Extraction and Analysis of Pharmaceutical Residues in Environmental Samples using SPE with LC-MS/MS.

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Declaration.

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of **Doctor of Philosophy** is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract.

Pharmaceuticals and personal care products (PPCPs) have recently emerged as a significant new class of organic micro-contaminants. Of recent years a number of reports detailing the presence of PPCPs in a variety of environmental matrices and compartments have been published in the peer-reviewed literature. However, in Ireland very little research has been conducted to determine the level of environmental contamination due to the presence of drug residues.

The primary focus of this research is to develop suitably sensitive analytical methods for the determination of residual PPCP contamination based upon solid phase extraction (SPE) and liquid chromatography mass spectrometry (LC-MS). Monolithic silica based stationary phases were used for the development of high performance liquid phase separations of common pharmaceuticals, the antifouling and anti-dandruff agent zinc pyrithione and a range of illicit drugs and abused pharmaceuticals. As a pre-requisite to all the developed methods, a SPE sample enrichment procedure was also developed focusing upon either off-line formats using modern hydrophilic lipophilic balanced polymeric phases or the use of column switching, whereby short reversed-phase monolithic micro-columns were applied as suitable traps for on-line preconcentration. Method performance data for all the developed methods were also determined and analytical detection limits were found to lie in the ngL^{-1} range. The developed methods were applied for the determination of the selected analytes in environmental aquatic samples.

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Poster Presentations:

J. Bones, K.V. Thomas and B. Paull, "*Development of Analytical Methods for the Determination of Pharmaceutical Residues in the Environment*", presented at:

- Royal Society of Chemistry Analytical research Forum 2004, University of Central Lancashire at Preston, July 19th to 21st 2004.
- The Third Biennial Conference on Analytical Science in Ireland, University College Cork, September 9th/10th 2004.
- The Second Annual National Symposium of the Irish Research Council for Science, Research and Technology, Croke Park Dublin, 2nd November 2004.

J. Bones and B. Paull, "*Dual Gradient Monolithic HPLC for the Routine Analysis of Pharmaceuticals in Environmental Samples*", presented at:

- Environ 2005, Institute of Technology Sligo, January 28th to 31st 2005.
- Pharmaceuticals in the Environment; Fate, Effects and Regulation, Society of Chemistry and Industry, London, 1st March 2005.

J. Bones, P.N. Nesterenko and B. Paull, "*Online Solid Phase Extraction and Dual Gradient Monolithic LC-MS for the Determination of Pharmaceuticals in the Environment*", presented at:

- HPLC 2005, Stockholm, Sweden, June 27th to June 30th 2005.
- The Royal Society of Chemistry Analytical Research Forum 2005, University of Plymouth, July 18th to 20th 2005.
- The Third Annual National Symposium of the Irish Research Council for Science, Research and Technology, Croke Park Dublin, 3rd November 2005.

J. Bones, P.N. Nesterenko, K.V. Thomas and B. Paull, "*Determination of Zinc Pyrithione in Environmental Samples using Online Solid Phase Extraction with LC-APCI-MS*", presented at:

- The Fourth Biennial Conference on Analytical Science in Ireland, Dublin Institute of Technology at Kevin Street, April 11th/12th 2004.

J. Bones, P.N. Nesterenko, K.V. Thomas and B. Paull, *"A Simple and sensitive Method for the Determination of Zinc Pyrithione in Environmental Samples"*, presented at:

- HPLC 2006, San Francisco, USA, June 19th to June 23rd 2006.

J. Bones, P.N. Nesterenko, K.V. Thomas and B. Paull, *"Column Switching LC/LC-APCI-MS for the Determination of Zinc Pyrithione and its Fate in Environmental Waters"*, presented at:

- The Royal Society of Chemistry Analytical Research Forum 2006, University College Cork, July 17th to 19th 2006.

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Warfarin; **12** Bezafibrate; **13** Flurbiprofen; **14** Diclofenac; **15** Ibuprofen; **16** Meclofenamic acid; **17** Mefenamic acid; **18** & **19** Gemfibrozil and Triclosan.

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4.8 TIC and EIC traces of river water spiked with 200ngL^{-1} using (A) positive and (B) negative electrospray polarity. *m/z Values:* Ketoprofen (255.0); Bezafibrate (362.1); Naproxen, (231.0); Fluoxetine (310.0); Ibuprofen (251.1); Clotrimazole (277.0); Gemfibrozil (121.1); Mefenamic acid (240.0); Meclofenamic acid (294.0); Indomethacin (312.0); Diclofenac (250.0); Flurbiprofen (199.0); Nimesulide and Warfarin both (307.0).

5.1 The structures of the pyrithione ligand tautomeric pair; (A) 2-mercaptopyridine-*N*-oxide and (B) *N*-hydroxypyridine-2-thione, (C) the structure of zinc pyrithione; (ZnPT) and (D) the structure copper pyrithione; (CuPT).

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5.17 The determination of ZnPT as CuPT in anti-dandruff shampoo samples diluted in river water as a waste matrix. The blue trace represents Dove Anti-Dandruff shampoo which contains 0.37% w/w ZnPT, the concentration corresponding to the blue trace is ~76 ngL^{-1}. The red trace represents Head and Shoulders Classic Clean shampoo which contains 0.80% w/w ZnPT, the concentration corresponding to the red trace is ~80 ngL^{-1}.

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6.3 Minimum resolution response surface for the optimisation of the pH and buffer concentration of the aqueous mobile phase. Experiments were performed using a 1 mgL^{-1} standard mixture of analytes.

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6.5 Resulting chromatograms from banknote analysis depicting; (A) the presence of ~24 ng of cocaine (blue trace) on a €5 note overlaid upon the EIC m/z 304 trace for the un-circulated €5 note (red trace) and (B) the presence of ~20 ng cocaine and ~4 ng of benzoylecgonine on a €20 banknote, (blue and red traces respectively).

6.6 Graphical representation of cocaine contamination on the analysed banknotes. The highest levels were detected on one €5 note and one €20 note as labelled in the above figure. It can also be seen that the levels of contamination are higher on the €20 and €50 notes than on the €5 and €10 notes.

7.1 The structure of (A) the Phenomenex Strata-X™ hydrophilic lipophilic balanced polymeric sorbent, (B) the additional strong cation

exchange functionality of the Strata-XC™ sorbent and (C) the weak cation exchange functionality of the Strata-XCW™ sorbent.

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7.3 Extracted ion chromatograms for a 200 ngL^{-1} mixed analyte spike solution prepared in river water collected from the River Boyne. *Peak identification:* 1 Morphine m/z 286 – m/z 268, T_R 4.1 mins; 2 MDMA m/z 194 – m/z 163, T_R 8.6 mins; 3 Benzoylecgonine m/z 290 – m/z 168, T_R 9.7 mins; 4 Ketamine m/z 238 – m/z 220, T_R 10.0 mins; 5 Cocaine m/z 304 – m/z 182, T_R 10.2 mins; 6 Cocaethylene m/z 318 – m/z 196, T_R 11.5 mins; 7 LSD m/z 324 – m/z 223, T_R 11.8 mins; 8 EDDP m/z 278 – m/z 249, T_R 12.5 mins; 9 Papaverine (Internal Standard) m/z 340 – m/z 202, T_R 13.9 mins; 10 Methadone m/z 310 – m/z 265, T_R 16.8 mins; 11 Temazepam m/z 301 – m/z 283, T_R 17.9 mins; 12 Fluoxetine m/z 301 – m/z 148, T_R 18.3 mins; 13 Diazepam m/z 285 – m/z 257, T_R 20.0 mins.

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Tables:

Table Number	Caption
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3.3 Dual gradient HPLC method performance data.

3.4 Sorbent selection performance data (calculated % recoveries).

3.5 Extraction repeatability and overall method precision calculated using 1 μgL^{-1} spikes in river water, (*actual spiking level was 2 μgL^{-1}).

3.6 Overall method sensitivity data calculated in two different sample matrices, (1L extraction volume), values quoted are in μgL^{-1}.

4.1 The chemical structures of the additionally included pharmaceuticals.

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4.4 Analyte recovery as a function of sample pH.

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4.6 Overall method precision and analyte recovery from two different sample matrices, (n = 10).

4.7 Tuned ESI-MS method parameters, (optimisation was performed using a nebuliser pressure of 55 psi, dry gas flow of 8 $Lmin^{-1}$ and a drying temperature of 300°C).

5.1 Optimised ion focusing APCI-MS parameters for ZnPT, (the optimisation was performed using a nebuliser pressure of 50.0 psi, a dry gas flow of 10.0 $Lmin^{-1}$, a drying temperature of 325°C, a corona voltage of +3200 V and an APCI temperature of 500°C).

5.2 Online SPE LC-APCI-MS method performance data.

5.3 TOC and metal content of the two certified sediment samples.

5.4 Calculated concentrations of CuPT in the aqueous and sediment phases after a 2 hour mixing period.

6.1 The chemical structures of the illicit drugs and abused pharmaceuticals chosen for study.

6.2 Optimised ion focusing parameters for ESI-MS/MS detection, (the optimisation was performed using a nebuliser pressure of 10.0 psi, a dry gas flow of 3.0 $Lmin^{-1}$ and a drying temperature of 300°C. Spectra were collected over the range 50 – 600 m/z).

6.3 Selected product ion transitions for MS/MS monitoring.

6.4 Method performance data.

6.5 Banknote analysis.

7.1 Wastewater treatment plants sampled for the presence of illicit drugs.

7.2 pK_a and Log *P*, (as theoretically calculated XLog*P*) data for the selected illicit drugs and pharmaceutical analytes.

7.3 Initial sorbent selection investigations; calculated analyte recovery for a 2 μgL^{-1} mixed spike using the sorbents and conditions mentioned in the text, (values quoted are mean recovery ± standard deviation, n = 3).

7.4 Calculated analyte recovery for the optimisation of the elution solvent.

7.5 Recovery data for extraction pH optimisation.

7.6 Method performance data for the developed SPE LC-MS/MS procedure.

7.7 Concentrations (ngL^{-1}) of illicit drugs detected in collected wastewater and surface water samples.

7.8 Cocaine loads within the sampled wastewater treatment plants.

7.9 Estimated community consumption of cocaine within the catchments served by the sampled wastewater treatment plants.

A List of Abbreviations Mentioned in the Text.

APCI	Atmospheric Pressure Chemical Ionisation
BOD	Biochemical Oxygen Demand
CE	Capillary Electrophoresis
CuPT	Copper (II) Pyrithione
DC	Direct Current Potential
DDD	Defined Daily Dose
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EDDP	2-Ethyldine-1,5-dimethyl-3,3-diphenyl pyrrolidine
EIC	Extracted Ion Chromatogram
EMEA	European Medicines Evaluation Agency
EPA	Environmental Protection Agency, (Irish)
ERA	Environmental Risk Assesment
ESI	Electrospray Ionisation
EtOAc	Ethyl Acetate
EU	European Union
FA	Formic Acid
FePT	Iron (III) Pyrithione
GAC	Granular Activated Carbon
GC	Gas Chromatography
GC-MS(/MS)	Gas Chromatography Mass Spectrometry, (tandem-MS)
GREAT-ER	Geography Referenced Regional Exposure Assessment Tool for European Rivers
HPLC	High Performance Liquid Chromatography or LC
HPTLC	High Performance Thin Layer Chromatography
IC	Ion Chromatography
ICP	Inductively Coupled Plasma
IMB	Irish Medicines Board

IMO	International Marine Organisation
IPA	Isopropyl Alcohol
IR	Infra-red Spectroscopy
K_D	Liquid-Solid Distribution Coefficient
K_{OC}	Organic Carbon Partition Coefficient
K_{OW}	Octanol Water Partition Coefficient
LC	Liquid Chromatography or HPLC
LC-MS(/MS)	Liquid Chromatography Mass Spectrometry, (tandem-MS)
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOEC	Lowest Observed Effect Concentration
LOQ	Limit of Quantitation
LSD	Lysergic Acid Diethylamide
MDMA	3,4-Methylene Dioxy Methamphetamine
MEC	Measured Environmental Concentration
MeCN	Acetonitrile
MeOH	Methanol
MES	Morpholino-Ethane Sulphonic Acid
MIPs	Molecularly Imprinted Polymers
MnPT	Manganese (II) Pyrithione
MS	Mass Spectrometry
MSDS	Material Safety Data Sheet
MtBE	Methyl *t*-Butyl Ether
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance Spectroscopy
NOEC	No Observed Effect Concentration
NOM	Natural Organic Matter
PAHs	Polynuclear Aromatic Hydrocarbons
PAPs	3'-Phosphoadenosine-5'-Posphosulphate
PEC	Predicted Environmental Concentration
PNEC	Predicted No Effect concentration
ppb	Parts Per Billion, (μgL^{-1})

PPCPs	Pharmaceuticals and Personal Care Products
ppt	Parts Per Trillion, (ngL^{-1})
QqQ	Triple Quadrupole Mass Analyser
QSAR	Quantitative Structural Activity Relationship
RF	Radio Frequency
RSD	Relative Standard Deviation, (%)
SPE	Solid Phase Extraction
TD	Thermal Desorption
TFA	Trifluoroacetic Acid
Δ^9-THC	Δ^9-Tetrahydrocannabinol
TIC	Total Ion Current Chromatogram
ToF	Time of Flight Mass Analyser
TRIS	*Tris*-(hydroxymethyl)-aminomethane
UDP	Uridine Diphosphate
UV	Ultra Violet
VWD	Variable Wavelength Detector
WWTP	Wastewater Treatment Plant
ZnPT	Zinc (II) Pyrithione

1.0 Pharmaceuticals and Personal Care Products – An emerging class of environmental pollutants.

1.1 Introduction:

Until relatively recently pharmaceuticals and personal care products (PPCPs), enjoyed anonymity as an undiscovered class of environmental micro contaminants. In 1985 Richardson and Bowron hypothesised that PPCPs may indeed enter the aquatic environment as a result of either industrial or human waste disposal to wastewater treatment plants (WWTPs) where they may totally biodegrade, partially biodegrade or persist. Therefore, as a consequence treated effluents that are discharged into receiving waters may contain residues of pharmaceutical compounds [1]. The concept that PPCPs may be present in the environment was a new one. However, the presence of PPCPs in the environment received little attention until the late 1990s and the advent of sophisticated analytical instrumentation and methodologies capable of detecting the ultra trace quantities of PPCPs likely to be present. Up to then research focused on classes of pollutants that could be readily analysed with confidence using traditional 'gold standard' gas chromatographic mass spectrometric (GC-MS) methods such as polynuclear aromatic hydrocarbons (PAHs), chlorinated organics and pesticides to name but a few [2]. The fact that PPCPs reflect an emerging class of organic micro pollutants is clearly demonstrated by Fig. 1.1, which depicts the number of peer-reviewed articles concerning PPCPs in the environment published within the last decade[1].

Pollution arising from PPCP presence presents an immense and troubling problem. In Ireland alone there are ~3,000 compounds licensed by the Irish Medicines Board (IMB), for market and use as human and veterinary medicines [3] and consequently each of these compounds along with their range of metabolites may possibly enter the environment. This number of ~3,000 only accounts for compounds that require a license for sale in this

[1] Fig. 1.1 was constructed by searching for term 'Pharmaceutical* AND Environment' within the ISI Web of Science 'Science Citation Index'. Returned results were screened for suitability. The search was last performed on 20th December 2006; the database was last updated 16th December 2006.

country, i.e. pharmaceuticals and medicines but overlooks those chemicals present in personal care products such as fragrances, hair products, soaps and detergents *etc.* all of which can be freely purchased 'off the shelf' or 'over the counter' in most shops and supermarkets.

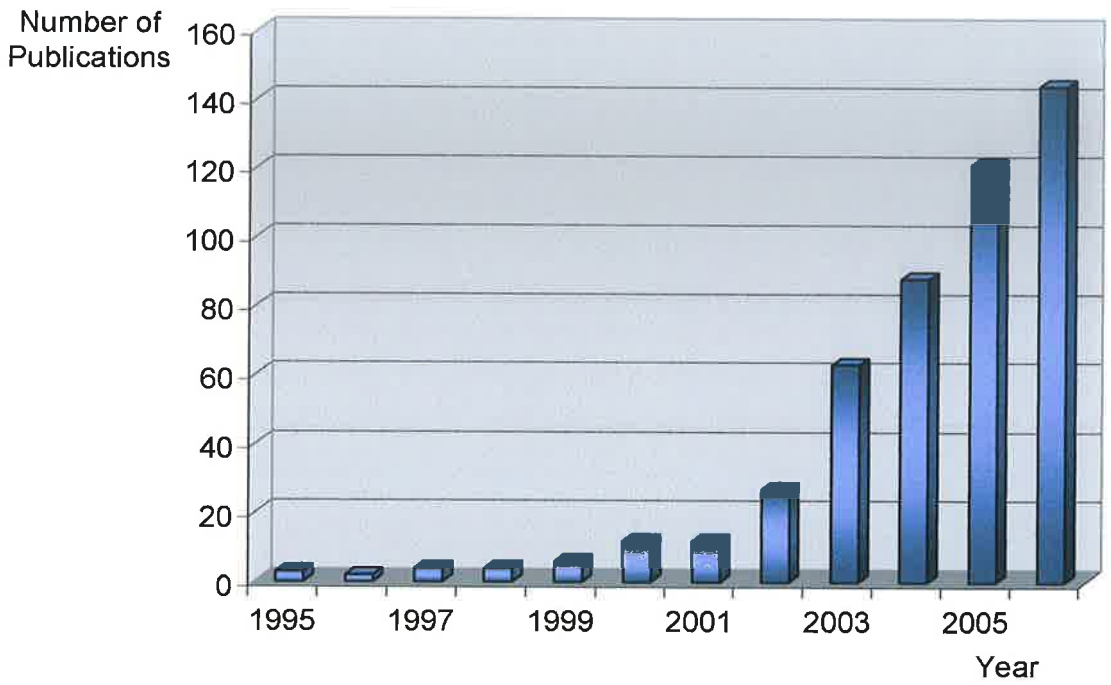


Figure 1.1: Peer-reviewed articles published within the period of 1995 to 2006 concerning pharmaceuticals in the environment, (see footnote on previous page for further information).

Ideally all anthropogenic chemicals that enter the environment should harmlessly biodegrade; however, pharmaceuticals by design are highly stable and potent biomolecules and to date very little is known about the possible impact they may have in the environment due to the lack of toxicological information. Another worrying concept connected with PPCP pollution is that unlike industrial chemicals or pesticides that are emitted from point sources during times of acute usage, PPCPs may be introduced into to the environment continually by humans wherever they may be. Therefore even areas that were once thought to be pristine, e.g. tourist attractions such as areas of natural beauty, due to lack of industry or agriculture may in fact be polluted with detectable amounts of PPCPs [4].

The alarming presence of PPCPs in the environment has resulted in the addition of two pharmaceuticals and one personal care product to the 'Oslo Paris Convention for the Protection of the Marine Environment of the North

East Atlantic - OSPAR' priority chemical list [5]. The said pharmaceutical substances are clotrimazole (antifungal agent) and diosgenin, (steroid hormone); the personal care product is musk xylene, (synthetic musk present in perfumes and fragrances). These substances were chosen due to their persistence, ability to bioaccumulate and also their toxicity and rank as highly as other pollutants such as heavy metals, organo halogens and biocides.

This section aims to present the reader with a concise review of current knowledge concerning the occurrence, fate and effects of pharmaceutically active compounds in the environment. Future prospects concerning the removal and avoidance of PPCP pollution will also be presented.

1.2 Sources of Pharmaceuticals in the Environment:

The detection of a multitude of pharmaceutical compounds in the environment posed the question of how such speciality compounds, specifically designed for use in human and veterinary medical practice could end up in ground and surface waters. When compared to other aquatic pollutants such as pesticide residues, the entry of pharmaceuticals into the environment depends on a number of integral factors [6]. These factors include the overall pharmaceutical consumption rate, the pharmacological fate of the drug within the body, the behaviour of the drug during the wastewater treatment process and the ability of the receiving water to provide adequate dilution [6,7,8]. Information concerning each factor is important when attempting to predict which pharmaceuticals may be present in the environment. Although it is acknowledged that the treatment of wastewater provides the biggest contribution to the environmental pharmaceutical load other sources including landfill leachate and aquacultural processes may also play their part. Wastes and effluents from pharmaceutical production are not, however, expected to be significant emitters due to 'Good Manufacturing Practice' regulations that require the manufacturing process to quantitatively account for all the intermediates and products during a particular synthesis [9]. Possible sources of entry of pharmaceutical compounds are visible in Fig. 1.2.

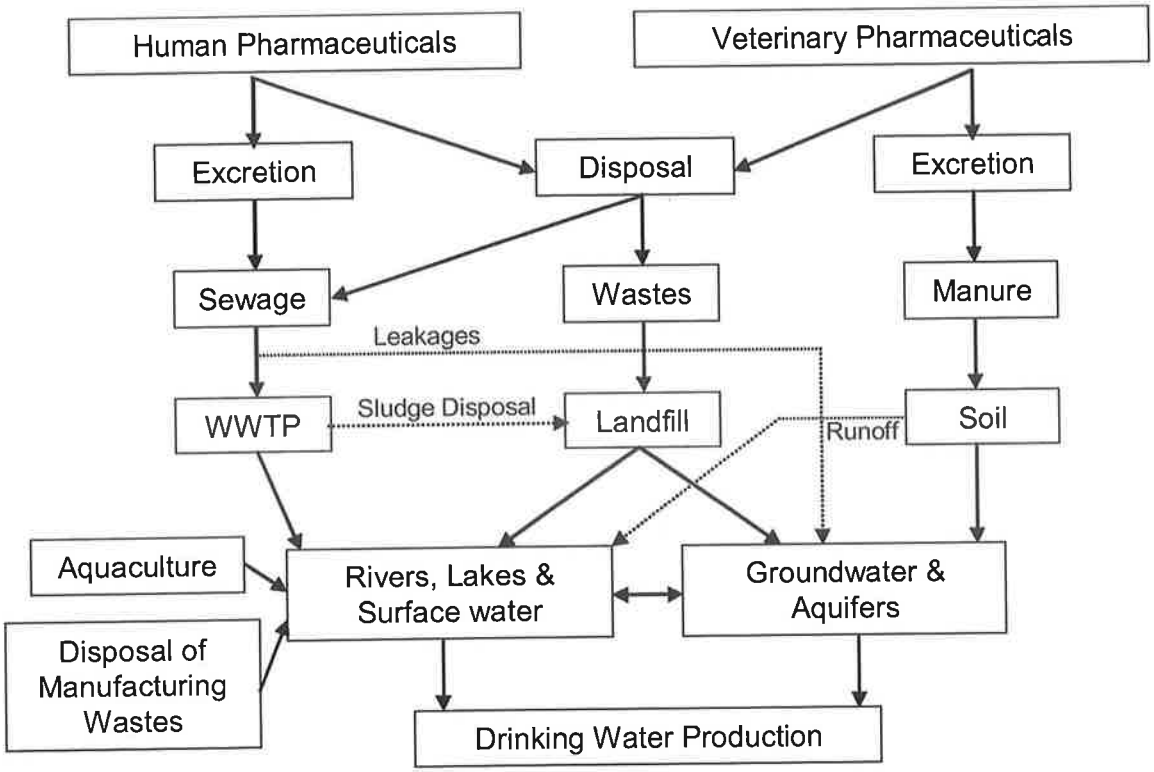


Figure 1.2: Possible sources and entry pathways of human and veterinary pharmaceuticals into the environment, adapted from, [6].

1.3 The Role of Drug Metabolism:

Pharmacokinetics is the branch of pharmacology that describes the processes affecting the absorption, distribution, metabolism and elimination of pharmaceutically active compounds in the body [10]. From the perspective of PPCPs as environmental pollutants the most important pharmacokinetic process is drug metabolism as it provides information as to whether:

- A drug will be metabolised within the body or be excreted in an unchanged form.
- If metabolism does occur, the proportions that will be excreted as parent molecule and metabolites.
- The types of metabolites that might be expected, i.e. which metabolic pathway dominates.

An important aspect of drug design is that of drug delivery, i.e. ensuring that the compound arrives at the desired site in the desired form to evoke its pharmacological effect. In order to cross cell membranes, pharmaceuticals must possess sufficient lipophilicity and consequently the primary function of drug metabolism is to transform these lipophilic compounds into more polar

metabolites that are suitable for elimination and excretion primarily through the kidneys via the urine [11].

Metabolism is an enzymatic process and involves transformation of the compound via Phase I and Phase II reactions. Phase I processes involve the functionalisation of the parent molecule in preparation for Phase II processes to occur and normally consists of the addition or activation of a reactive functional group on the parent molecule. Typical Phase I processes include oxidation, reduction, hydrolysis, hydration or dealkylation [12]. These reactions are predominantly governed by cytochrome P450 microsomal oxidase enzymes located in the endoplasmic reticulum of cells and require the presence of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH). Phase II processes involve the conjugation of an extremely polar moiety to the Phase I product leading to a hydrophilic drug conjugate that is readily removed by Glomerular filtration in the kidney. Typical conjugates include sugars or glucuronides, sulphate, amino acids, glutathione or acetyl groups [12]. A diverse group of enzymes regulate Phase II reactions individual to the conjugate, e.g. sulphotransferases, glucuronyltransferases *etc.* each requiring its own individual cofactor such as 3'phosphoadenosine-5'-phosphosulphate (PAPs), or uridine diphosphate (UDP), respectively [12]. An example of Phase I & II processes is depicted in Fig. 1.3 [11].

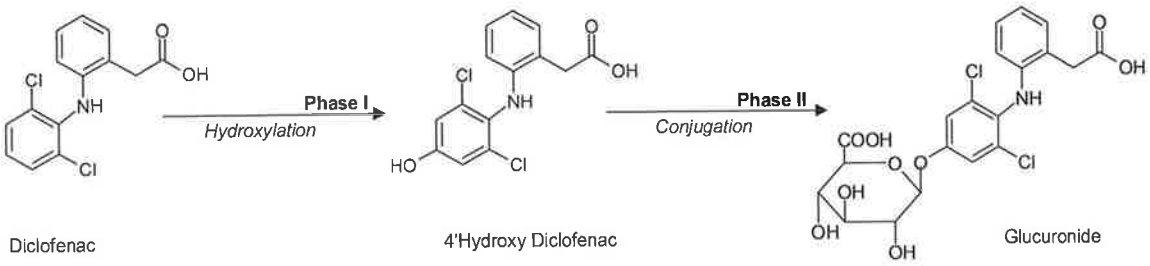


Figure 1.3: The metabolism of the non steroidal anti-inflammatory drug diclofenac by Phase I & II reactions, adapted from [11].

As demonstrated by Fig. 1.3 most pharmaceuticals are metabolised into a certain array of metabolites. However, the process becomes considerably more complicated as the enzymes involved in the metabolic reactions may be induced or inhibited by other chemicals to which a person may be exposed to either intentionally, accidentally or unknowingly through daily life [13]. Induction will increase elimination rates whilst conversely inhibition will reduce the rate of

elimination and promote retention of the parent compound within the body. Therefore, prediction of the most prominent form likely to be encountered in the environment is made increasingly difficult.

While the primary function of metabolism is to remove pharmaceutical compounds from the body, conversion by Phase I & II reactions may yield two ultimate outcomes. The first and more favourable of these is that the drug in question is rendered pharmacologically inactive and therefore, should it enter the environment it should be no major cause of concern. The second more worrying scenario is that metabolism converts the pharmaceutical compound into a more potent or toxic form, (through either pro-drug activation or parent compound conversion). Examples of more potent metabolites include the conversion of codeine and heroin into morphine whilst common drugs used in high quantities such as paracetamol are know to have highly toxic metabolites such as *N*-acetyl-*p*-benzo quinone imine [11].

1.4 The Treatment of Wastewater:

In the course of everyday life human activity consumes large quantities of water. The discharge from households and industry into drains and sewers is referred to as wastewater. Wastewater is on average >99.9% spent water with the other 0.1% comprising of dissolved and suspended solids [14]. The actual composition of wastewater is highly variable, however, likely components include micro organisms including pathogens, organic material, inorganic nutrients such as nitrogen and phosphorous compounds and metals [15]. Each of these components may exhibit their own unwanted effects if wastewater were discharged directly into ground and surface waters. Therefore, in an attempt to reduce the threat of pollution wastewater usually receives some form of treatment before final discharge into the environment. In Ireland the treatment of wastewater is governed by the Environmental Protection Agency Act of 1992 and more so by the 'Urban Wastewater Treatment Regulations 1994' passed to enact into Irish law EU directive 91/27/EEC [16]. Under Section 85 of the 1992 Act, discharges to sewers must be monitored and are licensed under the Integrated Pollution Control system to protect the receiving treatment plant and the general aquatic environment in the long run [16]. A schematic of wastewater treatment processes is depicted in Fig. 1.4.

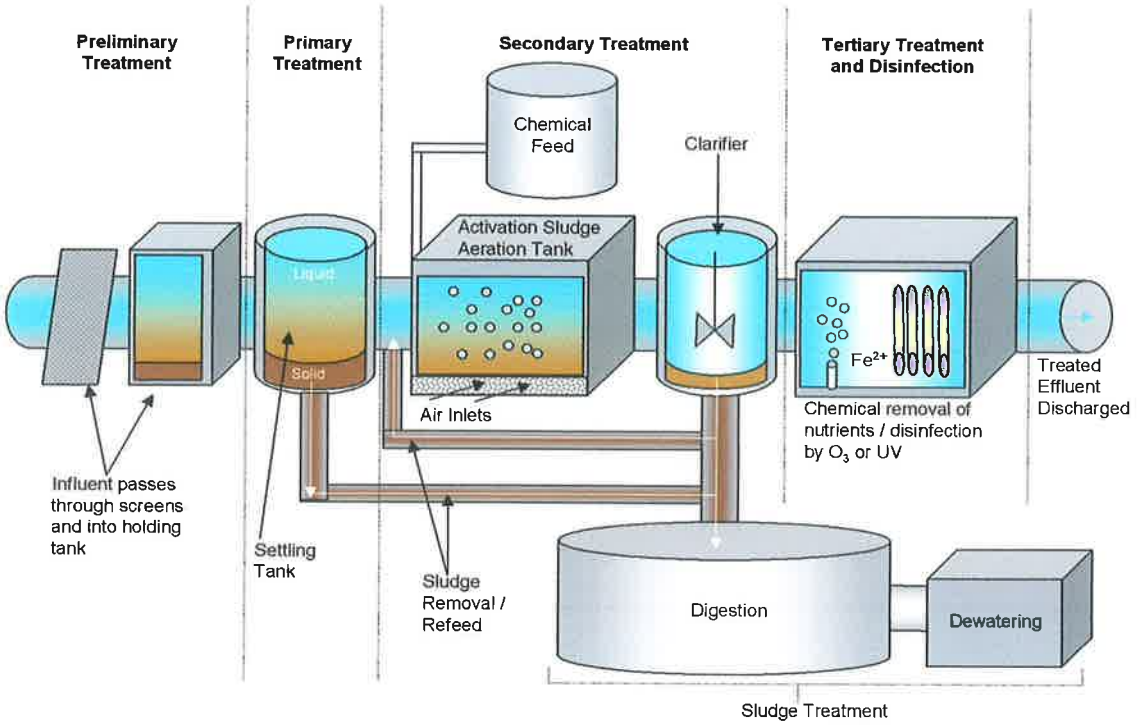


Figure 1.4: An overview of wastewater treatment, adapted from [14].

Wastewater treatment is a stepwise process of physical, biological and chemical means designed to remove the aforementioned wastewater components and protect the effluent receiving water body. Physical processes are usually involved in the preliminary and primary treatment stages. Preliminary treatment involves the filtration of influents to remove debris and large particles. The screened influent then passes into holding basins where the wastewater is held for sufficient periods of time to allow solids to settle to the bottom of the basin while organic matter such as oils, fats and greases float to the top. Solids may settle out in a variety of ways depending on their physical properties, i.e. size and density, formation of associated masses of particles or compression; whereby settling particles drag other dissolved solids downwards [16]. Both layers can then be physically removed before the next process occurs.

Biological treatment of wastewater occurs during the secondary stage, a process that is generally referred to as activated sludge treatment. Activated sludge consists of a complicated ecosystem of micro organisms ranging from heterotrophic and facultative bacterial species of *Achromobacter, Arthrobacter, Citromonas, Flavobacterium, Nitrobacter, Nitrosomonas* and *Pseudomonas* to higher protozoa such as *Amoeba*, *Opercularia* and *Trachelophyllum* and also rotifers and nematodes [17]. Activated sludge treatment involves the mixing of a

concentrated microbial population with wastewater under aerobic conditions, in order to provide both oxygen and a carbon source necessary for microbial respiration. Such conditions encourage high rates of microbial growth and consequently increased rates of microbial respiration leading to a reduction in the quantity of organic matter present within the wastewater [17]. Bacteria account for the highest proportion of microbes within the sludge and as bacterial cells grow they may produce a slime layer surrounding the cell wall. The slime layer imparts an absorptive surface onto the bacterial cell allowing for the formation of flocculated agglomerations of microbes, commonly referred to as 'flocs' [18]. The forming floc surface also absorbs colloidal and suspended matter; ionic substances may also be absorbed due to interaction with oppositely charged biomolecules within the bacterial cell wall [17]. Activated sludge treatment is a dynamic process as bacteria within a floc assimilate and utilise the adsorbed material, therefore producing free sites on the surface of the floc capable of adsorbing more and more of the wastewater matrix. However, a crucial factor is the treatment time, (or the hydraulic retention time) spent within the aeration tank in order to allow sufficient microbial activity; if the treatment time is not long enough, little organic material will be removed. The ecological make up of activated sludge is another important factor in maintaining a viable process. Protozoa species aid with treatment process by feeding off the bacterial populations thereby preventing the bacteria from reaching excessive lag phase numbers. Protozoal feeding also helps with the removal of suspended matter [17]. The final process involved with sludge treatment is clarification, i.e. liquid solid separation; the flocculated biomass is allowed to settle out of solution yielding a clarified effluent [16]. The effluent may then by subjected to tertiary treatment or be discharged into a receiving water body. The remaining sludge is either reintroduced into the aeration tank as bacterial innoculum or is itself inactivated and disposed [16].

Tertiary treatment of wastewater may be physical or chemical in nature. The objective of tertiary treatment processes is to remove non biodegradable organic materials, metals and nutrients present even after the primary and secondary processes. Chemical methods are used for the removal of eutrophication nutrients, i.e. nitrates and phosphates. Phosphates can be precipitated out of solution by the addition of calcium or iron [18], while nitrates can be converted to volatile ammonia at high pH which is easily purged from

solution by aeration. Nitrates may also be reduced at low pH to nitrogen gas or nitrous oxides [18]. Disinfection to inactivate any residual microbes, particularly pathogens may also be required and practices such as chlorination may also aid with the removal of some pharmaceuticals [19,20,21,22]. Non-biodegradable organics may be subjected to advanced oxidation using ozone, whilst metallic elements maybe precipitated out of solution by reaction with an appropriate chelating agent [23]. Physical methods such as UV radiation may also be used for non biodegradable organic removal.

An important parameter for determining the performance of the treatment process is a measurement of the dissolved oxygen concentration of the wastewater influent and effluents. The most common measurement performed is that of biochemical oxygen demand (BOD) which measures the quantity of oxygen required by microbes for organic matter consumption [16]. Each treatment stage is designed to reduce the BOD of the wastewater with the highest proportion as expected being removed by the activated sludge process. The more efficient the treatment process, the lower the expected BOD of the effluent.

1.5 The Behaviour of Pharmaceuticals in WWTPs:

Richardson and Bowron proposed three possible outcomes for pharmaceutical compounds during the treatment of wastewater [1]; mainly full degradation or partial degradation or persistence and discharge into the receiving environment with the treated effluents. The two most probable means for the removal of pharmaceuticals in wastewater treatment plants include microbial degradation either to produce compounds of lower molecular weight or ideally complete metabolism into CO_2 and H_2O, or the sorption of pharmaceuticals to particles and solid matter that can be removed by filtration or settling [4].

In 1996, Rogers reviewed the behaviour of many classes of organic contaminants including some pharmaceuticals in sewage sludge [24]. At the time, information concerning the behaviour of pharmaceuticals was mostly speculative due to lack of suitably sensitive analytical methods, however, it was suggested that the presence of pharmaceutical compounds need not be a cause of concern and it was acknowledged that many compounds, mostly antibiotics, were readily biodegradable [24]. The occurrence and behaviour of

pharmaceuticals in wastewater treatment plants was further investigated by Ternes in 1998 [25], who reported the presence of a multitude of drug residues from many pharmacological classes in the influent, effluent and receiving water of a municipal treatment plant near Frankfurt in Germany. By determining the difference between the concentrations detected in the plant effluent and influent the overall removal efficiency of the treatment process was estimated. It was reported that on average ≥ 60% of the detected drug residues were removed however, some compounds showed particularly low removal, e.g. the antiepileptic drug carbamazepine and clofibric acid, a metabolite of many lipid lowering agents and these compounds were ubiquitously present in the aquatic environment as a result [25]. Ternes also examined the presence and behaviour of highly polar and ionic compounds used as x-ray contrast media in the treatment plant and discovered that such chemicals were not removed during treatment and passed freely through the plant contaminating the receiving waters [26]. Other studies conducted have reported similar findings both in the United Kingdom [27] and Spain [28]. Carballa *et al.* focused upon sampling at each stage of the treatment process in an attempt to ascertain which treatment step provided the highest rate of removal, [28]. It was found that the degree of hydrophobicity of the analyte was important as more non polar compounds were observed to adsorb onto the primary and secondary sludge with more polar analytes remaining in the water phase and therefore, passing unhindered through the treatment plant [28]. Interestingly levels of some compounds, e.g. 17β-estradiol, were observed to increase after secondary treatment suggesting the cleavage of Phase II metabolites during biological processes. Information regarding the behaviour and removal of pharmaceutical compounds in wastewater treatment plants is of great importance when attempting to estimate the loading of such compounds into the environment. For example, Fischer and Borland estimate that between 15 to 30 tonnes of active pharmaceutical ingredients are released into the environment surrounding Sydney, Australia on a yearly basis [29] due to the insufficient treatment of wastes. With appropriate treatment these quantities could be significantly reduced.

The microbial processes leading to the removal of pharmaceuticals during wastewater treatment have not been fully investigated. Studies conducted have shown that the microbiological usage of pharmaceutical

compounds as carbon or nitrogen sources for metabolism occurs only in the absence of a primary substrate [30]. However, an equal probability exists that microbes may show no preference and metabolise pharmaceuticals even in the presence of a primary substrate depending on the affinity and resistance of the microbes enzymes to such pharmaceuticals. The removal rate of some pharmaceutical compounds in WWTPs is observed to increase with increased residence time [27], possible explanations for such an observation include the increased diversity of the microbial community with increasing sludge age or the ability of microbes to respond to limiting organic carbon availability [31].

Sorption to filterable solids during wastewater treatment has received more attention as it is generally a more understandable process than microbial degradation. Pharmaceuticals present in the aqueous phase of a WWTP may adsorb onto particulate and suspended material by hydrophobic interaction between nonpolar moieties of the molecule and lipid rich cell membranes of microbes or other agglomerations of fatty material on the sludge. Electrostatic interactions between oppositely charged groups on the pharmaceutical and the surface of microbes or particles may also be involved [31,32]. The distribution of pharmaceuticals between the aqueous phase and the solid phase is an equilibrium process, represented by solid water distribution coefficient; K_D, whereby;

$$K_D = \frac{C_{sorbed}}{C_{aqueous}} \qquad \text{(Eq. 1.1)}$$

C_{sorbed} and $C_{aqueous}$ are the concentrations of pharmaceutical in the solid and water phases respectively [32]. K_D values allow for the prediction of whether a substance will show appreciable adsorption or will preferentially remain in the aqueous phase. K_D values for several pharmaceuticals have been experimentally investigated but found to be quite low thereby suggesting that the drugs chosen would be expected to exhibit negligible adsorption and therefore, microbial degradation plays a major part in the removal of pharmaceuticals in actual treatment plants [33,34]. Another study performed by Urase and Kikuta attempted to estimate both the sorption and degradation of pharmaceuticals during activated sludge treatment [35]. They observed that the pH of the sludge played an important role in determining whether drugs would adsorb onto the sludge, however, microbial life would be eliminated under acidic conditions and therefore, the use of acidic conditions is not feasible. A

theoretical model for the prediction of probable concentrations and subsequent removal rates was described by Khan and Ongerth [36] for the 'Top 50' prescribed pharmaceuticals in Australia. Parameters included within the model included data on pharmaceutical quantities used, metabolic and excretory data, chemical and physical properties for each compound and operating data for the types of treatment plants involved. The model predicted that removal rates ranging from 14% for the antibiotic roxithromycin to 99% for the antihypertensive irbesartan.

Two other items which were observed to affect the behaviour of pharmaceuticals during the treatment process were rainfall and the infrastructure of the plant [25,37]. Ternes reported that the removal of several pharmaceuticals, predominantly analgesics, was significantly reduced during periods of increased rainfall [25]. It was also observed that the rate of removal took several days to recover to its previous level. It was suggested that the reasons for decreased pharmaceutical removal may include a reduction in microbial activity or a change in the sorption and flocculation due to increased fluid flow though the plant. Wolf *et al.* investigated the effect of sewer infrastructure in the German city of Rastatt and its role in the release of pharmaceutical compounds to the environment [37]. High concentrations of iodinated x-ray contrast media were detected in groundwater in the vicinity of sewer pipes illustrating that significant leakage of untreated sewage was occurring underneath the city and also that measurable quantities of pharmaceutical compounds were being introduced to groundwater as a result. Iodinated x-ray contrast media were therefore suggested as an anthropogenic maker species for monitoring the presence of untreated wastewater in environmental waters.

1.6 Pharmaceutical Disposal and Landfill Leachate:

According to Slack *et al.* approximately 60-70% of all municipal waste produced in the developed world is disposed of in landfill sites [38] and up to 5% of such wastes may contain hazardous materials originating solely from household use. However, as there is no legal definition of what constitutes hazardous household wastes and therefore, no enforced segregation procedures; it is difficult to accurately predict the quantities of hazardous substances being

placed in landfill sites [38]. Slack *et al.* conducted a survey in the United Kingdom in order to gauge the amounts of hazardous materials held in households and the disposal routes for such products including expired or unused pharmaceuticals [38]. It was discovered in the case of pharmaceuticals, the public did not understand that pharmaceuticals constituted a hazard or how to safely dispose of such waste. Only 19% of expired or unused pharmaceuticals were returned to pharmacies for proper disposal with ~50% being dumped in the bin and ultimately landfill and another ~20% being flushed down the toilet [38].

Emissions from landfill sites are normally gases, airborne particles or more importantly leachate in the case of pharmaceutical pollution. Leachate is expected to be complex and heterogeneous in composition depending on the types of wastes disposed in the landfill, contain both inorganic and organic constituents which pose a multitude of risks [39]. An important parameter in leachate analysis is the quantity of dissolved organic carbon (DOC), as it provides a surface for adsorption and also affects the mobility of metallic elements [39].

The first report of pharmaceutical compounds in landfill leachate was in 1995 when Holm *et al.* detected large amounts of sulphonamides, barbiturates and phenazone type analgesics in leachate plumes and groundwater in the vicinity of a landfill in Sweden that received both municipal waste and waste from pharmaceutical production over the course of its lifetime [40]. It was also observed that concentrations of the detected pharmaceuticals decreased with increasing distance from the landfill site. Phenazone type analgesics were also detected by Ahel and Jelicic in soils and groundwater below a Croatian landfill site [41]. It was estimated that the landfill site could contain up to 800 kg of phenazone and that such a level was adequate to maintain high levels of phenazone compounds in the groundwater surrounding the landfill for more than 100 years [41].

The design and upkeep of landfill sites is important in preventing the introduction of pharmaceuticals into the environment through leachate. A study of the organo-geochemical composition of seepage and leakage waters from a landfill site was undertaken by Schwarzbauer *et al.* using GC-MS [42]. More than 180 individual organic compounds were identified, mostly acids or molecules with polar groups. Pharmaceuticals detected in both seepage and

leakage water included ibuprofen, propylphenazone, clofibric acid and various sulfonamides [42]. Of these compounds propylphenazone was quantified to levels of 110-140 μgL^{-1}. The study concluded that the organic character of seepage water contained a mixture of natural and plant derived materials but also significant quantities of xenobiotic compounds suitable as tracer molecules for environmental contamination originating from landfill sites. A further study was performed by the same group to test this hypothesis [43]. It was observed that the selected marker species were indeed highly suitable for contamination tracing with propylphenazone and the insect repellent *N,N*-diethyl toluamide being detected in all samples taken surrounding the landfill site. It was also found that the concentration of the selected marker species decreased with increasing spatial distribution from the site. Sampling was performed at monthly intervals over the space of a year in order to assess time dependence on the concentration of the selected marker species. Only slight decreases were observed with detected levels remaining rather stable suggesting that landfills act as a constant source of pharmaceuticals into the environment.

1.7 Environmental Processes and Fate of PPCPs:

Once pharmaceutical compounds enter the environment the question exists as to their fate, i.e. are they transported along the watercourse and diluted to such levels whereby their presence becomes negligible, do they adsorb onto solids and accumulate over time leading to increased concentrations, or are they degraded or transformed into various other chemicals in the presence of sunlight. Few studies have been undertaken in an attempt to answer such questions but primary findings will be discussed in the following sections.

1.7.1 Environmental Transport:

Pharmaceuticals are predominantly introduced into the aquatic environment with treated wastewater at levels in the ngL^{-1} to the low μgL^{-1} range; the dilution of such residues, (and therefore, the dilution of the risk that they pose) depends upon the volume of the receiving water body and its ability to adequately disperse such chemicals. Ashton *et al.* investigated the introduction of drug residues into surface water from treatment plants in the United Kingdom [44]. Samples of surface water were collected upstream and downstream of the

plant along with the discharged effluent. In four instances, pharmaceuticals were detected prior to the treatment plant, the highest detection was the analgesic ibuprofen at a level of 181 ngL^{-1}. It was suggested that the detection of drug residues before the plant indicates that these chemicals were transported over a long range and therefore, have adequate stability to survive in the aquatic environment [44]. A statistical analysis of the concentrations of pharmaceuticals detected in the discharged effluent and receiving surface water was also performed and it was found that a positive correlation existed between the two, i.e. the levels of pharmaceuticals detected in surface water is a 'diluted' reflection of the quantities present in effluent which in turn is a reflection of overall usage of those particular pharmaceuticals [44].

Other studies conducted have also illustrated that pharmaceuticals can undergo long range transport in the aquatic environment. Thomas and Hilton detected fourteen pharmaceuticals in British estuaries of the Thames, the Tyne, the Mersey, the Tees and Belfast Lough [45] and suggest that the detection of pharmaceutical analytes in estuaries is a result of contaminated surface water infiltration. Two separate studies were conducted to determine pharmaceutical residues in the North Sea [46,47]. The North Sea is a particularly sensitive water mass which accepts rivers from the United Kingdom, Norway, Sweden, Denmark, Germany, Holland, Belgium and France. Clofibric acid was detected in both studies; it was observed that a concentration gradient existed from the mouth of certain rivers, in particular the Elbe, to the open sea and that levels detected were relatively stable over a considerable period of time and comparable with other "classic" pollutants such as Lindane [46,47].

A detailed study of the river Elbe in Germany was conducted by Wiegel *et al.* [48]. It was found that the river was heavily polluted with pharmaceutical residues due to the large number of WWTPs discharging into the river. Transport of pharmaceutical residues can be observed with increasing concentrations being detected with distance from the rivers source to its exit; ~700 km [48]. Levels of clofibric acid were detected in the rivers plume into its North Sea estuary and the authors conceded that the river is a significant source of clofibric acid in to the monitored marine environment.

The stability of pharmaceuticals in the environment is significant however, it is also acknowledged that due to a 'steady state' of introduction, levels entering the environment are sufficient to replace those being removed

[49]. Pharmaceutical residues therefore, lend themselves as appropriate marker species for tracking the transport and dilution of wastewater in the environment [50].

1.7.2 Photochemical Attenuation:

Knowledge concerning the fate of pharmaceutical compounds in the natural environment is essential when attempting to quantify the risk that they pose. Pharmaceuticals may be subject to both biotic processes, e.g. biological transformation and abiotic processes e.g. hydrolysis, photolysis or sorption in aquatic systems. Of the above processes, studies have shown that photolysis of pharmaceutical compounds in aquatic systems is significantly more important than other biotic and abiotic processes [51]. Two distinct pathways exist by which photolytic reactions may occur; direct photolysis, wherein a molecule upon the absorption of light becomes unstable and decomposes or indirect photolysis, wherein molecules interact with the reactive intermediate of another species produced by its absorption of light [52,53]. Research on the photochemical fate of pharmaceutical chemicals is limited, with only a handful of papers on the subject published to date. Of those only two have attempted to evaluate the fate of a variety of compounds while the majority have focused on a single analyte or chemically similar analytes.

The fate of the bactericide Triclosan was investigated in two separate studies. Lindstrom *et al.* examined the behaviour of Triclosan and methyl Triclosan in Swiss wastewater effluents and surface water [54]. They observed that Triclosan showed no appreciable sorption to sediments due to its presence in the phenolate anionic form at the pH of the lake water. The primary method of Triclosan removal from the lake was due to direct photolysis of the anionic form of Triclosan due to a spectral shift upon dissociation that allowed for significant overlap with the spectrum of natural light. It was also noticed that the levels of Triclosan present in the lake varied seasonally with sunlight intensity, with increased quantities present in the winter. In summer a stratification of the lake occurred leading to significant degradation of Triclosan in the surface layer [54]. Mezcua *et al.* focused upon the formation of 2,7- and 2,8-dibenzodichloro-*p*-dioxin as products of the photodegradation of Triclosan [55]. Again it was observed that only the dissociated form of Triclosan displayed significant photoactivity.

Buser *et al.* reported the rapid photodegradation of the non steroidal inflammatory diclofenac in Swiss lakes [51] and found that photolysis accounted for the removal of up to 90% of the pharmaceutical. Again, as was the case with Triclosan, sorption to suspended solids and sediments was discounted as an important attenuation process. Direct photolysis was found to be an extremely fast process with >95% removal in just 4 hours of exposure. Seasonal variations of light intensity were also examined and observed to follow the same patterns as Triclosan with higher concentrations being detected and predicted during the winter months. Schmitt-Jansen *et al.* also examined the photodegradation of diclofenac and noted the formation of six photoproducts, four more polar than the parent molecule and two less polar than diclofenac, within a period of 53 hours of irradiation using LC-UV [56]. The observed photoproduct peaks were the observed to decrease in intensity after such time and were completely absent after 144 hours of irradiation with natural sunlight. The chemical identification of the observed photoproducts was not undertaken. Similar research was also undertaken by Packer *et al.* [57], Lam *et al.* [58] and Zhang *et al.* [59].

The presence of antibiotic compounds in the environment has caused concern due to the possible development of microbial antibiotic resistance. Turiel *et al.* investigated the photochemical fate of two commonly used and potent quinolone and fluoroquinolone antibiotics; oxolinic acid and ciprofloxacin respectively [60]. It was noted that ciprofloxacin degraded much faster than oxolinic acid upon irradiation. The matrix in which the experiment was performed also played an import role as humic material was observed to decrease the rate of photodegradation [60]. Results suggested that quinolone antibiotics, due to their much slower rates of degradation may pose more of a risk to the development of microbial resistance than fluoroquinolones. However, attempts to identify the photoproducts of ciprofloxacin revealed that those compounds still contained the active centre of the molecule. Therefore it was suggested that in attempting to perform environmental risk assessments both the parent molecule and degradation products should be considered. Isidori *et al.* recommended the same approach as they investigated the ecotoxicity of both the anti-inflammatory naproxen and its photoproducts and the diuretic furosemide and its photoproduct and observed that the photoproducts were more toxic than the parent pharmaceuticals [61,62].

A detailed investigation into the direct and indirect photolysis of the selective serotonin reuptake inhibitor, fluoxetine, in surface waters was performed by Lam *et al.* [53]. The direct first order photolytic half life of fluoxetine was experimentally determined to be 7 days but the reaction was observed to proceed faster at alkaline pH. Degradation pathways were elucidated by mass spectrometric studies and three decomposition products were identified. Indirect photolysis was observed to proceed considerably faster than direct photolysis with an experimentally determined half life of 55.2 hours in reagent water and even lower values recorded in surface water [53]. The authors acknowledge that indirect photochemical reactions through hydroxyl radical mediated pathways could play an important role in the removal of fluoxetine from surface waters [53].

The role of innate photosensitisers such as nitrate and humic material on the photodegradation of a range of pharmaceuticals was investigated by Andreozzi *et al.* [52]. Humic material may act as a reactive intermediate due to its ability to produce hydroxyl radicals; however, it can also decrease the effect of radiation on other molecules by acting as a protective filter [52]. As was observed in other studies, seasonal variations affected the rates of photodegradation. The presence of nitrate caused an increase in the rate of photodegradation of pharmaceutical compounds due to the production of reactive hydroxyl radicals during the photolysis of nitrate. The noted effects of humic acid were twofold, humic materials appeared to act as photosensitisers and increase the photodegradation of the antibiotic ofloxacin, the sulphonamide sulfamethoxazole, the β-blocker propranolol and clofibric acid, while the opposite 'filtering' effect was observed to reduce the photodegradation of carbamazepine and diclofenac [52]. However, although humic materials add a degree of complexity to the photochemical process, it is still the most significant abiotic process affecting pharmaceuticals in the natural environment.

1.7.3 Sorption and Mobility in Solid Matrices:

Pharmaceuticals may adsorb onto solids during the treatment of wastewater and therefore, be removed with the sludge. While this process attenuates the levels of pharmaceuticals being discharged along with the treated effluent, a problem exists with the disposal of sludge, which as a result is likely to contain quite significant quantities of pharmaceuticals, e.g. a German study found

levels of Triclosan at ~50 ngL^{-1} in WWTP effluent and ~1200 ngg^{-1} in the corresponding sludge [63]. Therefore, the probability exists that pharmaceuticals may leach out under suitable conditions. A similar problem exists with veterinary medicines that may be introduced into the environment through the spreading of treated animal wastes on lands as fertiliser thereby contaminating soils, groundwater and surface water through overland flow [64,65].

The sorption of drug residues in the environment leads to increased localised concentrations of those particular analytes. One particular analyte known to accumulate is the antibacterial agent Triclosan. As discussed previously (section 1.7.2) , it has been demonstrated that at high pH Triclosan is readily photo degraded, but the associated form of the molecule is relatively stable, so much so that it has been shown to bioaccumulate in fish exposed to treated wastewater effluent and even in human milk [66]. However, conflicting reports were published by the manufacturers of Triclosan who claim that the molecule is not persistent [67]. The sorption of veterinary pharmaceuticals to soils was reviewed by Tolls [68] who reported that traditional approaches to describe sorption such as K_D, K_{ow} and K_{oc} that cater solely for sorption through hydrophobic interaction do not properly portray the sorption behaviour of drugs in soils. These parameters fail to account for hydrogen bonding, ion exchange and chelation, which are more important sorption and retention mechanisms for pharmaceuticals likely to be charged at soil pH, [68]. Christian *et al.* determined antibiotics in German soil, [69] and observed that one particular sulphonamide; sulfadimidine, was stable for long periods of time after application.

The potential for pharmaceuticals bound to soils to leach and contaminate groundwater has become the focus for many studies. Oppel *et al.* investigated the leaching behaviour of six drugs from two different soil columns of different pH and organic content [70]. The study reported that carbamazepine, diazepam, ibuprofen and ivermectin were retained on both soil columns, while clofibric acid and iopromide were determined solely in the leachate. Using radio-labelled standards it was possible to determine the depth penetration of the pharmaceuticals in the column. In the low pH high organic content soil the radioactivity was recorded only up to a depth of 5 cm whilst in the high pH low organic content soil penetration of up to 20 cm was noted [70]. It was concluded that the retained drugs pose little threat to groundwater as a

result of soil leachate and vice versa. The pH of the soil and the organic content were also important factors affecting the mobility of the monitored pharmaceuticals [71,72]. Kay *et al.* published two studies concerning the mobility of three veterinary antibiotics in soil treated with slurry [73,74]. Both studies detailed that oxytetracycline and the macrolide tylosin do not leach from soil with the application of slurry but the sulphonamide; sulfachloropyridazine was highly motile, with quantitative recovery of the applied quantity used during the study. The high mobility of the sulfonamide was attributed to macropores in the soil structure that allowed for unhindered transport to drainage systems and ultimately surface water [73]. The application of slurry was observed to cause an increase in soil pH but tillage of the soil prior to application was found to remove the risk for all the studied compounds [74]. Similar findings to the above studies were reported by Drillia *et al.* [75]. Again it was observed that the soil type and channels within the soil were important, however, simulated rainfall events were performed and it was observed that the flow and volume of rain affected the adsorption and the mobility of the drug through the soil. The higher the volume and flow, the less drug adsorbed, suggesting that high concentrations of pharmaceuticals may be released to surface and ground water during intense rainfall, although the increased volume will aid with dilution [75].

The processes affecting the concentrations and fate of pharmaceuticals in the environment are highly complex. Pharmaceuticals have adequate stability and can be transported over considerable distance through the water course. Soils may adsorb some pharmaceutical residues, however, such an effect was observed to concentrate levels in the uppermost topsoil layer [76]. Rainfall can affect sorption and lead to increased introduction of drug residues into groundwater. Photolysis is an important process in the removal of pharmaceuticals from the natural environment but is itself a complicated process as many transformation products may still contain active groups and express pharmacodynamic effects.

1.8 Assessing the Ecotoxicology and Risk posed by PPCPs:

The presence of pharmaceutical residues in the environment has caused concern as these chemicals are specifically designed to exhibit a pronounced biological effect on the organism for which they were developed. The possibility also exists that drug residues may also affect different species that express the same receptors or more worryingly cause unknown chronic toxicological effects in other unrelated organisms that may go unnoticed until it becomes too late. In Europe an environmental risk assessment (ERA) is required to be performed prior to a licence being granted to a particular pharmaceutical in an attempt to predict whether or not the drug poses a significant toxicological risk to the environment. The assessment is a legal obligation and enforced under EU Directive 93/39/EEC. A centralised agency, the European Medicines Evaluation Agency (EMEA), was also established under Regulation 2309/93/EEC with responsibility for the licence and registration of medical products for use within the EU [77]. Reviews of the above legal framework have been undertaken with the updated Directives 2001/82/EC and 2001/83/EC now enforced. These directives cover the licensing of veterinary and human medicines respectively and undertaking appropriate environmental risk assessments are primary functions of both documents [78].

The EMEA risk assessment procedure is a tiered process as depicted in Fig. 1.5 below.

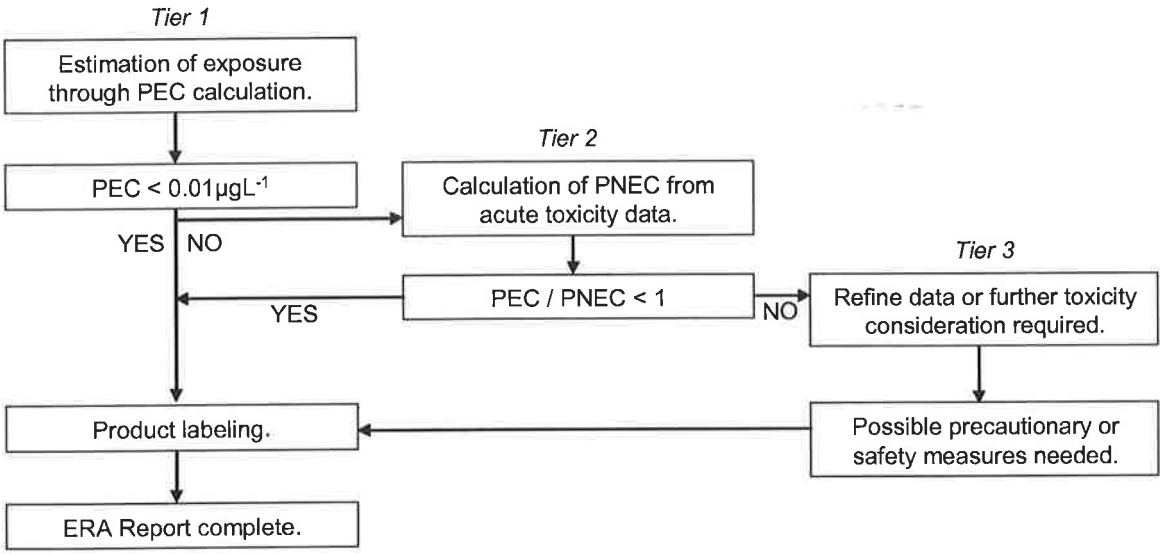


Figure 1.5: Schematic of ERA, adapted from [79].

The first tier of the ERA process involves the calculation of a crude predicted environmental concentration (PEC) for the pharmaceutical in question or its metabolites. PECs are usually calculated using Equations 1.2, (initial directive) or 1.3, (updated directive) below, which account for approximate quantities of the drug used, its removal rate and dilution in receiving water.

$$PEC = \frac{A \times (100 - R)}{365 \times P \times V \times D \times 100} \qquad \text{(Eq.1.2), [79].}$$

Where:

A is the amount used per year, ($kg.year^{1}$).

R is the removal rate.

P is the population considered.

V is the volume of wastewater produced per capita per day, (m^3).

D is the dilution factor in the receiving body.

OR:

$$PEC = \frac{DDD \times F_{pen}}{V \times D \times 100} \qquad \text{(Eq. 1.3), [80].}$$

Where:

DDD is the defined daily dose in mg per person per day.

F_{pen} is referred to as the penetration factor, i.e. the faction of the population being treated with the particular drug.

If the calculated PEC value is less that the 0.01 μgL^{-1} limit no further action is required and the investigation is complete. If, however, the calculated PEC is above the limit, the investigation proceeds to Tier 2 and the predicted no effect concentration (PNEC) is estimated by examining acute toxicity data performed on algae, Daphnia or fish and dividing the reported worst case endpoints, (i.e. EC_{50} or LC_{50}) by an assessment factor to impart a margin of safety [81]. If the PEC/PNEC ratio is less than one, i.e. the level of pharmaceutical that may exhibit a toxicological response is less than the predicted amount likely to be present, then the ERA is considered complete. If the ratio is greater than one, the third tier of the assessment should be performed which may involve carrying out chronic toxicity studies such as fish life cycle studies or the recommendation that the product is only administered and used in a controlled environment [77]. It is also noted the PEC limits are not set in stone and maybe changed accordingly to account for low dose high potency situations and vice versa. Also potential benefits out weigh

environmental concerns as even if the compound is found to pose a significant risk it will still be granted a licence if it is proved to be a highly effective medicine [77].

A number of studies have been undertaken to evaluate the risk posed by established pharmaceutical compounds to the environment. By evaluating PECs, analytes likely to pose an environmental risk can be identified. Castiglioni *et al.* undertook such an approach and using the results from developed analytical methods, compared the measured environmental concentration (MEC) with the calculated PECs [82]. It was observed that a refined PEC, corrected for metabolism and environmental half life provided a good estimation of the MEC, although PECs were generally higher than MEC. It was also observed that using the Equations 1.2 and 1.3 above to calculate crude PEC values leads to an over estimation. A similar investigation was performed by Ferrari *et al.* and again it was noted that the PEC was a useful approximation of the MEC [79]. However, in all cases the PEC and MEC were above the Tier 1 so a Tier 2 assessment was performed. The results of the Tier 2 assessment suggested that calculating the PNEC based upon acute toxicity bioassays may lead to confusing results and to avoid such situations PNEC values based upon chronic toxicity data may be more suitable. The reason being that the levels of pharmaceuticals in question are considerably lower than the minimum concentrations needed to exhibit a response in an acute toxicity bioassay [79]. Another ERA report by Christenen [83] found that three potent pharmaceuticals; 17-α-ethinylestradiol, phenoxymethylpenicillin and cyclophosphamide posed no threat to aquatic organisms or humans at the PEC. Similar research was also undertaken by Hernando *et al.* [84], Lissemnore *et al.* [85] and Pomati *et al.* [86].

Jones *et al.* assessed the 'Top 25' pharmaceuticals in the United Kingdom using the EMEA ERA approach in the aquatic compartment and attempted an assessment for sludge [87]. Using a lower Tier 1 limit of 1 ngL^{-1} it was necessary to perform a Tier 2 assessment for all pharmaceuticals on the list. Again it was noted that Tier 2 results can be ambiguous due to the use of acute rather than chronic toxicity data to calculate the PNEC but the PEC/PNEC ratio was less than unity for all but four compounds. Even in these situations it was acknowledged that calculations were performed using 'worst case scenarios' and likely environmental concentrations would be significantly

lower, thereby minimising the risk [87]. In the case of sludge, as no toxicity data was available, no risk assessment was performed, however, modelled results suggest that sorption and accumulation in sludge are not likely to be major processes [87]. Similar results were reported by Stuer-Lauridsen *et al.* who also noted the lack of toxicity data for sludge [88]. The calculation of PEC values for sludge were noted to be considerably more difficult as it requires masses of pharmaceutical consumed and masses of sludge produced. Large variations in the sludge PEC were observed depending on whether or not the value of K_D was corrected to include all forms present, (i.e. the inclusion of pK_a and pH into the expression). The results were found to vary by several orders of magnitude but it was noted that PEC values calculated using uncorrected K_D may simulate a 'worst case scenario' [88].

Often the ERA procedure is performed only for a single compound and the possibility that the cumulative or even synergistic effects by functionally similar compounds are ignored. Such possible effects were investigated by Cleuvers [89,90] using a 'concentration addition' estimation. It was concluded that acute effects due to pharmaceuticals are rather unlikely due to heterogeneous toxicity in the mixture of drugs tested and also that the likelihood for increased mixture toxicity is reduced if any of the compounds present is below its no observed effect concentration (NOEC). As this is the most likely scenario due to the ultra trace concentrations of pharmaceuticals suspected to be present, acute mixture toxicity is not expected but further research to evaluate chronic mixture toxicity is recommended [89]. Golet *et al.* estimated the exposure and risk of various fluoroquinolone antibiotics in Switzerland [91], using the combined concentration of fluoroquinolones detected to estimate the toxicity of a similar acting group of chemicals. The calculated risk quotients, (MEC/PNEC) was less than unity in both river water and WWTP effluent and therefore, it was reported that there was only a low risk of fluoroquinolones exhibiting adverse effects [91].

A prioritisation of veterinary medicines in the United Kingdom was performed by Boxall *et al.* using a developed ERA approach specific for animal treatments [92]. The assessment focused upon quantity used, format of usage, metabolic data and existing toxicity data where available. Based upon the prioritisation, eleven compounds received the top score and it was recommended that further studies be performed to asses their environmental

impact [92]. PEC values for veterinary medicines in soil were estimated by Blackwell *et al.* [93] and it was found that the PEC was a conservative estimate of the MEC and although it does not account for persistence or accumulation a sufficient margin of safety was included and therefore acute toxicity was not expected. Koschorreck *et al.* reported ERAs for three veterinary medicines [94]. In all cases Tier 1 & 2 exposures were required and for two compounds; a helmentic and a mastitis treatment, the active ingredients were found to pose a serious environmental threat after the Tier 2 assessment and warning levels were required to be fitted to the products. In the other case the Tier 2 exposure reported that the antibiotic posed no ecotoxicological threat [94].

Conceptual and computerised models have also been employed to asses the risk posed by pharmaceuticals. Conceptual ideas have attempted to link the pharmacodynamics with ecotoxicological risk posed [95,96]. It is proposed that such an assessment may be useful as unlike many other chemicals, a wealth of data exists for pharmaceuticals ranging from the activity for which the drug was designed, to mammalian data from clinical trials. No results exist to date using such conceptual approaches. Computerised models using quantitative structural activity relationships (QSARs), have also been reported [97,98,99]. QSARs allow for the generation of various types of data for a molecule based upon its structure and composition by comparison with similar molecules for which such information already exists [99]. The applicability of QSAR modelling was investigated by Sanderson *et al.* who ranked 2986 different pharmaceuticals relative to the risk they pose to algae, daphnids and fish [99]. It was found that the QSAR model was fast and provided reasonable PEC approximations but the results obtained could be improved by using a combination of models [99]. The Geography Referenced Regional Exposure Assessment Tool for European Rivers (GREAT-ER) model, was applied by Schowanek and Webb to simulate European rivers to pharmaceutical exposure [98]. It was found that the model again provided reasonable PEC estimations, however, the authors acknowledged that a refinement to include information from monitoring programs would improve the data in order to avoid deviations in the returned results [98].

Results from actual toxicity studies have also been reported in the literature. Laville *et al.* examined the effects of nine human pharmaceuticals on the enzyme activity of fish hepatocytes [100]. The pharmaceuticals were

classified and it was found that clofibrate, fenofibrate and fluoxetine were the most cytotoxic of the drugs studied and the majority of the pharmaceuticals exhibited enzyme inhibition. However, the concentrations required were >1000 times than those that are environmentally relevant [100]. Similar toxicological tests using cell cultures were also undertaken by Caminada *et al.* and Gagne *et al.* [101,102]. In the first instance, Caminada *et* al. reported effects at the mgL^{-1} concentration level for twenty one common pharmaceuticals and therefore, concluded that no risk was posed at environmentally relevant levels. Gagne *et al.* employed environmentally relevant concentrations by using treated wastewater as the matrix under investigation in their study and noted that the compounds investigated had the ability to exhibit an accelerating effect upon the rate of NADPH oxidation in the fish liver cells. A study into the estrogenic activity of thirty seven common pharmaceuticals was also performed using yeast cultures by Fent *et al.* [103]. Six of the investigated pharmaceuticals, i.e. cimetidine, fenofibrate, furosemide, paracetamol, phenazone and tamoxifen were observed to exhibit weak estrogenic activity at the high μgL^{-1} concentration level.

Mimeault *et al.* exposed goldfish to the lipid regulator gemfibrozil at an environmentally relevant concentration of 1.5 μgL^{-1} and observed bioaccumulation in the plasma of goldfish and also reduced the levels of circulating testosterone, thereby causing a disruption to the endocrine system of the test subjects [104,105]. A similar study reported feminisation of the 'fathead minnow' exposed to oestrogen in a life cycle test [106]. Emblidge and DeLorenzo investigated the risk posed by clofibric acid to three estuarine species; algae, crustaceans and fish, but concluded that clofibric acid poses no risk to the investigated species at relevant environmental levels [107]. Nalecz-Jawecki and Persoone examined the effect of twenty eight pharmaceuticals upon crustaceans and reported that seventeen were neither toxic nor slightly toxic at a concentration level of 200 mgL^{-1} [108].

Perhaps the most worrying effect was reported by Schwartz *et al.* and Ohlsen *et al.* who detail the presence of antibiotic resistant bacteria in biofilms on the distribution systems of wastewater, surface water and drinking water [109]. Bacteria carrying resistant genes were found in drinking water, this is unusual as the resistance genes detected are normally associated with enterobacteria detected in wastewater and WWTPs. Both studies suggest that

the transfer of drug resistant genes and plasmids via bacterial conjugation is extremely likely and therefore, the threat of further emergence of antibiotic and drug resistant microbes is increased due to the exposure to pharmaceuticals in the environment [109,110].

1.9 PPCPs and Drinking Water:

The presence of pharmaceutically active compounds in surface and groundwater poses a troubling concern as such systems are often used as supply for the production of potable water. Although the previous section focused upon the ecotoxicological risk caused by pharmaceutical residues to exposed marine organisms, the presence of drugs in drinking water would provide direct entry into human body for any residues present. To-date there have been very few published reports confirming the presence of pharmaceuticals in drinking water, the reason for such a small number may be two fold: firstly the concentrations present may be too low and therefore, beyond the detection limits of most current analytical methods or secondly pharmaceutical residues may be efficiently removed during drinking water treatment [111].

The presence of phenazone drugs and their metabolites in German drinking water was reported by Reddersen *et al.* [112] and Zuhlke *et al.* [113]. Levels detected in both studies were in the μgL^{-1} range using GC-MS for the analysis of samples of Berlin's potable water. Clofibric acid, the macrolide antibiotic tylosin and the benzodiazepine diazepam were detected in Italian drinking water, albeit in the low ngL^{-1} range [114]. The levels detected in finished potable water were higher than those measured in the river from which the source supply was taken and such an unusual fact was unexplained by the authors. Stackelberg *et al.* investigated the presence of organic compounds attributed in origin to wastewater infiltration in a drinking water treatment plant in the USA [115]. Pharmaceuticals were among the compounds detected, carbamazepine was present in the largest quantity at 0.258 μgL^{-1} along with caffeine and trace quantities of a metabolite of nicotine [115]. Analyses for pharmaceuticals were performed at the various stages of drinking water production in plants in Louisiana, USA and Ontario, Canada [116]. In both plants the analgesic naproxen was determined at ~60 ngL^{-1} in raw supply but

was absent from all finished samples suggesting that the drug was efficiently removed during the final disinfection process, (ozonation and chlorination).

Although the levels of drugs present in drinking water are extremely low the risk posed to humans through continual exposure needs to be assessed. Webb *et al.* estimated the lifetime exposure of the average person who drinks two litres of drinking water per day every day over the course of a seventy year lifespan for sixty pharmaceutical compounds [117,118]. Assuming the worst case scenario, the lifetime ingestion of pharmaceutically contaminated drinking water was less than the daily recommended dose for the vast majority of compounds investigated [118]. Similar findings were recently reported by Schwab *et al.* who employed an analogous model [119]. In the case where the lifetime exposure was greater than a recommended daily dose, (e.g. for ethinylestradiol and the x-ray contrast medium iopromide) the calculation could be refined and corrected to include metabolic and WWTP removal data. The question of the presence of antineoplastic and cytotoxic pharmaceuticals was also raised, as although these chemicals are used in the treatment of cancer, many are inherent carcinogens themselves and therefore, a significant risk is posed by any level, even the most minute exposure [118]. The general conclusion from both studies was that the risk is indeed low. However, further investigations need to be undertaken to asses the risk posed to more vulnerable groups of society such as infants, the elderly, dialysis patients *etc.* [111]. Despite the low risk public perception will be an important factor, to quote from Ref. [120], "As a friend of mine said, even if there's no toxicological problem, it makes you a little uncomfortable to know that you are drinking something that's been through someone else's kidneys".

