)-TLF was used as I.S for MFN and d\(_3\)-FLU was used as I.S for FLU-OH. The precision of the method, expressed as RSD values for the within-lab reproducibility of CPF, DCF, IBP, KPF, MFN and PBZ when fortified into milk was less than 16 % (Table 6.4).
precision of the method, expressed as RSD values for the within-lab reproducibility of MLX, FLU and TLF when fortified into milk was less than 10% except for FLU-OH which was less 25% (Table 6.4). A one way analysis of variance was carried out at each of the fortification levels to separate out estimates for within run, between run and total variance of the method and the results are shown in Tables 6.3 and 6.4. Commission Decision 2002/657/EC states that the precision for quantitative methods for mass fractions lower than 100 ng mL\(^{-1}\), the application of the Horwitz Equation gives unacceptable high values. Therefore, the RSD values for concentrations lower than 100 ng mL\(^{-1}\) shall be as low as possible.

Table 6.3: Intra- and inter-assay variation for accuracy of CPF, DCF, IBP, KPF, MFN, PBZ, in plasma

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<th>Analyte</th>
<th>Fortification level (ng mL(^{-1}))</th>
<th>Accuracy (%)</th>
<th>Within Run CV (%)</th>
<th>Between Run CV (%)</th>
<th>Total CV (%)</th>
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218
Table 6.4: Intra- and inter-assay variation for accuracy of CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX in milk

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<td>87</td>
<td>1.9</td>
<td>11.4</td>
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6.4.2.5 CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1 % is applied. The detection capability (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, were β = 5 %. In the case of non MRL substances CCα is
the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. 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). Blank plasma was fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng mL\(^{-1}\) set for CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX. CCα and CCβ were calculated in plasma using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with six replicates at three levels (5.0, 7.5 and 10.0 ng mL\(^{-1}\)). CCα values of 1.80, 0.58, 0.71, 0.87, 0.70 and 1.19 ng mL\(^{-1}\) were determined for CPF, DCF, IBP, KPF, MFN, and PBZ respectively. CCβ values of 3.1, 0.99, 1.22, 1.49, 1.20, 2.02 ng mL\(^{-1}\) were determined for CPF, DCF, IBP, KPF, MFN, and PBZ respectively (Table 6.5). Non-authorised substances in milk were fortified at 1, 1.5 and 2 times the minimum required performance level of 5 ng mL\(^{-1}\) set for CPF, DCF, IBP, KPF, MFN, and PBZ. CCα values of 2.11, 0.83, 0.47, 1.63, 0.92 and 0.55 ng mL\(^{-1}\) were determined for CPF, DCF, IBP, KPF, MFN, and PBZ (Table 6.6). CCβ values of 3.59, 1.41, 0.80, 2.77, 1.56 and 0.94 ng mL\(^{-1}\) were determined for CPF, DCF, IBP, KPF, MFN, and PBZ (Table 6.6). In the case of substances which have MRLs (MLX, FLU, FLU-OH and TLF) fortification was at 0.5, 1 and 1.5 times the corresponding MRL. The decision limit (CCα) is calculated by analysing the 18 milk samples fortified at the MRL over three days, and using the concentration at the permitted limit plus 1.64 times the standard deviation obtained to yield CCα. The detection capability (CCβ) of the proposed method was calculated from the CCα value plus 1.64 times the corresponding standard deviation. CCα values of 17.6, 42.89, 55.76 and 54.45 ng mL\(^{-1}\) were determined and CCβ values of 220
20.13, 45.78, 71.50 and 58.9 ng mL\(^{-1}\) were determined for MLX, FLU, FLU-OH and TLF in milk when fortified at their associated maximum residue limits.

Table 6.5: Calculated CC\(\alpha\) and CC\(\beta\) values for plasma

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<td>KPF</td>
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<td>1.40</td>
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<td>MFN</td>
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<tr>
<td>PBZ</td>
<td>1.19</td>
<td>2.02</td>
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Table 6.6: Calculated CC\(\alpha\) and CC\(\beta\) values for milk

<table>
<thead>
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<th>CC(\alpha) (ng mL(^{-1}))</th>
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<tr>
<td>MLX</td>
<td>67.47</td>
<td>20.13</td>
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</table>

6.4.2.6 Measurement Uncertainty

According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [SANCO 2004]. For the calculation of the extended uncertainty a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CC\(\alpha\), corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different plasma or milk sourced from different animals it was decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of the method in plasma was estimated at 47, 12, 15, 23, 19 and 26% for CPF, DCF, IBP, KPF, MFN and PBZ.
respectively. The measurement uncertainty of the method in milk was estimated at 50, 34, 36, 48, 20, 24, 16, 75, 24 and 30 % for CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX, respectively.

This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

### 6.4.2.7 Evaluation

The method developed in this study has been used to evaluate the presence of CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX in bovine milk and CPF, DCF, IBP, KPF, MFN and PBZ in plasma. In monitoring for these substances in either matrix at our laboratory it was possible to detect the precursor ion and two daughter ions (at 5 ng mL^{-1}) in multiple reaction monitoring mode except for IBP and KPF.

Furthermore the product ion ratio requirement was also met. The method has been carried out by different analysts under varying environmental conditions and the method was shown to be robust. To demonstrate the applicability of the method milk samples taken from animals treated with MLX and FLU-OH obtained from the Community Reference Laboratory in Berlin were tested. These samples had assigned values ranging from 5-15 ng mL^{-1}. The samples were analysed by the method developed in this study and all samples were found to contain 5 ng mL^{-1} of FLU-OH and 15 ng mL^{-1} of MLX.
6.5 Conclusions

A fast, simple, sensitive and selective LC-MS/MS method for the determination of CPF, DCF, IBP, KPF, MFN and PBZ in bovine plasma and CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX in bovine milk has been developed. The LC-MS/MS method provided quantitative confirmatory data for the analysis of bovine milk for CPF, DCF, MFN, PBZ, FLU, FLU-OH, TLF and MLX. The method developed allows for the analysis of a wide variety of drugs from different NSAID sub-classes such as CPF, IBP and KPF (arylpropionic acid derivatives), PBZ (pyrazolidinedione derivatives) DCF, MFN and TLF (anthranilic derivatives) and FLU and FLU-OH (nicotinic acid derivatives) and MLX (oxicam derivative). There is no published method available to the best of our knowledge for the simultaneous determination of authorised and non-authorised NSAIDs such as CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX in bovine milk that purifies sample extracts using Evolute™ ABN solid phase extraction cartridge procedure described in this study which is an improvement on previous work carried out utilising this cartridge chemistry. This is the first time that FLU, TLF, FLU-OH, IBP, KPF and MLX have been purified from milk using Evolute ABN™ solid phase extraction cartridges simultaneously with other NSAIDs. This is the first time that a method is available that meets the minimum requirements of 5 ng mL⁻¹ for IBP and KPF in milk. There is no published method available to the best of our knowledge for the simultaneous determination of CPF, DCF, IBP, KPF, MFN and PBZ in bovine plasma that purifies sample extracts using Evolute™ ABN solid phase extraction cartridges. This study describes the first such sensitive and selective methodology. This is also the first time that a rapid multi-residue methodology for the
above authorised and non-authorised NSAIDs has been validated according to Commission Decision 2002/657/EC [Commission Decision 2002] and the measurement uncertainty of the method has been described. This methodology shows that suitable sensitivity was obtained and that the method performs very well in terms of accuracy and within-laboratory reproducibility. The developed method was evaluated by comparison of results when method was performed by different analysts under different environmental conditions, using different batches of reagents and solid phase extraction cartridges. The results (unpublished data) were highly acceptable providing evidence of the development of a rugged analytical method in this study. Recently it was proposed by Community Reference Laboratories (CRLs) in Europe that laboratories should be capable of monitoring for NSAIDS at a level of 5 ng mL⁻¹ in EU member states where no MRL exists in plasma or milk and this study shows that these limits can be reached using the developed analytical strategy [SANCO 2004]. The objective of the work to anticipate the requirements of the future where risks could occur due to the administration of NSAIDs by developing a method to monitor for authorised and non-authorised NSAIDs simultaneously has been achieved. The objective of the work to validate an analytical strategy for these residues in bovine plasma and milk that meet EU target levels according to the requirements in Commission Decision 2002/657/EC therefore has also been achieved successfully.

6.6 ACKNOWLEDGEMENTS

The authors would like to thank staff at The State Laboratory, Ireland for their practical assistance.
Chapter 7: A hybrid liquid chromatography mass spectrometry strategy in a forensic laboratory for opioid, cocaine and amphetamine classes in human urine using a hybrid linear ion trap-triple quadrupole mass spectrometer

Published in Journal of Chromatography A, 1217, 2010, Pages 6857-6866
7.1 Abstract

A rapid method has been developed to analyse morphine, codeine, morphine-3-glucuronide, 6-monoacetylmorphine, cocaine, benzoylegonine, buprenorphine, dihydrocodeine, cocaethylene, 3,4-methylenedioxymphetamine, ketamine, 3,4-methylenedioxymethamphetamine, pseudoephedrine, lignocaine, benzylpiperazine, methamphetamine, amphetamine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and methadone in human urine. Urine samples were diluted with methanol:water (1:1, v/v) and sample aliquots were analysed by hybrid linear ion trap-triple quadrupole mass spectrometry with a runtime of 12.5 min. Multiple reaction monitoring (MRM) as survey scan and an enhanced product ion (EPI) scan as dependent scan were performed in an information-dependent acquisition (IDA) experiment. Finally, drug identification and confirmation was carried out by library search with a developed inhouse MS/MS library based on EPI spectra at a collision energy spread of 35±15 in positive mode and MRM ratios. The method was validated in urine, according to the criteria defined in Commission Decision 2002/657/EC. At least two MRM transitions for each substance were monitored in addition to EPI spectra and deuterated analytes were used as internal standards for quantitation. The reporting level was 0.05 µg mL\(^{-1}\) for the range of analytes tested. The regression coefficients (r\(^2\)) for the calibration curves (0-4 µg mL\(^{-1}\)) in the study were ≥ 0.98. The method proved to be simple and time efficient and was implemented as an analytical strategy for the illicit drug monitoring of opioids, cocaines and amphetamines in criminal samples from crime offenders, abusers or victims in the Republic of Ireland. To the best of our knowledge there are no hybrid LC-MS
applications using MRM mode and product ion spectra in the linear ion trap mode for opioids, cocaines or amphetamines with validation data in urine.

**Keywords:** Drugs of Abuse; Human Urine; Liquid Chromatography Hybrid Triple Quadrupole Linear Ion Trap (QTRAP) Liquid Chromatography Mass Spectrometry; Method Validation

### 7.2 Introduction

The analysis of drugs of abuse such as morphine (MOR), codeine (COD), morphine-3-glucuronide (M-3-G), 6-monoacetylmorphine (6-MAM), cocaine (COC), benzoylecgonine (BENZOYL), buprenorphine (BUPREN), dihydrocodeine (DHC), cocaethylene (COCA), 3,4-methylenedioxyamphetamine (MDA), ketamine, (KET) 3,4-methylenedioxymethamphetamine (MDMA) pseudoephedrine (PSEUDOEPH), lignocaine (LIGNO), benzylpiperazine (BZP), methamphetamine (METHAMP), amphetamine (AMP), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and methadone (METH) in urine is highly important as their illicit use is widespread. The molecular structure of these compounds is shown in Fig. 7.1. Acute intoxication of these drugs either alone or in combination with other drugs is well documented. Urine is a simple aqueous matrix that has been used frequently and is preferred for screening and identification of illicit drugs because the concentrations of drugs and their metabolites can be reasonably high [Rivier 2000]. Urine can be easily sampled and testing is non-invasive, the volume of sample is generally high and urine testing provides long detection windows for drug use, from several days for opiates and cocaines up to months for...
chronic cannabinoid use [Vandevenne et al., 2000]. The drawbacks of this matrix in regulatory monitoring are that the drug concentrations can be affected due to diurnal fluctuation and the effect of fluid intake, in post-mortem cases urine is not always available and to detect the effect of a drug blood needs to be analysed. The use of drugs analysed in this study alone or in combination with other drugs such as cannabis or alcohol is increasingly popular. 6-MAM is the specific metabolite of heroin in urine [Dams et al., 2003]. BZP has the reputation of producing amphetamine type effects [Herndon et al., 1992, Schechter et al., 1988] and is banned in Ireland since March 2009. BENZOYL, an inactive metabolite of COC has a longer half-life than COC [Capello-Peiro et al., 2005] additionally alcohol and COC are widely abused producing COCA having a longer halflife [McCance-Katz et al., 1993, Harris et al., 2003] than COC.

Various adulterants can be added to COC [Shannon et al., 1988, Risser et al., 2007] or to ecstasy tablets [Smith et al., 2002] but the type of drugs used for recreational use can change rapidly [Dillion et al., 2003, Jansen et al., 1993] with substances like KET becoming popular [Kohrs et al., 1998, Anis et al., 1983].

In our laboratory in the Republic of Ireland, the analysis of opioids, amphetamines and cocaines was carried out using three separate analytical procedures using three fulltime analysts and three different GC-MS instruments.

The procedures were well established and evaluated in a large number of external quality control schemes. GC-MS entails a great deal of sample preparation, requires longer chromatographic runtimes and GC-MS can lack sensitivity for certain drugs.

Work was undertaken in order to evaluate newer technology to improve overall analysis time in the laboratory and expand the classes of drugs for analysis. Sample preparation
procedures prior to analysis by LC-MS are generally more simplified. The aim of this study was to develop a fast, simple and reliable sample preparation procedure in urine to analyse 19 drugs representing drugs from the opioid, amphetamine and cocaine classes using a single sample preparation procedure and detection method that can be carried out by a single laboratory analyst. To date the simplest sample preparation procedures in urine in the literature for the determination of drugs included in this study such as AMP, MDA and MDMA [Andersson et al., 2008], MOR, M-3-G, M-6-G, COD, COD-6-glucuronide, ethyl morphine, M-6-G and 6-MAM [Gustavsson et al., 2007] and MOR, BENZOYL, 6-MAM, COC, COCA, METH and EDDP [Dams et al., 2003] were accomplished using direct injection. Alternatively KET has been filtered prior to direct injection [Chen et al., 2007] with subsequent LC-MS detection. To date drugs of abuse in this study have been analysed in urine by LC-ion TRAP MS [Chen et al., 2007, Tsutsumi et al., 2005, Wu et al., 2005, Cheng et al., 2006, Katagi et al., 1996], LC Tandem MS [Andersson et al., 2008, Gustavsson et al., 2007, Dams et al., 2003, Cheze et al., 2007, Concheiro et al., 2007, Kuwayama et al., 2008, Berg et al., 2009, Gergov et al., 2009, Musshoff et al., 2004, Concheiro et al., 2007] and hybrid LC-MS [Mueller et al., 2005]. Chromatographic runtimes were between 8 and 35 min, respectively. The LC-MS chromatography methods cover only a few analytes usually with long chromatographic run-times. There was a paucity of studies that used hybrid LC-MS technology in the literature as the majority of studies use LC-tandem MS. One such hybrid LC-MS technology is the 4000 QTRAP LC-MS from Applied Biosystems. This study evaluated the possibility of using this technology as a single detection technique to replace three separate GC-MS detection techniques. In the 4000 hybrid linear ion trap- triple
quadrupole mass spectrometer, Q3 can be utilized as a quadrupole or a linear ion trap with axial ion injection [Hager et al., 2002]. Operation as a triple quadrupole mass spectrometer is useful when high sensitivity and selectivity of MRM transitions is needed for example in quantitative analysis or targeted screening. Operation as a linear ion trap mass spectrometer is necessary when higher sensitivity in fullscan experiments is required. In EPI mode, Q1 is used to filter the precursor ions. Q2 acts as a collision cell to generate fragments while Q3 working in ion trap mode is used to scan product ions. This results in triple quadrupole MS like fragmentation pattern but with higher sensitivity. The QTRAP has a built in collision energy spread (CES) feature which allows collection of data at the different collision energies in one EPI spectrum.

