High Performance Liquid Chromatography-Tandem Mass Spectrometry of Pharmaceuticals and Personal Care Products in Environmental and Biological Matrices

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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

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List of Abbreviations:

APCI: Atmospheric pressure chemical ionisation

API: Active pharmaceutical ingredient

API: Atmospheric pressure ionisation

ASE: Accelerated solvent extraction

BEG: Benzoylecgonine

BOD: Biochemical oxygen demand

CEC: Capillary electro-chromatography

CH_2Cl_2: Dichloromethane

CID: Collisionally induced dissociation

DAD: Diode array detector

DBP: Dibutylphthalate

DC: Direct current

DEHP: Diethylhexylphthalate

DFC: Drug facilitated crime

DOM: Dissolved organic matter

EDC: Endocrine disrupting chemical

EDDP: 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine

EI: Electron impact

EIC: Extracted ion current

ELISA: Enzyme-linked immunosorbent assay

EMEA: European medicines evaluation agency

EMIT: Enzyme multiplied immunoassay technique

EOF: Electroosmotic flow

EPI: Enhanced product ion

ERA: Environmental risk assessment

ESI: Electrospray ionisation

EU: European Union

GC: Gas chromatography

GC-MS: Gas chromatography-mass spectrometry

GC-MS/MS: Gas chromatography-tandem mass spectrometry

GMP: Good manufacturing practice

GPC: Gel permeation chromatography

HCl: Hydrochloric acid

HETP/H: Height equivalent of a theoretical plate

HPLC: High performance liquid chromatography

HS-SPME: Headspace solid-phase micro-extraction

HT-UPLC: High temperature-ultra performance liquid chromatography

ICM: Iodinated contrast media

I.D.: Internal diameter

IDA: Information dependant acquisition

IPLC: Ion-pair liquid chromatography

IT: Ion-trap mass analyser

LC: Liquid chromatography

LC-MS: Liquid chromatography-mass spectrometry

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LLE: Liquid-liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

LPME: Liquid phase micro-extraction

LSD: Lysergic acid diethylamide

LSE: Liquid-solid extraction

MASE: Membrane assisted solvent extraction

MBR: Membrane bioreactor technology

MDA: Methylenedioxyamphetamine

MDMA: Methylenedioxymethamphetamine

MeCN: Acetonitrile

MEKC: Micellar electrokinetic chromatography

MeOH: Methanol

MIP: Molecularly imprinted polymers

MRM: Multiple reaction monitoring

MS: Mass spectrometry

MS^1: Single mode mass spectrometry

MS^2: Tandem mode mass spectrometry
MS/MS: Tandem mass spectrometry
NaOH: Sodium hydroxide
N: Number of theoretical plates per column
NMR: Nuclear magnetic resonance
NSAID: Non steroidal anti-inflammatory drug
oa-TOF: Orthogonal acceleration time-of-flight mass analyser
OSPAR: Oslo Paris convention for the protection of the marine environment of the North East Atlantic
OTC: Over the counter
PAH: Polycyclic aromatic hydrocarbons
PCB: Polychlorinated biphenyls
PEEK: Polyetheretherketone
PFPP: Pentafluorophenylpropyl
PLE: Pressurised liquid extraction
PPCP: Pharmaceutical and personal care product
PTFE: Polytetrafluoroethylene
PVDF: Polyvinylidine fluoride
q-LIT: Quadrupole linear ion-trap mass analyzer
QqQ: Triple quadrupole mass analyzer
q-TOF: Quadrupole time-of –flight mass analyser
RAM: Restricted access materials
RF: Radio frequency
RIA: Radioimmunoassay
RP-Reversed phase
RPLC: Reversed-phase liquid chromatography
SEC: Size exclusion chromatography
SFE: Supercritical fluid extraction
SIM: Single ion monitoring
SPE: Solid-phase extraction
SPME: Solid-phase micro-extraction
SRM: Selected reaction monitoring

SRT: Solids retention time

SS: Suspended solids

THC: Δ^9-tetrahydrocannabinol

THC-COOH: 11-nor-9-Carboxy-Δ^9-tetrahydrocannabinol

TIC: Total ion current

TLC: Thin layer chromatography

TOF: Time-of-flight mass analyser

TrBA: Tri-n-butylamine

UPLC: ultra performance liquid chromatography

USE: Ultrasonic solvent extraction

UV: Ultraviolet

UWWT: Urban wastewater treatment

WWTP: Waste water treatment plant

6-MAM: 6-Monoacetylmorphine

List of Poster Presentations:

Conference Poster Prize Winner

Martha Purcell, Leon Barron and Brett Paull

Extraction and analysis of hair samples for thirteen drugs of abuse and metabolites using solid phase extraction and liquid chromatography-mass spectrometry.

5th Biennial Conference on Analytical Science in Ireland, Waterford Institute of Technology, May 2008

Martha Purcell, Leon Barron and Brett Paull

Half-metre monolithic columns coupled to electrospray ion trap mass spectrometry for ultra high efficiency separations of pharmaceutical and personal care products.

5th Biennial Conference on Analytical Science in Ireland, Waterford Institute of Technology, May 2008

Martha Purcell, Leon Barron and Brett Paull

Ultra high efficiency liquid chromatographic separations of trace pharmaceuticals and personal care products in digested sludge using half metre columns.

RSC Analytical Research Forum 2008, University of Hull, UK, July 2008

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Abstract:

Pharmaceuticals and personal care products (PPCPs) have emerged in recent years as a new class of chemical and biological pollutants in our environment. In the search for suitably sensitive and specific techniques for detection of these compounds at very low concentrations, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as the new technique of choice. This work describes methods for screening and quantification of various pharmaceutical and illicit drug residues in solid and liquid environmental and biological matrices. Particular focus was given to efficiency of chosen stationary phase, sample preparation procedures and matrix effects which seriously affect the accuracy of LC-MS/MS measurements.

An analytical method was developed for the analysis of a range of illicit drugs in hair samples. The solid phase extraction procedure was optimised by comparison of the extraction efficiency of a range of commercially available cartridges. The method was validated and applied to the detection of cocaine traces in the hair of a recreational drug user.

In the area of high performance liquid chromatography (HPLC) the advantages of new monolithic stationary phases over traditional particle packed beds is becoming apparent. In this work, the performance of a half-metre monolithic column was characterised using van Deemter plots. The separation efficiency of the monolith was compared to that of a conventional particulate column with promising results. Investigations into loading capacity and peak capacity of the half-metre monolith also highlighted the suitability of the long monolith for the screening of large numbers of pharmaceutical compounds.

The half-metre monolithic column was applied to the analysis of pharmaceuticals and personal care products (PPCPs) in complex environmental and biological samples. The effects of ion suppression on mass spectrometric sensitivity for detection of pharmaceuticals in extracts of both soil and sludge were quantified for the long monolithic column and a particle packed column. The half-metre monolith demonstrated less ion suppression for the majority of analytes. The monolith was then applied to the determination of PPCPs in urine samples with minimal sample preparation.

Chapter 1.0: A Review of Analytical Techniques used for Determination of Pharmaceuticals and Personal care Products in Environmental and Biological Samples.

1.1 Introduction:

In the late 1990's pharmaceuticals and personal care products (PPCPs) began to receive attention as a newly emerging group of chemical pollutants which had previously been overlooked. These substances are of environmental interest as they are designed to have a biological effect and not enough is known about their fate after excretion from humans and animals. One of the earliest references to introduce pharmaceuticals as potential environmental pollutants was presented in 1985 by Richardson and Bowran. Here the presence of pharmaceuticals in human and industrial waste entering aquatic environments was introduced.[1] In the 1990's improved analytical instrumentation and techniques led to greater sensitivity meaning that the detection of trace levels of chemicals in environmental samples was no longer out of reach. The advent of these modern technologies such as soft ionisation interfaces for mass spectrometry e.g. electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) resulted in an exponential increase in publications dealing with the analysis of PPCPs from 2000 onwards. Originally most research was focused on identification and monitoring of the compounds particularly in aquatic environments.[2] More recently the scope of research has broadened to cover the treatment and fate of the organic pollutants in the environment. Other areas of interest include methods of PPCP removal as well as the analysis of more complex sample matrices such as soil and sludge.[3]

The term PPCP was first introduced in 1999 in a publication by Daughton and Ternes.[4] From this publication the term PPCP covered all pharmaceuticals either for human and veterinary use which are prescription or over the counter (OTC) products. The term also includes both licit and illicit drugs, active pharmaceuticals added to personal care products such as soaps, shampoo, sun block and perfumes, and nutraceuticals.[4] Endocrine disrupting chemicals (EDCs) are not included under the heading of PPCP in this work although it should be noted that some PPCPs are known to have EDC properties e.g. phthalates.[5] Pesticides are also excluded, however it should be noted that some compounds classified as pesticides e.g. triclosan and triclocarban are commonly present in everyday consumer products.[6] Metabolites of parent compounds must also be considered as relevant. During the metabolism and treatment of animal and human waste products, conjugates and transformation products are formed. These metabolites can have heightened solubility, increased, decreased or alternative biological effect and could also provide information regarding the total concentration of the parent drug reaching being excreted by a population.[7]

Drug development and usage is increasing all the time with pharmaceutical marketing consultants IMS Health reporting global prescription sales growth of 6.4% to 712 billion dollars in 2007.[8] In 2005, Germany reported 9,000 drug preparations approved for use in the EU. These pharmaceutical preparations were marketed in a total of 35,000 products.[9] Consumption of prescription medicines by humans represents a small fraction of total PPCP utilisation. Unapproved drug use and consumption of OTC remedies as well as cosmetics and other PPCPs which contain harmful compounds must also be considered. In addition to human medicines, veterinary medicines (e.g. growth promoters and those used for breeding purposes) and food supplements (e.g. nutraceuticals) employed in agriculture must be included.[6] From these origins a cocktail of potentially harmful chemicals is being released into the environment.

PPCP residues in environmental samples can originate from both anthropogenic and natural sources. Many of the most effective medicinal products are produced by microorganisms (e.g. antibiotics) or modelled on endogenous molecules (e.g. hormones). Pseudo-hormones are designed to mimic the action of naturally occurring molecules and so it is difficult to assign an environmental effect to one compound in particular. Ideally, all man-made PPCPs would be completely metabolised within the body or would be eliminated after excretion. However, pharmaceuticals are designed to be robust, stable molecules and it is now well known that metabolism is often incomplete and that these compounds are surviving wastewater treatment processes.[7] Environmental science has typically dealt with point source contamination where a large concentration of a pollutant is released e.g. in industrial effluent or as an industrial accident. This unfortunately is not the case with PPCPs which are continuously introduced into the environment in trace quantities by often large populations. In this way the origins of PPCP accumulation differs from more conventional pollutants in that their primary origin is impossible to define.[6]

The ‘Oslo Paris Convention for the Protection of the Marine Environment of the North East Atlantic’ (OSPAR) provides the current European legislation on the protection of the North East Atlantic marine environment. The convention regulates standards on eutrophication, release of hazardous and radioactive substances and oil and gas industries. OSPAR provides a list of priority chemicals that are considered harmful to the environment due to their persistence, accumulative abilities and environmental toxicity. Pharmaceuticals such as clotrimazole, (a common antifungal agent) and diosgenin, (steroid) were added to the above list in recent years. Additives to personal care products including xylene (a synthetic scent found in perfumes) and certain phthalates, dibutylphthalate (DBP) and diethylhexylphthalate (DEHP) were also labelled as priority chemicals.[10] These compounds are used to such a large extent in so many products that it is accepted that they are now almost ubiquitous.[11] Recently, research into the effects of pthalates on humans and animals has led to disturbing discoveries, such as adverse effects on the male sexual reproductive system and associations between phthalates and asthma.[12, 13] Other studies have revealed that children are particularly at risk.[14]

Daughton reported in a recent publication that approximately 100 PPCPs have been identified in a range of environmental matrices.[6] This figure may seem small when considering the extent and volume of PPCP consumption and use around the world. Several of these methods focus on a small quantity of structurally related compounds to allow for sensitive and robust determinations in a range of matrix types. When using broad screening approaches it is often the case that analytical methods are not sensitive enough to detect all target compounds. Furthermore, other PPCPs may not be consumed in sufficient quantities to produce a concentration detectable in environmental samples. In addition, these substances may be subjected to further transformation as they are excreted and treated in sewage treatment plants. The obvious upshot of this is the possibility for harmful metabolites and transformation products being present but undetected.[6] The aim of this Chapter is to provide an overview of origins, fate and effects of pharmaceuticals and other bioactive compounds in the environment.

1.2 Sources of PPCPs in the Environment:

Recent studies reporting worrying levels of a wide range of pharmaceuticals in ground and surface waters have stimulated interest in the sources and pathways by which these compounds reach the external environment. Conventionally, environmental concerns stemmed from contamination at point sources such as industrial processes, manufacturing and waste disposal sites. However it is now accepted that PPCPs are primarily introduced to the environment by the mass population.[15] Agricultural medicines such as antimicrobial compounds, growth promoters and drugs used in animal husbandry are metabolised by animals and excreted onto farmland. Manure is often applied as fertiliser resulting in possible exposure of sorbed PPCPs which may enter the soil or ground water environments through rain events.[7] Agricultural sources also include aquacultural processes including fish farming.