1.10 Technologies for the Removal of PPCPs:

In the previous section the second proposed reason for the low levels of pharmaceutical compounds detected in drinking water was attributed to the so called 'polishing' treatments used during production. Disinfection of treated water is essential to prevent the outbreak of disease and is normally performed using strong oxidants such as chlorine, ozone, UV, peroxide *etc.* The applicability of such established techniques for the removal of anthropogenic

chemicals, including pharmaceuticals, has attracted considerable attention of late.

The first report of the oxidative treatment of pharmaceuticals in water was in 2000 by Zwiener and Frimmel [121] who investigated the ability of ozone, and ozone along with hydrogen peroxide, to degrade three environmentally relevant pharmaceuticals. Results showed that the anti-inflammatory diclofenac was readily degraded at an exposed dose of 1 mgL^{-1}. Using a combination of ozone and hydrogen peroxide diclofenac was again removed and ibuprofen and clofibric acid that were previously persistent could be reduced to approximately 50% of their initial concentration [121]. The study also examined the role of DOM present in natural waters. Such natural materials may also react with hydroxyl radicals formed during ozone/H_2O_2 treatment in a scavenging manner, thereby reducing the efficiency of the oxidation process. In order to avoid such effects it was found that the quantity of ozone needed should equal the quantity of DOM present. Under such conditions the oxidative procedure was efficient in removing the drug residues present [121]. Ternes *et al.* examined the removal of pharmaceutical residues using four different processes common to drinking water treatment [122]. Both slow sand filtration under both aerobic and anoxic conditions and flocculation using ferric chloride were inefficient in removing pharmaceutical residues. Granular activated carbon (GAC), was efficient at removing three of the four investigated analytes, however, as expected the most polar; clofibric acid showed little affinity for the carbon sorbent. Ozonation was also found to be efficient but also highly selective. It was observed that specific doses were required to remove each investigated pharmaceutical [122] and in agreement with Zwiener and Frimmel, clofibric acid was stable even at high doses indicating that it may persist and be present in drinking water samples. Another paper investigating ozone applicability published by Ternes *et al.*, concerning the removal of pharmaceuticals and x-ray contrast media from wastewater, also confirmed that ozonation is a suitable tool for the removal of drug residues [123]. It was, however, noted that the ionic contrast agent diatrizoate was extremely persistent with a maximum removal of only 15% under extremely high ozone doses [123]. Ozonation was also examined as a feasible option for the removal of estrogenic activity during drinking water treatment [124]. By applying doses normally used during treatment, ~1 mgL^{-1} or higher, the three

estrogenic compounds investigated and their estrogenic activity was almost completely removed. It was estimated that the half life of the most potent oestrogen, 17-α-ethinylestradiol at pH 8 was in the order of 1 ms [124].

A potential problem with the oxidative treatment of pharmaceutical compounds is the formation of unknown oxidation by-products. The oxidation of diclofenac using ozone and UV/H_2O_2 was investigated by Vogna *et al.* [125]. Diclofenac degradation was found to be quite fast and efficient using both oxidation systems. Treatment with both oxidants was found to add hydroxyl groups to the molecule followed by cleavage of the carbon nitrogen bond to yield dihydrophenyl acetic acid, dichlorohydroquinone and dichloroaniline amongst others. By-products were identified by GC-MS, preparative thin layer chromatography (TLC) and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR). The identified oxidation by-products were further degraded into organic acids upon further ozone exposure [125]. Therefore, it may be concluded that oxidative treatment may have sufficient ability to completely remove pharmacological activity, however, diclofenac is a rather simple molecule and further research will indeed be necessary to evaluate the risk posed by the oxidation by-products formed during the degradation of more complex and potent chemicals.

The oxidation of clofibric acid using ozone and UV/H_2O_2 was performed by Andreozzi *et al.* [126]. The mineralisation of the compound under applied oxidant dose was monitored by the formation of chloride ions. As with previous studies it was observed that ozone degradation of clofibric acid was a faster process than UV/H_2O_2 but both oxidation systems were efficient. The degree of mineralisation was better under ozone with all chlorine quantitatively recovered as chloride indicating that no chlorinated oxidation by-products were formed [126].

The kinetics of the oxidation processes are important in attempting to evaluate the doses oxidant and contact times needed. Andreozzi *et al.* evaluated the kinetics of clofibric acid oxidation using the two aforementioned oxidants [126]. It was observed that the kinetics of ozonation depended greatly upon the pH at which the experiments were performed, i.e. at higher pH the clofibric anion is the dominant species in solution and this moiety was much more reactive with ozone than the associated parent acid [126]. The pH of the experimental solution during the UV/H_2O_2 oxidation system was found not to

play a major role. These findings can be explained by the fact that ozone is selective to certain functional groups while hydroxyl radicals are unselective and reacts with many sites on molecules [124]. The kinetics of the oxidation using UV/H_2O_2 were determined for two pharmaceutical intermediates by Lopez *et al.* [127], it was found that second order rate constants were quite large, (10^8-10^{10} $M^{-1}s^{-1}$) suggesting that the oxidation is quite efficient. The doses of oxidant required for complete substrate removal were significantly higher than those commonly used during disinfection treatments and therefore, maybe uneconomically feasible for large scale implementation [127]. The importance of pH on the kinetics and efficiency of the degradation were also noted by Balcioglu and Otker [128] and Huber *et al.*, [129] using ozone/H_2O_2 or ozone for the removal of antibiotics and mixed environmentally relevant pharmaceuticals respectively. It was found that antibiotics were efficiently removed at neutral pH, whilst at acidic pH the oxidation power of hydroxyl radicals was suppressed and at alkaline pH ozone depletion by cross reaction with hydroxide was observed [128]. As pH obviously affects the speciation of pharmaceuticals in aqueous solution Huber *et al.* reported that sample pH values in the range of 5-10 allowed for the most efficient degradation of the analytes investigated [129]. The proposed reason is that in this pH range many pharmaceuticals can be considered as strong nucleophiles and therefore, are more prone to attack by electrophilic ozone [129]. Second order rate constants calculated for most of the test pharmaceuticals were in average two to three times higher than for other investigated organic pollutants such as MtBE or trichloroethylene, as a consequence it is expected that the oxidative degradation of pharmaceuticals is expected to be extremely efficient [129]. In a separate investigation Huber *et al.* performed a pilot study to determine the applicability of ozonation for the oxidation of pharmaceutical contaminants in wastewater [130]. The effect of suspended material, that may scavenge ozone and hydroxyl radicals, was investigated but it was found that suspended matter did not affect the oxidation of the pharmaceutical analytes but as previously mentioned the ozone dose required was proportional to the quantity of DOM [130]. In practical terms it was observed that the ozone doses required were similar to those needed for microbe inactivation and therefore, ozonation or advanced oxidation procedures may indeed be a feasible option for the removal of pharmaceutical residues from both treated wastewater and drinking water.

Biological methods have also been investigated as more ecofriendly means for pharmaceutical removal. Using biofilms and biofilm reactors it was investigated whether environmentally relevant pharmaceuticals such as clofibric acid, ibuprofen and diclofenac could be degraded [131,132]. Biofilms consist of eukaryotic and prokaryotic microbes agglomerated through extracellular secretions and may be static or dynamic. Ibuprofen was readily biodegradable, however, as the concentration of ibuprofen decreased, its two metabolites, hydroxyl-ibuprofen and carboxy-ibuprofen, were observed to increase in solution [132]. The experiment was performed using both isomers of ibuprofen and it was noted that microbial biodegradation of the inactive *R*-isomer readily occurred whilst worryingly the pharmacologically active *S*-isomer was observed to be resistant to degradation. Clofibric acid was also unsuccessfully removed by the biofilm [132]. Zwiener and Frimmel also reported that clofibric acid and diclofenac were also resistant to biodegradation under aerobic conditions but a slight removal improvement was reported when the films were operated under anoxic conditions. The formation of hydroxyl-ibuprofen as a function of ibuprofen removal was also noted [131]. The applicability of biofilms therefore, appears to be limited when compared to the excellent removal efficiency of chemical oxidants.

1.11 Prevention is better than Cure:

As pharmaceuticals are continually being introduced into the environment along with treated wastewater effluents the problem appears to be persistent. Finding a solution will not be an easy task as the benefit to medicine outweighs environmental presence and risk. The advent of environmentally friendly pharmaceuticals is not going to happen in the short term considering the amount of time and money required for drug development. A concept introduced in Sweden may provide some promise. Pharmaceuticals were ranked based upon the threat they pose to the environment allowing doctors to prescribe, or patients to choose, more environmentally friendly treatments from existing medicines [133]. The use of the 'precautionary principle', which can be interpreted as the lack of scientific facts concerning a potential hazard not justifying inaction to prevent such a hazard [133], is inappropriate of pharmaceuticals for the above reasons.

As the majority of pharmaceuticals and their metabolites are excreted from the body through the urine, urine separation and subsequent treatment may be a viable for minimising the quantities of pharmaceuticals released to the environment [134]. Urine separation offers a number of potential benefits, primarily the removal of 80% of the nitrogen and 45% of the phosphate loading into wastewater treatment plants [135]. Larsen *et al.* note that in the absence of urine a carbon nitrogen balance is achievable in WWTPs and under such conditions the microbial population feeding on organic matter could successfully remove all nitrogen content [134]. Urine separation does not require massive capital investment to be performed, simply the change over from standard to so called 'NoMix' toilets which store the urine in a tank for later remote controlled discharge [134]. Rauch *et al.* developed a model for urine production in the average household that would help with planning the frequency of collection and subsequent treatment [136]. A report of urine separation in practice was also recently published [137]. A urine separation system was included when a student dormitory was being constructed. The system did not function as well as planned for nutrient removal, however, it was found that user attitudes and ignorance were to blame. Also the cost of implementing the system lay with the management company when compared with municipal sewer systems, whereby the expense is completely on the local authority. The author acknowledged that administrative change may be required to make the system more attractive. Urine separation is still hoped to be a prospect in minimising environmental contamination with pharmaceutical compounds, especially if used in conjunction with the aforementioned chemical oxidants [133].

An alternative use for spent activated sludge was proposed by Otero *et al.* [138] who used sludge for the removal of two dyes and phenol from water. The sludge underwent pyrolysis and acid activation and it was found that such treatment imparted high porosity and a large surface area to the new sorbent. Studies with the three chosen compounds revealed that they could be efficiently removed from water although the interaction mechanism was not evaluated. It was suggested that the material may be used for the successful removal of other organic micro pollutants.

It appears that finding an acceptable solution for the prevention of pharmaceutical compounds contaminating the aqueous environment is by no means an easy task and therefore, an area that requires significant research.

References:

1. M.J. Richardson & J.M. Bowron, *Journal of Pharmacy and Pharmacology* **37**, (1985), 1.

2. R.N. Reeve, *Introduction to Environmental Analysis*, (2002), J. Wiley & Sons, New York.

3. The complete list of licensed pharmaceuticals in Ireland as provided by the Irish Medicines Board, www.imb.ie, personal communication, January 2004.

4. C.G. Daughton & T.A. Ternes, *Environmental Health Perspectives*, **107**, (1999), 907.

5. OSPAR List of Chemicals for Priority Action; Update 2002. Reference number 2002-18, OSPAR 02/21/1-E Annex 5, last viewed 20th December 2006, available through, http://www.ospar.org/en/html/substances/dselected_subsances.html

6. C.G. Daughton, *Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues*, ACS Symposium Series, (2001), 1-37.

7. T. Heberer, *Journal of Hydrology*, **266**, (2002), 175.

8. T. Heberer, *Toxicology Letters*, **131**, (2002), 5.

9. R. Velagaleti *et. al. Environmental Health Perspectives,* (2002), **110**, 213.

10. S.P. Kourosh *et. al. Instant Pharmacology*, (1999), J. Wiley & Sons, New York.

11. H.P. Rang, M.M. Dale & J.M. Ritter, *Pharmacology*, 3rd Edition, (1995), 82-90, Churchill Livingstone Publishers, New York.

12. G.G. Gibson & P. Skett, *Introduction to Drug Metabolism*, Second Edition, (1994), 1-35, Blackie Academic & Professional Publishing, New York.

13. J.M. Ritter, L.D. Lewis, T.G.K. Mart, *A Textbook of Clinical Pharmacology*, 4th Edition, (1995), 37, Oxford University Press, London.

14. Wastewater Treatment Principles and Regulations, last viewed 20th December 2006, available through, http://ohioline.osu.edu/aex_fact/0768.html.

15. M. Henze, *Wastewater Treatment: Biological and Chemical Processes*, (2002), Springer, London.

16. Enviromental Protection Agency, *Wastewater Treatment Manuals*, 1997.

17. N.F. Gray, *The Biology of Wastewater Treatment*, 1989, 334-479, Oxford University Press, London.

18. L.M. Prescott, J.P. Harley & D.A. Klein, *Microbiology*, 4th Edition, 868-873, M^{C}Graw Hill Publishers, New York.

19. A. Kumar Gautam, S. Kumar & P. Sabumon, *Journal of Environmental Management*, Article in press (2006), doi: 10.1016/j.envman.2006.03.009.

20. S.T. Glassmeyer & J.A. Shoemaker, *Bulletin of Environmental Contamination and Toxicology*, **74**, (2005), 24.

21. M. Bedner & W.A. MacCrehan, *Chemosphere*, **65**, (2006), 2130.

22. T.E. Doll & F.H. Frimmel, *Catalysis Today*, **101**, (2005), 195.

23. W.J. Eilbeck & G. Mattock, *Chemical Processes in Wastewater Treatment*, 1987, 232-293, Chichester, New York.

24. H.R. Rogers, *The Science of the Total Environment*, **185**, (1996), 3.

25. T.A. Ternes, *Water Research*, **322**, (1998), 3254.

26. T.A. Ternes & R. Hirsch, *Environmental Science & Technology*, **34**, (2000), 2741.

27. R. Kanda, P. Griffin, H.A. James & J. Fothergill, *Journal of Environmental Monitoring*, **5**, (2003), 823.

28. M Carballa, F. Omil, J.M. Lema, M. Llompart, C. Garcia-Jares, I. Rodriguez, M. Gomez & T.A. Ternes, *Water Research*, **38**, (2004), 2918.

29. P.M.J. Fisher & R. Borland, *Journal of Cleaner Production*, **11**, (2003), 315.

30. P. Drillia, S.N. Dokianakis, M.S. Fountoulakis, K. Kornaros, K. Stamatelatou & G. Lyberatos, *Journal of Hazardous Materials*, **122**, (2005), 259.

31.T.A. Ternes, A. Joss, H, Siegrist, *Environmental Science &. Technology A-Page Magazine*, October 15th, (2004), 392A.

32. M.S. Diaz-Cruz, M.J. Lopez de Alda & D. Barcelo, *TrAC Trends in Analytical Chemistry*, **22**, (2003), 340.

33. T.A. Ternes, N. Herrmann, M. Bonerz, T. Knacker, H. Siegrest & A. Joss, *Water Research*, **38**, (2004), 4075.

34. J.T. Yu, E.J. Bouwer & M. Coelhan, *Agricultural Water Management*, **86**, (2006), 72.

35. T. Urase & T. Kikuta, *Water Research*, **39**, (2005), 1289.

36. S.J. Khan & J.E. Ongerth, *Chemosphere*, **54**, (2004), 355.

37. L. Wolf, I. Held, M. Eiswirth & H. Hotzl, *Acta Hydrochimica et Hydrobiologica*, **32**, (2004), 361.

38. R.J. Slack, P. Zerva, J.R. Gronow & N. Voulvoulis, *Environmental Science & Technology*, **39**, (2005), 1912.

39. R.J. Slack, J.R. Gronow & N. Voulvoulis, *The Science of the Total Environment*, **337**, (2005), 119.

40. J.V. Holm, K. Rugge, P.L. Bjerg & T.H. Christensen, *Environmental Science & Technology*, **29**, (1995), 1415.

41. M. Ahel & I. Jelicic, '*Phenazone Analgesics in Soil and Groundwater below a Municipal Solid Waste Landfill', Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues*, ACS Symposium Series, (2001), 100.

42. J. Schwarzbauer, S. Heim, S. Brinker & R. Littke, *Water Research*, **36**, (2002), 2275.

43. S. Heim, J. Schwarzbauer & R. Littke, *Environmental Chemistry Letters*, **2**, (2004), 21.

44. D. Ashton, M. Hilton & K.V. Thomas, *The Science of the Total Environment*, **333**, (2004), 167.

45. K.V. Thomas & M.J. Hilton, *Marine Pollution Bulletin*, **49**, (2004), 436.

46. S. Wiegel, J. Kuhlmann & H. Huhnerfuss, *The Science of the Total Environment*, **295**, (2002), 131.

47. H.R. Buser, M.D. Muller & N. Theobald, *Environmental Science & Technology*, **32**, (1998), 188.

48. S. Wiegel, A. Aulinger, R. Brockmeyer, H. Harms, J. Loffler, H. Reincke, R. Schmidt, B. Stachel, W. von Tumpling & A. Wanke, *Chemosphere*, **57**, (2004), 107.

49. D. Bendz, N.A. Paxeus, T.R. Ginn & F.J. Loge, *Journal of Hazardous Materials,* **122**, (2005), 195.

50. M. Clara, B. Strenn & N. Kreuzinger, *Water Research*, **38**, (2004), 947.

51. H.R. Buser, T. Poiger & M.D. Muller, *Environmental Science & Technology*, **32**, (1998), 3449.

52. R. Andreozzi, M. Raffaele & P. Nicklas, *Chemosphere,* **50**, (2003), 1319.

53. M.W. Lam, C.J. Young & S.A. Mabury, *Environmental Science & Technology*, **39**, (2005), 513.

54. A. Lindstrom, I.J. Buerge, T. Poiger, P.A. Bergqvist, M.D. Muller & H.R. Buser, *Environmental Science & Technology*, **36**, (2002), 2322.

55. M. Mezcua, M.J. Gomez, I. Ferrer, A. Aguera, M.D. Hernando & A.R. Fernandez-Alba, *Analytica Chimica Acta*, **524**, (2004), 241.

56. M. Schmitt-Jansen, P. Bartels, N. Adler & R. Altenburger, *Analytical & Bioanalytical Chemistry*, Article in press (2006), doi: 10.1007/s00216-006-0825-3.

57. J.L. Packer, J.J. Werner, D.E. Latch, K. McNeill & W.A. Arnold, *Aquatic Sciences,* **65**, (2003), 342,

58. M.W. Lam & S.A. Mabury, *Aquatic Sciences*, **67**, (2005), 177.

59. Y. Zhang, J.L. Zhou & B. Ning, *Water Research*, **41**, (2007), 19.

60. E. Turiel, G. Bordin & A.R. Rodriguez, *Journal of Environmental Monitoring*, **7**, (2005), 189.

61. M. Isidori, M. Lavorgna, A. Nardelli, A. Parallax, L. Preliterate & M. Rubin, *The Science of the Total Environment*, **348**, (2005), 93.

62. M. Isidori, A. Nardelli, A. Parrella, L. Pascarella & L. Previtera, *Chemosphere*, **63**, (2006), 785.

63. K. Bester, *Water Research*, **37**, (2003), 3891.

64. A.B.A. Boxall, P. Blackwell, R. Cavallo, P. Kay & J. Tolls, *Toxicology Letters*, **131**, (2002), 19.

65. P. Kay, P.A. Blackwell &. A.B.A. Boxall, *Chemosphere*, **59**, (2005), 951.

66. M. Adolfsson-Erici, M. Petterson, J. Parkkonen & J. Sturve, *Chemosphere*, **46**, (2002), 1485.

67. D. Sabaliunas, S.F. Webb, A. Haul, M. Jacob & W.S. Eckhoff, *Water Research*, **37**, (2003), 3145.

68. J. Tolls, *Environmental Science & Technology*, **35**, (2001), 3397.

69. T. Christian, R.J. Schneider, H.A. Farber, D. Skutlarek, M.T. Meyer & H.E. Goldbach, *Acta Hydrochimica et Hydrobiologica*, **31**, (2003), 36.

70. J. Oppel, G. Broll, D. Loffler, M. Meller, J. Rombke & T.A. Ternes, *The Science of the Total Environment*, **328**, (2004), 265.

71. D. Loffler, J. Rombke, M .Meller & T.A. Ternes, *Environmental Science & Technology,* **39**, (2005), 5209.

72. A.C. Hari, R.A. Paruchuri, D.A. Sabatini & T.C.G. Kibbey, *Environmental Science & Technology,* **39**, (2005), 2592.

73. P. Kay, P.A. Blackwell & A.B.A. Boxall, *Environmental Pollution*, **134**, (2005), 333.

74. P. Kay, P.A. Blackwell & A.B.A. Boxall, *Chemosphere,* **60**, (2005), 497.

75. P. Drillia, K. Stamatelatou & G. Lyberatos, *Chemosphere*, **60**, (2005), 1034.

76. E.M. Golet, I. Xifra, H. Siegrist, A.C. Alder & W. Geiger, *Environmental Science & Technology*, **37**, (2003), 3245.

77. J.O. Straub, *Toxicology Letters*, **131**, (2002), 137.

78. R. Clayton, *Analytica Chimica Acta*, **529**, (2005), 3.

79. B. Ferrari, R. Mons, B. Vollat, B. Frayasse, N. Paxeus, R. Lo Guidice, A. Pollio & J. Garric, *Environmental Toxicology & Chemistry*, **23**, (2004), 1344.

80. G. Huschek, P.D. Hansen, H.H. Maurer, D. Krengel & A. Kayser, *Environmental Toxicology*, **19**, (2004), 226.

81. J.P. Bound & N. Voulvoulis, *Chemosphere*, **56**, (2004), 1143.

82. S. Castiglioni, R. Fanelli, D. Calamari, R. Bagnati & E. Zuccato, *Regulatory Toxicology & Pharmacology*, **39**, (2004), 25.

83. F.M. Christensen, *Regulatory Toxicology & Pharmacology*, **28**, (1998), 212.

84. M.D. Hernando, M. Mecuza, A.R. Fernandez-Alba & D. Barcelo, *Talanta*, **69**, (2006), 334.

85. L. Lissemore, C. Hao, P. Yang, P.K. Sibley, S. Mabury & K.R. Solomon, *Chemosphere*, **64**, (2006), 717.

86. F. Pomati, S. Castiglioni, E. Zucatto, R. Fanelli, D. Vigetti, C. Rossetti & D. Calamari, *Environmental Science & Technology*, **40**, (2006), 2442.

87. O.A.H. Jones, N. Voulvoulis & J.N. Lester, *Water Research*, **36**, (2002), 5013.

88. F. Stuer-Lauridsen, M. Birkved, L.P. Hansen, H.C. Holten Lutzhoft & B. Halling-Sorensen, *Chemosphere*, **40**, (2000), 783.

89. M. Cleuvers, *Toxicology Letters*, **142**, (2003), 185.

90. M. Cleuvers, *Chemosphere*, **59**, (2005), 199.

91. E.M. Golet, A.C. Alder & W. Giger, *Environmental Science & Technology*, **36**, (2002), 3645.

92. A.B.A. Boxall, L.A. Fogg, P. Kay, P.A. Blackwell, E.J. Pemberton & A. Croxford, *Toxicology Letters*, **142**, (2003), 207.

93. P.A. Blackwell, A.B.A. Boxall, P. Kay & H. Noble, *Journal of Agriculture & Food Chemistry*, **53**, (2005), 2192.

94. J. Koschorreck, C. Koch & I. Ronnefahrt, *Toxicology Letters*, **131**, (2002), 117.

95. R. Lange & D. Dietrich, *Toxicology Letters*, **131**, (2002), 97.

96. J.P. Seiler, *Toxicology Letters*, **131**, (2002), 105.

97. H. Sanderson, D.J. Johnson, C.J. Wilson, R.A. Brain & K.R. Solomon, *Toxicology Letters*, **144**, (2003), 383.

98. D. Schowanek & S. Webb, *Toxicology Letters*, **131**, (2002), 39.

99. H. Sanderson, D.J. Johnson, T. Reitsma, R.A. Brain, C.J. Wilson & K.R. Solomon, *Regulatory Toxicology & Pharmacology*, **39**, (2004), 158.

100. N. Laville, S. Ati-Aissa, E. Gomez, C. Casellas & J.M. Porcher, *Toxicology*, **196**, (2004), 41.

101. D. Caminada, C. Escher & K. Fent, *Aquatic Toxicology*, **79**, (2006), 114.

102. G. Gagne, C. Blaise & C. Andre, *Ecotoxicology and Environmental Safety*, **64**, (2006), 329.

103. K. Fent, C. Escher & D. Caminada, *Reproductive Toxicology*, **22**, (2006), 175.

104. C. Mimeault, A.J. Woodhouse, X.S. Miao, C.D. Metcalfe, T.W. Moon & V.L. Trudeau, *Aquatic Toxicology*, **73**, (2005), 44.

105. C. Mimeault, V.L. Trudeau & T.W. Moon, *Toxicology*, **228**, (2006), 140.

106. R. Lange, T.H. Hutchinson, C.R. Croudace, F. Siegmund, H Schweinfurth, P. Hampe, G.H. Panter & J.P. Sumpter, *Environmental Toxicology & Chemistry*, **20**, (2001). 1216.

107. J.P. Emblidge & M.E. DeLorenzo, *Environmental Research*, **100**, (2006), 216.

108. G. Nalecz-Jawecki & G. Persoone, *Environmental Science & Pollution Research*, **13**, (2006), 22.

109. T. Schwartz, W. Kohnen, B. Jansen & U. Obst, *FEMS Microbiology Ecology,* **43**, (2003), 325.

110. K. Ohlsen, T. Ternes, G. Werner, U. Wallner, D. Loffler, W. Ziebuhr, W. Witte & J. Hacker, *Environmental Microbiology*, **5**, (2003), 711.

111. O.A. Jones, J.N. Lester & N. Voulvoulis, *TRENDS in Biotechnology*, **23**, (2005), 163.

112. K. Reddersen, T. Heberer & U. Dunnbier, *Chemosphere*, **49**, (2002), 539.

113. S. Zuhlke, U. Dunnbier & T. Heberer, *Journal of Chromatography A*, **1050**, (2004), 201.

114. E. Zucatto, D. Calamari, M. Natangelo & R. Fanelli, *The Lancet*, **355**, (2000), 1789.

115. P.E. Stackelberg, E.T. Furlong, M.T. Meyer, S.D. Zaugg, A.K. Henderson & D.B. Reissman, *The Science of the Total Environment*, **329**, (2004), 99.

116. G.R. Boyd, H. Reemtsma, D.A. Grimm & S. Mitra, *The Science of the Total Environment*, **311**, (2003), 135.

117. S.F. Webb, *'A data based perspective on the environmental risk assessment of human pharmaceuticals III – Indirect human exposure'* in 'Pharmaceuticals in the Environment' edited by K. Kummerer, 221, Springer, New York.

118. S. Webb, T. Ternes, M. Gilbert & K. Olejniczak, *Toxicology Letters*, **142**, (2003), 157.

119. B.W. Schwab, E.P. Hayes, J.M. Fiori, F.J. Mastrocco, N.M. Roden, D. Cragin, R.D. Meyerhoff, V.J. D'Aco & P.D. Anderson, *Regulatory Toxicology & Pharmacology*, **42**, (2005), 296.

120. P.S. Zurer, *Chemistry & Engineering News*, April 10th, (2003).

121. C. Zweiner & F.H. Frimmel, *Water Research*, **34**, (2000), 1881.

122. T.A. Ternes, M. Meisenheimer, D. McDowell, F. Sacher, H.J. Brauch, B. Haist-Gulde, G. Preuss, U. Wilme & N. Zulei-Seibert, *Environmental Science & Technology*, **36**, (2002), 3855.

123. T.A. Ternes, J. Stuber, N. Herrmann, D. McDowell, A. Reid, M. Kampmann & B. Teiser, *Water Research*, **37**, (2003),1976,

124. M.M. Huber, T.A. Ternes & U. von Gunten, *Environmental Science & Technology*, **38**, (2004), 5177.

125. D. Vogna, R. Marotta, A. Napolitano, R. Andreozzi & M. d'Ischia, *Water Research*, **38**, (2004), 414.

126. R. Andreozzi, V. Caprio, R. Marotta & A. Radovnikovic, *Journal of Hazardous Materials*, **103**, (2003), 233.

127. A. Lopez, A. Bozzi, G. Mascolo & J. Kiwi, *Journal of Photochemistry and Photobiology A: Chemistry*, **156**, (2003), 121.

128. I.A. Balcioglu & M. Otker, *Chemosphere*, **50**, (2003), 85.

129. M.M. Huber, S. Canonica, G.Y. Park & U. von Gunten, *Environmental Science & Technology*, **37**, (2003), 1016.

130. M.M. Huber, A. Gobel, A. Joss, N. Hermann, D. Loffler, C.S. M^{c}Ardel, T.A. Ternes & U. von Gunten, *Environmental Science & Technology*, **39**, (2005), 4290.

131. C. Zwiener & F.H. Frimmel, *The Science of the Total Environment*, **309**, (2003), 201.

132. M. Wnkler, J.R. Lawrence& T.R. Neu, *Water Research*, **35**, (2001), 3197.

133. T.A. Larsen, J. Lienert, A. Joss & A. Siegrist, *Journal of Biotechnology*, **113**, (2004), 295.

134. T.A. Larsen, I. Peters, A. Alder, R. Eggen, M. Maurer & J. Muncke, *Environmental Science & Technology*, May 1st, (2001), 192A.

135. J.A. Wilsenach &. M.C.M. van Loosdrecht, *Environmental Science & Technology*, **38**, (2004), 1208.

136. W. Rauch, D. Brockmann, I. Peters, T.A. Larsen & W. Gujer, *Water Research*, **37**, (2003), 681.

137. J.C. Berndtson, *Building and Environment*, **41**, (2006), 427.

138. M. Otero, F. Rozada, L.F. Calvo, A.I. Garcia & A. Moran, *Dyes and Pigments*, **57**, (2003), 55.

2.0 A Review of the Analytical Approaches used for the Determination of Pharmaceutical Residues in Environmental Samples.

2.1 Introduction:

Recent advances in analytical technology have allowed chemists to detect compounds at concentrations that were previously unattainable. The advent of commercially available interfaces for the coupling of LC with MS and reports of pharmaceutical compounds as environmental contaminants can be seen as two interrelated events. The traditional 'gold standard' GC-MS approach is not inherently suitable for the determination of pharmaceutical residues and consequently a derivatisation procedure must be incorporated into the procedure. Such an approach is not favourable as it increases the levels of variability of the method and also as derivatisation procedures are not specific the likelihood of multiple peaks for a single analyte with many functional groups increases, thereby making quantitation extremely complicated. The methods developed for the determination of pharmaceutically active compounds in the environment to date have focused upon the use of a three step procedure; i.e. sample preconcentration to increase method sensitivity, the use of liquid chromatography as an analytical separation method and finally specific detection using mass spectrometry. This section aims to review the theory and application of the analytical approaches used by researchers for the determination of trace residues of environmentally relevant pharmaceuticals to the present day.

2.2 Sample Preparation:

2.2.1 Considerations for sample preparation:

It is unfortunately all too often the case that in environmental samples the analytes of interest are present at concentrations below or near to the limits of detection of the technique used to perform the analytical measurement. This is especially true in the case of pharmaceutical residues, whereby levels detected to date have been in the low μgL^{-1} to the ngL^{-1} range [1]. In order to ensure that

there is confidence in the analytical measurement and that the generated results reflect the composition of the original sample, it is therefore, required that the sample preparation process leads to an enrichment of the analyte to a level more amenable for detection by the analytical technique being employed. Consequently, the resultant analyte signal is significantly higher than the baseline noise. Therefore, the first consideration for the inclusion of a sample preparation technique into the analytical method is to yield lower overall method detection limits, i.e. increased analytical sensitivity [2].

The second consideration for the inclusion of a sample preparation procedure into the analytical method may include the need to 'clean up' the sample by removing the matrix prior to analysis [2]. Environmental samples contain a high proportion of DOM as a result of natural processes that may interfere with the analytical determination, e.g. DOM may spoil chromatography columns or absorb at the same wavelengths used in spectrophotometric methods *etc.* Therefore, it is often necessary to completely isolate the analyte of interest from the matrix which may then be discarded in order to increase the selectivity of the method [3].

The last consideration for the inclusion of a sample preparation procedure may involve the exchange of the analyte from the natural matrix, e.g. water, into a solvent more compatible with the analytical technique to be used [2]. It is a necessity for techniques such as infrared spectroscopy (IR) or nuclear magnetic resonance spectroscopy (NMR) and GC that the analyte must be present in a specific environment prior to analysis, usually an organic solvent, (or deuterated solvent in the case of NMR) as the sensitivity of such techniques is negatively affected by the presence of water [2].

Although a sample preparation procedure may be necessary for any or all of the above reasons, the following conditions must also be fulfilled in order to ensure that it is an efficient and viable process. It is desired that the sample preparation procedure is convenient and easy to perform and relatively inexpensive, that sample loss as a result of the procedure is at an absolute minimum, that there is sufficient analyte selectivity and that the procedure does not lead to the creation of conditions that may adversely affect the analytical measurement, i.e. extremely acidic extracts may ruin chromatography columns [4].

2.2.2 The move towards solid phase extraction:

In the past, the primary method of extracting and preconcentrating aqueous samples was solvent extraction, referred to as liquid-liquid extraction; (LLE). LLE involves the distribution of analytes between two immiscible liquids, usually water and an organic solvent such as ether or hexane and is normally performed by agitating the solvents to ensure mixing and mass transport and then allowing the two immiscible phases to coalesce and separate [5]. Although LLE is relatively simple to perform and manipulate, it does suffer from a number of drawbacks which include the large consumption of organic solvents, the high costs caused by spent solvent disposal, problems with inefficient phase separation and emulsion formation that may lead to ruination of the sample. Another significant problem with LLE is the small preconcentration factors achievable. The ability of LLE to enrich sample components is governed by the ratio of the two liquid phases and therefore, it is impossible or impractical to extract large volumes of sample often needed for suitable analyte enrichment with small volumes of organic solvent [5]. As a consequence of the above factors the reported use of LLE for environmental analysis has declined, however, LLE based techniques are still popular for the processing of small volume samples of biological fluids [6,7].

A historical review of the advent of solid phase extraction (SPE) was presented by Liska in 2000 [8]. In the 1970's the first SPE procedures involved the use of activated carbon as a sorbent for the extraction of organic contaminants from water samples, chloroform was used as an eluent. However, the process was rather ineffective due to the low affinity of polar pollutants for the activated carbon sorbent and the inability of chloroform to elute high affinity compounds from the sorbent mass [8]. The first successful SPE methods applied the use of porous polystyrene polymers, (XAD resins) whereby up to 150L of water sample could be passed through the sorbent and then eluted using 15 mL of diethyl ether which in turn was reduced in volume and analysed by GC [9]. Such a method allowed for the determination of previously unmeasured organic contaminants, e.g. polynuclear aromatic hydrocarbons at the low μgL^{-1} levels in the aquatic environment. The first reports of silica based SPE materials were also published in the 1970's [8]. The use of porous polymers was limited until the 1980's and the advent of commercially available sorbents in pre-packed columns [9]. The 1990's saw the development of SPE disks and new sorbents based upon

immunoaffinity interaction and molecularly imprinted polymer supports (MIPs), which offered increased levels of selectivity and specificity previously unachievable [8]. The progress of SPE continues with the development and commercialisation of hydrophilic lipophilic balanced polymers such as Waters Oasis HLB™ that allow for the preconcentration of compounds displaying a broad spectrum of polarity in a single extraction [10], along with the availability of fully automated and instrument compatible SPE systems like Prospekt™ and Symbosis™ by Spark Holland [8] that allow for fast and more effective sample preparation.

The change from LLE to SPE based sample preparation techniques arose due to the lower costs involved with both equipment and solvents, (SPE was considered a 'greener' option compared with LLE) and the ease at which the procedure could be performed or even automated. SPE also offered chemists the ability to extract samples in the field allowing for easier sample transport and storage [11]. However, a number of limitations are also known. Because SPE involves the use of a synthetic sorbent, problems may arise with the batch-to-batch reproducibility of the product or the surface chemical modification. Also impurities present from the manufacturing process may adversely affect the performance of the sorbent [11]. As the sorbent bed is of limited smaller capacity compared to a bulk liquid phase as used in LLE, the possibility of sample loss exists due to overloading and breakthrough. The particulate nature of the sorbent bed and the use of frits can be prone to blockage under certain conditions or may permit channelling and therefore, poor sample sorbent interaction leading to low analyte retention [12]. Despite these limitations SPE has emerged as a preferred sample preparation option due to economic and safety reasons.

2.2.3 The four steps of SPE:

Regardless of the intended application, each SPE procedure consists of four basic steps, i.e., conditioning, sample adsorption, washing and elution, Fig. 2.1 following depicts the four steps of SPE in practice. Other sample pre-treatment procedures may also be necessary such as filtration to remove particulate matter and pH adjustment.

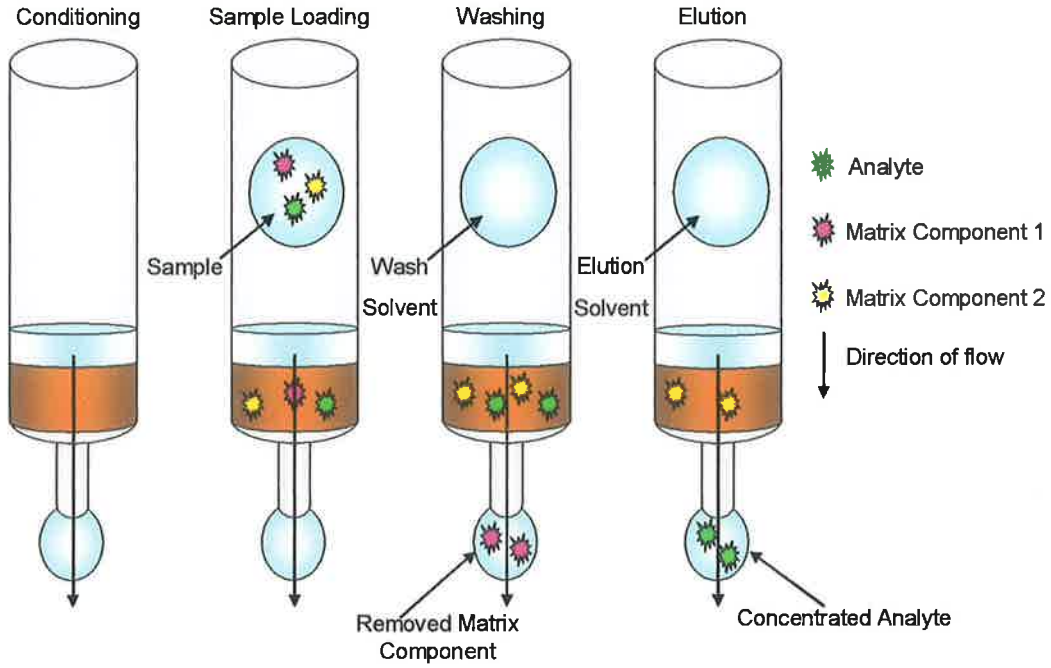


Figure 2.1: The four steps of SPE.

Each step of the SPE procedure is critically important in achieving a suitably concentrated and pure extract ready for instrumental analysis. Conditioning involves passing a water miscible solvent such as methanol or acetonitrile through the sorbent bed in order to prepare and 'wet' the active surface groups and therefore allow for efficient interaction with the sample [9]. The conditioning step also allows for the elution of any adsorbed organic impurities from the sorbent bed. The sorbent is then washed with water to remove the conditioning solvent prior to the introduction of the sample. Samples are passed through the sorbent bed usually under vacuum or pumped under pressure to allow for analyte retention by appropriate interaction with the sorbent, (types of sorbents and retention mechanisms will be reviewed later). The flow rate through the sorbent should be held constant over the course of sample introduction to avoid adverse affects on retention [9]. A washing procedure is then used to remove undesirable matrix components or excess salts and inorganics from the sorbent. The wash solvent conditions are usually optimised so that they remove the majority of matrix components without affecting the retention of the analyte of interest [2,9]. The last step of the SPE procedure involves elution of the analytes using a suitable solvent. The flow of the elution solvent is generally slower than the flow of the sample through the sorbent bed to ensure that all of the retained analyte is eluted. In order to maximise the preconcentration factor the volume of elution solvent required should be kept to a

minimum, usually three times the volume of the sorbent [11]. However, larger volumes may be used with subsequent volume reduction to yield a concentrated extract suitable for instrumental analysis. It is desirable that the analytes of interest have a retention factor (k) as close as possible to zero under the elution conditions to ensure complete recovery [9].

2.2.4 Theoretical considerations:

Solid phase extraction is a frontal chromatography whereby the sample, (acting as the mobile phase) is continuously fed into the stationary phase bed. In general the concentration of analyte present in the sample is quite low and therefore, the volume of sample that can be passed through the sorbent bed is determined by the breakthrough volume for the particular sorbent [11]. The breakthrough volume for a particular sorbent is determined from the breakthrough curve, (that may experimentally determined and is analogous to determination of chromatography column capacity), see Fig. 2.2 [13]. From Fig. 2.2 it can be seen that the analyte, of concentration X passing through the sorbent at a constant flow rate is quantitatively retained by the stationary phase until the capacity of the sorbent is exceeded. At this point the detection of analyte emerging from the sorbent is called the breakthrough volume; (V_B).

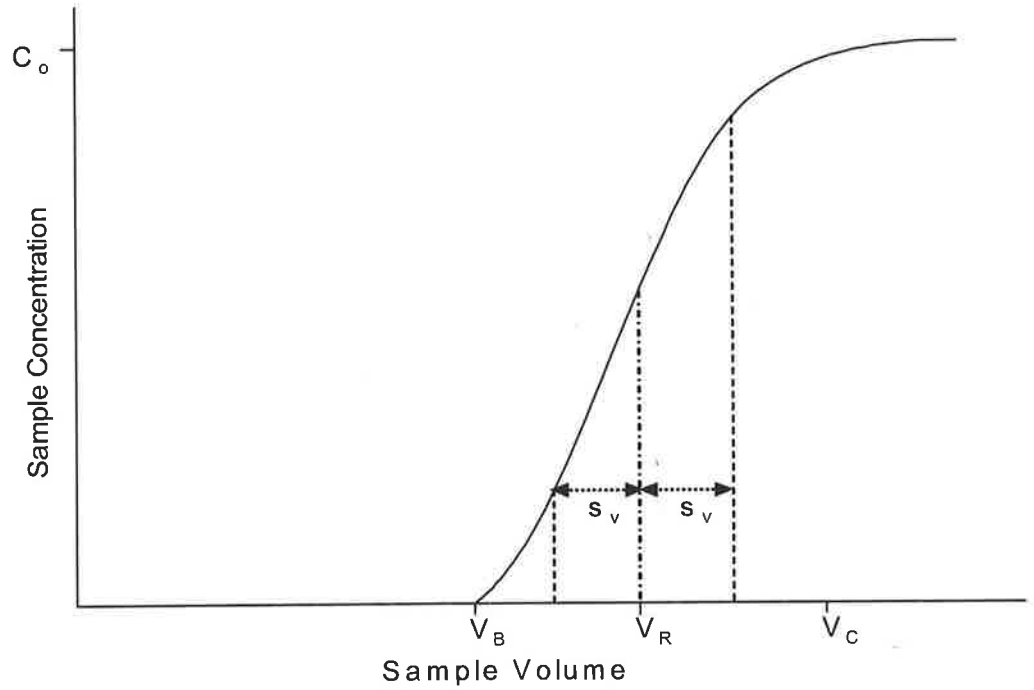


Figure 2.2: Typical breakthrough curve for SPE sorbent, adapted from [11].

It can also be seen that the resultant breakthrough curve is sigmoidal and a second point, V_C, represents the complete saturation of the sorbent analyte and therefore, the concentration of analyte entering the sorbent equals the concentration emerging from it [13]. The point of inflection of the breakthrough curve corresponds to the retention volume, V_R, as the first derivative of the breakthrough curve can be assumed to be a Gaussian distribution analogous to the peaks observed in elution chromatography [14]. It is desirable to choose a sorbent with a large breakthrough volume thereby allowing for a greater analyte enrichment.

The breakthrough volume may be mathematically related through the retention volume by the following equation [11,13,14]:

$$V_R = V_B + 2\sigma_V \qquad \text{(Eq. 2.1)}$$

Where σ_V is the standard deviation of the axial dispersion of the analyte through the sorbent bed and is calculated using:

$$\sigma_V = V_O(1+k)/\sqrt{N} \qquad \text{(Eq. 2.2)}$$

Where:

V_o is the inter particulate volume,

k is the retention factor,

N is the number of theoretical plates which is calculated using the following equation:

$$N = V_R(V_R - \sigma_V)/\sigma_V{}^2 \qquad \text{(Eq. 2.3)}$$

The values of N, *k* and V_R can be determined using SPE coupled to LC.

The pressure required to force the sample through the sorbent bed is described by the following equation [11].

$$\Delta P/L = \mu\eta\varphi/d_p{}^2 \qquad \text{(Eq 2.4)}$$

Where:

$\Delta P/L$ is pressure drop per unit length across the sorbent,

μ is the linear velocity of the sample,

η is the viscosity of the sample solution,

φ is the flow resistance parameter of the sorbent, (typically 10^3) and

d_p is the average sorbent particle diameter.

It can be seen from the above equation that the size of the particles affects the pressure generated, i.e. the smaller the particles the higher the pressure generated and vice versa.

The elution volume required for the recovery of greater than 99% of the analytes from the sorbent of bed volume V_m may be calculated by [13]:

$$V_E = V_M[1+k][1+(2.3/\sqrt{N})] \quad \text{(Eq. 2.5)}$$

The volume of solvent required may be reduced by minimising the inter particulate volume of the sorbent or by minimising the value of *k*.

2.2.5 Sorbent types and retention:

The popularity of SPE is reflected by the large choice of commercially available sorbents for all manner of applications. As with chromatography columns the majority of SPE sorbents are either silica or polymeric based. The retention mechanism depends upon the sorbent used, although reversed-phase sorbents are by far the most common, normal phase, ion exchange and mixed mode formats also exist. In order for efficient extractions leading to concentrated pure extracts it is desired that the sorbent used have a high active surface area in order to shift the equilibrium in favour of the solid phase and therefore, promote analyte retention. The interaction between the analytes and the stationary phase should be suitably strong to allow for retention but easily reversed to guarantee a high degree of analyte recovery. The sorbent should be pure and free from impurities that may leach out and contaminate the extract and the sorbent should allow for good contact between the sample and active groups [9].

Normal phase sorbents are available using inorganic oxides like silica, alumina or Florisil and allow for the retention of polar analytes based upon dipole-dipole or hydrogen bonding interaction [11]. Inorganic oxides offer a high active surface area for interaction and under pH conditions that promote the dissociation of surface hydroxyl groups allow the sorbent to be used as an ion exchanger [15]. However, drawbacks of inorganic oxides include pH instability and the possibility of irreversible adsorption or catalytic transformation of the adsorbed analyte [11]. The majority of applications of normal phase SPE have reported its use for the clean up of complex matrices and the removal of polar interferants prior to instrumental analysis [16,17,18].

Reversed-phase sorbents consisting of bonded phase silica, (alkyl silicas such as C_{18} or C_8) or polymeric based materials are widely used for the extraction of organic pollutants from aqueous solutions [15]. Retention is based upon either van der Waals interactions or π-π interactions with available groups [19]. Silica based sorbents are widely used, however, they suffer from limited pH

stability. Residual silanol activity can cause problems with the elution of basic analytes and breakthrough volumes can be small for polar analytes [15]. Polymeric supports using styrene or methacrylate as monomers and divinyl benzene as cross linking agents offer pH complete stability compared to silica based materials and the increased interaction and retention involving π-π interactions [20]. However, reversed-phase polymers are generally hydrophobic and can shrink or swell in some organic solvents [21]. The hydrophobicity of polymeric supports can be reduced by the inclusion of a hydrophilic co-monomer during the polymerisation process; successful examples of such an approach includes Waters Oasis HLB™ which incorporates the water wettable N-vinyl pyrrolidone monomer and can be used directly with aqueous samples and without the need for a sorbent conditioning step [10,21,22]. Solvents used for the elution of reversed-phase sorbents include methanol, acetonitrile, acetone amongst others. Reversed-phase sorbents have been used for the extraction of a wide variety of organics from numerous sample matrices [23].

Ion exchange sorbents retain analytes based upon electrostatic interactions between oppositely charged groups on the analyte and the sorbent surface. In general sulphonic or carboxylic acid groups are used as cation exchange groups while quaternary, secondary or primary amines are used as anion exchangers [24]. Both silica and polymeric based supports have been used in the preparation of ion exchange resins [11]. Ion exchange sorbents may also contain some reversed-phase groups thereby allowing the retention of more bulky organics containing ionisable groups. Another application of ion exchange sorbents has focused upon the removal of interfering ionic species prior to analysis by ion chromatography (IC) or capillary electrophoresis (CE) [25,26]. Such processes allow for the removal of nitrate and hydroxide, chloride and sulphate by passing the sample through sulphonic acid sorbents in the hydrogen, silver or barium form, respectively.

A rather underused alternative to ion exchange sorbents is to use ion-pair SPE whereby an ion pairing reagent is added to the sample in order to increase the retention of polar and ionic compounds using reversed-phase sorbents [27]. Typical ion pairing reagents used have included linear alkyl sulphonic acids for the extraction of basic analytes and amines or quaternary ammonium salts for the extraction of acidic analytes, concentrations used are generally in the milli-molar range [27]. Few reports of ion-pair SPE exist in the literature primarily due

to the extra uncertainty introduced by the inclusion of an ion pairing reagent, the extra extraction optimisation steps required and the possibility that the ion pairing reagent may negatively affect the chromatographic separation or may be incompatible with MS detection [27]. Reported applications of ion-pair SPE include the determination of naphthalene sulphonates in water using tetrabutylammonium bromide as the ion pairing reagent [28], the determination of pamoic acid in animal serum [29] and the extraction of the diabetes treatment metformin from plasma using sodium dodecyl sulphate as the ion-pairing reagent [30]. However, it is worth noticing that in each instance the ion-pair extraction was followed by determination using ion-pair HPLC with optical detection, thereby suggesting that the analytical method may have to be changed in order to facilitate the ion-pair extraction.

2.2.6 The SPE approach used for the determination of PPCPs:

As stated previously, due to the ultra trace levels of pharmaceutical compounds that may be present in the aquatic environment the preconcentration of samples prior to instrumental analysis is often a prerequisite and to facilitate this sample enrichment SPE has been the favoured approach reported to date. Reports of SPE in both the off-line format using disposable cartridges and in the on-line mode using short columns for the determination of drug residues have been reported in recent years.

Of the reported methods, those that use reversed-phase disposable cartridges in the off-line mode are in the majority. Sorbents used for the enrichment of drug residues from environmental matrices have included silica C_8 and C_{18} [31,32,33], cross linked polystyrene divinyl benzene [34,35,36], phenyl [37], silica C_2 for the extraction of antibiotics [38] and an immunosorbent for the extraction of sulfamethazine from soil and aquatic samples [39]. Passive sampling techniques have also been reported [40]. However, the greatest proportion of reported methods have focused upon hydrophilic lipophilic balanced polymers such as Waters Oasis HLB™ or Strata-X™ by Phenomenex, [41,42,43,44,45,46,47,48,49,50,51,52,53]. The popularity of such polymeric sorbents is reflected by the reasons discussed previously, i.e. the ability to extract compounds of varying polarity in a single extraction, increased retention and ease of use. Aguera *et al.* favoured the use of C_{18} over Oasis HLB™ for the extraction of Triclosan and biphenylol from sediment and wastewater due to

increased rates of analyte recovery determined when using the silica based material, however, it was also reported that the initial elution solvent used, (methanol) for the evaluation of the SPE sorbents was later replaced with acetone due to excessive retention of biphenylol on the C_{18} sorbent, however, the other sorbents investigated were not re-evaluated under the revised conditions [33]. In some instances the use of tandem SPE was also reported whereby a sacrificial sorbent was included to selectively retain possible matrix interferences [54,55,56]. Although such an approach may be useful for the clean up of particularly matrix rich samples, the extra costs involved and possible loss of target analytes due to retention on the sacrificial sorbent may make tandem SPE an unfeasible procedure.

A rather under investigated approach for the determination of ultra trace organic contaminants and particularly pharmaceutical residues is the use of on-line SPE. On-line methods offer greatly increased levels of sensitivity compared to off-line methods as the entire enriched sample components can be analysed instead of a small proportion of the concentrated extract. On-line methods also offer reduced sample handling and the greater possibility for complete automation. Chen *et al.* reported the use of on-line SPE using column switching for the determination of caffeine as an anthropogenic maker for the tracking of sewage effluent in natural waters [57]. Detection limits on the range of 0.1 μgL^{-1} were attainable from 50 mL of sample. Endocrine disrupting chemicals such as estrogens and alkyl phenols were also determined using an on-line SPE approach [58,59,60] and method detection limits in the pgL^{-1} range were frequently reported from moderate quantities of sample (250 mL) [59]. Sancho *et al.* used on-line SPE for the determination of 50 compounds comprising pesticides and their transformation products from water samples [61] and again limits of detection in the low ngL^{-1} range from just 1.3 mL of sample were attainable using LC-MS/MS.

From a theoretical standpoint, on-line SPE allows for the easier determination of breakthrough volumes compared to off-line extractions thereby allowing for a rapid optimisation of the sorbent and extraction parameters. A notable limitation of on-line methods involves the quantity of sorbent that can be used. As stated previously the pressure generated is proportional to the particle size, as are the number of theoretical plates available and therefore, the retention efficiency of the sorbent. Therefore, the use of long columns of small particles for

rapid extraction will be impractical due to the excessive backpressure generated as would the use of large particles for selective extractions due to low theoretical plate numbers consequently available. In order for on-line SPE to be a viable and successful process a careful examination of the sorbent material is essential.

2.3 Analytical Separation:

2.3.1 The importance of liquid chromatography:

An efficient separation of all analytes targeted for environmental monitoring is a vital component of the overall analytical approach. Due to the complexity of extracted samples and the lack of detection mechanisms with the ability to confidently identify and quantitate the analyte of interest in the presence of all other matrix components, the need for a chemical separation is paramount.

The concept of chromatographic separation was first introduced by Tswett in 1903 who reported the separation of plant pigments using calcium carbonate as an adsorbent [62]. Due to the inability of others to repeat the work of Tswett and language barriers, the method lay dormant for over thirty years until German scientists investigated and accepted its applicability for the purification of natural products [63]. A further problem with Tswetts work was the solubility of the adsorbent in water. However, in the 1940's Martin and Synge developed an alternative using partition chromatography based upon the distribution of an analyte between two liquid phases and followed on with the invention of GC in the early 1950's [63]. GC developed and matured rather quickly as an analytical technique due to the influence of the petrochemical industry but a major drawback of GC was its limited applicability for the determination of analytes with low volatility or thermal stability. A solution to these problems was offered by liquid chromatography and many experts in the GC field turned their attention to a resurgence of liquid phase separations [64]. Modern LC evolved due to the advance of small porous silica particles, chemically modified stable supports and more reliable and user friendly instrumentation [63]. Smaller and more reproducible particles leading to more efficient separations and faster analysis times were reported through the late 1970's to the 1990's. Just as the petrochemical industry had played a critical role in the development of GC, the pharmaceutical industry and its associated regulatory authorities were similarly important in the development of modern LC. It is now estimated that modern LC

instrumentation is the most important piece of equipment in analytical laboratories across the world after balances and pH meters [63]. The versatility and superior selectivity of LC along with the desire to perform faster and more efficient separations are the current driving forces for the continuous development and application of modern LC for the separation and determination of a wide variety of analytes.

Methods for the determination of pharmaceutical residues in environmental samples have focused primarily on the use of liquid chromatographic separations rather than the use of GC that was previously employed for the determination of the majority of environmentally relevant analytes. The reason why LC is preferred to GC for pharmaceutical analysis is as previously mentioned; pharmaceuticals are polar molecules of moderate molecular weight and lack volatility [65]. Reported methods for the determination of pharmaceutical residues reflect the superiority of LC for the determination of polar analytes.

2.3.2 Liquid chromatography - theoretical considerations:

Chromatography may be defined as the distribution of analytes between two phases; a stationary phase and a moving mobile phase. The separation arises due to differential rates of migration of the analytes through the chromatographic column based upon their affinity for either the stationary or the mobile phase [66]. In liquid chromatography the mobile phase consists of a solvent or a mixture of solvents whilst the stationary phase is a solid support with or without chemical modification. Depending upon the polarity of the stationary phase the separation is termed either normal phase; whereby the stationary phase is a polar solid and the mobile phase is a nonpolar solvent, or reversed-phase; whereby the stationary phase is a nonpolar support and the mobile phase is a polar solvent [67]. As reversed-phase separations are the most common, indeed as all the reported methods for the determination of pharmaceutical residues to date are based upon reversed-phase LC, normal phase LC will no longer be considered in this review.

The concept of analyte retention is demonstrated by Fig. 2.3. A three component mixture is injected onto the top of the column and is carried through the column by the continuous flow of mobile phase. The separation of the mixture arises due to differential migration through the column.

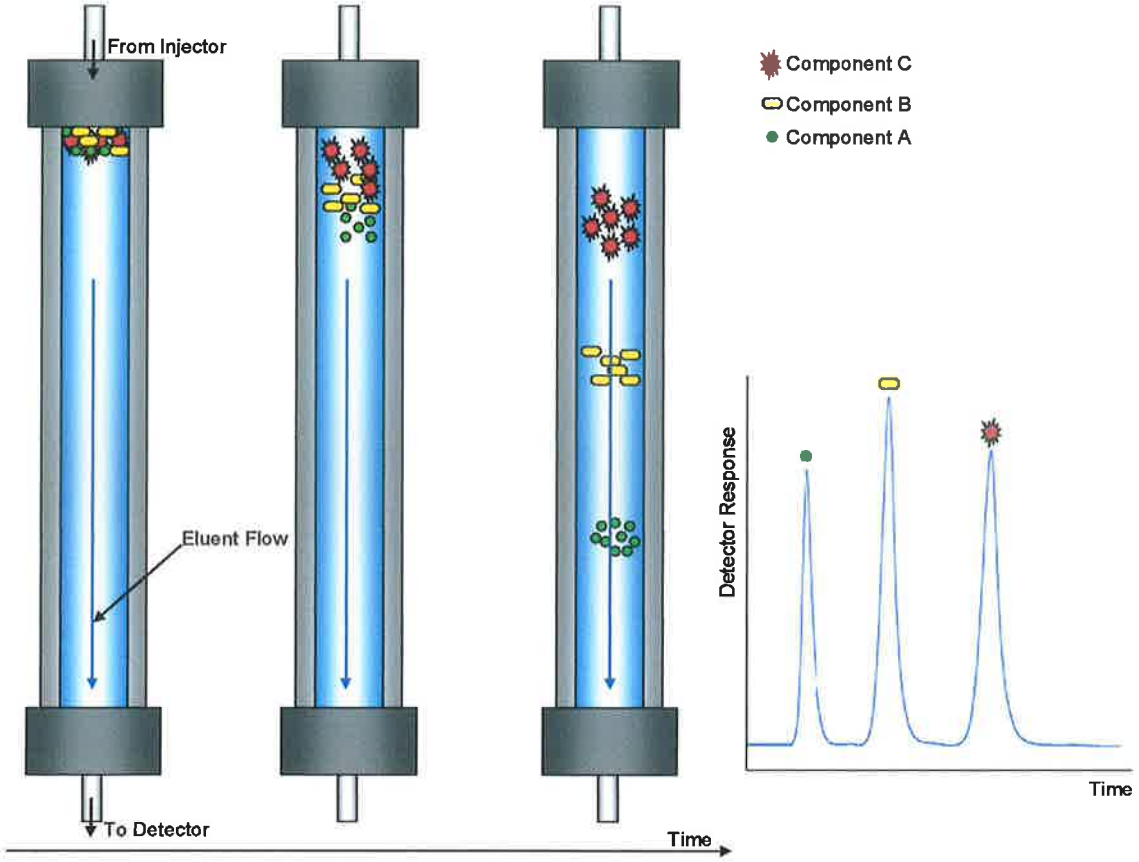


Figure 2.3: Hypothetical chromatographic separation adapted from [68].

The differential migration of analytes through the chromatographic column occurs as a result of the equilibrium distribution of each mixture component between the mobile and stationary phases [68]. Depending upon the position of the equilibrium, analytes with affinity for the mobile phase will elute earlier than those who show a greater affinity for the stationary phase as their path through the column is retarded due to interaction. The rates of differential migration can therefore be altered by changing either the composition of the mobile phase or the stationary phase [68]. The interactions that lead to retention are dependant upon the separation mode employed; see section 2.2.5 for a discussion of such interactions and mechanisms. In reversed-phase chromatography the most polar sample component will elute from the column first with the most nonpolar sample component being retained for the longest time. Therefore, by altering the polarity of the mobile phase using gradient elution, retention of polar components can be promoted by increasing the mobile phase polarity and in turn more hydrophobic components can be eluted by decreasing the polarity of the mobile phase.

The eluted peaks are detected in time as they pass from the column and the resulting plot of detector response versus time is referred to as a

chromatogram. The eluting peaks are generally bell shaped similar to the Gaussian distribution [69]. The amount of time it takes a component to elute from the column and be detected following injection is referred to as the retention time (T_R) and is characteristic for that component under the specific conditions used. The time taken for the mobile phase to pass through the column is assigned (T_O). The retention factor (*k*) calculated using the following equation, is a numerical representation of the actual quantity of time a component spends in the stationary phase,

$$k = \frac{T_R - T_O}{T_O} \qquad \text{(Eq. 2.6)}$$

Low values of *k* for a particular component indicate that the majority of its time is spent in the mobile phase and vice versa. It is generally desirable to have *k* values in the range of 1-5 which suggests that all analytes are suitably but not excessively retained on the column [67]. If two or more sample components have the same *k* value they will not be separated under the conditions used [69]. The ratio of retention factors of two sample components is called the separation factor (α), given as:

$$\alpha = \frac{k_2}{k_1} \qquad \text{(Eq. 2.7)}$$

The higher the value of α, the greater the degree of separation between the two components. If the value of α equals one, the two sample components will not separate and will therefore coelute [70].

The efficiency of the separation can be evaluated from the degree of resolution between the separated components calculated using the following equation:

$$R_S = \frac{2(T_{R,2} - T_{R,1})}{W_2 + W_1} \qquad \text{(Eq. 2.8)}$$

Where W is the baseline width of peaks 1 and 2 respectively.

The larger the value of R_S the more resolved the two peaks are. R_S value of 1.5 or greater suggests that the two components are completely baseline resolved [68]. A quantitative estimation of the efficiency of the column and therefore the separation is determined by calculating the number of theoretical plates (N) using the following equation:

$$N = 16\left(\frac{T_R}{W}\right)^2 \qquad \text{(Eq. 2.9)}$$

The concept of the theoretical plate was first presented by Martin and Synge in an attempt to elucidate the theory of the separation. The term arises from the theory of fractional distillation [67]. Each plate can be assumed to represent a single equilibrium distribution of the analyte between the mobile and stationary phases and therefore the higher the value of N, the greater the number of equilibrium distributions involved and consequently the more efficient the separation. The effective height of a theoretical plate (H) may be determined by dividing the length of the column by the theoretical plate count. The smaller the value of H the greater the efficiency of the peak.

The resolution of the separation is dependant upon the above variables and can be calculated mathematically using the following equation:

$$R_S = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_{av}}\right) \qquad \text{(Eq. 2.10)}$$

Where k_2 is the retention factor of peak 2 and k_{av} is the average retention factor of the two components.

From the above equation it can be seen that the resolution can be improved by increasing N, i.e. by increasing the column length, by altering the selectivity of the separation and increasing the retention of the analytes.

2.3.3 Band broadening in liquid chromatography:

Although samples are applied to chromatographic columns as discrete plugs the resultant peaks become distorted during their passage through the chromatographic system leading to the formation of bell shaped peaks similar to the Gaussian distribution. The diffusive variance associated with a Gaussian peak is described by the expression [70]:

$$\sigma^2 = 2D_m t \qquad \text{(Eq. 2.11)}$$

Where:

D_m is the diffusion coefficient and

t is the time spent within the chromatographic system.

From the above expression it can be deduced that the width of the chromatographic band is proportional to the square root of the retention time and therefore, increasing the retention time leads to the formation of broader peaks [70]. The actual processes that assist with the broadening of chromatographic peaks can be attributed to column and extra-column effects.

Extra-column effects are processes that cause the spreading of chromatographic bands outside the column and arise primarily due to mismatches in tubing diameter or large sources of dead volume within the system, e.g. large volume detector flow cells *etc.* Extra-column effects can be minimised by choosing suitable lengths of tubing of appropriately small internal diameter in order to minimise the loss of the resulting separation or the dispersion of the injected sample within the mobile phase prior to entering the chromatographic column.

The contribution of the chromatographic column to band broadening can be evaluated using the van Deemter equation which states that the separation efficiency, (in terms of H) is affected by the mobile phase flow rate and the column itself, [70]:

$$H \approx A + \frac{B}{\mu} + C\mu \qquad \text{(Eq. 2.12)}$$

Where:

A, B and C are constants for a column and stationary phase and

μ is the mobile phase linear velocity.

Therefore, from the above equation it can be seen that the mechanisms of band broadening are independent of, directly proportional and inversely proportional to the flow of mobile phase through the column [70].

The A term in Equation 2.12 is referred to as the multiple path term or Eddy diffusion and describes the multiple possible random paths of the molecule through the particulate bed leading to the distortion of the chromatographic band due to the fact that some molecules will take a shorter path than others and will consequently elute from the column earlier [69]. Such a fact is depicted pictorially in Fig. 2.4.

The B term in the van Deemter equation represents longitudinal diffusion along the axis of the column. Longitudinal diffusion arises due to the random distribution of sample molecules in all directions due to the existence of a concentration gradient, from an area of high concentration inside the band to areas of low concentration on the edges of the band [68,70]. The net result as can be seen in Fig. 2.4 again leads to the spreading of the chromatographic band.

The C term of the van Deemter equation accounts for the mass transfer of each analyte from the mobile phase to the stationary phase and back again.

From Fig. 2.4 it can be seen that there is a layer of mobile phase surrounding each particle that is essentially stagnant when compared to the mobile phase in the inter particulate spaces. Therefore, this stagnant layer of mobile phase may contribute to band broadening as molecules present in this layer are not moving at the same velocity when compared to molecules in the 'bulk' mobile phase [68].

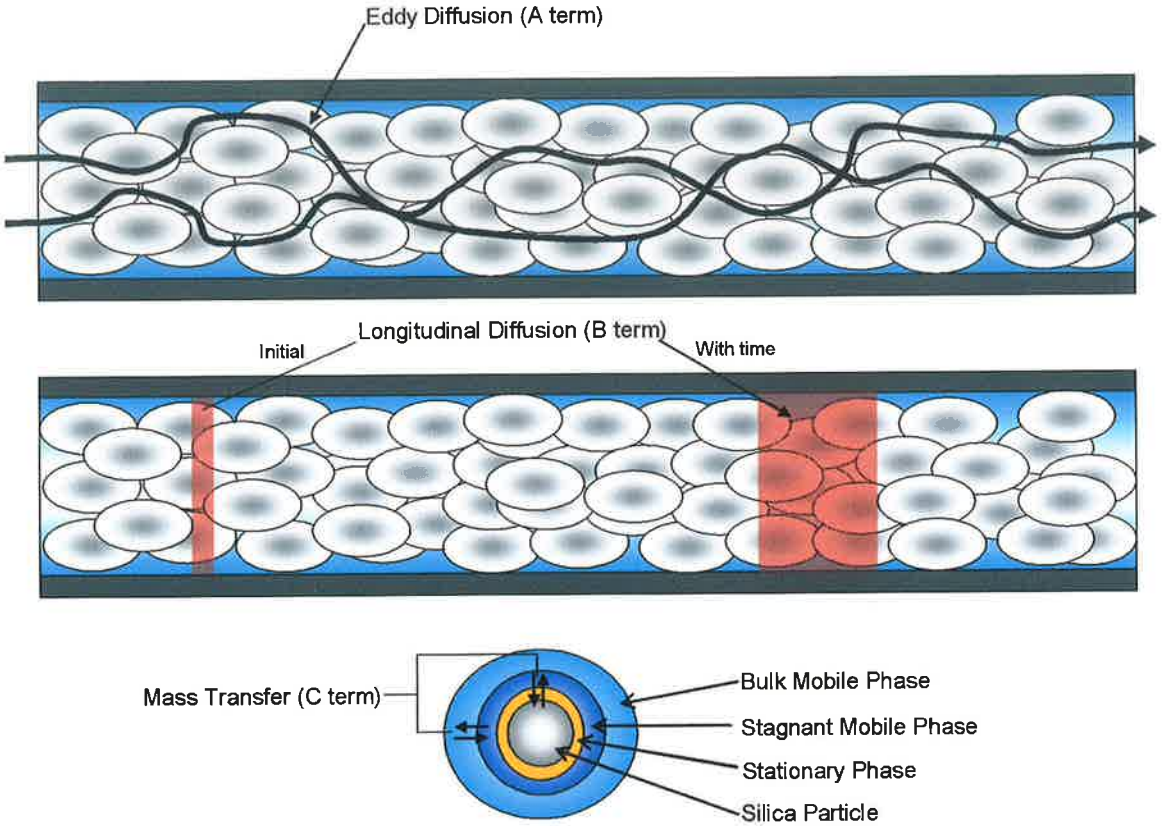


Figure 2.4: A pictorial representation of band broadening mechanisms, adapted from [68,69,70].

The stagnant mobile phase is also important as it impedes the interaction of molecules in the bulk mobile phase with the stationary phase, i.e. sample molecules must diffuse from the bulk into the stagnant layer and then from the stagnant layer onto and into the stationary phase and then back out again [68]. The net effect leads to a retardation of molecular movement again leading to a distortion of the chromatographic band. As these processes are continuously occurring as the sample passes long the length of the column it can be seen that the longer the column residence time the greater the effect of band broadening.

Therefore the effects of band broadening need to be minimised in order to maximise the efficiency and resolution of the separation and also to avoid problems with quantitation. A plot of H versus μ should in theory yield a minimum value of H that suggests the optimum linear velocity for maximum efficiency. Another alternative is offered by capillary or monolithic columns which reduce the

A and C terms of the van Deemter equation allowing for more efficient separations.

2.3.4 LC Instrumentation:

Instrumentation for LC consists of basic but integral parts, i.e. a pump capable of delivering solvents steadily at high pressures, a sample injection system, the chromatography column, a detector to monitor the column output and a data handling system. It is essential that all components of the system operate efficiently and reproducibly, e.g. disturbances in solvent delivery will manifest themselves as baseline noise in the detector output and therefore, cause a reduction in the sensitivity of the analysis.

A variety of pumping systems for LC have been developed ranging from displacement pumps whereby the solvent is forced out of the reservoir by external pressure to syringe pumps, however, the majority of modern LC pumps use double head reciprocating pumps, a schematic of which is shown in Fig. 2.5. Such a pump operates using sinusoidal dual piston movements that are 180^{o} out of phase and allows for a constant flow of solvent with minimal pulse disturbance [71]. Dual head reciprocating pumps also allow for the performance of flow gradients as the volume delivered can be changed rapidly be adjusting the displacement stroke volume or the stroke frequency [68].

The introduction of samples into a high pressure mobile phase as a discrete plug to avoid band broadening and also to avoid disturbing the mobile phase flow is usually performed using sample loop type injectors, e.g. Rheodyne injectors™ [71], (see Fig. 4.1). Injection is performed by loading the sample into a loop of defined volume when the valve is in the load position, by switching the valve to the inject position the mobile phase is redirected through the sample loop and carries the loaded sample onto the chromatographic column [72]. Loop injectors are also easily automated and are incorporated into autosamplers by many manufacturers. Column switching and selection can also easily be performed using sample loop type injectors.