A method by Mueller et al (2005) analyses 301 drugs qualitatively in blood and urine by 3200 QTRAP hybrid LC-MS with a chromatographic runtime of 30 min. Urine although mentioned in the manuscript data or validation results were not given. In addition one MRM transition was monitored in the study therefore MRM ratio's cannot be calculated if a situation arises and an EPI scan does not trigger successfully and re-injection of the sample would be necessary. Three EPI scans were also utilised in the study at three separate collision energies (CE) which increases the duty cycle. The advantage of the study by Mueller et al., [2005] is the capability to monitor a large number of compounds simultaneously in urine and blood however substances such as COCA, EDDP, BNZY and LIGNO were not analysed. The consumption of alcohol and COC is extremely popular and the detection of COCA is important in forensic toxicology because COCA has a longer detection window than COC [McCance-Katz et al., 1993, Harris et al., 2003]. This study describes the first hybrid triple quadrupole linear ion trap method with
MRM as survey scan and IDA and EPI scan as dependent scan for the determination of COCA, EDDP, BNZY and LIGNO. Furthermore this is the first time that the 19 forensically important drugs have been analysed simultaneously running two MRM's and a single EPI experiment in positive mode in urine providing information on high and low mass fragments in a single injection to the best of our knowledge. Drug identification was carried out by library searching with an in-house developed MS/MS library based on EPI spectra at a single CES of 35 ± 15 in positive mode. Validation of the method was based on Commission Decision 2002/657/EC [Commission Decision 2002], a validation protocol used in the field of veterinary drug residue monitoring. The decision states criteria on the agreement of retention times, base peak and diagnostic ions and relative abundances between the standards and the samples is essential. The decisions are based on the calculation of identification points (IPs) which depend on the analytical technique. In the case of banned substances the minimum number of IPs for a methodology to be considered reliable has to be equal to 4 or higher. This is achieved by monitoring a minimum of 4 selected ions per compound when working with single MS and a minimum of two selected reaction monitoring (SRM) transitions per compound in tandem MS. Thus running two MRM transitions gives 4 IPs and also obtaining an EPI spectra gives additional confirmatory information in this study. In this paper a fast, simple and reliable method is described for the simultaneous analysis of the 19 analytes in urine.
Structure a: EDDP

Structure b: Codeine

Structure c: Dihydrocodeine
Structure d: Methadone

Structure e: Morphine

Structure f: Cocaine
Structure g: 6-MAM

Structure h: Buprenorphine

Structure i: Benzoylegenine
Structure j: MDA

Structure k: MDMA

Structure l: Ketamine

Structure m: Amphetamine
Structure n: Pseudoephedrine

Structure o: Methamphetamine

Structure p: Cocaethylene

Structure q: Lignocaine
7.3 Experimental

7.3.1 Materials and methods

LC-MS grade water, methanol, propan-2-ol (HPLC) were obtained from Reagecon and formic acid was obtained from BDH (Merck, UK). Ammonium acetate was obtained from Sigma-Aldrich. MOR, M-3-G, COCA, COD, DHC, 6-MAM, METH, BUPREN, EDDP, COC, BENZOYL, LIGNO, MOR-d₆, COD-d₆, DHC-d₆, METH-d₉, M-3-G-d₃, BENZOYL-d₈, COC-d₃, METHAMP, KET, MDA, MDMA, PSEUDOEPH, BZP, AMP, METHAMP-d₁₄, MDA-d₅, MDMA-d₅, AMP-d₁₁ were purchased from LGC Standards (LGC, UK). Commercially prepared primary stock standards in solution were purchased from LGC standards available in concentrations ranging from 100-1000 µg mL⁻¹ except for BZP. A stock solution of BZP standard was prepared in methanol at a concentration of 1000 µg mL⁻¹. A working internal standard solution of MOR-d₆, COD-d₆, DHC-d₆, METH-d₉, M-3-G-d₃, BENZOYL-d₈, COC-d₃, METHAMP-d₁₄, MDA-d₅, MDMA-d₅, AMP-d₁₁ was prepared at a concentration of 2 µg mL⁻¹ (stable for 6 months). A intermediate standard solution (stable for 6 months) of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO was prepared at a concentration of
Injection solvent was water:methanol (1:1, v/v). All standards were stored at 4 °C in the dark. Injection solvent was water:methanol (1:1, v/v).

7.3.2 LC conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). The drugs were chromatographed on a 5 μm Phenomenex HYPURITY C8 column (4.6 × 100 mm) (AGB, Ireland) and the column temperature was maintained at 30 °C. A gradient was applied with water and methanol (95:5, v/v + 25 mM ammonium acetate) (A) and methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid) (B) (Table 7.1). The total run time was 12.5 minutes with a flow rate of 0.8 mL min⁻¹. The injection volume was 20 μL. The mass spectrometer used was a QTRAP™ 4000 with a TurboIonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.5 of Analyst software.

7.3.3 MS/MS/EPI

The analysis was performed using positive ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. Two transitions were used and the collision energy was optimised as shown (Table 7.2). The MRM MS/MS detector conditions were as follows: ion mode electrospray positive; curtain gas 25 psi; ion spray voltage 5000 V; temperature
650 °C; ion source gas 1 50 psi; ion source gas 2 50 psi; interface heater on; entrance potential 10 V; resolution Q1 unit; resolution Q2 unit; collision-activated dissociation CAD gas = medium

The strongest MRM transition and the CES spectra at 35 ± 15 for each substance were chosen from the enhanced product ion spectra (EPI mode) to set up the library. The IDA scan intensity threshold was set at 500 counts per second (cps). The dependent scan was an EPI scan which was carried out at the CES conditions before switching back to MRM mode. The resulting EPI spectra was then searched against the mass spectral library. The set up of the library was achieved as follows; the LC parameters described above were utilised and the injection volume was 20 μL; concentration of each substances was 0.1 μg mL⁻¹. Turbo Ion spray source in EPI scan mode with 60 V declustering potential. Q1 Resolution was unit. Dynamic fill time of the trap (Q3) was set. Curtain gas 25 psi; ion spray voltage 5000 V; temperature 650 °C; ion source gas 1 50 psi; ion source gas 2 50 psi; CAD medium; CES 35±15 V.

7.3.4 Urine samples

Urine obtained for use as negative controls were separated into 50 mL aliquots and stored at −20 °C. The urine was analysed in previous batches and urine found to contain no detectable residues of opioids, cocaines and amphetamines were used as negative controls.
Table 7-1: LC gradient profile for determination of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO

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Component A: water:methanol (95:5, v/v + 25 mM ammonium acetate)
Component B: methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid)
Table 7-2: MS/MS parameters for determination of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO

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<th>Compound</th>
<th>Transition</th>
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<th>Collision energy [V]</th>
<th>Collision exit potential [V]</th>
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<tr>
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<tr>
<td>6-MAM</td>
<td>300.0+193.0</td>
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<tr>
<td>328.0+190.0</td>
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</tbody>
</table>

Note: Matrix matched curves were used for quantification and deuterated internal standards were used as internal standards for all compounds.

7.3.5 Sample Preparation

Urine samples (100 µl) were aliquoted into 15 ml polypropylene tubes. The urine aliquots were fortified with internal standard at levels corresponding to 0.1 µg mL⁻¹ by adding a 100 µL portion of a 2 µg mL⁻¹ mix solution of MOR-d₆, COD-d₆, DHC-d₆, METH-d₉, M-
3-G-d₃, BENZYL-d₈, COC-d₃, METHAMP-d₁₄, MDA-d₅, MDMA-d₃ and AMP-d₁₁.

Samples were fortified at levels corresponding to 0.1, 0.5 and 1 μg mL⁻¹ by adding 40 μL of a 0.25 μg mL⁻¹ fortification solution and 20 and 40 μL portions of a 2.5 μg mL⁻¹ fortification solution. After fortification, samples were held for 15 min prior to the next analytical step. Methanol:water (1:1, v/v) (1800 μL) was added to the urine samples and vortexed (30 sec), centrifuged (3568 g, 5 min, 4 °C) and the supernatant was transferred to an autosampler vial. An aliquot (20 μL) was injected on the LC column.

7.3.6 Matrix-Matched Calibration

Matrix matched calibration curves were prepared and used for quantification. Control urine previously tested and shown to contain no residues was prepared as above (7.3.4).

Control urine sample (10 mL) was diluted with methanol:water (1:1, v/v) to 200 mL. A single urine sample was used for each calibration standard level. Urine samples (mL) were aliquoted into 50 mL polypropylene tubes and samples were fortified with internal standard at levels corresponding to 0.1 μg mL⁻¹ by adding a 100 μL portion of a 2 μg mL⁻¹ mix solution of MOR-d₆, COD-d₆, DHC-d₆, METH-d₆, M-3-G-d₃, BENZYL-d₈, COC-d₃, METHAMP-d₁₄, MDA-d₅, MDMA-d₃ and AMP-d₁₁. Calibration standard levels were fortified at levels corresponding to 0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 μg mL⁻¹ by adding 0, 20, 40, 100 μL portions of a 0.25 μg mL⁻¹ fortification solution and 20, 40, 80 and 160 μL portions of a 2.5 μg mL⁻¹ standard solution of MOR, M-3-G, COD, DHC, 5-MAM, METH, EDDP, BUPREN, COC, BENZYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO. After fortification, samples were held for 15 min prior to the vortexing and centrifugation procedure as described above.
The concentration of the drugs (μg mL⁻¹) were determined from the matrix
matched calibration curves. The calibration curves were calculated by linear regression,
plotting the response factor (peak area analyte/internal standard peak area of the strong
transition as a function of analyte concentration).

7.3.7 Method validation

For estimation of accuracy, blank urine samples were fortified with MOR, M-3-G, COD,
DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP,
PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO at 0.1, 0.5 and 1.0
μg mL⁻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. For
the estimation of the precision of the method, repeatability and within-laboratory
reproducibility was calculated. The decision limit (CCα) of the method was calculated
according to the ISO 11843 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. The detection capability (CCβ)
was calculated by adding 1.64 times the standard error to the CCα. Carryover was
investigated by analysing a blank solvent before and after each injection during validation
and routinely in each analytical batch. The stability of standard solutions was evaluated
by quantifying levels in an external quality control material over a six month period as a
QC is ran with every batch routinely. Short-term stability of extracts was performed by
analysing extracts held at 4 °C for 48 hours. Matrix effects were investigated by infusion
of all analytes (2.5 μg mL⁻¹) by an external syringe pump to a tee-connector at 10 μL mL⁻¹.
between the electrospray probe and the outlet of the analytical column with simultaneous injection of methanol:water (1:1, v/v). Subsequently 5 different blank matrices diluted in methanol:water (1:1, v/v) were injected on the analytical column. The specific ion transitions of the analytes in different blank matrices were recorded and any signal decreasing or increasing at the retention time of the investigated analyte was compared with the methanol:water (1:1, v/v) injection.

7.4 Results and Discussion

7.4.1 Preliminary experiments

In this study an analytical strategy was developed to analyse urine samples to detect drugs of abuse. The LC-MS/MS method using MRM mode and product ion spectra in the linear ion trap mode (Q3) was developed to provide unequivocal confirmatory data for the analysis of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO. The ionization of all drugs was studied in positive mode. The optimum conditions (declustering potential, collision energy, collision cell exit potential) were determined for each drug and the best diagnostic ions for MS/MS analysis were obtained and can be seen in Table 7.2. For a method to be deemed confirmatory 4 identification points must be obtained. In MRM (multiple reaction monitoring) mode this is achieved by monitoring one precursor ion (parent mass) and two daughter ions (corresponding to strong and weak ion) which is a suitable confirmatory method in accordance with 2002/657/EC [Commission Decision 2002]. Precursor and product ions for each analyte of interest were determined by direct infusion of single analyte solutions (1 µg mL\(^{-1}\) in methanol:water 1:1 v/v). Chromatographic tests were carried out using a 5 µm
HYPURITY C₈ column (4.6 x 100 mm). All analytes were eluted at a flow rate of 800 μL min⁻¹ and a runtime of 12.5 min per injection with good peak shape when using a mobile phase of water:methanol (95:5, v/v + 25 mM ammonium acetate) (A) and methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid) (B). The formic acid was used to assist the ionisation of the analytes in positive mode and improve peak shape by reducing peak tailing. The ammonium acetate assists with separation of the analytes. In the initial stages of development carryover was observed but a wash program was set up in the autosampler. No carryover problem was noted during validation and during routine use of the method when solvent blanks are analysed before and after samples. In MRM mode the possibility of cross talk of analytes with internal standards was evaluated after tuning by injection of standards on column singularly. Crosstalk occurs if two compounds co-eluting have similar fragment ions in two successive transitions in an MRM method. The degree of the problem depends on the instrument speed as the fragment ions pass through the collision cell rapidly enough to exit the cell before the same fragments of the next compound come in. However this can cause dead time making the scan cycle time significantly longer leading to fewer datapoints across the chromatographic peak. No issue with crosstalk was identified during the evaluation. A EPI experiment was set up in the Analyst 1.5 software. The strong MRM transition was chosen upon completion of tuning in MRM mode. The IDA scan intensity threshold was set at 500 cps (counts per second) in the instrument method. The dependent scan was an EPI scan set at 35 CES ± 15. One drawback of the Analyst 1.5 software in data dependent mode is that the software only allows a single preselected CE or CES for all analytes in an EPI experiment. It would be better if the software manufacturers in the future would
allow individual DP and CE settings per compound in EPI dependent scan mode. The resulting EPI spectra was searched against a mass spectra library. In Figure 7.2 chromatogram of COCA is shown. In Figure 7.3 the library spectrum of COCA and also the acquired library spectrum of COCA at a CES of 35 ± 15 including the library search fit values is shown. The fit value (Fit) gives information about the resemblance of the signals in the reference spectrum with those in the unknown spectrum. The reverse fit (Reverse Fit) gives information on the resemblance of the signals in an unknown spectrum with those in the reference spectrum. The purity (Purity) is a combination of both other values. For COCA in Figure 7.3 the purity value was greater than 94%. The stability of the solution standards was evaluated by quantifying levels in an external QC material over a six month period as the QC material was analysed with every batch routinely and is within specification. The short term stability of sample extracts was acceptable when extracts were stored at 4°C for 48 hours. In the initial stages of method development matrix suppression was witnessed at the beginning of the chromatographic runtime and as a result the eluent was diverted to waste initially to reduce the bulk of matrix components. To further reduce any possible matrix effects deuterated internal standards were used. During the evaluation period 233 samples were analysed by this LC-MS procedure and an established GC-MS procedure and results were in good agreement. Often in forensic toxicology cases it can be difficult for the pathologist to obtain large sample sizes. Preconcentration of urine during method development was not required based on the sensitivity achieved by the QTRAP method thus the urine samples (100 µL) were diluted 20 fold in methanol:water (1:1, v/v) and a good peak shape was achieved. It was envisaged that urine samples would not require extensive clean-up due
to its low protein and high aqueous content. The first advantage of the sample preparation procedure is a small sample volume is required. The second advantage is that the 19 drugs can be analysed rapidly and simultaneously using simple dilution with methanol:water (1:1, v/v). The third advantage is that the sample preparation procedure reduces the workload in monitoring for these substances in any laboratory and as a result a single analyst is capable of preparing a matrix matched curve and 50 samples in a single day. A fourth advantage is the savings in cost as there is no need to purchase costly hydrolysis reagents for the detection of drugs that are extensively metabolised. The first advantage of the hybrid LC-MS detection method developed in this study is the fast run-time of 12.5 min per injection allowing detection of 19 forensically important drugs. The second advantage of is that high and low concentrations of the drugs in urine samples can be identified, quantified and confirmed simultaneously in a single injection using EPI spectra. High levels of drugs can be detected routinely using EPI spectra and the samples do not need to be re-injected as EPI spectra can be used to unambiguously confirm overdose cases in a straightforward manner. The disadvantage of using MRM ratio’s only is that the sample will require dilution as a result of detector saturation and re-injection.