There are two main pathways by which the consumer introduces PPCP to the exterior environment. These pathways were defined as ‘involuntarily’ and ‘purposefully’ by Daughton.[6] Involuntary introduction occurs by excretion and washing of topical medicines or PCP’s via the sewage system. Many medicines are not fully metabolised within the body and are sometimes only slightly modified when they enter the sewage system. Purposely polluting refers to the disposal of out of date or unfinished medications into rubbish or sewage systems. By these routes bioactive PPCPs enter wastewater treatment plants (WWTPs) and landfills.[6] Not all PPCP residues are eliminated within the WWTP leading to contaminated effluents from these treatment plants flowing into surface waters. Landfill leachate is also an important point source as the products dumped at landfill sites have not been metabolised and arrive there chemically intact.[16] There are, however, some point sources which must be considered as sources of PPCP pollution such as leachate from landfill dumps, hospital effluent and accidental spills during manufacture. Drug production is no longer considered to be a significant source of the pollution due to Good Manufacturing Practice (GMP) regulations and the enormous costs associated with loss of active pharmaceutical ingredient (API).[7] The numerous routes of entry for PPCPs are outlined in **Figure 1.1**.

There are a number of factors that affect the introduction of PPCPs into the environment. These include the volume of the drug or compound produced and the amount consumed (dosage, regularity of consumption), the extent to which the parent drug is metabolised within the body and how readily it and its metabolites are excreted. Also the extent of metabolism within the WWTPs and finally the ability of the parent drug and its metabolites to adsorb onto soil and sludge.[3] The problem of environmentally persistent is exemplified by the common pharmaceutical clofibric acid which is alarmingly stable in aquatic environments and has even been detected in drinking water.[17, 18]

It is necessary to examine the many degradation pathways of PPCPs within animal bodies in order to understand their chemical structure when entering the environment. Studying the metabolic pathways of these compounds in the environment is difficult due to the cascade of metabolites originating from a single parent molecule. To predict the behaviour of PPCPs in the environment, physical and chemical properties need to be studied. Attributes such as the sorption coefficient (K_{dsolid}), the, octanol/water partition coefficient (K_{ow}) and the organic carbon normalisation coefficient (K_{oc}) can provide information on the hydrophobic tendencies of a pharmaceutical. However, it has been shown that sorption of pharmaceuticals to solids is a complex process and that other interactions such as cation exchange, bridging mechanisms and hydrogen bonding must also be considered.[3, 19] While there have been a number of publications detailing the fate and effects of PPCPs in aquatic environments[2, 4, 20], more focus is required in the field of solid matrices such as soil and digested sludge.

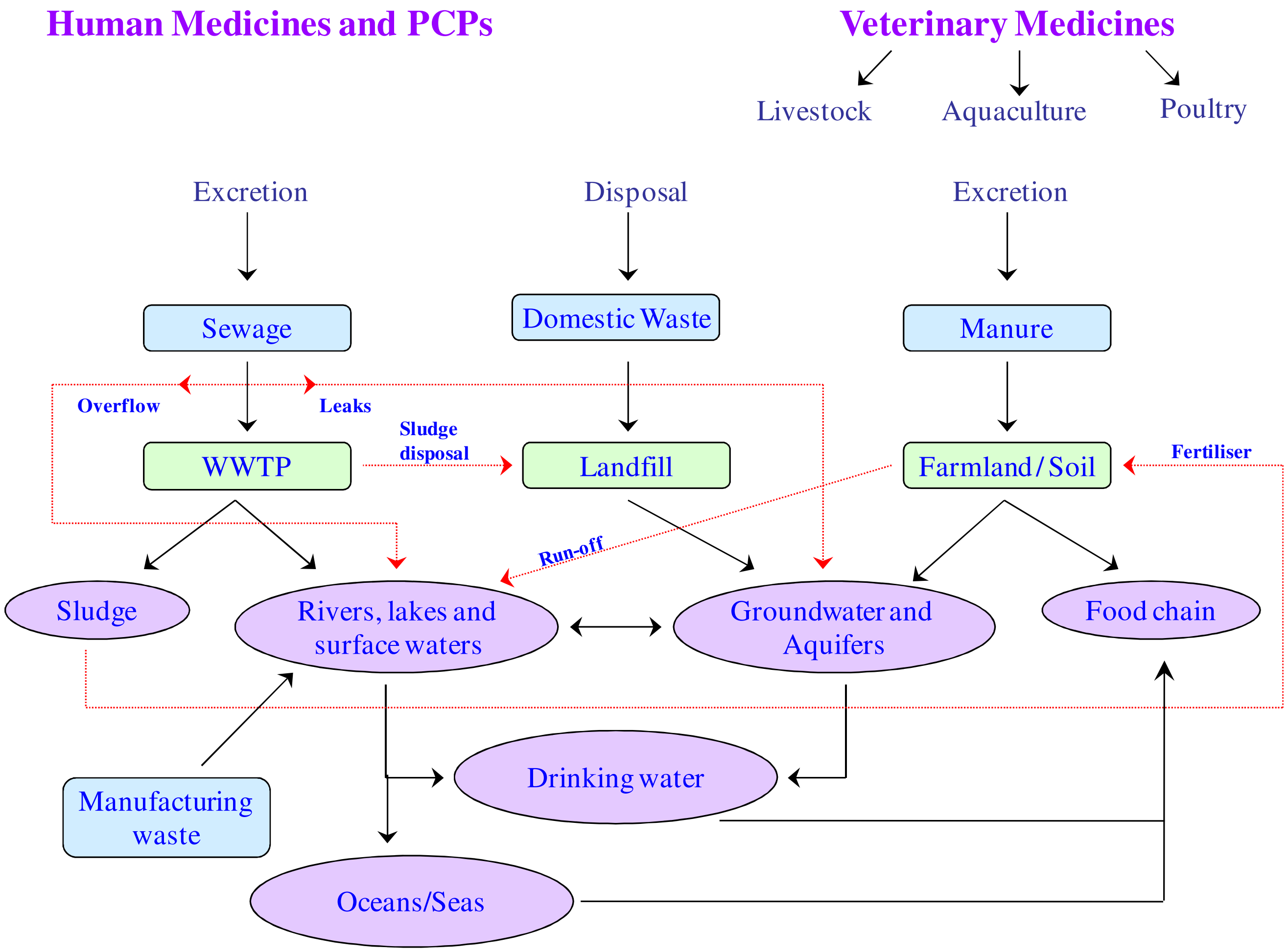

Figure 1.1: Primary sources and routes of human and veterinary pharmaceuticals into the environment, adapted from ref. 3

1.3 Drug Metabolism:

The topic of pharmaceutical metabolism is relevant to the understanding of the fate and effects of PPCP in the environment for a number of reasons. Study of metabolic pathways can yield information on the extent to which the parent drug is metabolised, the quantities of parent drug and metabolite that are formed and excreted and also the structure and bioactivity of the new metabolites can be discovered. Pharmacokinetics is the branch of pharmacology that deals with drug uptake, distribution, modification and excretion.[21] Drug metabolism takes place mainly in the liver where a series of enzymatic reactions alter the chemical structure of the drug, preparing them for excretion in urine.

In order for a drug to produce the desired effect, it must first travel to the site of action within the body. Manufacturing a drug to achieve this goal is one of the major challenges in drug design. The drug must partition through both hydrohilic and hydrophobic bodily compartments. In many cases the lipophilicity of a pharmaceutical is increased in order for it to cross lipid cell membranes. Lipophilic drugs are not easily excreted by the body and so they are transformed into more hydrophilic, (polar) metabolites that are more suited to elimination by the kidneys.[22]

The transformation of the drug molecule for excretion usually takes place in two steps known as the Phase I and Phase II reactions. The Phase I reaction serves to functionalise the drug structure by adding a reactive group to the molecule in preparation for the Phase II step. In Phase II, conjugation of the drug to a large bulky polar moiety occurs which labels it for excretion by the kidneys.[21]

Phase I reactions include oxidation, reduction, hydroxylation, deamination, dealkylation and hydrolysis.[21] Phase II reactions include glucuronidation, glycosidation, sulfation, methylation and acetylation. The polar conjugates employed in Phase II include sugars, amino acids, fatty acids, acetyl groups and sulphates. The Phase II reactions are controlled by a complex system of enzymes with each type of reaction requiring specific co-factors e.g. acetylation of sulfanilamides requires the enzyme acetyltransferase and co-factor acetyl-CoA.[21] The cytochrome P450 system is the most important group of metabolising enzymes and are located on the endoplasmic reticulum of cells particularly in cells of the liver, kidney and intestine.[21] An example of the Phase I and Phase II steps is presented in **Figure 1.2**.[22]

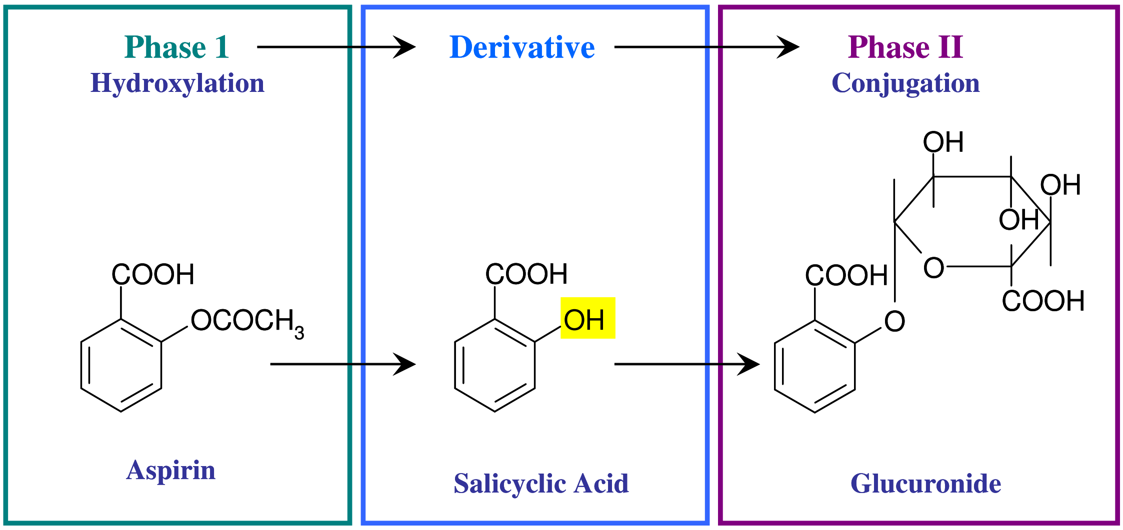

Figure 1.2: The Phase I and Phase II metabolism of aspirin, adapted from ref. 22

The metabolic reactions and pathways of many pharmaceuticals and commonly consumed medicines are well known, as depicted in **Figure 1.2**. However, within human populations, there are significant variations in metabolic enzymes, including cytochrome P450 enzymes.[22] This means that even if pharmacokinetic data has been obtained for a pharmaceutical, it cannot be universally applied to all individuals. Tracking metabolic processes become more complex when inhibiting and inductive factors are considered. Inducing substances cause an increase in the activity of metabolising enzymes resulting in increased elimination of the inducing drug itself or rapid biotransformation of other pharmaceuticals in the body. This is exemplified by ethanol which increases the activity of microsomal oxidase with repeated consumption.[22] Inhibition of metabolism is exemplified by the anti-ulcer drug cimetidine which binds to cytochrome P450, halting the enzymes activity.[21] All of these factors must be considered when answering the questions listed at the start of this section. Inhibiting agents will cause retention of therapeutics and medicines within the body while inducers will promote elimination of the compounds at an increased rate

PPCPs excreted from the body after metabolism as a pharmacologically inactive species are not of great concern. However, excreted drug metabolites are not always inactive. Pharmaceuticals may be designed to only become active after metabolism to overcome drug delivery issues e.g. prodrugs. Prodrugs are pharmaceuticals that are administered in an inactive form which produces an effect once metabolised. Human use of a prodrug is exemplified by the immunosuppressant azathioprine. Other pharmaceuticals produce more potent or toxic metabolites which are harmful to the body and may exert a toxic effect in the environment also. These harmful metabolites are exemplified by the hepatotoxic derivative of paracetamol *N*-acetyl-*p*-benzo quinine imine.[22] Also the immunosuppressant cyclophosphamide produces the toxic metabolite acrolein.[22]

1.4 The Wastewater Treatment Process:

Wastewater treatment plants receive polluted water from many different sources including domestic sewage from towns, industrial discharges or agricultural and landfill run-off. The chemical and biological composition of these wastewaters varies greatly.[23] Domestic wastewater is composed of 99.9% water with only 0.1% consisting of solid matter.[24] However, the composition of the wastewater that arrives from a range of sources at the WWTP will depend greatly on the industrial, agricultural and landfill catchment area.[23] Other components of wastewater include bacteria, organic matter, inorganic species containing nitrogen and phosphorous and pollutants such as pesticides, insecticides and heavy metals.[23]

The legislation in Ireland pertaining to wastewater treatment and management was entered into Irish law under the EU Directive 91/271/EEC.[25] This directive included the Environmental Protection Agency Act, 1992 and the Urban Waste Water Treatment Regulations, 1994 which provide the policies and standards that must be upheld in the treatment of wastewater.[25] The treatment plant itself is protected under the

1992 Act which ensures monitoring of influents so the performance of the plant is not affected.[25]

The wastewater treatment process is actually composed of a series of individual processes which each have different functions, but all are designed to reduce the concentration of pollutants in the water and prepare it for reintroduction to the environment. An outline of a WWTP is depicted in **Figure 1.3**. Preliminary treatment is the first step and involves mechanically removing debris and other floating material from the influent. The raw sewage arriving at the plant may contain wood, rags, paper, grit, large suspended matter and faecal matter.[23] These materials are removed so as not to damage machinery or cause blockages by filtering the water through screens or strainers.[23] The strained wastewater passes into large settlement tanks where it is left for an appropriate length of time to allow settleable solids (0.05-10 mm) to accumulate at the bottom of the tanks.[23] The settled sewage can then be physically removed from the bottom of the tank before the wastewater passes onto the next stage.