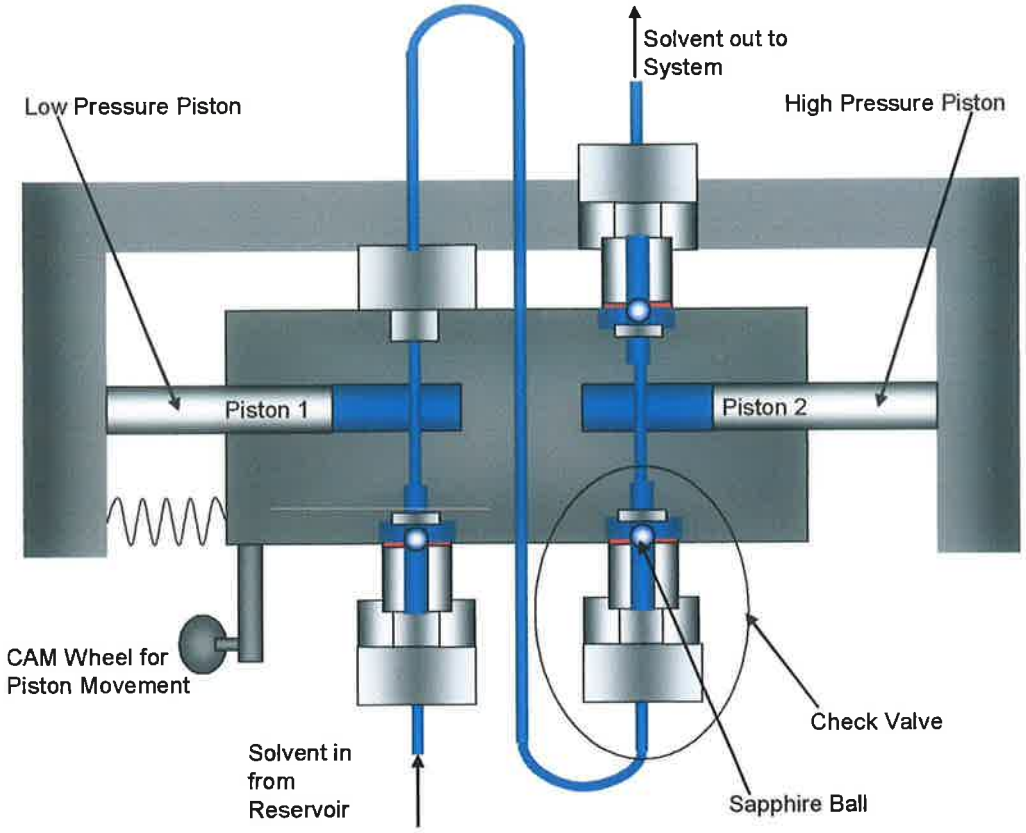


Figure 2.5: Dual head reciprocating pump, adapted from [73].

Detection of separated bands as they emerge from the chromatographic column is normally performed by monitoring a bulk property of the mobile phase, i.e. a change in refractive index due to the presence of dissolved solute or more selectively by monitoring a property of the solute itself, e.g. its UV absorption as it elutes from the column [68]. Detectors have been developed and commercialised that exploit some property of the analyte. However, regardless of the detector type there are a number of characteristics that a detector should ideally fulfil. These include; sensitivity, linearity, stability and reproducibility, a rapid response, selectivity and the detector must not contribute significantly to band broadening [72,74]. The detector used must also be compatible with the separation conditions, e.g. electrochemical detectors cannot be used with gradient elution or only volatile mobile phase modifiers may be used in conjunction with mass spectrometry [70]. Due to its versatility and fulfilment of many of the above conditions the UV detector is the most commonly used for the detection of pharmaceutical analytes, e.g. it is estimated that approximately 95% of all separations of pharmaceuticals using LC employ UV as the detection mechanism [65].

UV detection monitors the absorption of monochromatic light by the various solutes present in the mobile phase. The absorption of light is proportional to the concentration of analyte present according to the Beer-Lambert law which states [75]:

$$A = \varepsilon cL = \log \frac{I_o}{I} \qquad \text{(Eq. 2.13)}$$

Where:

A is the absorbance,

ε is the molar absorption coefficient, ($Lmol^{-1}cm^{-1}$),

c is the concentration, ($molL^{-1}$),

L is the light path, (cm),

I_o is the initial light intensity and

I is the sample attenuated light intensity.

A schematic of a UV detector is depicted in Fig. 2.6 following, the principle components include a light source such as deuterium (D_2) lamp for UV wavelengths or a tungsten halogen lamp for visible wavelengths, a monochromator for the selection of the appropriate wavelength, a flow cell that usually uses 'Z' type geometry to maximise the light path, light detectors such as photodiodes or photomultiplier tubes and electronic components to convert the resulting signal [74].

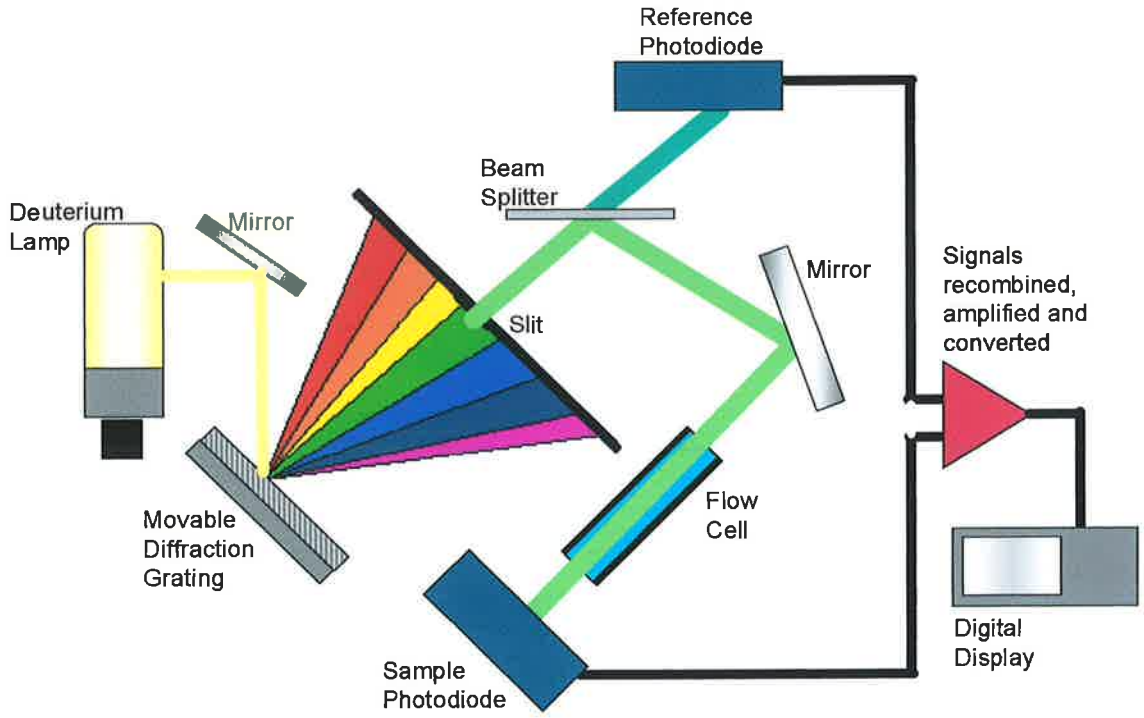


Figure 2.6: Schematic of UV detector, adapted from [73].

The popularity of UV detectors is due to their versatility. The majority of analytes contain some chromophoric moiety or indeed can be tagged with a chromophore to aid with their detection. UV detectors are quite sensitive with detection limits in the range of 100 pg - 1 ng achievable [72].

The use of photodiode array detectors adds an extra degree of selectivity to the analysis with the ability to record complete UV spectra over the course of the elution of the peak. Therefore, qualitative information may be obtained using spectral matching. Other advantages of the use of photodiode array detectors includes the assessment of peak purity and deconvolution of unresolved peaks. However, for such an approach to be successful it requires the unresolved components to have different UV spectra [74].

2.3.5 Column chemistry & monolithic silica:

Most modern forms of LC are performed using stationary phases consisting of a particulate support that may or may not be chemically modified. This is especially true in reversed-phase chromatography whereby columns are usually based upon silica functionalised via siloxane bonds with nonpolar hydrophobic organic chains such as C_8, C_{18} *etc.* The preparation of such reversed-phase materials is performed by reacting organochlorosilanes with silanol groups on the silica surface, for a detailed review see that of Kirkland [76]. The widespread use of silica as the support material is due to the ability to manufacture silica particles with suitably small diameters and a controlled pore distribution. Silica also has high mechanical strength and provides a large active surface area that promotes mass transfer [77]. However, the biggest limitation to the use of silica as a chromatographic support is its pH instability at acidic and basic pH values and also the presence of un-functionalised and un-endcapped silanol groups on the surface that interfere with the chromatographic process, leading to poor peak shapes [77]. Recent trends in LC have been driven by the need for fast and efficient separations in the fields of pharmaceutical and combinatorial chemistry research, metabolomics and proteomics [78]. To fulfil these aims columns with smaller particles and narrower internal diameters have been developed. However, limitations in the form of excessive backpressure generation or incapability with standard LC instrumentation can be encountered [78].

A relatively new development in the field of LC separations is the use of monolithic based stationary phases. Monoliths consist of a single piece of

homogenous phase and have been reportedly constructed using polymeric, (e.g. polymethacrylates) or silica based materials [79]. The history of polymeric monoliths has recently been reviewed by Tennikova and Reusch [80]. Monolithic silica was first reported as a chromatographic support by Japanese scientists at the 22nd International Symposium on High Performance Liquid Phase Separations and Related Techniques 1998, however, the technology was subsequently further developed and commercialised by Merck KGaA [79]. The preparation of monolithic silica columns is based upon sol-gel technology, which involves the hydrolysis and condensation of metal alkoxides to form ceramic like materials. A detailed account is beyond the scope of this text and therefore, the reader is referred to the reviews of Collinson [81] and Ishizuka *et al.* [82]. The commercially available monolithic columns are imparted a unique bimodal pore structure consisting of macropores of ~2 µm internal diameter and mesopores of ~13 nm internal diameter [83,84,85]. The macropores act as flow through pores whilst the mesopores provide a large active surface area on which the separation may occur. The bimodal pore structure also imparts a significantly higher permeability to the monolith when compared with particle packed columns [85]. The increased level of permeability may be attributed to the absence of inter particulate voids and pools of stagnant mobile phase that lead to the generation of higher backpressure in particle packed columns [86]. Therefore, monoliths generate less backpressure and allow for increased rates of mass transfer and as a consequence higher separation efficiencies compared to particle packed columns may be achieved [87,88]. Such properties allow for monolithic columns to be operated at high flow rates without compromising the separation efficiency. The adsorption capacity of monolithic columns may also be higher than their particle packed counterparts which allows for increased analyte stationary phase interaction and therefore, efficiency and selectivity on monolithic columns is often equivalent to that attainable on 3.5 µm particulate columns [89]. To date monolithic silica columns are only commercially available in either bare silica, C_8 or C_{18} phases whilst polymeric monoliths such as CIM™ disks are available in a choice of chemistries allowing for easy column coupling for multidimensional chromatography [86].

2.3.6 The LC separation approach used for PPCP determination:

As stated previously in section 2.1, all reported methods for the determination of pharmaceutical residues in environmental samples have focused upon the development of reversed-phase separation methods prior to MS with either electrospray (ESI) or atmospheric pressure chemical ionisation (APCI). In the vast majority of methods a C_{18} column was used along with a mobile phase consisting of water and either methanol or acetonitrile. Modifiers used for adjusting the pH or ionic strength have included acetic or formic acids or ammonium formate and ammonium acetate [38,45,48,49,50,90]. Blackwell *et al.* published two methods for the determination of three antibiotics again using a C_{18} column [55,56], a mobile phase consisting of tetrahydrofuran, acetonitrile and water containing trifluoroacetic acid (TFA), was used. In one instance, UV detection was used with multiple wavelength monitoring, i.e. 285, 355, 260 and 370 nm for extra selectivity whilst on the other occasion pre-column fluorescence derivatisation using fluorescamine as a fluorophore was employed [56]. Two reports concerning the use of C_8 phases were presented by Augeura *et al.* [33] for the determination of Triclosan and biphenylol in marine sediments and also by Miao *et al.* for the determination of carbamazepine and its metabolites. Augeura *et al.* used a mobile phase of ammonium hydroxide at pH 10.5 along with acetonitrile to promote the dissociation of Triclosan prior to ESI-MS determination, [33], however, it is worth recalling that the upper recommended pH limit of silica based materials is pH 7.5, (section 2.2.5). A complex mobile phase was used by Miao *et al.* [43] consisting of 2:3 methanol acetonitrile and ammonium acetate in water. Vanderford *et al.* employed a C_{12} column with a methanol water mobile phase modified with formic acid for the separation of acidic and basic pharmaceuticals, personal care products such as Triclosan and hormones. Detection was performed using ESI-MS, however, the mobile phase flow rate was 0.70 $mLmin^{-1}$, with no adverse affects on the ESI intensity as a result of the high flow rate being reported. Other detection mechanisms have also been reported, although, reports of such are scarce. Santos *et al.* report the use of fluorescence detection for the determination of pharmaceutical residues in treated wastewater [91].

Capillary LC-MS was used by van der Ven *et al.* for the determination of diazepam in aquatic samples [92]. The limit of detection for the analysis was approximately 0.1 $ngmL^{-1}$ for a 5 µL injection of un-preconcentrated sample into

the C_{18} capillary LC-MS system, as with normal and microbore methods a mobile phase consisting of ammonium acetate and methanol was employed but with a flow rate of only 15 $\mu L min^{-1}$. Another alternative approach was reported by Quintana and Reemtsma [93] who employed ion-pair LC with tandem MS detection for the determination of acidic pharmaceuticals. Tributylamine was used as the ion-pairing agent in a methanol water mobile phase and a phenyl-hexyl column. The ion-paring agent was indeed found to aid retention of acid drugs such as salicylic acid but its affects were less pronounced with decreasing analyte polarity and an incomplete separation of the analytes was reported. An ion chromatography approach was also reported by Sacher *et al.* who used anion exchange chromatography in conjunction with inductively coupled plasma mass spectrometric detection (ICP-MS) [94]. Reports of capillary electrophoretic separations are also rare, Buchberger *et al.* report the use of CE-MS for the determination of antidepressants in wastewater and surface water in association with SPE [95]. Detection limits reported concern only an instrumental validation; however, low $\mu g L^{-1}$ levels were easily achieved in a relatively fast run, less than 20 minutes per sample. It should be noted that the majority of published methods lack complete separation of the target analytes and in many cases excessive runtimes or unsuitable conditions are employed. Therefore, further research to improve the chromatographic performance of many monitoring methods needs to be undertaken.

2.4 Selective Detection using Mass Spectrometry:

2.4.1 The power of mass spectrometry:

Mass spectrometry; (MS) is the separation and identification of gaseous ions based upon their mass to charge ratio (m/z) [96]. MS is one of the most powerful tools available to the analytical chemist as it permits elemental and isotopic analysis, structural elucidation of molecules and surfaces and when used in conjunction with an analytical separation technique, a wealth of qualitative and quantitative data may be obtained for complex mixtures [96]. MS was pioneered through the research of Thomson and Aston, both Nobel Prize winners in physics and chemistry respectively for their work investigating the separation of non radioactive isotopes using electric and magnetic fields [97]. The technique received much attention and developed rapidly with the first commercial mass

spectrometer becoming available in 1948 [98]. Other significant developments in MS were the introduction of GC-MS instrumentation in 1956 followed by the development of liquid phase introduction techniques in the 1980s [98]. The most recent Nobel Prize winners for pioneering research in the MS field were Fenn and Tanaka in 2002 for the development of ionisation methods for the mass spectrometric analyses of biological macromolecules [98].

The importance of MS in environmental analysis, especially for the identification of pharmaceutical residues has been discussed by Daughton [99] who noted the necessity for highly sensitive and selective analytical methods when attempting to evaluate the sources of, and risks posed by drugs and also for the forensic identification of other previously unknown environmental organic micro contaminants. It is therefore, not surprising that the majority of published methods for determining the extent of environmental contamination with pharmaceutical residues report the use of either single or tandem MS detection based upon the ability to unambiguously identify compounds using mass spectral data.

Mass spectrometers consist of the following components, a sample introduction system, an ionisation source, a mass analyser and a detection system. A discernible operating characteristic of MS is the need for the instrument to be kept under high vacuum in order to ensure that ions reach the detector without undergoing unwanted gas phase collisions. Therefore, the incompatibility with LC that uses significant quantities of solvents over the course of the separation is clearly apparent. Other notable incompatibilities are that LC is suited to the analysis of polar non-volatile compounds, while MS requires the formation of gaseous ions and also the use of unsuitable mobile phase modifiers. The development of suitable interfaces to remove the mobile phase and convert dissolved solutes into gaseous ions was a necessity for the coupling of LC with MS. The interface serves two purposes; it facilitates the use of LC as a sample introduction technique for MS and also the use of MS as a chromatography detector. As a consequence MS must fulfil the requirements of a suitable LC detector [100]. Initial attempts to interface the two techniques focused upon the use of a moving belt interface. However, the technology rapidly improved with each new available interface superseding the previous [100]. Today only two interfaces are commonly used in LC-MS analyses, ESI and APCI. Both of these

have been applied to the determination of pharmaceutical residues and will now be reviewed.

2.4.2 Atmospheric pressure Interfaces for LC-MS; ESI & APCI:

As said previously in section 2.4.1, there is an apparent mismatch in the coupling of LC with MS and therefore, an interface is required not only to remove the mobile phase but also act as a link between the atmospheric pressure output of the LC and the high vacuum MS. The design of suitable interfaces must therefore include components to allow for liquid introduction, an atmospheric pressure region wherein the ionisation occurs, ionic sampling apertures, a vacuum interface and finally ion optics to ensure the ionised analytes reach the mass analyser [101]. The two interfaces currently used for the vast majority of LC-MS applications are the ESI and APCI interfaces.

The concept of ESI was first described by Fenn and co-workers in 1984 for the mass spectrometric analysis of proteins and macromolecules from the liquid phase [97]. ESI involves the transitory of ionised species from the liquid phase into the gas phase as individual entities [102]. The mechanism of electrospray can be seen in Fig 2.7. ESI involves passing a solution of the analyte, i.e. the column effluent, through a capillary to which a high potential is applied, the effect of the applied electric field causes the generation of a fine mist of highly charged droplets as the solution emerges from the capillary [102]. The charged droplets are attracted towards the endplate electrode due to the influence of both the potential difference and the pressure gradient that exists within the source, during this time the droplets reduce in size as a result of solvent evaporation and also due to 'coulomb explosions' that occur due to the build up of ions of one polarity on the surface of the drop, creating coulomb forces of repulsion that are greater than the surface tension of the liquid, (i.e. the Rayleigh limit) [100,103,104,105]. The net result is the formation of a smaller droplet rich in one ion polarity and the process reoccurs until the formation of extremely small droplets in the range of 3-10 nm diameter that are capable of producing gas phase ions upon fission [102,103,104,105]. The formed ions are then sampled using skimmer cones and transported through the ion optics to the mass analyser.

ESI is an extremely soft ionisation technique and yields the production of gaseous ions with the same charged state as was present in the liquid phase, the

process is also non energetic and as a consequence none or very little molecular fragmentation will occur [102]. The mechanism can be perceived to involve a step-wise removal of solvent molecules from the analyte without detrimentally affecting the confirmation of the analyte. As a result the technique is important in the investigation of biologically important macromolecules [103,104].

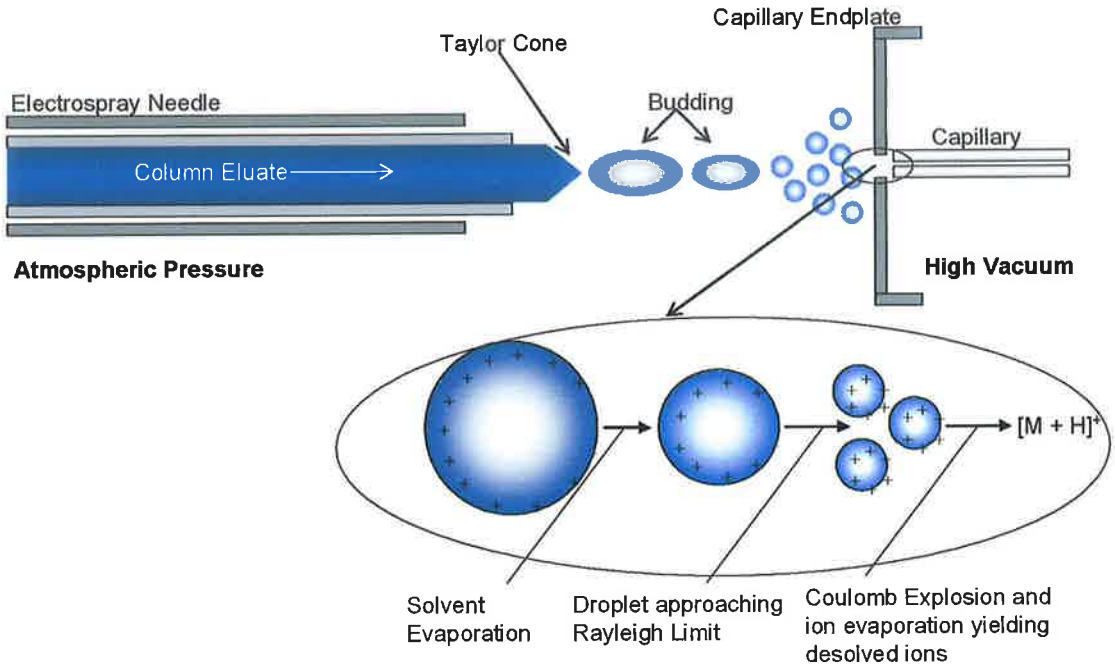


Figure 2.7: The mechanism of electrospray ionisation, adapted from [100].

Choosing suitable LC separation parameters is also extremely important when using ESI-MS as a chromatography detector. The flow rate used must be compatible with ESI as it affects the size distribution of the aerosol droplets and therefore, the level of charge on each droplet [104]. Mobile phase modifiers that are suitably volatile must be used to avoid build up of residues on the source components. Accordingly, it is worth recalling that the approach used by the majority of analysts for the determination of pharmaceutical residues has focused upon the use of microbore LC columns with mobile phases containing acetic or formic acids or their volatile ammonium salts (section 2.3.6 previous).

The production of ions by ESI is often heavily dependant upon solution chemistry and is highly suited to the analysis of ionic compounds, polar and neutral compounds that may be readily protonated or deprotonated by altering the solution conditions, e.g. pH and compounds that can be oxidised or reduced under the applied electric field at the capillary tip [104]. Pharmaceuticals are

therefore suited to ESI-MS analysis as they are often weak acids or bases and contain polarisable groups, e.g. carbonyl moieties *etc.*

For compounds that do not fulfil the above requirements APCI may be used as an alternative. In APCI the column effluent is nebulised and heated to form an aerosol. Ionisation is then achieved using a corona discharge (i.e. the ionisation of the nebuliser gas using a high applied voltage to the tip of a metallic electrode) [100,104,106,107]. The corona discharge ionises both analyte and eluent molecules and both species can react by gas phase ion molecule interactions such as proton transfer *etc.* as shown in Fig. 2.8, leading to ionisation of the analyte which is then sampled in an analogous manner to ESI [100,104,106,107]. As with ESI, APCI is a rather 'soft' process leading to the formation of molecular ions via protonation, deprotonation or adduct formation with mobile phase modifiers. [104].

A significant difference between the two interfaces is the LC flow rates that can be accommodated. As mentioned previously ESI demands lower flow rates in order to be efficient, however, APCI is compatible with flow rates normally used in conjunction with standard bore chromatography columns, even up to 2.00 $mLmin^{-1}$ [100]. Higher flow rates help prevent damage to the corona needle and the stability of the discharge. APCI is extremely useful for the ionisation of nonpolar to moderately polar compounds that posses a degree of volatility and thermal stability as common APCI performance temperatures are in the region of 350-500^{o}C [100]. APCI-MS has also been applied to the determination of drug residues in environmental samples, particularly for analytes that exhibit no ESI response, e.g. Loffler and Ternes used LC-APCI-MS for the determination of acidic analgesics and the parasiticide ivermectin in river sediments [34] and Vanderford *et al.* employed LC-APCI-MS for the analysis of hormones in aquatic samples, [41]. Other reported applications of LC-APCI-MS include the determination of antifouling agents [108], narcotics [109] and pesticides [110].

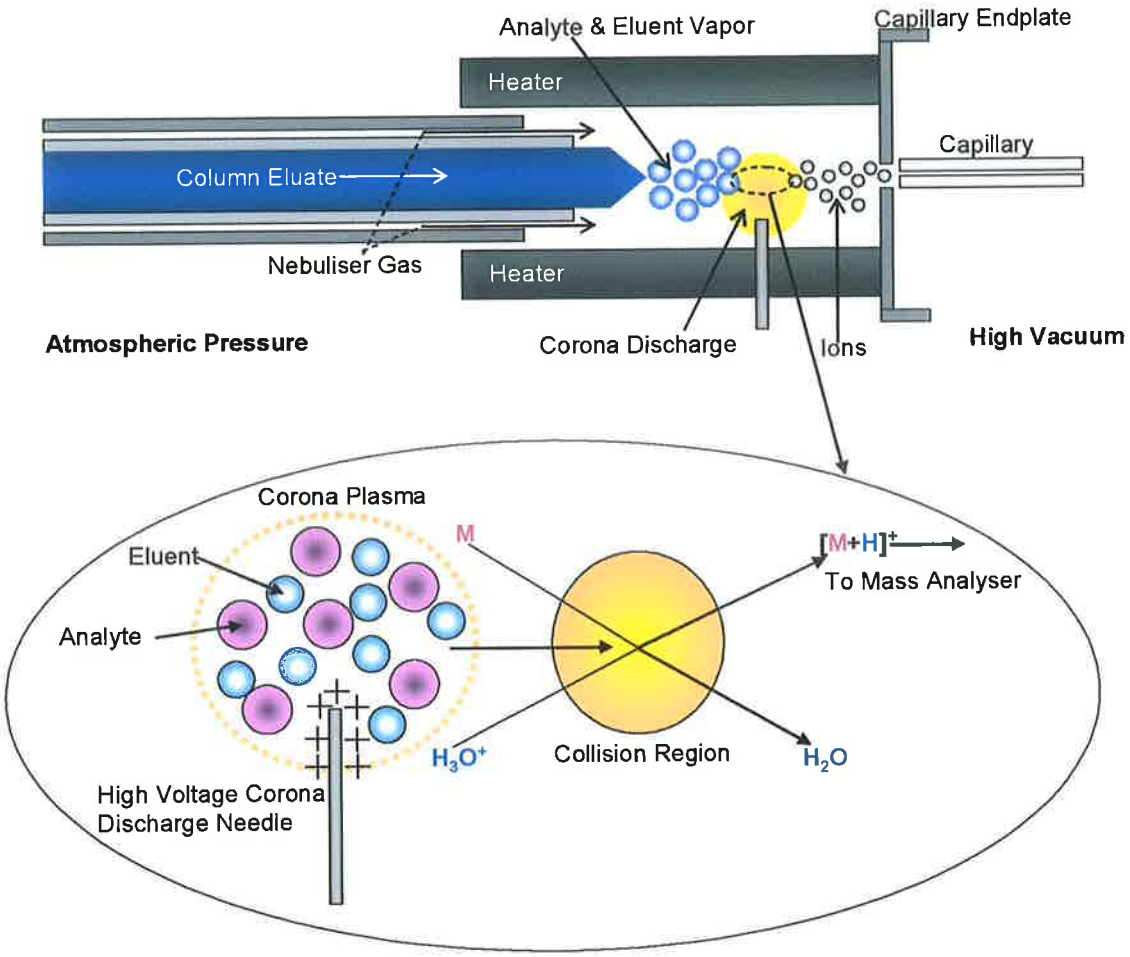


Figure 2.8: The mechanism of APCI, adapted from [98].

2.4.3 Mass analysers used in LC-MS:

After the interface has ionised the sample molecules or transferred them into the gas phase, they are then swept into the mass analyser as a result of potential differences and a vacuum gradient. The mass analyser separates ions based upon their m/z ratio. Mass analysers frequently reported in conjunction with liquid chromatography include the quadrupole, the ion trap and the time of flight mass analysers.

Quadrupole mass analysers consist of four rods that are arranged in a perfectly parallel orientation as depicted in Fig. 2.9. The rods are oppositely charged and have a fixed direct current (DC) potential and an oscillating radio frequency (RF) potential applied to them [111]. Introduced ions are passed along the centre of the quadrupole and by varying the RF potential ions of different m/z can be selectively allowed to pass through and reach the detector thereby permitting the collection of the mass spectrum [111].

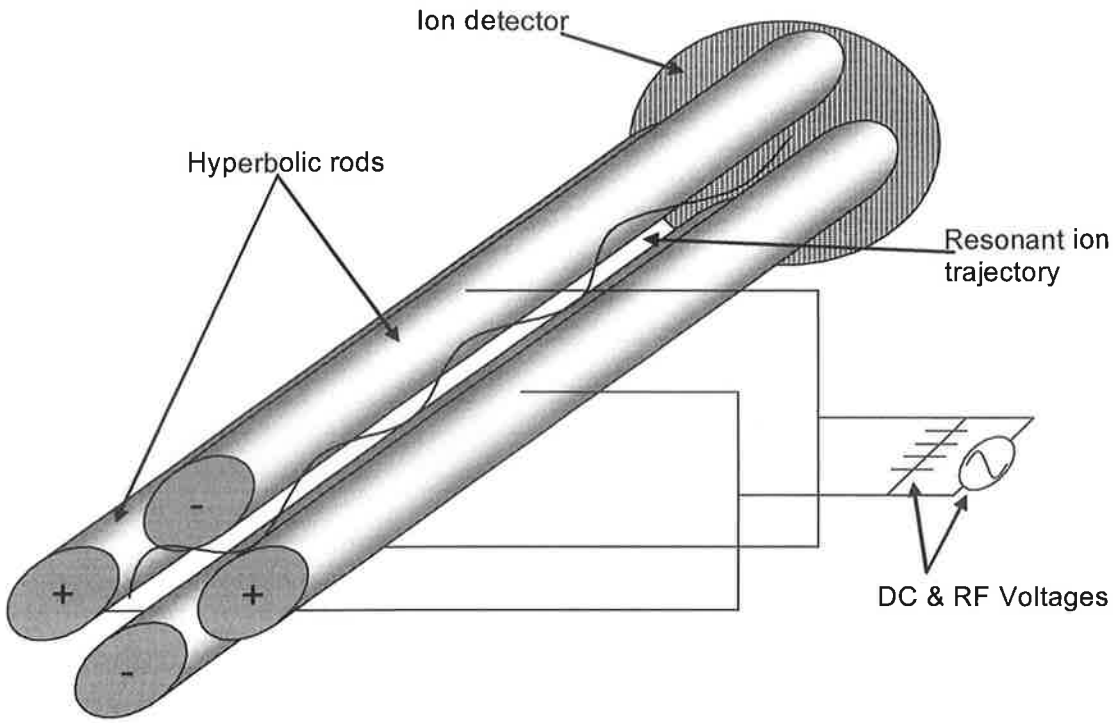


Figure 2.9: The quadrupole mass analyser, adapted from [112].

The equations describing the trajectory of an ion through the quadrupole field are extremely complex and beyond the scope of this text, however, the field experienced by ions as the travel through the quadrupole comprise both the constant and the alternating potentials described by the following equation [113].

$\varphi = +(U - V\cos\omega t)$ and $-\varphi = -(U - V\cos\omega t)$ (Eq. 2.14)

Where:

φ is the total applied potential,

U is the DC potential,

$V\cos\omega t$ is the applied RF potential of amplitude V and frequency ω.

Ions will resonate at particular values of $V\cos\omega t$ and will have a stable trajectory as shown in Fig. 2.9 therefore reaching the detector, whilst unstable ions collide with the rods, lose their charge and are removed from the mass analyser by the vacuum system [113].

Quadrupole mass analysers are widely used as mass analysers in both GC and LC-MS instruments due to their relatively low cost and their simplicity of operation, with single mass resolution readily achievable. Quadrupoles can also be coupled in series quite easily allowing for tandem MS measurements to be performed, i.e. QqQ instruments. In such an instance the first quadrupole acts as a mass filter allowing ions of particular m/z to pass through to the second quadrupole where they are fragmented by collisions with a gas. Fragmented ions

then pass into the third quadrupole where the fragmented ions are measured and identified [111]. Tandem MS measurements can be used to increase selectivity, sensitivity and structural elucidation, approaches frequently used in the articles cited previously for the determination of pharmaceutical residues, (section 2.3.6).

The ion trap mass analyser is a form of quadrupole and consists of two end-cap electrodes and a ring electrode as shown in Fig. 2.10.

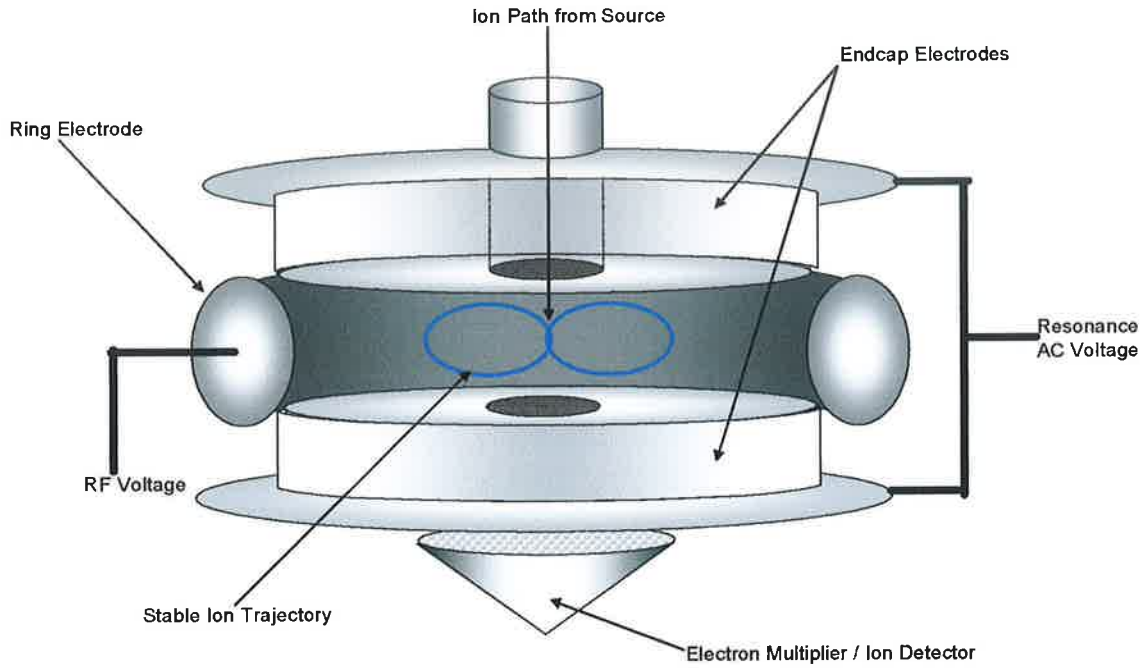


Figure 2.10: The ion trap mass analyser, adapted from, [98].

The ion trap operates using the same principles as the quadrupole mass analyser by the creation of a three dimensional quadrupole field upon the application of stable and variable potentials across the electrodes which allows for the trapping of all ions within the trap cavity. By ramping the RF potential ions of increasing m/z are ejected from the trap and detected [113]. Ions of similar charge will naturally repel each other inside the trap with a detrimental effect upon their trajectory, to avoid such an effect the trap is filled with helium at pressure of 10^{-3} torr which acts as a 'shock absorber' and removes excess energy by collision [113]. Ion traps are also widely used as mass selective chromatographic detectors as they are small and relatively inexpensive and offer unit mass resolution [111]. The ion trap also allows for tandem MS measurements to be performed by isolating the ion of interest within the trap during the first mass analysis, fragmenting it using an excitation pulse and helium collisions and then analysing the product ions in the normal scanning manner during the second mass analysis [111].

The time of flight; (ToF) mass analyser as shown in Fig. 2.11 operates on a much simpler principle than the previously discussed mass analysers. With ToF-MS ions are introduced directly from the source or if in a tandem instrument from the previous mass analyser as a controlled pulse, which imparts the same kinetic energy to all ions [111,113]. As they pass through the drift tube the ions separate based upon their mass, i.e. light ions travel faster than heavier ions and therefore, reach the detector first [98].

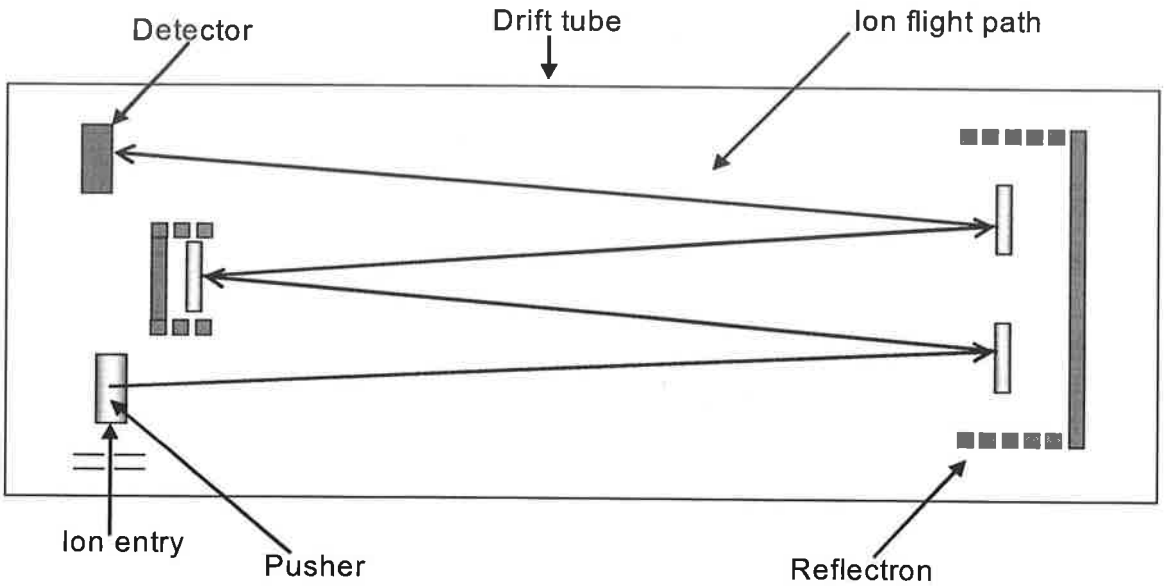


Figure 2.11: The time of flight mass analyser, adapted from [104].

Equation 2.15 describes the relationship between the flight time and ionic mass, [113]:

$$t^2 = \frac{m}{z}\left(\frac{d^2}{2V_s e}\right) \qquad \text{(Eq. 2.15)}$$

Where:

t is the time it takes to reach the detector,

d is the length of the drift tube,

V_s is the applied pulse potential and

e is the charge on an electron.

Therefore, from the above equation it can be seen that m/z can be calculated from the drift time as the values in brackets are constant. However, this also requires accurate time measurements for accurate mass measurement and fast electronics. The ToF analyser has no mass limits and high mass accuracy is readily achievable with extremely high sensitivity.

Few reports exist of the application of ToF-MS for the determination pharmaceutical residues in environmental samples. Marchese *et al.* examined the use of QqQ-MS and Q-ToF-MS for the LC-MS/MS determination of non steroidal anti inflammatory compounds in surface water [114] and it was found that the QqQ system was more sensitive with calculated detection limits lower than the Q-ToF system by a factor of 3. However, the Q-ToF system was more selective for the analysis of real samples as it was not as prone to matrix interference as the QqQ-MS [114]. Ferrer and Thurman reviewed the application of LC-ToF-MS for the determination of emerging environmental contaminants including pharmaceuticals and remarked that only two articles concerning drug residue analysis were reported, both of which were submitted for review [115]. It was however, noted that LC-ToF-MS is a powerful tool for the elucidation of previously unknown organic micro pollutants. Such an approach was further employed by Aguera *et al.* who demonstrated the ability to identify photo transformation products of the non steroidal anti-inflammatory drug diclofenac using ToF-MS. It was possible to perform accurate mass measurements with very low mass error and consequently identify thirteen previously unknown photoproducts of the drug [116]. Perez *et al.* performed a similar study using ion trap MS to determine and characterise metabolites of the x-ray contrast agent iopromide in activated sludge. Although ion trap MS lacks the same mass accuracy as ToF-MS it was still possible to identify four possible biodegradation products of iopromide in activated sludge using hydrogen deuterium exchange in order to increase the accuracy of the measurements [117].

2.4.4 Ion detectors:

Detection of the separated ions is the final parameter in the collection of mass spectral data. Typical ion detectors include electron multipliers, Faraday cups and array detectors. Electron multipliers operate in an analogous fashion to photomultiplier tubes in optical spectroscopy with signal multiplication arising due to the enhancement of charged particles across dynodes [113]. Electron multipliers consist of a Sn/SnO or Pb/PbO doped glass funnel that acts as a continuous dynode under a voltage gradient to attract electrons to the bottom of the multiplier, a gain of 10^7 electrons per ion is readily achievable [113].

With Faraday cup detectors the ion exiting the mass analyser strike a collector electrode that is surrounded by a Faraday cage, which captures

reflected ions or ejected secondary electrons [112]. The collector electrode and the Faraday cage are connected to a resistor and the analytical signal is recorded as the feedback current required across the resistor in order to maintain a base level potential [112]. Faraday cups are not as sensitive as electron multipliers and also exhibit a slower response and as a consequence are not suitable for rapid analysis, they are, however, relatively inexpensive.

Array detectors consist of a plate into which parallel cylindrical channels have been drilled and a potential difference is applied across the plate (inlet to outlet) [113]. Channels are coated with a semiconductor that acts as the continuous dynode, readily allowing gains of 10^5 to 10^8 per incident ion at a metallic collector anode. The plate can be used to detect ions of various m/z in different channels over the course of a single scan [113].

References:

1. T.A. Ternes, *TrAC Trends in Analytical Chemistry*, **20**, (2001), 419.
2. N.J.K. Simpson & M.J.M. Wells, *'Introduction to Solid Phase Extraction'*, in Solid Phase Extraction – Principles, Techniques and Applications, edited by N.J.K. Simpson, (2000), 1, CRC Publishers, London.
3. J. Pawliszyn, *'Unified Theory of Extraction'*, in Wilson & Wilson's Comprehensive Analytical Chemistry Volume XXXVII, 'Sampling and Sample Preparation for Field and Laboratory' edited by J. Pawliszyn, (2002), 253, Elsevier Science, Amsterdam.
4. H. Kataoka, *TrAC Trends in Analytical Chemistry*, **22**, (2003), 232.
5. F.F. Cantwell & M. Losier, *'Liquid-Liquid Extraction'*, in Wilson & Wilson's Comprehensive Analytical Chemistry Volume XXXVII, 'Sampling and Sample Preparation for Field and Laboratory' edited by J. Pawliszyn, (2002), 297.
6. E. Psillakis & N. Kalogerakis, *TrAC Trends in Analytical Chemistry*, **22**, (2003), 565.
7. S. Pedersen-Bjergaard, K.E. Rasmussen & T.G. Halvorsen, *Journal of Chromatography A*, **902**, (2000), 91.
8. I. Liska, *Journal of Chromatography A*, **885**, (2000), 3.
9. J.S. Fritz & M. Macka, *Journal of Chromatography A*, **902**, (2000), 137.
10. Waters Oasis Applications Notebook, (2003), Waters Corporation.
11. C.F. Poole, *'Principles and Practice of Solid Phase Extraction'*, in Wilson & Wilson's Comprehensive Analytical Chemistry Volume XXXVII, 'Sampling and Sample Preparation for Field and Laboratory' edited by J. Pawliszyn, (2002), 341.
12. Z.S. Ferrera, C.P. Sanz, C.M. Santana & J.J. Santana-Rodriguez, *TrAC Trends in Analytical Chemistry*, **23**, (2004), 496.
13. C.F. Poole, A.D. Gunatilleka & R. Sethuraman, *Journal of Chromatography A*, **885**, (2000), 17.

14. C.F. Poole & S.K. Poole, *'Theory Meets Practice'*, in Solid Phase Extraction – Principles, Techniques and Applications, edited by N.J.K. Simpson, (2000), 183.

15. C.F. Poole, *TrAC Trends in Analytical Chemistry,* **22**, (2003), 362.

16. M.R. Criado, D.H. Fernandez, I. Rodriguez Pereiro & R. Cela Torrijos, *Journal of Chromatography A*, **1056**, (2004), 187.

17. B. Shao, D. Dong, Y. Wu, J. Hu, J. Meng, X. Tu & S. Xu, *Analytica Chimica Acta*, **546**, (2005), 174.

18. J. Sunderland, A.M. Lovering, C.M. Tobin, A.P. MacGowan, J.M. Roe & A.A. Delsol, *International Journal of Antimicrobial Agents*, **24**, (2004), 511.

19. I. Ferrer & D. Barcelo, *TrAC Trends in Analytical Chemistry*, **18**, (1999), 180.

20. M.E. Leon-Gonzalez & L.V. Perez-Arribas, *Journal of Chromatography A*, **902**, (2000), 3.

21. J.J. Pesek & M.T. Matyska, *'SPE Sorbents and Formats'*, in Solid Phase Extraction – Principles, Techniques and Applications, edited by N.J.K. Simpson, (2000), 19, CRC Publishers, London.

22. C.W. Huck & G.K. Bonn, *Journal of Chromatography A*, **885**, (2000), 51.

23. V. Pichon, *Journal of Chromatography A*, **885**, (2000), 195.

24. N. Masque, R.M. Marce & F. Borrull, *TrAC Trends in Analytical Chemistry*, **17**, (1998), 384.

25. R. Saan-Nordhaus & J.M. Anderson, *Journal of Chromatography A*, **706**, (1995), 563.

26. P.R. Haddad, P. Doble & M. Macka, *Journal of Chromatography A*, **856**, (1999), 145.

27.M.C. Carson, *Journal of Chromatography A*, **885**, (2000), 343.

28. R.A. Gimeno, J.L. Beltran, R.M. Marce & F. Borrull, *Journal of Chromatography A*, **890**, (2000), 289.

29. M. Jorgensen, *Journal of Chromatography B*, **716**, (1998), 315.

30. S. AbuRuz, J. Millership & J. M^{c}Elnay, *Journal of Chromatography B*, **798**, (2003), 203.

31. S.N. Mahnik, B. Rizovski, M. Fuerhacker & R.M. Mader, *Chemosphere*, **65**, (2006), 1419.

32. T.A. Ternes, M. Bonerz, N. Herrmann, D. Loffler, E. Keller, B. Bago Lacida & A.C. Alder, *Journal of Chromatography A,* **1067**, (2005), 213.

33. A. Aguera, A.R. Fernandez-Alba, L. Piedra, M. Mezcua & M.J. Gomez, *Analytica Chimica Acta*, **480**, (2003), 193.

34. T. Vasskog, U. Berger, P-J. Samuelsen, R. Kallenborn & E. Jensen, *Journal of Chromatography A*, **1115**, (2006), 187.

35. D. Loffler & T.A. Ternes, *Journal of Chromatography A*, **1021**, (2003), 133.

36. S. Zuhlke, U. Dunnbier &T. Heberer, *Journal of Chromatography A*, **1050**, (2004), 201.

37. J.C. van de Steene, K.A. Mortier & W. E. Lambert, *Journal of Chromatography A*, **1123**, (2006), 71.

38. R. Lindberg, P.A. Jarnhimer, B. Olsen, M. Johansson & M. Tysklind, *Chemosphere*, **57**, (2004), 1479.

39. G.A. Grant, S.L. Frison & P. Sporns, *Journal of Agricultural & Food Chemistry*, **51**, (2003), 5367.

40. G.A. Mills, B. Vrana, I. Allan, D.A. Alvarez, J.N. Huckins & R. Greenwood, *Analytical & Bioanalytical Chemistry*, article in press 2006, doi.10.1007/s00216-006-0773-y

41. B.J. Vanderford, R.A. Pearson, D.J. Rexing & S.A. Snyder, *Analytical Chemistry*, **75**, (2003), 6265.

42. S. Yang, J. Cha & K. Carlson, *Rapid Communications in Mass Spectrometry*, **18**, (2004), 2131.

43. X.S. Miao & C.D. Metcalfe, *Analytical Chemistry*, **75**, (2003), 3731.

44. S. Yang & K.H. Carlson, *Journal of Chromatography A*. **1038**, (2004), 141.

45. S. Marchese, D. Perret, A. Gentili, R. Curini & F. Pastori, *Chromatographia*, **58**, (2003), 263.

46. S. Ollers, H.P. Singer, P. Fassler & S.R. Muller, *Journal of Chromatography A,* **911**, (2001), 225.

47. S. Weigel, R. Kallenborn & H. Huhnerfuss, *Journal of Chromatography A*, **1023**, (2004), 183.

48. S. Weigel, U. Berger, E. Jensen, R. Kallenborn, H. Thoresen & H. Huhnerfuss, *Chemosphere*, **56**, (2004), 583.

49. J.D. Cahill, E.T. Furlong, M.R. Burkhardt, D. Koplin & L.G. Anderson, *Journal of Chromatography A*, **1041**, (2004), 171.

50. M.J. Hilton & K.V. Thomas, *Journal of Chromatography A*, **1015**, (2003), 129.

51. N.M. Vieno, T. Tuhkanen & L. Kronberg, *Journal of Chromatography A*, **1134**, (2006), 101.

52. M. Gros, M. Petrovic & D. Barcelo, *Analytical & Bioanalytical Chemistry*, **386**, (2006), 941.

53. C. Hao, L. Lissemore, B. Nguyen, S. Kleywegt, P. Yang & K. Solomon, *Analytical & Bioanalytical Chemistry*, **384**, (2006), 505.

54. J.E. Renew & C.H. Huang, *Journal of Chromatography A*, **1042**, (2004), 113.

55. P.A. Blackwell, H.C. Holten Lutzhoft, H.P. Ma, B. Halling-Sorensen, A.B.A. Boxall & P. Kay, *Journal of Chromatography A*, **1045**, (2004), 111.

56. P.A. Blackwell, H.C. Holten Lutzhoft, H.P. Ma, B. Halling-Sorensen, A.B.A. Boxall & P. Kay, *Talanta*, **64**, (2004), 1058.

57. Z. Cheng, P. Pavelic, P. Dillon & R. Naidu, *Water Research*, **36**, (2002), 4830.

58. A. Motoyama, A. Suzuki, O. Shirota & R. Namba, *Rapid Communications in Mass Spectrometry,* **13**, (1999), 2204.

59. S. Rodriguez-Mozaz, M.J. Lopez de Alda & D. Barcelo, *Analytical Chemistry*, **76**, (2004), 6998.

60. M.J. Lopez de Alda & D. Barcelo, *Journal of Chromatography A*. **911**, (2001), 203.

61. J.V. Sancho, O.J. Pozo & F. Hernandez, *Analyst*, **129**, 38.

62. G.A. Guichon, *Analytica Chimica Acta*, **283**, (1993), 309.

63.H. Engelhardt, *Journal of Chromatography B*, **800**, (2004), 3.

64. K.K. Unger, *Journal of Chromatography A*, **1060**, (2004), 1.

65. R. Nageswara Rao & V. Nagaraju, *Journal of Pharmaceutical and Biomedical Analysis,* **33**, (2003), 335.

66. A. Rizzi, *'Retention and Selectivity'*, in The Handbook of HPLC edited by Katz *et al.* (1998), 1, Marcel Dekker, New York.

67. D.A. Skoog, D.M. West & F.J. Holler, *Fundamentals of Analytical Chemistry*, 7th Edition, (1996), 708, Saunders College Publishing, Philadelphia.

68. L.R. Snyder & J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, (1979), 16, J. Wiley & Sons, New York.

69. V.R. Meyer, *Practical High Performance Liquid Chromatography*, 2nd Edition, (1994), 17, Chichester Publishers, New York.

70. D.C. Harris, *Quantitative Chemical Analysis*, 5th Edition, (1999), 650, Freeman, New York.

71. H. Colin, G. Guichon & M. Martin, *'Liquid Chromatographic Equipment'*, in Practice of High Performance Liquid Chromatography edited by H. Engelhardt, (1986), 1, Springer, Berlin.

72. D.A. Skoog, F.J. Holler & T.A. Nieman, *Principles of Instrumental Analysis*, 5th Edition, (1998), 725, Saunders College Publishing, Philadelphia.

73. Seton Hall University On-line HPLC Textbook, last viewed 20th December 2006, available through: http://hplc.chem.shu.edu/book/html/intstrum.

74. W.T. Kok, *'Principles of Detection'*, in The Handbook of HPLC edited by Katz *et al.* (1998), 143, Marcel Dekker, New York.

75. B.E. Lendi & V.R. Meyer, *LC-GC Europe*, **18**, (2005), 156.

76. J.J. Kirkland, *Journal of Chromatography A*, **1060**, (2004), 9.

77. C.A. Doyle & J.G. Dorsey, *'Reversed-phase HPLC: Preparation and Characterisation of Reversed-phase Stationary Phases'*, in The Handbook of HPLC edited by Katz *et al.* (1998), 293, Marcel Dekker, New York.

78. G. Rozing, *LC-GC Europe*, June 2003.

79. R.E. Majors, *LC-GC Europe*, June 2003.

80. T.B. Tennikova & J. Reusch, *Journal of Chromatography A*, **1065**, (2005), 13.

81. M.M. Collinson, *TrAC Trends in Analytical Chemistry*, **21**, (2002), 30.

82. N. Ishizuka, H. Minakuchi, K. Nakanishi, N. Soga & N. Tanaka, *Journal of Chromatography A*, **797**, (1998), 133.

83. K. Cabrera, D. Lubda, K. Sinz &C. Schafer, *American Laboratory*, February 2001.

84. C. Schafer, K. Cabrera, D. Lubda, K. Sinz & D. Cunningham, *American Laboratory*, April 2001, 25.

85. F. Rabel, K. Cabrera & D. Lubda, *American Laboratory*, December 2000, 20.

86. F. Svec, *LC-GC Europe*, June 2003.

87. K. Cabrera, G. Weiland, D. Lubda, K. Nakanishi, N. Soga, H. Minakuchi & K.K. Unger, *TrAC Trends in Analytical Chemistry*, **17**, (1998), 50.

88. N. Tanaka, H. Kobayashi, N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Hosoya & T. Ikegami, *Journal of Chromatography A*, **965**, (2002), 35.

89. K. Cabrera, *Journal of Separation Science*, **27**, (2004), 843.

90. W. Ahrer, E. Scherwenk & W. Buchberger, *Journal of Chromatography A*, **910**, (2001), 69.

91. J.L. Santos, I. Aparicio, E. Alonso & M. Callejon, *Analytical Chimica Acta*, **550**,(2005), 116.

92. K. van der Ven, W. van Dongen, B.U.W. Maes, E.L. Esmans, R. Blust & W.M. de Coen, *Chemosphere*, **57**, (2004), 967.

93. J. B. Quintana & T. Reemtsma, *Rapid Communications in Mass Spectrometry*, **18**, (2004), 765.

94. F. Sacher, B. Raue & H-J. Brauch, *Journal of Chromatography A*, **1085**, (2005), 117.

95. M. Himmelsbach, W. Buchberger & C.W. Klampfl, *Electrophoresis*, **27**, (2006), 1220.

96. D.A. Skoog, F.J. Holler & T.A. Nieman, *Principles of Instrumental Analysis*, 5th Edition, (1998), 498, Saunders College Publishing, Philadelphia.

97. J.H. Beynon & A.G. Brenton, *An Introduction to Mass Spectrometry*, (1982), 1, University of Wales Press, Cardiff.

98. P. Gates, *The University of Bristol Mass Spectrometry Resource*, available on-line at www.chm.bris.ac.uk/ms, cited July 25th 2005.

99. C.G. Daughton, *Journal of the American Society for Mass Spectrometry*, **12**, (2001), 1067.

100. F. Lemiere, *'Interfaces for LC-MS', LC-GC Europe*, December 2001, 21.

101. W.M.A. Niessen, *Journal of Chromatography A*, **794**, (1998), 407.

102. S.J. Gaskell, *Journal of Mass Spectrometry*, **32**, (1997), 677.

103. P. Kebarle & Y. Ho, *'On the Mechanism of Electrospray Mass Spectrometry'*, in Electrospray Ionisation Mass Spectrometry, edited by R.B. Cole, (1997), 3, J. Wiley & Sons, New York.

104. B. Ardrey, *Liquid Chromatography-Mass Spectrometry: An Introduction*, (2003), 100, J. Wiley & Sons, New York.

105. A.P. Bruins, *Journal of Chromatography A*, **794**, (1998), 345.

106. J. Abian, *Journal of Mass Spectrometry*, **34**, (1999), 157.

107. W.M.A. Niessen, *'Developments in Interface Technology for Combined Liquid Chromatography, Capillary Electrophoresis and Supercritical Fluid Chromatography-Mass Spectrometry'*, in Applications of LC-MS in Environmental Chemistry, edited by D. Barcelo, (1996), 3, Elsevier Science, Amsterdam.

108. K.V. Thomas, *Journal of Chromatography A*, **833**, (1999), 105.

109. W. Weinmann, M. Goerner, S. Vogt, R. Goerke & S. Pollak, *Forensic Science International*, **121**, (2001), 103.

110. P.G.M. Kienhuis & R.B. Geerdink, *TrAC Trends in Analytical Chemistry*, **19**, (2000), 460.

111. F. Lemiere, *'Mass Analysers for LC-MS' LC-GC Europe,* December 2001, 14.

112. D.A. Skoog, F.J. Holler & T.A. Nieman, *Principles of Instrumental Analysis*, 5th Edition, (1998), 258.

113. E. de Hoffmann & V. Stroobant, *Mass Spectrometry: Principles & Applications*, 2nd Edition, (2001), 63, J. Wiley & Sons, New York.

114. S. Marchese, A. Gentili, D. Perret, G. D'Ascenzo & F. Pastori, *Rapid Communications in Mass Spectrometry*, **17**, (2003), 879.

115. I. Ferrer & E.M. Thurman, *TrAC Trends in Analytical Chemistry*, **22**, (2003), 750.

116. A. Aguera, L.A. Perez Estrada, I. Ferrer, E.M. Thurman, S. Malato & A.R. Fernandez-Alba, *Journal of Mass Spectrometry*, **40**, (2005), 908.

117. S. Perez, P. Eichhorn, M.D. Celiz & D.S. Aga, *Analytical Chemistry*, **78**, (2006), 1867.

3.0 Development of Dual Gradient Monolithic HPLC for the Determination of Pharmaceutical Residues in Environmental Samples.

3.1 Introduction:

A large number of HPLC methods have been published in the peer-reviewed literature for the determination of pharmaceutical substances spanning across analgesics [1,2,3,4,5,6], antibiotics [7,8], antifungal agents [9], lipid regulating agents [10,11], preservatives [12], personal care products [13,14], prospective drug candidates [15] to narcotics and banned or forensically interesting substances [16,17]. Stereo specific methods have also been reported but to a much lesser degree than standard assays [18,19,20]. The majority of reported methods have focused upon the use of reversed-phase particle packed columns and UV absorbance detection with the primary aims of the above studies being pharmacokinetic monitoring or finished and commercial product analysis. Many of the reported methods, while suited to the task at hand and often fully validated, are unsuitable for application to environmental monitoring or incompatible with MS due to the use of inorganic buffers in the mobile phase such as large amounts of phosphate, unsuitable flow rates, (flow rates of up to 0.7 $mLmin^{-1}$ were reportedly used with ESI [8]), lack of sensitivity and often lengthy runtimes for single compound analysis. Very few reports of the use of microbore columns exist, not even in conjunction with MS.

Pharmaceutical residues have been detected in a variety of environmental matrices, in both aqueous and terrestrial compartments, i.e. soils and sludge [21,22,23,24]. In contrast with the previous methods, the developed procedures for environmental pharmaceutical residue analysis are primarily based upon the use of small particle high resolution; (3-5 µm), microbore column LC-MS or LC-MS/MS to increase the sensitivity and specificity of the method. As a consequence of the use of MS detection, complete chromatographic separation of all analytes is no longer

crucial, however, in order to avoid adverse or unwanted effects upon the ESI intensity a suitable separation of the chosen analytes is still desired. A shortcoming of many of the reported methods is poor analytical separation. For example, four individual methods were required by Milton and Thomas [21] for the separation of thirteen pharmaceuticals leading to a single sample analysis time of greater than two hours, excluding re-equilibration time. In the case where a single chromatographic method was used it was often observed that a significant number of analytes were noticed to coelute [22,24]. In the case of Aguera *et al.* [23] the mobile phase employed was adjusted to pH 10.5 with hydroxide prior to use with an octyl silica column (it is worth remembering that the upper limit of silica based chromatography columns is usually pH 7.5 in order to prevent the stationary phase from dissolving, section 2.3.5). Therefore, it can be seen that although the methods published to date are suitably sensitive and fit for the purpose intended, an efficient chromatographic separation of the chosen analytes is often overlooked.

Monolithic silica columns are a relatively underused concept in environmental and pharmaceutical analyses. To date the majority of published reports have focused upon the use of monolithic silica columns to reduce analysis times and therefore, increase sample throughput [25,26,27]. However, monolithic silica columns also allow for more efficient separations with theoretical plate counts often far superior to those obtainable with 3.5 µm particulate columns due to higher permeability and increased rates of mass transfer [28,29]. Therefore, monolithic columns unlike their particle packed counterparts allow for highly efficient separations at high flow rates. The increased rate of column permeability, >80% compared to ~65% in a particle packed column, also allows for the use of significant flow gradients that are not possible due to excessive backpressure production in particle packed supports [30].

The aim of this initial research was to develop a high efficiency separation of chosen environmentally relevant pharmaceuticals using monolithic silica columns and to apply the developed method for the preliminary determination of pharmaceutical residues in environmental samples. A SPE procedure was also incorporated into the method in order to lower detection limits. In order to select a suitable sorbent various commercially available SPE cartridges based upon silica or polymeric supports were evaluated. Photodiode array detection was used for the

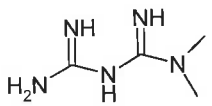
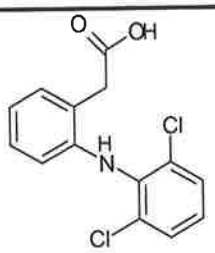
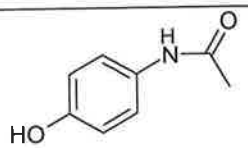
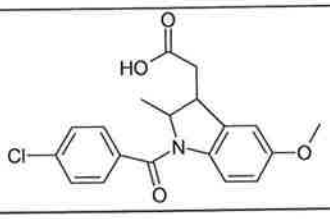
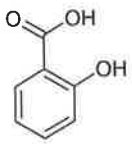
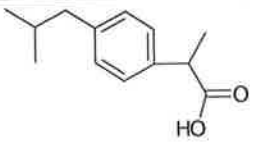
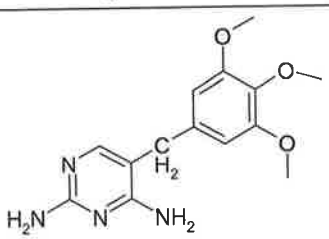
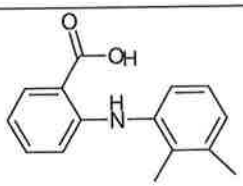
determination of sample extracts in an attempt to increase the specificity of the developed method.

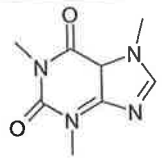
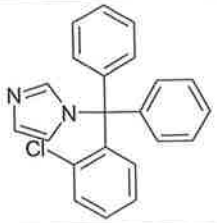
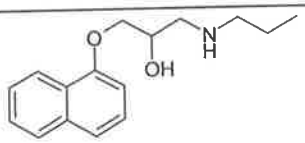
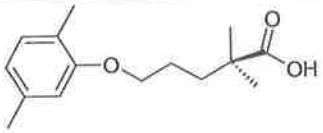
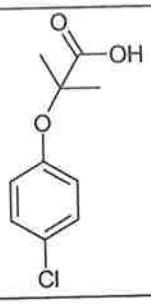
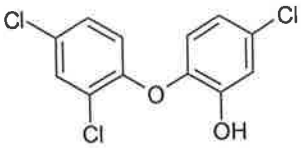
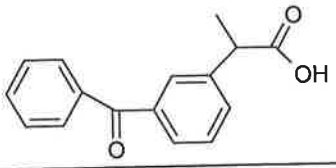
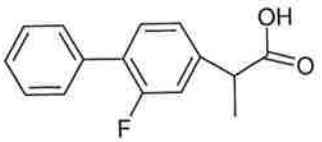
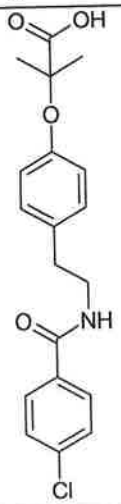
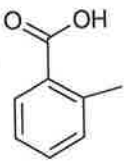
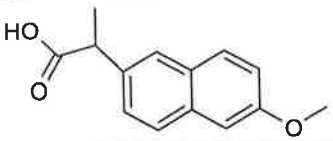
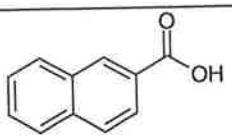
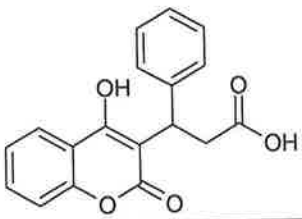
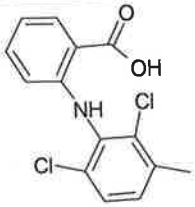
3.2 Experimental:

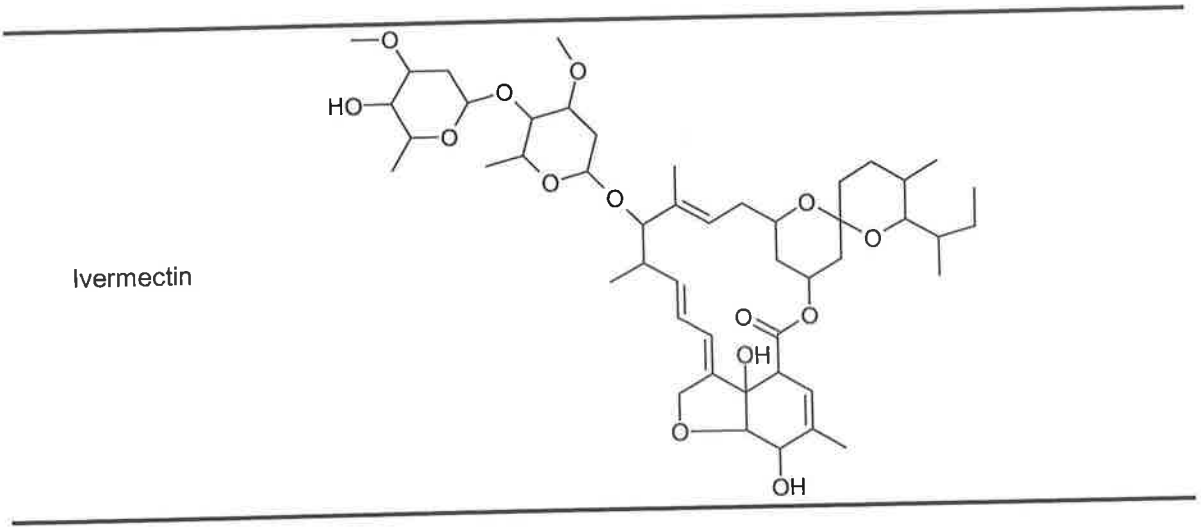
3.2.1 Analyte test group:

Analytes chosen for monitoring include those that showed prevalence in both the published literature and also the list of the most prescribed pharmaceuticals in Ireland for the year ending December 31st 2003 [31]. The chemical structures of the selected analytes are depicted in Table 3.1.

Table 3.1: Chemical structures of the pharmaceuticals and metabolites selected for study.

Analyte	Chemical Structure	Analyte	Chemical Structure
Metformin		Diclofenac	
Acetaminophen		Indomethacin	
Salicylic acid		Ibuprofen	
Trimethoprim		Mefenamic acid	

Caffeine		Clotrimazole	
Propranolol		Gemfibrozil	
Clofibric Acid		Triclosan	
Ketoprofen		Flurbiprofen	
Bezafibrate		*o*-Toluic acid	
Naproxen		2-Naphthoic acid	
Warfarin		Meclofenamic acid	



Ivermectin

3.2.2 Chemicals and reagents:

Reagent water used throughout, unless otherwise stated was obtained from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA) and was 18.2 MΩ or greater. Methanol (MeOH) was purchased from Labscan (Dublin, Ireland), acetone and ethyl acetate were obtained from Aldrich (Gillingham, UK). All solvents used were HPLC grade. Dichlorodimethylsilane, ammonium formate and formic acid were also purchased from Aldrich. BDH Analar grade sulphuric acid was used for sample pH adjustment (Poole, UK). Metformin hydrochloride, acetaminophen, salicylic acid, o-toluic acid, propranolol hydrochloride, clofibric acid, ketoprofen, diclofenac sodium salt, clotrimazole and 2-naphthoic acid were obtained from Aldrich (Steinheim, Germany). Trimethoprim, caffeine, naproxen and Triclosan were received from Fluka (Buchs, Switzerland). Bezafibrate, warfarin, flurbiprofen, indomethacin, ibuprofen sodium salt, meclofenamic acid sodium salt, mefenamic acid, gemfibrozil and ivermectin were all obtained from Sigma (Steinheim, Germany). All analytes were of a purity >95%.

Stock 1000 mgL^{-1} solutions of each analyte were prepared in MeOH and were stored as described on the material safety data sheets (MSDS) sheets for maximum stability, i.e. refrigerated if necessary and in the dark. In the case of salts, standards were prepared in terms of the parent analyte. These standards were periodically replaced. Working standards were prepared from these stock standards by appropriate dilution using MeOH.

3.2.3 Glassware preparation:

Prior to use all glassware was silanised by rinsing thoroughly with a 10% v/v solution of dichlorodimethylsilane in dichloromethane, followed by rinsing twice with dichloromethane and twice with MeOH.

3.2.4 Sample extraction:

A variety of sorbents were investigated as suitable stationary phases for sample extraction. These included Phenomenex Strata C_{18}-E™; 200 mg /3 mL, Phenomenex Strata-X™; 200 mg /6 mL, (Phenomenex, Macclesfield, UK), Merck LiChrolut EN™; 200 mg /3 mL, (Merck, Darmstadt, Germany), Waters Oasis HLB™; 200 mg /6 mL and Waters Oasis MAX™; 150 mg /6 mL, (Waters, Milford, MA, USA). From initial investigations the Phenomenex Strata-X™ and Oasis HLB™ sorbents demonstrated high analyte recovery and were therefore chosen for further study. Prior to extraction, 1 L water samples were filtered through Whatman GF/C glass micro fibre filters to remove particulate matter, (Whatman, Maidstone, UK). The filtrate was then spiked with surrogate standards (to yield an overall concentration of 2 μgL^{-1} of both *o*-toluic acid and meclofenamic acid) to assess extraction performance and adjusted to pH 4.0 with concentrated sulphuric acid. The SPE cartridge was conditioned with 6 mL of MeOH and 6 mL of water respectively. Samples were introduced by vacuum through Teflon tubing and extracted under an operating pressure of 20" Hg on a vacuum manifold. After complete sample introduction but without letting the cartridge run dry, the sorbent was washed with 1 mL of water that was discarded. The sorbents were then dried by vacuum aspiration for a minimum of 30 minutes. Elution was then performed using 10 mL of a 50% v/v solution of ethyl acetate in acetone [20]. The eluate was collected and reduced in volume to near dryness under a stream of N_2. The residue was reconstituted in 1 mL of internal standard solution (1 mgL^{-1} naphthoic acid in MeOH) and transferred to an autosampler vial for LC analysis.

3.2.5 Liquid chromatography:

A Hewlett Packard HP 1050 Series HPLC was used throughout and consisted of a quaternary pump with online vacuum degasser, a variable wavelength detector model 79853C and an autosampler. Agilent ChemStation for LC systems version

A.09.03 was used for system control and data analysis, (Agilent Technologies, Palo Alto, CA, USA). The analytical column used consisted of two Chromolith Performance RP18e; 100.0 x 4.6 mm monolithic octadecyl silica columns combined in series using a Chromolith column coupler. A 10.0 x 4.6 mm Chromolith RP18e guard cartridge was also incorporated to prevent spoilage of the analytical columns, (Merck, Darmstadt, Germany). A multi-step binary gradient, (Table 3.2) in conjunction with a flow gradient was employed for analyte elution using a mobile phase consisting of MeOH and 1 mM ammonium formate / formic acid buffer at pH 4.5. The temperature was held at 25°C throughout. A 20 µL injection volume was employed for both samples and standards. The total analysis time per injection was 70 minutes followed by a 10-minute re-equilibration period. Absorbance was monitored at 225 nm. At lower wavelengths the mobile phase itself showed significant absorbance and at higher wavelengths detector sensitivity for the majority of the pharmaceuticals was reduced.

Table 3.2: Liquid chromatographic method parameters.

Time (minutes)	Solvent A[1]	Solvent B[2]	Flow ($mLmin^{-1}$)
0	10	90	1.00
8	10	90	↓
15	52.5	47.5	↓
35	52.5	47.5	↓
40	65	35	↓
55	65	35	2.30
60	90	10	2.30
70	90	10	2.30

[1] Solvent A was MeOH.

[2] Solvent B was 1 mM ammonium formate buffer, pH 4.5.

3.3 Results and Discussion.

3.3.1 Methanol gradient:

Using an initial mobile phase consisting of MeOH and water adjusted to pH 3.0 using formic acid, simple linear gradients were investigated. Initial experiments involved starting conditions of 40% MeOH that was held isocratically for 10 minutes and then raised to 70%. However, results showed that for polar analytes such as trimethoprim, caffeine and paracetamol the initial percentage of MeOH in the mobile phase was too high, leading to subsequent poor retention. A number of peak pairs were also found to coelute, these included ketoprofen and clofibric acid, warfarin and bezafibrate and gemfibrozil and Triclosan.

Therefore, the starting percentage of MeOH was reduced in order to promote retention of polar analytes. It was found that at 10% MeOH, polar analytes like paracetamol could be retained for approximately 8 minutes. A series of individual linear gradients were then investigated to reduce the retention of all other less polar species and avoid the co-elution of the above mentioned peak pairs. To facilitate the complete elution and separation of all of the pharmaceuticals investigated, including ivermectin, the MeOH concentration had to be gradually increased over three individual linear gradients to 90% over a 75 min chromatographic run.

Fig. 3.1 depicts a 10 mgL^{-1} standard chromatogram (excluding ivermectin) recorded under optimum conditions, a near complete separation of all analytes is observable with the exception of gemfibrozil and Triclosan. The dashed line describes the % MeOH gradient used.