The method has been used since 2009 to replace three other analytical strategies in our laboratory for detection of opioid, cocaine and amphetamine drugs in urine. In addition full laboratory information management system (LIMs) connectivity of the analytical strategy has been achieved using Analyst 1.5 software as part of routine monitoring of forensic toxicology samples.
7.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [Commission Decision 2002] covering specificity, calibration curve linearity, accuracy, precision, decision limit (CCα) and detection capability (CCβ).

7.4.2.1 Specificity

The technique of liquid chromatography hybrid triple quadrupole linear ion trap (QTRAP) mass spectrometry itself offers a very high degree of selectivity and specificity. To establish the selectivity/ specificity of the method, urine samples (30) were fortified with the above drugs and also non-fortified samples were analysed. Interfering peaks were observed at the retention time of some of the analytes in the chromatograms of the non-fortified samples but the response was negligible when compared to the reporting level.

7.4.2.2 Linearity of the response

The linearity of the chromatographic response was tested with matrix matched curves using 8 calibration points in the concentration range of 0 to 4.0 μg mL⁻¹. In routine monitoring 25 analytical batches were analysed and the regression coefficients ($r^2$) for all the calibration curves were ≥ 0.98.

7.4.2.3 Accuracy

The accuracy (n=18) of the method was determined using human urine samples fortified at 0.1, 0.5 and 1.0 μg mL⁻¹ for in three separate assays was 84-113%.
7.4.2.4 Precision

The precision, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (0.1, 0.5 and 1.0 µg mL\(^{-1}\)) was less than 12 % (Table 7-3).

Table 7-3: Intra- and inter-assay variation for accuracy of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, EUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO.
7.4.2.5 CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1% is applied. The detection capability (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, were β = 5%. CCα and CCβ were calculated using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (0.1, 0.5 and 1.0 μg mL⁻¹). CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. 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). The full list of CCα and CCβ values are shown in Table 7.4. In our methodology CCα values of 0.03-0.05 μg mL⁻¹ and CCβ values of 0.04-0.09 μg mL⁻¹ were obtained. Although CCα and CCβ values are widely adopted in the field of veterinary drug residues. The use of these parameters was investigated as an alternative approach to limit of detection and limit of quantification in this study. Based on the calculated CCα level in this validation study samples were analysed to verify that the method can detect the calculated CCα level. In our methodology CCα values of 0.03-0.05 μg mL⁻¹ were obtained during validation. The lowest level in our matrix matched calibration curve standard is 0.05 μg mL⁻¹. This standard give acceptable signal-to-noise ratio’s for each compound and is used routinely as the reporting level.
7.4.2.6 Measurement Uncertainty

According to SANCO/2004/2726 rev 1, the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [SANCO 2004]. For the calculation of the extended uncertainty, a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the CCα, corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different urine sourced from different humans, it was decided to use a safety factor of 3.0 instead of 2.33. The measurement uncertainty of the method was estimated at 32, 26, 29, 27, 18, 16, 35, 30, 19, 28, 16, 20, 30, 18, 25, 17, 13, 31, and 20% for MOR, COD, M-3-G, 6-MAM, COC, BENZOYL, BUPREN, DHC, COC, MDA, KET, MDMA, PSEUDOEPH, LIGNO, BZP, METHAMP, AMP, EDDP and METH. This was determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

7.4.2.7 Evaluation

The method developed in this study has been used to evaluate the presence of MOR, COD, M-3-G, 6-MAM, COC, BENZOYL, BUPREN, DHC, COC, MDA, KET, MDMA, PSEUDOEPH, LIGNO, BZP, METHAMP, AMP, EDDP and METH in human urine in the Republic of Ireland in 2009. In monitoring for these substances at our laboratory drug identification was carried out by library search with a developed inhouse MS/MS library based on EPI spectra at a collision energy spread (CES) of 35 ± 15. Additionally, routinely it was possible to detect the precursor ion and two daughter ions (within a
single injection) in multiple reaction monitoring mode as well as generating an EPI spectra under collision energy spread conditions. The method has been carried out using different batches of urine, different QC material, by different analysts, using different batches of reagents, under varying environmental conditions and the method was shown to be robust. To demonstrate the applicability of the method incurred urine samples taken from subjects treated with MOR, COD and BENZOYL from the QC Reference Material were tested. These QC samples had values ranging from 0.25-0.37 μg mL\(^{-1}\) for MOR, 0.23-0.35 μg mL\(^{-1}\) for COD and 0.13-0.19 μg mL\(^{-1}\) for BENZOYL. The QC for MOR, COD and BENZOYL were found to be non-compliant as they contained levels above CCo\(_{\alpha}\) and the calculated concentrations were within the specified range of the QC material. Furthermore the EPI spectra confirmed unambiguously the presence of MOR, COD and BENZOYL as spectra matched the corresponding spectra in the library developed in-house. To further demonstrate the method applicability the method has been used to analyse a number of urine proficiency testing (PT) samples in which subjects were treated with MOR, BENZOYL, BUPREN, AMP, EDDP, METH and KET. The PT samples were analysed by the method developed in this study and were found to be non-compliant as they contained levels above the calculated CCo\(_{\alpha}\). The EPI spectra matched the corresponding spectra in the library developed in-house in Analyst 1.5 software and satisfactory Z-scores of below 1.4 were obtained for PT samples. Furthermore a PT sample negative for cocaines, opiates and amphetamines was analysed by this analytical strategy and was reported as being negative thus further ensuring that an accurate analytical strategy was developed. The method was also stringently evaluated in-house by comparison with established GC methods (3xGC-Ion Trap methods for opiates, cocaines...
and amphetamines) and running all incoming samples simultaneously with old and new analytical methods. The results (unpublished data) were acceptable. The developed analytical strategy performs very well in terms of accuracy and within-laboratory reproducibility.

7.4.2.8 Case Study

The described methodology has been applied in the laboratory since 2009 and positive drugs of abuse were identified in forensic criminal samples from crime offenders, abusers or victims using this method. The method has been used to analyse 233 samples from 01/03/2009 until 14/12/2009. The following substances were confirmed to be present in samples during this time period.

The presence of 6-MAM was confirmed in 110 samples. The presence of BENZOYL was confirmed in 91 samples. LIGNO was found in 78 samples. COC was found in 67 samples, METH was found in 65 samples. EDDP was found in 63 samples. AMP was found in 45 samples. COCA was found in 43 samples. MOR was found in 25 samples. BNZY was found in 21 samples. MDA was found in 17 samples. PSEUDOEPH and COD was found in 12 samples. MDMA was found in 7 samples. METHAMP was found in 6 samples and DHC was found in 3 samples during this time period. BENZOYL is the main metabolite of COC in urine and LIGNO is often used as an adulterant in COC. It can be concluded that 6-MAM which is a marker for heroin and COC dominates the picture during this time period. The results obtained using the analytical strategy developed in this study were reported in forensic cases in the Republic of Ireland.
Table 7.4: Calculated $CC\alpha$ and $CC\beta$ values in urine for of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP and LIGNO

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<th>$CC\beta$ (mg/mL⁻¹)</th>
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</tr>
<tr>
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</tr>
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</tr>
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Fig. 7-2: Chromatogram of Negative Control Urine fortified with 0.1 \( \text{ug.mL}^{-1} \) of \( \text{d}_3 \)-Cocaine (A) and fortified with 0.1 \( \text{ug.mL}^{-1} \) of Cocaethylene and at 0.1 \( \text{ug.mL}^{-1} \) with internal standard \( \text{d}_3 \)-Cocaine (B).

Fig. 7-3: Spectra of Cocaethylene
7.5 Conclusions

The developed strategy has been carried out using different batches of urine, different QC material, by different analysts, using different batches of reagents, under varying environmental conditions. The developed method shows good agreement with reference GC-MS methods (not shown). The advantage of a small sample size and the ability to confirm the identity of a wide variety of drugs in a single injection has important advantages for high sample throughput in a regulatory laboratory. Matrix effects studies were carried out and results have shown that utilizing a labelled internal standard, dilution of samples and a diverter valve minimised the effects. The accuracy of the method has been further certified as acceptable results were obtained by method comparison with PT samples and reference GC-MS methods. In conclusion the method shows that simple dilution of urine and analysis by hybrid LC-MS technology can present a rugged analytical strategy. There are no methods in the literature to the best of our knowledge that analyse the 19 drugs simultaneously in this study in urine representing opioids, cocaines or amphetamines by simple dilution and hybrid LC-MS using a hybrid linear ion trap-triple quadrupole mass spectrometer in MRM mode and product ion spectra in the linear ion trap mode. Therefore the aim of developing a fast, simple and reliable sample preparation and hybrid LC-MS strategy for opioids, cocaines and amphetamines in this study has been achieved successfully.

7.6 Acknowledgements

The authors would like to thank staff at The State Laboratory, Ireland for their practical assistance and for permission to publish this article.
Chapter 8: A new mixed mode solid phase extraction strategy for opioids, cocaines, amphetamines and adulterants in human blood with hybrid liquid chromatography tandem mass spectrometry detection

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8.1 Abstract

A rapid method has been developed to analyse morphine, codeine, 6-monoacetylmorphine, cocaine, benzoylegonine, dihydrocodeine, cocaethylene, 3,4-methylenedioxyamphetamine, ketamine, 3,4- methylenedioxymethamphetamine, pseudoephedrine, lignocaine, benzylpiperazine, methamphetamine, amphetamine, methadone, phenethylamine and levamisole in human blood. Blood samples were cleaned up using mixed mode solid phase extraction using Evolute CX™ solid phase extraction cartridges and the sample aliquots were analysed by hybrid triple quadrupole linear ion trap (QTRAP) mass spectrometry with a runtime of 12.5 min. Multiple reaction monitoring (MRM) as survey scan and an enhanced product ion (EPI) scan as dependent scan were performed in an information-dependent acquisition (IDA) experiment. Finally, drug identification and confirmation was carried out by library search with a developed inhouse MS/MS library based on EPI spectra at a collision energy spread of 35±15 in positive mode and MRM ratios. The method was validated in blood, according to the criteria defined in Commission Decision 2002/657/EC. At least two MRM transitions for each substance were monitored in addition to EPI spectra and deuterated analogues of analytes were used as internal standards for quantitation where possible. The method proved to be simple and time efficient and was implemented as an analytical strategy for the illicit drug monitoring of opioids, cocaines, amphetamines and adulterants in forensic cases of crime offenders, abusers or victims in the Republic of Ireland.
8.2 Introduction

Acute intoxication of drugs either alone or in combination with other drugs is well documented. In 2009 the veterinary drug LEV which is a veterinary anthelmintic drug has come into recent attention in the public health and medical communities as an alleged new cutting agent in adulterated cocaine. There have been a few cases first in Canada and then in the United States of patients with life-threatening cases of neutropenia/agranulocytosis as a result of LEV-adulterated cocaine [Zhu et al., 2009, Kinzie et al., 2009]. PHEN is a substance that can cause false positive results for amphetamines when immunoassay is used [Eichorst et al., 1991].

The molecular structure of these compounds are shown in Fig. 8.1.