The secondary treatment of wastewater is most commonly called the activated sludge process. Activated sludge consists of numerous co-inhabiting microorganisms, that are added to the wastewater to consume organic colloidal and dissolved matter. The organic matter is removed mainly by heterotrophic and autotrophic bacteria but also some fungi.[26] The bacteria found in the sludge can include individuals from the genera pseudomonas, bacillus, athrobacter, nocardia, acinetobacter and achromobacter. The sludge also contains protazoa belonging to three main groups: flagellates, ciliates and amoebae. Some of these eukaryotes feed off the organic matter in similar ways to the bacteria, but their main function is to prevent the stationary phase of the bacterial life cycle by grazing on the prokaryotes.[26] An activated sludge reactor consists of a mixture of aerated wastewater and activated sludge containing a high microbial population. The wastewater is supplied to specially designed tanks which are constantly aerated so as to provide optimum conditions for respiration to occur. The rate of microbial respiration in the tanks is increased and the amount of organic matter due to its metabolised to form CO_2, H_2O and new microbial biomass.[23]

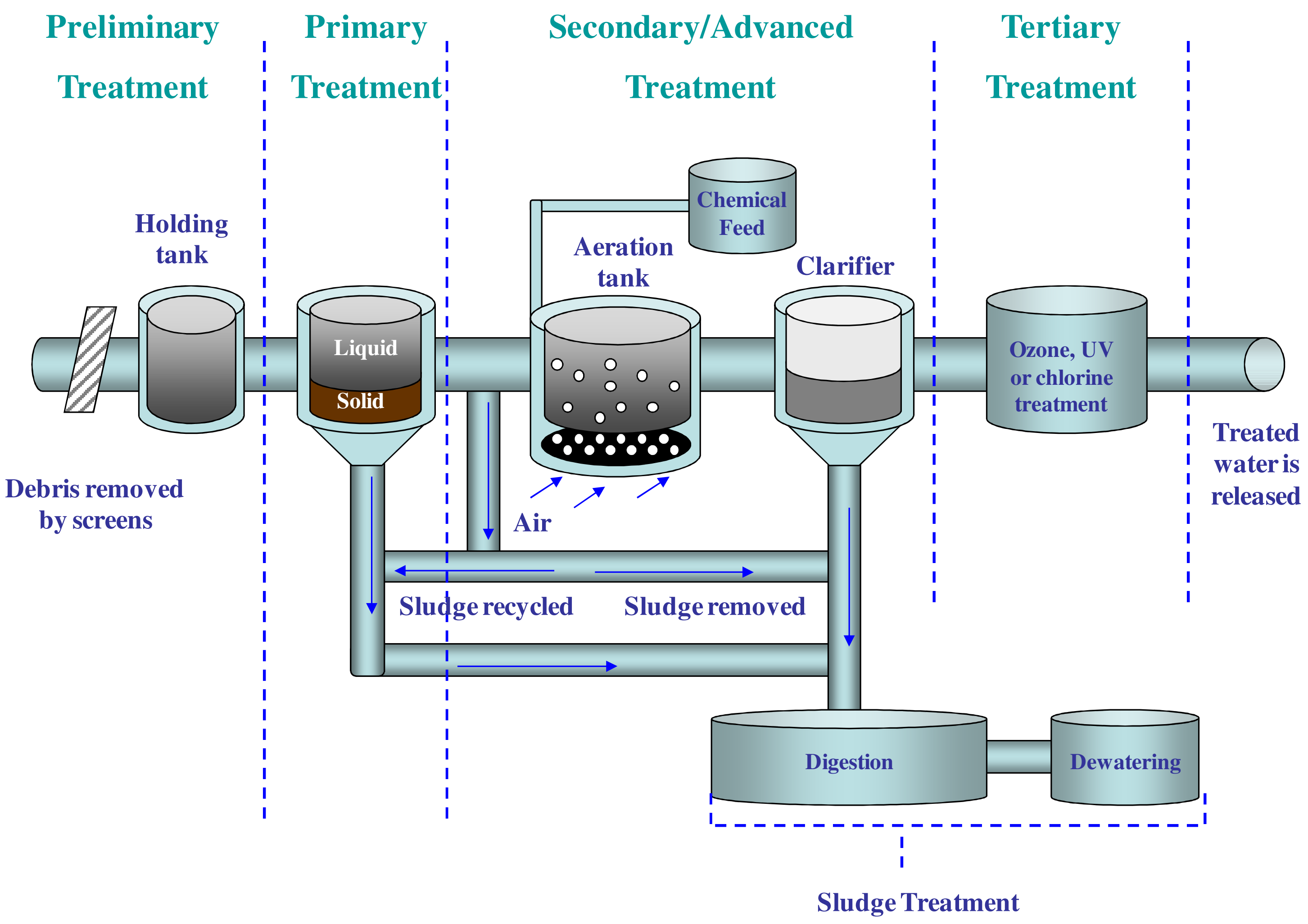

Figure 1.3: Outline of a waste water treatment plant, adapted from ref. 27

Another characteristic of secondary treatment is the formation of flocculated agglomerations (flocs) within the aeration tank. It is hypothesised that filamentous bacteria form the skeleton of the flocs and bacteria which produce slime layers form the bulk of the structure. The bacterial slime layer is composed of substances called exopolysaccharides which allow attachment to the filamentous backbone. The flocs expand and envelope other microorganisms and organic matter.[23] The flocs are an essential component of the treatment as they represent biological microcosms where the removal of organic matter is promoted by a number of mechanisms. Colloidal and suspended matter within the flocs is easily broken down into simple molecules by extracellular enzymes and transported into bacterial cells for metabolism. Other more complex substances are absorbed through the bacterial membrane. Respiratory pathways occur inside the bacterial cells resulting in the production of energy which is converted to new cellular matter.[26] Cellular matter is accumulated in the flocs which can be easily removed from the tanks. At the end of the treatment the sludge-wastewater mixture is passed into secondary settlement tanks to allow the solid flocculated material to be separated from the water. One of the most crucial functions of the floc is its ability to increase the rate of settlement within secondary settlement tanks.[23] The wastewater is then subjected to tertiary treatment while the sludge has a number of possible fates. These include reintroduction to the activated sludge process, inactivation and utilisation as an agricultural fertiliser or disposal through landfills or incineration.[25]

Tertiary treatment of wastewater deals with the removal of nutrients (nitrogen and phosphorous containing compounds) and elimination of any residual microbes via disinfection techniques. Nutrient removal is an essential process as it prevents eutrophication causing compounds reaching environmental waters and increasing the risk of excessive algal and aquatic plant growth.[25] A number of techniques are available for removal of nitrogen from wastewaters. Air stripping of ammonia involves lowering the pH of the water to form volatile ammonia gas which can be eliminated by aerating the tank. Aluminium based minerals called zeolites can be employed as ion exchangers for the removal of ammonium ions while the addition of chlorine to water converts the ions to nitrogen gas. Biological nitrification and denitrification are alternatives to the physical and chemical methods described above but require expensive alterations to the treatment plant.[25] A biological technique has also been developed for the removal of phosphates but the more common method of elimination involves the redox reaction of phosphate with metal ions (e.g. iron and aluminium) to produce an easily disposable precipitate.[25]

Disinfection is the final step and is carried out to eliminate any harmful, disease causing pathogens remaining in the water before its release to the environment. Disinfection techniques fall under three headings: chemical, physical and irradiation. Chemical techniques involve the addition of chlorine, ozone or hydrogen peroxide. Ozone is particularly effective against non-biodegradable organic matter. Physical techniques are mainly based around membranes with pore sizes for retention of bacteria, viruses and even ions. Finally irradiation with UV light can be employed to eliminate any remaining organic compounds.[25]

1.5 The Behaviour of PPCPs During the treatment Process:

Many different types of pharmaceutical compounds have been detected in WWTPs including blood-lipid regulators, antibiotics, antiepileptics, and tranquilisers.[4, 28, 29] The presence of these potentially damaging compounds in effluent entering environmental waters poses questions as to the efficiency of the treatment processes in the plant. Previous studies have indicated variations in removal of pharmaceuticals from wastewater with both high and low concentrations being released into the environment.[30, 31]

There are a number of operational factors which may affect the removal of PPCP from wastewater during its treatment. These include the biochemical oxygen demand (BOD), quantity of suspended solids (SS) loading, solids retention time (SRT), pH, and temperature.[32] The SRT is particularly important as a longer period of sludge treatment promotes the growth of microorganisms with a wider range of metabolising and transforming abilities. This can possibly lead to increased removal of PPCPs before the water is released back to the environment.[32]

The physical and chemical properties of each PPCP govern its fate within the WWTP. If the compound is biodegradable then its concentration in effluents may be lower. Important properties include hydrophobicity and volatility. Hydrophilic compounds such as acid, basic and neutral pharmaceuticals may be more likely to remain in aqueous media and therefore may be more difficult to eliminate from influent streams. Hydrophobic compounds may be more susceptible to adsorption onto sludge particles during treatment.[32]

There are numerous mechanisms by which pharmaceuticals could be degraded, however the most likely include: aerobic and anaerobic biodegradation, (during secondary treatment), chemical degradation, volatilisation and adsorption onto solid particles.[32] The more hydrophobic pollutants removal pathways may be predictable using the octanol-water partition co-efficient as previously reported by Rogers.[33] Those compounds with a higher log K_{ow} value could be more likely to adsorb onto solid matrices.[33]

Studies to date have indicated relatively low removal of pharmaceuticals, antibiotics and diagnostic agents used in x-rays from WWTPs.[34] Ternes *et al.* reported no affinity of acidic drugs for sludges during primary treatment processes using the solid-water distribution co-efficient (K_{d}).[35] Some removal of diclofenac and the fluoroquinolone antibiotics (e.g. ciprofloxacin and norfloxacin) was reported during primary treatment in separate studies.[35, 36] Some antibiotics exhibit high log K_{ow} values as does diclofenac, which may partly explain the higher levels of removal during primary processes.[32]

Many studies based on detecting pharmaceuticals in WWTPs have focused on acidic pharmaceuticals with concentrations in influent and effluent ranging from 300-23,400 ng/L and 24-2400 ng/L, respectively, (Ibuprofen being highest in both cases).[37-39] Other studies have focused on determining the amount of adsorption onto sludge which occurs during treatment. In 2001, removal of pharmaceuticals including diclofenac and carbamazepine were reported after just 15 minutes of contact with activated sludge.[40] Activated sludge treatment plants with low SRTs have demonstrated low levels of pharmaceutical removal and in some cases none at all in a number of publications.[39, 41] It is becoming increasingly clear that the length of the SRT within the plant influences the removal of PPCPs. This indicates that sorption of pharmaceuticals to solids is an equilibrium process which is highly dependent on time and independent of properties such as K_{ow} and K_{d}. Clara *et al.* reported an increase in elimination of ibuprofen from 0 % to 98 % by extending the SRT to 48 days.[39] Similar results have been reported by Buser *et al.* for WWTP that employ longer SRTs.[42] The theory that operational aspects of wastewater treatment affect the removal of drugs has also been investigated with activated sludge plants and oxidation ditch systems both reporting relatively high removals of acidic pharmaceuticals.[34, 37, 38] A WWTP employing a trickling filter treatment bed was less successful in the elimination of pharmaceutical residues.[37] However, some compounds do not adhere to these theories, discrepancies have been reported in removal data for diclofenac from wastewaters subjected to similar SRT periods. The concentration of the anti-inflammatory removed during treatment has varied from slight amounts to more than 70 %.[28, 39, 42] Investigations into the degradation of clofibric acid in a range of WWTPs with differing treatment techniques were also contradictory. Wide ranging levels of biodegradation were reported for conventional activated sludge plants, trickling filter establishments and plants with additional tertiary treatments.[37, 43] These contradictory findings indicate that the performance of sludge in WWTPs varies quite a lot as it cannot be reproducibly produced.