3.3.2 pH and buffer strength:

Using the previously optimised organic modifier the pH and the buffer strength of the aqueous proportion of the mobile phase was systematically varied to evaluate selectivity effects.

Buffers considered included ammonium formate and ammonium acetate for compatibility with possible MS detection. Of the buffered phases ammonium acetate demonstrated considerable absorbance at 225 nm and caused baseline disruption over the course of the gradient. Ammonium formate demonstrated a

considerably lower absorbance and although baseline disturbance was observed it was considerably less. Based on the above ammonium formate was chosen as the most suitable modifier.

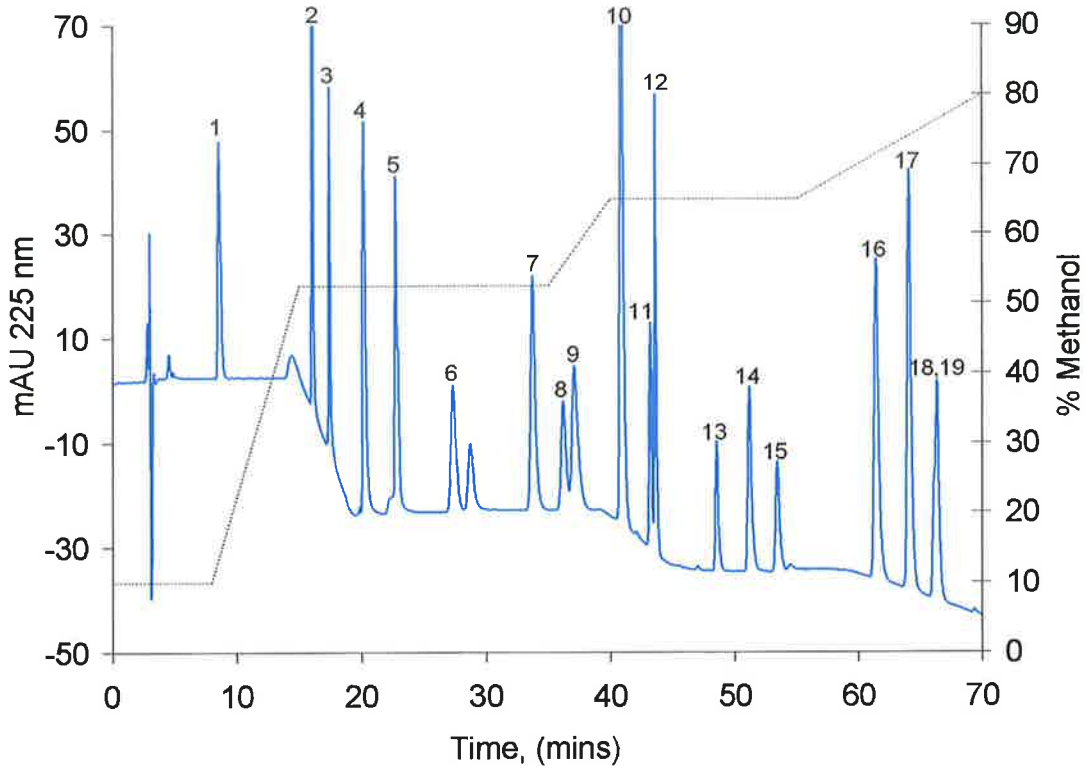


Figure 3.1: Optimised MeOH gradient separation of 19 pharmaceuticals on 20 cm reversed-phase silica monolithic column (pH 3.0 formic acid). Peak identification: **1** paracetamol; **2** trimethoprim; **3** caffeine; **4** salicylic acid; **5** *o*-toluic acid; **6** clotrimazole; **7** 2-naphthoic acid, (internal standard); **8** ketoprofen; **9** clofibric acid; **10** naproxen; **11** warfarin; **12** bezafibrate; **13** flurbiprofen; **14** diclofenac; **15** ibuprofen; **16** meclofenamic acid; **17** mefenamic acid; **18** & **19** gemfibrozil and Triclosan.

For optimisation, an experimental space was designed, governed by the buffering activity of formate, (pK_a = 3.75) and the desire to keep the ionic strength low enough to prevent salt precipitation at high proportions of organic solvent. Therefore, mobile phases containing ammonium formate concentrations of 1 to 5 mM, over the pH range of 2.5 to 5.5 were investigated. Twelve individual experiments were run within the above concentration and pH range. The resulting chromatograms were assessed using the resolution product criterion, R, calculated according to Equation 3.1 [32].

$$R = \prod_{i=1}^{n-1} \left(\frac{R_s(i,i-1)}{\frac{1}{n-1}\sum R_s((i,i-1)} \right) \qquad \text{(Eq. 3.1)}$$

Where:

n is the number of analytes present.

$R_s(i,i-1)$ is the resolution between peaks i and i + 1. Calculated using,

$$R_s = \frac{2(Tr_{i+1} - Tr_i)}{W_{i+1} + W_i} \qquad \text{(Eq. 3.2)}$$

Where:

Tr_{i+1} is the retention time of peak i + 1.

Tr_i is the retention time of peak i.

W_{i+1} is the baseline width of peak i + 1.

W_i is the baseline width of peak i.

R has values in the range of $0<R<1$. A value of 1 indicates that the resolution between all peaks is evenly distributed over the span of the separation and conversely a value of 0 indicates co-elution of two or more peaks somewhere within the chromatogram. From the calculated R-values a response surface was constructed, which is shown as Fig. 3.2. The optimum combination of pH and buffer strength was determined from the apex of the response surface within the experimental space.

The response curve depicts a mobile phase condition where all analytes were optimally resolved, this being 1 mM ammonium formate at pH 4.5. Fig. 3.3 depicts a standard chromatogram recorded under the optimised pH and buffer strength.

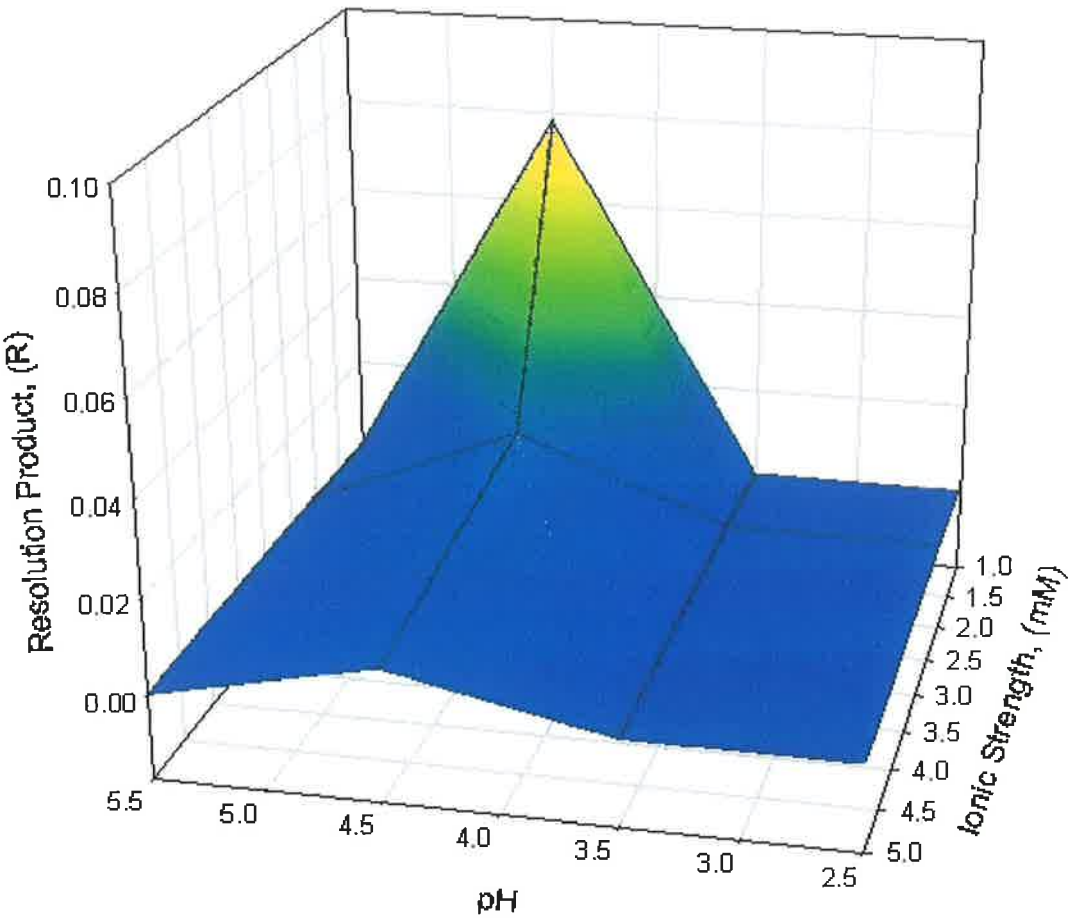


Figure 3.2: Resolution response surface for pH and buffer strength optimisation. Standard mixture as in Fig. 3.1.

A complete separation of all chosen analytes can be observed in the optimised chromatogram, including the previously co-eluting peak pair. It is also notable that a change in the retention order of certain basic compounds is also observable, for example clotrimazole is now retained for ~58 minutes as opposed to ~28 minutes at pH 3.0, and increase in retention of some 30 minutes due to the higher pH of the mobile phase, attributable to the deprotonation of the nitrogen atoms of the imidazole group leading to a neutral moiety as opposed to the cationic moiety that exists at the lower pH.

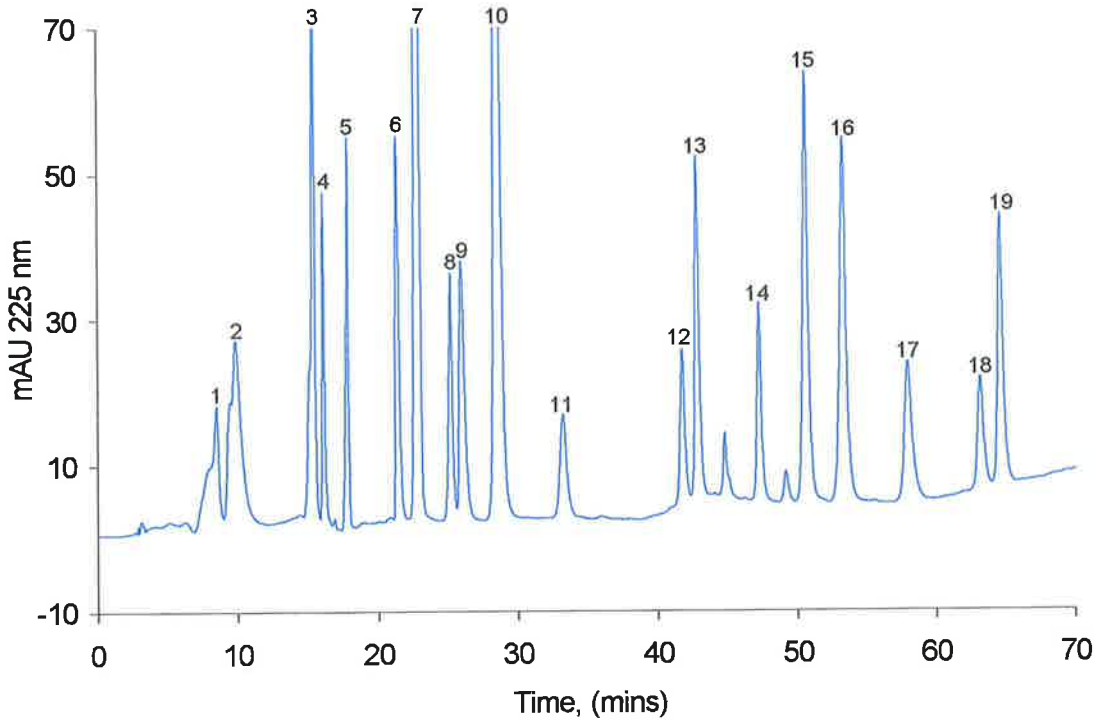


Figure 3.3: Optimised MeOH gradient separation of 19 pharmaceuticals on 20 cm reversed-phase silica monolithic column (pH 4.5, 1 mM formic acid). Peak identification: **1** paracetamol; **2** salicylic acid; **3** trimethoprim; **4** caffeine; **5** *o*-toluic acid; **6** clofibric acid; **7** 2-naphthoic acid, (internal standard); **8** ketoprofen; **9** bezafibrate; **10** naproxen; **11** warfarin; **12** flurbiprofen; **13** diclofenac; **14** ibuprofen; **15** meclofenamic acid; **16** mefenamic acid; **17** clotrimazole; **18** gemfibrozil; **19** Triclosan.

3.3.3 Combined solvent and flow gradients:

Although a complete separation of all pharmaceuticals in the optimisation standard is shown in Fig. 3.3, the overall analysis time is quite long at 90 minutes per injection (including re-equilibrium time). The high permeability of the porous monolithic columns is ideally suited for use at elevated flow rates. In this case operation under constant (isofluentic) elevated flow conditions were not suitable due to the desire to maintain resolution of early eluting peaks. Therefore, an approach first used by Paci *et al.* was investigated [33]. Paci *et al.* utilised a flow gradient combined with an elution gradient to reduce the retention of the anti-malarial agents chloroquine and proguanil on a 250.0 x 4.0 mm particulate C_{18} column. However, due to the pressure constraints of the particulate packed column used in the study, the flow could only be increased by 0.3 $mLmin^{-1}$. More recently, Paull *et al.* have reported more significant reductions in analysis times, together with increased efficiency, when a 'dual gradient' was applied to the separation of

UV absorbing anions on short monolithic column coated with a zwitterionic reagent [34]. In this case flow gradients from 1.0 to 6.0 $mLmin^{-1}$ were applied.

A number of flow gradient programs were investigated in combination with the optimised mobile phase gradient. Comparison of resultant separations showed that a continual linear flow gradient from 1 to 3 $mLmin^{-1}$ over the course of the run provided the best distribution of peaks across the entire chromatogram (with no detrimental effect upon resolution), combined with an approximate reduction in analysis time of 40 to 45%. Fig. 3.4 depicts a standard chromatogram obtained by separation using the dual gradient. Although not shown in Fig. 3.4, the dual gradient approach also allowed for the inclusion of ivermectin to the test mixture, which eluted after 65 minutes. Ivermectin is moderately lipophilic but it was found that an extremely high quantity of MeOH was required to elute it from the column under normal conditions.

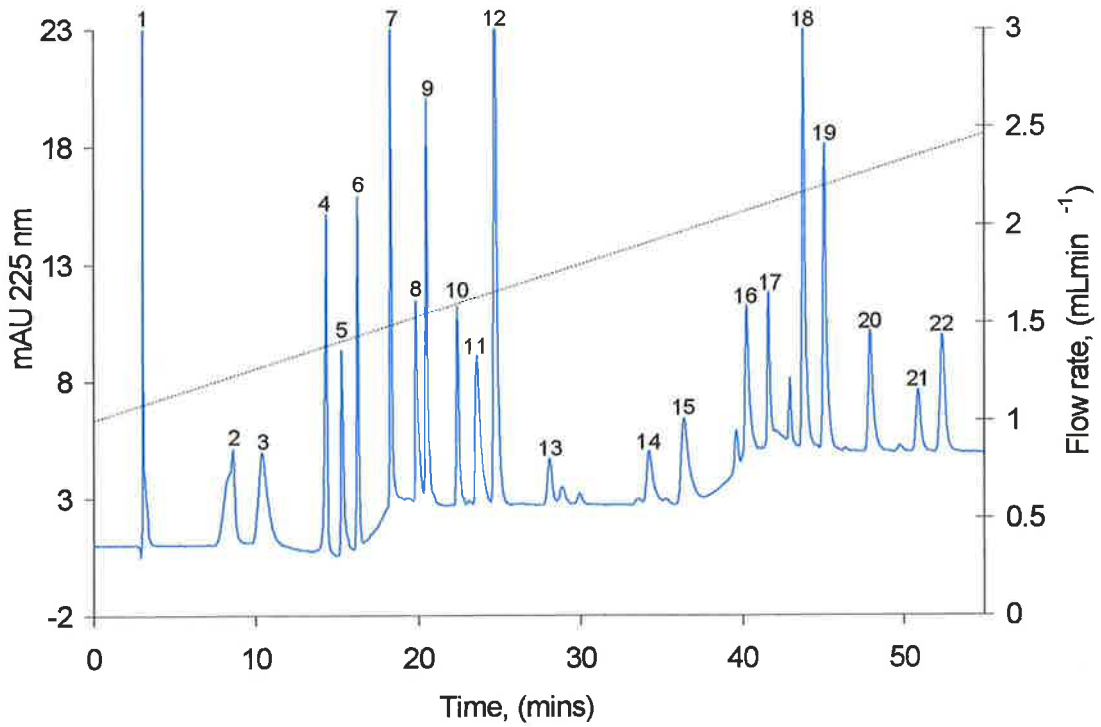


Figure 3.4: As Figure 3.3 with applied linear flow gradient from 1 $mLmin^{-1}$ at t=0 to 3 $mLmin^{-1}$ at t =75 minutes. Peak identification: **1** metformin; **2** paracetamol; **3** salicylic acid; **4** trimethoprim; **5** caffeine; **6** *o*-toluic acid; **7** propranolol; **8** clofibric acid; **9** 2-naphthoic acid, (internal standard); **10** ketoprofen; **11** bezafibrate; **12** naproxen; **13** warfarin; **14** flurbiprofen; **15** diclofenac; **16** indomethacin; **17** ibuprofen; **18** meclofenamic acid; **19** mefenamic acid; **20** clotrimazole; **21** gemfibrozil; **22** Triclosan.

3.3.4 Temperature effects:

Temperature is an important parameter in chromatographic separations that requires optimisation as fluctuations in temperature can drastically affect analyte retention. Temperature governs both the viscosity of the mobile phase and also the mobility of the analyte through the analytical column. For initial method development studies the column was kept isothermally at 25°C. The separation temperature was optimised over the range of 25 to 45°C in 5°C increments using a column oven. 45°C was chosen as the upper temperature limit as upon referral to the Chromolith column information, it is recommended not to heat the column greater than 45°C as higher temperatures can lead to strain and even breakage of the bond between the monolithic silica surface and the C_{18} ligand [35]. The resolution product was calculated for each resulting chromatogram.

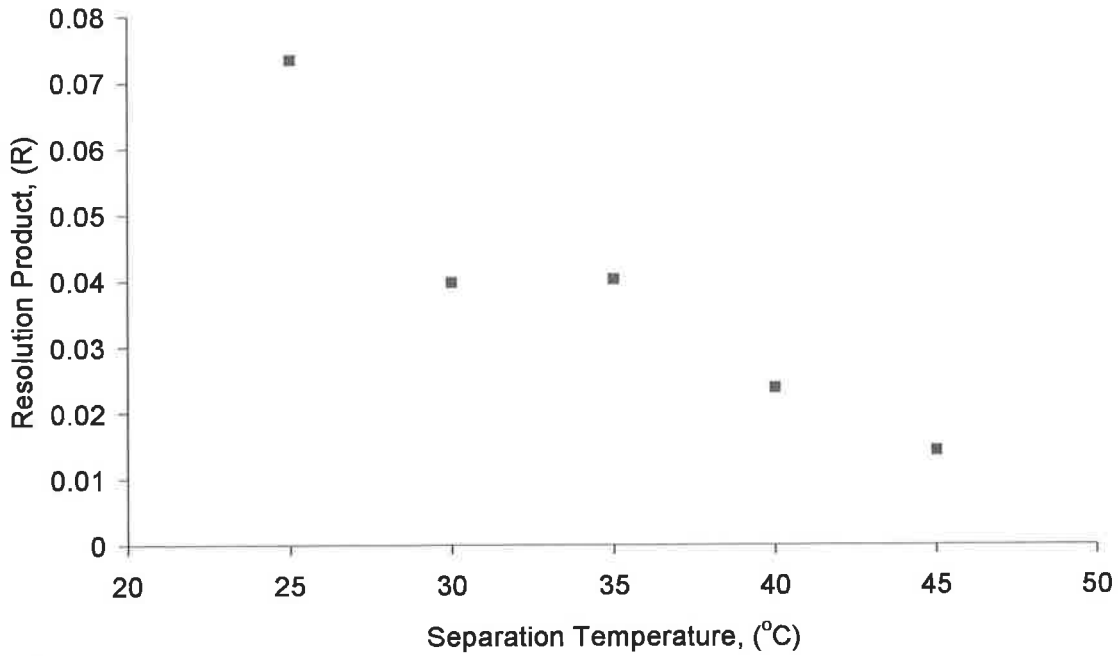


Figure 3.5: Investigation into the effect of increasing the operational separation temperature on the overall resolution product, (R). Separation conditions were as per those previously described in Table 3.2.

It can be seen that the resolution decreases as the temperature at which the separation is performed increases as demonstrated by Fig. 3.5. This can most probably be attributed to decreased analyte retention at elevated temperatures. The chromatographic efficiency was also observed to decrease as the separation temperature increased, theoretically it would have been expected to increase as

often by raising the operating temperature the kinetics of interaction are improved [36]. As the flow rate is constantly increasing, a uniform distribution of heat may not occur at elevated temperatures that may also lead to band distortion and therefore, decrease the efficiency of the separation.

3.3.5 Method validation:

Having developed and optimised the dual gradient separation the performance of the method was then investigated. In all instances method validation was performed according to the 'ICH Harmonised Tripartite Guidelines for the Validation of Analytical Procedures' [37]. The method was validated for linearity, reproducibility and repeatability and the instrumental limits of detection (LOD) and quantitation (LOQ) were calculated. The results for the validated parameters are presented in Table 3.3.

Table 3.3: Dual gradient HPLC method performance data.

Analyte	Linearity (R^2)	LOD (mgL^{-1})	LOQ (mgL^{-1})	Reproducibility (%RSD)	Repeatability (%RSD)
Metformin	0.9999	0.02	0.07	0.42	2.10
Paracetamol	0.9967	0.14	0.46	9.30	5.32
Salicylic acid	0.9999	0.22	0.72	2.37	7.97
Trimethoprim	0.9999	0.02	0.07	0.35	1.30
Caffeine	0.9982	0.09	0.29	1.91	3.41
o-Toluic acid	0.9999	0.06	0.19	0.75	1.03
Propranolol	0.9973	0.03	0.10	2.30	1.38
Clofibric acid	0.9955	0.02	0.06	3.28	3.59
2-Naphthoic acid, (I.S.)	-	-	-	-	-
Ketoprofen	0.9931	0.08	0.26	4.17	7.03
Bezafibrate	0.9954	0.06	0.21	1.09	7.17
Naproxen	0.9999	0.01	0.04	0.35	1.52
Warfarin	0.9999	0.24	0.81	7.49	10.24
Flurbiprofen	0.9999	0.24	0.81	0.91	11.10
Diclofenac	0.9998	0.15	0.49	9.70	15.09
Indomethacin	0.9997	0.10	0.33	2.02	9.31
Ibuprofen	0.9999	0.13	0.42	5.94	7.85
Meclofenamic acid	0.9999	0.04	0.13	0.61	2.47
Mefenamic acid	0.9999	0.05	0.15	2.10	3.68
Clotrimazole	0.9998	0.11	0.37	1.81	7.05
Gemfibrozil	0.9999	0.25	0.84	1.58	2.18
Triclosan	0.9999	0.12	0.40	2.74	3.70
Ivermectin	0.9996	0.12	0.40	4.20	3.52

Linearity was determined at five individual concentration levels within the range of 0.1 to 20 mgL^{-1}. A linear response was observed for each analyte as demonstrated by the regression coefficients that were all greater than $R^2 = 0.99$. The limit of detection was defined as a signal three times the standard deviation of the baseline noise and the limit of quantitation was defined as ten times the standard deviation of the baseline noise. Using the ChemStation software, version A.09.03, the 'peak-to-peak' baseline noise was measured for a triplicate blank injection, (HPLC grade MeOH) over a window of twenty times the peak width either side of each peak. The peak heights of a combined 0.5 mgL^{-1} standard were measured and from these measurements the LOD and LOQ for each analyte were determined. The reproducibility of the method was determined by performing six replicate injections of the same standard at the LOQ level using the same mobile phase. Reproducibility is acceptably low for most analytes, generally lower than 5% relative standard deviation (RSD) [37]. Repeatability was determined by the injection of six individual standards at the LOQ level using six fresh preparations of mobile phase for the separation. The calculated repeatability values are also quite low, <10% RSD for all analytes with the exception of flurbiprofen and diclofenac.

Paci *et al.* [33] acknowledge that dual gradient separations suffer from lower precision due to the more complex dynamic processes occurring during the separation, however, the observed reproducibility and repeatability values were within acceptable limits in this study.

3.3.6 Solid phase extraction:

3.3.6.1 Sorbent selection.

The chosen group of pharmaceuticals displayed a range of chemical properties, ranging from very hydrophilic to hydrophobic, and acidic to basic. Due to these distinct differences the selection of an extraction sorbent that showed good recoveries for all species was not trivial. Sorbents examined included those based upon standard octadecyl silica, hyper cross-linked styrene divinyl benzene, hydrophilic lipophilic balanced polymers and mixed functionality reversed-phase anion exchangers. Five commercial SPE phases were evaluated using recovery performance data for a 5 μgL^{-1} test mix of all analytes listed in section 3.2.1. The resultant data is presented in Table 3.4.

Table 3.4: Sorbent selection performance data (calculated % recoveries).

Analyte	Phenomenex Strata-C18E™	Phenomenex Strata-X™	Merck LiChrolut EN™	Waters Oasis HLB™	Waters Oasis MAX™
Paracetamol	-	11	76	19	15
Salicylic acid	-	79	49	70	58
Trimethoprim	20	98	44	89	65
Caffeine	14	90	82	76	289
o-Toluic acid, (S1)	6	62	53	57	47
Propranolol	13	78	36	72	54
Clofibric acid	66	89	83	77	65
2-Naphthoic acid, (I.S.)	-	-	-	-	-
Ketoprofen	71	85	77	79	23
Bezafibrate	71	86	81	78	46
Naproxen	66	83	76	78	22
Warfarin	74	89	95	90	27
Flurbiprofen	83	91	85	90	45
Diclofenac	63	75	80	72	15
Indomethacin	-	61	-	56	238
Ibuprofen	69	88	69	86	38
Meclofenamic acid, (S2)	72	85	83	75	65
Mefenamic acid	45	78	74	76	63
Clotrimazole	-	73	41	59	30
Gemfibrozil	76	93	100	86	70
Triclosan	66	79	79	75	45

From initial studies the hydrophilic lipophilic balanced sorbents, i.e. Waters Oasis HLB™ and Phenomenex Strata-X™ showed the greatest promise as relatively high recovery was observed across the range with these sorbents. The C_{18} sorbent demonstrated poor ability to extract and retain the more polar analytes. Recovery was generally good using the Merck LiChrolut EN™ sorbent, however, indomethacin was completely unretained on the styrene divinylbenzene resin, although it was retained on all of the other polymeric sorbents investigated in Table 3.4. As the majority of the pharmaceuticals were acidic, a mixed functionality reversed-phase anion exchange sorbent, Oasis MAX™ was investigated. The extraction was performed at pH 6.0 so as to promote the dissociation of carboxyl functionalities. Recovery for acidic analytes was low, generally <50% and recovery of basic and neutral analytes was also lower than that calculated from the reversed-phase sorbents, suggesting ion exclusion based upon electrostatic repulsion.

As the Oasis HLB™ and the Phenomenex Strata-X™ were chemically similar and share common retention chemistry, a *t*-Test was performed using the calculated percentage recovery values for a 5 μgL^{-1} spike standard to determine whether the two sorbents were significantly different using Microsoft Excel™. It was discovered that at the 95% confidence interval that there was no significant difference between the two sorbents (*t*-Experimental = 0.31 < *t*-Critical = 2.03, two tailed assuming equal variances).

3.3.6.2 Extraction pH.

A subset of six analytes was chosen to optimise extraction pH. The selected analytes were salicylic acid, clofibric acid, ketoprofen, diclofenac, ibuprofen and mefenamic acid. These solutes were selected as they span across the chromatographic separation. Buffer solutions were prepared within the pH range of 2-8 and spiked with 5 μgL^{-1} of each. Samples were extracted using the Oasis HLB cartridges and eluted with 10 ml MeOH, reduced in volume, reconstituted and analysed using the HPLC method. It was observed that an extraction pH of 4.0 provided optimum analyte recovery for all compounds.

3.3.6.3 Elution solvent.

Within the literature many SPE procedures recommend elution with MeOH. However, MeOH is difficult to reduce in volume under N_2 without significant sample loss and so alternative solvents were investigated. These included; acetone, acetonitrile, ethyl acetate, isopropyl alcohol (IPA), 50% v/v ethyl acetate in acetone [38], and 10% v/v MeOH in methyl *t*-butyl ether (MtBE) [39]. Using the Oasis HLB™ sorbent, a 5 μgL^{-1} mixed standard was extracted and eluted using each of the above solvents and solvent mixtures. Recovery from cartridges eluted with ethyl acetate and acetonitrile were unacceptably low, <50% for the test solutes. Recovery was in the range of 70-90% with IPA and acetone. For the mixed solvents, recovery was ~50% or less for cartridges eluted with 10% v/v MeOH in MtBE, however, 50% v/v ethyl acetate in acetone provided near quantitative recovery for all analytes, >90%. Of all the solvents examined the 50% v/v ethyl acetate in acetone mixture provided the highest recoveries, (due to increased solvent strength) and it is also easily reduced in volume under N_2 with minimal

splashing and sample loss. The recovery of salicylic acid was low in all extraction experiments, however, it is the most polar of the compounds examined (pK_a ~3.0). At this stage in the study it was noted that during the SPE procedural development that there was significant batch-to-batch variability with the Oasis HLB™ sorbent, reflected in some cases by large reductions in solute recovery. Such batch variability was not apparent with the Phenomenex Strata-X™ phase and therefore these cartridges were preferred in further sample extractions.

3.3.6.4 Elution volume.

The optimum volume of elution was determined by eluting an extracted 5 μgL^{-1} standard with ten 2 mL portions of 50 % v/v ethyl acetate in acetone. Each 2 mL fraction was collected, reduced in volume and reconstituted in 100 µL of MeOH. The relative recovery of each analyte was determined and plotted against the elution volume, Fig. 3.6. It was observed that 10 mL of elution solvent was sufficient to completely elute all compounds from the sorbent bed. It was also observed that as expected the more polar the solute the less solvent was required for complete elution.

3.3.6.5 Surrogate Performance.

Two surrogate standards were also included in the method at a concentration level of 2 μgL^{-1} to assess the extraction procedure. The recovery of *o*-toluic acid is quite low and outside the acceptable limits, (i.e. 70-130%, [37]) at 56%, while the recovery of meclofenamic acid is more acceptable at 80%. *o*-Toluic acid is the more polar of the pair and although it is not in itself a pharmaceutical compound, it was chosen as it shares a common functionality with many of the more polar pharmaceuticals. Meclofenamic acid was previously used as a surrogate standard by Clara *et al.* [40].

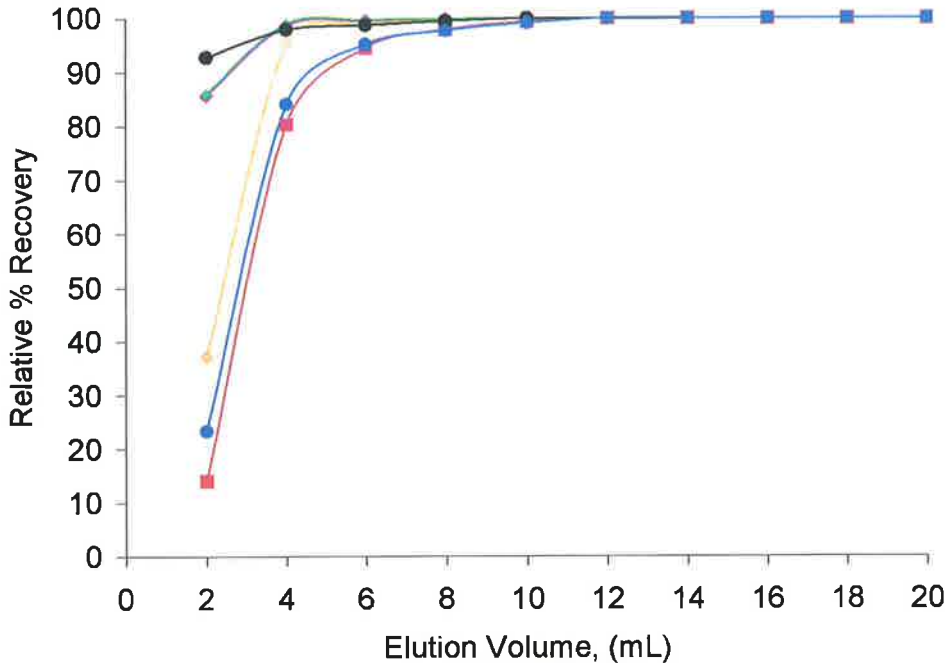


Figure 3.6: Plot of relative % recovery versus SPE elution volume, (mL) using Strata-X™ SPE cartridges and elution with 50 % v/v ethyl acetate in acetone. *Key:* black trace; ibuprofen, green trace; clofibric acid, pink trace; ketoprofen, orange trace; diclofenac, blue trace; mefenamic acid, red trace; salicylic acid.

3.3.6.6 Overall method performance data.

Using the optimised parameters six 1 μgL^{-1} spikes prepared in water collected from the River Boyne near Navan, Co. Meath, Ireland were extracted using the Phenomenex Strata-X™ cartridges and analysed using the dual gradient method. From the six replicate extractions the overall level of method precision was estimated (as the percentage RSD for the six replicate extractions). Unspiked aliquots of estuarine and river water were also extracted and analysed. The overall levels of sensitivity, (LOD and LOQ) were then calculated by determining the baseline noise in the unspiked extracts. Overall method performance data is presented in Tables 3.5 and 3.6.

Table 3.5: Extraction repeatability and overall method precision calculated using 1 μgL^{-1} spikes in river water, (*actual spiking level was 2 μgL^{-1}).

Analyte	% Recovery Repeatability, (n=6)	Method Precision, (% RSD, n=6)
Metformin	-	-
Paracetamol	-	-
Salicylic Acid	67 ± 19.8	29.38
Trimethoprim	-	-
Caffeine	-	-
o-Toluic Acid	45 ± 12.3	27.27
Propranolol	-	-
Clofibric Acid	77 ± 18.4	23.90
Ketoprofen	81 ± 15.6	19.32
Bezafibrate	67 ± 13.3	19.71
Naproxen	75 ± 13.7	18.21
Warfarin	97 ± 18.2	18.86
Flurbiprofen	77 ± 16.0	20.83
Diclofenac	73 ± 15.2	20.63
Indomethacin	72 ± 14.8	20.65
Ibuprofen	92 ± 17.2	18.56
Meclofenamic Acid	72 ± 13.0	17.95
Mefenamic Acid	75 ± 13.9	18.42
Clotrimazole	-	-
Gemfibrozil	72 ± 7.3	10.20
Triclosan	104 ± 24.4	23.57
Ivermectin	42 ± 5.3	12.65

Table 3.6: Overall method sensitivity data calculated in two different sample matrices (1 L extraction volume), values quoted are in μgL^{-1}.

Analyte	Estuarine Water		River Water	
	LOD	LOQ	LOD	LOQ
Metformin	0.35	1.17	-	-
Paracetamol	0.33	1.09	-	-
Salicylic Acid	0.66	2.21	0.18	0.62
Trimethoprim	0.03	0.11	-	-
Caffeine	0.27	0.91	-	-
o-Toluic Acid	0.13	0.44	0.17	0.58
Propranolol	0.04	0.13	-	-
Clofibric Acid	0.12	0.41	0.07	0.23
Ketoprofen	0.16	0.53	0.11	0.38
Bezafibrate	0.13	0.45	0.08	0.28
Naproxen	0.02	0.08	0.02	0.08
Warfarin	0.73	2.42	0.55	1.85
Flurbiprofen	0.41	1.36	0.32	1.06
Diclofenac	0.29	0.96	0.18	0.62
Indomethacin	0.09	0.31	0.15	0.49
Ibuprofen	0.19	0.64	0.33	1.12
Meclofenamic Acid	0.11	0.36	0.12	0.39
Mefenamic Acid	0.11	0.38	0.12	0.39
Clotrimazole	0.24	0.80	-	-
Gemfibrozil	0.46	1.52	0.37	1.24

Triclosan	0.23	0.78	0.14	0.46
Ivermectin	0.32	1.08	0.43	1.42

The determined recovery data from the Strata-X sorbent was generally in the acceptable range of 70-130% and for many analytes is near quantitative. As expected from the LC method development, the water solubility of the analytes limits its retention on the polymeric sorbent, i.e. the more hydrophilic the analyte the less retention. This fact is clearly demonstrated by metformin that shows little retention. The retention of basic analytes was generally lower than their acidic counterparts, which was due to the low pH at which the extraction was performed. Repeatability was generally in the range of 10-20% RSD for the six replicate extractions which is acceptable considering the complexity of the method and the number or steps involved before achieving a concentrate extract. The levels of precision achievable will depend upon the influence of the sample matrix, i.e. interference from component of the matrix present, possible co-elution *etc.* Analyte recovery was also noted to be higher from estuarine water samples compared to fresh water samples, this is attributed to the increased ionic strength of the estuarine sample and resulting 'salting out' effects. The homogeneity of the extraction material is also important in providing repeatable recovery values. Based upon the levels of repeatability calculated it can be deduced that the Strata-X sorbent is quite homogenous with acceptable inter batch repeatability and the sample matrix is not excessively preconcentrated under the method conditions allowing for clear identification of the target analytes.

The calculated LOD and LOQ values in a real sample matrix are on average in the low to sub μgL^{-1} range therefore suggesting that the developed method is suitably sensitive for its intended purpose. It can be observed that components of the sample matrix that are extracted along with the target analytes reduce the sensitivity of the method, this is particularly visible for trimethoprim and propranolol amongst others which exhibit strong responses under standard conditions but were completely obscured by co-extracted matrix components. It is also interesting to note that the calculated detection and quantitation limits are lower in estuarine water than in river water. This may be due to lower matrix content or the influence of increased ionic strength on the extraction compared to river water. The

calculated sensitivity data also compares well with other methods developed for the determination of pharmaceutical residues from complex environmental samples. For example Blackwell *et al.* reported detection limits in the range of 0.25–0.35 μgL^{-1} for the determination of three antibiotics in surface water samples, however, a much more complicated extraction procedure was employed and detection was performed at longer wavelengths that would not be expected to suffer as much from matrix interference [41]. Similarly Gonzalez-Barreiro *et al.* employed photochemically induced fluorescence for the determination of ten pharmaceuticals in wastewater and detection limits were in the region of 2-120 μgL^{-1} [42]. It can therefore, be seen that the developed method compares favourable with other reported LC procedures with optical detection.

3.3.7 Application to environmental samples:

Grab samples were collected in silanised 2 L amber glass bottles from areas in north Co. Dublin and Co. Meath, Ireland namely the Malahide Estuary (which receives treated effluent from sewage treatment plants in north Co. Dublin), in the vicinity of sewage sea outfalls near Howth and Portmarnock, and from the River Boyne downstream of the Navan Wastewater Treatment Centre in Co. Meath (>40,000 population equivalents). A sample of potable water from the laboratory was also analysed. Samples were filtered and extracted as previously described in section 3.2.4 and analysed using the dual gradient method, Table 3.2. A 1 L portion of each sample was also spiked with 1 μgL^{-1} of each pharmaceutical and extracted and analysed using the dual gradient method. None of the chosen analytes were detected in any of the samples collected. However, the spiked samples showed very clear peaks for all of the pharmaceuticals added at the expected retention times. Fig. 3.7 depicts the resulting chromatogram of extracted spiked Malahide Estuary sample.

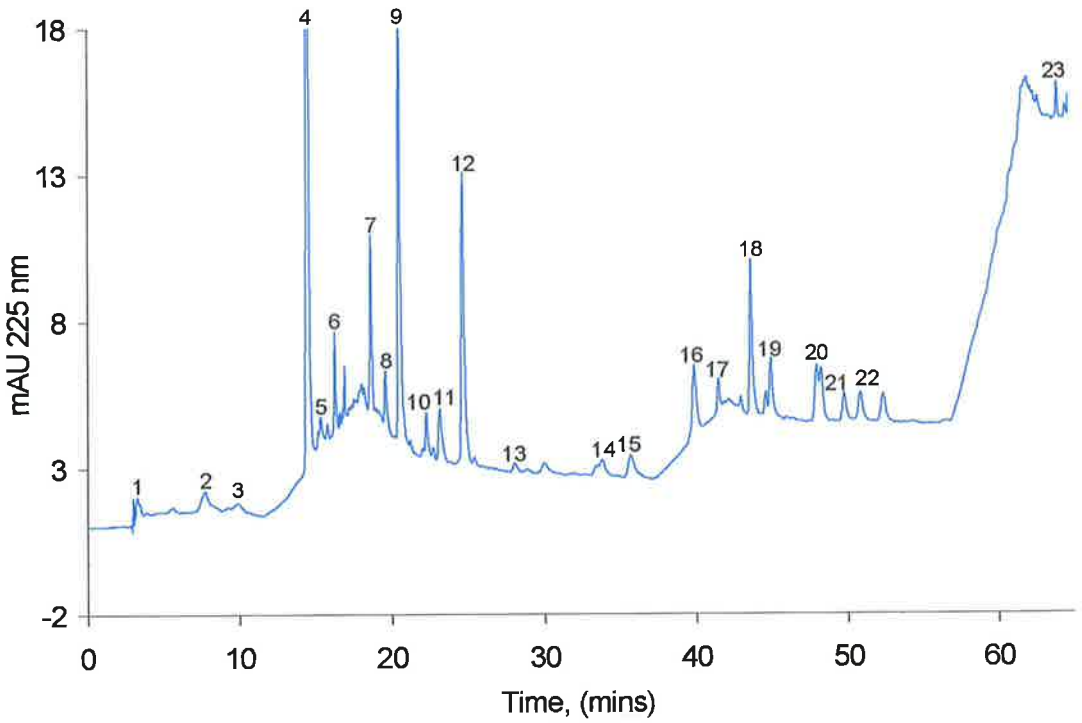


Figure 3.7: Chromatogram of the extracted 1 μgL^{-1} spiked Malahide Estuary sample. Separation conditions as in Figure 3.4. Peak identification: **1** metformin, **2** paracetamol, **3** salicylic acid, **4** trimethoprim, **5** caffeine, **6** *o*-toluic acid, **7** propranolol, **8** clofibric acid, **9** 2-naphthoic acid, (internal standard, **10** ketoprofen, **11** bezafibrate, **12** naproxen, **13** warfarin, **14** flurbiprofen, **15** diclofenac, **16** indomethacin*, **17** ibuprofen, **18** meclofenamic acid, **19** mefenamic acid, **20** clotrimazole*, **21** gemfibrozil*, **22** Triclosan, **23** ivermectin*, (*Actual spiking level: 2 ppb).

Samples and spikes were also analysed using a photodiode array detector and a portion of an extracted contour plot is displayed in Fig. 3.8. It can be seen that distinguishable UV spectra can be obtained even from ultra trace levels on complex matrices when suitable preconcentration is employed. Preliminary qualitative data can be achieved by UV library searching if available and therefore the method may be of use in environmental laboratories where LC-MS instrumentation is not available.

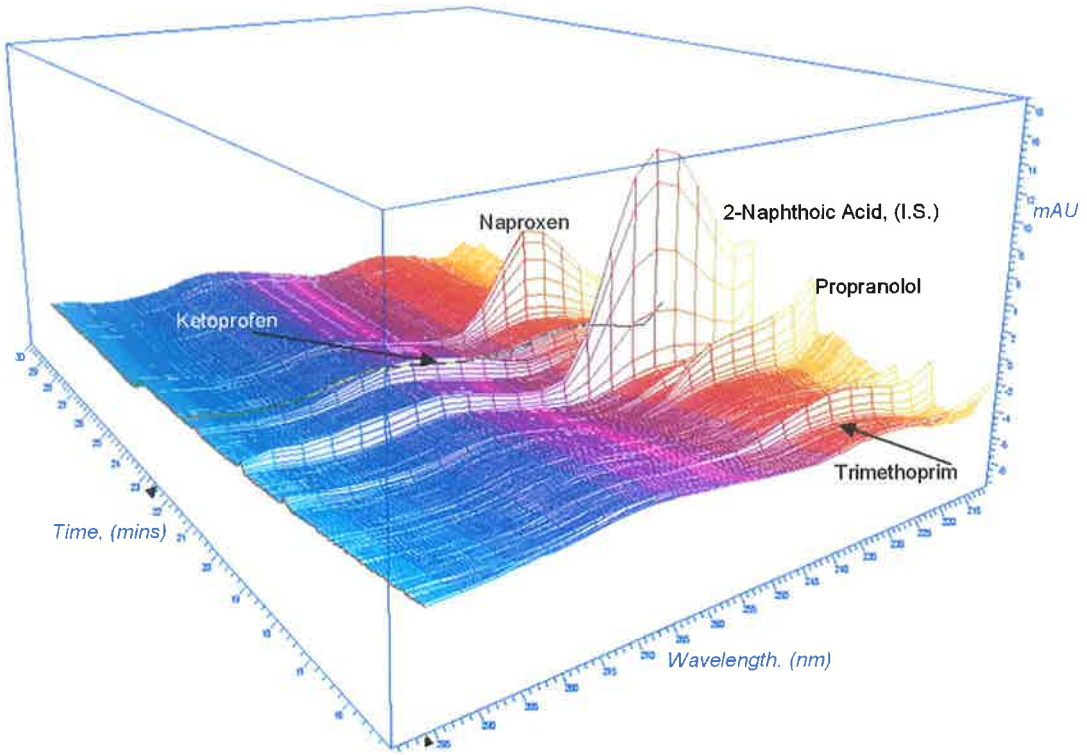


Figure 3.8: Partial contour plot of spiked Malahide estuary sample, distinguishable UV spectra can be obtained even for ultra trace residues with sample preconcentration using SPE.

3.4 Conclusions:

A simple method has been developed that allows for the preconcentration and determination of acidic and basic pharmaceuticals. Using monolithic columns a highly efficient separation is achievable with calculated theoretical plate counts far superior to those attainable using particulate columns. Acceptable SPE recoveries for the majority of analytes permit the lowering of instrumental detection limits by one thousand fold thereby allowing low μgL^{-1} levels to be determined using LC-UV. When combined with photodiode array detection preliminary qualitative information is achievable especially if a spectral library search function is available. The method has been applied to a variety of environmental waters. The method may become useful to those who wish to determine the selected pharmaceuticals in environmental samples but who do not have access to LC-MS instrumentation.

References:

1. M.J. Martin, F. Pablos & A.G. Gonzalez, *Talanta*, **49**, (1999), 453.

2. E. Mikami, T. Goto, T. Ohnom H. Matsumato & M. Nishida, *Journal of Pharmaceutical and Biomedical Analysis,* **23**, (2000), 917.

3. Y. Sun, K. Takaba, H. Kido, M.N. Nakashima & K. Nakashima, *Journal of Pharmaceutical and Biomedical Analysis*, **30**, (2003), 1611.

4. J. Dey, A. Mohanty, S. Roy & D. Khatua, *Journal of Chromatography A*, **1048**, (2004), 127.

5. C. Arcelloni, R. Lanzi, S. Pedercini, G. Molteni, I. Fermo, A. Pontiroli & R. Paroni, *Journal of Chromatography B*, **763**, (2001), 195.

6. T. Hirai, S. Matsumoto & I. Kishi, *Journal of Chromatography B*, **692**, (1997), 375.

7. C. Akay & S.A. Ozkan, *Journal of Pharmaceutical and Biomedical Analysis,* **30**, (2002), 1207.

8. M. Dubois, D. Fluchard, E. Sior & Ph. Delahunt, *Journal of Chromatography B*, **753**, (2001), 189.

9. E.M. Abdel-Moety, F.I. Khattab, K.M. Kelani & A.M. AbouAl-Alamein, *Il Farmaco*, **57**, (2002), 931.

10. E. Gonzalez-Penas, S. Agarraberes, A. Lopez-Ocariz, E. Gacia-Quetglas, M.A. Campanero, J.J. Carballal & J. Honorato, *Journal of Pharmaceutical and Biomedical Analysis,* **26**, (2001), 7.

11. E.J. Park, D. Lee, Y.G. Shin, D.D. Lantvit, R.B. van Breemen, A.D. Kinghorn & J.M. Pezzuto, *Journal of Chromatography B*, **754**, (2001), 327.

12. E. Mikami, T. Goto, T. Ohno, H. Matsumoto & M. Nishida, *Journal of Pharmaceutical and Biomedical Analysis*, **28**, (2002), 261.

13. D.J. Schakel, D. Kalsbeek & K. Boer, *Journal of Chromatography A*, **1049**, (2004), 127.

14. A. Piccoli, J. Fiori, V. Andrisano & M. Orioli, *Il Farmaco*, **57**, (2002), 369.

15. Y. Hsieh, J.M. Brisson, G. Wang, K. Ng & W.A. Korfmacher, *Journal of Pharmaceutical and Biomedical Analysis*, **33**, (2003), 251.

16. K. Pihlainen, E. Sippola & R. Kostiainen, *Journal of Chromatography A*, **994**, (2003), 93.

17. R.C. Schneider & K.A. Hovar, *Chromatographia*, **57**, (2003), 287.

18. S.C. Tan, S.H.D. Jackson, C.G. Swift & A.J. Hutt, *Journal of Chromatography B*, **701**, (1997), 53.

19. G.A. Jacobson, F.V. Chong & N.W. Davies, *Journal of Pharmaceutical and Biomedical Analysis,* **31**, (2003), 1237.

20. X.W. Teng, S.W.J. Wang & N.M. Davies, *Journal of Pharmaceutical and Biomedical Analysis,* **33**, (2003), 95.

21. M.J. Hilton & K.V. Thomas, *Journal of Chromatography A*, **1015**, (2003), 129.

22. S. Yang, J. Cha & K. Carlson, *Rapid Communications in Mass Spectrometry*, **18**, (2004), 2131.

23. A. Aguera, A.R. Fernandez-Alba, L. Pedra, M. Mezcua & M.J. Gomez, *Analytica Chimica Acta*, **480**, (2003), 193.

24. D. Loffler & T.A. Ternes, *Journal of Chromatography A*, **1021**, (2003), 133.

25. A.M. van Nederkassel, A. Aerts, A. Dierick, D.L. Massart & Y. Vander Heyden, *Journal of Pharmaceutical and Biomedical Analysis,* **32**, (2003), 233.

26. J.H. Smith & H.M. M^{c}Nair, *Journal of Chromatographic Science*, **41**, (2003), 209.

27. A. Bugey & C. Staub, *Journal of Pharmaceutical and Biomedical Analysis*, **35**, (2004), 555.

28. V.V. Tolstihov, A. Lommen, K. Nakanishi, N. Tanaka & O. Fiehn, *Analytical Chemistry*, **75**, (2003), 6737.

29. N. Tanaka, H. Kobayashi, N. Ishizuka, H. Minakuchi, K. Nakanishim K. Hosoya & T. Ikegami, *Journal of Chromatography A*, **965**, (2002), 35.

30. C. Schafer, K. Cabrera, D. Lubda, K. Sinz & D. Cunningham, *American Laboratory*, April 2001, 25.

31. General Medical Services (Payments) Board report for year ending 31st December 2003, available through www.gmspb.ie

32. K.L. Ng, B. Paull, P.R. Haddad & K. Tanaka, *Journal of Chromatography A*, **850**, (1999), 17.

33. A. Paci, A.M. Caire-Maurisier, A. Rieutord, F. Brion & P. Clair, *Journal of Pharmaceutical and Biomedical Analysis*, **27**, (2002), 1.

34. B. Paull, C. O'Riordain & P. Nesterenko, *Chemical Communications*, **2**, (2005), 215.

35. Merck product literature, "General Chromolith Information and Applications" received with columns.

36. R.G. Wolcott, J.W. Dolan, L.R. Snder, E.R. Bakalyar, M.A. Arnold & J.A. Nichols, *Journal of Chromatography A*, **869**, (2000), 211.

37. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Tripartite Guideline, '*Validation of Analytical Procedures: Methodology*', available on-line at http://www.nihs.go.jp/dig/ich/quality/q2b/q2bstep4.pdf, multiple consultations.

38. S. Ollers, H.P. Singer, P. Fassler & S.R. Muller, *Journal of Chromatography A*, **911**, (2001), 225.

39. B.J. Vanderford, R.A. Pearson, D.J. Rexing & S.A. Snyder, *Analytical Chemistry*, **75**, (2003), 6265.

40. M. Clara, *Water Research*, **38**, (2004), 947.

41. P.A. Blackwell, H.C. Holtan Lutzhoft, H.P. Ma, B. Halling-Sorenson, A.B.A. Boxall & P. Kay, *Journal of Chromatography A*, **1045**, (2004), 111.

42. C Gonzales-Barreiro, M. Lores, M.C. Casais & R. Cela, *Journal of Chromatography A*, **993**, (2003), 29.

4.0 On-line Preconcentration of Pharmaceutical Residues from Large Volume Water Samples using Short Reversed-Phase Monolithic Cartridges Coupled to LC-UV-ESI-MS.

4.1 Introduction:

Within the last decade pharmaceuticals and personal care products have emerged as a new class of organic micro contaminants that are frequently detected in the aquatic environment [1,2]. As a result, the number of methods appearing in the peer-reviewed literature for their determination has increased. Due to the ultra trace levels of pharmaceuticals that may be present, normally sub parts per billion [3] a sample enrichment procedure is necessary in order to attain the desired levels of analytical sensitivity. The foremost approach reported to date has been the use of solid phase extraction (SPE) techniques predominantly using disposable cartridges in the off-line mode [4,5,6]. A drawback of off-line SPE procedures is that they can be quite cumbersome to perform, often requiring many steps before reaching a concentrated extract suitable for instrumental analysis, of which only a small proportion is actually injected onto the chromatographic column. In contrast on-line SPE techniques offer increased sensitivity with reduced sampling handling as all the extracted components can easily be introduced to the analytical column. Reported detection limits in the pgL^{-1} range are not uncommon [7,8]. One of the driving forces for on-line SPE methods is higher throughput and faster analysis as required for pharmacokinetic and metabolomic studies [9]. The formats of on-line SPE reported in the literature vary from column switching techniques [10,11,12,13,14,15,16,17,18] to automated SPE instruments coupled to liquid chromatographs [8,19,20,21]. With automated systems only a small portion of the extract is actually injected onto the analytical column compared with complete introduction by mobile phase back flush when column switching is employed. Therefore it would be more appropriate to refer to such procedures as automated

SPE rather than true on-line SPE. Although on-line SPE is an attractive prospect it is not without limitations. Extraction columns are generally quite small and therefore can only contain a limited quantity of sorbent leading to problems with analyte retention [10]. Sorbents are generally particulate in nature and small particles with a large active surface area may be required. However, the speed of the extraction may then be limited due to the excessive backpressure generated. Sorbents must also be chosen with high analyte affinity thereby allowing higher volumes of sample required for increased sensitivity to be extracted without the possibility of analyte breakthrough. Application of on-line SPE and automated SPE include the preconcentration of pharmaceuticals [10,18], estrogens and endocrine disruptors [8,20] and nonylphenol [14] with subsequent LC and photodiode array or MS detection.

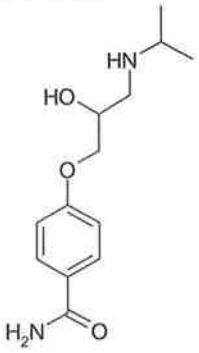
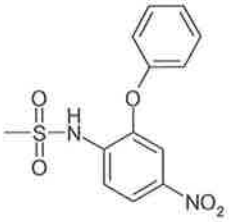
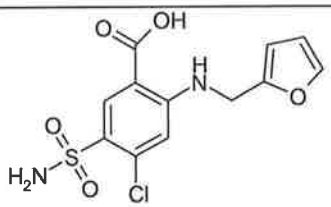
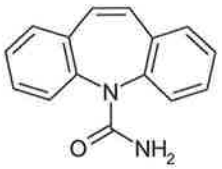
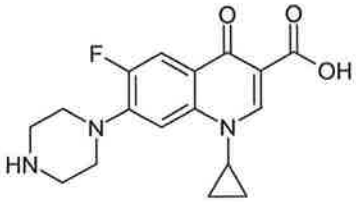
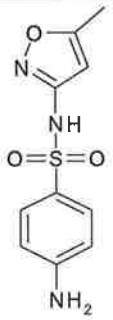
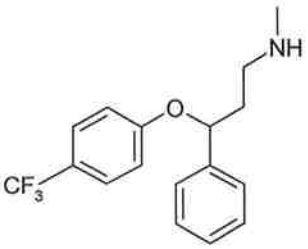
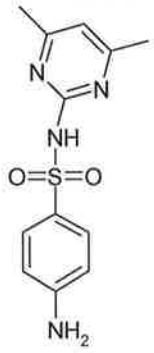
Monolithic silica consists of a unique bimodal pore structure, (macropores of ~2 μm and mesopores of ~13 nm, section 2.3.5) which imparts high porosity and a higher available active surface area to the column [22]. Such high porosity also allows monolithic columns to be used at elevated flow rates without the generation of excessive backpressure. The approach taken with this research has focused upon the use of short reversed-phase monolithic silica columns for the on-line SPE of acidic, basic and neutral pharmaceutical residues from aquatic samples. The monolithic column allows for the rapid extraction of a large sample volume at relatively low pressure. A longer reversed-phase monolithic column was employed for the analytical separation using the method developed in Chapter 3.0. Coupling the on-line SPE-LC procedure with ion trap MS increased the selectivity of the method.

4.2 Experimental:

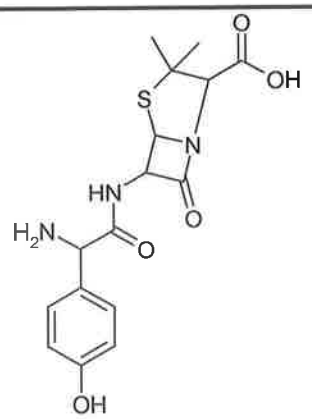
4.2.1 Analyte selection:

Analytes chosen for monitoring include those that showed prevalence in both the published literature and also the list of the most prescribed pharmaceuticals in Ireland for the year ending December 31st 2003 [23]. In addition to the analytes mentioned in Chapter 3.0 Table 3.1, the pharmaceuticals depicted in Table 4.1 were also included.

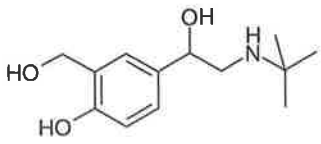
Table 4.1: The chemical structures of the additionally included pharmaceuticals.

Analyte	Chemical Structure	Analyte	Chemical Structure
Atenolol		Nimesulide	
Furosemide		Carbamazepine	
Ciprofloxacin		Sulfamethoxazole	
Fluoxetine		Sulfamethazine	

Amoxicillin



Salbutamol



4.2.2 Chemicals and reagents:

Chemicals and reagents used in this study were as per section 3.2.2 unless otherwise stated. Ciprofloxacin was received from Fluka, (Buchs, Switzerland). Atenolol, salbutamol, amoxicillin, sulfamethoxazole, sulfamethazine sodium salt, furosemide, carbamazepine, nimesulide and fluoxetine were all obtained from Sigma, (Steinheim, Germany). All pharmaceuticals were of a purity >95%.

Stock 1000 mgL^{-1} solutions of each pharmaceutical were prepared in MeOH with the exception of ciprofloxacin which was prepared in water. In the case of salts, standards were prepared in terms of the parent analyte. These standards were periodically replaced. Working standards were prepared from these stock standards by appropriate dilution using MeOH.

Buffer solutions used for the optimisation of the extraction pH were prepared by mixing appropriate amounts of acid and its conjugate base for formate and acetate, respectively. In the case of MES and HEPES weighed proportions of salt were dissolved in ~900 mL Milli-Q water and titrated to the desired pH using either 1 M HCl or 1 M NaOH.

4.2.3 Glassware preparation:

Silanisation was performed as described in section 3.2.3.

4.2.4 On-line SPE:

On-line SPE was performed using a Rheodyne 7000 six-port column-switching valve (Cotati, CA, USA). The extraction column was a Merck Chromolith RP18e

guard cartridge; 10.0 x 4.6 mm i.d. monolithic silica (Merck, Darmstadt, Germany). The experimental set-up is depicted in Fig. 4.1 following.

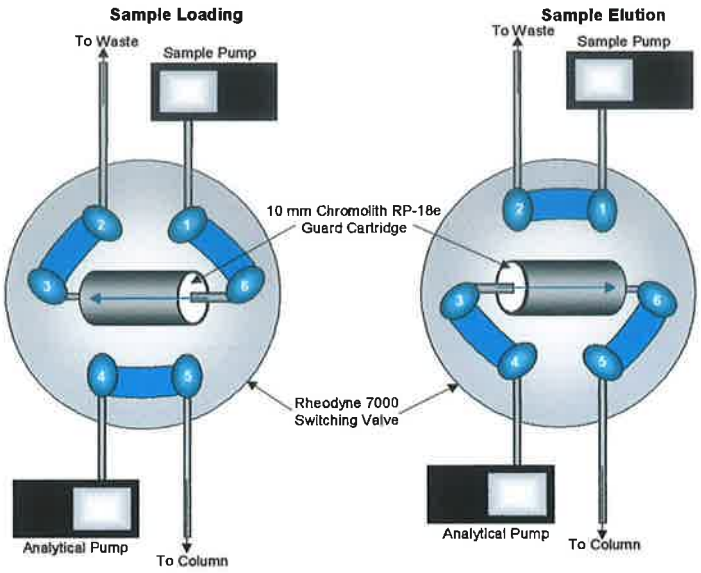


Figure 4.1: On-line SPE experimental set-up.

A Merck Hitachi LaChrom L-7100 pump was used for sample delivery. Prior to extraction the short monolithic column was conditioned with 20 mL of MeCN and 20 mL of water at flow rates of 5 $mLmin^{-1}$. Samples were filtered through Whatman GF/C glass fibre filters to remove suspended matter and a 500 mL aliquot was transferred to a silanised glass bottle and adjusted to pH 4.0 using concentrated sulphuric acid. Samples were pumped through the extraction column at a rate of 10 $mLmin^{-1}$. Following complete extraction the column was washed with 20 mL of 10% v/v acetonitrile (MeCN) in water at a rate of 10 $mLmin^{-1}$. Elution was performed by back flushing with mobile phase for 25 minutes of the chromatographic dual gradient run. After such time the valve was switched back to the load position for the next extraction.

4.2.5 LC-UV & LC-MS Analysis:

HPLC instrumentation unless otherwise stated was as per section 3.2.5. LC-MS analysis were performed using an Agilent 1100 series HPLC consisting of a binary pump with on-line vacuum degasser and a photodiode array detector, model G1315B. The LC system was coupled to a Bruker Daltonics esquireLC ion trap MS

complete with an atmospheric pressure ESI interface. Agilent ChemStation version A.06.01, (Agilent Technologies, Palo Alto, CA, USA) and Bruker Daltonics esquire control version 6.08, (Bruker Daltonics, Coventry, UK), were used for LC-MS system control, Bruker Daltonics DataAnalysis version 3.0 was used for data processing. For ESI optimisation solutions of analyte were directly infused into the MS using a Cole Parmer 74900 series syringe pump at a rate of 300 μLh^{-1} (Cole Parmer, Vernon Hills, IL, USA).

Analytical separations were performed using two Merck Chromolith Performance RP18e; 100.0 x 4.6 mm i.d. monolithic silica columns joined in series using a Chromolith volumeless column coupler (Merck KgaA, Darmstadt, Germany). Details of the dual gradient separation parameters were previously reported, (Table 3.1). Prior to entering the ESI interface the flow was split ~1/20 using an UpChurch Scientific graduated micro splitter valve, model P-470, (UpChurch Scientific, Oak Harbor, WA, USA).

4.3 Results & Discussion:

4.3.1 Liquid chromatography:

The chosen set of pharmaceutical analytes is quite chemically diverse spanning across acidic, neutral and basic chemicals. The pK_a values and both experimentally determined and theoretical octanol water partition coefficients (Log *P* and XLog *P*), data of the selected pharmaceuticals can be seen in Table 4.2 following.

During previous LC method development it was observed that the pH of the mobile phase exhibited the most pronounced effect on the selectivity of the separation. It was found that a pH of 4.5 provided sufficient separation of all chosen analytes. Additional pharmaceuticals have been included because a complete separation of all analytes is not completely necessary when accompanied by MS detection. Fig. 4.2 depicts the separation of a 5 mgL^{-1} mix standard of the chosen pharmaceuticals with UV detection at 225 nm, for the purposes of clarity the set of chosen analytes was subdivided and each resulting chromatogram is offset to easily permit the resulting peaks to be identified.

Table 4.2: pK_a and Log *P* data for the selected pharmaceutical analytes.

Analyte	pK_a [24]	Log *P* and theoretical (XLog *P*) [25].
Metformin	12.40 [26]	-, (0.56)
Atenolol	9.16 [27]	0.16, (0.46)
Salbutamol	5.90 [28]	-, (1.44)
Paracetamol	9.50 [29]	0.46, (0.45)
Salicylic Acid	3.50	2.36, (2.43)
Amoxicillin	2.40	0.87, (0.03)
Trimethoprim	6.60	0.91, (0.65)
Sulfamethazine	2.65	0.89, (0.46)
Caffeine	14.00 [30]	-0.07, (-0.50)
Sulfamethoxazole	5.70	0.89, (0.68)
Ciprofloxacin	6.38	-, (1.94)
Furosemide	3.90 [27]	2.03, (1.41)
Propranolol	9.49	3.48, (3.03)
Clofibric Acid	-	2.58, (2.58)
Carbamazepine	13.90	2.45, (2.30)
Ketoprofen	4.45	3.12, (3.22)
Bezafibrate	3.60	-, (3.85)
Nimesulide	6.50 [31]	-, (2.51)
Naproxen	4.20	3.18, (2.84)
Warfarin	5.05 [32]	2.60, (2.96)
Fluoxetine	8.70 [33]	-, (3.82)
Flurbiprofen	4.33 [27]	4.16, (3.76)
Diclofenac	4.15	-, (3.91)
Indomethacin	4.50 [26]	4.27, (4.18)
Ibuprofen	4.51	3.97, (3.64)
Meclofenamic Acid	-	-, (4.32)
Mefenamic Acid	4.20 [34]	5.12, (4.16)
Clotrimazole	6.12	-, (6.14)
Gemfibrozil	-	-, (3.56)
Triclosan	8.10 [35]	4.76, (4.96)
Ivermectin	-	-, (3.41)

From Fig. 4.2 it can be observed that the use of simultaneous organic solvent and flow gradients allows for the efficient separation of a broad spectrum of analytes within a reasonable amount of time (65 minutes per injection). The previously optimised mobile phase conditions are also expected to be readily adaptable to ESI as Ahrer *et al.* reported that concentrations of mobile phase additives greater than 2 mM can often lead to a decrease in mass spectral intensity [36]. The peak shape of the early eluting peaks is attributed to band distortion as a result of solvent effects, as the standard was prepared in a stronger reversed-phase solvent, i.e. MeOH, than the conditions encountered by the analytes under initial mobile phase conditions. The polarity of pharmaceutical compounds is clearly observable from Fig. 4.2 with the majority of analytes being eluted from the

reversed-phase column within the first twenty minutes of the chromatographic run, suggesting perhaps that other stationary phase chemistries such as C_8, phenyl or even ion exchange sorbents may warrant investigation to improve the retention and increase selectivity for such early eluting analytes.

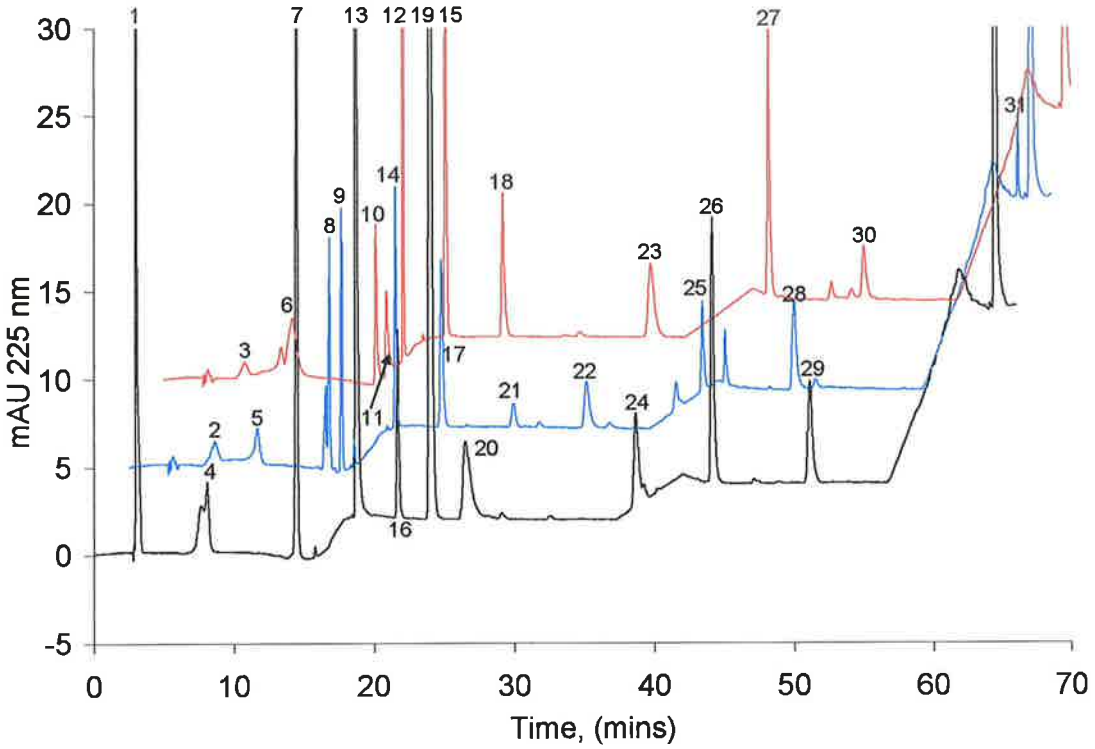


Figure 4.2: Standard separation of chosen pharmaceuticals. *Peak identification:* **1**; Metformin, **2**; Atenolol, **3**; Salbutamol, **4**; Acetaminophen, **5**; Amoxicillin, **6**; Salicylic acid, **7**; Trimethoprim, **8**; Sulfamethazine, **9**; Sulfamethoxazole, **10**; Caffeine, **11**; Ciprofloxacin, **12**; Furosemide, **13**; Propranolol, **14**; Clofibric acid, **15**; Carbamazepine, **16**; Ketoprofen, **17**; Bezafibrate, **18**; Nimesulide, **19**; Naproxen, **20**; Fluoxetine, **21**; Warfarin, **22**; Flurbiprofen, **23**; Diclofenac, **24**; Indomethacin, **25**; Ibuprofen, **26**; Meclofenamic acid, **27**; Mefenamic acid, **28**; Clotrimazole, **29**; Gemfibrozil, **30**; Triclosan, **31**; Ivermectin. The blue trace is offset by 2.5 minutes in time and 5 mAU, the red trace is offset by 5 minutes in time and 10 mAU for the purposes of clarity, separation conditions as per Table 3.1.

4.3.2 On-line SPE – optimisation of the sample loading flow:

The unique bimodal pore structure of monolithic silica allows for the use of elevated flow rates without the generation of excessive backpressure normally encountered when using particulate-based media under the same conditions. In this study, the use of a short monolith as an on-line concentrator column with LC was investigated, configured as shown in Fig. 4.1. On-line SPE using short monolithic trap columns offers the following potential advantages over using an off-

line SPE approach. Firstly, despite the surface area of the short monolith being ~350 m^2g^{-1} compared to higher surface areas for commercial packed SPE cartridges (Oasis HLB = 700 m^2g^{-1}, LiChrolut EN = 1000 m^2g^{-1}), it was envisaged the relatively small dead volume within the monolithic phase and the comparatively small flow through channels would result in equal or improved extraction efficiencies for analytes exhibiting some reasonable degree of hydrophobicity. Secondly, the system would permit rapid sample loading, equivalent to the loading rates used with off-line SPE using commercial cartridges, but also permit automated washing and elution steps. Thirdly, the elution of the retained analytes would be possible using the LC mobile phase and the direct back flushing of the analytes and sharp sample bands onto the analytical column possible, without any analyte loss. Finally, the system allowed for simultaneous loading of the next sample during the chromatographic separation of the previous sample. Such a feature allows for the reduction of analysis times significantly, in this instance the desire was to use short monolithic columns for the rapid extraction of reasonably large volumes of sample. In order to determine the maximum possible flow rate, reagent water was pumped through the extraction column at various flow rates and the resultant backpressure was measured. Fig. 4.3 depicts the average of ten replicate backpressure measurements at each of the different flow rates.

From the Fig. 4.3 it can be seen that the backpressure generated increases linearly as the flow rate increases. The maximum recommended operating pressure according to the product literature is not more than 200 bar [37] and therefore, from Fig. 4.3 it is estimated that the maximum possible flow rate is ~19 $mLmin^{-1}$ via linear extrapolation. However, the maximum flow rate possible with the Merck Hitachi pump was 10 $mLmin^{-1}$ thereby limiting the minimum possible extraction time. The inserted error bars indicate acceptable flow reproducibility that suggests that the flow of sample through the column should be homogenous.

The effect of flow rate on analyte recovery was measured by extracting 500 mL portions of 200 ngL^{-1} mixed analyte standards prepared in Milli-Q water and adjusted to pH 4.0 with sulphuric acid. Flow rates investigated were as in Fig. 4.3 above and all extractions were performed in duplicate. Table 4.3 following lists the calculated recovery values at each of the investigated sample loading flow rates.