**Structure 1: Levamisole**

![Structure 1: Levamisole](image)

**Structure 2: Phenethylamine**

![Structure 2: Phenethylamine](image)

*Figure 8.1 Structures of Levamisole and Phenethylamine*
The analysis of these and drugs such as morphine (MOR), codeine (COD), 6-monoacetylmorphine (6-MAM), cocaine (COC), benzoylcgonine (BENZOYL), dihydrocodeine (DHC), cocaethylene (COCA), 3,4-methylenedioxyamphetamine (MDA), ketamine, (KET) 3,4-methylenedioxyamphetamine (MDMA) pseudoephedrine (PSEUDOEPH), lignocaine (LIGNO), benzylpiperazine (BZP), methamphetamine (METHAMP), amphetamine (AMP) and methadone (METH) in blood is of vital importance in forensic toxicology. Blood is an important matrix as provides a sample screen of toxic substances present in the body at the time of collection. In our laboratory in the Republic of Ireland, the analysis of opioids, amphetamines and cocaines in blood was carried out using three separate sample preparation procedures and three different GC-MS instruments with additional derivatisation procedures. LEV was not monitored previously but the procedures were well established in blood but time consuming. The aim of this work was to develop a single fast, simple and reliable sample preparation procedure in blood to analyse the 18 drugs in this study. A study carried out by Juhascik et al. [2009] investigated whether switching from an established liquid liquid partitioning (LLE) sample preparation procedure in blood to a solid phase extraction (SPE) sample preparation procedure was feasible for drugs of abuse. The study found that SPE had lower limits of detection for a wider range of drugs, was capable of detecting drugs that were previously not detectable by LLE and was shown to be a faster technique than LLE. Further benefits of the technique of SPE include reduction of matrix effects, the ability to automate the sample preparation procedure, dual retention mechanisms, improved sorbent chemistries for rigorous wash and elution protocols, improved sensitivity and decreases exposure and costs due to hazardous solvents. The main
challenge of SPE is the large number of parameters that need to be adjusted to optimise
the sorbent chemistry performance. To date sample preparation procedures in whole
blood in the literature using LC coupled to various detection systems for the
determination of drugs included in this study was achieved by solvent extraction

[Juhascik et al., 2009, Shima et al., 2008], liquid/liquid extraction [Juhascik et al., 2009,
Gergov et al., 2009, Mueller et al., 2005, Gergov et al., 2003, Logan et al., 1987] and SPE
[Mueller et al., 2005 Johansen et al., Bogusz et al., 1997, Dams et al., 2002, Decaestecker
et al., 2003, Chen et al., 1993, Gerostamoulos et al., 1995]. SPE sorbent chemistries
utilized were reverse phase [Decaestecker et al., 2003, Bogusz et al., 1997,
Gerostamoulos et al., 1995], cation exchange [Decaestecker et al., 2003, Johansen et al.,
2007, Dams et al., 2002] strong/weak mixed mode cation exchange sorbents [Dams et al.,
2002, Decaestecker et al., 2003, Chen et al., 1993] and polymeric sorbents utilizing Oasis
HLB [Decaestecker et al., 2003]. A new mixed mode SPE sorbent chemistry technology
was developed by Biotage™ with an optimised pore size and was evaluated as a single
purification strategy for the 18 drugs in blood. To date drugs in our study have been
analysed using LC coupled to different detectors including LC-diode array and
fluorescence detectors [Dams et al., 2002], LC-electrochemical detector [Logan et al.,
1987, Chen et al., 1993, Gerostamoulos et al., 1995]. LC-MS [Gergov et al., 2009,
Bogusz et al., 1997], LC-MS/MS [Shima et al., 2008, Gergov et al., 2003, Johansen et
al., 2007, Decaestecker et al., 2003, Che’ze et al., 2007] and hybrid LC-MS [Mueller et
al., 2005]. The benefits of the technique of LC-MS are that it does not require
derivatisation, can analyse both free and conjugated drugs simultaneously, can analyse
thermolabile drugs directly, short chromatographic runtimes, easy online coupling to SPE
and sample preparation procedures prior to analysis by LC-MS are generally more simplified. The disadvantage is the possibility of matrix effects. Evaluation of the literature showed the majority of studies in blood analysed blood by tandem LC-MS with chromatographic runtimes varying from 10 to 30 min. There are limited studies using hybrid LC-MS technology such as the 4000 QTRAP LC-MS system available from Applied Biosystems. The hybrid LC-MS method in this study was based on work undertaken at our laboratory for drugs of abuse in urine [Dowling et al., 2010]. The second aim of this study was to extend the method to include LEV and PHEN. In the 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer, Q3 can be operated as a quadrupole or as a linear ion trap with axial ion injection [Hager et al., 2002]. The instrument also has the capability to perform a large number of survey scans because it has a linear acceleration collision cell (LINAC) [Sasaki et al., 2007] that enables ions to be transported through the system rapidly. Further information relating to this type of LC-MS is previously described [Dowling et al., 2010]. A method developed by Mueller et al [2005] analyses 301 drugs qualitatively in blood and urine by 3200 QTRAP hybrid LC-MS with a chromatographic runtime of 30 min. Blood was mentioned in the manuscript but no validation results were given and results were qualitative only. A disadvantage was that only one MRM transition is monitored and if a situation arises where the EPI scan does not trigger re-injection of samples would be necessary. Three EPI scans in addition are utilised at three separate collision energies (CE) which increases the duty cycle in the study and substances such as COCA, BNZY, LEV and PHEN were not analysed in blood. This study describes a new single solid phase extraction sample preparation procedure using Evolute ABN™ CX in blood for the analysis of COCA.
BNZY, LEV and PHEN and the 14 other drugs with detection by hybrid LC-MS running two MRM's and a single CES at $\pm 15$ in positive mode. Validation was based on Commission Decision 2002/657/EC [Commission Decision 2002] and in-house procedure and drug identification was achieved by library searching based on EPI spectra at a single CES of $35 \pm 15$ in positive mode. The method is being used as an analytical strategy in the Republic of Ireland in forensic cases.

8.3 Experimental

8.3.1 Materials and Reagents

LC-MS grade water, ethyl acetate, methanol, propan-2-ol (HPLC) were obtained from Reagecon and formic acid was obtained from BDH (Merck, UK). Ammonium acetate and ammonium hydroxide were obtained from Sigma Aldrich. Evolute™ ABN CX solid phase extraction cartridges were obtained from Biotage (Biotage, UK).

MOR, COCA, COD, DHC, 6-MAM, METH, BUPREN, EDDP, COC, BENZOYL, LIGNO, LEV, PHEN, MOR-d$_6$, COD-d$_6$, DHC-d$_6$, METH-d$_9$, M-3-G-d$_7$, BENZOYL-d$_8$, COC-d$_3$, KET- d$_4$, PSEUDOEPH- d$_3$, 6-MAM- d$_6$, EDDP- d$_3$, BNZY- d$_8$, METHAMP, KET, MDA, MDMA, PSEUDOEPH, BZP, AMP, METHAMP-d$_{14}$, MDA-d$_5$, MDMA-d$_5$, AMP-d$_{14}$ were purchased from LGC Standards (LGC, UK). Commercially prepared primary stock standards in solution were purchased from LGC standards available in concentrations ranging from 100-1000 $\mu$g mL$^{-1}$ except for BZP. A stock solution of BZP standard was prepared in methanol at a concentration of 1000 $\mu$g mL$^{-1}$. A working internal standard solution of MOR-d$_6$, COD-d$_6$, DHC-d$_6$, METH-d$_9$, M-3-G-d$_3$, BENZOYL-d$_8$, COC-d$_3$, KET- d$_4$, PSEUDOEPH- d$_3$, 6-MAM- d$_6$, EDDP- d$_3$, BNZY- d$_8$, 263
METHAMP-d\textsubscript{14}, MDA-d\textsubscript{5}, MDMA-d\textsubscript{5}, AMP-d\textsubscript{11} was prepared at a concentration of 2 \(\mu\)g mL\textsuperscript{-1} (stable for 6 months). A intermediate standard solution (stable for 6 months) of MOR, COD, DHC, 6-MAM, METH, EDDP, BUPREN, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP, LIGNO, LEV and PHEN was prepared at a concentration of 10 \(\mu\)g mL\textsuperscript{-1} (stable for 6 months). Standard fortification solutions (stable for 6 months) were prepared in methanol at a concentration of 1.25 \(\mu\)g mL\textsuperscript{-1} and 5 \(\mu\)g mL\textsuperscript{-1} from the 10 \(\mu\)g mL\textsuperscript{-1} intermediate stock solution. All standards were stored at 4 °C in the dark. Injection solvent was water:methanol (50:50, v/v). 50 mM ammonium acetate, 2% formic acid and 100 % methanol were used as solid phase extraction wash solvents. 5% ammonium hydroxide in ethyl acetate (70:30, v/v) was used as the solid phase extraction elution solvent. Injection solvent was water:methanol (50:50, v/v).

Generic manufacturer’s SPE procedure (Procedure A) involved dilution of blood 1:5 with 50 mM ammonium acetate buffer at pH 6.0, sonication of the samples (10 min), centrifugation (3000 rpm, 10 min) and passing the supernatant under gravity through an SPE cartridge preconditioned with methanol (3 mL) and ammonium acetate buffer (3 mL). The cartridges were washed with ammonium acetate buffer (2 mL), methanol (2 mL) and eluted with 5% ammonium hydroxide: methanol (3 mL). Modified Generic manufacturer’s SPE procedure (Procedure B) consisted of an acid wash step using 2 % formic acid being introduced prior to the methanol wash step to ensure that all drugs were ionised and retained on the cartridge prior to the high organic wash step. Wash solvent volumes were increased from 2 to 3 mLs. A drying step of 20 minutes was introduced also. Elution solvent study (Procedure C) involved using procedure B but elution with
ethyl acetate:methanol:ammonium hydroxide (68:25:2, v/v/v) or (70:25:5, v/v/v). Ethyl acetate:ammonium hydroxide (98:2, v/v) or (95:5, v/v). Methylene chloride:isopropanol:ammonium hydroxide (78:15:2, v/v/v) or (80:15:5, v/v/v). Elution volumes of 3 x 2 mL were used.

8.3.2 LC conditions

The LC consisted of an Agilent 1200 Rapid Resolution LC equipped with a G1312B Binary pump, G1316B-HiPALS SL autosampler and a G1316B-TCCSL column oven (Agilent Ireland). The drugs were chromatographed on a 5 μm Phenomenex HYPURITY C8 column (4.6 x 100 mm) (AGB, Ireland) and the column temperature was maintained at 30 °C. A gradient was applied with water and methanol (95:5, v/v + 25 mM ammonium acetate) (A) and methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid (B) (Table 8.1). The total run time was 12.5 minutes with a flow rate of 0.8 mL min⁻¹. The injection volume was 20 μL. The mass spectrometer used was a QTRAP 4000 with a TurboIonSpray source from Applied Biosystems (Applied Biosystems/MDS-Sciex, Canada). The MS was controlled by version 1.5 of Analyst software.

8.3.3 MS/MS/EPI parameters

The analysis was performed using positive ion electrospray MS/MS in multiple reaction monitoring (MRM) mode. Two transitions were used and the collision energy was optimised as shown (Table 8.2). The MRM MS/MS detector conditions were as follows: ion mode electrospray positive; curtain gas 25 psi; ion spray voltage 5000 V; temperature 650 °C; ion source gas 1 50 psi; ion source gas 2 50 psi; interface heater on; entrance
potential 10 V; resolution Q1 unit; resolution Q2 unit; collision-activated dissociation CAD gas = medium. The strongest MRM transition and the CES spectra at 35 ± 15 for each substance were chosen from the enhanced product ion spectra (EPI mode) to set up the library. The dependent scan was an EPI scan which was carried out at the CES conditions before switching back to MRM mode. The resulting EPI spectra was then searched against the mass spectral library. The set up of the library was achieved as follows; the LC parameters described above were utilised and the injection volume was 20 µL; concentration of each substance was 0.1 µg mL⁻¹.

Table 8.1: Gradient profile for determination of MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN

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<td>12.5</td>
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Component A: water:methanol (95:5, v/v + 25 mM ammonium acetate)
Component B: methanol:propan-2-ol (97.95:2, v/v + 0.05 mM % formic acid)
Table 8.2: MS/MS parameters for determination of MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN

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| Note: Matrix matched curves were used for quantification of all compounds.

8.3.4 Blood samples

Blood obtained for use as negative controls were separated into 50 mL aliquots and stored at -20 °C. The blood was analysed by the methodology described in this paper to ensure it was negative before being used in validation studies.
8.3.5 Sample preparation

Blood samples (500 μL) were aliquoted into 50 mL polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 0.2 μg mL⁻¹ by adding a 100 μL portion of a 2 μg mL⁻¹ mix solution of MOR-d₆, COD-d₆, DHC-d₆, METH-d₆, M-3-G-d₃, BENZOYL-d₈, COC-d₃, KET- d₄, PSEUDOEPH-d₃, 6-MAM- d₆, EDDP- d₃, METHAMP-d₁₄, MDA-d₅, MDMA-d₅ and AMP-d₁₁. Samples were fortified at levels corresponding to 0.05, 0.075 and 0.1 μg mL⁻¹ by adding 20, 30 and 40 μL of a 1.25 μg mL⁻¹ fortification solution. After fortification, samples were held for 15 min prior to extraction. Ammonium acetate (50 mM, 5 mL) buffer pH 6 (adjusted with concentrated formic acid) was added and the samples were sonicated (10 min). The samples were centrifuged (3568 g, 10 min, 4 °C) and the supernatant was transferred to a clean polypropylene tube. The sample extracts were further purified by mixed mode cation exchange solid phase extraction using Evolute™ CX SPE cartridges. Sample extracts were loaded onto the cartridges (preconditioned with methanol (3 mL)) and ammonium acetate buffer (50 mM, 3 mL). The cartridges were washed with ammonium acetate buffer (3 mL), 2% formic acid (3 mL), methanol (3 mL) and then dried using a vacuum pump (20 min). The cartridges were eluted with 5% ammonium hydroxide in ethyl acetate: methanol: 70:25, v/v (3 x 2 mL). The eluates were reduced to dryness under nitrogen at 40 °C before re-dissolving in 500 μL of methanol:water (50:50, v/v). An aliquot (20 μL) was injected onto the LC column.
8.3.6 Matrix-Matched Calibration

Matrix matched calibration curves were prepared and used for quantification. Control blood previously tested and shown to contain no residues was prepared as above (8.3.4). Control blood samples (500 μL) were weighed into 50 mL polypropylene tubes. Samples were fortified with internal standard at levels corresponding to 0.2 μg mL⁻¹ by adding a 100 μL portion of a 2 μg mL⁻¹ mix solution of MOR-d₆, COD-d₆, DHC-d₆, METH-d₉, M-3-G-d₃, BENZOYL-d₈, COC-d₃, KET- d₄, PSEUDOEPH- d₃, 6-MAM- d₆, EDDP- d₃, METHAMP-d₁₄, MDA-d₅, MDMA-d₅ and AMP-d₁₁.

Samples were fortified at levels corresponding to 0, 0.025, 0.05, 0.1 and 0.25 μg mL⁻¹ by adding 0, 10, 20, 40 and 100 μL portions of a 1.25 μg mL⁻¹ standard solution. Samples were fortified at the 0.5, 1.0 and 2.0 μg mL⁻¹ calibration levels by adding 50, 100 and 200 μL portions of a 5 μg mL⁻¹ standard solution. After fortification, samples were held for 15 min prior to extraction procedure as above (8.3.5). The concentration of the drugs (μg mL⁻¹) were determined from the matrix matched calibration curves.

8.3.7 Method validation

For estimation of accuracy, blank blood samples were fortified with MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET, METHAMP, LIGNO, LEV and PHEN at 0.05, 0.075 and 0.1 μg mL⁻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. For the estimation of the precision of the method, repeatability and within-laboratory reproducibility was
calculated. The decision limit (CCα) of the method was calculated according to the ISO 11843 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. The detection capability (CCβ) was calculated by adding 1.64 times the standard error to the CCα. Matrix effects were investigated by infusion of all analytes (2.5 μg mL⁻¹) by an external syringe pump to a tee-connector at 10 μL mL⁻¹ between the electrospray probe and the outlet of the analytical column with simultaneous injection of methanol:water (1:1, v/v) only and subsequently with blank matrix diluted in methanol:water (1:1, v/v) onto the analytical column. The specific ion transitions of the analytes were recorded and any signal decreasing or increasing at the retention time of the investigated analyte was compared with the methanol:water (1:1, v/v) injection.