An important study carried out by Jones *et al.* in 2007 investigated the sorption of pharmaceuticals paracetamol, ibuprofen, propranolol, mefenamic acid and salbutamol to biosolids in a UK WWTP. Although elimination rates were high (~90%) for all target analytes, ng/L concentrations were still detected in effluent except for propranolol. The conclusion drawn from this study was that sorption of the selected analytes to sludge was not the primary mechanism of removal and that microbial metabolism was more likely.[44] More recently several PPCPs from a wide range of therapeutic classes were detected in the influent and effluent of Irish WWTPs.[45]

Antibiotic residues have been detected in the influent and effluent waters of a number of WWTPs across Europe, Canada and the USA.[46-48] The antibiotics detected included sulfonamides, tetracyclines, macrolides, and fluoroquinolones.[48] Influent and effluent concentrations of five antibiotics ranged from 0.04-1.30 μg/L in American WWTPs,[48] and sulfamethoxazole concentrations as high as 400 ng/L have been reported in Germany.[46] Overall, activated sludge treatments have shown relatively successful removals of antibiotics.[41, 48] A study of wastewater samples taken during different seasons demonstrated that lower concentrations of antibiotics were detected in the early spring and summer representing the months receiving the most precipitation.[48, 49] These results are in agreement with earlier studies which highlighted reduced removal of pharmaceuticals during period of high rainfall.[28] Similar concentrations of the fluoroquinolones have been detected at WWTP sites in Switzerland and Canada.[47, 50] Sorption to sewage sludge is thought to play an important role in the removal of antibiotics from wastewater. Tetracyclines are prone to complexation with metal ions forming stabilised complexes which could bind to suspended matter in the sludge.[32] This may explain low concentrations of tetracycline in some German WWTPs,[46] while other studies with particularly short SRTs reported median concentrations of ~ 150 ng/L.[47] This result suggests that biodegradation is also an important factor in removal of antibiotics and should not be ignored. However, recent studies into the antibiotic removal from the Pearl River Delta in South China have indicated that fluoroquinolones are eliminated due to adsorption to sludge.[51] This was demonstrated by direct analysis and detection of fluoroquinolones in sludge biomass. Macrolides and sulfonamides were found to survive the treatment process.[51] One possible future problem associated with antibiotic presence during the activated sludge process is inhibition of bacterial function as demonstrated by Dokianakis *et al*.[52]

Diagnostic chemicals are another branch of PPCPs which have been detected in municipal influents and effluents. Six diagnostic chemicals employed for x-rays were detected at particularly high levels in a German WWTP where the maximum level exceeded 3000 ng/L.[53] Once again the SRT was judged to play an important role in removal of these compounds as concentrations removed increased with increasing SRT in a study by Kreuzinger *et al.*[41] Tertiary treatments in WWTPs such as ferric chloride, lime and aluminium sulphate coagulation were shown to remove less than 25% of PPCPs from drinking water.[54] Tertiary treatments involving oxidation with chlorine and ozone showed better results, however, this study focused mainly on endocrine disrupting chemicals and hormones, further work is required to investigate the effects of these processes on pharmaceuticals.[54]

New techniques are being developed to improve the quality of wastewater before it is released back to the environment. These include membrane bioreactor technology (MBR) and advanced oxidation processes such as ozonation and photocatalysis.[55] MBR has already showed promising results for the removal of acidic, neutral and basic pharmaceuticals, however there are still issues with some persistent compounds such as carbamazepine.[41, 56] These new techniques will be expanded upon in a later section of this review.

1.6 PPCP Disposal and Leaching from Landfill Sites:

The consumer is often unsure what to do with unused or expired medications. Traditionally discarding the drugs to the sewage system was common practice and is still common as it avoids any risk of children reaching them.[57] However concern is mounting about the concentrations of pharmaceuticals being detected in aquatic environments and some governments are advising their public to dispose of the unused medications with household waste. In an attempt to prevent further contamination of water systems, the United States White House Office of National Drug Control Policy released a statement in 2007 advising consumers to avail of any possible drug take-back programs or to add the medicines to their household waste.[58] As a result of this, any hazardous materials are transported to landfill sites to join the rest of the municipal solid waste (MSW). In many modern landfill sites, leachates are collected and treated in

WWTPs resulting in many pharmaceuticals entering the aquatic environments, which these new guidelines were aiming to protect. A recent survey conducted by Musson *et al.*, indicated that almost 50 % of consumers are disposing of pharmaceuticals as solid waste and not using the sewage system route.[59]

Hazardous substances can be released from landfill sites in a number of ways including gaseous emissions of volatile compounds, airborne particulate matter and leachate of liquid seepages into land around or below the site.[60] Landfill leachate is a complex matrix composed of organic and inorganic elements including xenobiotic organic compounds and toxic heavy metals. These substances pose a risk to the environment and to humans when they seep from landfills into ground and surface waters. This occurs despite recent advances in landfill design such as barrier systems.[60] The chemical composition of landfill leachate has been studied by Schwarzbauer *et al.* between two studies in 2002 and 2004.[61] Over 180 individual organic compounds were identified within the seepage water of a landfill facility in Germany. Common degradation products of carbohydrate, peptides and other biological molecules were identified but xenobiotic compounds were also present including pharmaceuticals, pesticides and halogenated aromatics.[61] Among the detected compounds were the pharmaceuticals ibuprofen, propylphenazone, clofibric acid and many of the sulphonamide antibiotics. In the second study certain organic compounds were chosen and monitored within the seepage as markers of pollution originating from the landfill site. It was noted that the concentrations of the compounds decreased with increasing distance from the waste facility.[62] It is obvious that landfill leachates are an important source of PPCPs within the environment. Greater efforts need to be taken in future to update landfill sites with barrier systems, treat leachate as it is produced using proven methods or organise separate collection of pharmaceuticals for incineration.[57]

1.7 Fate of PPCPs and Environmental Risk Assessment:

The fate of pharmaceuticals once released into the environment it is difficult to predict. There are several processes that can occur: photochemical conversion to form other compounds, dilution and transportation within the aquatic environment and sorption onto solid matrices such as soil and sewage sludge which is of particular importance in this work. PPCPs enter sewage sludge as already discussed, in the WWTPs and are often applied within the biosolid as agricultural fertilisers. The PPCPs may enter and persist within the soil or leach into water systems depending on a number of factors.

The sorption and persistence of a pharmaceutical in solid matrices depends on its capacity for sorption, its resistance to photo-degradation and its affinity for water, which if high will cause seepage from the solid to surface waters.[3] Natural waters may also also act as a semi-solid matrix due to a high dissolved organic matter (DOM) load, allowing sorption to occur. The distribution of a drug between a solid and a liquid is described by calculating the ratio of drug concentration in solid and liquid at equilibrium, known as the distribution co-efficient, (K_d). This is the simplest method available, however it has been shown to vary for some highly hydrophobic compounds perhaps due to its dependance on pH.[63] The variation in data can be reduced by the calculation of the normalised sorption coefficient, (K_{oc}) which is a ratio of K_d to the organic carbon content of the solid.[63] K_{oc} can be easily calculated using the octanol-water partition co-efficient which is an easily obtained physiochemical parameter, making K_{oc} the preferred tool for investigation of environmental risk.[64] However, these co-efficients still vary quite considerably for a given compound in different types of solid sample.[65] These variations cannot be simply explained by the differing organic content of the soil samples. There are other mechanisms of binding at work such as complexation, ion-exchange and hydrogen bonding.[65]

A recent study of digested sludge from a WWTP and soil which had been enriched with the resulting biosolid from the WWTP were analysed for traces of 27 PPCPs.[66] Significant levels of triclosan, warfarin and carbamazepine were identified in the digested sludge samples, while lower but still relevant concentrations of triclosan were found in the fertilised soil.[66] These results confirm earlier studies which reported triclosan at ~1200 ng/g in digested sludge from a WWTP in Germany.[67] Soil column studies have also been conducted to determine if drugs remain sorbed to soil or leach into water systems. Oppel *et al.* determined that diazepam, carbamazepine and ibuprofen were all highly retained on the soil while clofibric acid seeped out in leachate.[68]

It is important to assess the toxicological effects that PPCPs have on the environment and on individual species. Pharmaceuticals are designed to exert a specific effect on an individual, however when released into the environment they could affect other organisms. The use of medicinal products in the EU is regulated by the European Medicines Evaluation Agency (EMEA). [69] An environmental risk assessment (ERA) must be carried out under EU Directive 93/39/EEC to investigate any toxicological risks associated with a drug before it can be licensed for human or veterinary use.[69]

1.8 Sample Preparation Techniques:

1.8.1 Introduction:

In recent years technological advances have allowed scientists to detect analytes at lower concentrations than was ever possible before. Modern interface technologies altered the standard analytical techniques used for pharmaceutical analysis by linking liquid chromatography (LC) to mass spectrometry (MS) for the first time. This new technique has replaced gas chromatography-mass spectrometry (GC-MS) as the 'work-horse' of analytical science particularly with respect to PPCPs in the environment. GC-MS is not suitable for determining many pharmaceuticals and requires complex derivatisation steps. Modern environmental analysis concerning PPCPs generally consists of three procedures, sample preparation, liquid chromatographic separation, mass selective detection and data interpretation. The theory of LC and MS were discussed in Chapter 1 of this work. This section aims to describe the theory and processes involved in modern sample preparation techniques and review the analytical techniques which have been used by scientists to date for the analysis of pharmaceutical and illicit drugs in solid environmental matrices and biological matrices such as hair and urine.

1.8.2 The goals of sample preparation:

The function of the sample preparation step of an analytical procedure is to separate the analyte of interest from the sample matrix components. In the case of environmental samples, the matrices are often very complex, but also the target analytes may be present at very low concentrations, a fact which is particularly true for PPCPs. The same challenges are faced with biological samples such as urine and hair. These samples contain high levels of organic matter which can contaminate equipment such as chromatographic columns or interfere with detection. Therefore, sample preparation must not only increase the concentration of the target analyte to a level detectable by modern instruments, but it must selectively increase only the target analyte concentration and and to a certain degree eliminate the matrix components. This is known as sample clean-up in which the analyte is extracted from very 'dirty'or complex sample types to reduce matrix interference and improve method sensitivity.[70] During sample preparation, analytes may also be transferred to a solvent which is more amenable to the next step of the procedure, e.g. the analytical separation. Techniques such as GC require volatile samples and nuclear magnetic resonance (NMR) requires deuterated sample environments. Additionally, the sample preparation technique should not render the sample highly acidic or basic as this can also spoil chromatographic columns.[71] The clean-up procedure should be relatively inexpensive, rugged, easy to perform and result in minimal analyte loss.

1.8.3 Solid phase extraction (SPE):

Solid phase extraction (SPE) is a sample preparation technique that involves passing a gas or liquid over a solid sorbent, which has an affinity for the target analyte within the sample. The analyte is retained on the sorbent while matrix components pass through to waste. The analyte is then retrieved by elution in a suitable solvent or by thermal desorption.[72] SPE fulfills the requirements previously outlined in Section *2.8.2*, as it can enrich the sample, can minimise most of the matrix content and allows the transfer of analytes to a suitable solvent for analysis. These attributes have made SPE one of the most common preparation techniques in many scientific fields including pharmaceutical, environmental and biological analysis.[72] However SPE was not always the technique of choice and there are alternative techniques also employed such as LLE.