Recovery was calculated based upon peak area comparison with the direct injection of a 5 mgL^{-1} standard.

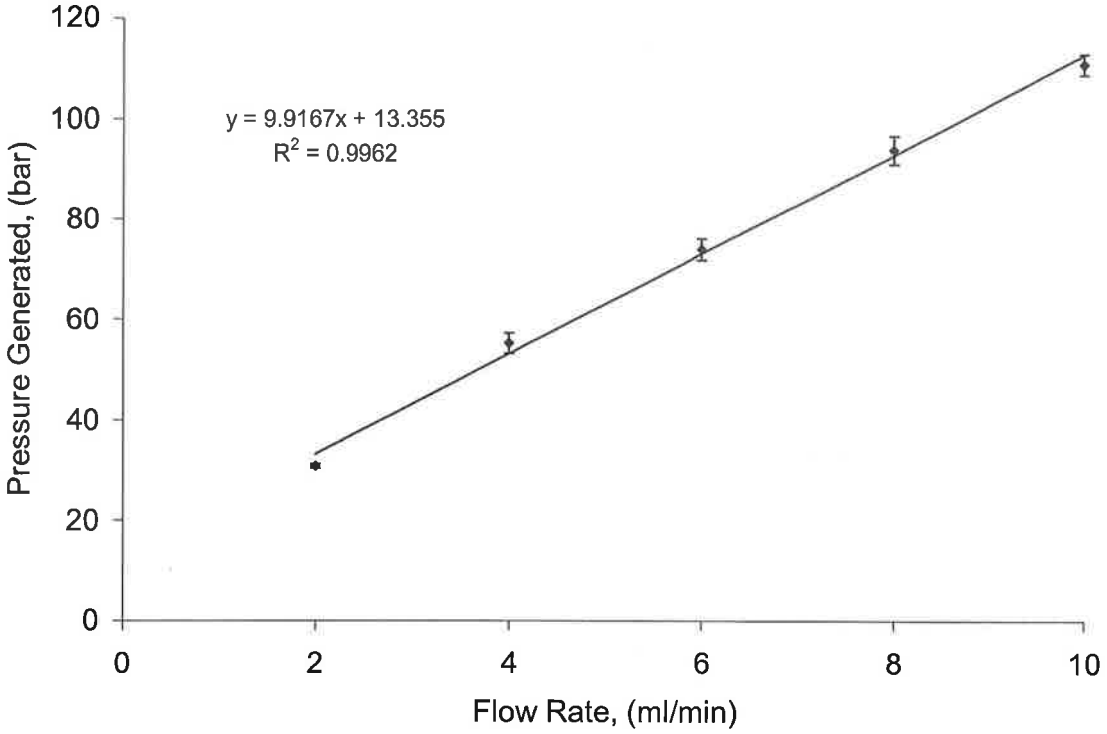


Figure 4.3: Extraction column backpressure generated at various flow rates of Milli-Q water.

Table 4.3: The effect of sample loading flow rate upon analyte recovery, sample size 500 mL.

Analyte	% Recovery at different sample loading flow rates, ($mLmin^{-1}$)				
	2.00	4.00	6.00	8.00	10.00
Metformin	0	0	0	0	0
Atenolol	0	0	0	0	0
Salbutamol	0	0	0	0	0
Paracetamol	0	0	0	0	0
Salicylic Acid	0	0	0	0	0
Amoxicillin	0	0	0	0	0
Trimethoprim	4	3	4	5	3
Sulfamethazine	8	4	3	5	3
Caffeine	0	9	5	5	4
Sulfamethoxazole	8	5	4	8	7
Ciprofloxacin	0	38	12	16	15
Furosemide	20	19	29	16	20
Propranolol	24	34	49	50	48
Clofibric Acid	6	57	53	30	10
Carbamazepine	105	33	75	75	74
Ketoprofen	>130	98	108	102	105
Bezafibrate	90	130	101	124	80
Nimesulide	>130	90	116	119	77
Naproxen	99	93	109	105	109
Warfarin	>130	>130	123	>130	94

Fluoxetine	69	92	97	101	92
Flurbiprofen	64	126	95	>130	82
Diclofenac	30	28	85	90	65
Indomethacin	103	85	100	96	112
Ibuprofen	106	118	83	99	130
Meclofenamic Acid	57	103	75	83	63
Mefenamic Acid	80	84	87	84	92
Clotrimazole	43	98	28	14	20
Gemfibrozil	75	109	87	72	69
Triclosan	54	55	61	60	66
Ivermectin	-	-	-	-	-

From Table 4.2 it can be observed that there is little recovery of the more polar analytes from the extraction column suggesting that there is insufficient interaction between these polar compounds and the reversed-phase sorbent. This also suggests that the pH at which the extraction is performed will be of critical importance in attempting to promote retention of such analytes. However, as was observed in Fig. 4.2, these analytes also elute extremely early from the analytical column, within starting mobile phase conditions of maximum aqueous modifier. Therefore, as previously suggested, the use of more polar or ionic stationary phases may be needed to sufficiently retain and extract these compounds. This is an unfortunate observation as it is expected that these analytes that clearly demonstrate high aqueous affinity are those that are expected to show prevalence in discharged wastewater effluent and the receiving aquatic system.

In a number of instances the percentage analyte recovery was greater than 130%, particularly in the case of warfarin. Such excessive recovery is attributed to co-adsorbed trace organic contaminants present in the reagent water. Based upon a mass balance approach there is a 25000-fold magnification of both analytes and matrix and therefore, impurities that are normally at such minute levels suddenly become significant interferants and leading to problems with peak integration. Such effects were particularly prevalent at lower flow rates. It was also observed that due to the increased path through the on-line SPE system retention times slightly increased when compared to those recorded by direct standard injection. In most instances such an effect caused no problems except in the case of ivermectin that elutes just before a system peak, (later identified using ESI-MS/MS to be diethyl hexyl phthalate, m/z 391.1, a plasticizer contaminant present in the MeOH used)

during standard injection and was observed to coelute with the system peak in the chromatograms of on-line SPE extracts.

Analyte recovery, with the exception of the aforementioned polar analytes was quite high, on average >70% and near quantitative in many cases. For the majority of analytes recovery was unaffected by increasing the rate of extraction, thereby allowing for reduced extraction times. Practicality must also be taken into consideration and therefore, it was decided to use a flow rate of 10 $mLmin^{-1}$ for sample extraction for all further development.

4.3.3 On-line SPE – optimisation of the sample pH:

In an attempt to increase the retention of the more polar pharmaceuticals the extraction pH was optimised. As the extraction column is a silica-based material and according to the product literature is only stable within the range of pH 2 - 7.5 [37], pH values in the range of 3 – 7 were investigated using appropriate buffer solutions prepared in Milli-Q water. Table 4.4 lists analyte recovery at each of the experimental pH values.

Table 4.4: Analyte recovery as a function of sample pH, sample size 500 mL.

Analyte	% Analyte recovery				
	pH 3 (formate)	pH 4 (formate)	pH 5 (acetate)	pH 6 (MES)	pH 7 (HEPES)
Metformin	0	0	0	0	0
Atenolol	0	0	0	0	0
Salbutamol	0	0	0	0	0
Paracetamol	0	0	0	0	0
Salicylic Acid	0	0	0	0	0
Amoxicillin	0	0	0	0	0
Trimethoprim	4	6	9	25	33
Sulfamethazine	5	3	3	3	1
Caffeine	5	8	7	7	0
Sulfamethoxazole	7	9	6	4	2
Ciprofloxacin	36	77	40	15	0
Furosemide	48	36	16	5	0
Propranolol	58	40	49	82	71
Clofibric Acid	40	11	4	0	0
Carbamazepine	88	97	84	84	75
Ketoprofen	93	82	98	51	28
Bezafibrate	92	94	87	83	100
Nimesulide	83	104	123	85	32
Naproxen	73	78	73	16	11
Warfarin	73	112	99	90	0

Fluoxetine	46	58	77	58	70
Flurbiprofen	92	90	98	0	0
Diclofenac	100	105	98	46	0
Indomethacin	70	67	107	110	79
Ibuprofen	110	93	101	35	51
Meclofenamic Acid	58	70	90	85	0
Mefenamic Acid	68	68	85	87	71
Clotrimazole	24	35	48	93	60
Gemfibrozil	75	96	92	86	87
Triclosan	59	57	59	91	74
Ivermectin	-	-	-	-	-

As was noticed during the flow rate study no appreciable recovery of early eluting analytes was observed. These analytes may require more extreme acidic or alkaline conditions in order to exhibit retention on the C_{18} phase. However, because of the chemical diversity of the analytes, extractions at acidic pH would promote the protonation of both acidic and basic analytes and thereby convert alkaline compounds into the BH^+ cationic form reducing their interaction with the reversed-phase sorbent. The opposite would be expected at more alkaline pH values. An example of such effects is demonstrated by clotrimazole, the imidazole ring of the compound is protonated at acidic pH values but as the pH is increased the percentage recovery is also observed to increase due to the conversion of clotrimazole to a neutral moiety.

From Table 4.4 it was decided to extract all samples at pH 4.0 as at this pH an acceptable balance between all analyte recovery was observed, e.g. small but distinguishable peaks for some of the polar analytes such as caffeine were observable whilst for most other analytes including basic compounds recovery was quite high. Compounds that are more hydrophobic demonstrate a greater tolerance to changes in the pH. For example, the recovery of gemfibrozil varies only slightly as the pH at which the extraction was performed changed. As was previously observed ivermectin could not be distinguished due to co-elution with the diethyl hexyl phthalate contaminant peak.

4.3.4 Matrix removal:

For any on-line extraction technique, preconcentration of matrix components is an important factor. In an attempt to examine the suitability of the method, laboratory tap water was spiked with 200 ngL^{-1} of the chosen analytes and extracted at pH

4.0. However, the recorded UV chromatogram displayed a rather large broad peak (~20 minutes wide). This peak is attributed to excessive preconcentration of the sample matrix, (natural organic matter, humic and fulvic acid, tannins *etc.*) and was observed to totally swamp all analyte peaks and also cause suppression of the ESI signal during initial MS work. Previously reported methods for matrix removal in the literature have focused upon changing the pH at which the extraction is performed [38], sample dilution [39], or the addition of cationic surfactants to the sample prior to extraction [40]. However, such approaches are impractical in this instance. Traditionally in off-line SPE methods the sorbent is washed with water containing small portions of organic solvent and this procedure was further investigated due to the ease at which it could be performed on-line.

For the development of the wash solvent composition 500 mL aliquots of tap water were extracted under the previously optimised conditions. Upon complete sample introduction the short monolithic trap column was linked directly to the UV detector and analytes were desorbed using a linear gradient of 100% water to 100% solvent in 30 minutes. Both MeCN and MeOH were investigated but preliminary data showed that MeCN removed a larger proportion of the matrix in a smaller period of time than MeOH and was therefore, preferred for further development. Upon examination of the ChemStation solvent profile it was observed that at 20% MeCN the majority of the matrix components had been desorbed see Fig. 4.4 (A). However, as the experiment was performed under gradient conditions it was necessary to repeat it under isocratic conditions with a defined lower proportion of organic solvent, in this case 10% MeCN was investigated. Again a 500 mL aliquot of tap water was extracted and this time the extraction column was washed with 10% v/v MeCN in water at 1 $mLmin^{-1}$, see Fig. 4.4 (B). From this investigation it was observed that the majority of the adsorbed matrix components were removed directly upon the introduction of the wash solution and that after a period of 20 minutes of washing nearly all the retained matrix components were completely removed from the extraction column. The effect of the washing procedure on the retention of the analytes was also investigated by preconcentrating a 200 ngL^{-1} standard prepared in reagent water. Upon complete introduction the extraction column was washed with 10% v/v MeCN

for 20 minutes, after such time the percentage of MeCN was increased in a linear step to ensure complete analyte elution, Fig. 4.5 depicts the resulting UV trace.

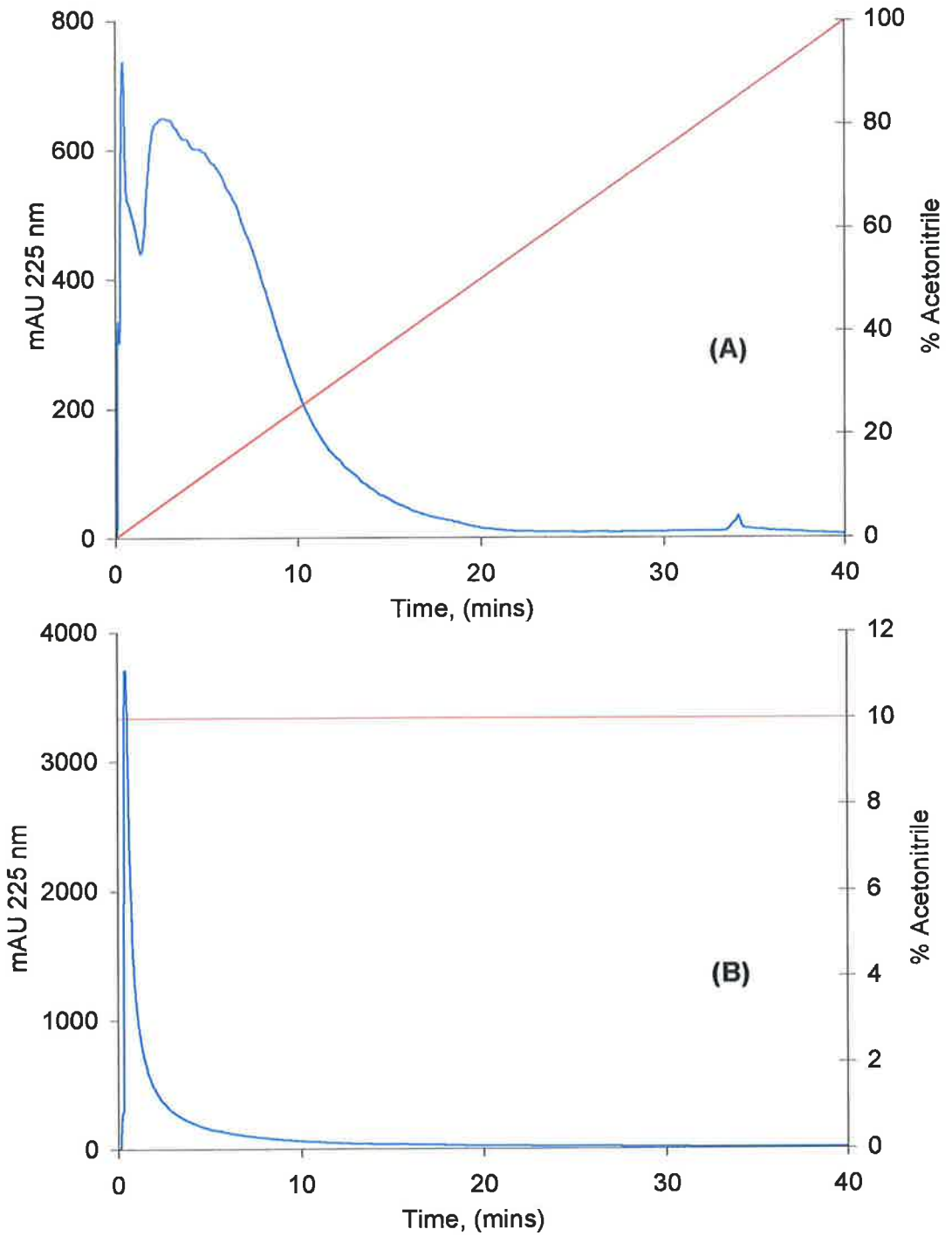


Figure 4.4: Matrix removal using MeCN washes; (A) depicts the determination of the % MeCN required for the removal of the sample matrix. (B) depicts the removal of the preconcentrated matrix under the chosen isocratic solvent wash conditions (10% MeCN).

From Fig. 4.5 it can be observed that the retention of some analytes is indeed affected by the washing procedure with peaks present at the beginning of the chromatogram. These analytes that are observed to elute in the early stages of the chromatogram elute in the same region as the majority of the matrix and as a result are omitted from further study. However, a significant proportion of the analytes is completely retained and was only observed to elute as the percentage of organic solvent was increased. The higher the degree of hydrophobicity of the compound the higher its tolerance to the washing procedure, it is expected that the wash solvent lacks sufficient strength to elute nonpolar analytes from the 1 cm extraction column. The volume of wash solvent required can also be estimated from Fig. 4.5, it is clear that analyte retention is unaffected for at least 20 minutes under conditions of 10% MeCN pumped through at a rate of 1 $mLmin^{-1}$. Again considering the porosity of the monolithic extraction column it was possible to perform the washing procedure at an elevated flow rate of 10 $mLmin^{-1}$, thereby allowing for a reduction in the analysis time.

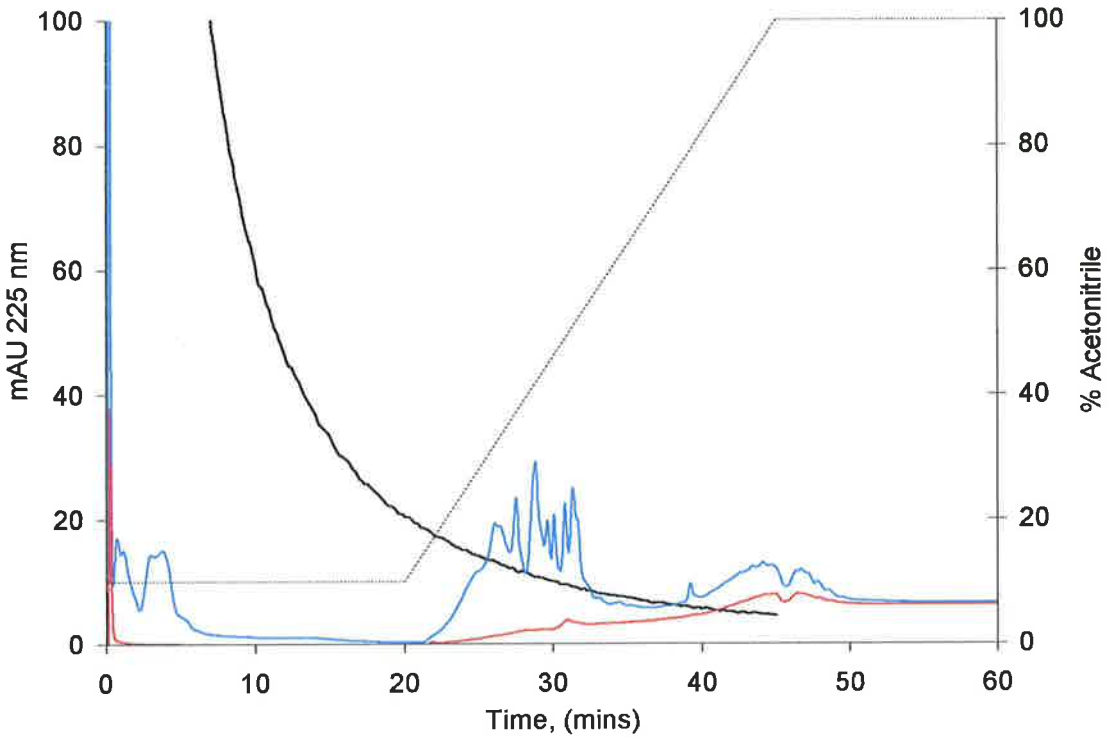


Figure 4.5: On-line SPE wash development, depicting the effect of the matrix removal wash procedure upon the retention of the analytes on the short monolithic extraction column, a MeCN gradient (dashed line), was used to completely remove the retained components from the trap column. The black trace depicts the matrix profile, the blue trace depicts the elution profile of the retained analytes, (200 ngL^{-1}) and the red trace depicts a 'blank extraction' of reagent water.

4.3.5 Extraction to extraction carryover:

Due to the high rate of matrix magnification, problems with extraction to extraction carryover may be expected, leading to contamination or matrix build up and surface spoilage within the monolithic trap column. The possibility of such carryover was investigated by extracting a 500 mL portion of tap water without a matrix removal wash step, i.e. a 'worst case scenario' approach. The sample was eluted by mobile phase back flush onto the analytical column. At the end of the chromatographic run the analytical column was analysed for residual matrix using the dual gradient method without injection while the extraction monolith was conditioned as normal and then examined for any residual contamination also using the dual gradient method. Fig. 4.6 is an overlay of the resulting UV traces.

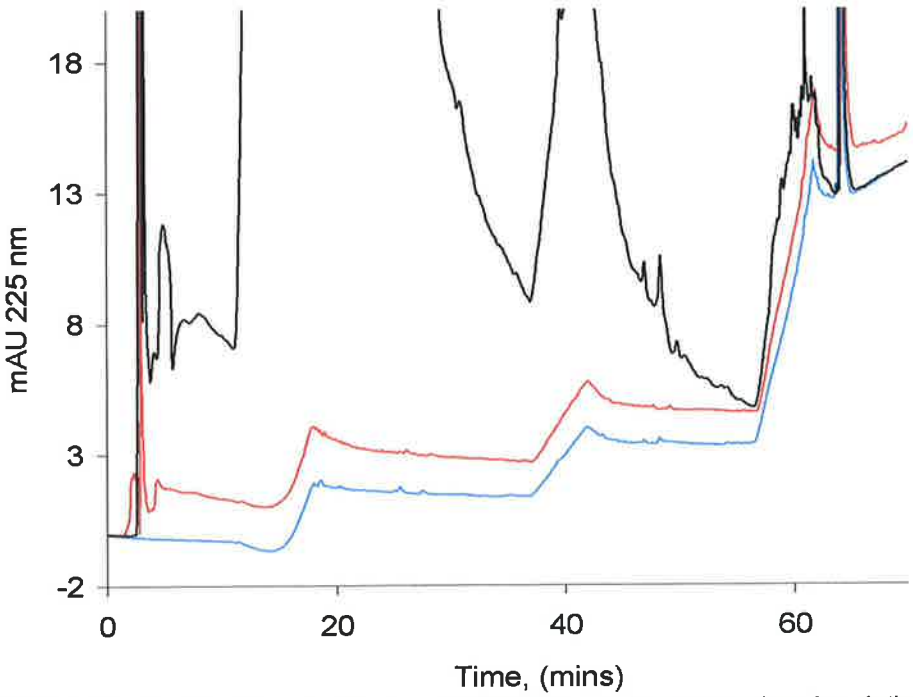


Figure 4.6: Extraction to extraction carryover. The black trace depicts the elution of the 'unwashed' sample matrix from the extraction and analytical columns. The red trace depicts the resulting UV trace and shows that there is no residual matrix contamination on the analytical column. The blue trace is the resulting UV trace of the reconditioned monolithic trap.

From the above figure it is clear that extraction to extraction carryover is negligible with no residual matrix peaks being observed in either the analytical or extraction column traces. No residual matrix carryover is expected as during the separation the percentage MeOH is increased to 90% at a flow rate of 2.3 $mLmin^{-1}$

while the extraction column is conditioned with 20 mL of MeCN prior to extraction suggesting that both sets of solvent conditions are sufficient to completely remove any adsorbed sample or matrix from both the C_{18} phases.

4.3.6 Method performance:

The performance of the on-line SPE dual gradient LC method was evaluated using two distinct sample matrices, river water taken from the River Boyne in Co. Meath, Ireland and potable water obtained from the tap in the laboratory. The levels of natural organic matter present in these sample types is expected to be quite different with a greater proportion expected in the river water samples. Overall method detection limits were determined in each sample matrix, LODs are quoted as three times the standard deviation, (n = 3) of the peak-to-peak baseline noise while the LOQs are quotes as ten times the standard deviation, (n = 3) of the peak-to-peak baseline. Method performance data quoted was calculated from UV chromatograms as preliminary attempts to couple the method with ESI-MS using the flow splitter showed that there was a rather high degree of variability with the MS signal and therefore the method is semi quantitative using UV detection and qualitative when accompanied by ESI-MS. The calculated LOD and LOQ values calculated by comparison with 200 ngL^{-1} spikes also prepared in both river and potable water samples are presented in Table 4.5 following.

The calculated detection and quantitation limits are rather low suggesting that the on-line SPE procedure imparts much greater sensitivity to the method due to the higher levels of preconcentration obtainable and complete sample introduction when compared to levels observed when off-line SPE methods are employed. It can also be observed that the matrix of the sample exhibits a pronounced effect on sensitivity, limits are lower in potable water compared to river water and also components of the river water matrix were observed to coelute and mask the responses of carbamazepine, fluoxetine and warfarin making their detection at such ultra trace levels impossible by UV alone. Detection limits are in some instances greater than two orders of magnitude lower than those previously reported by other researchers for similar pharmaceutical analytes in similar matrices using optical detection [41,42,43] and comparable if not superior to many limits quoted using LC-MS and LC-MS/MS methods with off-line SPE [44].

Table 4.5: Overall method sensitivity using on-line SPE, values in μgL^{-1}, sample size 500 mL.

Analyte	River Water		Potable Water	
	LOD	LOQ	LOD	LOQ
Carbamazepine	-	-	0.01	0.03
Ketoprofen	0.05	0.17	0.05	0.16
Bezafibrate	0.06	0.21	0.02	0.07
Nimesulide	0.07	0.24	0.02	0.07
Naproxen	0.01	0.03	0.004	0.01
Fluoxetine	-	-	0.05	0.01
Warfarin	-	-	0.04	0.12
Flurbiprofen	0.20	0.68	0.06	0.19
Diclofenac	0.11	0.38	0.04	0.12
Indomethacin	0.17	0.56	0.03	0.09
Ibuprofen	0.09	0.30	0.03	0.08
Meclofenamic acid	0.03	0.09	0.01	0.03
Mefenamic acid	0.03	0.09	0.01	0.04
Clotrimazole	0.15	0.48	0.08	0.27
Gemfibrozil	0.14	0.46	0.05	0.17
Triclosan	0.09	0.29	0.03	0.11
Ivermectin	0.32	1.06	0.17	0.55

Overall method precision was determined by performing ten replicate extractions of 200 ngL^{-1} analyte spikes prepared in both river and potable water samples. Method precision data is presented in Table 4.6 and is quoted as the %RSD based upon peak area measurement for the ten replicate extractions in each matrix. The average analyte recovery was also determined in each matrix by comparison with the direct injection of a 5 mgL^{-1} standard.

Overall method precision shows higher variability with increased levels of sample matrix, the %RSD values are higher in river water when compared to those for the potable water spikes. Precision is <20% RSD for the majority of analytes in both matrices with some exceptions, notably clotrimazole in river water and naproxen in potable samples. The levels of precision attainable with on-line SPE are expected to be dependant on the homogeneity of the sample matrix, analytes with small responses such as warfarin and clotrimazole demonstrate this fact and are more affected by the presence of small matrix peaks than analytes that exhibit a larger UV response.

Table 4.6: Overall method precision and analyte recovery from two different sample matrices, (n = 10), sample size 500 mL.

Analyte	River Water		Potable Water	
	Precision (% RSD)	Recovery	Precision (% RSD)	Recovery
Carbamazepine	-	-	14.42	9 ± 1
Ketoprofen	20.92	71 ± 15	15.90	18 ± 3
Bezafibrate	16.35	64 ± 11	21.42	42 ± 9
Nimesulide	18.17	96 ± 17	7.12	107 ± 8
Naproxen	13.02	88 ± 11	47.60	41 ± 19
Fluoxetine	-	-	37.94	52 ± 20
Warfarin	-	-	25.62	100 ± 26
Flurbiprofen	16.05	65 ± 10	9.08	86 ± 8
Diclofenac	22.02	60 ± 13	5.72	75 ± 4
Indomethacin	34.57	56 ± 19	24.57	74 ± 18
Ibuprofen	17.08	89 ± 15	4.55	73 ± 3
Meclofenamic acid	30.13	68 ± 20	6.02	77 ± 5
Mefenamic acid	20.31	79 ± 16	3.13	86 ± 3
Clotrimazole	44.55	37 ± 17	9.31	58 ± 5
Gemfibrozil	11.09	86 ± 10	10.53	81 ± 9
Triclosan	10.51	102 ± 11	6.61	78 ± 5
Ivermectin	38.41	23 ± 9	18.78	19 ± 4

The calculated rates of recovery are acceptably high for the majority of analytes. Interestingly ivermectin that was indistinguishable from the system peak is again completely resolved with the inclusion of the matrix removal step allowing for its unhindered determination. There does not appear to be a trend when correlating the calculated recovery to the type of sample matrix with some analytes exhibiting higher rates of recovery in matrix rich river water than in tap water and vice versa. The recovery of naproxen from tap water was much lower with higher variability when compared to the rate calculated for the same spiking level in river water. A possible explanation for the lower recovery and variability may include the reaction of naproxen with residual chloride present from the drinking water disinfection process. Boyd *et al.* [45] investigated the removal of naproxen during water chlorination and reported that naproxen is highly reactive with residual chlorine to form a range of chlorinated disinfection by-products. The recovery of ketoprofen and bezafibrate are also considerably lower from tap water than from the river samples perhaps resulting from elution during the matrix removal wash.

From the ten replicate extractions the reusability of the extraction column was estimated by examining the stability of analyte peak areas. It was found that

the column displays excellent stability for the extraction of potable water samples with no significant variation of peak area for the majority of analytes over the course of the ten extractions with the exception of naproxen as previously discussed. For the extraction of the river water samples the column displayed no great disparity for the first eight extractions but decreases in peak area were observed for extraction numbers nine and ten respectively. Therefore, it is concluded that the maximum column usage for the extraction of matrix rich samples such as river water should not exceed eight extractions but for samples with lower natural organic matter content the column can be used a minimum of ten times with no noteworthy problems. This makes the on-line SPE method economically viable, as the extraction columns are similar in price to SPE cartridges. The superior sensitivity levels also make the method more attractive.

4.3.7 Direct infusion mass spectrometry:

Prior to coupling the on-line SPE-LC method with MS detection an optimisation of the ESI and ion trap focusing parameters was performed by directly infusing solutions of each individual analyte prepared in 1:1 MeOH 1 mM ammonium formate pH 4.5 into the MS. These parameters were automatically fine tuned using the Bruker esquire software, version 6.08, for stated m/z values. Analytes were infused using both negative and positive ion polarity; that which provided the highest intensity was selected for further study. Optimised parameters under both polarities are presented in Table 4.7. An average of the optimised focusing parameters under each ion polarity was then taken for use with the coupled SPE-LC-UV method.

Table 4.7: Tuned ESI-MS method parameters, (optimisation was performed using a nebuliser pressure of 55 psi, dry gas flow of 8 $Lmin^{-1}$ and a drying temperature of 300°C).

Parameter	(+) ESI Determined Value	(-) ESI Determined Value
Capillary Voltage (V)	-4500.00	4500.00
End Plate Offset (V)	-590.10	-517.20
Capillary Exit Offset (V)	70.60	-54.10
Skim 1 (V)	25.40	-22.20
Skim 2 (V)	6.20	-6.50
Octopole (V)	2.50	-1.80
Octopole Δ (V)	2.10	-2.00
Octopole RF (Vpp)	138.50	-175.00

Trap Drive	34.10	35.20
Lens 1 (V)	-2.60	2.30
Lens 2 (V)	-41.80	36.10

Simple spectra were recorded in most instances, under positive ion polarity the majority of analytes exhibited a $[M+H]^+$ pseudomolecular ion, e.g. carbamazepine, ketoprofen, bezafibrate, naproxen and fluoxetine while for clotrimazole no significant pseudomolecular ion response was observed. The predominant ion for clotrimazole was recorded at m/z 277.0 suggesting the loss of the imidazole moiety from the molecule. Ibuprofen also exhibited no pseudomolecular ion response, the most intense signal being present at m/z 251.1 suggesting the formation of a formic acid adduct of ibuprofen $[M+HCOOH]^+$. The monitoring of ketoprofen, bezafibrate and naproxen using positive ion conditions is also unusual, these compounds are acidic in nature and have more usually been reported under (-)ESI conditions and under the mobile phase conditions used these analytes are expected to be partly deprotonated (pK_a values of 4.45, 3.6 and 4.2 respectively [24]), thereby the formation of positive pseudomolecular ions seems strange. The intensity of the pseudomolecular ions was higher in (+)ESI than when the same standard was infused under (-)ESI and therefore (+)ESI was chosen for further study. Under negative ion polarity again all analytes exhibited a pseudomolecular $[M-H]^-$ ion with the exception of flurbiprofen whose most intense signal corresponded to the decarboxylation of the molecule, $[M-COOH]^-$. For diclofenac and indomethacin the $[M-COOH]^-$ ion was significantly more intense than the $[M-H]^-$ ion and was therefore more suitable for extracted ion chromatogram (EIC) generation. Gemfibrozil was produced two distinct ions corresponding to a $[M-H]^-$ molecular ion at m/z 249.1 and a $[M-C_7H_{12}O_2]^-$ ion at m/z 121.1 with the latter displaying higher intensity and therefore being chosen as more suitable for EIC generation. Both Triclosan and ivermectin exhibited no response when infused under both negative and positive ion polarity as these analytes are neutral and nonpolar. Previous reports of ivermectin analysis by LC-MS have focused upon the use APCI [46] which is more suitable for the determination of higher molecular weight non-polar organics. Triclosan has been determined using negative ion ESI-MS but requires the use of high pH mobile phases, (> pH 7.5) for significant ion formation [47,48].

Due to the flow mismatch between the on-line SPE-LC method and ESI-MS the volume of column effluent must be reduced prior to entering the ESI source, this was performed using a variable flow splitter. To optimise the flow splitter injections of a 10 mgL^{-1} mixed analyte standard were performed at various splitter settings using the dual gradient LC method now complete with MS detection. The resulting EIC peak areas were monitored at each splitter setting until a maximum was recorded. At this optimum splitter setting the volume of eluent entering the ESI source at both the initial and final flow rates was determined by weight allowing for an approximate split ratio to be calculated. It was determined that the split ratio was approximately 1/20 at both flow rates. The reproducibility of the splitting process was also investigated by performing six replicate injections of a 10 mgL^{-1} standard at the optimised splitter setting. The % RSD for the six replicate injections was quite high (max. 25.5% for bezafibrate) and therefore there is a significant degree of variability introduced by the requirement to split the eluent flow from the monolithic analytical column. Due to this high degree of variability mass spectral measurements were only used for qualitative determinations to complement any peaks observed in the UV chromatogram.

The previously unoptimised conditions of nebuliser pressure and dry gas flow rates along with the drying temperature were then determined from values recommended by the esquire software now knowing the approximate volumes of mobile phase entering the ESI source. The values adopted were a nebuliser pressure of 30 psi with a dry gas flow of 8 $Lmin^{-1}$ and a drying temperature of 325°C. These parameters were used for all further LC-MS analyses.

4.3.8 Analysis of samples using on-line SPE-DG-LC-MS:

Samples of water were collected from the River Boyne in Co. Meath, Ireland in the vicinity downstream of the Navan Wastewater Treatment Centre that caters for a population equivalent of ~40,000 along with samples of potable water from the laboratory were analysed using the developed on-line SPE-LC-MS method. Aliquots of each sample were spiked with 200 ngL^{-1} of the chosen pharmaceuticals and also analysed (Table 3.1 and Table 4.1). None of the chosen analytes were detected in either sample type however, clear sharp distinguishable peaks are observed for each analyte in the UV chromatograms of both spiked sample types.

Fig. 4.7 depicts the resulting UV traces for 200 ngL^{-1} spikes in both river and potable water samples. The expanded region of the chromatogram illustrates the sensitivity if the developed method and also shows the resolution of the spiked pharmaceuticals from a number of unknown peaks present within the river water and to a lesser extent the potable water sample. The excellent retention reproducibility between the two sample types is also clear from the overlaid sample chromatograms.

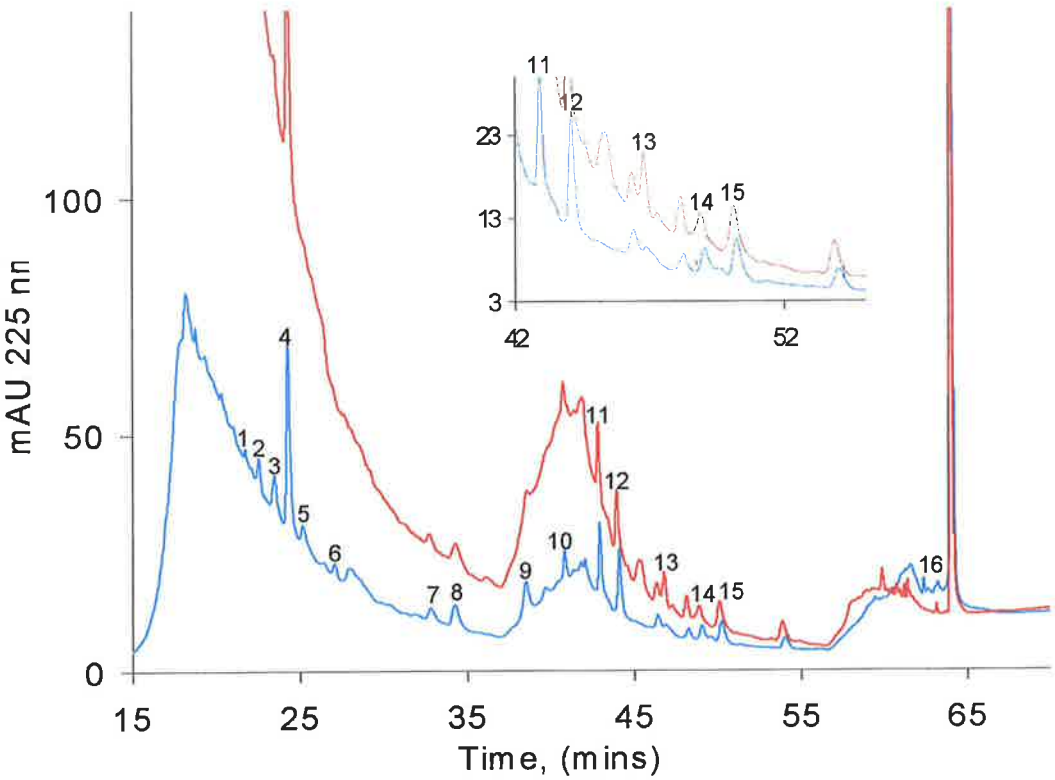


Figure 4.7: Overlay chromatogram of 200 ngL^{-1} analyte spikes in river, (red trace) and potable water, (blue trace). *Peak Identification:* **1**; ketoprofen, **2**; bezafibrate, **3**; nimesulide, **4**; naproxen, **5**; fluoxetine, **6**; warfarin, **7**; flurbiprofen, **8**; diclofenac, **9**; indomethacin, **10**; ibuprofen, **11**; meclofenamic acid, **12**; mefenamic acid, **13**; clotrimazole, **14**; gemfibrozil, **15**; Triclosan, **16**; ivermectin. Separation conditions as per Table 3.1.

Fig. 4.8 depicts the total ion current chromatogram (TIC) and EIC traces for blank and spiked river water. The excellent selectivity imparted by MS is clearly visible with defined analyte peaks clearly visible even in regions of high matrix interference in the EIC chromatograms. Both fluoxetine and warfarin can be detected without matrix interference unlike with UV detection where the identification of these analytes was obscured. The ability to obtain such clear mass

spectra in regions of simultaneous matrix elution illustrates the success of the washing step that reduces this background to acceptable levels. The advantage of the simultaneous use of UV and ESI-MS detection as shown in Figs. 4.7 and 4.8 in overcoming problematic background interferences incurred during ultra trace analyses is clear in this case.

4.4 Conclusions:

A highly sensitive and selective LC-MS method has been developed for the determination of acidic, neutral and basic pharmaceuticals in complex environmental samples using monolithic silica columns. The short monolithic extraction column allows for the rapid extraction of rather large sample volumes with acceptably high analyte recovery. The method was found to be repeatable and detection limits were in the low ngL^{-1} range. In order to avoid excessive matrix preconcentration and subsequent ESI signal a simple solvent wash step that could easily be performed on-line was developed. It was found that such a procedure effectively reduced the matrix signal by ~90% therefore minimising the matrix effect upon the ESI signal. Due to the necessity to split the column effluent prior to entering the ESI interface the method is semi-quantitative using UV detection and qualitative by MS. The method was successfully applied to environmental waters.

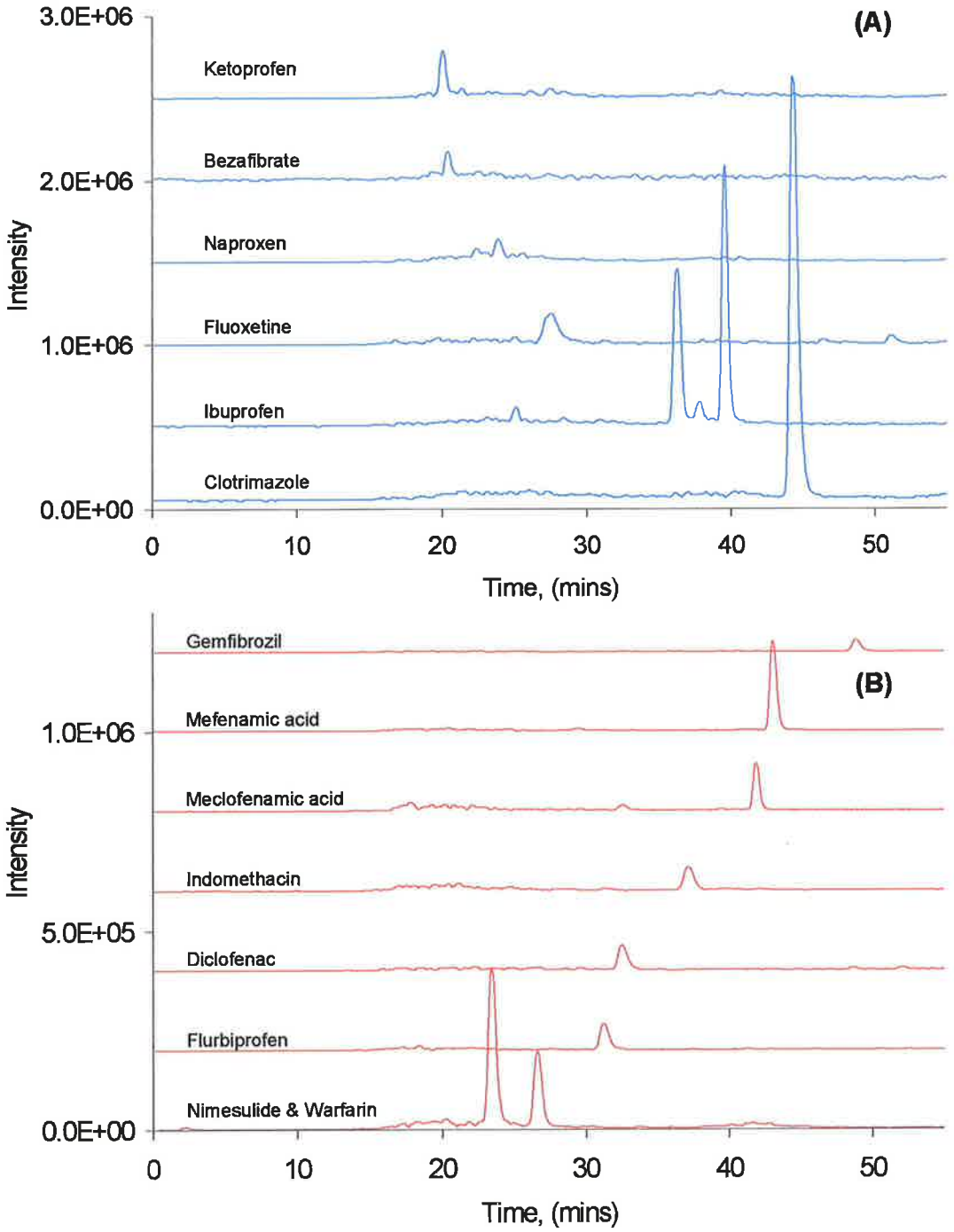


Figure 4.8: TIC and EIC traces of river water spiked with 200ngL^{-1} using (A) positive and (B) negative ESI polarity. *m/z Values:* ketoprofen (255.0); bezafibrate (362.1); naproxen, (231.0); fluoxetine (310.0); ibuprofen (251.1); clotrimazole (277.0); gemfibrozil (121.1); mefenamic acid (240.0); meclofenamic acid (294.0); indomethacin (312.0); diclofenac (250.0); flurbiprofen (199.0); nimesulide and warfarin both (307.0).

References:

1. C.G. Daughton & T.A. Ternes, *Environmental Health Perspectives*, **107**, (1999), 907.

2. B. Halling-Sorenson, S. Nors Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Holten Lutzhoft & S.E. Jorgensen, *Chemosphere*, **36**, (1998), 357.

3. T.A. Ternes, *TrAC Trends in Analytical Chemistry*, **20**, (2001), 419.

4. T.A. Ternes, M. Bonerz, N. Herrmann, D. Loffler, E. Keller, B. Bago Lacida & A.C. Alder, *Journal of Chromatography A*, **1067**, (2005), 213.

5. B.J. Vanderford, R.A. Pearson, D.J. Rexing & S.A. Snyder, *Analytical Chemistry*, **75**, (2003), 6265.

6. S. Yang, J. Cha & K. Carlson, *Rapid Communications in Mass Spectrometry*, **18**, (2004), 2131.

7. K. Inoue, M. Kawaguchi, F. Okada, Y. Yoshimura & H. Nakazawa, *Analytical & Bioanalytical Chemistry*, **375**, (2003), 527.

8. S. Rodriguez-Mozaz, M.J. Lopez de Alda & D. Barcelo, *Analytical Chemistry*, **76**, (2004), 6998.

9. E. Koster & B. Ooms, *LC-GC*, December 2001, Column Watch, 2.

10. Z. Cheng, P. Pavelic, P. Dillon & R. Naidu, *Water Research*, **36**, (2002), 4830.

11. J.V. Sancho, O.J. Pozo & F. Hernandez, *Analyst*, **129**, 38.

12. H. Zeng, Y. Dend & J.T. Wu, *Journal of Chromatography B*, **788**, (2003), 331.

13. H. Egle, R. Trittler & K. Kummerer, *Rapid Communications in Mass Spectrometry*, **18**, (2004), 2871.

14. A. Motoyama, A. Suzuki, O. Shirota & R. Namba, *Rapid Communications in Mass Spectrometry*, **13**, (1999), 2204.

15. C.R. Mallet, Z. Lu, J. Mazzeo & U. Neue, *Rapid Communications in Mass Spectrometry*, **16**, (2002), 805.

16. S. Souverain, M. Mottaz, S. Cherkaoui & J.L. Veuthey, *Analytical & Bioanalytical Chemistry*, **377**, (2003), 880.

17. Y.Q. Xia, D.Q. Liu & R. Bakhtiar, *Chirality*, **14**, (2002), 742.

18. E. Pitarch, F. Hernandez, J. ten Hove, H. Meiring, W. Niesing, E. Dijkman, L. Stolker & E. Hogendoorn, *Journal of Chromatography A*, **1031**, (2004), 1.

19. Y. Alnouti, K. Srinivasan, D. Waddell, H. Bi, O. Kavetskaia & A.I. Gusev, *Journal of Chromatography A*, **1080**, (2005), 99.

20. M.J. Lopez de Alda & D. Barcelo, *Journal of Chromatography A*. **911**, (2001), 203.

21. Y.C. Barrett, B. Akinsanya, S.Y. Chang & O. Vesterqvist, *Journal of Chromatography B*. **821**, (2005), 159.

22. K. Cabrera, *Journal of Separation Science*, **27**, (2004), 843.

23. General Medical Services (Payments) Board report for year ending 31st December 2003, available through www.gmspb.ie

24. J. Beausse, *TrAC Trends in Analytical Chemistry*, **23**, (2004), 753.

25. VCCLAB: The virtual Computational Chemistry Laboratory, ALlogPs 2.1 LogP On-line Calculation Software, viewed 24th October 2005, http://146.107.217.178/lab/alogps/index.html.

26. RxList, The Internet Drug Index, viewed 21st October 2005, http://www.rxlist.com/.

27. Advanced Chemistry Development Laboratories, ACD/pK_a DB vs. Experiment - A comparison of predicted and experimental values, viewed 21st October 2005, http://www.acdslabs.com/products/phys_chem_lab/pka/exp.html%231.

28. I.G. Dougal, D. Harper, D.M. Jackson & P. Leff, *The British Journal of Pharmacology*, **4**, (1991), 1057.

29. W.K. Chiang, R.Y. Wang, 'Acetaminophen Toxicity' viewed 24th October 2005, http://www.hypertension-consult.com/secure/textbookarticles/textbook/121_acetaminophen.htm.

30. Spectrum Laboratories, Chemical Fact Sheet CAS 58082, viewed 21st October 2005, http://www.speclab.com/compound/c58082.htm

31. K.D. Rainsford, *Rheumatology*, **38**, (1999), 4.

32. R.J. Julkunen, *Journal of Pharmacy and Pharmacology*, **6**, (1978),493.

33. Eli Lilly & Co. Material Safety Data Sheet, Olanzapine Fluoxetine Combination Capsules, viewed 21st October 2005, http://www.ehs.lilly.com/msds/msds_olanzapine_fluoxetine_combination_capsules.html.

34. National Poisons Information Service Centre in the United Kingdom, Summary for UKPID Mefenamic acid, viewed 21st October 2005, http://www.intox.org/databank/documents/pharm/mefena/ukpid07.htm.

35. C. Tixier, H.P. Singer, S. Canonica & S.R. Muller, *Environmental Science and Technology*, **36**, (2002), 3482.

36. W. Ahrer, E. Scherwenk & W. Buchberger, *Journal of Chromatography A*, **910**, (2001), 69.

37. Merck product literature, "General Chromolith Information and Applications" received with columns, Janurary 2005.

38. V. Pichon, C. Cau Dit Coumes, L. Chen, S. Guenu & M.C. Hennion, *Journal of Chromatography A*, **737**, (1996), 25.

39. A. Deng, M. Himmelsbach, Q.Z. Zhu, S. Frey, M. Sengl, W. Buchberger, R. Niessner & D. Knopp, *Environmental Science & Technology*, **37**, (2003), 3422.

40. M. Ibanez, Y. Pico & J. Manes, *Journal of Chromatography A*, **823**, (1998), 137.

41. P.A. Blackwell, H.C. Holten Lutzhoft, H.P. Ma, B. Halling-Sorenson, A.B.A. Boxall & P. Kay, *Journal of Chromatography A*, **1045**, (2004), 111.

42. P.A. Blackwell, H.C. Holten Lutzhoft, H.P. Ma, B. Halling-Sorenson, A.B.A. Boxall & P. Kay, *Talanta*, **64**, (2004), 1058.

43. C. Gonzalez-Barreiro, M. Lores, M.C. Casais & R. Cela, *Journal of Chromatography A*, **993**, (2003), 29.

44. M. Petrovic, M.D. Hernando, M.S. Diaz-Cruz & D. Barcelo, *Journal of Chromatography A*, **1067**, (2005), 1.

45. G.R. Boyd, S. Zhang & D.A. Grimm, *Water Research*, **39**, (2005), 668.

46. D. Loffler & T.A. Ternes, *Journal of Chromatography a*, **1021**, (2003), 133.

47. A. Aguera, A.R. Fernandez-Alba, L. Piedra, M. Mecuza & M.J. Gomez, *Analytica Chimica Acta*, **480**, (2003), 193.

48. W. Hua, E.R. Bennett & R.J. Letcher, *Environment International*, **31**, (2005), 621.

5.0 Column Switching Liquid Chromatography with Atmospheric Pressure Chemical Ionisation Mass Spectrometry for the Determination of Zinc Pyrithione and its Fate in the Aquatic Environment.

5.1 Introduction:

5.1.1 Background information:

From January 1st 2008 the use of antifouling paints containing organotin biocides will be completely banned in accordance with the International Marine Organisation (IMO), Convention on the Control of Harmful Anti-fouling Systems [1]. In Europe, from the same date, under Regulation 782/2003, ships registered, operating under the authority of, or entering the port of Member States will be legally required to bear a certificate stating that they have no coating containing organotin compounds or must possess a secondary coating which acts as a protective barrier to prevent organotin compounds from leaching into the surrounding waters, [1]. As replacements for organotin compounds a number of organic booster biocides have appeared on the market. These organic booster biocides have attracted increased analytical attention due to their inherent environmental toxicity and the ability of some chemicals such as Irgarol 1051 and diuron to persist in the environment and perhaps bioaccumulate [2].

However, one particular organic booster biocide; zinc pyrithione; (*bis*-(*N*-oxopyridine-2-thionato) zinc(II)) has received little attention due to the lack of sufficient analytical methods for its determination. Originally synthesised by Shaw *et al.* in 1950 as a functional analogue of the antibiotic aspergillic acid the pyrithione ligand was found to be considerably more potent against *Staphylococcus*, *Klebsiellia* and *Bacillus* species than was aspergillic acid [3,4]. Pyrithione exists as a tautomeric pair, (see Fig. 5.1) and reacts with some transition metal ions via both the sulphur and the oxygen atoms to form complexes with five membered chelation rings [5].

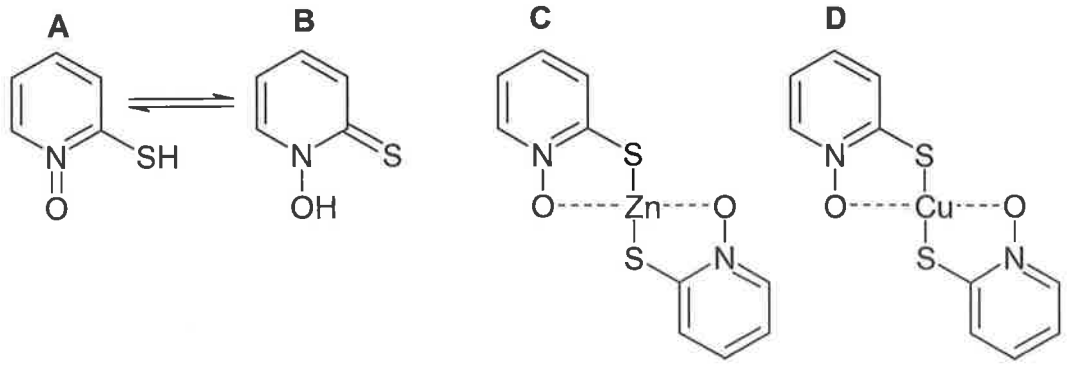


Figure 5.1: The structures of the pyrithione ligand tautomeric pair; (**A**) 2-mercaptopyridine-*N*-oxide and (**B**) *N*-hydroxypyridine-2-thione, (**C**) the structure of zinc pyrithione (ZnPT) and (**D**) the structure copper pyrithione (CuPT).

The use of zinc pyrithione (ZnPT) as an organic booster biocide is a relatively new concept, to date the majority of ZnPT produced has been used as either the active ingredient in anti-dandruff shampoo or as an additive in cosmetics and dermatitis treatments. In Sweden for example it was estimated that at least 10 tonnes of ZnPT were consumed in the anti-dandruff shampoo sold in 2003 while only 2.4 tonnes of ZnPT were used as an organic booster biocide in marine anti-fouling paints [6]. The permitted levels of ZnPT usage are a maximum of 4% w/w in marine paints and a maximum of 1% w/w in anti-dandruff shampoo [7].

Pharmacologically ZnPT acts upon the cell membrane causing depolarisation via inactivation of the primary proton pump and other membrane ion channels due to the interaction of ZnPT with, and consequent disaggregation of, the membrane phospholipid phosphatidyl ethanolamine [8,9,10]. Also Guthery *et al*. note that the dipole structure of the molecule creates pseudoquaternary ammonium groups capable of providing another mode of antimicrobial activity and therefore due to the multimodal activity of ZnPT, microbial resistance is unlikely to occur [8]. As a consequence the use of personal care products containing ZnPT would be expected to increase.

5.1.2 Environmental entry and fate ZnPT:

Two distinct routes into the aquatic environment exist for ZnPT that depend principally upon the mode of usage. In the case of marine antifouling paints ZnPT may leach directly from a painted ship surface into the surrounding water. The rate

of ZnPT leaching has been estimated by Turley *et al.* to be 1-3 $\mu gcm^{-2}d^{-1}$ for long lasting paint formulations or 3-11 $\mu gcm^{-2}d^{-1}$ for self-polishing paints [11]. Using this information further, Turley *et al.* modelled a PEC of 0.04 μgL^{-1} for ZnPT. Based upon the above leach rates, coated ship surfaces would be expected to act as continuous diffuse sources of ZnPT and other booster biocides into the environment. However, it was noted by Thomas *et al.* that the rate of introduction of booster biocides into the environment significantly increased during high pressure cleaning of painted ship surfaces [12]. Such practices can account for a significant proportion of the total quantity of biocide introduced into the surrounding water over the course of the cleaning event, but could, however, be minimised by adopting improved cleaning practices [12].

In two separate studies Thomas *et al.* report that paint particles released into the environment during high pressure cleaning operations may act as further sources of booster biocides into the aquatic environment [13,14]. It was found that booster biocides introduced to sediment in the form of paint particles persisted for a longer period than those introduced as a result of direct leaching [13]. The paint particle was also observed to release booster biocides during dissolution and therefore, cause "hot spots" of biocide pollution in docks and marinas *etc.*

As stated previously in section 5.1.1, the primary use of ZnPT is in anti-dandruff shampoo and other personal care products and therefore, ZnPT and free pyrithione may be introduced into the aquatic environment via municipal household wastewater where such products are used. It would also be expected due to the topical only administration and short contact times of such products that metabolic conversion would not occur. Galvin *et al.* estimate that based upon a rate of production of 100-200L wastewater per person per day, possible resulting concentrations of pyrithione being introduced into the aquatic environment are in the region of 1×10^{-7} M, (~0.32 μgL^{-1}) and consequently household wastewater is a significant source of pyrithione with possible pollution of receiving waters [15].

Once introduced into the environment the question is posed as to the fate of ZnPT, ideally it should readily degrade and therefore, pose minimal risk to aquatic biota. Reports concerning the environmental fate of ZnPT are mixed, however, a general consensus exists within the published literature that photochemical attenuation is by far the most important removal process for ZnPT and other

pyrithiones. Maraldo and Dahllof examined the photolytic behaviour of both ZnPT and CuPT in seawater [16] and reported that the photolytic half-lives for the aforementioned complexes were ~8.3 and ~7.1 minutes respectively. A loss in the toxicity of the two complexes to the test bacterial species was also noted upon exposure of sample solutions to light. Biotic degradation was also examined during the study but it was noted that compared to photochemical attenuation biotic degradation was an insignificant process for the removal of the pyrithione complexes [16]. Maraldo and Dahllof concluded that wavelengths in the region of 320-355 nm were the most efficient in the removal of the pyrithione complexes. However, in environmental systems the dept of penetration of such wavelengths, i.e. the turbidity of the water, and also the photon flux will be the limiting factors governing the overall removal of the pyrithione complexes from the water column, [16]. Turley *et al.* also examined the photochemical behaviour of ZnPT and CuPT in the environment [17]. It was determined that the pyrithione complexes rapidly degraded upon exposure to light accompanied with a resultant loss in toxicity. The photochemical products of both ZnPT and CuPT were identified as 2-pyridine sulphonic acid and CO_2. Based upon available toxicity data for 2-pyridine sulphonic acid it was concluded that little environmental risk was posed by the photoproducts, (no observed effect concentration (NOEC) for 2-pyridine sulphonic was 5460 μgL^{-1}) [17].

Pyrithione released into the aquatic environment in either a free or complexed form would be expected to interact with endogenous metal ions. Galvin hypothesised that free pyrithione introduced into surface water might accumulate as the manganese (II) complex (MnPT) [15], however, it was previously reported that pyrithione forms its most stable complex with Cu(II) and therefore, all pyrithione introduced into the aquatic environment may complex with or transchelate to form CuPT, i.e. pyrithione complex formation follows Irving-Williams behaviour [18]. Formation constants for pyrithione metal complexes can be found in Ref. [18].

With a reported Log K_{ow} of 9.33 it would be expected that ZnPT would accumulate in sediments due to very low aqueous solubility [19]. However studies to determine the extent of the adsorption of ZnPT onto sediments are scarce. In contrast to what would be expected, Turley *et al.* proposed that free or complexed

pyrithione will not persist or accumulate in sediment due partly to rapid photochemical attenuation in the water column but also through anaerobic degradation of any pyrithione adsorbed to the sediment via reduction of the *N*-oxide group [11]. It was also proposed that the reason for sediment detoxification of pyrithione was due to either a high microbial population within the sediment or sediment surface catalysed degradation [11]. Similarly Galvin *et al.* hypothesised that pyrithione might associate with suspended natural organic matter (NOM) within the water column [15]. It was thought the carbonaceous NOM surface may play a part in the adsorptive oxidative detoxification of pyrithione and that such a process might be important in waters where photochemical attenuation did not efficiently occur [15].

5.1.3 Toxicity of ZnPT:

Although mentioned previously that ZnPT introduced into the aquatic environment is expected to be rapidly degraded, concern still exists due to the inherent toxicity of ZnPT. As was the case with its environmental fate, published reports concerning the toxicity of ZnPT vary from declaring that the highest risk posed by ZnPT arises solely from the Zn(II) ion due to the photochemical instability of the pyrithione ligand [20], to those that claim that ZnPT poses a more significant ecotoxicological risk than organotin compounds [21]. However, a large proportion of published toxicity studies have noted that ZnPT may adversely affect aquatic biota at environmentally relevant concentrations. Most studies have focused upon determining the toxicological risk posed to single species using laboratory based tests. For example, Kobayashi and Okamura determined the effect of ZnPT and CuPT upon the development of the sea urchin [21]. It was discovered that the NOEC levels for ZnPT and CuPT were 0.01 fgL^{-1} and 1 pgL^{-1} respectively compared with tributyl tin oxide that was found to exhibit a NOEC level of 10 ngL^{-1} [21], with significant teratogenic effects noted upon fish larvae at levels of 5 μgL^{-1}. Teratogenic effects were also reported in sea urchin and mussel by Bellas *et al.* at similar concentrations [22]. Goka determined the effect of ZnPT using commercial anti-dandruff shampoo samples upon the development of fish species [23]. It was found that exposure to ZnPT resulted in embryotoxic and teratogenic abnormalities, namely curved vertebral columns. The author also concluded that

fish exposed to domestic wastewater containing ZnPT from anti-dandruff shampoo were highly at risk [23]. Other studies conducted also found ZnPT and CuPT to be the most toxic of all compounds currently in use as booster biocides in marine paints [24,25]. A field study was also performed by Sanchez-Bayo and Goka to determine the effect of ZnPT on Japanese medaka fish in an experimental rice field, unlike the previous laboratory tests chronic teratogenic effects were not observed, presumably due to loss of ZnPT to the environment, prompting the authors to believe that the actual risk posed by ZnPT may be overestimated by laboratory based studies [19].

The effect of ZnPT and CuPT upon the bacterial community of sediment was examined by Groth Petersen *et al.* who noted that in particular nitrogen cycling was effected with an increase in nitrification and a decrease in denitrification [26]. It was thought that the changes in the nitrate flux occurred due to the effect of ZnPT on nitrifying bacteria, which are mostly Gram negative species and are more susceptible to the membrane activity of pyrithione [26]. Other endpoints investigated included a phosphate flux, which was observed to increase upon exposure to ZnPT and also DNA content that was also observed to increase. The reason for the increase of both endpoints was thought to be due to death and degradation of cells as a result of pyrithione exposure [26].

Structural activity relationship (SARs) testing was also investigated by Doose *et al.* [27], who examined the toxicological effects of ZnPT and a number of structural analogues to rat leukemic cells. It was discovered that analogues containing the *N*-hydroxythioamide group exhibited similar toxicity to ZnPT and therefore, it was noted that deactivation of this functionality was necessary to ensure the toxicity threat was fully removed [27]. Based upon the results of the SARs study it was decided that the environmental fate and effects of ZnPT, CuPT, iron (III) pyrithione (FePT) and a decomposition product *bis*-(2-pyridinyl)-disulfide,1,1'-dioxide (PT_2) should also be investigated prior to the recommendation of ZnPT as a safer and greener option to organotins [27].

5.1.4 Analytical methods for the determination of ZnPT:

In many of the aforementioned studies a concluding remark noting the absence of suitable analytical methods for the determination of ZnPT in both cosmetic and

environmental matrices has been made and indeed analytical procedures in the published literature are few and far between. Chromatographic techniques for pyrithione and ZnPT determination are not commonplace due to problems that will be discussed here. Instead, researchers have focused upon the use of electrochemical techniques such as polarography, voltammetry and amperometry. However, such methods are unsuitable for the determination of ZnPT in environmental samples due to the lack of analytical sensitivity and also problems with electrode fouling [28,29,30,31,32].

Difficulties have been reported concerning the chromatographic analysis of pyrithione complexes due to problematic unwanted interactions with the silica stationary phase. The majority of researchers have reported that ZnPT readily transchelates with metallic impurities present in the silica stationary phase such as $Fe^{2+/3+}$ leading to severe peak tailing or adsorption of the pyrithione complex on to the silica stationary phase [33]. The effect of silanol activity on the chromatography of pyrithione complexes has, however, been overlooked and would be expected to be of high significance due to the presence of the pyridyl moieties of the ligand. Attempts to avoid such unwanted interactions have focused upon the derivatisation of ZnPT with fluorescent reagents such as 5-dimethylaminonaphthalene-1-sulphonylaziridine (DNS-A) or 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), which apparently stabilise the pyrithione complex and aid with detection [34]. More commonly ZnPT is converted to the more stable CuPT by the addition of Cu(II) that thereby facilitates easier chromatography [33,35]. Due to the higher formation constant for CuPT it is expected that the transchelation of ZnPT to CuPT should be rapid and efficient [18]. Other problems noted with the chromatographic analysis of pyrithione complexes have included the oxidation of pyrithione by the silica stationary phase yielding PT_2 over the course of the chromatographic run [36].

To date only one method has appeared in the literature with the desired levels sensitivity for the determination of ZnPT in the aquatic environment. Using LC-APCI-MS Thomas determined the levels of ZnPT in marinas in the southern United Kingdom [37]. However, in all instances no ZnPT was detected above the limit of detection (20 ngL^{-1}). Sample preparation was performed using copper chelation LLE to extract any ZnPT present as CuPT thereby facilitating more efficient chromatography. Recently another method based upon LC-APCI-MS has

appeared that claims to allow for the direct analysis of ZnPT using a phenyl stationary phase [38]. However, the method is limited to concentrations in the range of 3.5-10 mgL^{-1} and therefore is not directly applicable to the determination of ZnPT in the environment.

This research therefore aims to do the following:

- The development of a suitably sensitive validated analytical method using LC-APCI-MS for the determination of ZnPT in the environment.
- Investigate cleaner preconcentration chemistries based upon SPE for the trace enrichment of ZnPT prior to LC-APCI-MS analysis and,
- Investigate the fate of ZnPT when exposed to common environmental constituents.

5.2 Experimental:

5.2.1 Chemicals and reagents:

Chemicals and reagents unless otherwise state were as per section 3.2.2. Zinc pyrithione (95%) was received from Sigma-Aldrich, (Steinheim, Germany) as was copper sulphate pentahydrate (99%), copper (II) acetate (98%), 2,6-pyridine dicarboxylic acid (99%), sodium chloride (ACS reagent grade), formic acid (95-97%), ammonium formate (99.995%), ammonium acetate (99%), disodium hydrogen phosphate (99%), MES (98%), pyridine (99%) and phenol (ACS reagent grade). Ferric nitrate nonahydrate, hydrochloric acid, nitric acid, sulphuric acid, glacial acetic acid and ammonia solution (33%) all AnalaR grade were received from BDH Chemicals Ltd. (Poole, UK). Copper (II) nitrate trihydrate and anhydrous sodium sulphate were both received from Riedel de Haen, (Seelze, Germany). Potassium nitrate was purchased from Merck KGaA, (Darmstadt, Germany).

Stock 1000 mgL^{-1} solutions of ZnPT were prepared in dichloromethane and stored in the refrigerator in darkness. These stock standards were replaced monthly. Buffer solutions used for the optimisation of the extraction pH were prepared by mixing the appropriate amounts of acid and its conjugate base for formate and acetate respectively. In the case of MES weighted proportions of salt

were dissolved in ~900mL Milli-Q water and titrated to the desired pH using either 1M HCl or 1M NaOH.

5.2.2 LC-APCI-MS analysis:

LC instrumentation unless otherwise stated was as per section 3.2.5 for method development and section 4.2.5 for LC-MS analyses. The LC system was coupled to a Bruker Daltonics esquireLC ion trap mass spectrometer complete with an atmospheric pressure chemical ionisation source. For APCI optimisation, solutions of analyte in dichloromethane were infused using a Cole Parmer 74900 series syringe pump at a rate of 600 μLh^{-1}, (Cole Parmer, Vernon Hills, IL, USA) into a flow of MeOH at a rate of 190 $\mu Lmin^{-1}$ from the LC pump through a mixing tee and then into the APCI source. Gradient separations were performed on a Merck Chromolith Performance RP18e; 100.0 x 4.6 mm i.d. monolithic silica column, (Merck KGaA, Darmstadt, Germany) with a mobile phase of MeOH and 10 mM ammonium acetate. Quantitation was performed upon the extracted ion chromatogram (EIC) m/z 316.0 traces while the ratio of EIC m/z 316.0 to EIC m/z 318.0, (~100:45) was used for qualitative confirmation.

5.2.3 On-line SPE:

On-line SPE was performed as previously described in section 4.2.4. Extraction columns investigated included a 10.0 x 4.6 mm i.d. column packed with imminodiacetic acid; (IDA) functionalised silica, (Nucleosil 7µm, pore size 100Å, prepared and packed by Prof. Pavel N. Nesterenko) and a Phenomenex Onyx C_{18} guard cartridge; 10.0 x 4.6 mm i.d. monolithic silica, (Phenomenex, Macclesfield, UK). The experimental set-up was previously described in Fig. 4.1. A Merck Hitachi LaChrom L-7100 isocratic pump was used for sample delivery at flow rates of either 6 $mLmin^{-1}$ for the IDA column or 10 $mLmin^{-1}$ for the C_{18} monolithic column. Prior to use the IDA column was washed with 200 mM HNO_3 to remove any retained metallic impurities followed by conditioning with 10 mM ammonium acetate / acetic acid buffer pH 4.7 to promote dissociation of the acidic functionalities. Depending on the desired choice of extraction chemistry the IDA column was used directly after buffering or was modified with a solution of 20 mM Cu^{2+} to facilitate the use of immobilised metal affinity interaction. The C_{18}

monolithic column was conditioned with 20 mL MeCN and 20 mL of water respectively prior to use. From initial investigations only the C_{18} monolithic column demonstrated any applicability and was therefore, used for all further study. Environmental samples were filtered through Whatman GF/C glass fibre filters to remove particulate matter and adjusted to pH 7.0 prior to extraction. A 200 mL portion of sample was extracted using the C_{18} monolithic column and elution was performed using mobile phase back flushing onto the analytical monolithic column. Methods for the removal of matrix interference will be discussed later.

5.3 Results & Discussion:

5.3.1 Liquid chromatography:

Central to the development of a chromatographic method for the determination of ZnPT was the correct selection of the analytical column. As previously mentionedin section 5.1.4, is was necessary to identify a stationary phase that was essentially free from metallic impurities and also exhibited very low silanol activity. Of these two parameters the activity of residual silanol groups was deemed to be the more critical as metallic contamination could either removed by washing or suppressed by the addition of additives to the mobile phase. In order to assess the silanol activity of possible analytical columns a pyridine phenol test was performed according to Ref. [39]. Ideally, pyridine should elute from the column before phenol, symmetrically with minimal tailing. Active silanols cause excessive retention and tailing of the pyridine peak. Co-elution of pyridine and phenol, although undesirable indicates an acceptable level of silanol activity [39]. Fig. 5.2 displays the resulting traces for the pyridine phenol test performed upon a Chromolith Performance RP18e 100.0 x 4.6 mm monolithic silica column, a Waters Symmetry 50.0 x 2.1, 3.5 µm ODS column, a Hypersil ODS 50.0 x 2.1, 3 µm column and a Hypersil BDS 250.0 x 3.0 mm, 5 µm column.

As can be seen from Fig. 5.2 the Chromolith Performance RP18e monolithic column out performs all the particle packed columns with a narrow co-eluting peak observed for pyridine and phenol. Of the particle packed columns the Waters Symmetry column appears to be the most inert, again a single peak was observed for pyridine and phenol with more tailing present when compared with the

monolithic column. The effect of uncapped silanol groups is clearly depicted in the trace of the Hypersil ODS column whereby an extremely broad peak can be observed for pyridine. A similar observation was noted for the Hypersil BDS column, which surprisingly showed quite high silanol activity considering the column is marketed for the separation of basic analytes. Due to these results the monolithic column and the Waters Symmetry column were selected for further study.