8.4 Results and Discussion

8.4.1 Development and optimisation experiments

In this study a methodology was developed as a tool for the analysis of drugs in forensic cases in the Republic of Ireland using a new solid phase extraction sorbent technology and the drugs were detected by hybrid LC-MS. The hybrid LC-MS/MS method was based on previous method developed in the author’s laboratory for urine analysis (Dowling et al., 2010) using MRM mode and product ion spectra in the linear ion trap mode (Q3) however in addition LEV and PHEN were monitored allowing the analysis of 18 drugs simultaneously in a single injection. The ionisation of the drugs was studied in positive mode. The optimum conditions (declustering potential, collision energy,
collision cell exit potential) were determined and the best diagnostic ions for MS/MS analysis were obtained (Table 8.2). For a method to be deemed confirmatory 4 identification points must be obtained. In MRM (multiple reaction monitoring) mode this is achieved by monitoring one precursor ion (parent mass) and two daughter ions (corresponding to strong and weak ion) in accordance with 2002/657/EC [Commission Decision]. Precursor and product ions were determined by direct infusion of single analyte solutions (1 μg mL\(^{-1}\) in methanol:water (50:50, v/v)). Chromatography conditions were described in section 8.3.2. An EPI experiment was set up in the Analyst 1.5 software. The strong MRM transition was chosen upon completion of tuning in MRM mode. The dependent scan was an EPI scan. One of the drawbacks of the Analyst 1.5 software in data dependent mode was that the software only allows a single preselected CE or CES for all analytes in an EPI experiment. It would be more advantageous if individual DP and CE settings per compound could be set. The dependent scan was an EPI scan and experiments to evaluate the optimum CES conditions for each analyte showed that using simultaneously different settings improved fragmentation patterns. The optimum CES conditions for amphetamine, cocaine classes and adulterants was 35±15 however for certain opioids a 50 CES ±15 gave improved fragmentation. Due to the ability of the software to only set one CES value. A value of 35±15 was chosen and the spectra generated under these conditions were utilisable for opioids. Evaluation of EPI spectra of a sample peak and spectra obtained from analysis of reference standards in a mass spectral library was subsequently carried out.

A new mixed mode solid phase extraction technology was launched by Biotage called Evolute™ ABN CX. The cartridge is a mixed mode resin based cation exchange SPE
sorbent with an optimised pore size that minimises retention of high molecular weight matrix components. In this study a sample size of 500 μL was chosen based on the sensitivity required. Preliminary studies were carried out using the generic solid phase extraction protocol (Procedure A) obtained from the sorbent manufacturer using ultra pure water spiked with target compounds at a concentration of 0.25 μg mL⁻¹. The results showed that poor recoveries were obtained for KET, BENZOYL and LIDO. Collection of wash solvents in the generic procedure and analysis showed that KET, BENZOYL and LIDO were recovered at a high percentage in methanol. The generic manufacturer’s procedure was modified as described (Procedure B). Studies showed that the methanol wash solvent contained no analytes upon addition of acid wash step. The addition of the drying procedure is important when elution solvents are non-polar to maintain a good recovery. Subsequent work was carried out to evaluate the best elution solvent (Procedure C) for the range of drugs tested. The results of the elution study showed that the best recoveries were obtained when a solution of ethyl acetate:methanol:ammonium hydroxide (70:25:5, v/v/v) was used.

The blood purification procedure developed as described in the experimental section has the following advantages as reduces the workload in monitoring for these substances at our laboratory replacing three separate sample preparation methodologies, the ability to automate the SPE procedure, the option to collect the methanol wash fraction for the analysis of neutral and acidic compounds or to fractionate using different types of elution solvent thus widening the potential number of analytes that can be detected in a single injection. Very stringent procedure and the strategy can be adopted for additional matrices such as vitreous humor, muscle and urine (unpublished data) and strategy can be
used with other detection techniques. The new purification strategy produces extremely clean extracts and the optimised pore size of the cartridges ensures the whole blood samples passed unhindered through the cartridges making the method very fast. Furthermore the matrix of blood alone is a very complicated matrix so a thorough sample purification procedure is essential and a 100% methanol wash step ensures clean sample extracts.

A hybrid LC-MS detection method developed as described has advantages such as replacing three separate detection technologies for opioid, cocaine and amphetamine classes in blood using GC-MS, a fast run-time of 12.5 min per injection and the ability to analyse LEV and PHEN and 16 important drugs simultaneously in blood, high and low concentrations of drugs in blood samples can be identified, quantified and confirmed simultaneously in a single injection with no need for re-injection as EPI spectra can be used to unambiguously confirm overdose cases in a straightforward manner. analysis of PHEN and the amphetamine class simultaneously allows identification of false positive results for amphetamines reducing significant time spent on sample re-analysis to identify this and the elimination of derivatisation steps. The disadvantage of using MRM ratios only instead of MRM-to-EPI experiments is that the sample will require dilution as a result of detector saturation and re-injection. The method developed has been used in 2010 for detection of opioid, cocaine, amphetamine and adulterant drugs in blood in forensic cases in the Republic of Ireland. In addition full laboratory information management system (LIMS) connectivity of the analytical strategy has been achieved using Analyst 1.5 software as part of routine monitoring of blood forensic toxicology samples at our laboratory.
8.4.2 Validation study

Validation of the method was according to procedures described in Commission Decision 2002/657/EC [Commission Decision 2002] covering specificity, calibration curve linearity, accuracy, precision, decision limit (CCα) and detection capability (CCβ).

8.4.2.1 Specificity

The technique of liquid chromatography hybrid triple quadrupole linear ion trap (QTRAP) mass spectrometry itself offers a very high degree of selectivity and specificity. To establish the selectivity/specificity of the method, blood samples were fortified with the above drugs and also non-fortified samples were analysed. No interfering peaks were observed at the retention time of some of the analytes in the chromatograms of the non-fortified samples when compared to the reporting level.

8.4.2.2 Linearity of the response

The linearity of the chromatographic response was tested with matrix matched curves using 8 calibration points in the concentration range of 0 to 2.0 µg mL\(^{-1}\). The regression coefficients (\(r^2\)) for all the calibration curves used in this study were \(\geq 0.99\).

8.4.2.3 Accuracy

The accuracy (\(n=18\)) of the method was determined using human blood samples fortified at 0.05, 0.075 and 0.10 µg mL\(^{-1}\) in three separate assays was 80-103%.
8.4.2.4 Precision

The precision of the method, expressed as RSD values for the within-lab reproducibility at the three levels of fortification (0.05, 0.075 and 0.10 µg mL\(^{-1}\)) was less than 16\% (Table 8.3).
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8.4.2.5 CCα and CCβ

The decision limit (CCα) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. In general, for non-MRL substances an α equal to 1 % is applied. The detection capability (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, were β = 5 %. CCα and CCβ values obtained were shown in Table 8.4 and calculated using the intercept (value of the signal, y, were the concentration, x is equal to zero) and the standard error of the intercept for a set of data with 6 replicates at 3 levels (0.05, 0.075 and 0.1 µg ml⁻¹). CCα is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. 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).

Table 8.4: Calculated CCα and CCβ values in blood for MOR, COD, DHC, 6-MAM, METH, COC, BENZYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN

<table>
<thead>
<tr>
<th>Substance</th>
<th>CCα (µg ml⁻¹)</th>
<th>CCβ (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>0.014</td>
<td>0.024</td>
</tr>
<tr>
<td>COD</td>
<td>0.015</td>
<td>0.025</td>
</tr>
<tr>
<td>DHC</td>
<td>0.016</td>
<td>0.026</td>
</tr>
<tr>
<td>6-MAM</td>
<td>0.020</td>
<td>0.030</td>
</tr>
<tr>
<td>METH</td>
<td>0.025</td>
<td>0.035</td>
</tr>
<tr>
<td>COC</td>
<td>0.030</td>
<td>0.040</td>
</tr>
<tr>
<td>BENZYL</td>
<td>0.030</td>
<td>0.040</td>
</tr>
<tr>
<td>COCA</td>
<td>0.035</td>
<td>0.045</td>
</tr>
<tr>
<td>BZP</td>
<td>0.040</td>
<td>0.050</td>
</tr>
<tr>
<td>METHAMP</td>
<td>0.045</td>
<td>0.055</td>
</tr>
<tr>
<td>LIGNO</td>
<td>0.050</td>
<td>0.060</td>
</tr>
<tr>
<td>PSEUDOEPH</td>
<td>0.055</td>
<td>0.065</td>
</tr>
<tr>
<td>AMP</td>
<td>0.060</td>
<td>0.070</td>
</tr>
<tr>
<td>KET</td>
<td>0.065</td>
<td>0.075</td>
</tr>
<tr>
<td>MDA</td>
<td>0.070</td>
<td>0.080</td>
</tr>
<tr>
<td>MDMA</td>
<td>0.075</td>
<td>0.085</td>
</tr>
<tr>
<td>LEV</td>
<td>0.080</td>
<td>0.090</td>
</tr>
<tr>
<td>PHEN</td>
<td>0.085</td>
<td>0.095</td>
</tr>
</tbody>
</table>
8.4.2.6 Measurement Uncertainty

According to SANCO/2004/2726 rev 1 the within laboratory reproducibility can be regarded as a good estimate of the combined measurement uncertainty of individual methods [SANCO 2004]. For the calculation of the extended uncertainty a safety factor is required. The within laboratory reproducibility should be multiplied by a value of 2.33 and this should be used when determining the $CC_\alpha$, corresponding to a confidence level of 99%. As the only source of variation during the validation was the different days and different blood sourced from different humans it was decided to use a safety factor of 3.0 instead of 2.33. The values are shown in Table 8.5 and were determined by calculating the within laboratory reproducibility of the method, followed by multiplication of the within laboratory reproducibility by the safety factor of 3.0.

Table 8.5: Calculated measurement uncertainty values in blood for MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN

<table>
<thead>
<tr>
<th>Substance</th>
<th>Measurement uncertainty</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>0.1</td>
</tr>
<tr>
<td>COD</td>
<td>0.3</td>
</tr>
<tr>
<td>DHC</td>
<td>0.2</td>
</tr>
<tr>
<td>6-MAM</td>
<td>0.1</td>
</tr>
<tr>
<td>METH</td>
<td>0.2</td>
</tr>
<tr>
<td>COCA</td>
<td>0.2</td>
</tr>
<tr>
<td>BENZOYL</td>
<td>0.2</td>
</tr>
<tr>
<td>COCA</td>
<td>0.1</td>
</tr>
<tr>
<td>BZP</td>
<td>0.1</td>
</tr>
<tr>
<td>METHAMP</td>
<td>0.1</td>
</tr>
<tr>
<td>LIGNO</td>
<td>0.1</td>
</tr>
<tr>
<td>PSEUDOEPH</td>
<td>0.1</td>
</tr>
<tr>
<td>AMP</td>
<td>0.1</td>
</tr>
<tr>
<td>KET</td>
<td>0.1</td>
</tr>
<tr>
<td>MDMA</td>
<td>0.1</td>
</tr>
<tr>
<td>LEV</td>
<td>0.1</td>
</tr>
<tr>
<td>PHEN</td>
<td>0.1</td>
</tr>
</tbody>
</table>
8.4.2.7 Evaluation

The analytical strategy in this study has been used to evaluate the presence of MOR, COD, DHC, 6-MAM, METH, COC, BENZOYL, COCA, BZP, METHAMP, LIGNO, PSEUDOEPH, AMP, KET, MDA, MDMA, LEV and PHEN in human blood in the Republic of Ireland in 2010. In monitoring for these substances at our laboratory drug identification was carried out by library search with a developed inhouse MS/MS library based on EPI spectra at a collision energy spread (CES) of 35 ± 15. Additionally routinely it was possible to detect the precursor ion and two daughter ions (within a single injection) in multiple reaction monitoring mode as well as generating an EPI spectra under collision energy spread conditions. The method has been carried out using different batches of blood, different QC material, using different batches of reagents, under varying environmental conditions and the method was shown to be robust. To demonstrate the applicability of the method incurred blood samples taken from subjects treated with AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC from the QC Reference Material were tested. The QC for AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC were found to be positive as they contained levels above CCα and the calculated concentrations were within the specified range of the QC material. Furthermore the EPI spectra confirmed unambiguously the presence of AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC as spectra matched the corresponding spectra in the library developed in-house. To further demonstrate the method applicability the method has been used to analyse a number of PT schemes. In addition the method was also stringently evaluated in-house by comparison with established GC methods (3 × GC-Ion Trap methods for opiates,
cocaines and amphetamines) and running all incoming samples simultaneously with old established methods and this new analytical strategy. The developed analytical strategy performs very well in terms of accuracy and within-laboratory reproducibility.

8.5 Case study

The described methodology has been applied in the laboratory since 2010 and positive drugs of abuse were identified in forensic cases from drug overdoses, suicidal or accidental poisonings using this method. The method has been used to analyse 40 blood samples received for toxicological analysis. The following substances were confirmed to be present in these samples. The presence of METH and MOR was confirmed in 9 samples. The presence of BENZOYL and LIGNO was confirmed in 6 samples, COD was found in 5 samples, PHEN was found in 4 samples, COC was found in 3 samples, 6-MAM, DHC, MDMA and LEV were found in 2 samples and COCA, PSEUDOEPH and MDA were each found once. It can be noted during the evaluation period that the adulterants LEV and LIGNO were identified. In 2009 the veterinary drug LEV which is a veterinary antihelminthic was being used as an alleged new cutting agent in adulterated cocaine. Figure 8.2 shows the EPI library spectrum of the veterinary drug and adulterant LEV which was found in a positive sample also with cocaine in Ireland. Information on fit, reverse fit and purity is previously described [Dowling et al., 2010]. It can be concluded that METH and MOR were the most prevalent substances in the 40 samples studied.
Fig. 8.2 Chromatogram of LEV positive with EPI spectra used for confirmation
8.6 Conclusions

The present investigation confirms that the sample preparation procedure using Evolute ABN CX™ solid phase extraction cartridges and detection using hybrid triple quadrupole/linear ion trap mass spectrometer in blood can be used for the confirmation of opioids, cocaine, amphetamines and adulterants simultaneously.

There are no quantitative confirmatory methods in the literature to the best of our knowledge that analyse 18 drugs simultaneously in this study using this sample preparation procedure and hybrid LC-MS using 4000QTRAP in MRM mode and product ion spectra in the linear ion trap mode and this study is the first. The sample preparation procedure produces extremely clean extracts and the volume of sample required is only 500 μL. The advantage of the analytical strategy at our laboratory is that it replaces 3 separate sample preparation procedures utilising 3 different GC instruments and that the sample preparation is dramatically simplified omitting extraction, hydrolysis, derivitisation steps and sample analysis time is reduced. The method has been carried out using different batches of blood, different QC material using different batches of reagents, under varying environmental conditions and the method was shown to be rugged. The developed method shows good agreement with well established reference GC-MS methods (not shown) at our laboratory. The advantage of a small sample size and the ability to confirm the identity of a wide variety of drugs in a single injection has important advantages for high sample throughput in a regulatory laboratory. Matrix effects studies were carried out and no suppression effects were evident. The accuracy of the method has been further certified as the quantitative and qualitative results were obtained by method comparison with PT samples and reference GC-MS methods. The
The primary advantage of the developed analytical methodology is the quantitation and confirmation of a wide range of forensically important drugs can be carried out using a single analytical strategy and a single analyst with a short analysis time. Therefore a reliable and fast sample preparation and detection strategy for opioids, cocaines, amphetamines and adulterants has been achieved successfully.

8.7 ACKNOWLEDGEMENTS

The authors would like to thank staff at The State Laboratory, Ireland for funding and permission to publish this article.
CHAPTER 9

CONCLUSIONS AND FUTURE WORK
9.1 Overview

Fast, robust and unequivocal confirmatory LC-MS methods were developed for the determination of drugs in animal and human matrices.