SPE was first introduced as a replacement for the then popular preparation technique of liquid-liquid extraction (LLE). LLE is a type of solvent extraction that involves two immiscible liquids, usually aqueous phase and an organic solvent. The analytes partition into each phase based on their solubilities, hydrophobic molecules will be distributed in the organic solvent while polar and ionic species will partition into the aqueous phase.[73] LLE was the standard method of sample preparation up until the 1980's when SPE became a widely used laboratory technique. There are a number of drawbacks associated with LLE, large volumes of expensive solvents are consumed which are difficult to dispose. Emulsions can be formed during the procedure particularly with biological samples such as urine which lead to ruination of the sample. Additionally LLE produces low preconcentration factors when compared to SPE. It is not feasible to extract a large volume of sample with a small volume of organic solvent in LLE, an essential step in environmental analysis as the analytes are present at such low concentrations.[73]

In the early 1950's activated charcoal was employed for the preconcentration of organic compounds in large volumes of water.[74] However, this technique did not become popular due to irreversible adsorption for groups of analytes and a weak affinity for others.[74] The late 1960's and early 1970's saw the advent of porous polymer sorbents based on polystyrene and polyacrylate polymer beads. These types of sorbent were very successful in the analysis of organic compounds in water samples.[74] During the 1970's bonded phases employing a silica back-bone became of interest with the increasing popularity of LC. Soon after, disposable cartridges were introduced which improved the ease and availability of the technique. In the 1990's automation of SPE was developed along with mixed-mode sorbents, immunosorbents, restricted access sorbents and multi-well plate formats for multiple samples.[72]

SPE techniques have their own drawbacks which cannot be ignored. Manufacture of the sorbents is not always a reproducible process and leads to variations in retention of compounds. Also the small amount of sorbent within the column or tube can become overloaded when extracting large volumes of sample and lead to loss of analytes due to breakthrough. Impurities introduced during manufacture can also affect the sample preparation process and the sorbents can also become blocked leading to low recoveries.[72]

1.8.4 The practice and theory of SPE:

All SPE procedures are composed of four steps regardless of samples and sorbent type. These steps involve sorbent conditioning, sample loading, washing and analyte elution. **Figure 1.4** below outlines the four steps of the SPE technique. Firstly, the sorbent is washed usually with an organic solvent such as methanol or acetonitrile to remove any contaminants from manufacturing and to allow 'wetting' of the sorbent functional groups. Other conditioning steps involving acidic or basic solutions may also be employed to ionise functional groups. Equilibration is carried out by passing an aqueous solvent through the SPE column.[75]

A vacuum is generally employed to allow more rapid sample loading, especially for large sample volumes. The sample is passed through the solvent at a constant rate, without allowing the sorbent to dry out, to allow sufficient time for the analytes to interact with the sorbent. During this step the analyte is either retained on the sorbent as matrix components flow through or the analyte is eluted while the matrix species are retained. The washing procedure is carried out using a suitable solvent to remove any interfering matrix components and to dry the sorbent more quickly. Finally the analytes can be eluted in a suitable solvent.[75] the volume of eluting solvent used should be as small as possible to maximise the preconcentrating effect of the technique while ensuring that all of the analyte is removed.[72]

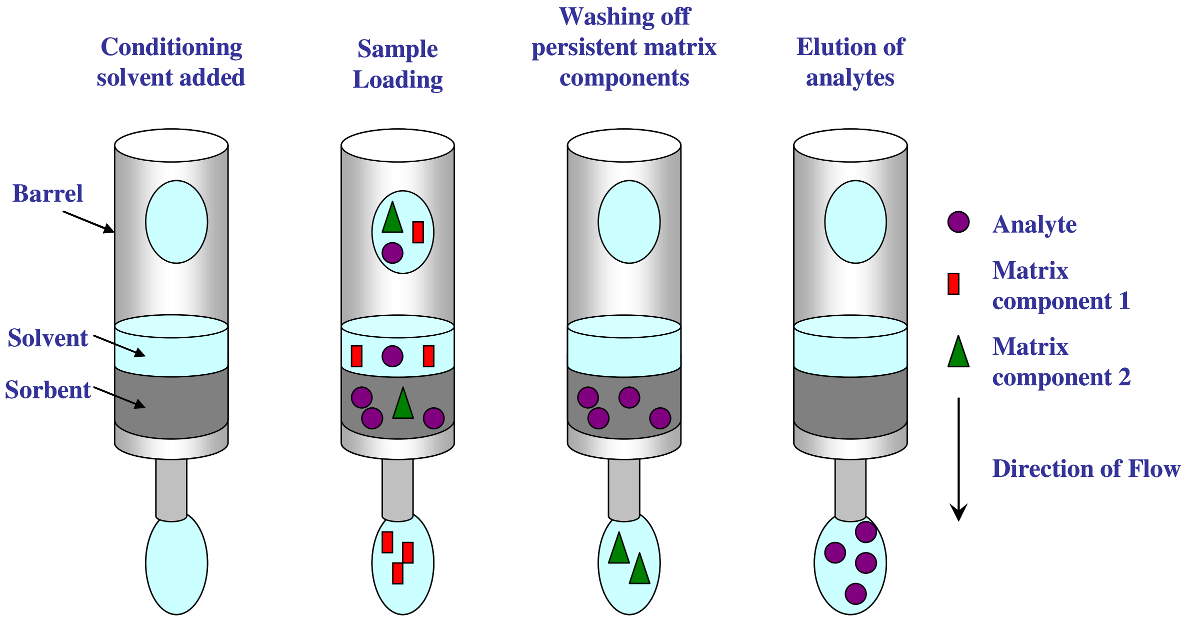

Figure 1.4: Diagram outlining the four steps of SPE, adapted from ref. 76

The theoretical processes that occur during SPE are comparable to those in liquid chromatography as bothcan be described as frontal techniques. Large volumes of sample are usually loaded due to the low concentrations of analyte within them. The amount of analyte that can be isolated depends on the breakthrough volume (V_B) of the sorbent.[72] The breakthrough volume can be determined by passing sample through the sorbent continuously until analyte is detected coming through the sorbent without being retained. At this point during the technique the analyte is no longer being quantitatively retained on the sorbent.[72] The point at which the concentration of analyte entering the barrel and the concentration leaving it are equal indicates that the retention capacity is saturated. This means that sorbent adsorption sites are saturated with analyte or matrix components or its retention capacity has been exceeded. The point of inflection on the curve is known as the retention volume (V_R).[72]

The breakthrough volume can be calculated using the following equation.[72]

$$V_B = V_R - 2.3\sigma_V \qquad \text{(Eqn. 1.1)}$$

Where σ_V is the standard deviation of the axial dispersion of the analyte through the SPE sorbent.[72] The volume of eluting solvent required to achieve 99% analyte recovery (V_E) is given by:

$$V_E = V_M\,[1+k][1+(2.3/\sqrt{N})] \qquad \text{(Eqn. 1.2)}$$

Where V_M is the interparticle volume of the sorbent, k is the retention factor and N is the number of theoretical plates.[72]

1.8.5 Types of SPE sorbent and retention of analytes:

A large selection of SPE sorbents are commercially available today, which is testament to the popularity and success of the technique. The type of retention mechanism involved depends on the sorbent chosen. Reversed-phase sorbents are the most universally used, however ion-exchange, normal phase and mixed-mode are also common. The choice of sorbent is based on the nature of the analyte and the sample. The type of sample matrix (organic/aqueous), the nature of the analyte, (polar, non-polar, ionised) the type of ionisation, (strong/weak or acidic/basic) must all be considered.[72] The stationary phase chosen should also be porous and allow sufficient interaction between analytes and active groups. The interaction between the analyte and the functional group of the sorbent should be reversible so the analyte can be eluted but suitably strong to allow retention.

Normal phase adsorbents are made up of the inorganic oxides such as silica gel, alumina and Florisil, which is a magnesium silicate. The advantages of normal phases are their highly active groups and large surface area. Careful buffering of solutions allow for ion-exchange applications.[72] Analyte preconcentration occurs due to hydrogen bond and dipole interactions. Unfortunately these sorbents are subject to irreversible retention and catalytic degradation of analytes.[72] Applications of normal phase sorbents include fractionation of different lipid types,[77] preconcentration of herbicides[78] and clean-up of complex matrices before analytical separation.[79]

Reversed-phase sorbents include the bonded silicas and porous polymers sorbents, both of which have been used extensively for the preconcentration of organic compounds in aqueous matrices.[80] Retention of analytes on reversed-phase sorbents occurs due to π- π interactions and van der Waals forces. The silica backbone is most commonly functionalised with long alkyl chains e.g. C_8 and C_{18} groups. Longer chains promote retention of smaller molecules while shorter chains are employed for macromolecule retention.[80] Other silica functionalising groups include 3-cyanopropyl and 3-aminopropyl which selectively extract analytes due to polar interactions. Despite their success, silica based sorbents suffer from pH sensitivity and low breakthrough volumes for small polar compounds. Silanol sites that remain uncapped can irreversibly retain basic compounds causing low recoveries.[80] Porous polymers hold solutions to both of these problems as they contain no residual silanol groups and they can withstand the full pH range. The polymers are most commonly manufactured from styrene and divinylbenzene monomers which demonstrate stronger π- π interactions and exhibit a higher surface area.[81] One disadvantage associated with polymer sorbents is low level of interaction with aqueous samples due to their hydrophobicity. This problem is being addressed by surface modification with polar groups such as sulfonates or addition of a polar monomer to the polymerisation process.[81] This approach is exemplified by the commercially available Oasis HLB product which is easily water-wettable.[82]

Inorganic and organic ions can be removed from an array of matrices (usually aqueous) using ion-exchange sorbents. As with many other types of compound specific stationary phases in SPE, e.g. molecular recognition, restricted access and immunoaffinity sorbents, ion-exchange SPE is based on modification of the normal and reversed-phase sorbents.[80] Both silica and polymer backbones can be employed once modified with a cation or anion exchanging group. Retention occurs due to electrostatic interaction between analyte and sorbent functional group. Some of the most common functional groups include tertiary and quaternary amines for anion exchange and carboxylic or sulphonic acid groups for cation retention.[72]

Mixed-mode sorbents consist of ion-exchange functionalities combined with hydrophobic alkyl chains for retention of ionisable compounds that also exhibit a non-polar character. These types of sorbents have found particular use for pharmaceutical and clinical analysis due to the acidic and basic functional groups ofeten found in pharmaceuticals and illicit drugs.[83] Preconcentration of pharmaceuticals in biological and environmental matrices using this type of phase results in excellent reduction of matrix components, providing extracts suitable for chromatographic analysis.[80]

1.8.6 Pressurised liquid extraction:

The inherent homogeneity of solid samples renders it difficult to reproducibly extract and detect pharmaceutical residues. The high organic load of matrices such as hair, soil and sludge mean matrix effects are a particular problem in the development of analytical techniques. Generally, an extraction process coupled with SPE clean-up and preconcentration is required to allow accurate qualitative and more importantly quantitative information about drug residues in environmental and biological matrices to be obtained.

Pressurised liquid extraction (PLE) or accelerated solvent extraction (ASE) was introduced in 1995 by Dionex Inc. as a new extraction technique for organic compounds from solid or semi-solid environmental, food and industrial matrices.[84] The principle of the technique is that high temperature solvents at elevated pressures should produce improved extraction efficiency due to faster kinetics than at ambient temperature and atmospheric conditions.[85] There are two processes that occur during the extraction process that should lead to improved extraction of organic compounds, solubility and enhanced mass transfer coupled with distortion of equilibria.[85]

At high temperatures the solvents used in ASE have a higher solubilising capacity and dissolve more organic compounds within the sample. Mass transfer or diffusion rates are improved due to the higher temperatures and introduction of fresh solvent also improves extraction rates as the concentration gradient between the solvent and the sample matrix is increased.[85] Surface equilibria within the sample can be disrupted by both the elevated temperatures and pressures. Increased thermal energy can disturb intermolecular forces such as hydrogen bonding and van der Waal's forces between matrix adsorption sites and target analytes. Additionally, the decreased extraction solvent viscosity results in more efficient diffusion into the solid matrix, both of these effects result in improved analyte extraction.[85] Elevated temperatures would not be possible if high pressure did not allow the solvents to remain in the liquid phase. High pressure is also an important factor in the penetration of sample matrix.[85]

A description of the ASE setup is given in **Figure 1.5**. The sample (1-30 g) is measured into a stainless steel extraction cell and tightly sealed. The extraction solvent (e.g. 50 : 50 methanol : water) is pumped into the cell and extraction takes place at temperatures up to 200 °C and pressures of up to 3000 psi. The solvent remains stationary in the cell for a short period and is then purged using firstly fresh solvent and, secondly, a gas (N_2). The extract is collected in a glass sample vial.[84] Sample extraction times and volumes of solvent used are smaller than those employed in other more conventional techniques such as Soxhlet extraction.[85] ASE has been approved by the US EPA as an extraction technique for organic compounds such as polychlorinated biphenyls (PCB's), polyaromatic hydrocarbons (PAH's) and pesticides from soil.[86]