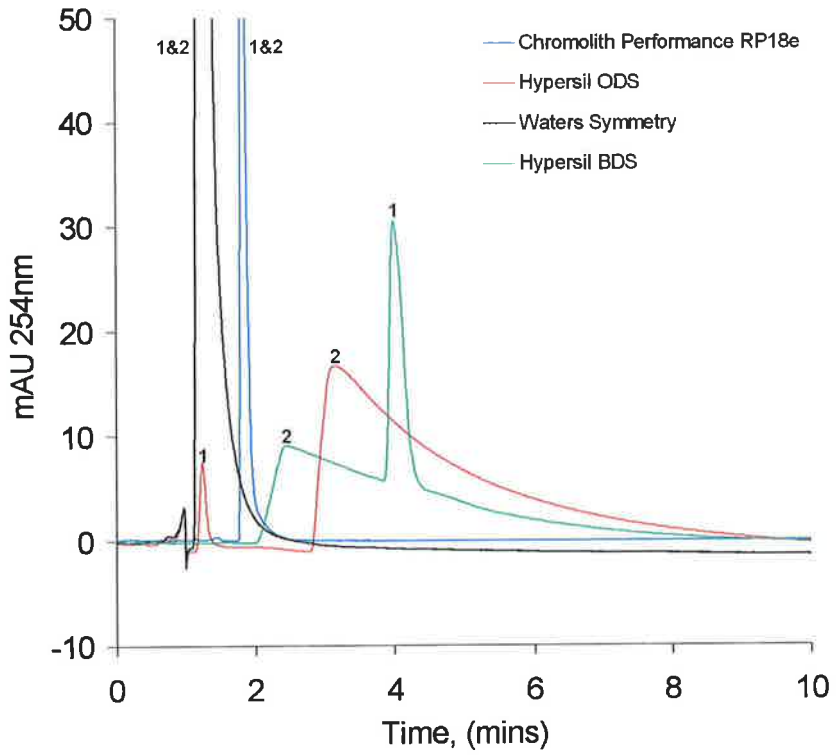


Figure 5.2: Overlaid pyridine phenol test chromatograms to examine the silanol activity of perspective analytical columns. *Test conditions:* mobile phase 50% MeCN in water with UV detection at 254 nm, column temperature 40^{0}C, concentrations; 0.5 µLmL^{-1} pyridine and 4 mgmL^{-1} phenol. *Peak Identification*: **1** Phenol, **2**, Pyridine.

Initial gradient method development was performed using the Waters Symmetry column but metallic contamination within the silica substrate was encountered. Fig. 5.3 demonstrates the effect of metallic impurities within the silica substrate upon the ZnPT peak, i.e. the ZnPT peak is diminished in size and is fronted. Metallic contamination was removed by washing the stationary phase with a selective chelating agent, in this case a solution of 10 mM 2,6-pyridine dicarboxylic acid (dipicolinic acid) pH 4.0 as was used as previously by Elefterov *et*

al. for the elution of excessively retained transition metals from an IDA modified silica column [40]. The dipicolinic acid wash successfully removed metallic contamination from the silica substrate of the Waters Symmetry column as upon re-injection of the same ZnPT standard solution a large sharp symmetrical peak was observed. Therefore, a dipicolinic acid wash was performed whenever a change in the response of ZnPT was observed. No such effects were observed when using the Chromolith column presumably due to the high purity monolithic silica substrate and also the superior level of endcapping, thereby minimising the number of available silanol groups to interact with and retain metal ions via ion exchange. It is also worth noting that the Chromolith column contains no metal components.

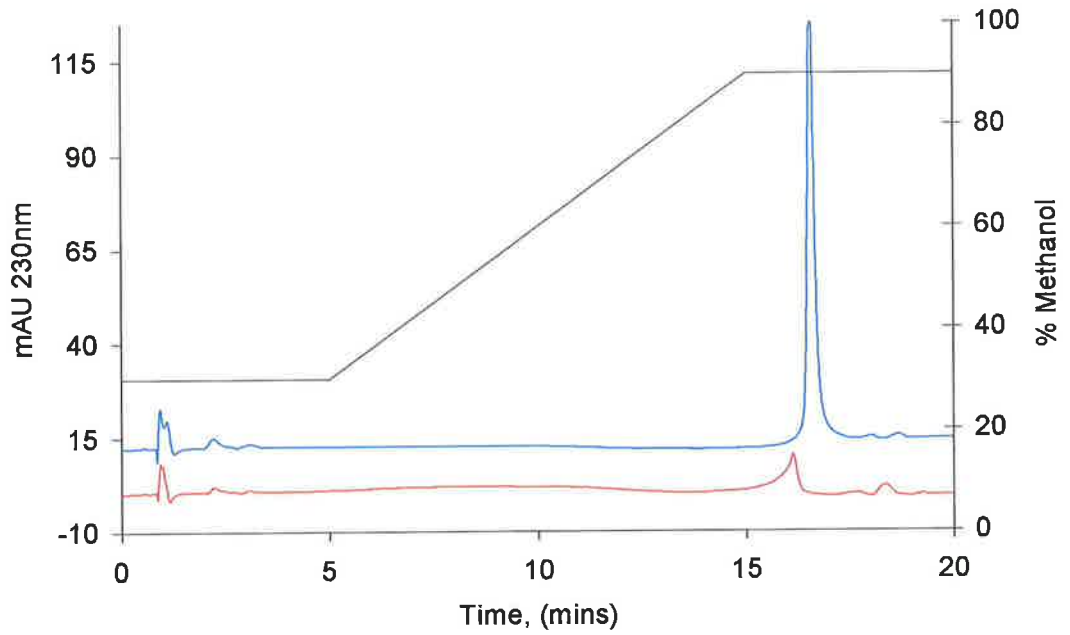


Figure 5.3: The effect of stationary phase metal contamination upon ZnPT, the bottom trace depicts a 20 mgL^{-1} ZnPT injection prior to washing the stationary phase with dipicolinic acid, the upper trace depicts an injection of the same 20 mgL^{-1} standard ZnPT solution after washing the stationary phase with dipicolinic acid. The MeOH gradient used is depicted by the black line, T_R ZnPT = 16.60 minutes.

Initial investigations into the LC analysis of ZnPT suggested that a rather high proportion of organic solvent was required to elute the complex from the column. This observation correlates well with the reported Log K_{ow} of ZnPT = 9.33. [19]. Using a mobile phase consisting of MeOH and acetate buffer pH 4.7 simple linear gradients were examined and in each instance >70% MeOH was required to

elute the ZnPT peak. Of the gradients examined a rapid linear sweep changing from 30% MeOH to 90% MeOH in one minute was initially chosen as it produced a sharp ZnPT peak with a retention time of approximately 8.3 minutes. However, it was later found when using MS detection that the rapid linear gradient was a significant source of ion suppression as the sharp increase in MeOH caused a focusing effect of retained matrix components and therefore, a shallower multi-step gradient was developed as shown in Fig. 5.4.

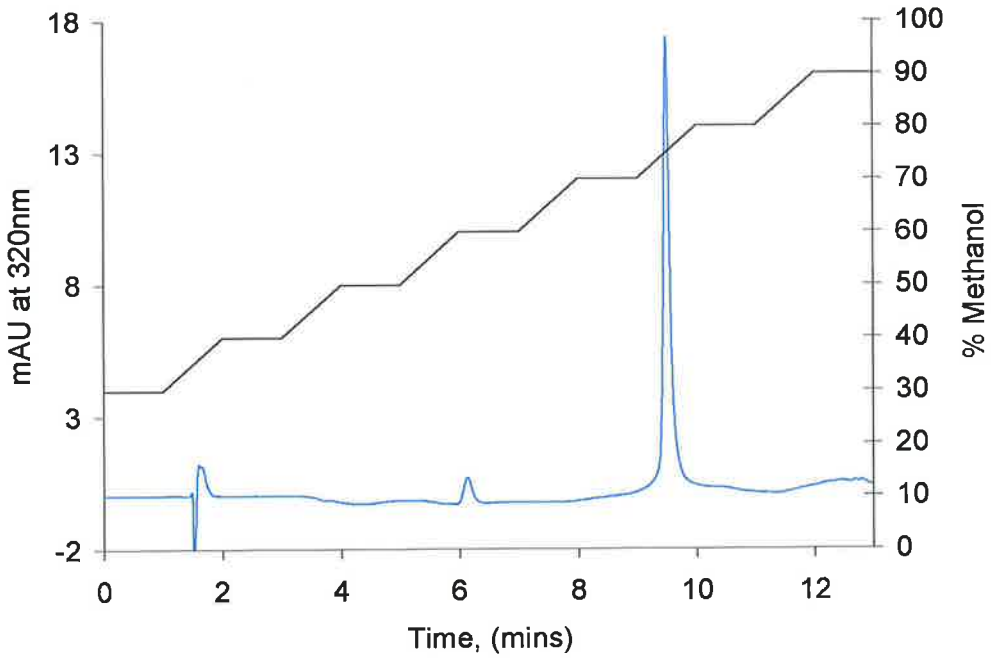


Figure 5.4: A 5 mgL^{-1} injection of ZnPT on the Chromolith Performance RP18e monolithic column using the multi-step gradient, T_R ZnPT = 9.2 minutes.

From the above figure it can be seen that the multi-step gradient still allows for the relatively rapid determination of ZnPT with a sharp peak observed at approximately 9.2 minutes. However, when the LC method was later used in conjunction with APCI-MS detection it was observed that the acetate buffer MeOH mobile phase on the monolithic column yielded a peak that was slightly fronted as shown in Fig. 5.5. The reason for the fronting was unclear. However, upon changing the acetate buffer to a solution of ammonium acetate the fronting was completely removed, most probably due to an increase in the conditional stability constant of the complex with the increased pH of the aqueous modifier of the mobile phase, i.e. pH 6.5 as compared to pH 4.7 when using the ammonium

acetate / acetic acid buffer. Therefore, the optimised LC conditions used for the determination of ZnPT in environmental samples were a mobile phase of MeOH and 10 mM ammonium acetate with a step-wise gradient on the Chromolith Performance monolithic column.

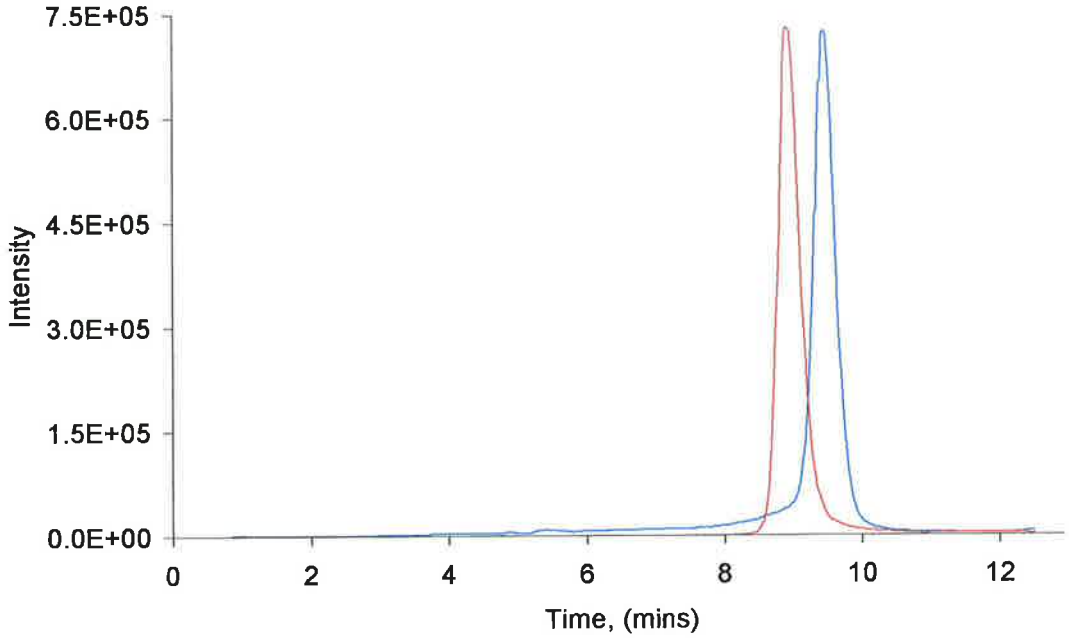


Figure 5.5: 5 mgL^{-1} ZnPT standard injections using LC-APCI-MS on the Chromolith Performance RP18e monolithic column. The blue trace was recorded using a mobile phase of MeOH and 10 mM ammonium acetate / acetic acid buffer pH 4.7, the red trace was recorded using 10 mM ammonium acetate pH 6.5. The calculated USP asymmetry value for the red trace was 0.95.

5.3.2 APCI-MS study of transition metal pyrithione complexes:

In order to determine the optimum ionisation and ion focusing parameters a solution of 1000 mgL^{-1} ZnPT in dichloromethane was split 1/20 with MeOH from the LC pump and infused into the mass spectrometer. These parameters were automatically fine tuned using the Bruker esquire software for the ZnPT pseudomolecular ion; m/z 317.0. The ZnPT solution was infused using both ESI and APCI in both positive and negative modes, however, ZnPT gave no ESI response with either positive or negative ion polarity, only positive polarity APCI gave an appreciable signal. The optimised ion focusing parameters are listed in Table 5.1.

Table 5.1: Optimised ion focusing APCI-MS parameters for ZnPT, (the optimisation was performed using a nebuliser pressure of 50.0 psi, a dry gas flow of 10.0 L/min, a drying temperature of 325°C, a corona voltage of +3200 V and an APCI temperature of 500°C).

Parameter	Measured Value
Capillary Voltage (V)	-4500.00
End Plate Offset (V)	-684.00
Capillary Exit Offset (V)	50.00
Skim 1 (V)	15.00
Skim 2 (V)	5.90
Octopole (V)	2.64
Octopole Δ (V)	1.48
Octopole RF (Vpp)	201.60
Trap Drive	40.30
Lens 1 (V)	-2.90
Lens 2 (V)	-49.50

The resultant APCI-MS spectrum for ZnPT is depicted in Fig. 5.6, which depicts two significant ions, the pseudomolecular $[M+H]^+$ ion at m/z 317.0 and another ion at m/z 221.2.

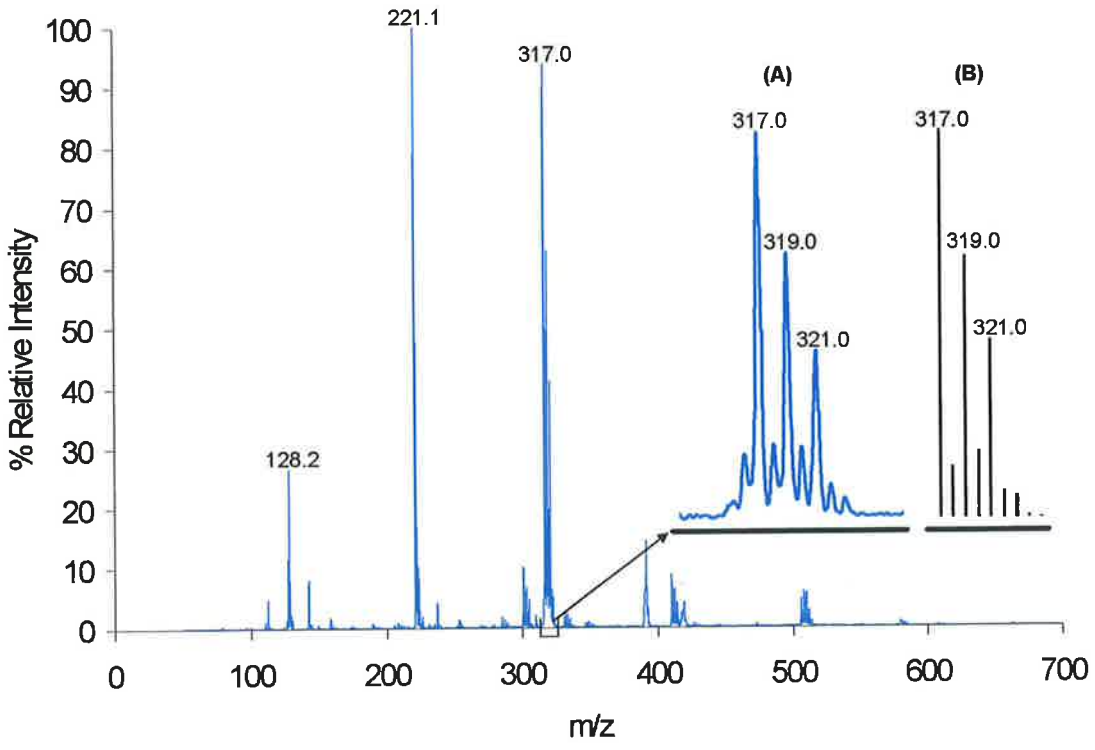


Figure 5.6: APCI-MS spectrum of ZnPT showing the $[M+H]^+$ pseudomolecular ion at m/z 317.0 and a decomposition product ion at m/z 221.1. Inset A shows the expanded isotopic pattern of the pseudomolecular ion and Inset B depicts the theoretical isotopic pattern as calculated by the Sheffield Chemputer re-plotted using Microsoft Excel™ [41].

The expanded isotopic pattern for the pseudomolecular ion at m/z 317.0 is inserted as Insert A in Fig. 5.6 and clearly shows the isotope pattern of elemental zinc with $[M+H]^+$, $[M+H+2]^+$ and $[M+H+4]^+$ ions in the approximate ratio of 100:60:40, it can also be seen that the recorded experimental spectrum correlates well with the expected theoretical spectrum as calculated using the University of Sheffield on-line Chemputer [41], which again shows an isotope pattern characteristic of the central zinc atom. The ion at m/z 221.2 was also observed by Thomas [37] although no definitive molecular structure was assigned. In an attempt to elucidate the identity of the species at m/z 221.2 tandem mass spectrometry was performed that yielded MS/MS daughter ions at m/z 111.2 and m/z 187.1, with the ion at m/z 111.2 being significantly more intense. Upon studying the isotope patterns for all the ions it was concluded that the complex at m/z 221.2 did not contain a metallic element and the daughter ion at m/z 111.2 suggests that the molecule fragments by splitting in half. From a literature search for possible decomposition products of pyrithione it is proposed that the species at m/z 221.2 is pyridine disulfide and the daughter ion at m/z 221.2 arises from α-cleavage of the disulfide bond as shown in Fig. 5.7.

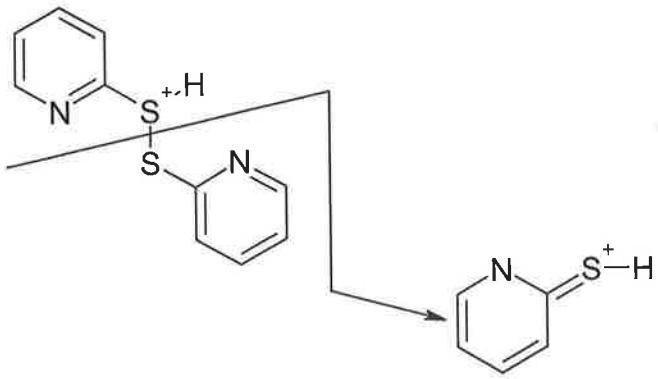


Figure 5.7: Proposed structure of the ion at m/z 221.2 and the MS/MS daughter ion at m/z 111.2.

Other metal pyrithione complexes were prepared by LLE of ZnPT with solutions of Cu^{2+}, Fe^{3+} and Mn^{2+} and dichloromethane, the APCI-MS spectra of the formed complexes, of which CuPT (golden) and FePT (violet) were strongly coloured, were then recorded in a similar manner as described for ZnPT. Fig. 5.8 depicts the recorded spectrum of CuPT. Again as was observed for ZnPT, the only significant ion present is the pseudomolecular CuPT $[M+H]^+$ ion at m/z 316.0, the

decomposition product at m/z 221.1 can also be observed although at a much lower intensity than was present in the ZnPT APCI-MS spectrum. Inserted into Fig. 5.8 are the experimentally recorded and theoretical Chemputer isotopic pattern of the pseudomolecular ion, both of which clearly show the characteristic copper isotopes with $[M+H]^+$ and $[M+H+2]^+$ ions in the approximate ratio of 100:45. In the case of FePT no pseudomolecular ion was observed in the resulting spectrum, the only significant ion present was at m/z 127.2 that corresponds to $[pyrithione+H]^+$, therefore, suggesting that the FePT complex is quite labile and readily decomposes within the APCI source. A similar observation was observed for MnPT, whereby the only significant ions present in the spectrum were m/z 127.2, 221.1 and 317.0 which correspond to $[pyrithione+H]^+$, pyridine disulfide and the ZnPT pseudomolecular ion respectively, suggesting that the manganese complex does not readily form in solution and therefore accumulation of MnPT in the environment as proposed by Galvin *et al.* would not be expected to occur [15].

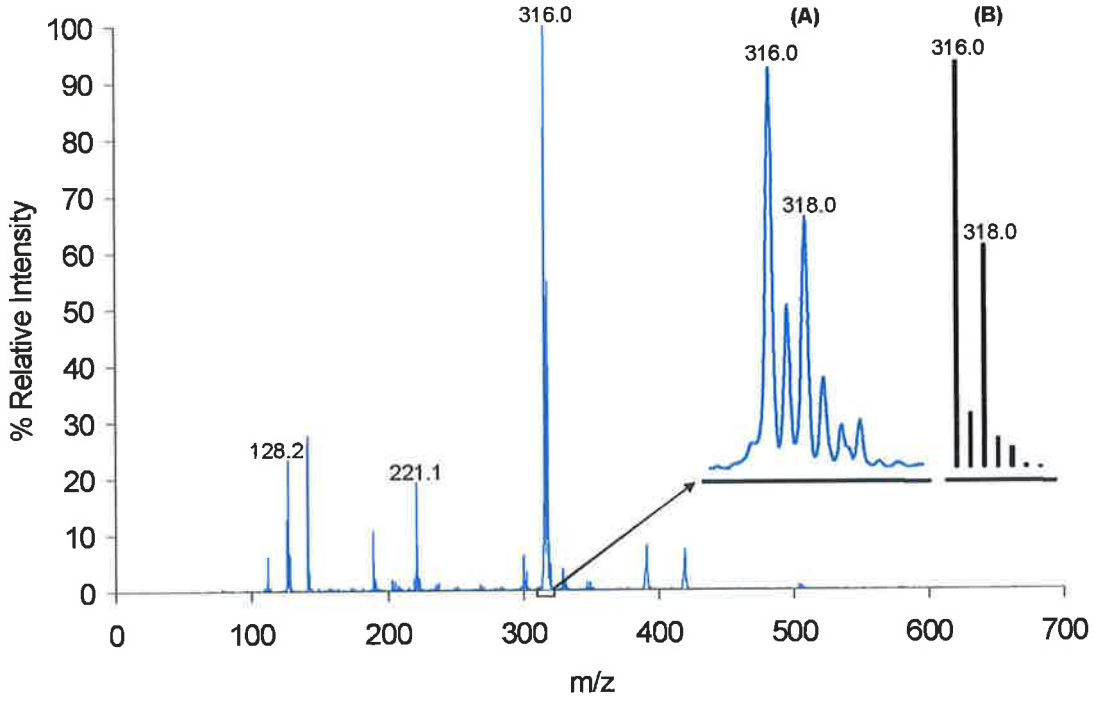


Figure 5.8: APCI-MS spectrum of CuPT showing the $[M+H]^+$ pseudomolecular ion at m/z 316.0. Inset (A) shows the expanded isotopic pattern of the pseudomolecular ion and Inset (B) depicts the theoretical isotopic pattern as calculated by the Sheffield Chemputer re-plotted using Microsoft Excel™ [41].

Based upon the APCI-MS study it was concluded that only ZnPT and CuPT were of relevance for further study due the lability or instability of the FePT and MnPT complexes. It is worth noting that the CuPT and ZnPT pseudomolecular ions only differ by one mass unit and also co-elute under the chosen LC conditions and therefore, in an attempt to find exploitable differences between the complexes MS/MS was performed. Under tandem conditions ZnPT yielded a daughter ion at m/z 192.0 that corresponds to the loss of a pyrithione ligand from the molecule and an ion at m/z 127.2, i.e. $[pyrithione+H]^+$. Further tandem transitions of the ion at m/z 192.0 yielded another daughter ion at m/z 174.0 suggesting dehydration of the species at m/z 192.0. CuPT did not yield any daughter ions of significant intensity under MS/MS, therefore, suggesting the greater stability of CuPT, which correlates well with previously reported data [18]. It was therefore concluded that tandem MS was a viable option for distinguishing ZnPT and CuPT even with co-elution on a LC column.

An optimisation of the APCI source ionisation parameters, i.e. applied corona voltage and APCI reaction temperature was performed upon linking the LC method with APCI-MS detection using repeat injections of a 5 mgL^{-1} solution of ZnPT. The corona voltage was optimised in the region of +1000 V to +3400 V and a sigmoidal relationship was observed between the resulting intensity and the applied voltage, see Fig. 5.9 (A). Insignificant intensity was observed below +1000 V whilst above +3400 V excessive current was generated within the source. The optimum applied voltage was deemed to be +2600 V above which point no significant increase in intensity was recorded. Therefore, +2600V was used as the optimum applied corona voltage for all subsequent analysis. The APCI temperature was optimised in the range of 200 to 500°C using 25°C intervals, see Fig. 5.9 (B). A linear increase in intensity with increasing APCI temperature was observed up to ~325°C followed by a linear decrease in intensity with further increases in the APCI temperature, suggesting that at temperatures exceeding 325°C ZnPT may begin to decompose. A temperature of 325°C was adopted as both the optimum APCI reaction temperature and also the dry gas temperature set point in order to maintain isothermal conditions within the source.

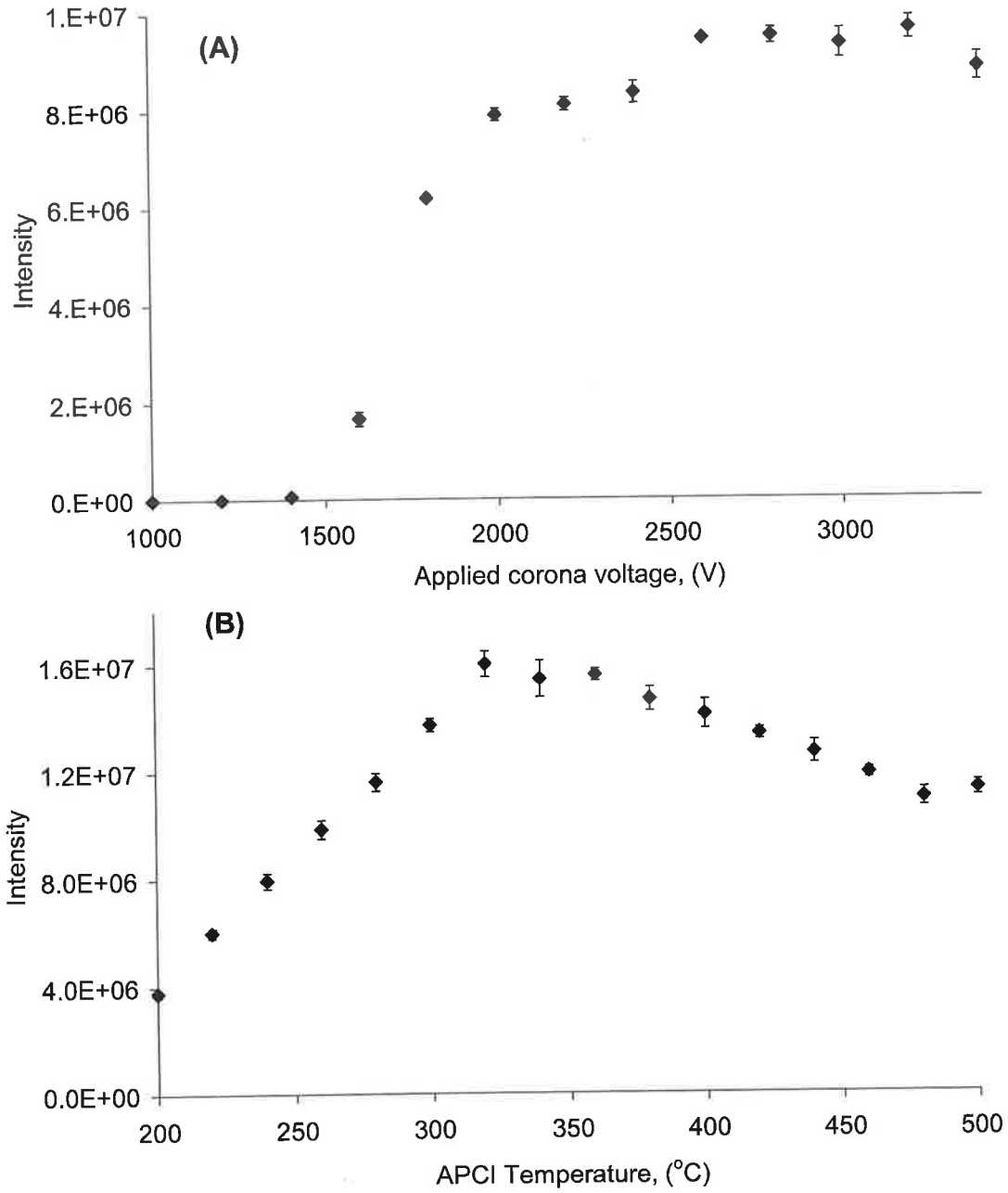


Figure 5.9: Optimisation of the APCI ionisation parameters, (A) corona voltage optimisation and (B) APCI temperature optimisation, inserted y-error bars are the standard deviation of the three replicate measurements.

5.3.3 Selection of the extraction chemistry:

As remarked previously, methods in the literature concerning the determination of ZnPT in environmental samples are limited. The method of Thomas [37] appears to be the primary reference for those wishing to examine the presence of ZnPT. However, the method uses large sample volumes and LLE for sample enrichment

with toxic dichloromethane. Little research has to date been focused upon the development of preconcentration using SPE for the trace enrichment of ZnPT prior to instrumental analysis. Extraction chemistries identified that may offer applicability include immobilised metal affinity chromatography (IMAC), chelating phases and reversed-phase interaction due to the presence of a central metal ion within and the hydrophobicity of the pyrithione complex. IMAC and chelating phases were identified as more selective sorbents, for example IMAC has recently been used for the selective trace enrichment and characterisation of naturally occurring complexing ligands of both copper and aluminium from soil and marine water samples [42,43,44,45,46,47]. The original aim was therefore, to use copper modified IDA functionalised silica for the IMAC extraction of ZnPT with transchelation to the more stable CuPT occurring on the stationary phase as it was hoped that such an extraction technique would be less prone to the co-extraction of NOM matrix components as compared with reversed-phase sorbents. However, initial extractions appeared to show retention of ZnPT but no elution of CuPT, even when using abrasive conditions such as 200 mM H^+ and therefore, an investigation was performed in order to determine the exact mechanism of the extraction.

The behaviour of ZnPT during its passage through the sample pump was examined and it was found that at the low sampling flow initially used of 1 $mLmin^{-1}$ only approximately 39% of the ZnPT present was actually introduced onto the extraction column. Fig. 5.10 depicts an overlay of EICs for the pseudomolecular ZnPT ion (m/z 317.0) at various sampling points on the Merck sample pump. Although not shown, EIC traces were also generated for the $[pyrithione+H]^+$ ion at m/z 127.1 to see if the complex disintegrated but in each instance no peak was observed. Therefore, the exact degradation mechanism of ZnPT within the sample pump remains unclear. The experiment was repeated using CuPT and a similar response was observed. In order to ascertain whether the contact time within the pump had an affect upon the degradation of the pyrithione complexes the above experiment was repeated using the max loading flow rates for both the IDA silica column, i.e. 6 $mLmin^{-1}$ and the C_{18} monolithic column, i.e. 10 $mLmin^{-1}$. It was observed that in the case of ZnPT and CuPT when pumping at 6 $mLmin^{-1}$ that a degree of degradation still occurred although it was considerably less than at 1 $mLmin^{-1}$ meaning that a proportion of analyte would be lost prior to extraction when

using the IDA silica column either modified with Cu^{2+} for IMAC or un-modified allowing for pure chelation chemistries.

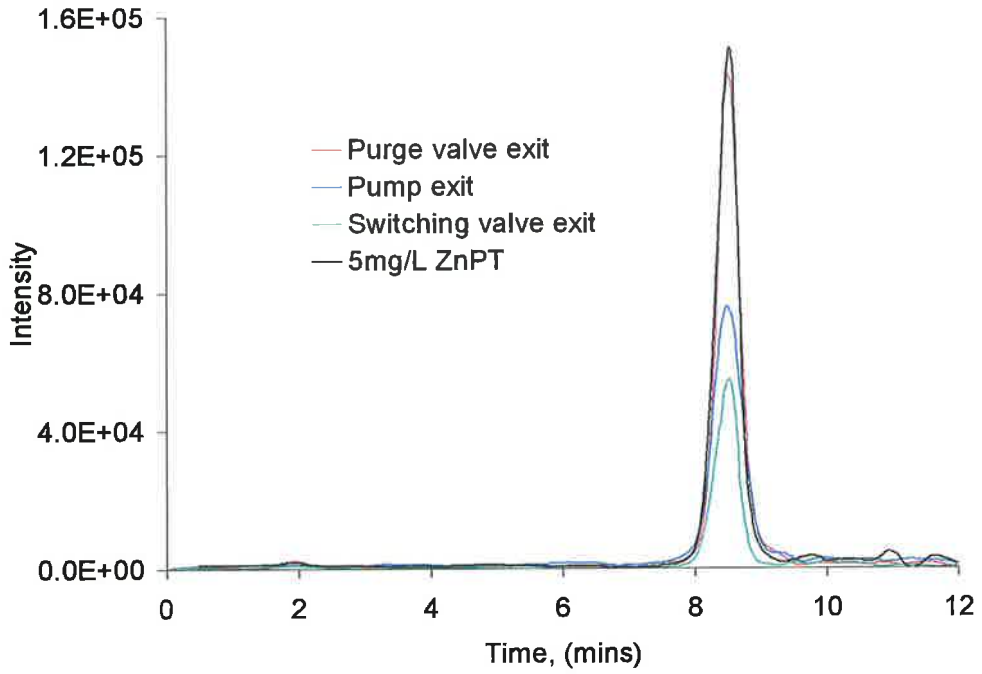


Figure 5.10: Overlay of EIC m/z 317.0 traces showing the degradation of ZnPT upon its passage through the Merck sample pump at a flow rate of 6mLmin^{-1}.

In contrast, when pumping the pyrithione complexes through the sample pump at 10 mLmin^{-1}, i.e. the maximum flow rate of the Merck pump, an insignificant amount of degradation was noticed for both ZnPT and CuPT, thereby suggesting that the contact time within the sample pump is a critical parameter when considering the degradation of both ZnPT and CuPT, however, in all instances CuPT was observed to show a larger amount of degradation than ZnPT.

With the knowledge of the instability of the pyrithione complexes during sample pumping simple qualitative retention experiments were performed in order to evaluate which of the previously mentioned extraction chemistries showed the most promise for use as a ZnPT trace enrichment procedure prior to LC-APCI-MS analysis. In each instance 10 mL aliquots of 2 mgL^{-1} solutions of ZnPT and CuPT were extracted using previously conditioned IMAC, un-modified IDA and the C_{18} monolithic column and the loading effluents were tested for the presence of the pyrithione complexes using LC-UV. Elution from both the IMAC and the un-modified IDA was performed using 200 mM HNO_3, excess acid was neutralised

using concentrated ammonia solution, an excess of Cu^{2+} was added and the solution was then tested for the presence of CuPT. With the C_{18} monolithic column elution was performed using MeOH.

Using an IMAC interaction on the copper modified IDA silica column ZnPT exhibited approximately 50% retention while CuPT exhibited a much higher degree of retention, approximately 90%. It was expected that ZnPT would show a much higher affinity for the immobilised Cu^{2+} ion due to the higher formation constant of the copper pyrithione complex compared to the zinc pyrithione complex thereby suggesting fast mass transfer kinetics and it was also expected that it would be more thermodynamically unfavourable to form CuPT on the IMAC surface from CuPT in solution due to entropic effects. In the extracts of both pyrithione complexes no reformed CuPT was determined suggesting that the acidic conditions used did not elute the retained complex or formation of the copper pyrithione complex did not occur even upon pH adjustment and the provision of an excess of Cu^{2+} ions.

Using the un-modified IDA silica column a similar trend was observed; retention of both ZnPT and CuPT was noted, with more CuPT appearing to be retained than compared with ZnPT. However, upon testing the extracts no CuPT was present in either solution. The absence of CuPT in the tested extracts is again attributed to the destruction of retained ZnPT and CuPT using concentrated acid to elute the complexes from the IDA surface followed by no formation of CuPT upon neutralisation and addition of Cu^{2+}.

Using the short C_{18} monolithic column, when tested, the loading effluents for both ZnPT and CuPT showed no presence of either complex while the MeOH extracts demonstrated considerable peak magnification thereby suggesting that the C_{18} phase showed a considerable prospect for preconcentration and was hence selected for further study. An additional bonus noted was the possibility of performing on-line preconcentration using column switching as the elution conditions tested in the preliminary experiments matched those of the analytical mobile phase. On-line SPE attempts did provide positive promise with minimal sample handling and therefore, this approach was pursued.

5.3.4 Optimisation of column switching LC extraction conditions:

In order to maximise the recovery of ZnPT from aqueous samples using the C_{18} monolithic column, an optimisation of the extraction pH and the ionic strength of the sample was performed. The sample pH was optimised in the working range of the monolithic silica column, i.e. pH 2-7, by extracting solutions of 1 μgL^{-1} ZnPT prepared in either pH adjusted water or buffer solutions. The resulting percentage recovery, calculated by peak area comparison with a 5 mgL^{-1} standard injection was then plotted versus the extraction pH as can be seen in Fig. 5.11 (A). The plot appears to show no definitive trend although the percentage recovery increases in a somewhat linear fashion between pH 5 and pH 7. Given that the pK_a of the mercaptan group of the pyrithione ligand is ~4.6 [36], low recovery of ZnPT would be expected as observed at pH values less than the pK_a, i.e. in this study pH 2-4, due to protonation of the mercaptan group and a consequent reduction in the overall stability of the ZnPT complex. The increase in the percentage recovery observed in the region of pH 5-7 is therefore, attributed to an increase in the overall stability of the pyrithione complex with increasing solution pH. Based upon Fig. 5.11 (A) the optimum extraction pH is clearly pH 7. Such an observation was welcomed as most environmental waters are generally in the range of pH 7-8 meaning that minimal sample handling would be necessary and also previously published reports suggested that the co-extraction of NOM containing acidic functionalities on the C_{18} monolithic column should be reduced.

Fig. 5.11 (B) depicts the effect of increasing the salt concentration of the extraction solution upon the percentage recovery of ZnPT. It was observed that the percentage recovery of ZnPT increased with increasing salt concentration due to a salting out effect i.e. reduced solubility in a saline matrix. Such behaviour would be expected due to the hydrophobicity of the pyrithione complex (Log K_{ow} = 9.33 [19]) and its low solubility in aqueous solutions. From Fig. 5.11 (B) it can be seen that the recovery of ZnPT is almost quantitative at salt concentrations greater than 1 M NaCl, however, it must be noted that the above experiments were performed in reagent water lacking an innate matrix and therefore, for environmental samples it was proposed to adjust the salt concentration to 0.5 M NaCl for fresh water samples with no adjustment speculated for marine samples.

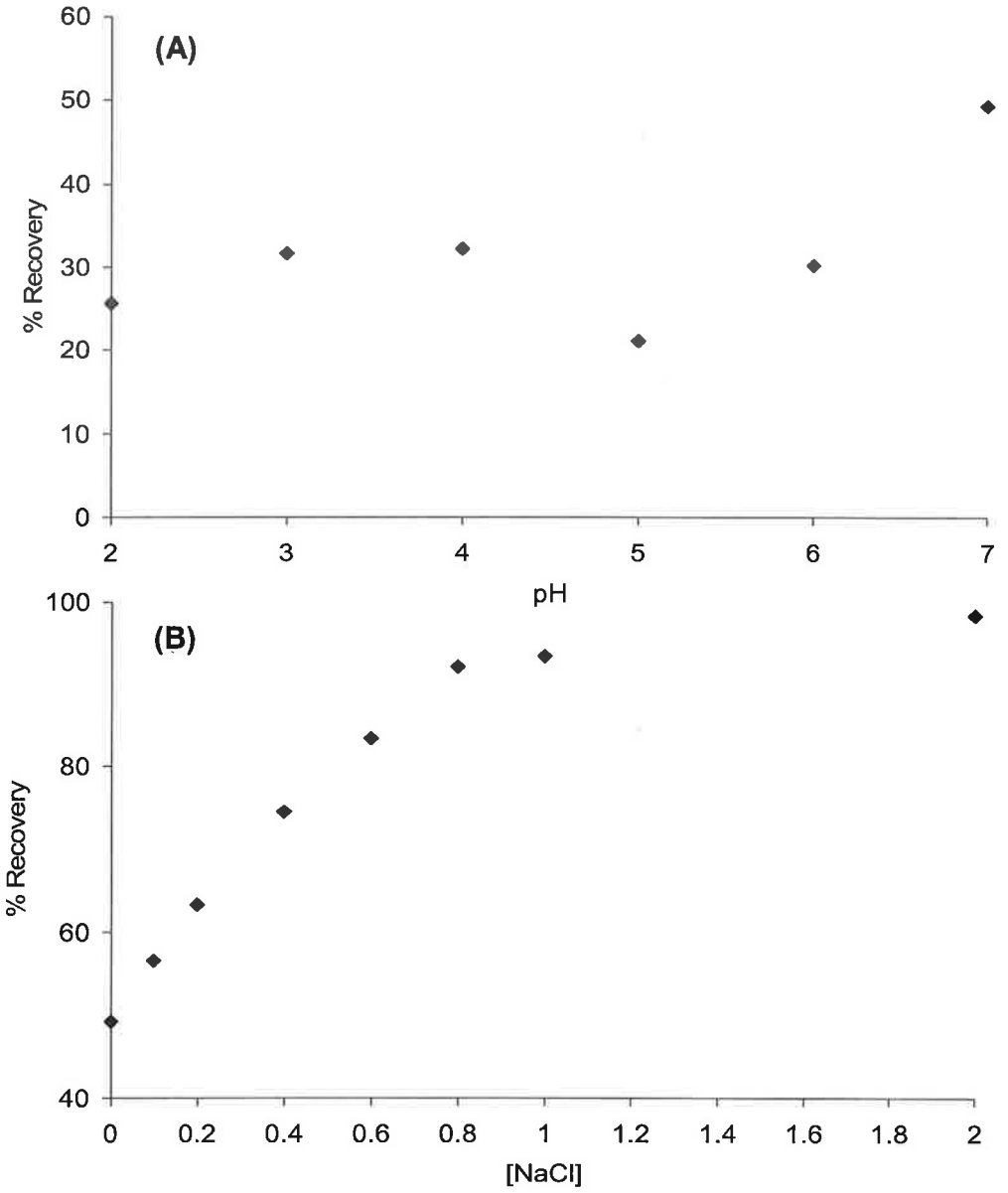


Figure 5.11: Optimisation of the C_{18} extraction parameters, (A) the sample pH and (B) the salt concentration. In each instance an increase in the % recovery is observed with increasing pH and [NaCl].

5.3.5 Matrix removal:

Primary on-line SPE investigations with real sample matrices yielded the problem of excessive NOM extraction along with ZnPT and consequent APCI-MS ion suppression. The co-extracted NOM was observed to elute from the analytical column in the same region as the ZnPT peak with large intensity due to the presence of a significant ion at each m/z value. As mentioned previously the LC gradient and APCI ionisation parameters were altered in an attempt to minimise

APCI ion suppression. However, the natural matrix was still found to be problematic. Therefore, the development of a matrix removal procedure was deemed necessary.

As was performed in Section 4.3.4 a solvent wash step was developed due to the ease at which such procedures can be performed on-line. As mentioned previously the most exploitable parameter for the extraction of ZnPT is its hydrophobicity with no secondary equilibria to be considered when extractions are performed at pH neutral. Therefore, the retention of ZnPT and the percentage of organic solvent required for elution from the C_{18} monolithic column were investigated. It was found using a 1 μgL^{-1} extracted solution that ZnPT was retained upon the C_{18} monolithic concentrator column for approximately 4.4 minutes and ~45% MeOH was require for elution. Knowing the elution conditions simple experiments to determine the wash solvent composition and the wash duration were then carried out. Using isocratic conditions it was observed that extracted ZnPT could be retained completely up to and including 40% MeOH in the mobile phase with a linear increase to 90% required for complete elution, however, when greater than 20% MeOH was used a peak was present at the start of the extracted molecular ion chromatograms in addition to the ZnPT peak, which was found to increase with increasing percentages of MeOH. Extracted solutions of 1 μgL^{-1} in reagent water were exposed to the wash solution for increasing periods of time in order to evaluate the maximum wash duration without affecting the retention of ZnPT. The maximum wash solvent exposure duration was found to be 20 minutes at a flow rate of 1 $mLmin^{-1}$, with longer exposures the resulting ZnPT peak was significantly diminished in intensity. The optimum wash conditions were therefore, 20 mL of 20% MeOH in 80% 10 mM ammonium acetate. Due to the bimodal pore structure and increased permeability of the C_{18} monolithic column it was however, possible to perform the wash procedure in 2 minutes using the maximum pump flow rate of 10 $mLmin^{-1}$.

The retention of NOM using a river water sample without the addition of 0.5 M NaCl was then examined upon exposure to the optimised wash solvent, (at a flow rate of 1 $mLmin^{-1}$) with the majority of the retained matrix observed to elute as a large peak at the beginning of the chromatographic run within the first 3 minutes. Therefore, it appeared that the solvent wash conditions were successful. However,

upon the addition of 0.5 M NaCl as previously optimised it was observed that even with the wash procedure a significant proportion of NOM was extracted and retained due to the increased ionic strength of the extraction solution and not removed with washing. The presence of the salt was also found to cause problems with the extraction pump such as increased backpressure generation *etc.* and consequently is was decided not to add salt to sample solutions prior to extraction in order to prevent damage to the sample pump.

In order to assess the applicability of the wash procedure a 1 μgL^{-1} ZnPT spike in laboratory tap water was prepared, extracted and washed and the resulting EIC m/z 316.0 trace is inserted following. It is clear from Fig. 5.12 that even with the inclusion of the wash procedure a significant proportion of NOM is still retained on the C_{18} monolithic column and interfering with the LC-APCI-MS analysis of ZnPT.

Possible solutions to the matrix interference problem identified in the literature included increasing the extraction pH to promote dissociation of acidic NOM functionalities [48]. However, the extraction was already being performed at pH 7, i.e. near the upper stability limit for the silica monolith and therefore such a solution was not viable. The addition of detergents or chemical reducing agents such as $NaBH_4$ to the extraction solution were also recommended but in the interest of simplicity and to prevent unwanted reactions or foiling of the APCI source such procedures were avoided [49,50].

A more feasible option identified in the literature appeared to be the use of a sacrificial strong anion exchange sorbent (SAX) prior to the extraction column, i.e. the C_{18} monolithic column, for the retention of NOM containing dissociated acidic functionalities [51,52,53]. Due to the absence of an acidic functionality within the ZnPT molecule it was decided that the use of a sacrificial SAX sorbent might be a possibility due the ease of inclusion of a SAX pre-column prior to the C_{18} monolithic extraction column. Initial investigations were focused upon using a polymeric SAX sorbent, (Alltech Sample Prep Anion Exchange) packed into a 50.0 x 4.0 mm polymeric column housing. The backpressure generated by the dual column was measured using reagent water at various flow rates in order to ascertain as to whether the dual column could be safely used at the required loading flow of 10 $mLmin^{-1}$. It was found that at 10 $mLmin^{-1}$ the resulting backpressure was

acceptable at ~110 bar. A 1 μgL^{-1} ZnPT spike prepared in reagent water was prepared and extracted through the dual SAX-C_{18} column and elution was performed through the C_{18} monolithic column using mobile phase back flushing, however no ZnPT was present in the resulting trace. It was expected that the ZnPT present was retained upon the polystyrene substrate of the polymeric SAX particles due to hydrophobic interaction. Also as the SAX sorbent was polymeric and therefore unsuitable for use with organic solvents it was decided that the SAX sorbent must be silica based (Si-SAX).

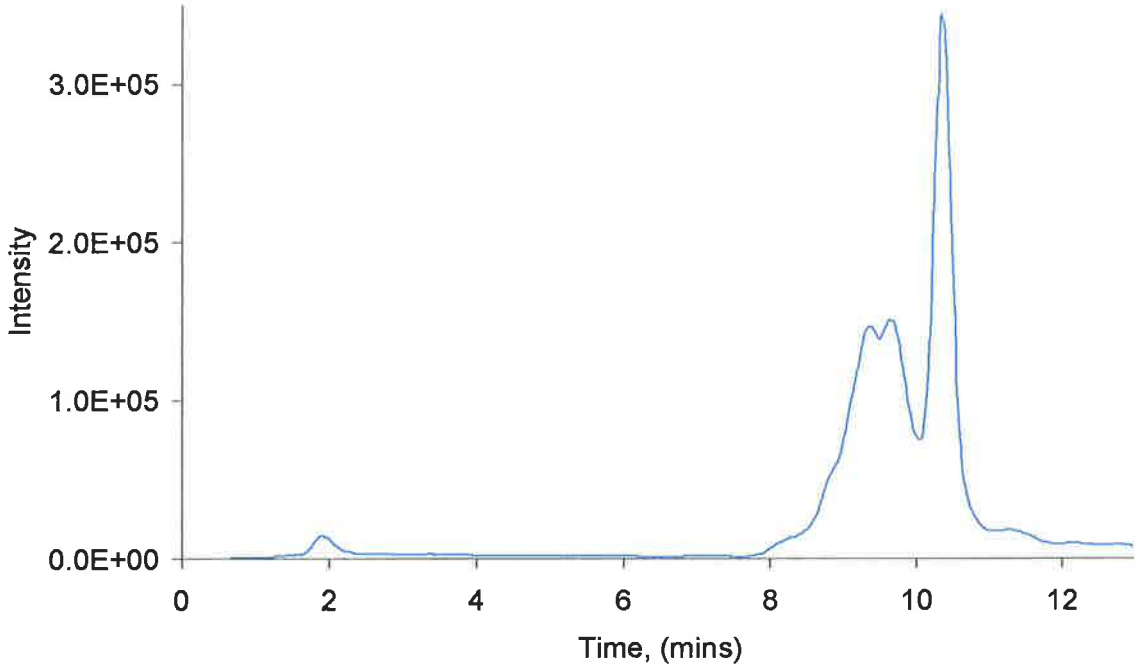


Figure 5.12: 1 μgL^{-1} ZnPT spike in laboratory tap water depicting the matrix interference even after the use of a solvent wash procedure.

The Si-SAX sorbent used was Vydac 301SC anion exchange silica packed into a 33.0 x 4.6 mm stainless steel column housing. As was the case with the polymeric SAX the backpressure generated using reagent water at the required sample loading rate and was found to be acceptable at ~120 bar. Prior to usage the Si-SAX column was conditioned with 0.2 M ammonium acetate and water. In order to estimate the system suitability a 1μgL^{-1} ZnPT solution prepared in reagent water was extracted through the Si-SAX-C_{18} tandem column with elution via mobile phase back flush performed through the C_{18} monolithic column only, the resulting trace is shown in Fig. 5.13.

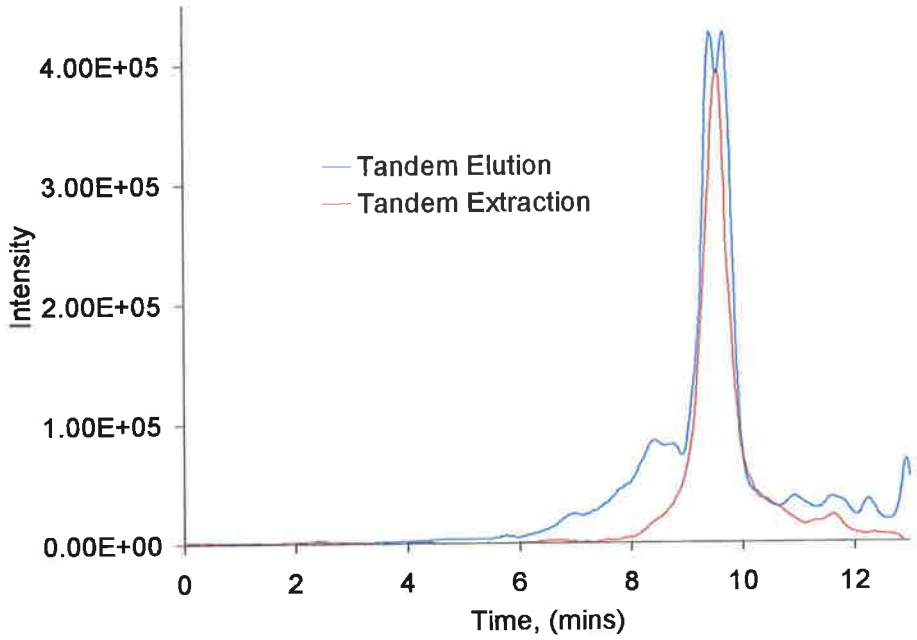


Figure 5.13: Overlay of EIC m/z 316 trace depicting the extraction of 1 μgL^{-1} ZnPT using the Si-SAX-C_{18} tandem column.

The Si-SAX-C_{18} column was found to function effectively as can be seen from Fig. 5.13, with a distinct peak clearly visible for ZnPT (the red trace). Retention was evaluated and it was found that the C_{18} monolithic column is solely responsible for the retention of extracted ZnPT with only a negligible peak observed when the Si-SAX column was eluted. The blue trace in Fig. 5.13 depicts the resulting chromatogram when tandem extraction followed by elution through the tandem column was performed, it can be seen that the ZnPT peak appears to be slightly split upon passage through the tandem column. However, no such splitting was observed when elution was performed only through the C_{18} monolithic column. It is also worth noting that when elution through the tandem column was performed the baseline of the resulting trace was considerably noisier that when elution was performed through the C_{18} monolithic column only. It is suspected that the reason for the increased level of baseline noise is due to the elution of loosely bound NOM on the Si-SAX sorbent with subsequent introduction onto the analytical column. In order to avoid such affects elution was performed through the C_{18} monolithic column only.

Having devised and optimised methods of matrix removal, i.e. washing and the use of a sacrificial Si-SAX sorbent, the applicability of the on-line SPE

procedure for the preconcentration of ZnPT from river water was examined. Fig. 5.14 following depicts a 1 μgL^{-1} ZnPT spike prepared in river water and extracted under optimum conditions. A clear sharp intense peak can be seen with minimal matrix interference thereby suggesting that the Si-SAX sorbent efficiently removes problematic NOM that was still retained using a solvent wash procedure alone.

A draw back of the Si-SAX sorbent was, that due to its efficient removal of NOM it became rapidly soiled and caused large increases in the operating backpressure, e.g. within 2-3 extractions. As a consequence it was decided to dilute samples with reagent water ten fold prior to extraction in order to increase the lifetime of the Si-SAX sorbent. Sample dilution increased the lifetime of the Si-SAX column considerably with no adverse effects upon reproducibility observed even after ten extractions thereby making the method more economically viable. Dilution also led to a decrease in the baseline noise due the reduced quantity of NOM present in the extraction solution.

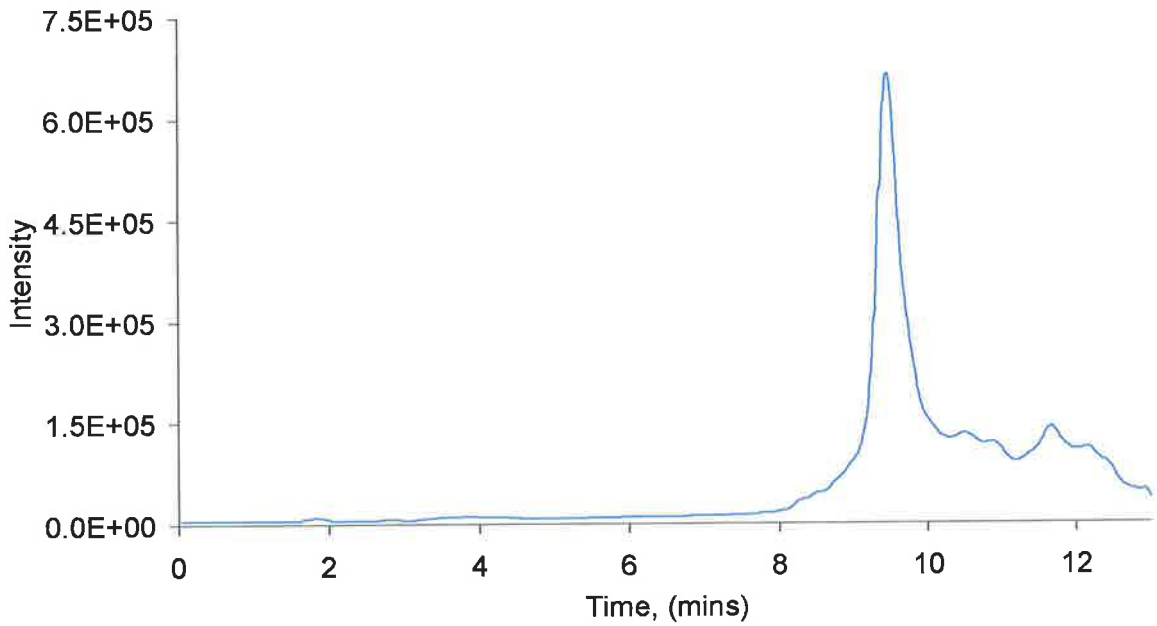


Figure 5.14: EIC m/z 316 chromatogram depicting the extraction of 1 μgL^{-1} ZnPT from river water.

Using optimised conditions a 10 μgL^{-1} ZnPT spike was prepared in river water, diluted ten fold and extracted. Possible sample to sample carryover was then evaluated by checking each component of the analytical system after elution and reconditioning. From Fig. 5.15 it can clearly be seen that there is negligible sample to sample carryover with no traces of ZnPT detected on either the

extraction or the analytical C_{18} monolithic columns and therefore, the possibility of cross contamination is expected to be quite small.

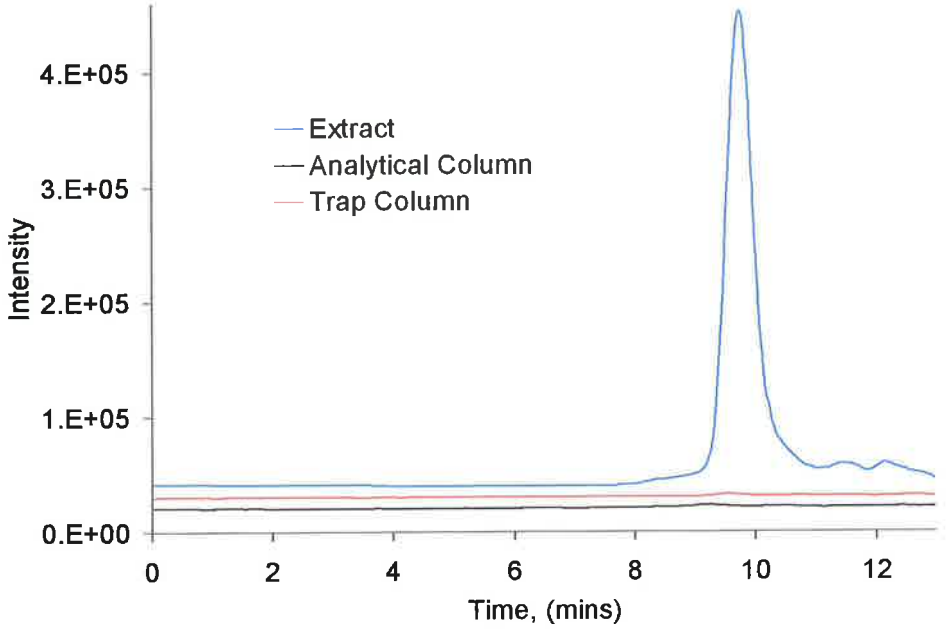


Figure 5.15: Overlay of EIC m/z 316 traces depicting minimal sample to sample carryover on the analytical and extraction columns.

5.3.6 Speciation in environmental samples; ZnPT or CuPT:

It was noted that when using APCI-MS detection that ZnPT introduced into the system via standard injection or by column switching yielded a more intense ion at m/z 316.0 than m/z 317.0. This observation was somewhat confusing as the ZnPT pseudomolecular ion previously observed using direct infusion was present at m/z 317.0 (Fig. 5.6). Upon examining the isotope pattern of resulting LC-APCI-MS peaks it was clear that the species present was CuPT and not ZnPT, i.e. ZnPT appears to transchelate within the LC system to form CuPT. Such an effect was also observed by Doose *et al.* who noted that ZnPT injected using LC-ESI-MS was detected only as PT_2 and FePT, therefore, it appears that ZnPT will automatically transchelate with other available metal ions present to form other metal pyrithione complexes [36]. Transchelation to form CuPT was also exploited by Thomas [37] for the LC-APCI-MS analysis of ZnPT and such an approach was noted by Doose *et al.* as a reasonable option for the chromatographic analysis of ZnPT [36]. Therefore, in order to ensure unavoidable but quantitative transchelation to CuPT it was deemed necessary to add a small excess of Cu^{2+} ions to all solutions after

dilution prior to extraction, in this instance a 20 fold excess of Cu^{2+} was judged to be adequate. Preliminary investigations were performed using copper (II) nitrate, copper (II) sulphate and copper (II) acetate and the different counter ions were found to have a distinct effect on the resulting CuPT peak.

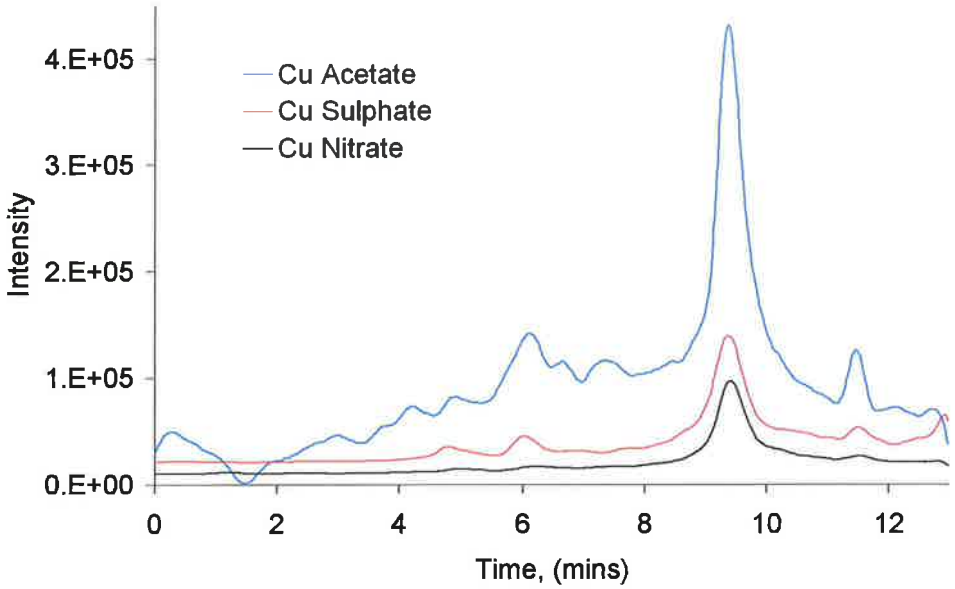


Figure 5.16: The effect of different copper salts upon the transchelation of ZnPT to CuPT: extracted EIC m/z 316 traces depicting a 1 μgL^{-1} ZnPT extract to which a 20 fold excess of Cu^{2+} had been added, samples prepared in river water.

It is clear from Fig. 5.16 that copper (II) acetate allowed for the transchelation of ZnPT to CuPT without affecting the APCI-MS response whereas both the sulphate and nitrate salts had more profound negative effects upon the resulting CuPT peak, the reason for such an effect remains unclear as both copper (II) sulphate and copper (II) nitrate were used to transchelate ZnPT by Thomas [37]. Due to this observation only copper (II) acetate was used for in further studies.

5.3.7 Method performance:

The performance of the on-line SPE LC-APCI-MS was evaluated using river water as a real sample matrix collected from the River Broadmeadow prior to its entry into the Malahide estuary near Swords in north Co. Dublin, Ireland. Before usage, the river water was filtered through Whatman GF/C glass fibre filters to remove suspended material. Spike solutions were prepared in the filtered river water,

diluted with reagent water with the addition of 20 μgL^{-1} Cu^{2+} and pH adjusted prior to extraction. Method performance data is presented in Table 5.2.

Table 5.2: On-line SPE LC-APCI-MS method performance data.

Parameter	Result
Sensitivity Validation:	
Limit of detection, (ngL^{-1})	18
Limit of quantitation, (ngL^{-1})	62
Method Precision, (%RSD, n = 10)	27
Linearity, (R^2)	0.9802
Recovery, (%, n = 3)	72 ± 9

The limits of detection and quantitation were calculated as three and ten times the standard deviation of the baseline noise respectively for blank extractions of 200 mL aliquots of diluted Broadmeadow river water. The signal to noise ratio was calculated by peak height comparison with a 1 μgL^{-1} ZnPT spike, (as CuPT) also prepared in river water. The calculated limits indicate an acceptable level of analytical sensitivity as it was previously mentioned that the postulated ZnPT PEC in the aquatic environment was 0.04 μgL^{-1} [11]. It should also be noted that the sensitivity of the method can be improved by extracting a larger volume of sample, therefore, yielding a larger preconcentration factor. Such an approach may not be feasible due to subsequent increases in analysis times, greater proportions of NOM extraction leading to more rapid Si-SAX spoilage *etc.* The calculated LOD also compares well with that obtained by Thomas [37] who achieved a LOD of 20 ngL^{-1} ZnPT, however, the sample volume extracted was 2 L as opposed to the 200 mL, (after dilution) extracted using the on-line SPE LC-APCI-MS method. Solvent use and waste generated is also minimised.

The calculated levels of precision and recovery also compare well with the method of Thomas, where overall method precision and recovery were 17% and 77% respectively [37], whereas using the on-line SPE LC-APCI-MS method precision and recovery were found to be 27% and 72% respectively. Considering the complexity of the sample matrix the calculated levels or precision and analyte recovery are deemed acceptable. Linearity was determined in the region of 0.25 to

10 μgL^{-1} prior to dilution and the calculated regression coefficient was R^2 = 0.9802. Although not ideal, considering that the overall method of precision was 27% the R^2 was also deemed acceptable. From the determined method performance data it was concluded that the developed on-line SPE LC-APCI-MS method was sufficiently sensitive and suitable for the determination of ZnPT in aqueous environmental samples.

5.3.8 Application to samples and shampoo analysis:

Samples of fresh and sea water were collected from the Malahide estuary in north Co. Dublin and analysed for ZnPT content using the developed and optimised on-line SPE LC-APCI-MS method. However, no ZnPT was detected in any of the collected samples. In order to examine the applicability of the method, two well known brands of anti-dandruff shampoo known to contain ZnPT as the active ingredient were purchased and diluted in river water as a model waste matrix and analysed using the developed method. Dilutions of the shampoo samples of 1000-fold (Head and Shoulders™, Proctor & Gamble) and 500-fold (Dove™, Unilever) were first prepared, with an aliquot of each further diluted 10^4-fold in river water. The spiked river water samples were finally diluted 1/10 with reagent water prior to analysis. As was previous 20 μgL^{-1} Cu^{2+} was added to the sample in order to ensure transchelation to CuPT. Resulting traces for the on-line SPE LC-APCI-MS analysis of the shampoo samples in river water can be seen in Fig. 5.17.

From Fig. 5.17 it should be seen that anti-dandruff shampoo is a significant emission source of ZnPT into the environment via municipal wastewater. The quantities of shampoo used for the above experiment, (250 mg Head and Shoulders and 500 mg Dove) were significantly smaller than those likely to be regularly used by consumers and in this instance even with 10^8 fold dilution ZnPT, as CuPT, could still be detected in a waste matrix. The similarity in peak areas found was highly encouraging, given the 10^8-fold dilution of the original samples, the ultra trace concentration and the complexity of the sample matrix involved. Due to the probability of regular usage and even unwarranted usage of anti-dandruff shampoo by the public, it is proposed that household wastewater is a more significant source of ZnPT into the aquatic environment than marine antifouling booster biocide paints.

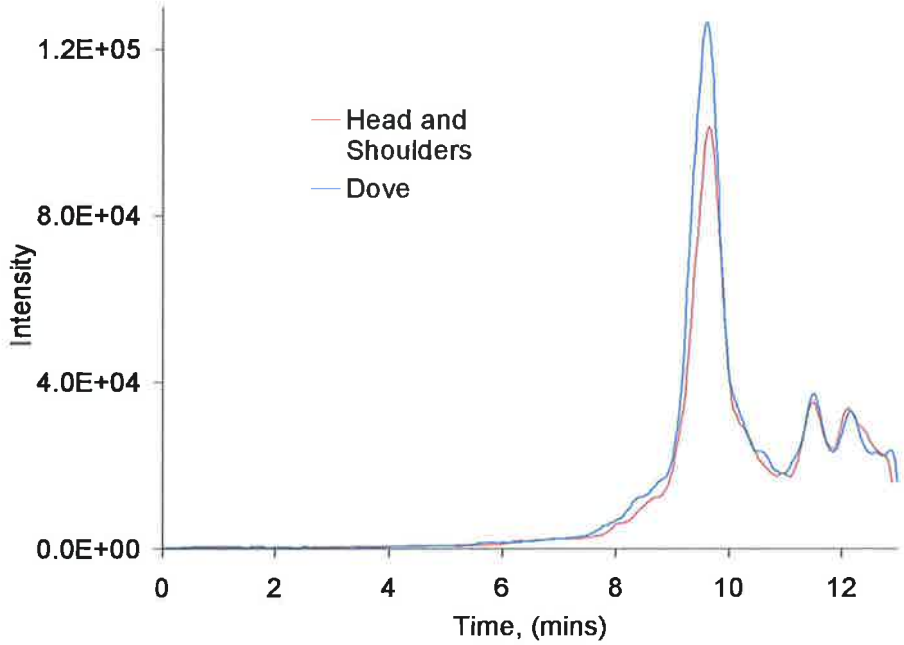


Figure 5.17: The determination of ZnPT as CuPT in anti-dandruff shampoo samples diluted in river water as a waste matrix. The blue trace represents Dove Anti-Dandruff shampoo which contains 0.37% w/w ZnPT, the concentration corresponding to the blue trace is ~76 ngL^{-1}. The red trace represents Head and Shoulders Classic Clean shampoo which contains 0.80% w/w ZnPT, the concentration corresponding to the red trace is ~80 ngL^{-1} [6].

5.3.9 An assessment of the environmental fate of ZnPT and CuPT:

As no ZnPT, as CuPT, was present in the collected water samples an investigation into the possible fate of the pyrithione complexes was performed. Having noted the effect of the copper salt counter ion previously, it was decided to investigate the effect of contact with common anions, sorption to suspended materials, sediment partioning and photochemical attenuation.

Solutions of 5 mgL^{-1} ZnPT were prepared in water containing varying concentrations of chloride, sulphate, nitrate and phosphate in the range of 5-20 mgL^{-1} and 5 mgL^{-1} Cu^{2+}. The solutions were then analysed using LC-UV in order to examine the effect of initial contact and then reanalysed after both 24 and 48 hours respectively in order to determine the effect of contact time. Of the anions tested only sulphate had an immediate effect upon CuPT contact with a reduction of the resulting CuPT peak area with increasing sulphate concentration. Nitrate, chloride and phosphate had no pronounced effect upon immediate contact. Contact time also appeared to have no effect upon the formed CuPT with a general reduction in peak area with time in both control and anion containing solutions. Therefore, ZnPT

as CuPT in the environment appears to be unaffected by the presence of inorganic anions, even though some ions such as nitrates have been previously observed to act as photosensitisers to organic micro-pollutants no such effects were observed in this study, [54].

The sorption of pyrithione metal complexes onto suspended materials was also evaluated. 1 L aliquots of collected river and sea water samples were filtered through Whatman GF/C filter papers, air dried and then eluted using a solvent mixture of 50:50 dichloromethane MeOH. The solvent was filtered through glass wool and evaporated to near dryness under a gentle stream of N_2. Resulting residue was reconstituted with 200 µL MeOH and analysed using direct injection LC-APCI-MS. Both the filter paper and the glass wool used for filtration were also checked for cross contamination. However, no CuPT was detected on any of the extracted filter papers thereby suggesting that CuPT does not adsorb onto suspended media in the aqueous phase.

Reports concerning the adsorption of pyrithione metal complexes onto sediment in the published literature are quite scarce and of those published there appears to be a consensus that pyrithione metal complexes will rapidly degrade in the aqueous phase and the likelihood of any sediment adsorption is therefore, quite small. Any pyrithione metal complexes that do adsorb onto sediment are thought to be degraded via either sediment catalysed degradation or microbial degradation *etc.* [11,15,17]. Due to the attenuation of incident light with depth, there exists a probability that any pyrithione metal complexes that reach sufficient depth to intimately interact with sediment may indeed adsorb due to the absence of wavelengths of light thought to cause photolytic degradation. Therefore, using two certified reference sediments obtained from the National Research Council of Canada, the adsorption of ZnPT onto the sediment phase was determined. The two sediment reference materials varied in their total organic carbon content (TOC) and also their metal content, of particular importance were the concentrations of copper and zinc, values for which are inserted in Table 5.3.

Table 5.3: TOC and metal content of the two certified sediment samples.

Component	BCSS-1	PACS-1
C (%)	2.19 ± 0.09	3.69 ± 0.11
Zn ($\mu g.g^{-1}$)	119 ± 12	824 ± 22
Cu ($\mu g.g^{-1}$)	18.5 ± 2.7	452 ± 16
Sampling Location	*Baie des Chaleurs, Gulf of St. Lawrence*	*Esquimalt Harbour British Colombia*

Using amber glass sample vials, 5 mL of 5 mgL^{-1} ZnPT in water was added to 0.5 g of sediment and repeatedly shaken by hand several times over the course of two hours. For each sediment sample the behaviour of a control and two ZnPT solutions were examined. An aliquot of the aqueous phase was then withdrawn and placed in an amber vial for subsequent LC-APCI-MS analysis. The remaining aqueous phase was removed via filtration and the sediment sample was air dried overnight in the dark. The sediment was then transferred to another amber sample vial and extracted with 5 mL of 50:50 dichloromethane MeOH. The solvent solution was filtered through nylon filters, reduced in volume under N_2 and finally reconstituted with 200 µL of MeOH before LC-APCI-MS analysis. Both the nylon and paper filters used were also analysed by LC-APCI-MS in order to examine any possible sources of cross contamination. The concentration of CuPT was determined from a prepared five point calibration curve and can be viewed in Table 5.4 following.