The research in the veterinary drug residue field on non-steroidal anti-inflammatory drugs covered a wide range of NSAIDs in milk and plasma matrices. The research in the post-mortem forensic toxicology field covered a wide range of drugs including opioid drugs, cocaine, amphetamines and adulterant drugs in human blood and urine. Multi-residue analytical protocols in each matrix for the analysis of a wide range of forensically important drugs were developed.

All methods in the veterinary drug residue field and the post-mortem forensic toxicology field were validated in accordance with Commission Decision 2002/657/EC.

Some of the analytical protocols in the veterinary drug residue section of the thesis have been implemented for routine use in the Veterinary Toxicology section at the State Laboratory and also in the National Residue Control Program in Ireland for residue monitoring of NSAIDs. They have been accredited for use as such in accordance with the ISO17025 standard. All of the analytical protocols in the Post-mortem Forensic Toxicology section of the thesis have been implemented for routine use in the Human Toxicology Section at the State Laboratory and also in the Post-Mortem Forensic Toxicology Monitoring Program in Ireland. The drugs of abuse methods are used to provide toxicological results to the coroners of Ireland and also to the State Pathologist and results have been utilised in court in forensic cases. In addition the analytical strategies have been rigorously tested in international proficiency testing programs. Also
as part of this research in the post-mortem forensic toxicology work of the laboratory, a
survey was carried out in chapters 7 and 8 to investigate the prevalence of a number of
drugs of abuse in a variety of post-mortem urine and blood samples in Ireland.
9.1.1 NSAIDS in Bovine Milk

- This investigation focused on the development of a fast, simple and selective analytical protocol for four NSAIDs in bovine milk using GC-MS/MS.

- It was proposed by Community Reference Laboratories (CRLs) in Europe that laboratories monitoring for NSAIDs should be capable of monitoring for KPF, DCF and PBZ in bovine milk at a level of 5 ng ml\(^{-1}\) and IBP at a level of 10 ng ml\(^{-1}\) in EU member states[SANCO 2007]. No analytical methods were available for the determination of these substances at the legislative levels set and the research was undertaken in order to detect the proposed levels in milk.

- The method developed in this study was based on a method developed by Vinci et al. [2006] but adapted to include deuterated analogues of IBP, DCF and PBZ and the LC-MS detection was replaced with GC-MS/MS coupled with a derivatisation step for detection of IBP, KPF and DCF. The advantage of the additional derivatisation step was the ability to produce two daughter ions for the determination of IBP whereas by LC-MS only precursor (parent) and one daughter ion for these compounds could be obtained [Vinci et al., 2006].

- The developed method had also been adopted by the National Reference Laboratory in Ireland for the confirmatory analysis of NSAIDs in samples taken by veterinary inspectors for the Department of Agriculture and used by a number of analysts. The method was accredited according to the ISO17025 standard [ISO 17025].
9.1.2 Firocoxib in Bovine Milk

- This investigation focused on the development of a fast, simple and selective analytical protocol for one NSAID in bovine milk using RRLC-MS/MS.

- This study was concerned with the development of a confirmatory method for firocoxib (FIRO) which is a newly licensed NSAID in horses [MRL Opinion 38346206] and has become available on the market under the trade name Previcox since 2007 [EMEA 082] and Equioxx since 2008 [EMEA V-142-en1]. FIRO cannot be used in mares in which milk is intended for human consumption. FIRO has no MRL established in bovine species. It is anticipated that due to the large increase in NSAID use in recent years that this substance may be used to treat food producing animals other than equines. Off label application of a veterinary drug compound to cows that produce milk for human consumption in the Republic of Ireland and the European Union is illegal. FIRO has been shown to be comparable in efficacy to meloxicam and carprofen [EMEA 082] and also been shown to be comparable in efficacy to phenylbutazone [Doucet et al., 2008]. In the case of carprofen and meloxicam these substances are licensed for use in horses and cattle, therefore it cannot be excluded that FIRO would not be used in cattle.

- The developed method involves the addition of acetonitrile to bovine milk followed by clean-up using Evolute™ ABN solid phase extraction (SPE) cartridges and analysis by RRLC-MS/MS. There were no methods available for the determination of FIRO in bovine milk prior to this research.
The method performs very well in terms of accuracy and within-laboratory reproducibility.
9.1.3 Non-authorised NSAIDs in Milk

- This investigation focuses on the analysis of non-authorised NSAIDs at the time of publication (except carprofen) in milk. The development of a rapid analytical method at the National Reference Laboratory for NSAIDs in Ireland was undertaken to provide an analytical tool to monitor for non-authorised substances in bovine milk.

The NSAIDs selected in this study were chosen to allow a method to be developed for the analysis of a wide variety of non-authorised drugs from different NSAID sub-classes.

- A newly developed water wettable polymer based Evolute™ ABN (acidic basic neutral) solid phase extraction (SPE) sorbent was investigated in the research and a new procedure was developed using this sorbent for the purification of CPF, DCF, MFN, NIFLU, NAP, OXYPHEN, PBZ and SUXI from milk.

- Rapid Resolution Liquid Chromatography (RRLC, trade name given by Agilent Technologies) was selected as the separation technique and a new protocol for determination of NSAIDs using RRLC-MS in the study was developed. The technology allowed for rapid analytical results, increased speed of method development times and utilised less solvent.

- The developed method in this research was the most sensitive available to the best of our knowledge and the theoretical values calculated for the CCα according to ISO 11843 [ISO 11843] calibration curve procedure were shown experimentally
to be valid by spiking milk at levels below the CCα values calculated to illustrate the sensitivity.

- It was proposed by Community Reference Laboratories (CRLs) in Europe that laboratories should be capable of monitoring for NSAIDs at a level of 5 ng mL\(^{-1}\) in EU member states where no MRL exists in milk. The objective of this work was to develop a method that is capable of the determination of NSAID residues in bovine milk below 5 ng mL\(^{-1}\) were no MRL exists.

- To demonstrate the applicability of the method milk samples taken from animals treated with CPF, DCF and PBZ from the Community Reference Laboratory in Berlin were tested. These samples had values ranging from about 4-13 ng mL\(^{-1}\). The samples were analysed by the method developed in this study and all samples were found to be non-compliant as they contained levels above the calculated CCα and also met the confirmatory criteria for both ion ratio and relative retention time.
9.1.4 Simultaneous Analysis of Authorised and Non-Authorised NSAIDs in Milk

- This investigation focuses on the analysis of authorised and non-authorised NSAIDs simultaneously in milk and non-authorised NSAIDs in bovine plasma.

- The extraction of the NSAIDs from plasma was based on methods developed by Gowik et al., (1998) and by Vinci et al., (2006) but modified with the addition of acetonitrile. The extraction of NSAIDs in milk was based on a method previously developed at the author's laboratory [Dowling et al., 2009].

- In this study an improved purification procedure was developed using Evolute ABN™ solid phase extraction cartridges for the analysis of a wider range of NSAIDs including authorised and non-authorised NSAIDs in bovine milk. The purification of NSAIDs from the plasma and milk extracts was investigated initially using a solid phase extraction procedure previously developed at the author's laboratory using Evolute ABN™ cartridges (chapter 5 methodology) but the original procedure was not satisfactory for the additional range of new analytes in this study. The NSAID FLU-OH was poorly recovered when the method previously developed at our laboratory was utilised. Elution studies were performed to ascertain where losses were occurring. The cartridges were eluted with different compositions and volumes of solvents including, 3 mL diethyl ether:hexane:acetonitrile (45:45:10, v/v/v), 3 mL diethyl ether:hexane:acetonitrile:methanol (45:45:5:5, v/v/v/v), 1.5 mL diethyl ether:hexane (80:20, v/v-elution 1) and 1.5 mL acetonitrile:methanol (90:10, v/v-elution 2), 1.5 mL acetonitrile:methanol 90:10, v/v-elution 1) and 1.5 mL diethyl ether:hexane (80:20, v/v-elution 1) and 1.5 mL acetonitrile:methanol (90:10, v/v-elution 2).
ether: hexane 50:50, v/v-elution 2), 3 mL methanol and 3 mL diethyl ether:hexane:acetonitrile:methanol (37.5:37.5:20:5 v/v/v/v). The results showed that elution of the cartridge with a solvent composition containing n-hexane:diethyl ether:acetonitrile: methanol (45:45:7:3, v/v/v/v, 2 x 1.5 mL) gave the best results for all the analytes tested in this study.

The developed solid phase extraction procedure was suitable for the purification of six NSAIDs in bovine plasma.

An improved liquid chromatography tandem mass spectrometry detection technique was developed to analyse ten NSAIDs simultaneously with a run-time of 15 min. Chromatographic tests were carried out to evaluate the suitability of the 1.8 µm Agilent Eclipse Plus C18 column (3.0 x 50 mm) and the LC mobile phase utilised in this study when additional NSAIDs were added. The tests showed that the internal standards of DCF and FLU overlapped in each internal standard transition when the analytes were eluted with a flow rate of 750 µL min⁻¹ and a run time of 6.5 min per injection. A study was performed using the same composition of mobile phase A and B. The times in the gradient were adjusted, the flow rate was reduced and the chromatographic runtime was extended to separate the internal standard of DCF and FLU. This resulted in the analytes being eluted with good peak shape when using a mobile phase of water containing 0.001 M acetic acid and acetonitrile (90:10, v/v + 0.001 M acetic acid) (A) and acetonitrile (B) with a flow rate of 0.5 µL.min⁻¹ and a runtime of 15 min. The internal standards d₄-DCF and d₃-FLU were completely separated under these conditions.
The methodology is capable of meeting the 5 ng mL\(^{-1}\) set for NSAIDs in plasma set by the EU. Moreover the values determined for the decision capability (CC\(_{\alpha}\)) in this study were lower than those previously reported for CPF, DCF, IBP, KPF, MFN and PBZ substances in plasma by LC-MS/MS.

FLU, TLF, FLU-OH, IBP, KPF and MLX were extracted from milk using Evolute ABN\(^{TM}\) solid phase extraction cartridges. The method meets the target level of 5 ng mL\(^{-1}\) for IBP and KPF in milk for the first time. There are no other analytical methods that monitor for authorised and non-authorised NSAIDs in milk as well as FLU and FLU-OH simultaneously in milk that meet the stringent validation requirements according to Commission Decision 2002/657/EC.

After validation of this method, the EU changed the legislation for DCF and an MRL of 0.1 ng mL\(^{-1}\) was set in milk. Preliminary spiking studies at the new MRL for DCF were carried out using the developed analytical strategy in this study. Results showed that this analytical strategy was sensitive enough to detect DCF at this level.

The applicability of the method for use on a variety of types of milk samples was demonstrated. To demonstrate the applicability of the method milk samples taken from animals treated with MLX and FLU-OH obtained from the Community Reference Laboratory in Berlin were tested. These samples had assigned values ranging from 5-15 ng mL\(^{-1}\). The samples were analysed by the method developed in this study and all samples were found to contain 5 ng mL\(^{-1}\) of FLU-OH and 15 ng mL\(^{-1}\) of MLX.
The methods were implemented into the National Monitoring Programme in Ireland for the determination of CPF, DCF, IBP, KPF, MFN and PBZ in bovine plasma and CPF, DCF, IBP, KPF, MFN, PBZ, FLU, FLU-OH, TLF and MLX in bovine milk at the National Reference Laboratory for NSAIDs and accredited according to ISO17025 Standard [ISO17025].

The method developed allows for the analysis of a wide variety of drugs from different NSAID sub-classes such as CPF, IBP and KPF (arylpropionic acid derivatives), PBZ (pyrazolidinedione derivatives) DCF, MFN and TLF (anthranilic derivatives) and FLU and FLU-OH (nicotinic acid derivatives) and MLX (oxicam derivatives).

Additionally the method published in 2010 in Food Additives and Contaminants has been adopted in an inter-laboratory study by the Community Reference Laboratory (CRL) in Germany for NSAIDs. The CRL recommend the analytical methodology be implemented by EU National Reference Laboratories for NSAIDs. A training workshop on the method was held in 2010 at the CRL headquarters in Germany and regulatory laboratories within EU member states were trained on the NSAID methodology which became available as a result of this research.
9.1.5 Drugs of Abuse in Urine

- This study focused on the development of a hybrid liquid chromatography-mass spectrometry strategy in a forensic laboratory for opioid, cocaine and amphetamine classes in human urine using a hybrid linear ion trap-triple quadrupole mass spectrometer.

- The analysis of drugs of abuse such as morphine (MOR), codeine (COD), morphine-3-glucuronide (M-3-G), 6-monoacetylmorphine (6-MAM), cocaine (COC), benzoylecgonine (BENZOYL), buprenorphine (BUPREN), dihydrocodeine (DHC), cocaethylene (COCA), 3,4-methylenedioxymethamphetamine (MDA), ketamine, (KET), 3,4-methylenedioxymethamphetamine (MDMA), pseudoephedrine (PSEUDOEPH), lignocaine (LIGNO), benzylpiperazine (BZP), methamphetamine (METHAMP), amphetamine (AMP), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and methadone (METH) in urine is highly important as their illicit use is widespread and the aim of this study was to develop a fast, simple and reliable sample preparation procedure in urine to analyse 19 drugs from the opioid, amphetamine and cocaine classes using a single sample preparation procedure and detection method that can be carried out by a single laboratory analyst.

- There was a paucity of studies that used hybrid LC-MS technology in the literature as the majority of studies use LC-tandem MS. One such hybrid LC-MS technology is the 4000 QTRAP LC-MS from Applied Biosystems.

- This is the first time that the 19 forensically important drugs have been analysed simultaneously running two MRM’s and a single EPI experiment in positive
mode in urine providing information on high and low mass fragments in a single
injection to the best of our knowledge. Drug identification was carried out by
library searching with an in-house developed MS/MS library based on EPI spectra
at a single CES of 35 ± 15 in positive mode. Validation of the method was based
on Commission Decision 2002/657/EC [Commission Decision 2002], a validation
protocol used in the field of veterinary drug residue monitoring.

- The LC-MS/MS method using MRM mode and product ion spectra in the linear
ion trap mode (Q3) was developed to provide unequivocal confirmatory data for
the analysis of MOR, M-3-G, COD, DHC, 6-MAM, METH, EDDP, BUPREN,
COC, BENZOYL, COCA, BZP, PSEUDOEPH, MDMA, MDA, AMP, KET,
METHAMP and LIGNO.

- Often in forensic toxicology cases it can be difficult for the pathologist to obtain
large sample sizes. Preconcentration of urine during method development was not
required based on the sensitivity achieved by the QTRAP method thus the urine
samples (100 μL) were diluted 20 fold in methanol:water (1:1, v/v) and a good
peak shape was achieved.

- The first advantage of the sample preparation procedure is that a small sample
volume is required. The second advantage is that the 19 drugs can be analysed
rapidly and simultaneously using simple dilution with methanol:water (1:1, v/v).