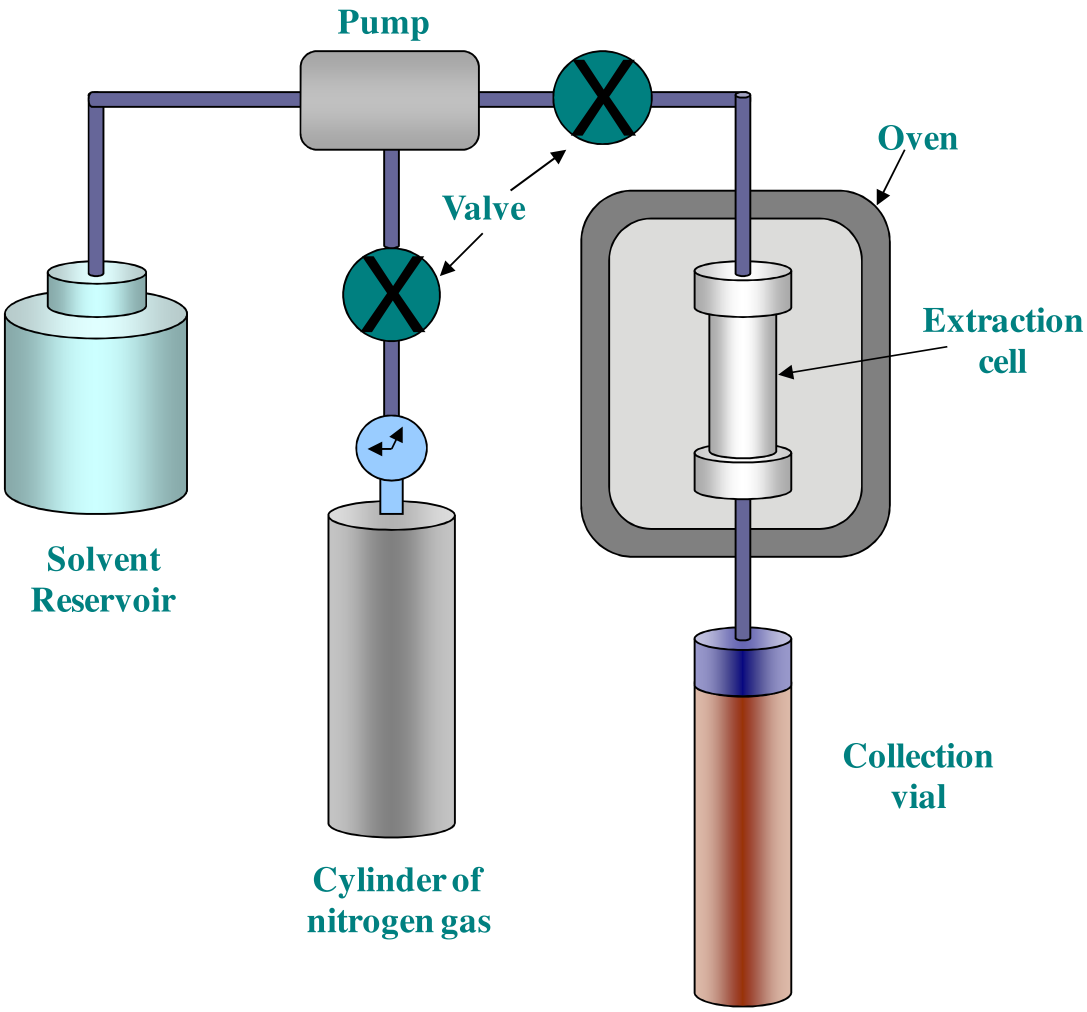

Figure 1.5: Diagram of accelerated solvent extraction apparatus, adapted from ref. 84

1.9 Analysis of PPCPs in Complex Environmental Matrices:

1.9.1 Introduction:

PPCPs have attracted much interest in recent years as an emerging class of contaminants due to their extensive human and veterinary usage and their numerous entry routes into the environment. The need to study the distribution, occurrence and fate of these compounds in environmental matrices has led to numerous analytical methodologies being proposed for their analysis. These proposed procedures however have focused almost exclusively on sample clean-up usually involving SPE coupled to either LC or GC with single or tandem mode MS.[87] Detecting pharmaceutical residues in environmental matrices is particularly challenging for a number of reasons. The concentration of the compounds is generally very low. Furthermore, many of the compounds are thermally unstable and may also interact with components of the environmental matrix making extraction even more difficult.[88] To date, the majority of research has focused on drinking, surface and ground waters, as well as influent and effluent of WWTPs with a number of review papers being published solely on this topic.[29, 89, 90] It is challenging to quantitatively extract trace residues from aquatic samples, however new advances in SPE technologies have improved recoveries of analytes and allowed for multi-residue analysis of different families of drugs simultaneously.[87]

The extraction and detection of PPCPs from solid environmental matrices (e.g. soil, sediment, sludge) is of great importance, particularly as antibiotics reaching the environment may lead to drug-resistant bacteria. The effects of PPCPs in the environment have been reported particularly in the fish farming industry due to contaminated sediments.[3] Additionally, sludge applied in agriculture and the receiving soil are subject to accumulation of persistent PPCPs leading to a possible detrimental effect on plant life.[91, 92] Leaching of PPCPs from soil may also be responsible for deleterious effects on aquatic organisms.[3] Solid environmental matrices require more selective extraction and sample-preconcentration due to variable composition and high organic content. The aim of this section is to discuss the analytical techniques for analysis of PPCPs in complex environmental matrices with emphasis on solid samples such as sludge and soil. Particular attention is to be given to sample preparation, LC-MS and LC-MS/MS.

1.9.1 Analytical techniques for determination of PPCPs in the aquatic environment:

Modern analytical techniques for determination of PPCP traces in aquatic samples involve sample preconcentration, gas or liquid chromatographic separation and selective detection using MS. Today, with the advent of new polymeric, multi-mode SPE sorbents along with improvements in liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) technologies, multi-analyte determinations are possible.[93] These, so called 'multi-residue methods, are in demand due to the continuously growing number of PPCPs being identified in the aquatic environment. An analytical method that allows detection of a range of analytes reduces analysis time, sampling time, costs less and provides an overall picture of the state of the environment.[87]

Concentrations of PPCPs in environmental samples are generally in the ppm to ppb range depending on the type of sample.[88] Sample preparation techniques therefore must involve a high enrichment factor if the target analytes are to be detected. In recent years, pre-treatment of aqueous samples has moved away from traditional LLE, with solid phase microextraction (SPME) and SPE becoming the commonly used preconcentration techniques. SPME is particularly useful when employed with GC-MS. Very small volumes of sample are required and there are no solvents involved providing high concentration factors of organic compounds. The pharmaceuticals can also be desorbed directly from the SPME fibre into the GC instrument which further simplifies the sample preparation.[94, 95] GC is only applicable to separation of volatile analytes which limits its use in multi-residue analysis, particularly for larger analytes such as antibiotics. Additionally, SPME can suffer from low sorption capacity of fibres leading to incomplete extraction of analytes.[96]

A wide range of SPE sorbents have been applied to the detection of different categories of pharmaceuticals and other medicinal products in recent years. Reversed-phase C_{18} cartridges have been used to extract antibiotics, analgesics and non-steroidal anti-inflammatory drugs (NSAID's).[97] Ion exchange sorbents have been employed for extraction of antiobiotics, analgesics, beta-blockers and blood-lipid regulators.[98] Polymeric sorbents have been used for enrichment of analgesic, anti-epiletics and NSAID's in surface waters.[99] One of the main challenges facing the analyst when developing a multi-residue method is extraction of all analytes simultaneously. The physico-chemical properties of pharmaceuticals determine their extraction efficiency for a given sorbent.[87] Analytes with a wide range of properties such as pK_a and Log K_{OW} will have differing levels of enrichment. Often when conducting multi-residue analytical methods, mixed-mode sorbent types are needed or two types of extraction cartridges are placed in sequence for extraction of all analytes however, this may lead to high levels of matrix components also being enriched.[87]

New polymeric sorbents such as Waters Oasis are distinguishable from other types by their use for molecules with a wide range of polarities, which allow for improved interaction with analytes in aqueous samples. These characteristics are due to a hydrophilic pyrrolidone monomer being included at manufacture.[87] This sorbent has been used for extraction of acidic and neutral pharmaceuticals simultaneously.[100, 101] The extraction can be carried out at a neutral pH with a lesser need for acidification.[100] Elimination of the acidification step means no risk of hydrolysis of analytes within the sample.[102]

A number of studies have been carried out to compare the extraction efficiencies of different types of sorbent for a diverse group of PPCPs.[99, 103] Lin *et al.* investigated the extraction efficiency of a C_{18} reversed-phase sorbent and two polymeric sorbents (including Oasis HLB) for pharmaceuticals in WWTP effluent and riverwater.[99] The Oasis HLB cartridges reported higher and more reproducible extraction efficiencies than the other sorbents at neutral pH.[99] Alternative sample preparation techniques which have been applied to PPCP analysis in aqueous matrices are membrane assisted solvent extraction (MASE) and hollow fibre liquid phase micro-extraction (LPME). Both methods demonstrated good detection limits but reported that method precision needed improvement.[104, 105]

Similar results were obtained by Gros *et al.* who compared two polymeric, one hydrophobic reversed-phase and one mixed mode cartridge with cation exchange and reversed-phase properties (Oasis MCX).[103] In this study, 29 pharmaceutical analytes were chosen with a range of pK_a values from 4-9. The extractions were carried out at neutral pH in order to identify the best extraction method without sample pre-treatment.

The mixed-mode cartridge reported high recoveries of the acidic pharmaceuticals at neutral pH, but the basic analytes were not retained.[103] This cartridge is generally employed at low pH where basic compounds are protonated and can be retained due to cation exchange. The acidic and neutral compounds are enriched by the reversed-phase groups. The second polymeric sorbent (Lichrolut ENV) performed badly and is also generally employed at low pH. The C_{18} sorbent demonstrated good retention of most analytes but was still inferior to the Oasis HLB sorbent which was subsequently chosen as the optimum sorbent for the analysis of surface waters and WWTP influent and effluent.[103]

The majority of pharmaceutical analysis in aquatic environmental matrices has been carried out on C_{18} or C_8 reversed-phase high performance liquid chromatography (HPLC) columns.[88] MS detection provides excellent selectivity so that complete resolution of analyte peaks is not a requirement for detection, however, if possible, it does increase sensitivity by reducing ion suppression effects.[93] Mobile phases commonly consist of an aqueous phase (water) and organic phase (usually methanol or acetonitrile). The aqueous phase often contains a mobile phase additive to stimulate ESI ionisation or to achieve consistent retention times.[93] Analytical separations of acidic pharmaceuticals and anti-inflammatory drugs generally employed volatile additives e.g. formic acid, ammonium acetate and ammonium formate.[106-108] Analysis of antibiotics sometimes requires mobile phase additives to increase sensitivity of the mass spectrometric detection. This has previously been achieved by the addition of ammonium acetate, ammonium formate, oxalic acid and formic acid.[46, 92, 109] More basic drugs such as beta-blockers are usually analysed using a more neutral mobile phase.[110] More complex separation techniques such as ion-pair liquid chromatography (IP-LC) have been employed for the analysis of NSAID's and triclosan in surface and wastewaters. Quintana *et al.* added ion-pairing agent tri-n-butylamine (TrBA) to the mobile phase and achieved increased signal intensities and stable retention times.[111] Ultra performance liquid chromatography (UPLC) has also been applied to these complex environmental samples.[112, 113] These analytical methods reported separations of up to 29 analytes in 10-15 minutes and reductions in peak width when compared to conventional HPLC analysis. Detection limits were in the low ng/L level which is comparable to HPLC analysis methods, although despite the shorter analysis times UPLC still has only seen limited application in this area.[88, 112, 113]

Mass spectrometry has been the detection technique of choice for analysis of pharmaceutical residues in aqueous sample for many years. In single mode MS, molecular ions isolated at a single m/z in positive or negative ion modes are measured in a process known as single ion monitoring (SIM). Over the past two decades, LC-MS technologies have greatly advanced in reliability, sensitivity and selectivity. In modern day laboratories, detection limits in the ng/L range and lower are commonplace.[87] LC-MS/MS is now the standard detection technique for pharmaceuticals in environmental matrices.[93] Selected reaction monitoring (SRM) allows unequivocal identification of compounds in complex samples. The triple quadrupole (QqQ) mass analyser is the most frequently used mass analyser with popular interface technologies being ESI and APCI.[114, 115] These soft ionisation techniques suffer from matrix effects particularly when analysing a complex sample such as wastewaster of WWTP influent and effluent.[87] These matrix effects can lead to enhancement or suppression of signal intensity.[89] A number of strategies have been introduced in recent years to minimise these effects. A combination of adding of internal standards (e.g. stable isotope surrogate standards) and standard addition calibration are most commonly used although it has also been demonstrated that reducing the flow of solvent and sample entering the ion source can increase signal intensities and reduce matrix effects by up to 60% for many PPCPs including the NSAID's.[116, 117] Another common mass analyser is the 3D ion-trap allows for easy identification of compounds in multiple reaction monitoring (MRM) modes. This instrument has been successfully applied to the determination of 20-30 pharmaceuticals in river and wastewater simultaneously with detection limits in the ng/L range.[45, 118]

Advanced hybrid mass spectrometers have now become widely available such as the quadrupole time-of-flight (q-TOF) and the orthogonal acceleration time-of-flight (oa-TOF) mass analysers.[87] These are useful in quantification of environmental PPCP residues due to their high resolution and ability to provide accurate mass measurement data for precursor and product ions.[88] The elucidation of the metabolic pathways of PPCPs in the environment is of crucial importance to toxicological studies. Some metabolites may have even more potent biological effects than the parent compound and must be identified and removed from the aquatic environment.[87] Eichorn *et al.* employed the q-TOF analyser to identify antibiotic metabolites which had been formed due to biodegradation activities of sludge on trimethoprim.[119] Stolker *et al.* also reported the excellent capability of the q-TOF to provide improved qualitative information and improved selectivity over the more conventional triple quadrupole.[98]

1.9.2 Analytical techniques for determination of PPCPs in the solid environmental matrices:

The analysis of solid matrices for antibiotics and pharmaceuticals is important mainly due to the development of antibiotic resistant bacteria, which are exposed in soils, sludges and sediments. If these microorganisms develop resistance there is a possibility they will no longer be eliminated when treated with antibiotics and in this way may be more harmful to humans.[120] PPCPs in solid matrices however are particularly challenging to isolate and enrich without co-extraction of matrix components and usually require laborious, time-consuming extraction and analysis techniques. The analytical methods designed for this purpose were reviewed by Diaz-Cruz *et al.* in 2003 and Xia *et al.* in 2005.[3, 121] In this work, recent advances in this field will be discussed. A list of analytical techniques, their operating procedures and validation information is provided in **Table 1.1**

Table 1.1: Analytical techniques employed for the extraction, sample clean-up and determination of PPCP residues in solid environmental matrices.