Table 5.4: Calculated concentrations of CuPT in the aqueous and sediment phases after a 2 hour mixing period.

	BCSS-1	PACS-1
Aqueous phase, (mgL^{-1})	0.11	0.00
Sediment phase, ($mgkg^{-1}$)	0.43	0.86
Sediment:aqueous phase ratio	3.91	-
Amount of residue in aqueous phase, ($\mu g\ vial^{-1}$)	0.55	0.00
Amount of residue in sediment phase, ($\mu g\ vial^{-1}$)	0.22	0.43
% Initial dose in aqueous phase	2.20	0.00
% Initial dose in sediment phase	0.88	1.72

From Table 5.4 it can be seen that the measured concentrations of CuPT in each phase differ for the two sediment samples, see Fig. 5.18. The reason for the difference is attributable to the TOC content of the sediments with a higher proportion of ZnPT adsorbed and transformed on the PACS-1 sediment that

contains larger quantities TOC and metals. Therefore, based upon the above findings it can be estimated that distribution ratio for the BCSS-1 sediment is ~4:1 and consequently if intimate contact occurs between water containing pyrithione metal complexes and sediment, the sediment would be expected to preferentially concentrate the pyrithione metal complex from the water due to the high partition coefficient of ZnPT. Such an effect is illustrated more clearly with the PACS-1 sediment whereby all of the detected CuPT was found solely on the sediment phase. However, upon conversion of the measured data to a percentage initial dose, it can be seen that approximately 97% of ZnPT introduced into the experimental vial disappeared over the course of the experiment. Similar observations were also noted by Turley *et* al. who found that ZnPT rapidly converted into 2-pyridine sulphonic acid when also performing sorption experiments as a result of light exposure [17]. From such data it can be deduced that ZnPT and CuPT present in the environment are unlikely to accumulate on sediment due to the existence of a more important removal mechanism despite their high partitioning behaviour [17]. However, the need therefore, still exists for further research to determine the effect of pyrithione metal complexes on sediment biota and to also determine the actual fate of pyrithione metal complexes adsorbed onto sediment.

As mentioned previously, photochemical attenuation appears to be the most important removal mechanism of pyrithione metal complexes introduced into the aquatic environment. However, of the photochemical attenuation studies reported in the literature, the majority were performed using bioassays and primarily in a marine water matrix. As observed in this study, anti-dandruff shampoo appears to be a more significant source of ZnPT introduced into the aquatic environment, especially into fresh water systems, than marine antifouling paints and also ZnPT introduced into the environment will transchelate to form CuPT. Therefore, a brief study was performed to examine the photochemical attenuation of CuPT in filtered river water. A 10 mgL^{-1} solution of CuPT in river water, along with a control sample was placed in a clear glass bottle an exposed to natural sunlight on the laboratory window during April 2006. Sacrificial samples were withdrawn from the clear glass bottle into amber vials and immediately frozen until LC-APCI-MS analysis. The relative presence of CuPT was plotted against time as in Fig. 5.19 following. The

resulting plot correlates well with previously published data [11,16,17,37] with a relatively rapid decline of CuPT observed, the estimated half life from the graph is ~45 minutes.

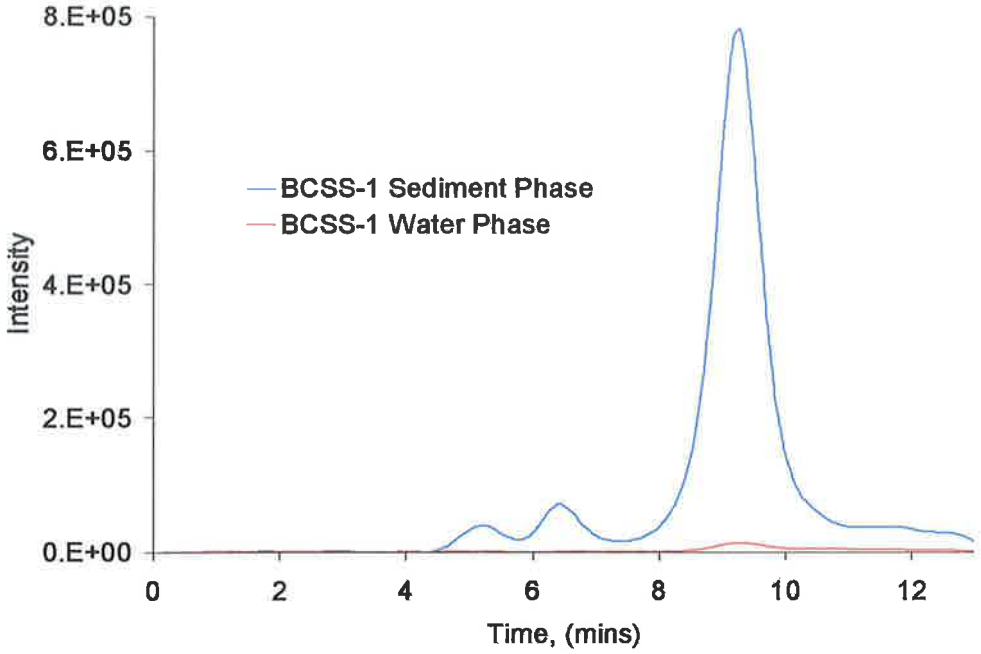


Figure 5.18: Overlaid EIC m/z 316 traces for the analysis of the BCSS-1 extracted and preconcentrated sediment phase and the BCSS-1 water phase.

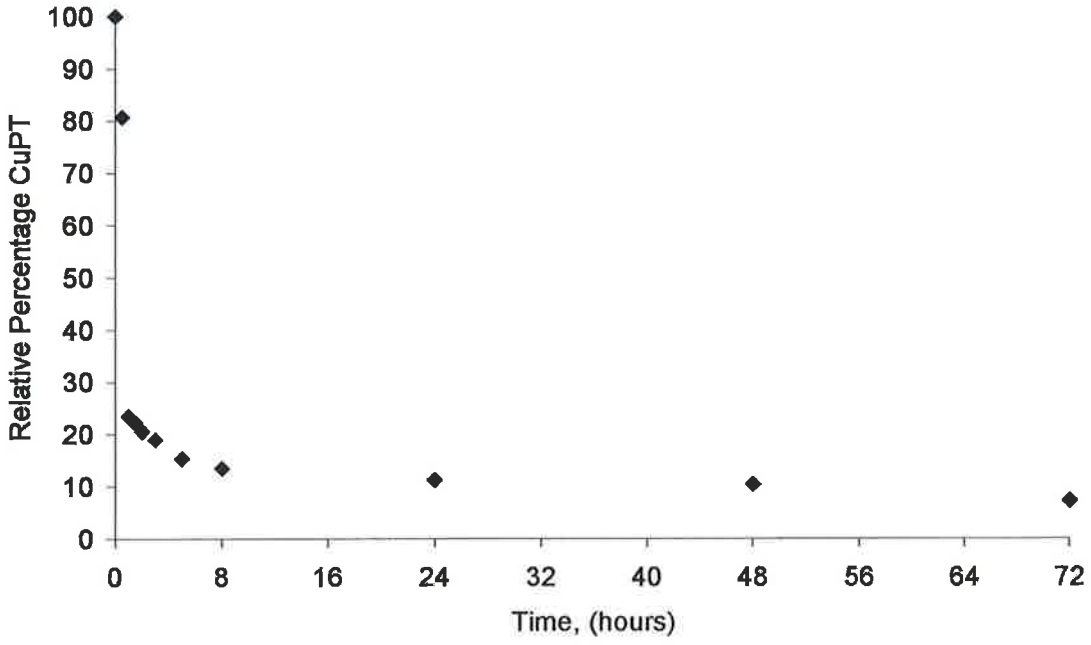


Figure 5.19: Degradation of CuPT in filtered river water upon exposure to natural sunlight under laboratory conditions.

Although CuPT appears to initially rapidly disappear it can be seen from the above plot that the rate of photochemical attenuation appears to decrease with increasing time, consequently as ZnPT is likely to be constantly introduced into the aquatic environment via untreated municipal wastewater, the probability exists that ZnPT may at times reach detectable concentrations depending on the input flux of ZnPT, the availability of Cu^{2+} and also the light exposure of receiving waters. However, in waters with high light intensity and high mixing rates it is expected that photochemical attenuation is likely to be an extremely efficient removal process for introduced metal pyrithione complexes.

5.4 Conclusions:

A column switching LC method in conjunction with APCI-MS detection has been developed for the extraction and analysis of ZnPT, (as CuPT) in aquatic environmental samples. Monolithic silica columns were chosen for use for the analytical separation due to the low silanol activity and lack of metallic contamination and also for the performance of on-line SPE due the ability to use high flow rates without the generation of excessive backpressure. Excessive matrix co-extraction and consequent ion suppression was found to be problematic and required the use of both a sacrificial Si-SAX sorbent prior to the C_{18} extraction column and a subsequent wash step for the removal of NOM. The method was validated in a real sample matrix and high levels of sensitivity with acceptable analyte recovery and reproducibility attained. The method is likely to be of use to those who wish to monitor the presence of ZnPT in the aquatic environment.

Based upon results of an environmental fate study it appears that ZnPT will transchelate to form CuPT in environmental waters. CuPT appears to be unaffected by the presence of inorganic anions and does not appear to adsorb onto suspended solids. Photochemical attenuation appears to be the most important removal mechanism of CuPT. However sediment adsorption has also been shown to occur, particularly in sediments with a high proportion of TOC. It is therefore concluded that ZnPT introduced into the environment e.g. via municipal wastewater is rapidly degraded in well illuminated waters with a high rated of mixing, e.g. fast flowing streams and rivers. Further research needs to be conducted to determine

the behaviour and removal of pyrithione metal complexes during municipal wastewater treatment, the characterisation and risk evaluation of degradation products in water and sediments should also be performed.

References:

1. Activities of the European Union, "*Maritime safety: prohibition of organotin compounds on ships*", viewed February 13th 2006, http://europa.eu.int/scadplus/leg/en/lvb/124256.htm.
2. S.M. Evans, A.C. Birchenough & M.S. Brancato, *Marine Pollution Bulletin*, **40**, (2000), 204.
3. W.A. Lott and E. Shaw, *Journal of the American Chemical Society*, **71**, (1949), 70.
4. E. Shaw, J. Berntstein , K. Losee & W.A. Lott, *Journal of the American Chemical Society*, **72**, (1950), 4362.
5. B.L. Barnett, H.C. Kretschmar & F.A. Hartman, *Inorganic Chemistry*, **16**, (1977), 1834.
6. The Swedish Society for Nature Conservation, "*The Investigation of Zinc Pyrithione in Dandruff Shampoo*" (2004), viewed March 30th 2006, http://www.snf.se/pdf/rap-hmv-dandruffeng.pdf
7. N. Voulvoulis, M.D. Scrimshaw & J.N. Lester, *Applied Organometallic Chemistry*, **13**, (1999), 135.
8. E. Guthery, L.A. Seal & E.L. Anderson, *American Journal of Infection Control*, **33**, (2005), 15.
9. E. Ermolayeva & D. Sanders, *Applied and Environmental Microbiology*, **61**, (1995), 3385.
10. R.J. Knox, N.S. Magoski, D. Wing, S.J. Barbee & L.K. Kaczmarek, *Journal of Neurobiology*, **60**, (2004), 411.
11. P.A. Turley, R.J. Fenn & C.J. Ritter, *Biofouling*, **15**, (2000), 175.
12. K.V. Thomas, M. M^{c}Hugh & M. Waldock, *The Science of the Total Environment*, **293**, (2002), 117.
13. K.V. Thomas, M. M^{c}Hugh, M. Hilton & M. Waldock, *Environmental Pollution*, **123**, (2003), 153.
14. K.V. Thomas, S.J. Blake & M.J. Waldock, *Marine Pollution Bulletin*, **40**, (2000), 739.
15. R.M. Galvin, J.M. Rodiguez Mellado & M. Ruiz Montoya, *European Water Management*, **1**, (1998), 61.
16. K. Maraldo & I. Dahllof, *Marine Pollution Bulletin*, **48**, (2004), 894.
17. P.A. Turley, R.J. Fenn, J.C. Ritter & M.E. Callow, *Biofouling*, **21**, (2005), 31.
18. P-J. Sun, Q. Fernando & H. Freiser, *Analytical Chemistry*, **36**, (1964), 2485.
19. F. Sanchez-Bayo & K. Goka, *Aquatic Toxicology*, **74**, (2005), 285.
20. I.K. Konstantinou & T.A. Albanis, *Environment International*, **30**, (2004), 235.
21. N. Kobayashi & H. Okamura, *Marine Pollution Bulletin*, **44**, (2002), 748.
22. J. Bellas, A. Granmo & R. Beiras, *Marine Pollution Bulletin*, **50**, (2005), 1382.
23. K. Goka, *Environmental Research*, **81**, (1999), 81.
24. H. Okamura, T. Watanabe, I. Aoyama & M. Hasobe, *Chemosphere*, **46**, (2002), 945.
25. J. Karlsson & B. Eklund, *Marine Pollution Bulletin*, **49**, (2004), 456.

26. D. Groth Petersen, I. Dahllof & L.P. Nielsen, *Environmental toxicology and Chemistry*, **23**, (2004), 921.
27. C.A. Doose, J. Ranke, F. Stock, U. Bottin-Weber & B. Jastorff, *Green Chemistry*, **6**, (2004), 259.
28. A.F. Krivis, E.S. Gazda, G.R. Supp & M.A. Robinson, *Analytical Chemistry*, **35**, (1963), 966.
29. L-H. Wang, *Electroanalysis*, **12**, (2000), 227.
30. D.S. Mackie, C.M.G. van den Berg & J.W. Readman, *Analytica Chimica Acta*, **511**, (2004), 47.
31. M. Ruiz Montoya, R.M. Galvin & J.M. Rodriguez Mellado, *Electroanalysis*, **10**, (1998), 1030.
32. Y. Shih, J-M. Zen, A.S. Kumar & P-Y. Chen, *Talanta*, **62**, (2004), 912.
33. R.J. Fenn & M.T. Alexander, *Journal of Liquid Chromatography*, **11**, (1988), 3403.
34. N. Voulvoulis, M.D. Scrimshaw & J.N. Lester, *Chemosphere*, **38**, (1999), 3503.
35. K. Nakajima, T. Yasuda & I. Nakazawa, *Journal of Chromatography*, **502**, (1990), 379.
36. C.A. Doose, M. Szaleniec, P. Behrend, A. Muller & B. Jastorff, *Journal of Chromatography A*, **1052**, (2004), 103.
37. K.V. Thomas, *Journal of Chromatography A*, **833**, (1999), 105.
38. Y. Yamaguchi, A. Kumakura, S. Sugasawa, H. Harinos, Y. Yamada, K. Shibata & T. Senda, *International Journal of Environmental Analytical Chemistry*, **86**, (2006), 83.
39. Intersil Quality Control Manual Chromaographic tests, www.intersil.com/technical_data/inertness.pdf, viewed
40. A.I. Elefterov, S.N. Nosal, P.N. Nesterenko & O.A. Shpigun, *Analyst*, **110**, (1994), 1329.
41. The University of Sheffield Chemputer isotope calculator, http://winter.group.shef.ac.uk/chemputer, viewed April 29th 2006.
42. C. Baldwin, J. Cumming & A.T. Timperman, *The Analyst*, **130**, (2005), 318.
43. A.R.S. Ross, M.G. Ikonomou & K.J. Orians, *Marine Chemistry*, **83**, (2003), 47.
44. F.C. Wu, R.D. Evans & P.J. Dillon, *Analytica Chimica Acta*, **452**, (2002), 85.
45. I. Paunovic, R. Schulin & B. Nowack, *Journal of Chromatography A*, **1100**, (2005), 176.
46. A.S. Gordon, B.J. Dyer, R.A. Kango & J.R. Donat, *Marine Chemistry*, **53**, (1996), 163.
47. J.R. Donat, R.A. Kango & A.S. Gordon, *Marine Chemistry*, **57**, (1997), 1.
48. V. Pichon, C. Cau Dit Coumes, L. Chen, S. Guenu & M.C. Hennion, *Journal of Chromatography. A*, **737**, (1996), 25.
49. M. Ibanez, Y. Pico & J. Manes, *Journal of Chromatography A*, **823**, (1998), 137.
50. A. Di Corcia, A. Constantino, C. Crescenzi & R. Samperi, *Journal of Chromatography A*, **852**, (1999), 465.
51. J.E. Renew & C-H. Huang, *Journal of Chromatography A*, **1042**, (2004), 113.
52. I. Ferrer, D. Barcelo & E.M. Thurman, *Analytical Chemistry*, **71**, (1999), 1009.
53. R.J.C.A. Steen, A.C. Hogenboom, P.E.G. Leonards, R.A.L. Peerboom, W.P. Confino & U.A.Th. Brinkman, *Journal of Chromatography A*, **857**, (1999),157.
54. R. Andreozzi, M. Raffaele & P. Nicklas, *Chemosphere*, **50**, (2003), 1319.

6.0 Comparison of Monolithic and Sub 2µm Particle Packed Columns – Application to the Determination of Drug Contamination on Irish Euro Banknotes.

6.1 Introduction:

It has recently been reported that the use of illicit drugs by Irish society, in particular cocaine, has escalated dramatically. For example, one national newspaper stated that cocaine usage alone had increased some 829% in the period of 2001 to 2005 with cannabis resin usage rising approximately 750% based upon drug seizure data [1]. The illicit drug trade is big business to those involved and conservatively estimated to be worth close to €650 million annually [2]. Official An Garda Síochána statistics available concerning offences committed under The Misuse of Drugs Act 1984 corroborate media reports and show that while cannabis is still the most commonly abused illicit drug by Irish society, (accounting for ~65% of all offences), cocaine has now overtaken amphetamines and opiates to become the second most commonly abused drug in the country [3]. Statistics mentioned are, however, thought to greatly underestimate the actual levels of illicit drug usage within the community as they only reflect those cases where a prosecution under the Misuse of Drugs Act 1984 has occurred and, therefore, do not include usage data concerning social and recreational users.

Gauging the actual levels of drug use in the country appears to be an onerous task. Traditionally survey based methodologies have been used but such techniques are inadequate as they are often targeted at specific subgroups within the population, e.g. known drug addicts. Such methods are also susceptible to bias either positive or negative, depending upon the social group studied. For example, it would be expected that a general survey would be negatively biased and therefore, underestimate the actual use due to the fact that recreational drug users would not be expected to openly admit their behaviour, especially when it involves breaking the law. As a consequence survey based methods are likely to report

anecdotal information and speculation rather than scientific facts [4]. Another method previously used for the estimation of drug use has focused upon the numbers of people presenting themselves for drug treatment. It was also recently reported that such a phenomenon is no longer mainly confined to the capital city with numbers seeking treatment in regions outside the Eastern Regional Health Authority having almost trebled [5]. However, the problem exists that the Irish treatment system has long been focused upon opiates and may not be sufficiently equipped to deal with the recent surge in cocaine usage [4].

Previously published reports have demonstrated that paper currency in general circulation may be contaminated with detectable levels of illicit materials, most commonly cocaine [6,7,8,9]. Such contamination is likely to occur due to rolled up bank notes being used to 'snort' substances or as a result of transfer during drug deals [10,11,12]. Cross contamination between notes during counting processes in financial institutions is also possible as it has been demonstrated that nanogram quantities of cocaine can be transferred from contaminated to other bank notes using both manual and automated counting procedures [13]. Although not a quantitative measure, the presence of illicit substances on bank notes in general circulation provides an indication that such substances are being used within the community under study. Recently, Zucatto *et al.* have proposed monitoring for the presence of illicit drug residues in surface waters arising from human use, which appears to offer a more robust method for estimating drug use within the community [14].

A limited number of analytical methods for the determination of drug contamination on banknotes are available in the published literature and may be divided into two groups; those which use GC-MS or GC-MS/MS [6,8,9] and those which directly analyse banknotes using thermal desorption tandem MS (TD-MS/MS) [13,15,16,17]. Surprisingly, due to the polarity of many illicit substances, only one method based upon liquid phase separation techniques is available in the peer-reviewed literature, [18]. Xu *et al.* explored the possibility of using CE with electrochemiluminesence detection (CE-ECL) for the determination of heroin and cocaine on Chinese currency. While the method was successfully applied, it is worth noting the lack of specificity compared to MS based methods. In the case of GC-MS/MS based methods a sample preparation step is necessary in order to

elute the compounds of interest from the surface of the banknote. Esteve-Turrillas *et al.* reviewed sample preparation procedures that usually involve washing the note with organic solvents and recommended that in the case of Euro banknotes MeOH should be used, as destructive problems previously encountered when using other organic solvents were completely avoided [7]. In some cases a SPE procedure has also been incorporated into the method in order to remove co-extracted compounds such as inks, oils fats and greases and cosmetics *etc.* [8]. TD-MS/MS methods have the advantage of simpler sample preparation, whereby dust from the banknotes is trapped on filter meshes and then directly desorbed into the source of the MS [15]. Although it would be considered that such a process would lead to complete destruction of the sample and therefore, remove the possibility for replicate analysis, the authors claim that "the process does not remove all material and further analyses may be preformed on any of the meshes" [15]. Consequently, it appears that although TD-MS/MS methods provide the possibility to analyse a greater number of notes simultaneously, the desorption procedure is inefficient and therefore, does not provide an accurate reflection of the actual levels of drug contamination on circulating currency. Tandem MS detection is commonly used due to the ability to attain a structural fingerprint for the analytes of interest and therefore, the ability to unequivocally identify the compounds present in a sample, often a necessity if the analytical results are to be presented as evidence in court [19].

Current advances in the field of LC are driven by the requirement for fast high throughput separations and also the requirement to achieve maximum selectivity, i.e. the ability to separate all compounds present in a complex mixture in order to permit their quantitation and identification [20]. To facilitate the above requirements, recent advances in column technology have included the introduction of sub 2 μm particle packed and monolithic type stationary phases. Other attempts to achieve faster more efficient analyses have focused upon the use of high separation temperatures in order to reduce the viscosity of the mobile phase but such an approach is however, not desirable due to potential problems with both analyte and stationary phase stabilities at elevated temperatures [21]. The advantages of reducing the size of stationary phase particles are widely known, namely improved efficiency with faster analysis times and greater

sensitivity [20,22]. However, such gains are often offset by, or unattainable due to the increased backpressure needed to pump solvents through columns containing very small particles. In addition to the generation of excessive backpressure, detectors capable of fast responses and rapid sampling rates are also required in order to achieve the maximum benefit from the chromatographic analysis. Another problem generated by the excessive backpressure required when using sub 2 µm particles is that of frictional heating and the generation of non uniform temperature gradients within the column and therefore, the resulting effects upon retention and efficiency [23,24]. The effect of frictional heating and very high operating pressures upon behaviour of analytes is currently attracting attention.

In contrast to sub 2 µm particulate media, monolithic silica columns, which allow for the achievement of high efficiency without the generation of excessive backpressure, are now available, (section 2.3.5). Although monolithic columns are therefore, an attractive prospect they are not without their limitations, namely higher solvent consumption, lack of available stationary phase chemistries and also until recently lack of direct compatibility with MS detection [20]. However, the recent introduction of a 3 mm i.d. monolithic silica reversed-phase column on to the market helps to overcome the above limitations, most importantly facilitating easier coupling to MS.

The objective of this study was first to directly compare a commercially available 1.8 µm particle packed column with recently available 3 mm i.d. monolithic silica columns for the reversed-phase separation of strongly basic compounds, i.e. illicit drugs. Secondly, this work developed, for the first time, an optimised LC-MS/MS method based upon the new monolithic phases for the determination of illicit drug and abused pharmaceuticals and its application to the evaluation of contamination on Irish Euro banknotes in current circulation in the greater Dublin area, with the aim of establishing an indication of drug use, particularly cocaine, within the community.

6.2 Experimental:

6.2.1 Chemicals and reagents:

Reagent water used throughout this study was obtained from a Millipore Milli-Q water purification system, (Millipore, Bedford, MA, USA) and was 18.2 MΩ or greater. MeOH, MeCN and dichloromethane were received from Labscan (Dublin, Ireland), all solvents used were HPLC grade. Cocaine hydrochloride, morphine sulphate salt pentahydrate, methadone hydrochloride, ketamine hydrochloride, heroin and Δ^9-tetrahydrocannabinol methanolic solution (Δ^9-THC) were purchased under license from Sigma-Aldrich (St. Louis, MO, USA). Benzoylecgonine hydrate, cocaethylene, D-amphetamine sulphate salt, temazepam, diazepam, carbamazepine, fluoxetine hydrochloride, lysergic acid diethylamide (LSD), papaverine hydrochloride, 3,4-methylenedioxymethamphetamine hydrochloride (MDMA) and 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine perchlorate (EDDP) were purchased under license from Sigma-Aldrich (Poole, UK). The chemical structure of each drug is depicted in Table 6.1. AnalaR grade glacial acetic acid and hydrochloric acid were obtained from BDH Chemicals (Poole, UK). Ammonium acetate, (ACS reagent grade) was purchased from Riedel de Haën (Sleeze, Germany) and ammonium hydroxide solution, (LC-MS additive grade) was obtained from Fluka (Steinheim, Germany).

Individual 100 mgL^{-1} stock solutions of each chemical were prepared in MeOH and were stored at 4°C in the dark. Working solutions were prepared from the individual stock standards using water as a diluent. In the case of salts, standard solutions were prepared in terms of the parent analyte. Stock solutions were retained for the duration of the research, working solutions were prepared fresh prior to use.

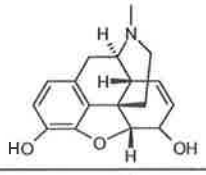
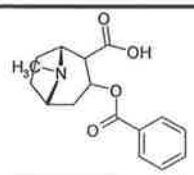
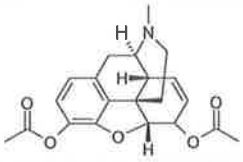
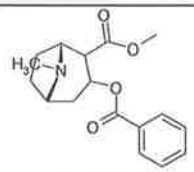
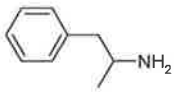
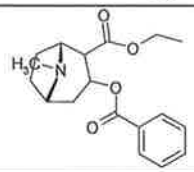
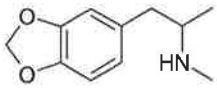
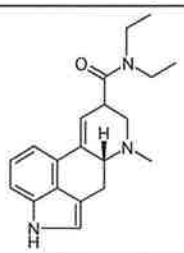
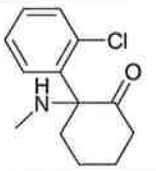
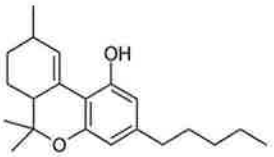
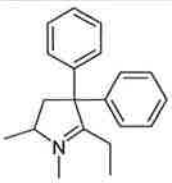
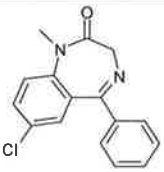
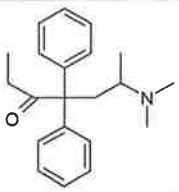
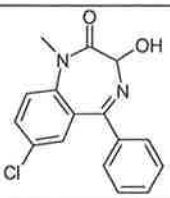
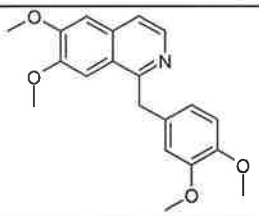
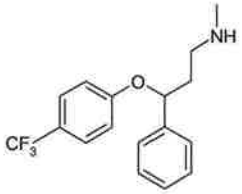
6.2.2 Glassware preparation:

Silanisation was performed as per section 3.2.3.

6.2.3 LC-MS/MS analysis:

LC instrumentation used was as per section 3.2.5 and 4.2.5.

Table 6.1: The chemical structures of the illicit drugs and abused pharmaceuticals chosen for study.

Analyte	Structure	Analyte	Structure
Morphine		Benzoylecgonine	
Heroin		Cocaine	
Amphetamine		Cocaethylene	
MDMA		LSD	
Ketamine		Δ^9-THC	
EDDP		Diazepam	
Methadone		Temazepam	
Papaverine		Fluoxetine	

6.2.4 Chromatographic conditions:

Columns investigated included a Zorbax Rapid Resolution HT Eclipse XDB-C_{18} 50.0 x 2.1 mm i.d. 1.8 µm column (Agilent Technologies, Palo Alto, CA, USA) and Phenomenex Onyx monolithic C_{18} 100.0 x 3.0 mm i.d. columns, (Phenomenex, Macclesfield, Cheshire, UK). In order to obtain a 200.0 x 3.0 mm i.d. column, two monolithic columns were connected in series using an Onyx column coupler. For column comparison studies an isocratic mobile phase consisting of 60:40 MeOH water was used for the separation of a mix standard containing 1 mgL^{-1} carbamazepine, temazepam and diazepam. For the separation of the complete mix of illicit drugs a multi-step linear gradient of 5 mM ammonium acetate, pH 4.5 and MeOH was used. The initial mobile phase conditions were 20% MeOH increased linearly to 30% in 2 minutes, followed by another linear increase in 3 minutes to 50% MeOH, then to 80% MeOH over the next 15 minutes with a final linear increase to 95% MeOH in 2 minutes, which was then held isocratically for the remainder of the chromatographic run. A re-equilibration time of 8 minutes was applied. In all instances the injection volume was 20 µL.

For least squares weighted regression analysis of van Deemter plots the Solver add-in of Microsoft Excel™ as previously described by Harris was used [25].

6.2.5 Mass Spectrometry:

For ion optic optimisation, 20 mgL^{-1} solutions of analyte in 1:1 MeOH 5 mM ammonium acetate buffer, pH 4.5 were directly infused into the MS using a Cole Parmer 74900 series syringe pump at a rate of 120 μLh^{-1} (Cole Parmer, Vernon Hills, IL, USA). All parameters were automatically optimised using the Bruker esquire software for the pseudomolecular ions of all analytes. An average of all recorded values was then used in order to achieve the ionisation and focusing parameters for use in conjunction with LC-MS/MS, (Table 6.2).

Table 6.2: Optimised ion focusing parameters for ESI-MS/MS detection, (the optimisation was performed using a nebuliser pressure of 10.0 psi, a dry gas flow of 3.0 $Lmin^{-1}$ and a drying temperature of 300°C. Spectra were collected over the range 50 – 600 m/z).

Parameter	Measured Value
Capillary Voltage (V)	-4500.00
End Plate Offset (V)	-603.00
Capillary Exit Offset (V)	74.20
Skim 1 (V)	23.50
Skim 2 (V)	9.40
Octopole (V)	4.40
Octopole Δ (V)	3.17
Octopole RF (Vpp)	203.40
Trap Drive	66.90
Lens 1 (V)	-4.30
Lens 2 (V)	-87.80
Auto MS/MS Fragmentation Amplitude (V)	1.0
Isolation Width (m/z)	4.0

Tandem MS was performed by generating product ion spectra for the pseudomolecular ion of each analyte. Quantitative LC-MS/MS measurements were performed using the resulting peak areas in the EICs for each of the analyte pseudomolecular ions, the tandem product ion transitions listed in Table 6.3 were used for qualitative confirmation.

Table 6.3: Selected product ion transitions for MS/MS monitoring.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)
Morphine	286	268
Amphetamine	136	119
MDMA	194	163
Benzoylecgonine	290	168
Ketamine	238	220
Heroin	370	268
Cocaine	304	182
Cocaethylene	318	196
LSD	324	223
EDDP	278	249
Papaverine	340	202
Methadone	310	265
Fluoxetine	310	148
Temazepam	301	283
Diazepam	285	257
Δ^9-THC	315	259

6.2.6 Extraction of banknotes:

Euro banknotes analysed in this study were collected from a branch of a national bank on the north side of Dublin and extracted using an adaptation of the method of Esteve-Turrillas *et al.* [7]. Briefly, notes were placed in a clean 40 mL amber glass sample tube to which 10 mL of MeOH was added. The vial was then sealed and vortexed at high speed for one minute. The MeOH was then removed to a clean 12 mL sample vial and reduced in volume to near dryness under a gentle stream of nitrogen. The dried extract was then reconstituted in 250 µL of initial mobile phase (80:20 5 mM ammonium acetate MeOH) and analysed using the developed LC-MS/MS method.

6.3 Results and Discussion:

6.3.1 Comparison of monolithic and 1.8 µm particle packed columns:

The available stationary phases and column lengths were examined for optimum chromatographic efficiency using a mixture of the three structurally related tricyclic basic drugs; carbamazepine, temazepam and diazepam. Experimental conditions were maintained identical throughout all investigations. The resulting peak width at half height was used for all efficiency calculations. Fig. 6.1 depicts the resulting van Deemter curves in terms of plate height (H) and plate number (N) determined for each of the stationary phase configurations using diazepam as the test analyte.

As can be seen from Fig. 6.1(A) the 1.8 µm particle packed column offers higher efficiency due to its lower H_{min} value than the 10 cm and 20 cm monolithic columns respectively. As stated previously by Cabrera, the chromatographic efficiency of monolithic columns is comparable to that of 3.5 µm particles and therefore, the observation that H_{min} decreases with a reduction in the particle size of the stationary phase can clearly be seen from Fig. 6.1 (A) [26,27]. The optimum linear velocity is higher when using the monolithic columns compared to the 1.8 µm particle packed column, optimum values were determined to be 0.40 mms^{-1} on the 1.8 µm particle packed column, 0.52 mms^{-1}

on the 10 cm monolithic column and 0.98 mms^{-1} on the 20 cm monolithic columns.

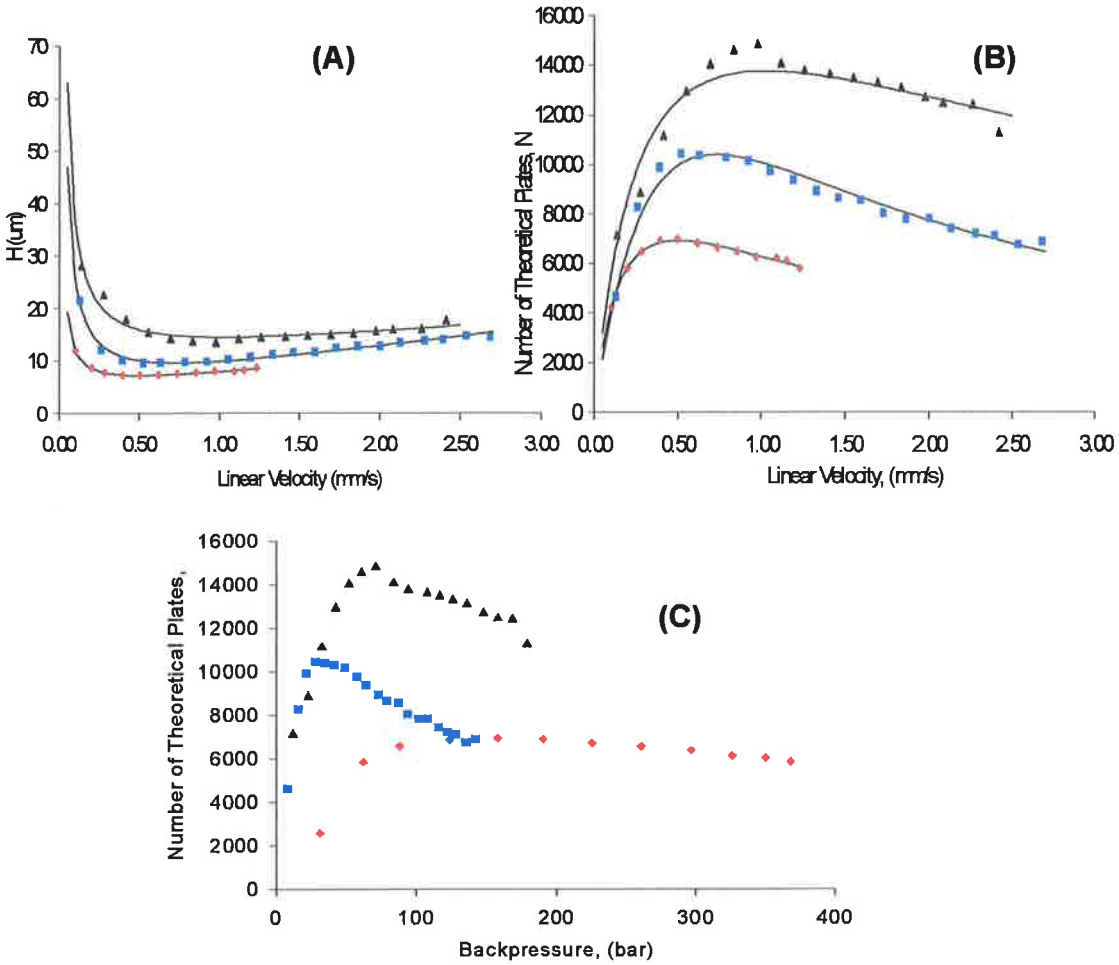


Figure 6.1: (A) Plot of plate height (H) versus mobile phase linear velocity using diazepam as the test analyte, (B) Plot of plate number (N) versus mobile phase linear velocity and (C) Plot of plate number (N) versus the generated backpressure on the 1.8 µm column (♦), the 10 cm monolithic column (■) and the 20 cm monolithic column (▲).

Fig. 6.1 (B) depicts plate number plotted versus the linear velocity of mobile phase. From Fig. 6.1 (B) it can be seen that a significantly higher number of theoretical plates is attainable when using the monolithic columns compared to the 5 cm 1.8 µm particle packed column. With the 20 cm monolithic phase it can be seen that over twice the number of theoretical plates can be obtained at ~twice the linear velocity, compared to the particle packed column. Fig. 6.1 (C) is a plot of plate number versus column backpressure generated. It can clearly be seen that the primary advantage of the monolithic columns over

the 1.8 µm particle packed column is the ability to provide significantly higher N at considerably lower operating pressures. With the 20 cm monolithic phase, optimum N (~15000) was achieved at only 75 bar, compared to ~150 bar on the particle packed column (N = ~7000).

6.3.2 Kinetic behaviour of monolithic and 1.8 µm particle packed columns:

An alternative method of comparing liquid chromatographic supports of different geometries and sizes is to use so-called kinetic plots, whereby traditional van Deemter couples such as (μ_o,H) and (μ_o,N), where μ_0 is the linear velocity of the mobile phase, are converted into kinetic parameters that take into account the column permeability, the mobile phase viscosity and the maximum pressure drop across the column [28,29].

Fig. 6.2 (A) is a plot of t_o versus plate number and allows for the direct visualisation of the range of plate numbers (N) wherein different supports of different geometries can provide faster separations or increased levels of chromatographic efficiency than the other. It can be seen that for fast separations requiring relatively low efficiency the monolithic columns and the 1.8 µm particle packed column all behave similarly. An interesting observation is that the 10 cm monolith and the 1.8 µm particle packed column appear to exhibit almost identical plate generation behaviour, however, as was previously mentioned the monolithic columns generate significantly less operating pressure. Kinetic plots are constructed using the maximum pressure drop (ΔP_{max}) for the system, for the 1.8 µm column ΔP_{max} was set to 400 bar while for the monolithic columns a value of 200 bar was applied. Therefore, it appears that the 10 cm monolithic column is a more suitable option for rapid, moderately efficient separations (i.e. N ~10,000) compared to sub 2 µm column technologies. The 10 cm monolith and the 1.8 µm column also appear to provide higher numbers of plates more rapidly than the 20 cm monolithic column, although the inclusion of the column coupler to generate the 20 cm monolithic column may be the reason for such an observation, due to the introduction of additional void volume into the chromatographic system.

A noteworthy characteristic of Fig. 6.2 (B) is its similar appearance to the van Deemter curves presented in Fig. 6.1 above. However, the advantage of the kinetic plot over the van Deemter plots is the clearly defined minimum that corresponds to the optimum attainable number of theoretical plates (N_{opt}) at the minimum separation impedance (E_o). From a detailed discussion of the kinetic plot method by Desmet *et al.* [30], it was noted that $E_o \sim H^2$ and therefore, the minimum of the E_o versus N/N_{opt} plot occurs at the same mobile phase linear velocities as mentioned in section 6.3.1. However, in this case the minimum corresponds to a point where the system generates the optimum number of theoretical plates at its best possible kinetic performance / pressure cost ratio [30]. Therefore, from Fig. 6.2 (B) it can be seen that the 10 cm monolithic column offered higher N_{opt} values at lower E_o values, than the 20 cm monolithic column and the 1.8 µm column. Such an observation can be explained as the shorter monolith would be expected to exhibit a higher degree of permeability than the longer monolithic column and in turn the particle packed column. However, all the available information should be considered when deciding on the actual analytical column to be used and based on the all the data presented, it was determined that the 20 cm monolithic column, which offered the highest N at acceptable mobile phase flow rates and column backpressures provided the best performance for the proposed application.

6.3.3 Liquid chromatography of illicit drugs:

Using the 20 cm monolithic column a gradient separation of the chosen analytes was investigated. An optimisation of the pH and buffer concentration of the aqueous proportion of the mobile phase was systematically performed using an experimental design approach to evaluate selectivity effects. For optimisation, an experimental space was designed, governed by the buffering activity of ammonium acetate and the desire to keep the buffer concentration low enough to be compatible with ESI-MS. Therefore, mobile phases containing ammonium acetate concentrations of 5 to 20 mM, over the pH range of 2.5 to 6.5 were investigated, with a total of twelve experiments run within the above concentration and pH range. The resulting chromatograms were assessed using the resolution product criterion, *R*, calculated according to Equation 6.1, [31].

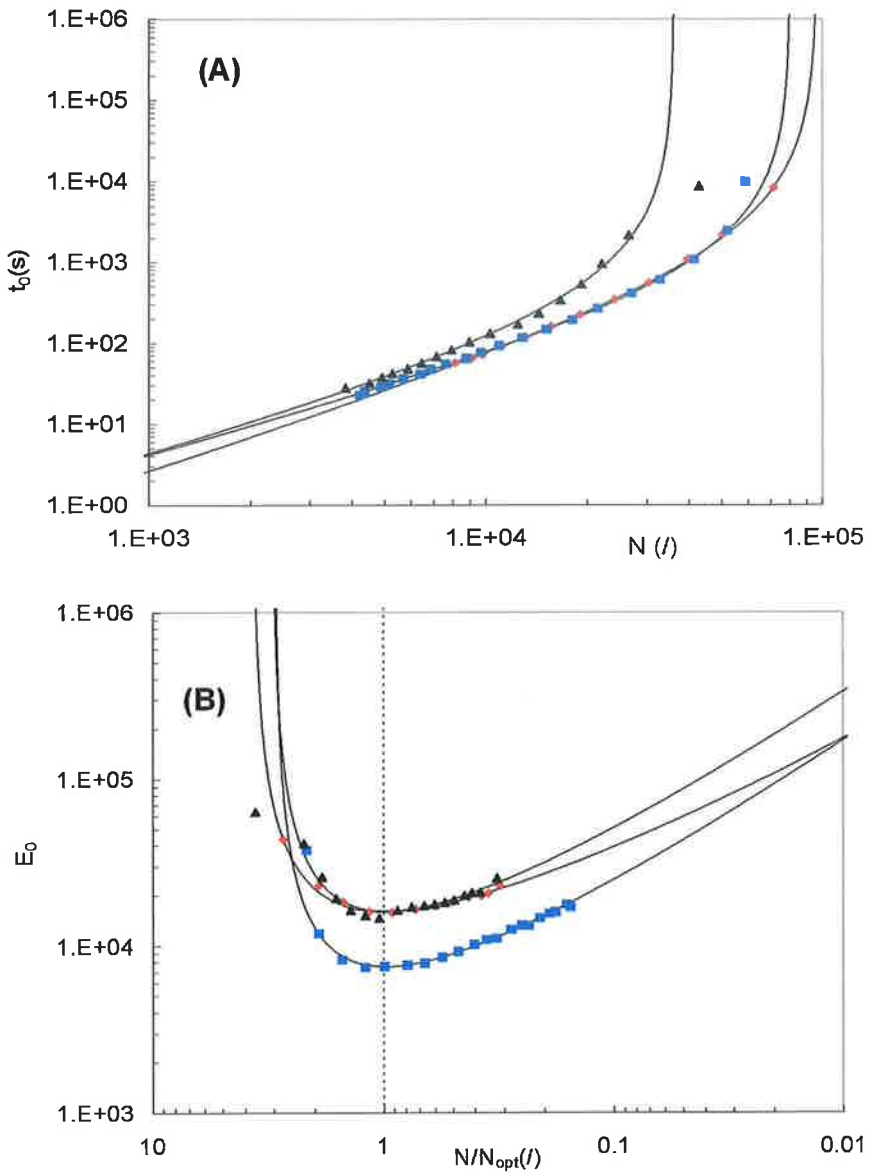


Figure 6.2: Kinetic comparison of the examined stationary phases; (A) a plot of t_o versus the pressure drop limited plate number (N), and (B) a plot of the t_o based separation impedance E_o versus the ratio of pressure drop limited plate number (N/N_{opt}). Symbols: 1.8 µm column (♦), the 10 cm monolithic column (■) and the 20 cm monolithic column (▲).

$$R = \prod_{i=1}^{n-1} \left(\frac{R_s(i, i-1)}{\frac{1}{n-1}\sum R_s(i, i-1)} \right) \qquad \text{(Eq. 6.1)}$$

Where:

n is the number of analytes present and

R_s is the resolution between peaks *i* and *i-1*, calculated using peak width measured at baseline.

The calculated *R* values a minimum resolution response surface as shown in Fig. 6.3 was constructed for the separation of the 16 illicit drugs and metabolites listed in Table 6.1.

From Fig. 6.3 it can be seen that two sets of possible optima were generated from the optimisation experiments. Of these the lower buffer concentration optimum was further refined, resulting in final mobile phase conditions of 5 mM ammonium acetate, pH 4.5. MeOH gradient conditions were as described earlier under (section 6.2.4 chromatographic conditions). Under the optimum mobile phase conditions a complete separation of most analytes was achieved with the exception of the ketamine and heroin and the cocaethylene and LSD peak pairs wherein a slight degree of co-elution still occurred, see Fig. 6.4. Δ^9-THC was strongly retained, however, the total run time of only 30 minutes per injection for full screening of all 16 drugs was deemed acceptable.

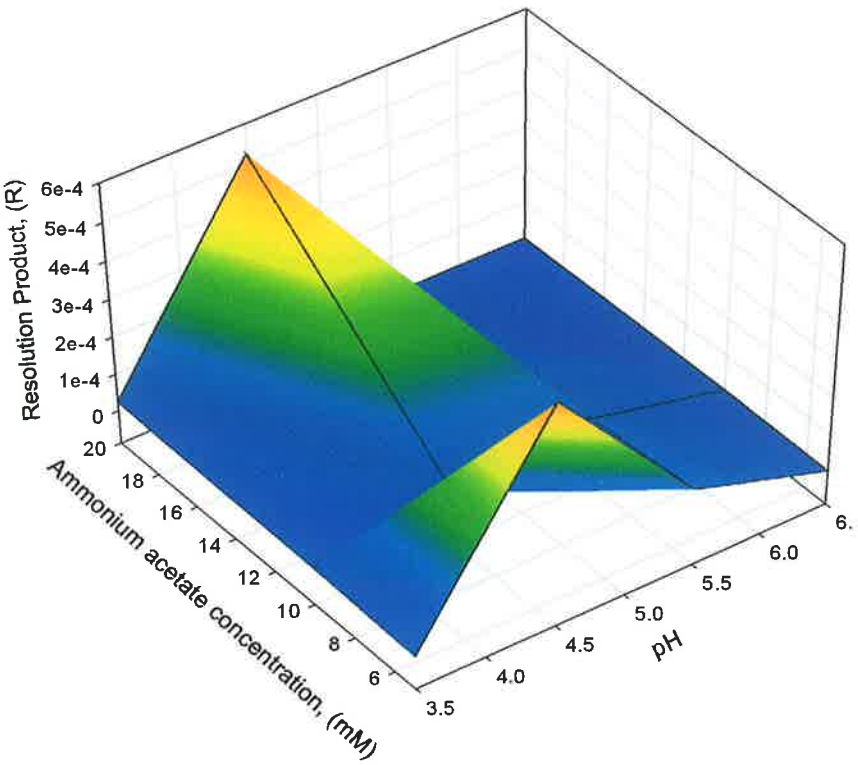


Figure 6.3 Minimum resolution response surface for the optimisation of the pH and buffer concentration of the aqueous mobile phase. Experiments were performed using a 1 mgL^{-1} standard mixture of analytes.

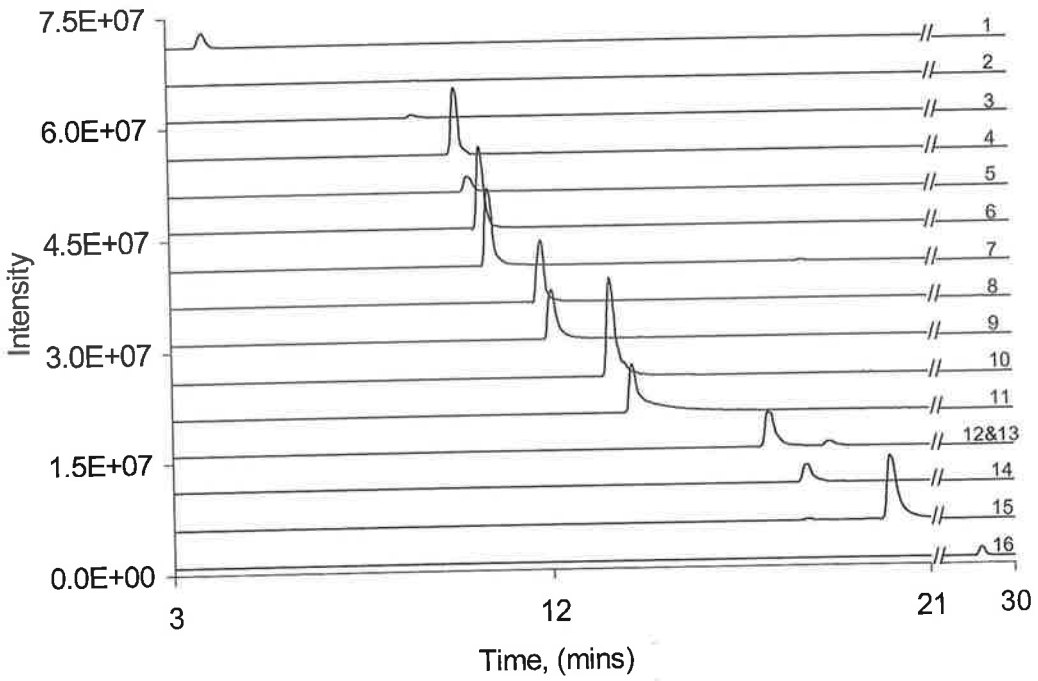


Figure 6.4: Separation of a 1mgL^{-1} standard solution of all analytes using a multistep gradient of MeOH and 5 mM ammonium acetate, pH 4.5 on the 200.0 mm x 3 mm i.d. monolithic column at a flow rate of 0.35 mLmin^{-1} using ESI-MS/MS detection. The traces have been offset for the purpose of clarity. *Peak identification*, **1** morphine, **2** amphetamine, **3** MDMA, **4** benzoylecgonine, **5** ketamine, **6** heroin, **7** cocaine, **8** cocaethylene, **9** LSD, **10** EDDP, **11** papaverine, **12** methadone, **13** fluoxetine, **14** temazepam, **15** diazepam and **16** Δ^9-THC.

6.3.4 MS/MS Detection:

For the detection of most drugs separated simple easily interpretable spectra were recorded, i.e. only pseudomolecular ions $[M+H]^+$ were observed. In the case of amphetamine and MDMA the product ions generated are as a result of the loss of ammonia and methyl amine respectively from the precursor pseudomolecular ions. Cocaine and its structural analogues benzoylecgonine and cocaethylene were all observed to lose 122 mass units from the parent molecule corresponding to the loss of the benzoic acid functionality from the molecules. The MS/MS transition of morphine is thought to be due to loss of water from the parent molecule while for heroin the MS/MS transition occurs due to loss of both acetyl groups as CH_3CO followed by a dehydration to yield the same product ion as morphine at m/z 268. Methadone is thought to lose dimethyl amine to yield its product ion at m/z 265, its metabolite EDDP loses 29 mass units corresponding to the loss of the ethyl group while papaverine is

thought to lose a dimethoxy phenyl group to yield the product ion at m/z 202. The product ion of LSD is suspected to arise due to the loss of $CON(C_2H_5)_2$ from the molecule. Ketamine and temazepam both undergo dehydration to yield their product ions while the MS/MS transition of diazepam occurs due to the contraction of the 7 membered ring to a 6 membered ring with the loss of CO to yield the ion at m/z 257 [32]. Δ^9-THC is thought to under go a similar transition with ring contraction and loss of $HOC(CH_3)_2$ resulting in the product ion at m/z 259. The MS/MS transition of fluoxetine arises as a result of $CF_3C_6H_4O$. All of the recorded MS/MS transitions were significantly intense, with in most cases complete fragmentation of the precursor ion. These unequivocal chemical fingerprints were used for unambiguous identification of resulting peaks when analysing banknote samples.

6.3.5 Method performance:

Prior to application, the performance characteristics of the developed LC-MS/MS method were determined. Linearity was assessed from prepared five point calibration curves in the region of 0.01 to 5 mgL^{-1} equating 0.2 to 100 ng $note^{-1}$. Repeatability was determined by performing six replicate injections of a 0.10 mgL^{-1} mixed standard solution while reproducibility was examined using six individually prepared 0.5 mgL^{-1} mixed analyte solutions. The limits of detection and quantitation were defined as signals corresponding to 3 and 10σ respectively of the baseline noise. The determined performance characteristics are listed in Table 6.4. Linearity was demonstrated in all cases with $R^2 > 0.99$ with the exception of amphetamine, heroin, papaverine and Δ^9-THC. The calculated levels of precision were also deemed acceptable, in general repeatability was <5% RSD while reproducibility was in the range of 5-10% RSD. The method was also determined to be suitable sensitive with detection and quantitation limits in the pg-ng $note^{-1}$ range. The levels of sensitivity achieved in this study using LC-MS/MS out perform previously published methods using GC-MS/MS [7] and CE-ECL [18], wherein an instrument validation was performed. It is a noteworthy comment that many of the published methods do not report method performance data. The superior

sensitivity data obtained in this study further supports the use of LC-MS/MS for the determination of illicit drugs due to the majority of such chemicals occurring as charged species in solution.

Table 6.4: Method performance data.

Analyte	Linearity	Repeat.*	Reproduc.*	LOD	LOQ	Recovery
	R^2	%RSD	%RSD	pg note^{-1}	pg note^{-1}	%
Morphine	0.9945	2.54	3.98	22	74	26 ± 3
Amphetamine	0.9898	5.18	6.80	1010	3367	64 ± 7
MDMA	0.9976	4.02	7.02	195	651	48 ± 17
Benzoylecgonine	0.9981	1.25	7.65	4	14	37 ± 1
Ketamine	0.9995	3.00	8.58	18	60	65 ± 1
Cocaine	0.9997	3.69	4.93	3	14	87 ± 8
Heroin	0.9876	2.91	11.29	5	17	53 ± 5
Cocaethylene	0.9991	4.79	9.66	5	17	54 ± 9
LSD	0.9998	3.33	7.16	10	33	28 ± 3
EDDP	0.9954	3.26	6.15	3	9	74 ± 10
Papaverine	0.9873	8.09	11.97	4	15	79 ± 9
Methadone	0.9995	2.97	4.51	9	31	44 ± 3
Temazepam	0.9998	4.69	6.81	21	71	32 ± 2
Fluoxetine	0.9992	5.01	5.65	52	175	24 ± 4
Diazepam	0.9994	2.33	4.81	5	16	30 ± 6
Δ^9-THC	0.9866	8.60	4.91	150	502	71 ± 24

*Repeatability and reproducibility have been abbreviated for the purposes of fit.

The recovery of the test materials was also assessed using a similar procedure to that as previously described by Esteve-Turrillas *et al.* [7] Recovery experiments were performed using €5 banknotes which were first extracted as previously described (section 6.2.6) in order to avoid any possible cross contamination (n = 3). The cleaned banknote was spiked with 125 ng of all analytes prepared in MeOH, after sufficient drying time the note was extracted and the percentage recovery was determined by comparing the peak areas recorded for the spiked note with a standard injection. The percentage recoveries were observed to vary for each analyte, with cocaine and methadone demonstrating quite high levels of recovery, but analytes such as LSD and diazepam exhibiting poor recovery from the banknote. The reason for the poor recovery of some compounds compared to others, particularly those that are structurally analogous, is unclear. As the majority of the test analytes are basic in nature the possibility that mixed mode retention via adsorption and ion

exchange on the cotton surface of the banknote was investigated by using MeOH containing either 0.1% v/v acetic acid or 0.1% v/v ammonium hydroxide in the extraction procedure. However, the inclusion of the acidic or basic modifiers into the extraction solution had no positive affect on the determined percentage recoveries and therefore, further research into extraction techniques is recommended. It should, however, be mentioned that many of the test analytes are administered or taken in tablet form and therefore, minimal residue would be expected on banknotes in such cases.

6.3.6 Application to banknotes:

The developed LC-MS/MS method was then applied to the determination of illicit drug and abused pharmaceutical residue contamination on Euro banknotes. Where possible, notes of Irish origin were preferentially collected, (serial numbers beginning with the Irish identifier, letter 'T'), in an attempt to locally bias the sample, although it must be mentioned that the history of the sampled notes is unknown and they may indeed been outside Ireland and used in other Euro zone countries. New un-circulated €5, €10 and €20 notes were also obtained from the bank to serve as method blanks. Samples were extracted as previously described (section 6.2.6) and the re-constituted extracts analysed using the developed LC-MS/MS method (section 6.2.4). Fig. 6.5 shows some typical chromatograms for used (A) 5 and (B) 20 Euro banknotes, overlaid with chromatograms from un-circulated blanks.

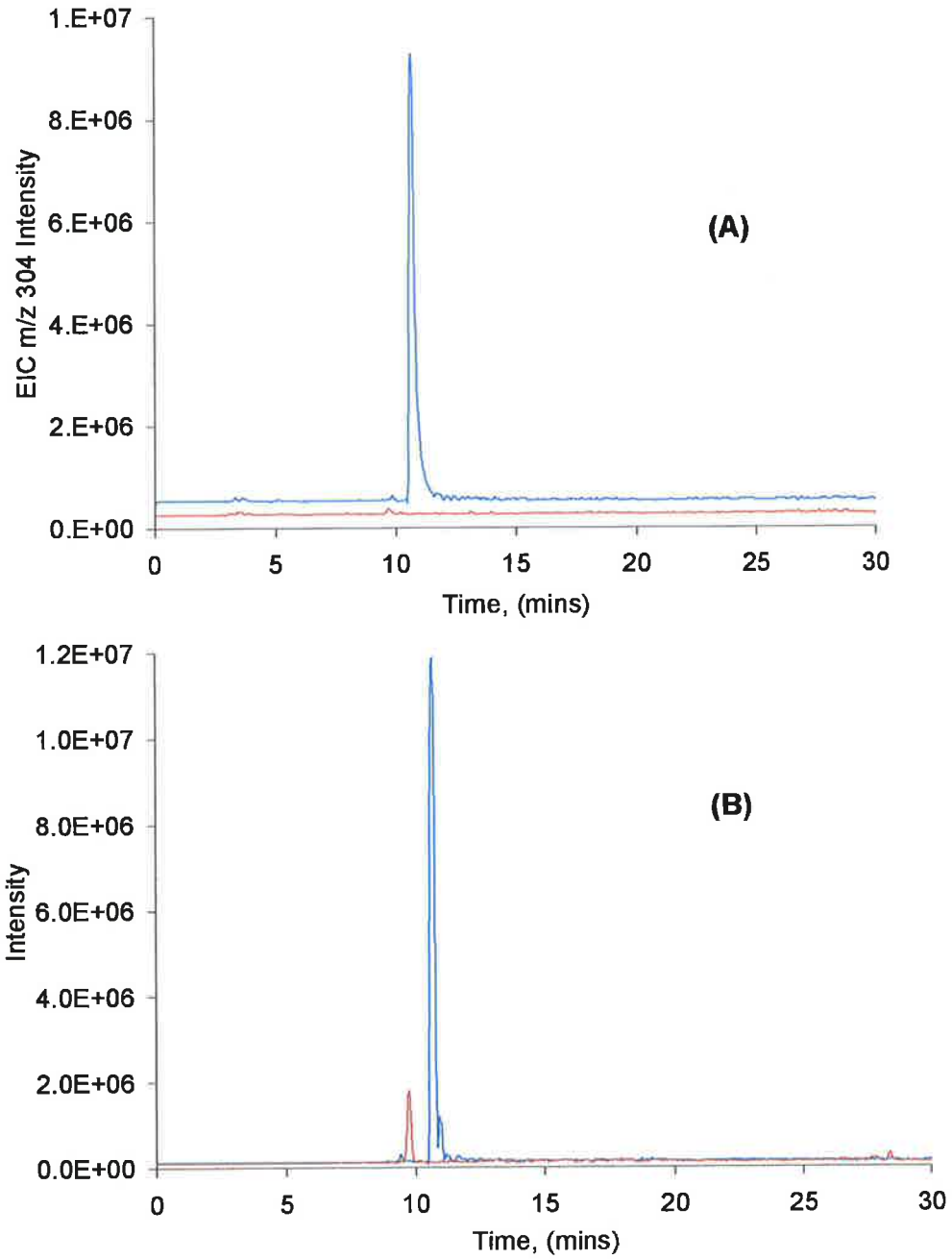


Figure 6.5: Resulting chromatograms from banknote analysis depicting; (A) the presence of ~24 ng of cocaine (blue trace) on a €5 note overlaid upon the EIC m/z 304 trace for the un-circulated €5 note (red trace) and (B) the presence of ~20 ng cocaine and ~4 ng of benzoylecgonine on a €20 banknote, (blue and red traces respectively).

All of the 45 banknotes tested positive for trace quantities of cocaine with the exception of the 3 un-circulated controls. Exact results are listed in Table 6.5 and shown graphically as Fig. 6.6. In all, two of the notes; a €5 and a €20 note; appear to be contaminated with significant levels of cocaine that would

suggest direct contact with the drug, either through drug dealing or more probably when using the note to 'snort' the narcotic. From Fig. 6.6 it can be seen that significantly higher average levels of cocaine are present on the higher denomination notes compared to the smaller €5 and €10 banknotes. The presence of benzoylecgonine, the primary breakdown product of cocaine, suggests aging of the cocaine residue on the surface of the note, perhaps due to hydrolysis of cocaine residues as a result of exposure to moisture. Although not currently known, it is thought that the rate of cocaine breakdown and the consequent rate of formation of benzoylecgonine could provide a time frame concerning the contamination of the note with cocaine. However, the presence of benzoylecgonine may also be due to contamination of the note via contact with the urine of a cocaine user. Trace cocaine contamination on the remaining notes is suspected to have occurred as a result of transfer during the counting processes within financial institutions as previously demonstrated by Carter *et al.* [13].

Traces of heroin were also detected on three of the analysed notes along with trace levels of cocaine, two €5 and one €10 note. The levels of heroin detected were in one case less than the levels of cocaine, in one case approximately equal and in the final case greater then the quantity of cocaine determined.

Table 6.5: Banknote analysis.

Serial Number	Cocaine (ng note^{-1})	Benzoylecgonine (ng note^{-1})	Heroin (ng note^{-1})
	€5 Banknotes		
T15874943298 – Un-circulated.	-	-	-
T15180843102	0.20 ± 0.01	-	-
T15186514803	23.91 ± 0.32	0.84 ± 0.02	-
T15051087366	4.11 ± 0.07	-	-
T15250805205	0.09 ± 0.01	-	-
T15151331679	2.39 ± 0.12	-	-
T15101438289	0.43 ± 0.13	-	-
T15137112939	2.77 ± 0.68	-	-
T15613930023	0.35 ± 0.06	-	-
T15229068657	0.67 ± 0.10	-	-
T15016543359	2.67 ± 0.16	-	-

T15282288987	6.05 ± 0.68	-	-
T15204866307	2.23 ± 0.04	-	-
T15639048267	1.07 ± 0.14	-	-
T15012889105	7.90 ± 0.86	-	1.56 ± 0.46
T15118170486	286.66 ± 40.57	1.71 ± 0.21	-
T15066106161	28.28 ± 0.81	-	-
T15141438195	0.89 ± 0.15	-	-
T15220803669	3.48 ± 0.21	-	13.62 ± 0.21
T15051938415	0.42 ± 0.07	-	-
T15033617439	0.64 ± 0.15	-	-
	€10 Banknotes		
T27286582281 – Un-circulated.	-	-	-
T24712617075	6.02 ± 0.08	-	6.75 ± 0.54
T23923994712	19.17 ± 0.71	2.98 ± 0.13	-
T24836124858	0.84 ± 0.15	-	-
T23979554331	4.23 ± 0.16	-	-
T24692863812	4.18 ± 0.31	-	-
T24852294537	0.95 ± 0.08	-	-
T24742606866	0.38 ± 0.16	-	-
T23931481452	0.43 ± 0.07	-	-
T25751663349	14.40 ± 2.18	2.47 ± 0.02	-
T24611116416	0.07 ± 0.02	-	-
	€20 Banknotes		
S12765607531 – Un-circulated.	-	-	-
X04803373901	19.66 ± 0.35	3.51 ± 0.01	-
S12906683881	0.70 ± 0.10	-	-
V09275921032	5.98 ± 1.00	-	-
X03284468993	8.69 ± 1.85	1.48 ± 0.13	-
U47400639503	25.19 ± 0.25	2.27 ± 0.15	-
S13260546565	576.14 ± 18.26	12.78 ± 0.78	-
P06454506576	8.88 ± 1.11	-	-
S10844734174	14.78 ± 0.50	1.58 ± 0.10	-
T31907250933	0.78 ± 0.13	-	-
T32083554147	5.61 ± 1.88	0.71 ± 0.01	-
	€50 Banknotes		
V15951910873	2.09 ± 0.07	0.76 ± 0.01	-
Z33472745721	32.47 ± 0.30	-	-
V20707197421	13.68 ± 1.29	-	-
X27532668296	0.25 ± 0.12	-	-
U08132346032	23.20 ± 1.09	3.86 ± 0.09	-

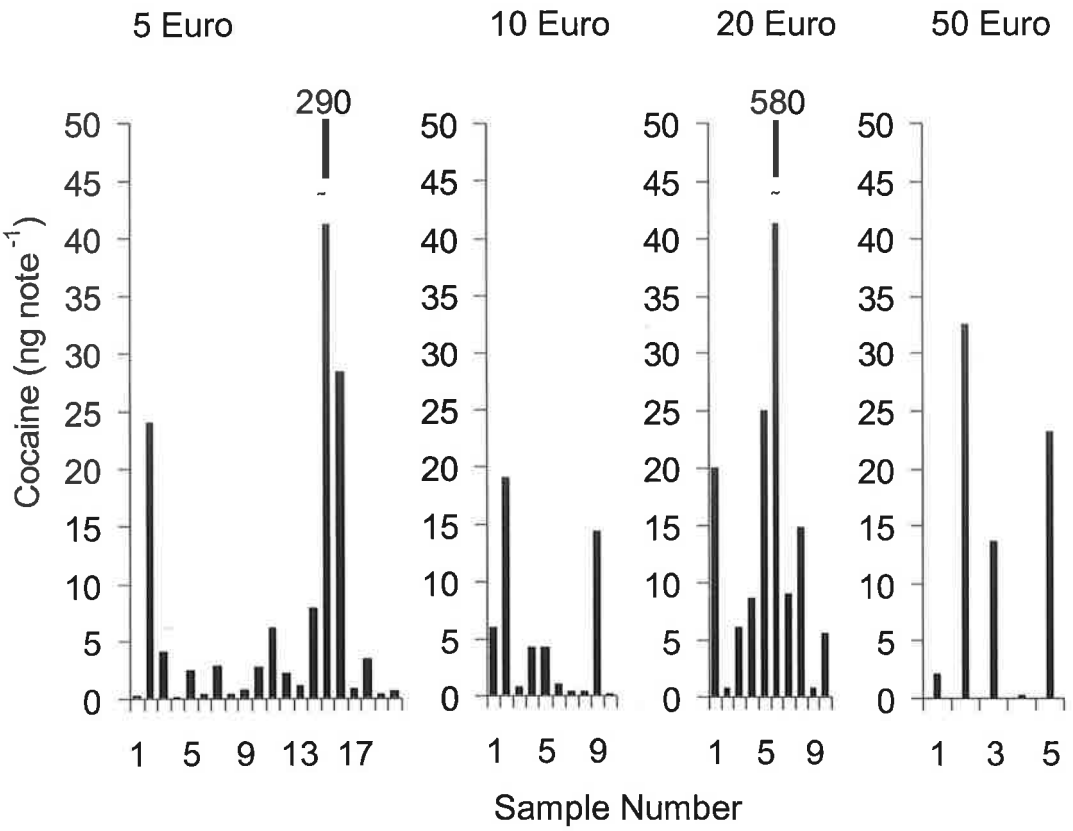


Figure 6.6: Graphical representation of cocaine contamination on the analysed banknotes. The highest levels were detected on one €5 note and one €20 note as labelled in the above figure. It can also be seen that the levels of contamination are higher on the €20 and €50 notes than on the €5 and €10 notes.

6.4 Conclusions:

A highly sensitive and relatively rapid monolithic LC-MS/MS separation of illicit drugs and abused pharmaceuticals preceded by simple sample preparation has been developed and successfully applied for the determination of such contamination on Irish Euro banknotes collected from a branch of a national bank on the north side of the city. Trace levels of cocaine were found on all notes tested. In some instances traces of benzoylecgonine, the breakdown product of cocaine was also determined. Interestingly, traces of heroin were also found on 3 of the 45 samples. The presence of such illicit material on the studied banknote samples provides an indication of the presence of such narcotics within the community. However, this initial study does not attempt to draw sociological or geographical conclusions regarding the use of narcotics

within the community and therefore, any such conclusions should only be considered with caution.

References:

1. M. O'Halloran, *'Cocaine seizures rise eight fold"*, The Irish Times, Saturday October 1st 2005.

2. O. Kelly, *"Illegal drugs trade worth E650 million annually"*, The Irish Times, Thursday December 22th 2005.

3. An Garda Síochána Annual Report 2005, *'Drug Offences Statistics'*, pages 65-69, www.garda.ie/angarda/statistics/report2005.html, viewed July 27th 2006.

4. The National Advisory Committee on Drugs, *'An Overview of Cocaine use in Ireland'*, 2003, available on-line www.nacd.ie/publications/prevalence_cocaine.html, viewed July 27th 2006.

5. E. Burke-Kennedy, *'Drug-use treatment outside East region soars'*, The Irish Times, July 29th 2004.

6. J.C. Hudson, *Journal of the Canadian Society of Forensic Science*, **22**, (1989), 203.

7. F.A. Esteve-Turrillas, S. Armenta, J. Moros, S. Garrigues, A. Pastor & M. de la Guardia, *Journal of Chromatography A*, **1065**, (2005), 321.

8. A.J. Jenkins, *Forensic Science International*, **121**, (2001), 189.

9. D. Song, S. Zhang & K. Kohlhof, *Journal of Chromatography A*, **731**, (1996), 355.

10. J. Oyler, W.D. Darwin & E.J. Cone, *Journal of Analytical Toxicology*, **20**, (1996), 213.

11. D. Paradis, *RCMP Gazette*, **59**, (1997), 20.

12. K.A. Ebejer, J. Winn, J.F. Carter, R. Sleeman, J. Parker & F. Korber, *Forensic Science International*, Article in press (2006), doi: 10.1016/j.forsciint.2006.06.039.

13. J.F. Carter, R. Sleeman & J. Parry, *Forensic Science International*, **132**, (2003), 106.

14. E. Zucatto, C. Chianbrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea & R. Fanelli, *Environmental Health*, **4**, (2005), 14.

15. R. Sleeman, I. Fletcher, A. Burton, J.F. Carter & D.J. Roberts, *Analyst*, **124**, (1999), 103.

16. K.A. Ebejer, R.G. Brereton, J.F. Carter, S.L. Ollerton & R. Sleeman, *Rapid Communications in Mass Spectrometry*, **19**, (2005), 2137.

17. D.J. Roberts, J.F. Carter, R. Sleeman & I.F.A. Burton, *Spectroscopy* Europe, **9/6**, (1997), 24.

18. Y. Xu, Y. Gao, H. Wei, Y. Du & E. Wang, *Journal of Chromatography A*, **1115**, (2006), 260.

19. M.A. Huestis & M.L. Smith, *Drug Discovery Today: Technologies*, **3**, (2006), 49.

20. J.R. Mazzeo, U.D. Neue, M. Kele & R.S. Plumb, *Analytical Chemistry*, December 2005, **77**, 460A.

21. H. Chen & C. Horvath, *Journal of Chromatography A*, **705**, (1995), 3.

22. D.T.T. Nguyen, D. Guillarme, S. Rudaz & J-L. Veuthey, *Journal of Separation Science*, **29**, (2006), 1836.

23. J.E. MacNair, K.C. Lewis & J.W. Jorgenson, *Analytical Chemistry*, **69**, (1997), 983.

24. A. de Villiers, H. Lauer, R. Szucs, S. Goodall & P. Sandra, *Journal of Chromatography A*, **1113**, (2006), 84.

25. D.C. Harris, *Journal of Chemical Education*, **75**, (1998), 119.

26. K. Cabrera, *Journal of Separation Science*, **27**, (2004), 843.

27. D.T.T. Nguyen, D. Guillarme, S. Rudaz & J-L. Veuthey, *Journal of Chromatography A*, **1128**, (2006), 105.

28. G. Desmet, P. Gzil & D. Clicq, *LC-GC* Europe, July 2005, 403.

29. G. Desmet, P. Gzil, D. Cabooter & D. Clicq, *The Kinetic Plot Method*, available to download from http://wwwir.vub.ac.be/chis/KineticPlot/, last viewed 23rd October 2006.