The third advantage is that the sample preparation procedure reduces the
workload in monitoring for these substances in any laboratory and as a result a
single analyst is capable of preparing a matrix matched curve and 50 samples in a
single day. A fourth advantage is the savings in cost as there is no need to
purchase costly hydrolysis reagents for the detection of drugs that are extensively metabolised.

The first advantage of the hybrid LC-MS detection method developed in this study is the fast run-time of 12.5 min per injection allowing detection of 19 forensically important drugs. The second advantage of is that high and low concentrations of the drugs in urine samples can be identified, quantified and confirmed simultaneously in a single injection using EPI spectra. High levels of drugs can be detected routinely using EPI spectra and the samples do not need to be re-injected as EPI spectra can be used to unambiguously confirm overdose cases in a straightforward manner. The disadvantage of using MRM ratio's only is that the sample will require dilution as a result of detector saturation and re-injection.

In addition full laboratory information management system (LIMS) connectivity of the analytical strategy was achieved using Analyst 1.5 software as part of routine monitoring of forensic toxicology samples.

To demonstrate the applicability of the method incurred urine samples taken from subjects treated with MOR, COD and BENZOYL from the QC Reference Material were tested. These QC samples had values ranging from 0.25- 0.37 μg mL⁻¹ for MOR, 0.23- 0.35 μg mL⁻¹ for COD and 0.13- 0.19 μg mL⁻¹ for BENZOYL. The QC for MOR, COD and BENZOYL were found to be present as samples contained levels above CCα and the calculated concentrations were within the specified range of the QC material. Furthermore the EPI spectra confirmed unambiguously the presence of MOR, COD and BENZOYL as spectra
matched the corresponding spectra in the library developed in-house. To further demonstrate the method applicability the method has been used to analyse a number of urine proficiency testing (PT) samples in which subjects were treated with MOR, BENZOYL, BUPREN, AMP, EDDP, METH and KET. The PT samples were analysed by the method developed in this study and were found to be non-compliant as they contained levels above the calculated CCa. The EPI spectra matched the corresponding spectra in the library developed in-house in Analyst 1.5 software and satisfactory Z-scores of below 1.4 were obtained for PT samples. Furthermore a PT sample negative for cocaines, opiates and amphetamines was analysed by this analytical strategy and was reported as being negative thus further ensuring that an accurate analytical strategy was developed.

The method was also stringently evaluated in-house by comparison with established GC methods (3 ×GC-Ion Trap methods for opiates, cocaines and amphetamines) and running all incoming samples simultaneously with old and new analytical methods. The results (unpublished data) were acceptable. The developed analytical strategy performs very well in terms of accuracy and within-laboratory reproducibility.

The described methodology has been applied in the laboratory since 2009 and positive drugs of abuse were identified in forensic criminal samples from crime offenders, abusers or victims using this method. The method has been used to analyse 233 samples at the time of publication.

The developed strategy has been carried out using different batches of urine, different QC material, by different analysts, using different batches of reagents.
under varying environmental conditions and 1000’s of samples have been analysed using the strategy and replaced the analysis of all urine samples in Ireland for drugs of abuse.

- The advantage of a small sample size and the ability to confirm the identity of a wide variety of drugs in a single injection has important advantages for high sample throughput in a regulatory laboratory. Matrix effects studies were carried out and results have shown that utilizing a labelled internal standard, dilution of samples and a diverter valve minimised the effects. The accuracy of the method has been further certified as acceptable results were obtained by method comparison with PT samples and reference GC-MS methods. In conclusion the method shows that simple dilution of urine and analysis by hybrid LC-MS technology can present a rugged analytical strategy.

9.1.6 Drugs of Abuse in Blood

- This chapter describes a multi-residue hybrid LC-MS strategy for the determination of opioid, cocaine, amphetamines and adulterants in human blood.

- A new mixed mode SPE sorbent chemistry technology was developed by Biotage™ with an optimised pore size and was evaluated as a single purification strategy.

The 18 drugs in blood included morphine (MOR), codeine (COD), 6-monoacetylmorphine (6-MAM), cocaine (COC), benzoylecgonine (BENZOYL), dihydrocodeine (DHC), cocaethylene (COCA), 3,4-methylenedioxyamphetamine (MDA), ketamine (KET), 3,4-methylenedioxymethamphetamine (MDMA), pseudoephedrine (PSEUDOEPH), lignocaine (LIGNO), benzylpiperazine (BZP),
methamphetamine (METHAMP), amphetamine (AMP) and methadone (METH) and are of vital importance in forensic toxicology.

* Evaluation of the literature showed the majority of studies in blood were using tandem LC-MS with chromatographic runtimes varying from 10 to 30 min. There are limited studies using hybrid LC-MS technology such as the 4000 QTRAP LC-MS system available from Applied Biosystems. The hybrid LC-MS method in this study was based on work undertaken at our laboratory for drugs of abuse in urine [section 9.1.5]. The second aim of this study was to extend the method to include levamisole (LEV) and phenethylamine PHEN.

* Collision Energy Spread (CES) conditions for each analyte were investigated and showed that using simultaneously different settings improved fragmentation patterns. The optimum CES conditions for amphetamine, cocaine classes and adulterants was 35±15 however for certain opioids a 50 CES ±15 gave improved fragmentation. Due to the ability of the software to only set one CES value a value of 35±15 was chosen and the spectra generated under these conditions were usable for opioids.

* The developed method was capable of detecting the presence of 18 drugs within a runtime of 12.5 minutes in a single injection.

* Validation was based on Commission Decision 2002/657/EC [Commission Decision 2002] and in-house procedure and drug identification was achieved by library searching based on enhanced product ion (EPI) spectra at a single CES of 35 ± 15 in positive mode.
Preliminary studies were carried out using the generic solid phase extraction protocol (Procedure A-chapter 8) obtained from the sorbent manufacturer using ultra pure water spiked with target compounds at a concentration of 0.25 µg mL⁻¹. The results showed that poor recoveries were obtained for KET, BENZOYL and LIDO. Collection of wash solvents in the generic procedure and analysis showed that KET, BENZOYL and LIDO were recovered at a high percentage in methanol. The generic manufacturer’s procedure was modified as described (Procedure B-chapter 8). Studies showed that the methanol wash solvent contained no analytes upon addition of an acid wash step. The addition of the drying procedure is important when elution solvents are non-polar to maintain a good recovery.

Work was carried out to evaluate the best elution solvent (Procedure C-chapter 8) for the range of drugs tested. The results of the elution study showed that the best recoveries were obtained when a solution of ethyl acetate:methanol: ammonium hydroxide (70:25:5, v/v/v) was used.

The blood sample preparation procedure has the advantages of reducing the workload in monitoring for these substances at our laboratory replacing three separate sample preparation methodologies, the ability to automate the SPE procedure, the option to collect the methanol wash fraction for the analysis of neutral and acidic compounds or to fractionate using different types of elution solvent thus widening the potential number of analytes that can be detected in a single injection. The procedure is very stringent and the strategy can be adopted.
for additional matrices such as vitreous humor, muscle and urine (unpublished data) and strategy can be used with other detection techniques.

• The hybrid LC-MS procedure has advantages such as replacing three separate detection technologies for opioid, cocaine and amphetamine classes in blood using GC-MS at our laboratory, a fast run-time of 12.5 min per injection and the ability to analyse LEV and PHEN and 16 important drugs simultaneously in blood. Other advantages include high and low concentrations of drugs in blood samples can be identified, quantified and confirmed simultaneously in a single injection with no need for re-injection as EPI spectra can be used to unambiguously confirm overdose cases in a straightforward manner. In addition the analysis of PHEN and the amphetamine class simultaneously allows identification of false positive results for amphetamines reducing significant time spent on sample re-analysis to identify this and the elimination of derivatisation steps. The disadvantage of using MRM ratios only instead of MRM-to-EPI experiments is that the sample will require dilution as a result of detector saturation and re-injection.

• In addition full laboratory information management system (LIMS) connectivity of the analytical strategy has been achieved using Analyst 1.5 software as part of routine monitoring of blood forensic toxicology samples and has been implemented into our regulatory laboratory.

To demonstrate the applicability of the method incurred blood samples taken from subjects treated with AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC from the QC Reference Material were tested. The QC for
AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC were found to be positive as they contained levels above CCα and the calculated concentrations were within the specified range of the QC material. Furthermore, the EPI spectra confirmed unambiguously the presence of AMP, MDA, MDMA, METHAMP, COC, BENZOYL, MOR, COD and DHC as spectra matched the corresponding spectra in the library developed in-house. To further demonstrate the method applicability the method has been used in a number of PT schemes. In addition the method was also stringently evaluated in-house by comparison with established GC methods (3 × GC-Ion Trap methods for opiates, cocaines and amphetamines) and running all incoming samples simultaneously with old established methods and this new analytical strategy. The developed analytical strategy performs very well in terms of accuracy and within-laboratory reproducibility.

• A case study was performed and the described methodology has been applied in the laboratory since 2010 and positive drugs of abuse were identified in forensic cases from drug overdoses, suicidal or accidental poisonings using this method. The method had been used to analyse 40 blood samples received for toxicological analysis at the time of publication and has subsequently replaced all other drugs of abuse methods for analysis of blood in Ireland in The State Laboratory.

• The developed strategy has been carried out using different batches of blood, different QC material, by different analysts, using different batches of reagents, under varying environmental conditions.
• The present investigation confirms that the sample preparation procedure using Evolute ABN CX™ solid phase extraction cartridges and detection using hybrid triple quadrupole/linear ion trap mass spectrometer in blood can be used for the confirmation of opioids, cocaines, amphetamines and adulterants simultaneously.

• There were no quantitative confirmatory methods in the literature for the 18 drugs analysed simultaneously in this study using this sample preparation procedure and hybrid LC-MS in MRM mode and product ion spectra in the linear ion trap mode.

• This research developed the first such strategy for incorporation into a forensic toxicology laboratory. The described methodology has been applied in the laboratory since 2010 and positive drugs of abuse were identified in forensic criminal samples from crime offenders, abusers or victims using this method.

9.2 Recommendations and Future Work

In the veterinary drug monitoring focusing on NSAIDs there is on-going public interest in relation to these drug residues in food worldwide. A study published by Azzouz et al., [2011] in milk showed that NSAIDs were identified in raw, whole, semi-skimmed and skimmed bovine milk products in combination with other drugs. The study in 2011 found KPF and PBZ in raw bovine milk detected with estrone and 17β-estradiol. In whole bovine milk NIFLU, NAP, FLU, KPF, DCF, PBZ, MFN were detected with florfenicol, estrone, 17β-estradiol, 17α-ethinylestradiol. In semi-skimmed bovine milk NIFLU and PBZ were found with triclosan, estrone and 17β-estradiol. In skimmed bovine milk
NIFLU, NAP, FLU, KPF, PBZ and DCF were found with pyrimethamine, estrone, 17β-estradiol, florfenicol and 17α-ethinylestradiol.

This study identifies that there this is an on-going problem in NSAIDs in milk and other veterinary drug residues in edible matrices and more methodologies are required. The papers published in this thesis and in the literature focus primarily on acidic NSAIDs but there is very limited methodology available for the determination of basic NSAIDs.

Subsequently further work upon completion of papers published for this thesis was undertaken focusing, in particular, on the basic NSAIDs in milk and a new methodology was published by Dowling et al., [2011]. There is a need to extend regulatory analyses to include basic NSAIDs in muscle and other matrices outlined in EU legislation.

Development of a multi-residue method for the determination of acidic and basic NSAIDs simultaneously in a variety of matrices of food producing animals would be highly beneficial as there are no methodologies available yet to the best of our knowledge.

There is also a concern over the environmental impact of the agricultural practices and use of NSAIDs in food producing animals. Since the 1990’s the Gyps vulture population has significantly dropped across the Indian subcontinent [Gilbert et al., 2002; Prakash et al., 2003; The Peregrine Fund, 2004]. Three species in particular Gyps bengalensis, G. indicus and G. tenerirostris have been known to be affected. Diclofenac has shown a direct correlation with renal failure by Oaks et al., [2004] in Gyps bengalensis. The numbers of these species have declined by more than 97% since 2002 [Prakash et al., 2003, Green et al., 2004]. All three of these species are now on the critically endangered
list by the World Conservation Union. This is quite significant given that G. bengalensis was ten years previously one of the commonest large raptor's in the world. There is a huge amount of evidence showing that the catastrophic vulture decline is caused by the NSAID diclofenac which was widely used to treat livestock across the subcontinent [Green et al., 2004, Oaks et al., 2004, Schultz et al., 2004]. As a result of the problems with NSAIDs as environmental contaminants a manuscript was written on “Potential application and future monitoring of unconventional wildlife specimens in wildlife forensic and regulatory investigations” by Ngoia et al., and the work on NSAIDs, veterinary drug monitoring by Dowling et al., was incorporated (under review). The article discusses wildlife forensics and the need to evaluate alternative matrices for NSAIDs and the need for a legislative framework similar to veterinary drug residues monitoring in the European Union for wildlife forensic monitoring in the future. Naidoo et al., [2009] has stated that diclofenac has been largely regarded as the most devastating environmental toxicant in recent times. Future work needs to be undertaken in order to set up NSAIDs methodologies in these alternative matrices and set up target levels for legislation in these matrices.

Further work completed as a result of the NSAIDs research published in this thesis has been in the availability internationally of the NSAIDs methodologies for use in other veterinary drug residue laboratories. Additionally the method published in 2010 in Food Additives and Contaminants had been adopted in an inter-laboratory study by the Community Reference Laboratory (CRL) in Germany for NSAIDs. The CRL recommend the analytical methodology be implemented by EU National Reference Laboratories for NSAIDs in EU member states. A training workshop on the method was held in 2010 at
the CRL headquarters in Germany and regulatory laboratories within EU member states were trained on the NSAID methodology developed as a result of the work described in this thesis. Future work will involve helping other regulatory laboratories to set up analytical methods for these substances.

As a result of the published methodology in this thesis Dubreil et al., [2011] has completed and published further work. This group utilised the methodology published in 2010 in Food Additives and Contaminants. They carried out studies on mobile phase and their results showed the best option for their research were the conditions developed in article published in 2010 in Food Additives and Contaminants as part of this thesis. Dubreil et al., [2011] incorporated this into their NSAID analysis performed at Veterinary Drug Residues Unit, Laboratory of Fougeres, ANSES (National Agency for Food Environment and Occupational Health Safety), France.

In 2012 work is being undertaken by the United States Food and Drug Agency (USFDA) utilising the NSAIDs paper published in J. Chrom A in 2009 as a basis for developing a NSAIDs method in milk at their site in Colorado.

There are very few studies available for licensed drugs to evaluate the effect of processing on residues and there is a need to develop such methodologies for the determination of NSAIDs. As mentioned earlier it is necessary to obtain more accurate estimates of consumer exposure to residues or possible breakdown products. The effects
of cooking and processing procedures on NSAID drug residues should be evaluated in
the future.

There has been very little research on NSAIDs in edible products although NSAID are
widely used in veterinary medicine. The reason for requiring such experiments is that
residues in animal food products at the retail level and sampling in import/exports is
restricted to muscular tissue, milk, eggs and honey and more research is required in this
area in the future. Edible product analysis is important as it also allows quantification in
the consumable part of the animal below the maximum residue limit (MRL).