Compounds	**Matrix**	**Extraction procedure**	**Clean-up procedure**	**LC column**	**Mobile Phase**	**Detection**	**%Relative/ Absolute Recovery**	**LOQ**	**Detected levels**	**Ref**
Amphetamine	Activated sludge	Ultrasonic solvent extraction (USE)	Oasis HLB	C_{18}-RP	50 mM formic acid + MeOH	API-IT	~90%	2 µg/Kg	5-300 µg/Kg	[122]
Pharmaceuticals, and ICM	Activated + digested sludge	USE	Oasis MCX C_{18} SPE	C_{18}-RP	5 mM NH_4Ac or acetic acid + MeCN	ESI + API-QqQ	~40-119% (all analytes incl.)	20-50 µg/Kg	<1mg/Kg	[123]
Antibiotics-fluoroquinolones, trimethoprim, sulfamethoxazole	Anaerobic digested sludge	Two step USE	Filtration through membrane syringes	C_{18}-RP	0.1% formic acid + MeCN	ESI-IT	14-86%		0.1-4.4 mg/Kg	[124]
Antibiotics-sulfonamides, macrolides, trimethoprim.	Activated sludge	PLE	Oasis HLB	C_{18}-RP	1% formic acid + MeOH	ESI-QqQ	78-142%	3-41 µg/Kg	12-197 µg/Kg	[125]
Antibiotics-fluoroquinolones	Municipal sewage sludge + sediment	USE	Chromabond Tetracycline	C_{18}-RP	50 mM formic acid + MeOH	API-IT	80-105%	1-7 ng/L	30-510 µg/Kg	[126]
Antibiotics-fluoroquinolones	Activated + digested sludge + soil	PLE	Mixed phase cation disk SPE	RP-Amide C_{16}	25 mM o-H_3PO_4 + MeCN	FLD	75-94%	0.18-0.45 mg/Kg	1.4-2.4 mg/Kg	[127]

Table 1.1: Contd.

Acidic pharmaceuticals, antibiotics + ivermectin	River sediment	USE	Oasis MCX, Lichrolute En + C_{18}	C_{18}-RP	20 mM oxalic acid + MeCN	APCI + ESI-QqQ	80-110%	0.4-8 µg/Kg		107
Carbamazepine, metabolites + caffeine	WWTP biosolid	PLE	Oasis HLB	C_8-RP	10 mM NH_4Ac with 0.1% formic acid + MeOH/ MeCN	ESI-QqQ	80-92%	0.2-1.7 µg/Kg	2-258 µg/Kg	128
Tetracyclines + tylosin	Fertilised soil	Liquid-solid extractrion (LSE)	none	C_{18}-RP	0.5% formic acid with 1 mM NH_4Ac + MeCn/MeOH	ESI-IT	~40-70%	~5 µg/Kg	Up to 200 µg/Kg	92
Estrogens	Digested + activated sludge	USE	GPC and silica gel clean-up	GC capillary	n/a	GC-IT	73-104%	2-4 µg/Kg	17-49 µg/Kg	129
Phenylureas, triazines + chloroacetanilides	Soil	PLE	none	C_{18}-RP	MeCN + water	ESI + APCI-IT	~60-120%	0.3-22 µg/Kg		130
Sulfonamides + trimethoprim	Liquid manure	LLE	Aminopropyl SPE	C_{18}-RP	0.1% Formic acid, 1 mM NH_4Ac + MeCN	ESI + APCI-QqQ	77-91%	5 µg/Kg	10-270 µg/Kg	131
Synthetic musks	Biosolid	PLE	GPC and silica gel clean-up	GC capillary	n/a	EI-IT	>80%	0.2-1.9 µg/Kg	409-7897 µg/Kg	132

Table 1.1: Contd.

Insecticide + pesticides	Soil and digested sludge	PLE	Florisil SPE	HP5MS GC capillary	n/a	GC-IT	>92%	3-62 µg/Kg	38-210 µg/Kg	[133]
Sulfonamides, tetracycline + tylosin	Pig manure	PLE	LLE + anion exchange SPE	C_{18}-RP	Formic acid, water + MeOH	ESI-QqQ	~70%	10-100 µg/Kg	2-30 mg/Kg	[134]
Estrogens + progestrogens	River sediments	USE	Sep-Pak Plus C_{18} SPE	C_{18}-RP	MeCN + water	ESI-MS	64-100%		Low µg/Kg range	[135]
Multi-residue	Biosolid + enriched soils	PLE	Oasis HLB	C_{18}-RP	10 mM NH_4Ac + MeCN	ESI-IT	≥60%	~50 µg/Kg	5-20 mg/Kg	[66]
Antibiotics, macrolides + ionophores	Soil	PLE	Diol SPE	C_{18}-RP	100 mM NH_4Ac + MeCN	APCI-QqQ	43-118%	0.6-5.3 µg/Kg	0.7 µg/Kg	[136]
Tetracyclines, macrolides and sulfonamides	Soil	PLE	Isolute SAX + Oasis HLB	C_{18}-RP	Formic acid, water + MeOH	ESI-QqQ	50-100%	1.5-5 µg/Kg	10-55 µg/Kg	[137]
Triclosan + triclocarban	Municipal sewage sludge + biosolid	PLE	Oasis HLB	C_{18}-RP	Water + MeOH	ESI-QqQ	90-103%	5 +0.5 µg/Kg	0.62-11.55 mg/Kg	[138]
Sulfonamides	Aged soil	PLE	PVDF filtration	C_{18}-RP	1 mM acetic acid in water + MeCN	ESI-QqQ	41-93%	>15 µg/Kg	>50 µg/Kg	[139]

Sample extraction techniques for solid matrices must be efficient and selective for the target analytes due to their low concentration and the high organic load present in the sample. Low recoveries will result from inefficient extraction of analytes or co-extraction of matrix components. From **Table 1.1,** PLE has become the most commonly used extraction technique for soils. However, Hamscher *et al.* employed liquid-solid extraction with no SPE clean-up step for the determination of tetracyclines in soil.[92] Method recoveries were calculated at a range of spiking concentrations and were generally in the 60-70% range for all analytes except tetracycline. This study provides evidence that tetracycline is a stable pharmaceutical pollutant which may have an affinity for soil particles.[92] The last three studies described in **Table 1.1** employed PLE for the extraction of antibiotics from soil however very different extraction solvents and clean-up techniques were employed.[136, 137, 139] Schlüsener *et al.* employed ammonia in methanol, Jacobsen *et al.* used citric acid buffer and methanol with tandem SPE and Stoob *et al.* extracted with acetonitrile : water at pH 8.8. The tandem SPE involving an anion exchanger (SAX) and a hydrophilic-lipophilic polymer (HLB) has also been termed 'mixed-mode SPE' and worked to remove negatively charged organic material and preconcentrate the analytes on the polymer sorbent.[137] Despite the differences in analytical procedure, the recoveries and limits of quantitation for the three methods are quite comparable. PLE has also been applied to the extraction of pharmaceuticals from soil to determine the effect of recycled wastewater being used for irrigation.[140] Kinney *et al.* also employed water : acetonitrile as an extraction solvent and determined acidic and neutral compounds e.g erythromycin, carbamazepine and fluoxetine.[140]

Sludges are generally analysed directly from the WWTP (activated sludge) and have >95% water content, or in a processed form known as dewatered sludge or biosolid.[141] Activated sludges are sometimes treated as liquids and subjected to LLE followed by gel permeation chromatography (GPC) and SPE clean-up, particularly for the analysis of polycyclic and nitro-aromatic musks.[142] The traditional preparation technique for solid samples such as the digested sludges was soxhlet extraction which is still commonly used.[142] However, the thermal instability of many pharmaceutical residues as well as laborious lab work and higher solvent usage also renders this technique outdated when compared to more modern techniques such as USE and PLE.[143] It is evident from **Table 1.1,** that ultrasonic solvent extraction (USE) and PLE are now the most commonly used extraction techniques for liquid and solid sludges. Both sludges are filtered and lyophilised (freeze-dried) prior to extraction to give them solid sample characteristics.[129] Elimination of excess moisture in the biosolids has also been performed using diatomaceous earth to absorb the water before extraction.[128] In the case of PLE, extraction temperature plays a big role in the efficiency of the technique. This was exemplified in the work of Barron *et al.* where a decrease in extraction efficiency was noted for a range of pharmaceuticals above and below 60 °C.[66]

Miao *et al.* extracted carbamazepine and its metabolites (10,11-dihydro-10,11-epoxycarbamazepine, 11-dihydro-10,11-epoxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine and 10,11-dihydro-10-hydroxycarbamazepine) from raw and treated WWTP biosolid using acetone and water followed by SPE on Oasis HLB cartridges with excellent recoveries of 80-92%.[128] This study helped to elucidate the degradation pathways of carbamazepine within the WWTP. The study showed that carbamazepine was not effectively removed from wastewater during treatment and that it and its metabolites distribute within the aqueous phase rather than the solid phases during WWTP treatment [128]

PLE was also applied to the removal of antibiotics in lyophilised sludge by Göbel *et al.* in 2005 and reported good recoveries for macrolides, sulfonamides and trimethoprim.[125] A study of extraction solvent including methanol, acetonitrile and acetone indicated that the most efficient mixture was 50 : 50 methanol : water. A study of pH also indicated that hydrophobic interaction may be responsible for sorption of macrolides to sludge.[125] The optimised PLE method was also compared to a USE method for extraction of the same analytes at two different operating pHs. Both methods provided efficient extraction, however, under both conditions the PLE method reported higher recoveries with Oasis HLB as an SPE sorbent. The recoveries at pH 7 and pH 4 were comparable but at pH 7 were more reproducible illustrating that extracts can be directly enriched after PLE on the polymer cartridges.[125]

Golet *et al.* employed a phosphoric acid and acetonitrile PLE extraction solvent in the analysis of zwitterionic fluoroquinolone antibiotics including ciprofloxacin and norfloxacin.[127] An acidic solvent provided the best extraction because at low pH both the anionic binding sites on the sludge and the antibiotics were protonated causing electrostatic repulsion and better extraction. USE was tested initially as an alternative pre-treatment technique but reported poor recovery data. Clean-up was performed using a mixed-phase cation exchange SPE disk and resulted in overall good method recoveries from sludge and sludge treated soil.[127] Synthetic musks have also been identified in biosolids using PLE in sample treatment with n-hexane and ethyl acetate as extraction solvents.[132] GC-MS is still employed for the analysis of these compounds due to their volatile nature. However, a more complex sample clean-up involving GPC and silica gel columns is required prior to analysis. The GPC step serves to separate the musk fraction for co-extracted compounds. The synthetic fragrances were found to accumulate in biosolids rather than aqueous sludges and were also identified in soil that had been treated with the biosolid.[132]

Ternes *et al.* designed an analytical method for the determination of acidic and neutral pharmaceuticals, musks and iodinated contrast media (ICM) in activated and digested sludges.[123] USE was employed for the extraction of all analytes with 100% methanol while PLE was also used to withdraw the polycyclic aromatic musks using methanol mixed with acetone. A range of clean-up techniques were employed including cation exchange (for acidic drugs) and reversed-phase SPE sorbents. The PLE and USE methods demonstrated comparable extraction efficiency for two musks (galaxolide and tonalide) from both sludge samples.[123] Relative recoveries greater than 70% were reported for the acidic pharmaceuticals using USE while extraction of the neutral drugs was less reproducible. Although effective, this method involved many different extraction techniques to detect a wide range of analytes.[123] More recently, Barron *et al.* developed a multi-residue procedure for the analysis of 27 acidic and basic pharmaceuticals and antibiotics in biosolid and biosolid treated soils.[66] The method involved PLE extraction with Oasis HLB clean-up and reported recoveries ≥60% for 20 of the analytes in both soil and sludge matrices. High concentrations of the antibacterial triclosan were identified in environmental samples and were even present 3-4 months after fertilisation of soil with the contaminated biosolid.[66]