30. G. Desmet, D. Clicq & P. Gzil, *Analytical Chemistry*, **77**, (2005), 4058.

31. K.L. Ng, B. Paull, P.R. Haddad & K. Tanaka, *Journal of Chromatography A*, **850**, (1999), 17.

32. S. M°Clean, E.J. O'Kane & W.F. Smyth, *Electrophoresis*, **21**, (2000), 1381.

7.0 Using Environmental Analytical Data to Estimate Levels of Community Consumption of Illicit Drugs and Abused Pharmaceuticals.

7.1 Introduction:

As mentioned previously in Section 6.1, there has of late, been increased media attention concerning the escalating use of illicit drugs, in particular cocaine, by Irish society [1]. Such media reports appear to be corroborated by official seizure data from An Garda Síochána, wherein a search of press releases available through their website reveals no less than eighteen major hauls of cannabis, cocaine, opiates and amphetamines in the period of January to November 2006, including the largest ever seizure of heroin in this country [2]. However, it was also previously noted that current methods for the estimation of illicit drug use within Irish society often suffer from significant shortcomings. For example, performing surveys that focus upon specific subgroups known to abuse drugs, or attempting to gauge cocaine abuse by measuring the numbers presenting themselves for treatment for cocaine addiction into an opiate focused and ill-equipped system. Of course, any information regarding the extent of the 'drug problem' in Ireland is inherently valuable, especially to those governing the allocation of resources to ensure adequate funding to the health services and law enforcement in order to aid in combating a growing problem. However, the predicament exists that current approaches to estimate drug consumption often, if not always, do not contain information regarding the use of illicit drugs by social and recreational users and hence, the picture of the 'drug problem' presented in the public domain may not provide an accurate reflection of the actual state of affairs.

Several analytical procedures exist in the peer-reviewed literature for the determination of illicit drugs. The majority of these methods are however, focused on personal testing as part of usage or abstinence monitoring and toxicological studies rather than community estimations of current usage. Due to the scope of

such studies the matrix investigated has always been biological in nature, spanning across plasma and serum [3,4,5,6,7,8], to urine [9,10,11] and other bodily fluids such as saliva and sweat [12,13,14,15,16]. Hair analysis has also become a recent focus in abstinence monitoring programs due to the ability to place a time frame to usage periods in accordance with standard hair growth rates and consequently a number of methods have also appeared detailing procedures for the determination of illicit drug residues in hair [17,18,19,20,21]. To a lesser extent, reports for the determination of mother to child substance transfer have also been published, wherein meconium was the biological matrix under investigation [22,23]. A common feature of the published methods is the widespread use of LC in conjunction with either ESI or APCI with MS/MS detection allowing for separation followed by unequivocal detection and the garnering of structural information. Detailed discussions concerning the quality assurance of generated LC-MS/MS data used for confirmatory analysis as part of monitoring and toxicological studies have also been published [10,24]. However, although the above methods are all suitably sensitive and selective for the analyte set investigated, all procedures rely heavily upon invasive sampling procedures in order to determine the desired information about the person under investigation.

In the past decade, a number of reports concerning the presence of pharmaceuticals and other medicines in the aquatic environments have entered the public domain, wherein classes of pharmaceuticals ranging from common over the counter painkillers and analgesics such as ibuprofen and paracetamol to specialist cytotoxic agents and x-ray contrast media have been detected in both fresh and marine aquatic systems at trace and ultra-trace levels [25,26,27,28,29,30]. In some instances the MECs demonstrated a statistical relationship concerning the levels of the detected pharmaceutical in the environment and that consumed by the population. For example Ashton *et al.* determined the levels of common pharmaceuticals, e.g. diclofenac, ibuprofen, propranolol *etc.* in treated wastewater and also in the effluent receiving waters and found that levels detected downstream of the wastewater treatment plant statistically correlated to levels detected in the plant's discharged effluent and a further statistical correlation may exist to actual levels of said drug usage within the community [31].

The detection of pharmaceutically active compounds within the aquatic environment as a direct result of treated wastewater discharge further highlights the "intimate, immediate and inseparable relationship between humans and their environment through personal use of chemicals" [32]. Daughton further hypothesised that in a similar manner to medicinal compounds, residues of illicit drugs may also be detected in the aquatic environment as a result of human usage and hence, discharge of both parent compounds and metabolites into aquatic systems is likely to occur via the municipal wastewater treatment system [32]. It is also speculated that wastewater treatment plants are the primary emitters of such compounds into the environment, as due to the effort, risk and cost involved in obtaining and importing illicit drugs, improper disposal and dumping would not be expected to occur.

The first report concerning the presence of illicit materials in treated wastewater appeared in 2004, whereby low ngL^{-1} of methamphetamine and 3,4-methylenedioxy methamphetamine (MDMA or 'ecstasy') were individually detected in the effluent of two monitored wastewater treatment plants in Nevada and South Carolina in the United States, respectively. Although no further research was conducted in this instance, it was acknowledged that the detection of these two illicit drugs corroborated evidence from the United States Drug Enforcement Agency (USDEA) that both chemicals were becoming increasingly problematic in the metropolitan areas surrounding the sampled plants [33]. More recently amphetamine was also detected in sewage sludge by Austrian researchers in the low μgkg^{-1} range, however, caution must be used when interpreting such results as amphetamine and similar compounds may also be presented in wastewater treatment plants as metabolites of prescription drugs used in the treatment of Parkinson's disease [34].

It was also previously noted that the determination of illicit drug residues in wastewater could provide an opportunity to non-invasively determine community wide consumption of such substances. Pioneering research on this front was published by Italian researchers in 2005 where levels of cocaine and its primary metabolite, benzoylecgonine, detected in surface waters and treated wastewater could be used to statistically estimate consumption of the parent narcotic within the community under study [35]. The approach used was to convert MECs to daily

doses per thousand population equivalents (PEq) using available information regarding the volume of wastewater through the treatment plant per day and a correction factor of 2.33 to adjust benzoylecgonine MECs in favour of the parent compound, cocaine [35]. Based upon their environmental monitoring data, Zucatto *et al.* estimated that on average within the general population approximately 7 doses per 1000 PEq were consumed daily which was further refined to 27 doses per 1000 PEq aged in the range of 15-34 years [35]. This research received considerable media attention and further sampling was also undertaken along the River Thames in London, although the results of this study have not yet been published, [36,37]. More recently a publication has emerged from the same group where the levels of a broad suite of illicit drugs were determined using isotope dilution LC-ESI-MS/MS in the treated effluents of wastewater treatment plants in Milan, Italy and Lugano, Switzerland [38]. In both plants the majority of the target analytes were detected in the influent samples, while reduced quantities were detected in the treated effluent, suggesting the existence of removal mechanisms with in the treatment plant. Interestingly, the Milan wastewater treatment plant appeared to be extremely efficient at removing illicit drug residues with most compounds, including cocaine, being removed to levels below the LOQ of the developed analytical method [38]. Although no 'community consumption' calculations were reported in this publication, the authors acknowledge that the results obtained could indeed be used to estimate levels of drug use within the area under study.

The aims of this research are therefore, to develop a suitably sensitive analytical method for the determination of illicit drug residues, in particular cocaine, in treated wastewater and receiving waters using SPE-LC-MS/MS. Where residues of illicit drugs are detected it is hoped to estimate community consumption data for the said narcotic using the approach of Zucatto *et al.* [35]. Treatment plants identified for study include that in Ringsend, Dublin, Ireland, which currently serves ~1.7 million PEq along with smaller plants serving areas in the north and south of the capital city. Treated effluent from wastewater treatment plants in rapidly expanding 'commuter' towns surrounding Dublin such as Navan, Co. Meath and Leixlip, Co. Kildare were examined.

7.2 Experimental:

7.2.1: Chemicals and reagents.

Chemicals and reagents used in this study were as per section 3.2.2 and section 6.2.1 unless otherwise stated. Individual 100 mgL^{-1} stock solutions of each chemical were prepared in MeOH and were stored at 4°C in the dark. Working solutions were prepared from the individual stock standards using water as a diluent. In the case of salts, standard solutions were prepared in terms of the parent analyte. Stock solutions were retained for the duration of the research, working solutions were prepared fresh prior to use.

Buffer solutions used for the optimisation of the extraction pH were prepared by mixing the appropriate amounts of acid and its conjugate base for formate and acetate respectively. In the case of MES and TRIS weighted proportions of salt were dissolved in ~900mL Milli-Q water and titrated to the desired pH using either 1M HCl or 1M NaOH.

7.2.2: Glassware preparation.

Silanisation was performed as per section 3.2.3.

7.2.3: Sample Collection.

24 hour composite samples of treated effluent were obtained from the wastewater treatment plants listed in Table 7.1, (with the exception of the Shanganagh treatment works where only a grab sample could be obtained). Corresponding influent samples were also collected from the Ringsend wastewater treatment plant in Dublin. Grab samples of receiving water were obtained immediately downstream of the treatment plants during each sampling campaign in an attempt to investigate the effect of dilution within the aquatic environment.

Samples from the plants (Table 7.1) were collected during the week beginning November 20th 2006. Samples were extracted within 24 hours of collection and the dried SPE cartridge was immediately frozen pending instrumental analysis.

Table 7.1: Wastewater treatment plants sampled for the presence of illicit drugs.

Treatment Plant	Governing Authority	Type of Treatment	No. of Population Equivalents	Through Flow, ($Lday^{-1}$)
Ringsend	Dublin City Council	1^{y}, 2^{y}.	1,700,000	5.00×10^{8}
Swords	Fingal County Council	1^{y}, 2^{y}.	48,000	1.00×10^{7}
Shanganagh	Dun Laoighre Rathdown County Council	1^{y} only.	65,000	2.20×10^{7}
Leixlip	Kildare County Council	1^{y}, 2^{y}.	80,000	3.00×10^{7}
Navan	Meath County Council	1^{y}, 2^{y}.	40,000	1.05×10^{7}

Key: 1^{y}; primary treatment, 2^{y}; secondary treatment using activated sludge.

7.2.4: Solid phase extraction.

Sorbents investigated for sample extraction included Phenomenex Strata-X™, Strata-XC™ and Strata-XCW™, all 200 mg sorbent mass pre-packed into 6 mL cartridges (Phenomenex, Macclesfield, Cheshire, UK). From initial investigations the Strata-XC™ sorbent provided the highest degree of analyte recovery and hence, was considered for further study. Prior to extraction 500 mL aqueous samples were filtered through Whatman GF/C glass micro-fibre filters to remove particulate matter (Whatman, Maidstone, UK). The filtrate was then adjusted to pH 6.0 using concentrated HCl. Prior to use the SPE cartridge was conditioned with 2 x 6 mL MeOH and 2 x 6 mL water, respectively. Samples were introduced by vacuum through Teflon tubing and extracted under an operating pressure of 20" Hg on a vacuum manifold. After complete sample introduction but without letting the cartridge run dry, the sorbent was washed with 50 mL of 10% v/v MeOH in 100 mM formic acid. Finally, 500 µL of glacial acetic acid was then added to each cartridge, which was allowed to slowly percolate through the sorbent bed in order to aid with dehydration. The sorbents were then dried by vacuum aspiration for a minimum of 30 minutes. Elution was performed using 10 mL of 5% v/v ammonium hydroxide in 1:1 acetone ethyl acetate, the elution solvent was allowed to slowly percolate through the sorbent bed and was collected in a 12 mL glass vial. The collected eluate was then reduced in volume to near dryness under a gentle stream of N_2 with heating if necessary. The resulting residue was then reconstituted in 200 µL of 30% v/v MeOH in 5 mM ammonium acetate pH 4.5 fortified with 0.1 mgL^{-1} papaverine that served as an internal standard for quantitation and transferred to

an autosampler vial containing a low volume polypropylene insert for LC-MS/MS analysis.

7.2.5: Liquid chromatography tandem mass spectrometry.

LC-MS/MS analyses were performed as described in section 6.3.4. The optimised ion optic parameters were as previously reported in Table 6.2; the nebuliser pressure was set at 30.0 psi with a dry gas (N_2) flow rate of 8.0 $Lmin^1$ and a drying temperature of 325°C. MS/MS transitions monitored were previously mentioned in Table 6.3. Product ion MS/MS transitions were used for qualitative confirmation, quantitation was also performed using the area from the resulting product ion peak. Analytical separations were performed on a 200.0 x 3.0 mm i.d. Phenomenex Onyx monolithic C_{18} column, (Phenomenex, Macclesfield, Cheshire, UK) using the multi-step linear gradient of MeOH and 5 mM ammonium acetate pH 4.5 developed in Chapter 6.0.

7.3 Results & Discussion:

7.3.1 SPE sorbent selection:

The illicit drugs and abused pharmaceuticals chosen for study comprise a set of analytes that are weak to moderately basic, with the exception of Δ^9-THC which is uncharged and also span across a broad range of polarities as can be seen from the pK_a and the octanol water partition coefficient data (Log *P*) values in Table 7.2 following.

Due to the expectation that the majority of the chosen analytes would be presumed to exist in their protonated cationic form in solution mixed mode cation exchange sorbents, both weak and strong, were investigated along with the previously used hydrophilic lipophilic balanced polymeric sorbent. The sorbents used were from the Phenomenex Strata-X family of polymeric functionalised phases for analyte enrichment, the structures of which are shown in Fig. 7.1.

Table 7.2: pK_a and Log *P*, (as theoretically calculated XLog*P*) data for the selected illicit drugs and pharmaceutical analytes.

Analyte	pK_a	Log *P*
Morphine	9.85 [39]	0.96 [40]
Amphetamine	9.80 [41]	1.76 [42]
MDMA	9.90 [43]	-0.32 [44]
Benzoylecgonine	2.25, 11.2 [45]	1.29 [46]
Ketamine	7.50 [47]	2.88 [42]
Cocaine	8.60 [48]	2.31 [42]
Heroin	7.60 [49]	1.69 [42]
Cocaethylene	-	-
LSD	7.80 [50]	2.10 [42]
Methadone	9.10 [51]	3.92 [42]
EDDP	-	4.76 [42]
Papaverine	8.07 [52]	3.00 [42]
Temazepam	-	2.99 [42]
Fluoxetine	7.37, 4.69 [53]	4.65 [42]
Diazepam	3.40 [51]	2.92 [42]
Δ^9-THC	10.60 [54]	6.48 [42]

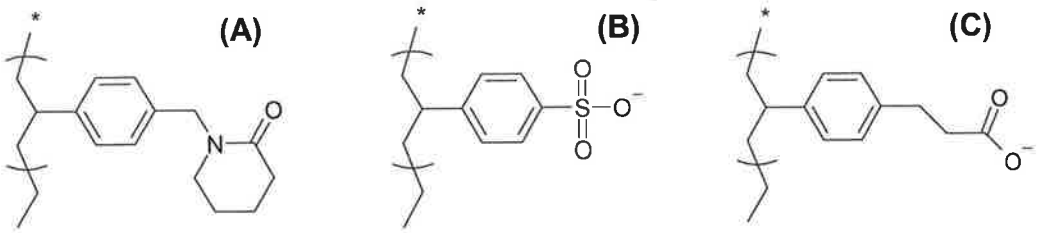


Fig. 7.1: The structure of (A) the Phenomenex Strata-X™ hydrophilic lipophilic balanced polymeric sorbent, (B) the additional strong cation exchange functionality of the Strata-XC™ sorbent and (C) the weak cation exchange functionality of the Strata-XCW™ sorbent [55].

In order to ascertain which of the above sorbents provided the highest degree of analyte recovery, a 500 mL aliquot of a 2 μgL^{-1} mixed analyte spiked solution prepared in reagent water was extracted using each of the above sorbents. The solution pH was adjusted to pH 7.0, 2.0 and 5.0 for the Strata-X™, Strata-XC™ and Strata-XCW™ sorbents, respectively. Elution was performed using 10 mL of MeOH or in the case of the mixed mode cation exchange sorbents, 10 mL of 5% v/v ammonium hydroxide in MeOH. The percentage analyte recovery in each instance was determined by comparison of the resulting peak areas with those of a directly injected 2 mgL^{-1} mixed standard, see Table 7.3.

Table 7.3: Initial sorbent selection investigations. Calculated analyte recovery for a 2 μgL^{-1} mixed spike using the sorbents and conditions mentioned within the text, (values quoted are mean % recovery ± standard deviation, n = 3).

Analyte	Strata-X	Strata-XC	Strata-XCW
Morphine	143 ± 7	124 ± 6	-
Amphetamine	-	-	-
MDMA	44 ± 1	88 ± 1	75 ± 5
Benzoylecgonine	78 ± 2	70 ± 2	58 ± 1
Ketamine	70 ± 3	89 ± 5	66 ± 2
Cocaine	102 ± 1	115 ± 1	94 ± 2
Heroin	22 ± 1	26 ± 1	13 ± 1
Cocaethylene	94 ± 1	106 ± 2	91 ± 2
LSD	56 ± 4	52 ± 1	57 ± 1
Methadone	29 ± 1	97 ± 1	71 ± 1
Temazepam	84 ± 1	80 ± 1	73 ± 1
Fluoxetine	30 ± 1	71 ± 1	61 ± 1
Diazepam	82 ± 1	81 ± 1	70 ± 1
Δ^9-THC	-	-	-

From Table 7.3 it can be seen that the highest degree of analyte recovery was achieved when using the Strata-XC™ mixed mode string cation exchange sorbent with acceptably high recovery of all analytes with the exception of heroin. Although it cannot be said with certainty, it is assumed that heroin hydrolysed to morphine under the acidic conditions used, hence the excessive recovery of morphine. The sorbent selection study was performed using LC with UV detection at 230 nm and therefore, the cases in which analyte recovery greater then 100% may have arisen due to co-elution with unknown peaks. High levels of analyte recovery were also achieved with the weak mixed mode Strata-XCW™ sorbent. However, recovery was generally less than the Strata-XC™ sorbent with the exception of LSD where results achieved with the three investigated sorbents were similar. When using the Strata-X™ sorbent, which exploits both reversed-phase and hydrogen bonding as mechanisms of retention, analyte retention can be observed to increase with increasing levels of hydrophobicity. Analytes such as MDMA and methadone, which are expected to exist as cations in solution under the experimental conditions exhibited low levels of retention as expected. It must also be mentioned that EDDP was not included in the above study as it was received at a later date. In all cases no appreciable recovery of both amphetamine and Δ^9-THC was achieved. Although the reasons for such are not inherently clear, it has previously been noted that amphetamine is readily purged from solution during

solvent evaporation using nitrogen and often requires acidification of the elution solvent in order to prevent such effects [34]. Δ^9-THC was expected to be neutral in solution under the experimental conditions, the reported pK_a value corresponds to the dissociation of the phenolic group of the molecule, and therefore, retention was expected when using the Strata-X sorbent. Δ^9-THC exhibits high retention during the chromatographic analysis and therefore, the possibility of excessive retention on the extraction sorbent was investigated. However, when the Strata-X sorbent was eluted with larger volumes of solvent, still no recovery of Δ^9-THC was noted. As a result of these observations both amphetamine and Δ^9-THC were omitted from further study as their enrichment appeared unfeasible using the SPE approach.

7.3.2 Elution solvent selection:

As the chosen Strata-XC sorbent contains the same sorbent 'backbone' as the Strata-X sorbent which was previously used for the enrichment of pharmaceutical residues in Chapter 3.0, it was decided to investigate whether the elution solvent of 5% v/v ammonium hydroxide in MeOH as recommended by the product literature [55] could be replaced with 5% v/v ammonium hydroxide in 1:1 acetone ethyl acetate as used previously due to both the increased solvent strength and ease at which the acetone ethyl acetate mixture can be reduced in volume under nitrogen. To determine which solvent system provided the optimum levels of analyte recovery, 1 μgL^{-1} spiked solutions of chosen analytes prepared in reagent water were adjusted to pH 2.0 using HCl and extracted using the selected Strata-XC sorbent. After complete sample introduction and sorbent drying SPE cartridges were individually eluted with 10 mL the aforementioned solvent systems that were then reduced in volume under nitrogen, reconstituted in internal standard solution and analysed using LC-MS/MS. Analyte recovery was determined by comparison with the resulting peak areas of a directly injected 1 mgL^{-1} mixed standard, see Table 7.4.

Table 7.4: Calculated analyte recovery for the optimisation of the elution solvent.

Analyte	5% v/v NH_4OH in MeOH	5% v/v NH_4OH in 1:1 Acetone Ethyl Acetate
Morphine	53	8
MDMA	107	60
Benzoylecgonine	87	122
Ketamine	132	109
Cocaine	164	87
Heroin	0	27
Cocaethylene	100	73
LSD	33	43
EDDP	62	62
Methadone	93	83
Fluoxetine	31	36
Temazepam	81	90
Diazepam	83	77

In most instances, analyte recovery was again acceptably and comparably high. However, recoveries obtained when using 5% v/v NH_4OH in MeOH were in some instances excessively high, for example, in the case of cocaine, which was almost double that obtained when using the 5% v/v NH_4OH in 1:1 acetone ethyl acetate elution solvent system. Another observation noted was that there was no recovery of heroin when using 5% v/v NH_4OH in MeOH. In an attempt to ascertain whether or not there was a significant difference between the levels of analyte recovery obtained using both solvent systems a students *t*-test was performed using Microsoft Excel assuming unequal variances, (after a previously performed *F*-Test, wherein $F_{experimental}$ 0.669 was found to be greater than $F_{critical}$ 0.403 at the 95% level of confidence). From the performed *t*-Test it was found that there was no significant difference between the levels of analyte recovery obtained using either elution solvent system, ($t_{experimental}$ 0.765 < $t_{critical}$ 2.052 at the 95% level of confidence) and therefore, for all subsequent investigations elution was performed using 5% v/v NH_4OH in 1:1 acetone ethyl acetate.

Having selected the elution solvent, the minimum volume of solvent required for complete analyte elution was then determined. A Strata-XC cartridge was successively eluted with fifteen 1 mL portions of 5% v/v NH_4OH in 1:1 acetone ethyl acetate, each of which in turn was reduced in volume under nitrogen, reconstituted and analysed using LC-MS/MS. The relative recovery of each analyte was determined and plotted against the volume of elution solvent as depicted in

Fig. 7.2. For the purposes of clarity Fig. 7.2 has been simplified and only depicts the resulting elution profiles of cocaine, methadone and temazepam, however, identical traces were recorded in the case of all of the investigated analytes. From Fig. 7.2 it can be seen that 8 mL of solvent is sufficient to remove all retained analyte from the sorbent bed, however, an elution volume of 10 mL was chosen for all further investigations in order to ensure completeness.

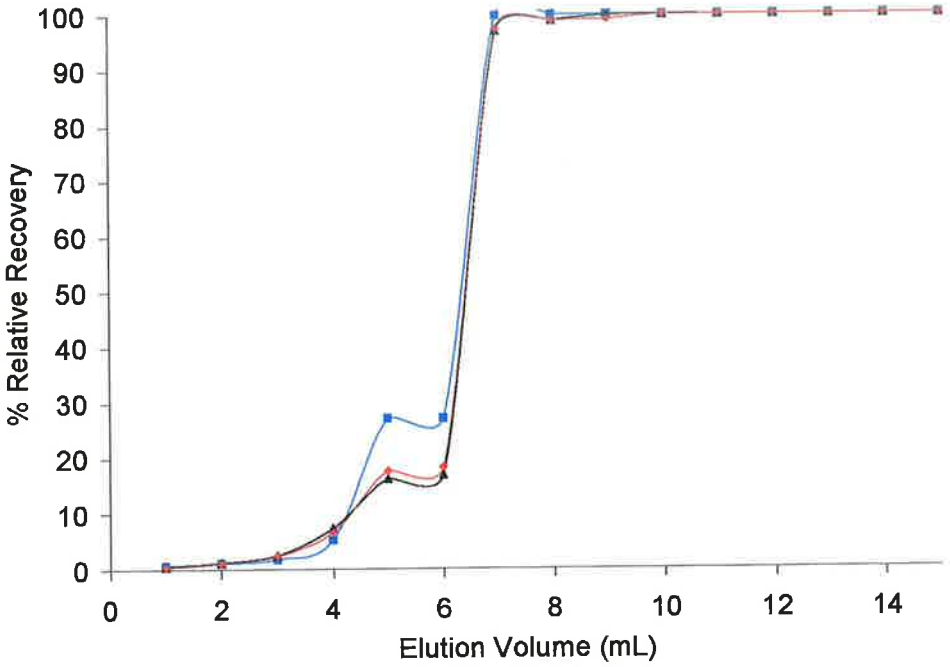


Figure 7.2: Plot of % relative recovery versus SPE elution volume using Strata-XC SPE cartridges and elution with 5% v/v NH_4OH in 1:1 acetone ethyl acetate. *Key:* black trace; methadone, blue trace; cocaine, red trace; temazepam.

7.3.3 Extraction pH optimisation:

The pH of the extraction solution was next optimised in order to determine the sample loading pH that provided the highest levels of analyte recovery. 500 mL aliquots of 1 μgL^{-1} spiked solutions were prepared in 10 mM buffer solutions and extracted using the Strata-XC™ sorbent. Extractions were performed at pH 2 using reagent water adjusted with HCl, pH 3 and 4 using ammonium formate buffer, pH 5 using ammonium acetate buffer, pH 6 and 7 using MES and pH 8 using TRIS. After elution with 10 mL of 5% v/v NH_4OH in 1:1 acetone ethyl acetate, solvent removal and reconstitution, the extract was analysed using LC-MS/MS and as before, the levels of analyte recovery were determined by area comparison with a 1 mgL^{-1} standard, the calculated recovery values are inserted as Table 7.5.

Table 7.5: Recovery data for extraction pH optimisation.

Analyte	% Recovery						
	pH 2	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
Morphine	5	6	16	2	6	7	14
MDMA	62	45	55	67	62	62	38
Benzoylecgonine	113	90	112	99	81	106	55
Ketamine	70	61	62	71	70	68	73
Cocaine	11	15	7	33	54	49	0
Heroin	22	11	5	33	56	55	0
Cocaethylene	36	38	31	57	69	69	2
LSD	40	46	48	48	60	60	88
EDDP	51	40	39	62	63	40	45
Methadone	60	63	59	74	76	74	43
Fluoxetine	43	25	32	41	47	52	17
Temazepam	79	71	87	81	82	92	61
Diazepam	78	72	85	85	82	94	71

Analyte recovery was observed to vary quite significantly with the pH of the extraction solution and in most instances there appears to be no significant trend between the determined levels of analyte recovery and the pH at which the extraction was performed. It was expected that recovery would increase with deceasing solution pH as all analytes under investigation are weakly basic and therefore, by lowering the solution pH they should become positively charged, thereby facilitating increased interaction with the mixed mode cation exchange Strata-XC™ sorbent. Such an effect was expected to be of particular significance for benzoylecgonine, which exists as a zwitterion in neutral solution, whereby performing the extraction at an acidic pH should result in protonation of the acidic functionality of the molecule (pK_a 2.25) and therefore, minimise any possible electrostatic repulsion from the similarly charged sulphonic acid functionality of the extraction sorbent. However, such an effect appears to be absent, with acceptably high recovery of benzoylecgonine determined at investigated pH values in the range of pH 2.0-7.0.

Upon examination of the recovery data in Table 7.5, it was decided that pH 6.0 appeared to be the optimum pH for sample extraction, as levels of recovery for the majority of the chosen analytes were acceptable. It must be mentioned that the recovery of both morphine and fluoxetine was particularly low in all instances. Morphine is a hydrophilic basic compound and it is thought that significant breakthrough of retained morphine occurred from the sorbent during the extraction.

Fluoxetine on the contrary is weakly basic and relatively non polar and therefore, it was expected that higher levels of recovery would be achieved under the experimental conditions employed. In other cases where recovery was found to be less that 70% it was hoped that desired levels of analytical sensitivity would still be achieved when using the previously developed LC-MS/MS method for the determination of preconcentrated residues.

7.3.4 Matrix removal using selective washing:

Having optimised the SPE loading and elution procedures attention was then focused upon the development of a selective washing step in order to achieve cleaner 'matrix free' extracts. The approach undertaken focused upon the determination of the volume of wash solution to which the retained analytes could be exposed without the occurrence of significant breakthrough and hence, analyte loss from the sorbent bed. From the product literature it was recommended that washing be performed with solutions containing low proportions of organic solvent and also when using mixed mode cation exchangers the wash solution should also be acidic in order to 'lock' retained basic analytes onto the sulphonic acid sorbent [55]. Based upon these guidelines, the wash solvent investigated was a solution of 10% v/v MeOH in 0.1 M formic acid. In order to examine the effect this solution on the retained analytes, a 500 mL aliquot of a 5 μgL^{-1} spiked solution, prepared in MES buffer pH 6.0, was extracted using the Strata-XC™ SPE cartridges. The spiking level was deliberately higher than usually used during method development so as any analyte breakthrough during the sorbent washing procedure was clearly detectable upon LC-MS/MS analysis. Without allowing the cartridge run dry, the sorbent was successively washed with twenty 1 mL portions of the wash solution, each of which was collected and individually determined using LC-MS/MS. After washing the sorbent with the first 20 mL of wash solution, a further 3 x 10 mL washings were performed leading to a total of 50 mL solvent washing altogether. The final mL of wash solution was collected and analysed in each of these instances. Upon complete sorbent washing, 500 µL of glacial acetic acid were then added to the sorbent and allowed to percolate slowly through the packed bed in order to aid with drying. In an attempt to ascertain as to whether or not the addition

of the glacial acetic acid had any effect upon analyte retention, the 500 µL portion added was also collected and subsequently determined using LC-MS/MS.

The EICs for both the pseudomolecular ion and the MS/MS product ion transitions were generated for each of the test analytes in all of the determined wash solutions and also the 500 µL addition of glacial acetic acid. However, no traces of any analyte were detected in any of the collected wash solution fractions. Based upon this finding it was suggested that the sorbent could be washed with 50 mL of the 10% v/v MeOH in 0.1 M formic acid solution without any significant analyte loss. Knowing the minimum volume of wash solvent that could be applied to the sorbent bed, the effect of washing upon a real sample matrix, in this case river water collected from the River Boyne, near Navan, Co. Meath, Ireland was then investigated. Fig. 7.3 depicts the resulting chromatogram for a 200 ngL^{-1} spike in river water adjusted to pH 6.0.

From Fig. 7.3 it can clearly be seen that the washing procedure appears to be highly effective in removing any retained matrix components as large distinguished peaks, corresponding to the MS/MS product ion transitions, can easily be detected at a low spiking level in a real sample matrix. It is worth noting that the recorded baseline in each EIC is extremely stable and relatively free of noise, therefore, it was hoped that high levels of analytical sensitivity could be achieved, even when using a moderate sample size of 500 mL.

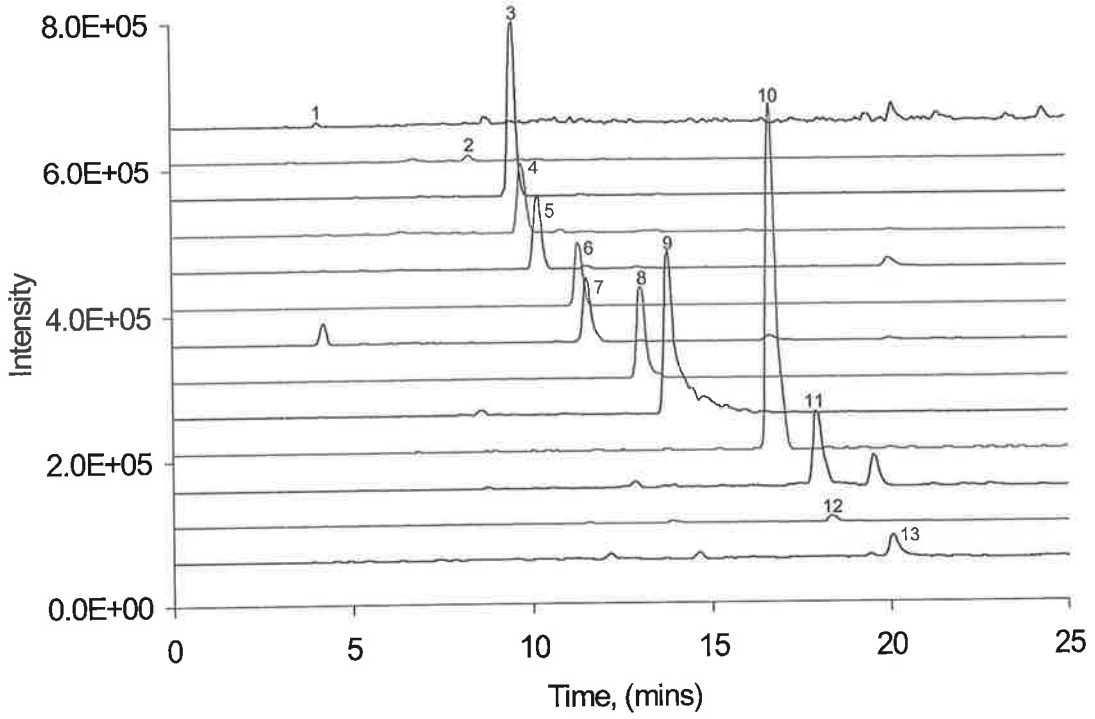


Figure 7.3: Extracted ion chromatograms for a 200 ngL^{-1} mixed analyte spike solution prepared in river water collected from the River Boyne. *Peak identification:* **1** Morphine m/z 286 – m/z 268, T_R 4.1 mins; **2** MDMA m/z 194 – m/z 163, T_R 8.6 mins; **3** Benzoylecgonine m/z 290 – m/z 168, T_R 9.7 mins; **4** Ketamine m/z 238 – m/z 220, T_R 10.0 mins; **5** Cocaine m/z 304 – m/z 182, T_R 10.2 mins; **6** Cocaethylene m/z 318 – m/z 196, T_R 11.5 mins; **7** LSD m/z 324 – m/z 223, T_R 11.8 mins; **8** EDDP m/z 278 – m/z 249, T_R 12.5 mins; **9** Papaverine (Internal Standard) m/z 340 – m/z 202, T_R 13.9 mins; **10** Methadone m/z 310 – m/z 265, T_R 16.8 mins; **11** Temazepam m/z 301 – m/z 283, T_R 17.9 mins; **12** Fluoxetine m/z 301 – m/z 148, T_R 18.3 mins; **13** Diazepam m/z 285 – m/z 257, T_R 20.0 mins.

7.3.5 Method performance:

Prior to application, the performance characteristics of the developed SPE LC-MS/MS method were determined using Boyne river water as a sample matrix. Linearity was assessed using seven point curves prepared by extracting mixed analyte spike solutions in the region of 0.01 to 1 μgL^{-1} (concentrations quoted are those prior to extraction). Repeatability was determined by performing six replicate injections of a 0.20 μgL^{-1} mixed extract while reproducibility was examined using six individually extracted 1 μgL^{-1} mixed analyte solutions. The limits of detection and quantitation were defined as signals corresponding to 3 and 10σ respectively of the baseline noise in each of the extracted MS/MS production ion transition traces. The determined performance characteristics are listed in Table 7.6.

Table 7.6: Method performance data for the developed SPE LC-MS/MS procedure.

Analyte	Linearity	Repeatability	Reproducibility	LOD	LOQ	Recovery
	R^2	%RSD	%RSD	ngL^{-1}	ngL^{-1}	%
Morphine	0.9951	7.10	4.13	257	856	4 ± 0
MDMA	0.9967	4.92	5.22	7	22	52 ± 1
Benzoylecgonine	0.9940	4.53	5.70	1	2	53 ± 3
Ketamine	0.9975	6.44	1.73	1	4	51 ± 3
Cocaine	0.9980	6.64	2.15	1	2	56 ± 2
Cocaethylene	0.9535	6.93	5.79	1	5	65 ± 3
LSD	0.9967	5.29	5.96	3	10	51 ± 3
EDDP	0.9929	6.07	3.69	2	7	59 ± 2
Methadone	0.9771	8.08	2.34	4	14	55 ± 0
Fluoxetine	0.9621	6.19	6.74	93	312	33 ± 2
Temazepam	0.9978	7.79	7.21	7	23	59 ± 3
Diazepam	0.9923	7.56	6.95	38	127	55 ± 3

Levels of determined linearity were excellent within the range of concentrations extracted, calculated regression coefficients greater than 0.99 determined were in all cases with the exception of cocaethylene, methadone and fluoxetine, although even in these instances regression coefficients greater than 0.95 were achieved. The upper limit of linearity was set at 1 μgL^{-1} as it was previously reported by Castiglioni *et al.* that deviations from linearity were observed when similar levels were exceeded when using MS/MS detection [38]. Extraction repeatability was determined using a low level spiked standard, 200 ngL^{-1} and was on average in the range of 5-8% RSD for the six replicate extract injections. Reproducibility was determined using a higher spiking level and was in the region of 2-6% RSD for the six replicate extractions, both determined levels of precision compare well with those of Castiglioni *et al.* who achieved levels of precision <10% for repeatability and <5% for reproducibility using low mgL^{-1} level mixed analyte solutions prepared in wastewater, however, without any preconcentration [38].

Analyte recovery was determined to be in the region of 50-65% for the most analytes, although morphine demonstrated insignificant recovery and fluoxetine also exhibited relatively low affinity for the Strata-XC™ sorbent, both such observations were previously noted. Recovery values determined in this study are considerably lower than that achieved by Castiglioni *et al.* wherein analyte recovery in the region of 85-112% was achieved for illicit drugs using Waters Oasis MCX™, a mixed reversed-phase cation exchange sorbent similar to the Strata-XC™ sorbent. Although the levels of analyte recovery were low, extraction repeatability denoted

by the quoted standard deviation values in Table 7.6 were acceptable. The reason for the determined low levels of analyte recovery is not clear, although, the use of the large volume of wash solvent appears not to be to blame, as no breakthrough from the sorbent was determined during the development of the selective washing step.

An unfortunate consequence of the low levels of analyte recovery was a possible limitation on the levels of analytical sensitivity attainable during the study. However, it should be noted from Table 7.6 that the levels of analytical sensitivity obtained are still suitable for the determination of ultra-trace quantities of any illicit drug residues that may be present in wastewater and surface waters, even when using a moderate sample volume of 500 mL. Detection limits presented in Table 7.6, calculated as 3σ of the peak-to-peak baseline noise in each of the extracted ion chromatograms for each analyte MS/MS transition using river water as a sample matrix were all found to lie in the low ngL^{-1} region. LODs calculated compare favourably with those achieved by both Zucatto *et al.* and Castiglioni *et al.* who then successfully determined residues of illicit drugs in treated wastewater and surface water in both Italy and Switzerland [35,38]. Again the LODs for morphine and fluoxetine are higher than those achieved for other analytes but this is directly attributed to their low levels of recovery during the extractive enrichment step. The calculated LOD for diazepam was also high. It is thought that this is due to the low intensity of the m/z 257 MS/MS product ion. However, similar sensitivity data was obtained when calculated using the EIC for the pseudomolecular ion; m/z 285, (LOD: 36 ngL^{-1}, LOQ: 120 ngL^{-1}) and therefore, due to the higher level of specificity imparted, it is recommended that the MS/MS product ion would still be used for all quantitative purposes.

7.3.6 Determination of illicit drug residues in wastewater and surface waters:

The developed analytical method was then applied for the determination of illicit drug residues in wastewater treatment plants and their receiving waters in Dublin and also in the surrounding counties of Meath and Kildare, both of which have experienced large increases in population in the last decade. In Dublin, wastewater treatment plants investigated included the Ringsend Wastewater Treatment Works which is located at the mouth of the River Liffey on the south side of Dublin Bay

and caters for all of the greater Dublin metropolitan area (1.7 million PEq), the Swords Wastewater Treatment Works in north County Dublin, which discharges into the River Broadmeadow prior to its entry into the Malahide Estuary and the Shanganagh Wastewater Treatment Works which is located in south County Dublin, which discharges via a sea outfall 1.6 km offshore from Killiney Beach into the Irish Sea. Samples of treated effluent were also collected from Leixlip Wastewater Treatment Works in County Kildare which serves the towns of Leixlip, Maynooth, Celbridge and Kilcock, from which treated effluent is discharged directly into the River Liffey and the Navan Wastewater Treatment Centre in County Meath which serves the town of Navan and discharges treated effluent into the River Boyne. All of the plants sampled employ both primary and secondary treatment using activated sludge with the exception of the Shanganagh Wastewater Treatment Works which offers primary screening only prior to discharging the crude wastewater into the sea. The Ringsend Wastewater Treatment Works also offers tertiary treatment using ultraviolet irradiation, however, tertiary disinfection is only employed during the bathing season (May to September) and therefore, samples collected were not exposed to UV light [56]. Official weather data for the weekend of the 18/19th November was obtained from The Irish Meteorological Service wherein total precipitation at the Dublin Airport monitoring station was 5.3 mm [57]. Collected samples were analysed as previously described and levels of analytes detected are presented in Table 7.7.

Table 7.7: Concentrations (ngL^{-1}) of illicit drugs detected in collected wastewater and surface water samples.

Sample	Concentration Detected, (ngL^{-1})				
	Morphine	Benzoylecgonine	Cocaine	EDDP	Temazepam
Ringsend Influent	-	290 ± 11	489 ± 117	-	320 ± 56
Ringsend Effluent	< LOQ	22 ± 4	138 ± 20	48 ± 1	126 ± 14
Swords Effluent	874 ± 86	-	-	206 ± 10	-
River Broadmeadow	-	-	25 ± 7	-	-
Shanganagh Effluent	-	31 ± 18	77 ± 25	-	-
Killiney Beach	-	-	-	-	-
Leixlip Effluent	< LOQ	-	47 ± 10	9 ± 1	106 ± 3

River Liffey	-	-	33 ± 11	-	-
Navan Effluent	452 ± 86	-	111 ± 15	67 ± 10	-
River Boyne	-	-	-	-	-

From the list of selected analytes only morphine, benzoylecgonine, cocaine, EDDP and temazepam were detected in the collected wastewater and surface water samples. Morphine was detected in relatively high concentrations in the treated effluents of the Swords and Navan wastewater treatment plants. However, such levels are more likely to be attributable to medicinal use of morphine and related opiates rather than consumption of illicit heroin. Morphine was not detected in the influent of the Ringsend Wastewater Treatment Works but was present at a detectable level below the LOQ in the corresponding effluent sample. Such an observation may occur due to cleavage of glucuronide metabolites of morphine during the treatment process [26,38].

Benzoylecgonine and cocaine were detected in both the influent and effluent samples of the Ringsend Wastewater Treatment Works and also in the grab sample collected from the Shanganagh Wastewater Treatment Works. Cocaine was also detected at low ngL^{-1} levels in the treated effluent of the Navan and Leixlip treatment plants. The presence of cocaine and its primary urinary metabolite benzoylecgonine provides a reliable indication of human consumption of cocaine. However, unlike previous reports from Zucatto *et al.* and Castiglioni *et al.* the levels of cocaine detected in the present study were higher than those of benzoylecgonine [35,38]. Previous pharmacokinetic studies reveal that cocaine is readily metabolised into benzoylecgonine and therefore, it was expected that higher quantities of benzoylecgonine should be present compared to those of cocaine [58]. A reason for the low levels of benzoylecgonine detected may be due to higher rates of removal via either degradation or sorption during the treatment process compared to cocaine. Based upon the levels of both benzoylecgonine and cocaine detected in the influent and effluent of the Ringsend Wastewater Treatment Works it is estimated that the removal rates within the plant on the day of sampling were 93% and 72% for benzoylecgonine and cocaine, respectively. Levels detected compare well with those reported by Castiglioni *et al.* when analysing the effluents of wastewater treatment plants in Italy and Switzerland [38].

Interestingly, cocaine was also detected in the samples (collected near the point of discharge) of the River Broadmeadow and the River Liffey. However, the levels detected were lower than those detected in the treated effluent samples therefore, suggesting dilution within the receiving water. It would be expected that levels detected would decrease significantly with increasing distance from the discharge point. Such an effect can be seen in the case of the River Boyne wherein the sample, in which no illicit drug residues were detected, was collected approximately 4 km downstream from the Navan Wastewater Treatment Centre.

EDDP, the primary metabolite of methadone, was detected in a number of effluent samples in low ngL^{-1} concentrations, although methadone was not detected in any of the collected samples. The presence of EDDP in the absence of methadone is somewhat surprising, Castiglioni *et al.* detected EDDP in the presence of methadone in a ratio of ~2:1 in both treatment plants studied [38]. However, EDDP exists as a charged species in solution and would therefore, be expected to persist in the aqueous phase during wastewater treatment and be detected in the final effluents.

Temazepam was detected in both the influent and the effluent samples of the Ringsend Wastewater Treatment Works and also in the effluent of Leixlip Wastewater Treatment Works. Temazepam is a prescribed medication and was included in the list of target analytes along with diazepam as both sedatives have the potential for abuse. The presence of temazepam in the wastewater samples is thought, like morphine, to be a result of medicinal and therapeutic use rather than illicit or abused consumption. As was noted for both benzoylecgonine and cocaine, the level of temazepam detected in the Ringsend treatment plant decreased from influent to effluent suggesting a removal of approximately 60% via either sorption or degradation during the passage of temazepam through the treatment works.

For the purposes of illustration, Fig. 7.4 depicts MS/MS product ion chromatograms showing (A) the presence of benzoylecgonine and cocaine in the influent and effluent of the Ringsend Wastewater Treatment. The excellent retention time reproducibility should also be noted from Fig. 7.4 (A) along with the clear reduction of the levels of both substances detected in the effluent sample compared to the influent sample. Fig. 7.4 (B) depicts the suspected presence of EDDP in the effluent sample collected from the Navan Wastewater Treatment

Centre. The term 'suspected' is used only due to the absence of methadone in the sample. However, Fig. 7.4 (B) unequivocally shows the presence of trace levels of EDDP in the collected sample.

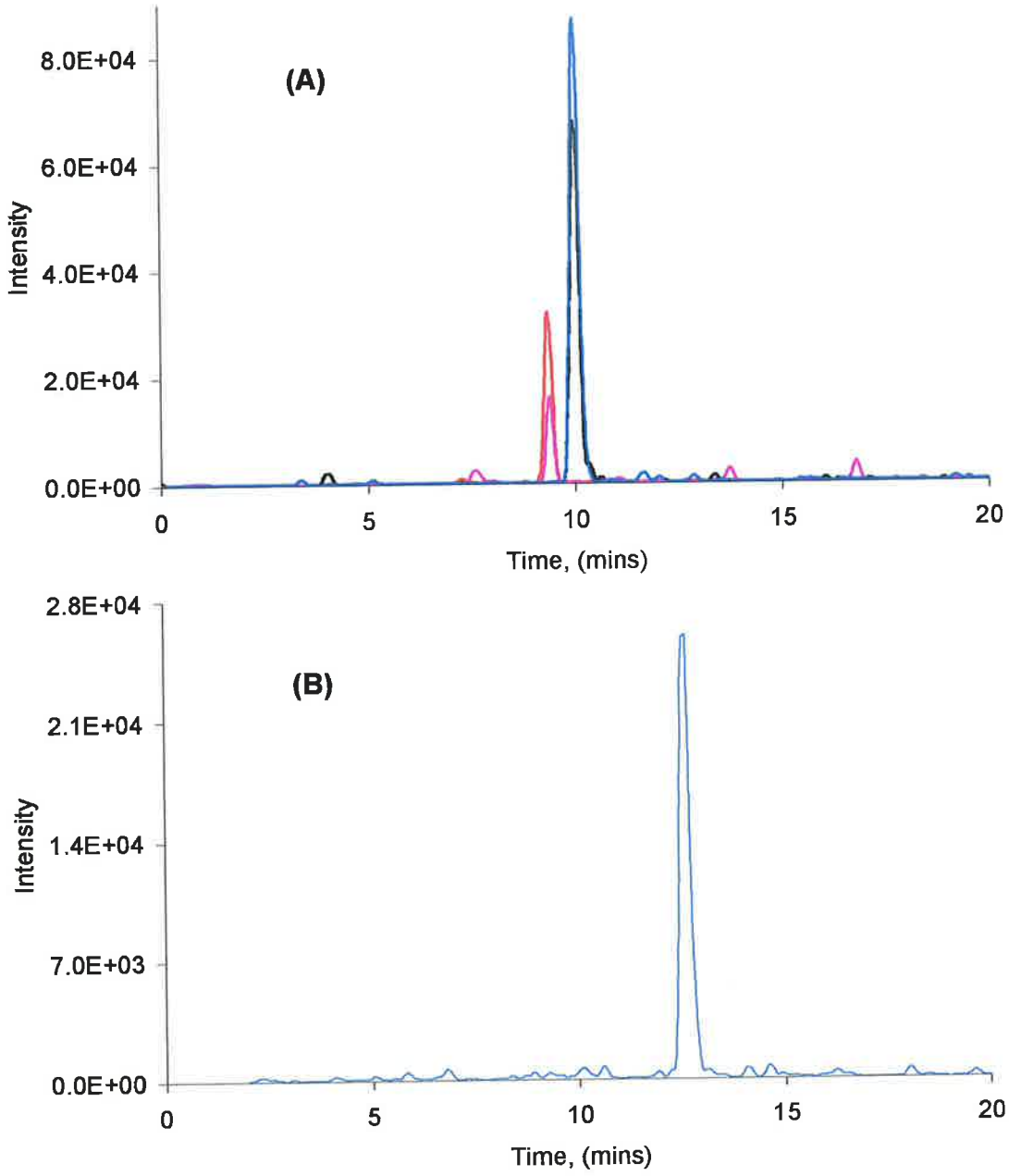


Figure 7.4: (A) The presence of ngL^{-1} quantities of benzoylecgonine and cocaine in the influent and effluent of the Ringsend Wastewater Treatment Works, *key*: benzoylecgonine influent; red trace, benzoylecgonine effluent; pink trace, cocaine influent; blue trace, cocaine effluent; black trace, and (B) the presence of ngL^{-1} quantities of EDDP in the treated effluent of the Navan Wastewater Treatment Centre.

7.3.7 Estimation of community consumption of cocaine from environmental data:

Having detected levels of illicit drugs, in particular cocaine, in the effluents of the examined wastewater treatment plants, an attempt was made to estimate consumption of cocaine within the community served by those treatment works. As mentioned previously, the presence of morphine and temazepam in treated effluent is most likely to be attributed to medicinal and therapeutic use of such compounds and therefore, it would be inappropriate to relate such levels to heroin consumption or benzodiazepine abuse, respectively. Calculations were not performed on measured EDDP concentrations due to the absence of the parent compound methadone, and also a lack of available pharmacokinetic information regarding the transformation of methadone.

The approach used in this instance is based upon that of Zucatto *et al.* however, as previously mentioned, cocaine was found in greater quantities in this study than its primary urinary metabolite benzoylecgonine and was therefore used for the performance of community consumption calculations rather than benzoylecgonine [35]. From the levels of cocaine detected it is possible to calculate the load of cocaine within the treatment plant on the day in which the sample was collected simply by multiplying the analytical data by the flow through the plant, such data is presented in Table 7.8.

However, a further refinement of the data can be performed when considering the pharmacokinetic behaviour of cocaine, wherein only approximately 10% of the parent dose ingested is excreted as cocaine via the urine [59]. Therefore, based upon such information, the data in Table 7.8 can be further refined as displayed in column 3 of Table 7.8. Even though the calculated cocaine loads may seem high, it is thought, that results generated are actually conservative when considering consumption of the drug within the community under study as a high probability exists that analyte degradation or removal within the treatment plant or wastewater transport system may have occurred prior to collection of the sample. The cocaine load value quoted for the Ringsend Wastewater Treatment Works refers to that determined in the influent sample in order to minimise extra variability such as analyte removal during treatment *etc.* Although the value for the Ringsend Wastewater Treatment Works appears high it should be remembered that the plant caters for the entire Dublin metropolitan area, (1.7 million PEq) and

also the sample corresponds to the weekend, a time in which cocaine consumption would be thought to be at a maximum.

Table 7.8: Cocaine loads within the sampled wastewater treatment plants.

Wastewater Treatment Plant	Cocaine Load ($g.day^{-1}$)	Refined Cocaine Load ($g.day^{-1}$)
Ringsend	224.50	2245.00
Shanganagh	1.69	16.90
Leixlip	1.41	14.10
Navan	1.17	11.70

From the cocaine load within the plant it is possible to estimate the use of cocaine by the population catchments served by the wastewater treatment plants. Combining the data from Tables 7.1 and 7.8 the estimated use of cocaine within the community is presented in Table 7.9.

Table 7.9: Estimated community consumption of cocaine within the catchments served by the sampled wastewater treatment plants.

Catchment	Estimated Cocaine Consumption	
	Per 1000 PEq ($g\ day^{-1}$)	Per 1000 PEq ($doses\ day^{-1}$)
Ringsend	1.44	14.38
Shanganagh	0.26	2.61
Leixlip	0.18	1.76
Navan	0.29	2.91

The results presented in Table 7.9 compare favourably with those previously published by Zucatto *et al.* [35]. The dose per day data presented in Table 7.9 were calculated based upon information present in [59], which states that one dose equals approximately 100 mg cocaine. Zucatto *et al.* further refined their data to include a positive bias against young adults in the age bracket of 15 to 34 years old due to a higher consumption of cocaine by persons within this age bracket when compared to the general population. It was hoped to undertake a similar transformation in this study. However, official statistics available from The Central Statistics Office concerning the population census 2006 are only preliminary in nature. It is stated in the opening lines of the available preliminary report that record increases in population have been recorded within the four year period

since the last census in the catchment areas studies, particularly due to the influx of young immigrants and therefore, it was felt that it would be incorrect to use the data from the previous census conducted in 2002 [60].

The levels of cocaine consumption estimated in this approach suggest that large quantities of the drug are being consumed within Dublin but it can also be said that the problem of cocaine consumption is not wholly confined to the capital city but present in smaller towns outside Dublin. The detection of cocaine and its urinary metabolite in the wastewater treatment plants adds weight to the assumption that the presence of cocaine arises due to human consumption and not improper disposal of the narcotic. The problem exists in Ireland that there are currently no reliable statistics regarding cocaine consumption to compare the generated data against. The developed analytical procedure could be applied for the routine monitoring of cocaine consumption within the community in order to assess sociologically whether usage trends are stable, thereby indicating regular use and possibly addiction within the community or whether trends are sporadic, which may indicate whether cocaine lives up to its 'party drug' reputation. It is also felt that the developed method and any results generated provide a much more reliable snapshot of community consumption of cocaine compared to survey based population investigations due to the non invasive sampling employed in the current study.

7.4 Conclusions:

A suitably sensitive and validated analytical method using SPE LC-MS/MS for the determination of residues of illicit drugs in wastewater and surface water has been described. Cocaine was detected in samples of wastewater and subsequent receiving waters collected from treatment plants in Dublin city and surrounding counties. From the environmental analytical data it was possible to estimate community consumption of cocaine within the catchment areas of the sampled wastewater treatment plants. Estimations of cocaine consumption as doses per 1000 PEq per day compare well with previously published data from Italian researchers. From the estimated consumption data it is concluded that cocaine is widely used within the country at present and that such an observation is not solely

confined to the capital city. The present study provides a precedent in Ireland concerning drug consumption within the community and it is therefore, recommended that the approach presented here should be used for the continuous 'real time' monitoring of illicit drug consumption within society by regulatory bodies. The method is cheaper and faster to apply than survey based methodologies and offers a new prospect to social scientists and those with a key interest in monitoring the Irish drug problem.

References:

1. M. O'Halloran, *'Cocaine seizures rise eight fold"*, The Irish Times, Saturday October 1st 2005.
2. The Press Releases Section of the website of An Garda Síochána, viewed 5th December 2006, http://www.garda.ie/angarda/press.html.
3. G. Singh, V. Arora, P.T. Fenn, B. Mets & I.A. Blair, *Analytical Chemistry*, **71**, (1999), 2021.
4. R.J. Lewis, R.D. Johnson, K.M. Angier & R.M. Ritter, *Journal of Chromatography B*, **806**, (2004), 141.
5. F. Sadeghipour & J-L. Veuthey, *Journal of Chromatography A*, **787**, (1997), 137.
6. M. Concheiro, A. de Castro, O. Quintela, M. Lopez-Rivadulla & A. Cruz, *Journal of Chromatography B*, **832**, (2006), 81.
7. M.R. Moeller, S. Steinmeyer & T. Kraemer, *Journal of Chromatography B*, **713**, (1998), 91.
8. H.Z. Rofael & M.S. Abdel-Rahman, *Journal of Applied Toxicology*, **22**, (2002), 123.
9. K-C. Wang, T-S. Shih & S-G. Cheng, *Forensic Science International*, **147**, (2005), 81,
10. B. Maralikova & W. Weinmann, *Journal of Chromatography B*, **811**, (2004), 21.
11. V. Bianchi & G. Donzelli, *Journal of Chromatography B*, **675**, (1996), 162.
12. R. Dams, C.M. Murphy, R.E. Choo, W.E. Lambert, A.P. de Leenheer & M.A. Huestis, *Analytical Chemistry*, **75**, (2003), 798.
13. P. Kintz, M. Villain, M. Concheiro & V. Cirimele, *Forensic Science International*, **150**, (2005), 213.
14. M. Wood, M. Laloup, M. del Mar Remirez Fernandez, K.M. Jenkins, M.S. Young, J.G. Ramaekers, G, de Boeck & N. Samyn, *Forensic Science International*, **150**, (2005), 227.
15. K.A. Mortier, K.E. Maudens, W.E. Lambert, K.M. Clauwaert, J.F. van Bocxlaer, D.L. Deforce, C.H. van Peteghem & A.P. de Leenheer, *Journal of Chromatography B*, (2002), 321.
16. D.A. Kidwell, J.C. Holland & S. Athanaselis, *Journal of Chromatography B*, **713**, (1998), 111.
17. K.M. Clauwaert, J.F. van Bocxlaer, W.E. Lambert, E.G. van den Ecekhout, F. Lemierem, E.L. Esmans & A.P de Leenheer, *Analytical Chemistry*, **70**, (1998), 2336.
18. K.B. Scheidweiler & M.A. Huestis, *Analytical Chemistry*, **76**, (2004), 4358.

19. F. Musshoff, F. Driever, K. Lachenmeier, D.W. Lachenmeier, M. Banger & B. Madea, *Forensic Science International*, **156**, (2006), 118.

20. M. Cheze, G. Duffort, M. Deveaux & G. Pepin, *Forensic Science International*, **153**, (2005), 3.

21. M. Villain, M. Concheiro, V. Cirimele & P. Kintz, *Journal of Chromatography B*, **825**, (2005), 72.

22. J. Gareri, J. Klein & G. Koren, *Clinica Chimica Acta*, **366**, (2006), 101.

23. S. Pichini, R. Pacifici, M. Pellegrini, J. Lozano, J. Murillo, O. Vall & O. Garcia-Algar, *Analytical Chemistry*, **76**, (2004), 2124.

24. D. Thieme & H. Sachs, *Analytica Chimica Acta*, **492**, (2003),171.

25. C.G. Daughton & T.A. Ternes, *Environmental Health Perspectives*, **107**, (1999), 907.

26. T.A. Ternes, *Water Research*, **322**, (1998), 3254.

27. M.J. Hilton & K.V. Thomas, *Journal of Chromatography A*, **1015**, (2003), 129.

28. S. Zuehlke, U. Duennbier & T. Heberer, *Analytical Chemistry*, **76**, (2004), 6548.

29. P.H. Roberts & K.V. Thomas, *Science of the Total Environment*, **356**, (2006), 143.

30. P.H. Roberts & P. Bersuder, *Journal of Chromatography A*, **1134**, (2006), 143.

31. D. Ashton, M. Hilton & K.V. Thomas, *Science of the Total Environment*, **333**, (2004), 167.

32. C.G. Daughton, *"Illicit Drugs in Municipal Sewage"* in *"Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues"*, ACS Symposium Series, (2001), 348, The American Chemical Society.

33. T.L. Jones-Lepp, D.A. Alvarez, J.D. Petty & J.N. Huckins, *Archives of Environmental Contamination and Toxicology*, **47**, (2004), 427.

34. A. Kaleta, M. Ferdig & W. Buchberger, *Journal of Separation Science*, **29**, (2006), 1662.

35. E. Zucatto, C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea & R. Fanelli, *Environmental Health*, **4**, (2005), 14.

36. J. Orr & N. Goswami, '*River of cocaine*' The Telegraph, London, November 6th 2005.

37. K. Ross, *Analytical Chemistry*, 'AC Detective Article' January 1st 2006, **78**, 13.

38. S. Castiglioni, E. Zucatto, E. Crisci, C. Chiabrando, R. Fanelli & R. Bagnati, *Analytical Chemistry*, **78**, (2006), 8421.

39. Morphine Products information, www.rxlist.com/cgi/generic/ms.htm, viewed July 12th 2006.

40. Morphine information, HIV/OI Chemical Biological Database, http://chemdb2.niaid.nih.gov/struct_search/all/url_search.asp?aids_no=001987, viewed July 12th 2006.

41. FDA Adderall XR Capsules product information, www.fda.gov/cder/foi/label2005/0213035009lbl.pdf, viewed July 12th 2006.

42. Virtual Computational Chemistry Laboratory LogP Calculator, (XLogP Data), http://146.107.217.178/lab/alogps/start/html, viewed July 12th 2006.

43. S. Pichini, *"Distribution of 3,4-Methylenedioxy Methamphetamine in non conventional matrices and its application in clinical toxicology"*, www.tdx.cesce.es/TDX-0119106-195119/, viewed July 12th 2006.

44. S. Seidel, E.S. Singer, H. Just, H. Farhan, P. Scholze, O. Kudlacek, M. Holy, K. Koppatz, P. Kirvanek, M. Freissmuth & H.H. Sitte, *Molecular Pharmacology*, **67**, (2005), 140.

45. G. Skopp & L. Potsch, *International Journal of Legal Medicine*, **112**, (1999), 213.

46. Benzoylecgonine information, HIV/OI Chemical Biological Database, http://chemdb2.niaid.nih.gov/struct_search/all/url_search.asp?aids_no=001987, viewed July 12th 2006.

47. J.F. MacDonald, M.C. Bartlet, I. Mody, P. Pahapill, J.N. Reynolds, M.W. Slater, J.H. Schneiderman & P.S. Pennefather, *Journal of Physiology*, **432**, (1991), 483.

48. L. Barkley Barnett, *"Toxicity, cocaine"*, http://www.emedicine.com/emerg/topic102.htm, viewed July 12th 2006.

49. D.A. Kidwell & F.P. Smith, *"Susceptibility of PharmChek Drugs of Abuse Patch to Environmental Contamination"*, http://www.ncjrs.gov/pdffiles/nij/195986.pdf, viewed July 12th 2006.

50. S. Kerrigan, Clinical & Forensic Technology News March 2000, http://www.eaacc.org/cftn/issues/2000/mar00.pdf, viewed July 12th 2006.

51. D.L. Roerig, R.R. Dahl, C.A. Dawson & R.I. Warp, *Drug Metabolism and Disposition*, **12**, (1984), 536.

52. H. Choe, Y-K Lee, Y-T Lee, H. Choe, S-H Ko, C-U Joo, M-H Kim, G-S Kim, J-S Eun, J-H Kim, S-W Chae & Y-G Kwak, *The Journal of Pharmacology and Experimental Theraputics*, **304**, (2003), 706.

53. Eli Lilly Product MSDS, http://www.ehs.lilly.com/msds/msds_olanzapine_fluoxetine_combination_capsules.htm, viewed July 12th 2006.

54. Wikipedia Δ^9-THC information, http://en.wikipedia.org/wiki/tetrahydrocannabinol, viewed July 12th 2006.

55. Phenomenex product information, '*Polymeric Sorbents for Sample Preparation*', received with cartridges purchased from Phenomenex, Macclesfield, United Kingdom.

56. Dublin Drainage Consultancy, *'Greater Dublin Strategic Drainage Study – Assessment of Existing Wastewater Treatment Works'*, July 2002, available online at http://www.fingalcoco.ie/YourLocalCouncil/Services/Waterservices/GreaterDublinStrategicDrainageStudy/GDSDS/Appendix%20E-Assesment%20of%20TW/WWTS%assesment%20report.pdf, last viewed December 15th 2006.

57. Dublin Airport weather station data for the 18/19/20th November 2006 available through http://www.meteireann.ie/climate/daily-data.asp, viewed 18th December 2006.

58. J. Ambre, *Journal of Analytical Toxicology*, **9**, (1985), 241.

59. O.H. Drummer, *Forensic Science International*, **142**, (2004), 101.

60. The Central Statistics Office, *'Census 2006 – Preliminary Report'*, available through http://www.cso.ie, viewed December 21st 2006.

8.0 Overall Conclusions & Future Research Direction.

8.1 Overall Conclusions:

In the research presented here, efficient and sensitive methods for the determination of pharmaceuticals and illicit drugs residues in environmental samples, based upon monolithic silica column technology were developed. In the first instance a highly efficient dual gradient LC separation of a selection of environmentally relevant pharmaceuticals was developed using a 20 cm monolithic C_{18} column. It was found that when combined with SPE, detection limits were in the sub μgL^{-1} range and the method was applied to the monitoring of the selected drugs in a variety of environmental matrices. The use of PDA detection imparted a greater degree of selectivity to the developed method allowing for the collection of UV spectra at the ultra trace level from which semi-qualitative data could be obtained. The method may be particularly useful to those who wish to monitor pharmaceutical residues but who do not have access to LC-MS instrumentation.

The second developed method involved the adaptation of the dual gradient LC method with the coupling to ESI-MS to increase detection specificity. The use of short monolithic columns as online SPE extraction traps was also evaluated and it was found that detection limits in the low ngL^{-1} region were readily achievable. It was however, noted that excessive matrix magnification was a major problem but the incorporation of a simple solvent wash step was found to remove the preconcentrated matrix by >80%. A major limitation to the use of monolithic silica columns in conjunction with ESI-MS detection was encountered as due to the flow mismatch and the need for the incorporation of a splitting device. Therefore, ESI-MS could only be used to provide qualitative information and could not be used as a quantitative detector.

Monolithic silica columns were also effectively used for the development of an analytical procedure for the determination of the highly toxic antifouling and anti-dandruff agent zinc pyrithione in environmental waters. Due to the low silanol activity of the high purity monolithic silica stationary phase, easier LC was facilitated, wherein previous problems concerning tailing and non specific interaction with metal ion contamination were completely avoided. Short

monolithic columns were successfully employed for the rapid trace enrichment of low ngL^{-1} levels of ZnPT from water samples. The inclusion of both a sacrificial silica strong anion exchange sorbent prior to the monolithic trap column and a solvent wash procedure aided with the minimisation of APCI-MS ion suppression by co-extracted matrix components. Using the developed method it was found that ZnPT transchelates with Cu^{2+} ions to form the more stable CuPT complex in the environment. The formed CuPT is rapidly degraded upon exposure to sunlight, with and experimentally determined half life of 45 minutes under laboratory conditions. It therefore, appears that ZnPT, (as CuPT) does not pose an environmental problem provided that the rates of photolytic removal exceed the rates of introduction.

Two methods were then presented concerning non invasive analytical procedures for the estimation of illicit drug use within the community. A comparison of the efficiency and kinetic behaviour of newly available 3.0 mm i.d. monolithic silica reversed-phase columns and sub 2 µm particle packed columns was also undertaken and it was observed that the monolithic columns were considerably more efficient chromatographic supports with significantly lower operating pressures than the sub 2 µm particle packed columns. The first approach to estimate drug use within the community focused upon the determination of illicit drug contamination of banknotes in general circulation using simple sample preparation followed by LC-MS/MS analysis. 45 notes of different denominations were collected along with three un-circulated controls. Trace levels of cocaine were detected on all of the investigated samples accompanied by benzoylecgonine in many instances. Traces of heroin were found on three lower denomination notes. Of the levels of cocaine detected, it appears that the majority of contamination is thought to result due to transfer during counting in financial institutions. Two notes out of the 45 tested showed levels significantly high to suggest direct contact with the drug; most likely the note was used to snort cocaine. To further refine the estimation, a solid phase extraction procedure was developed in order to increase the sensitivity of the analytical procedure and samples of wastewater and surface water were analysed for the presence of illicit drug residues. In cases where drugs were detected, it was possible to apply an adaptation of the approach previously presented by Italian researchers for the estimation of cocaine consumption in both weight and doses per 1000 population equivalents. Based upon the

generated analytical data it was estimated that 14 100 mg doses of cocaine were consumed per 1000 population equivalents in the twenty four hour period of the collected Ringsend Wastewater Treatment Works composite sample, which caters for the entire Dublin metropolitan area. It is thought that the developed analytical method should be further applied for the routine monitoring of illicit drug consumption within the community by the State and relevant interested authorities.

Interesting data was generated using each of the developed methods. With regard to the samples tested, levels of illicit drug residues were detected more often than levels of medicinal pharmaceuticals. However, the reason for such an observation lies in the matrices tested and also the detection mechanisms employed. The methods developed concerning the use of LC-MS/MS for the determination of the illicit drug residues highlight the need for both the sensitivity and specificity imparted by mass selective detection. The lack of analyte detection when using the dual gradient methods is a combination of the use of optical detection to determine ultra trace residues in samples wherein high dilution of any possible pharmaceutical input is guaranteed, i.e. fast flowing rivers. Although not applied to treated wastewater, a level of confidence exists that traces of the investigated analytes would indeed have been found as the levels of sensitivity attainable with the dual gradient methods is suitable for the monitoring of such.

Another notable conclusion from the research undertaken in the present study is that of the unwanted effects of the sample matrix. Co-extraction and magnification of the sample matrix presents a limiting problem, especially when mass selective detection is employed as problems such as ion suppression are readily encountered, thereby adversely affecting sensitivity and detection in general. It was also noted that distinct matrix diversity exists across the range of samples investigated, with some more problematic than others. A solution encountered in the literature is often to develop methods that are matrix specific, i.e. one for wastewater, one for surface water, etc. However, such an approach increases both the time and cost of the analysis and is therefore, not a feasible solution. The need therefore, exists for the development of more specific extraction chemistries and procedures. For example, as was previously mentioned in Chapter 5.0, the incorporation of a sacrificial anion exchange sorbent prior to the reversed-phase trap column radically minimised the matrix

suppression of the APCI-MS signal. When using polymeric SPE cartridges efforts should be focused upon sample washing and clean up, again referring back to Chapter 7.0 the effect of a selective washing procedure allowed for almost complete removal of the sample matrix with no observed effects on analyte recovery or the ESI-MS/MS signal. It is therefore, recommended to those developing methods for trace enrichment of organic micro contaminants in complex matrices that selective sample clean up will be of paramount importance in order to achieve the desired levels of sensitivity.

The advantages offered by monolithic silica columns, in particular the newly available 3.0 mm i.d. column are also worth mentioning again. The high purity of the silica substrate and low silanol activity of the functionalised stationary phase should be noted, especially for the chromatography of basic compounds and as demonstrated in Chapter 5.0 labile compounds such as metal ligand complexes. As shown in Chapters 6.0 the primary advantage of monolithic silica columns lies within their high inherent porosity and therefore, the ability to use longer columns with high chromatographic efficiency. It is hoped that further reduction in the 'bore size' of the monoliths and a wider array of stationary phase chemistries will soon become commercially available.

One last question to be posed concerns the detection of pharmaceutically active compounds in the environment. It can be suggested that the levels detected are so small, they pose no environmental risk or toxicological threat. This indeed may be true; it is only with recent advances in analytical chemistry that the detection of such residues has become possible, even though the introduction of such compounds to the environment has been occurring for years previous. Although the presence of many drug residues at low environmental concentrations may be harmless, the ability to demonstrate the presence of pharmaceutically active compounds in the environment is still important. As shown in this research, it affords to opportunity to non invasively monitor community behaviour and consumption of drugs and other substances. The threat, however, exits in cases where the presence of high potency low dose compounds such as anti-neoplastic agents and hormones and therefore, the development and application of methods for the determination of such are of high importance. With continuing advances in analytical methodologies and instrumentation we may soon approach the situation of 'seek and you shall find'.

8.2 Future Research Direction:

The following areas have been identified as possibly requiring further research and may be of relevance to those undertaking similar studies:

- The development of more specific and selective extraction methods is an area which undoubtedly requires increased attention by the analytical community. The availability of high performance sorbents such as the hydrophilic lipophilic balanced polymers has increased the applicability of SPE. However, as discovered in this study, these sorbents also magnify the matrix significantly and therefore, the development of complimentary wash steps to selectively remove or minimise matrix retention is an area that requires attention.
- The inclusion of metabolites into the analytical methods should also be addressed. This will require availability of both information and reference standards for pharmaceutical manufacturers. A current limitation in the area is the lack of reference standards, especially of pharmaceuticals that are still under patent.
- It is felt that the non invasive community consumption approach presented in Chapter 7.0 should be routinely applied by the State and relevant authorities or interested bodies in order to gather accurate data regarding the use of illicit drugs within the community. The method presented is specific and sensitive, a further refinement could, however, focus upon the substitution of the Phenomenex Strata-XC™ SPE sorbent used by the Waters Oasis MCX™ SPE sorbent in order to see if analyte recovery and hence analytical sensitivity can be further improved.
- The behaviour of pharmaceutically active compounds during the wastewater treatment process also requires further investigation, particularly the adsorption of drug residues to solids and estimations of partitioning behaviour. Other environmental processes, such as photo transformation of pharmaceutical residues should also be examined, thereby allowing for the refinement of analytical methods to include relevant compounds, e.g. photoproducts rather than parent compounds etc.