Methods developed in edible matrices in order to achieve reliable quantitation should
create the calibration curves in matrix and adequate internal standards should be used to
correct for ESI response. Detection of NSAIDs using LC-MS particularly in food
analysis is still quite limited. There are even more limited studies utilising high resolution
mass spectrometry. Further research for the future could focus on identifying new
metabolites of NSAIDs drugs in different food animal products (e.g metabolomic
studies).

The main objectives of the research work and for the future on NSAIDs should be to
address the food safety issues concerning NSAIDS and edible matrices by developing
new analytical strategies and apply these to ascertain answers to key questions. In
addition, the effect of NSAIDs in the environment which is a new emerging difficulty
due to use of these substances in food producing animals needs to be addressed.
Furthermore the evaluation of the presence of these substances in the aquatic environment requires study.

In the post-mortem forensic toxicology monitoring field detection systems utilising hyphenation of a gas chromatograph and mass spectrometer had become the gold standard for analysis in the post-mortem forensic toxicology area. In the post-mortem forensic toxicology discipline liquid chromatography mass spectrometry has become increasingly valuable and rapidly methodologies are transferring from LC-UV or GC-MS. LC-MS is helping to close the gaps in knowledge with respect to hydrophilic, thermolabile and non-volatile analytes that previously could not be analysed by the most widely used technique of GC-MS. In forensic laboratories LC-MS is advantageous because direct analysis of aqueous solutions containing thermolabile, non-volatile and hydrophilic compounds can be performed. In the future the use of LC-MS and GC-MS in post-mortem forensic toxicology field will however still be complementary in the regulatory laboratory. In post-mortem forensic toxicological analysis the drugs are generally unknown. Therefore in the future the analytical methods utilised should cover a wide range of compounds and their metabolites and in addition allow for unequivocal identification. The papers published in this thesis and in the literature focus on traditional drugs of abuse and adulterants in blood and urine. There has been an explosion of new alternative designer drugs being utilised and the numbers and types are always rapidly changing. It is a difficult challenge to set up analytical methodologies to ascertain designer drug presence in post-mortem samples and in the future work should be done to incorporate these.
Another general problem in the analysis of new designer drugs is the lack of quality controlled analytical reference standards making it impossible to record mass spectra for these drugs to incorporate into the database/libraries. It will be necessary to synthesise new designer drugs within forensic laboratories in order to respond quickly.

New drugs of abuse also cause a problem in post-mortem forensic toxicology as often there is little known about the metabolism and toxicity of these substances and whether they can contribute to the cause of death. It will be necessary to perform metabolism studies and toxicity studies in order to understand their effects in the future.

It would also be useful if there was a more proactive approach within the forensic toxicology field for scientists to set up protocols in order to develop incurred quality control material for these substances for distribution between regulatory laboratories in order to test new analytical strategies that are developed.

Further work carried out subsequent to that reported in this thesis focused in particular on the designer cannibinoid CP47, 497. A dilute and shoot methodology in human urine was published by Dowling et al., [2011] detailing the limitations of developing methodologies for designer drugs. CP47, 497 is a synthetic cannibinoid and is functionally similar to cannabis. The synthetic cannabinoids are considerably more potent than cannabis and were never intended for human use. However certain manufacturers were marketing these substances for human consumption under the identity of incense while satisfying the authorities by writing in very small print “not for human consumption” on the packaging initially so they could not be banned. These herbal products were sold under trade names
such as “Spice Gold”, “Spice Diamond” and “Spice Silver” to name but a few. CP47, 497 has been banned in the Republic of Ireland. The analysis of new designer drugs is a new area and difficulties in their analysis include the availability of incurred material, reference standards, appropriate isotopically labelled reference standards, lack of knowledge of measurement ranges in human tissues and lack of validation protocols. In the future it will be necessary to rapidly develop methods for the determination of new designer drugs to address the gaps in knowledge due to lack of analytical protocols.

There is also a need to monitor traditional drugs of abuse and new designer drugs simultaneously to identify their mis-use as there are very few analytical protocols available that can do this. In 2011 research was presented at a joint meeting of the Society of Forensic Toxicologists and The International Society of Forensic Toxicologists. The work focused on the new designer cannabinoids analysed simultaneously with cannabis and its metabolites in human blood. There is a need to use such protocols to include the most up-to-date information on changes in drug patterns from traditional to new designer drugs in the future.

In the analysis of drugs of abuse there is a need to include drugs in the analytical protocols that are known to cause false positives or negatives in order to be in a position to interpret immunoassay results more effectively. Work should be undertaken to obtain knowledge of the main problematic compounds and these should be included in confirmatory procedures.
There is a need to improve interpretation of urine test results for opiates as test results for semi-synthetic opioids can be difficult because of complex biotransformation of parent drug to metabolites that are also commercially available that can be abused. It would be useful in the future if normetabolites such as norcodeine, norhydrocodone and noroxycodone, which are unique metabolites that are not commercially available, are incorporated simultaneously in drug detection strategies. As a result detection of normetabolites in specimens not containing parent drug provides final evidence that the parent drug was consumed and will reduce the potential for false negatives that would occur without tests for these unique metabolites.

There is a need to perform surveys of the incidence of positive/negative samples for immunoassay with new designer drugs in order to identify if there is an epidemic of use that is occurring but not been identified in forensic cases or if these new drugs are affecting the immunoassay/LC-MS procedures.

There is a need for forensic laboratories to have stronger links with emergency room medical personnel in order to analyse samples that present in accident and emergency to ascertain if designer drugs give clinical symptoms that may lead to bizarre, suicidal or criminal behaviour in order to alert the general public to the potential dangers of use of such substances or new substances.
The above are issues related specifically to classes of drugs studied in this thesis. In the veterinary drug residue monitoring field and post-mortem forensic toxicology field there are common areas that need to be addressed for the future.

There have been many applications in the use of LC-MS in both fields however the majority of applications use LC-MS or LC-tandem MS/MS. There are two main LC-MS protocols that are being utilised. The first approach is the development of multi-residue procedures using quadrupole, ion trap or hybrid mass spectrometers which generate product ion spectra that are subsequently searched against libraries of reference mass spectra recorded on the same apparatus. A major drawback of this approach at present is that the LC-MS reference libraries generally cannot be used on different LC-MS apparatus due to insufficient reproducibility of LC-MS/MS spectra obtained with different instruments. In the future it would be advantageous if manufacturers could develop transferable libraries.

Another drawback in both fields in LC-MS analysis is the lack of information on metabolite spectra for comparison in libraries. It would be better if more information on metabolites was available in commercial libraries obtained from instrument manufacturer’s.

Some manufacturer’s have set up a software feature labelled as “scheduled MRM” which allows for the setting of time windows for transitions in the survey scan. This allows for increasing the number of transitions in the survey scan allowing for detection of a wider...
number of drugs while minimising the cycle time of the MS method. In the future it would be advantageous if vendor software manufacturers would improve software allowing for an increased number of transitions thus allowing the development of wide ranging multi-residue LC-MS detection methods in both fields.

It was noted in research carried out in this thesis focusing on the detection of drugs of abuse utilising hybrid LC-MS systems that monitoring of peaks did not trigger information dependent acquisition (IDA) mode or the quality of the enhanced product ion (EPI) spectra was not sufficient. In addition polarity switching, if required, could not be performed because the software was too slow and separate injections would be necessary. In the future the LC-MS hardware and software available for analytical laboratories from various manufacturers needs to be continually improved thus allowing regulatory laboratories the possibility to develop rapid targeted sensitive multi-residue methods for a wide number of compounds and improving capabilities for positive/negative switching in a single injection when running MRM to EPI experiments.

Another LC-MS approach is based on high resolution mass spectrometry. Compounds are identified by comparison with accurate mass. The advantage of using this approach is that in theory searching of samples for compounds where no reference standard is possible. It is important to note that utilising accurate mass and isotopic patterns alone is not enough for unequivocal confirmation. It is better to set up reference mass database using theoretical mass data and retention time data. Additionally it is useful to generate fragments of compounds thus providing additional structural information. A difficulty of
such databases using theoretical mass data and retention time data is that information is only available for a limited set of data. There is also a difficulty in differentiating between isomeric compounds. Another drawback of such databases in relation to new designer drugs is often it is not possible to have knowledge of the metabolic pathway utilised and knowledge of metabolites is limited.

At present software manufacturers are developing metabolite prediction software. This would allow the predicted compounds to be added to the database. Other software tools are in development for the prediction of fragmentation patterns for which no reference standards are available. In the future it would be useful if dependable software tools for metabolite and fragmentation patterns could be developed to help with drug analysis and to support accurate mass based compound identification in both fields.

There is also a need for studies to be performed to evaluate how reliable these software prediction tools are in real samples without reference standards in regulatory laboratories.

In databases that have been developed at present there is a wide range of compounds and it is not uncommon for a large number of compounds to have the same molecular formulae giving rise to a large hit list being formed. It is recommended that software tools need to be developed in order to shorten the hit lists and perhaps this might be achieved by identifying the presence of metabolites.

Some work has begun using a metabolomic approach where a predefined set of functional groups can be calculated for each candidate compound. In addition mass shifts from major biotransformation reactions can be calculated and the peaks with respective
masses can be searched in the data files of real samples. In the future metabolomic protocols will need stringent testing in real samples in both fields in order to ascertain the validity of such an approach.

In some laboratories, work has been carried out developing screening procedures using accurate mass and in addition retention time information in the search protocol and recording at least two spectra. One spectrum leaves the intact pseudomolecular ion and one spectrum which contains a unique pattern or fingerprint thus allowing for unequivocal confirmation. This approach appears worthwhile. The difficulties arise as using different voltages to collect spectra increases the MS cycle time. It will be important to slow the chromatography in order to achieve a sufficient amount of data points across the peak. This results in longer run-times for analysis which reduces the response in emergency toxicology situations. It would be advantageous if instrument vendors could improve the hardware and software in high resolution LC-MS technologies in the areas above so the next generation LC-MS instruments can improve sample throughput. Although the use of high resolution mass spectrometry has been more limited in the field than tandem LC-MS it is envisaged that in the future this approach will become much more prevalent as an analytical tool in regulatory laboratories.

The above issues are difficulties encountered with the analytical detection tools provided by manufacturers of the detection technology to provide reliable LC-MS results. Validation protocols to be utilised with these detection systems and the particular sample pre-treatment steps are crucial. In the sample preparation area analytical protocols that
focus on generic extraction procedures allowing for the concurrent detection of large
tumbers of compounds from a wide variety of classes is expected to rapidly expand.
Sample preparation methodologies traditionally involved liquid/liquid extraction, protein
precipitation and solid phase extraction in the vast majority of methods independent of
the sample matrix. Newer approaches utilising dilute-and-shoot preparation should be
used for the urine matrix for the future. Blood samples are usually analysed using protein
precipitation or a combination of precipitation and centrifugation. In the future sample
preparation methods will reduce the volume of sample required, minimise the number of
steps thus reducing the analytical time and solvent consumption and eliminating the need
for chlorinated organic solvents. Sample preparation SPE or other sorbent technology
formats could be reduced to 96 well-plate format. In addition automated SPE or newer
sorbent technologies e.g supported liquid extraction (SLE) or online SPE approaches
could be utilised. The number of sorbent technologies is rapidly expanding and newer
sorbent technologies such as molecular imprinted polymers and SLE to name but a few
are becoming available allowing for clean extracts while lowering limits of detection.
The aim is the development of generic extraction protocols for a wide variety of drugs.
The drawback of generic extraction is that unavoidably more matrix components are
extracted which lead to matrix effects.
Matrix effects refer to “the direct or indirect alteration or interference in response due to
the presence of unintended analytes or other interfering substances in the sample” The
presence of a matrix effect can have two consequences within the sample. The signal can
be enhanced (ion enhancement) due to matrix effect or the signal can be reduced (ion
suppression) this increases the probability that a sample could be false compliant or false
non-compliant. Currently there is no consensus as to how matrix effects experiments should be performed and it is important for the future that the matrix effect experimental protocol be established in both fields. In addition setting of widely accepted criteria for this parameter needs to be addressed. No criteria exist within the validation framework of Commission Decision 2002/657/EC for matrix effects in veterinary drug residue monitoring.

The validation criteria for mass spectrometry in Commission Decision 2002/657/EC were adopted in this thesis for veterinary drug residues and post-mortem forensic toxicology research. The criteria within Commission Decision 2002/657/EC does not include TOF-MS, medium to high resolution of approximately 10,000 FWHM. Due to high resolution mass spectrometry approach becoming a valuable analytical tool in regulatory laboratories it will be necessary to include criteria within Commission Decision 2002/657/EC.

In addition it would be helpful at a national level if laboratories could add spectra of new designer drugs to a database that other regulatory laboratories could have access to.
9.3 List of Presentations at International Conferences based on Thesis and Future Work

G. Dowling, L. Williams, R. Jones and L. Regan A new supported liquid extraction strategy with liquid chromatography mass spectrometry detection for the determination of S018, lWH073, CP47, 497-C8, JWH200, JWH019, HU210 and JWH250 (2012), Society of Forensic Toxicologists (SOFT) 2012, Boston, USA

G. Dowling, L. Regan Drugs of abuse in Urine Abstract under submission! (2012), United Kingdom and Ireland Association of Forensic Toxicologists (UKIAFT) 2012, Belfast, United Kingdom

G. Dowling, E. Dixon, A. Murphy and L. Regan A rapid sample preparation procedure utilizing hybrid quadrupole linear ion trap mass spectrometry detection as an analytical strategy for controlled drugs in blood and application to forensic cases in Ireland, (2011), SOFT/TIAFT 2011, San Francisco, USA

G. Dowling, L. Williams, R. Jones and L. Regan Evaluation of Supported Liquid Extraction (SLE) for the determination of cannabis and synthetic cannabinoids in blood. (2011). SOFT/TIAFT 2011 San Francisco, USA

G. Dowling and E. Malone A confirmatory method for the determination of basic non-steroidal anti-inflammatory drugs in bovine plasma by liquid chromatography tandem mass spectrometry, In Sixth International Symposium in Hormone and Veterinary Drug Residue Analysis, 2010, Ghent, Belgium, 1-4 June 2010


G. Dowling and L. Regan Confirmatory analysis of phenylbutazone in bovine plasma by gas chromatography tandem mass spectrometry, In Third International Symposium on Recent Advances in Food Analysis, 2007, Prague, Czech Republic, 7-9 November 2007
9.4 List of Publications as a Result of Research

Future Work Based on Thesis Research


Thesis Research


G. Dowling and L. Regan Confirmatory analysis of firocoxib in bovine milk by rapid resolution liquid chromatography tandem mass spectrometry, *Journal of Chromatography B*, 5-6 (2009), 541-546

CHAPTER 10

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The following papers in thesis are as a result of my own work.


G. Dowling and L. Regan Confirmatory analysis of firocoxib in bovine milk by rapid resolution liquid chromatography tandem mass spectrometry, *Journal of Chromatography B*, 5-6 (2009), 541-546


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