Acidic pharmaceuticals along with antibiotics and parasiticide ivermectin were extracted from river sediments using USE.[107] Acetone and acetic acid was employed for the removal of acidic drugs with clean-up on Oasis MCX at reduced pH to ensure ionisation. The USE method was reported to extract up to 92% of the ibuprofen from spiked sediment and the method recoveries for the entire USE-SPE procedure ranged from 80-110%.[107] The exception to this was bezafibrate which also demonstrated low recoveries for Ternes *et al.*[123] Alternative extraction techniques include microwave assisted extraction (MAE) which was employed for the extraction of triclosan from sludge and sediments by Morales *et al.* in 2005.[144] The target analytes were extracted in acetone and methanol under the influence of a microwave field, method recoveries ranged from 78-106%. Recently, Xia *et al.* reviewed analytical techniques used for extraction of PPCPs from solid environmental samples and deemed PLE to be the most efficient method in terms of extraction time and solvent consumption.[121]

GC-MS and gas chromatography-tandem mass spectrometry (GC-MS/MS) have been the standard methods of analysis in environmental studies for years. However, with the emergence of this new group of pharmaceutical pollutants that includes thermolabile, non-volatile and highly polar compounds, LC has become more suitable.[120] GC is however still employed for the analysis of synthetic musks, pesticides and hormones in solid environmental samples as depicted in **Table 1.1**.[129, 132, 133] The application of biological assays has also been limited in this field perhaps due to insufficient sensitivity and specificity. Radioimmunoassays (RIA) were applied to the detection of anti-microbial residues in animal manure. The results indicated that when compared to a more conventional LC-ESI-MS technique, the RIA had insufficient sensitivity to detect low levels of the residues.[145]

Reversed-phase LC is by far the most popular chromatographic separation mode due to its versatility and applicability to a wide range of analytes. The stationary phase of choice (from **Table 1.1**) is composed of C_{18} or C_8 chains on a silica particle packing.[120] Retention in reversed-phase chromatography is due to hydrophobic interaction between molecule and the alkyl chains of the stationary phase. Octadecyl chains contain a higher hydrophobic organic load than C_8 phases, which are also popular, and often result in more efficient separations.[143] Low flow rates (0.1-0.5 mL/min) are required with ionisation sources such as ESI and so narrow-bore columns (e.g. 2-3 mm i.d.) have become common for chromatographic separations. Additionally, low flow rates have been shown to improve matrix effects.[143]

Methanol and acetonitrile are the most commonly used mobile phases, particularly for the separation of acidic and neutral pharmaceuticals and are usually used individually. However; Miao *et al.* combined the two organic solvents for the analysis carbamazepine in sludges and reported good retention and separation.[128] The pH of LC mobile phase plays a big role in the efficiency of the separation. At high pH acidic pharmaceuticals are completely dissociated and may not be well retained by reverse-phase sorbents. Low pH is more suited to the separation of acidic analytes but for multi-residue methods the pH must also be suited to basic analytes which require a higher pH to avoid ionisation and decreased retention. In general the pH of the mobile phase should be approximately 2 units from the pK_a of the analyte.[143] Another factor in mobile phase composition is compatibility with the MS detector. Volatile buffers such as ammonium acetate and ammonium formate can be added at low concentrations to produce reproducible retention times and encourage ionisation in the MS interface but can also result in suppression of the signal intensities.[146] For acidic drugs ammonium acetate concentrations from 2-20 mM are preferred to achieve stable retention and acceptable levels of ion suppression.[97] Tri-butylamine can also be added to mobile phases as an ion-pairing agent to increase sensitivity for anionic pharmaceuticals. Other common mobile phase additives include formic acid and acetic acid which are employed for acidification of mobile phase in analysis of antibiotics.[92, 125, 126, 131] Shortening of analysis time is also an important factor in multi-residue analysis as long run times are often a problem when targeting so many analytes.[147]

Analysts are constantly seeking to increase the speed of HPLC analysis without losing efficiency of separation. There are several aspects of the HPLC method that can be considered when attempting to shorten analysis time. Most obviously, increasing flow rates can achieve more rapid analyses. However, higher flow results in higher column backpressures, which restrict the flow rate to one that is within the pressure limitation of the HPLC system, usually about 400 bar. At higher flow rates there is less time for mass transfer processes to occur in the column and this can lead to a reduction in column efficiency.

Another way of reducing run-time is to decrease the length of the analytical column. Retention times can be cut using this method but unfortunately, less stationary phase means less plates and column performance suffers. These short columns are usually packed with smaller particles (sub 2 µm) to overcome this problem. The smaller particles provide a greater surface area and hence greater N values while enhancing mass transfer due to smaller diameters. However, particle diameter is inversely proportional to column backpressures and so this approach also has its limits.

Increasing the column temperature can also be advantageous. Even though the range of temperatures that can be used is limited by stationary phase and solute stability, it is still useful. Mobile phase viscosity is reduced at high temperatures which broadens the range of flow rates that can be employed. Additionally, the rate of mass transfer is increased leading to advances in separation efficiency.

Modern techniques known as ultra-fast LC or UPLC are now commonplace in analytical publications. These new technologies employ expensive, ultra-high pressure pumps to produce high flow rates through columns containing sub 2 µm particles. Although high values of N have been recorded and analysis times are shorter due to high flow rates, these systems require specialised HPLC components, deal with excessively high column backpressure, allow only minute sample injection volumes and suffer from frictional heating.[148] Petrovic *et al.* and Kasprzyk-Horden *et al.* have both developed multi-residue techniques for almost 30 pharmaceuticals across a wide range of therapeutic classes using UPLC with promising results.[112, 113]

Particle packed beds as stationary phases suffer from high operating backpressures and slow mass transfer rates. From the evidence discussed in the section on fast HPLC it is obvious that a replacement is needed. In recent years much attention has been focused on the production of columns from a single monolithic block of media of either an organic or inorganic material. These monolithic columns are composed of cross-linked polymers or blocks of continuous silica that are porous in nature.[148] Monolithic columns are beneficial to HPLC analyses because they allow for faster flow rates and therefore more rapid analysis times due to their macroporous structure. Nakanishi *et al.* published one of the first papers on the production of silica monolithic rods which had a biporous structure of large pores in the micrometre range and smaller 'meso-pores' in the nanometre range.[149, 150] The monoliths rigid structure has enhanced permeability and so higher flow rates are permitted without the hindrance of high backpressures. This porosity and large surface area have a second advantage, mass transfer kinetics occur more rapidly in the monolithic columns leading to less band broadening at high flow rates. Additionally, capacity for large sample volumes is also increased by the presence of the miniscule meso-pores in the monolithic structure.[148]

UV and fluorescent detection have been employed for the analysis of PPCPs in solid matrices and up until a few years ago were still the detection techniques of choice.[127, 135, 151] Kinney *et al.* applied LC-ESI-MS to the determination of acidic and neutral pharmaceuticals in soils. The specificity of this technique can be improved to mimic that of an LC-MS/MS instrument by increasing the exit voltage across the needle in the electrospray chamber. This increased energy causes the molecular ions to fragment and is known as collisionally induced dissociation (CID).[143] This approach to increasing specificity was utilised by Cahill *et al.* for the analysis of drugs in water samples.[152] However, CID can lead to co-extracted compounds and matrix components in the complex samples also fragmenting within the ion source which affect the sensitivity and accuracy of the measurement.

The two most common interfaces used in LC-MS/MS of environmental solids are carried out at atmospheric pressure, i.e. ESI and APCI. From **Table 1.1** the electrospray technique has been used most often for analysis of solid samples but APCI is also common.[153] For certain analytes such as the acidic pharmaceuticals, ESI has proven to be a more sensitive technique as demonstrated by Stolker *et al.*[98], however, both ionisation procedures are suitable for analysis of neutral and basic analytes.[106] The most commonly used mass analysers used in LC-MS/MS, from **Table 1.1** are the triple quadrupole (QqQ) and the ion-trap (IT). These instruments can be operated to fragment the parent drugs molecules and allow unequivocal identification of compounds.[120] Even more advanced quadrupole-TOF instruments are now becoming widely available and provide high resolution analysis not possible on the aforementioned analysers.[143]

The limits of quantification (LOQ) for analysis of PPCPs in sludge samples using IT and QqQ mass analysers given in **Table 1.1** demonstrate that both techniques provide comparable levels of sensitivity. Methods using both instruments have reported LOQs of between 0.2 and 50 µg/Kg for pharmaceuticals, antibiotics and synthetic musks. These results illustrate the versatility and specificity of tandem MS for pharmaceutical analytes even in complex matrices. **Table 1.1** also depicts the applicability of these techniques to the determination of a very diverse group of analytes analysis in real environmental samples, many of which were detected in the µg/Kg or ng/g range. Although not yet demonstrated for solid matrices, the advantages of the new q-TOF mass analyser have already been demonstrated. Stolker *et al.* employed both a q-TOF and QqQ for the analysis of acidic drugs and antibiotics in environmental water samples.[98] The q-TOF allowed for all analytes to be screened and identified using the exact masses of the product ions in one run.[98] Loffler *et al.* demonstrated that different ionisation techniques can be employed depending on the nature of the compounds.[107] In this study, APCI-MS/MS was employed for acidic pharmaceuticals while the antibiotics were analysed using ESI-MS/MS. Limits of quantification (LOQs) were comparable though slightly lower for the pharmaceuticals.[107] Schlusener *et al.* employed APCI-MS/MS in SRM mode for the analysis of macrolides (e.g. erythromycin) and ionophores (veterinary antibiotics) in manure and reported very low limits of detection (LODs) of 0.2-1.6 µg/Kg.[136] There is growing concern in MS/MS analysis about the criteria for confirmation of pharmaceutical residues in environmental samples to avoid false positives being reported. The European Commission Guidelines now state that at least two SRM transitions must be monitored for correct identification of a compound in an environmental matrix when using a conventional QqQ instrument. Mass analysers such as the q-TOF are becoming increasingly popular as their ability to produce full scan product ion spectra simplifies analysis.[147]

One of the pitfalls of MS/MS analysis is the matrix effect. It affects data quality in LC-MS, GC-MS, GC-MS/MS and LC-MS/MS. The matrix effect leads to differences in analyte signal when compared to a standard solution. In GC-MS and GC-MS/MS analysis matrix effects are most commonly caused by co-extracted material in the sample that elutes at the same time as the analyte or has a similar m/z value as the target compound. This problem can also be seen in LC analysis and can be partially solved by improving the separation step. In LC-MS and LC-MS/MS analysis, the major drawback is the susceptibility of API interfaces such as ESI and APCI to ion suppression or enhancement caused by interfering matrix components. The co-extracted compounds can be organic or inorganic and interact with the analytes during sample preparation or during ionisation in the interface.[88] The extracted interferences can vary between samples and so the matrix effect in a series of samples is not uniform or predictable. The signal suppression or enhancement that occurs can affect linearity, precision and accuracy of quantitative data from LC-MS/MS analysis.[147] Ion suppression is probably the most common effect observed in environmental analysis and has become one of the most challenging issues for analytical scientists. It is caused by interfering compounds in the ion source which reduce the efficiency of desolvation and desorption processes resulting in a varying amount of charged analyte reaching the detector.[147] Environmental samples such as soil and sludge pose a severe risk of matrix effects in reversed-phase liquid chromatography (RPLC) due to a higher organic load and are also very unpredictable due to inhomogeneity of the samples.[88]

A number of studies have indicated that APCI is affected by matrix effects to a lesser extent than ESI.[106, 154] Zuehlke *et al.* reported that highly polar pharmaceuticals were strongly suppressed when employing ESI whereas APCI reported recoveries close to 100%.[154] Similar results were reported by Schlüsener *et al.* for the detection of steroid hormones and antibiotics in the influent and effluent of sewage plants.[155] The two ionisation techniques were compared and a much higher matrix effect was reported for ESI even after extensive sample-clean-up with SPE and size exclusion chromatography (SEC). Unfortunately, ionisation of all analytes was not possible when using APCI and so ESI had to be used for quantification.[155] It is a well known fact that APCI is not as suitable ionisation technique for very polar compounds as ESI and so the matrix effect associated with ESI must be dealt with.[143]

Matrix effects can also be caused by contamination of the internal parts of the instrument. With ESI, the end plate of the source can become dirty, particularly when analysing complex samples such as sludge. As analysis progresses, the matrix components that adhere to the end plate can enter the source resulting in suppression or enhancement. The unpredictability of matrix effects is exemplified by another study conducted by Ingrand *et al.* for steroid hormones also in the effluents of a WWTP.[156] In this study, APCI and ESI were compared in both positive and negative mode and ESI negative mode was chosen as the most sensitive technique for analysis.[156] Pfeifer *et al.* employed both ionisation techniques for the analysis of antibiotics in manure.[131] The ionisation efficiency of the two techniques was comparable. Sodiated adducts were observed when using ESI and the intensity of signals varied with the amount of sodium in the sample. Both enhancement and suppression were observed for analytes when using ESI while only enhancement was reported for APCI analysis.[131] The increase in matrix effect with increasing complexity of sample matrix was exemplified in a study by Miao *et al.* which compared signal suppression in a range of sample matrices including influents, effluents, surface water and analytical grade water.[157] LC-ESI-MS/MS analysis exhibited increasing ion suppression from surface water to effluent to influent while no effect was observed for the analytical grade water.[157] High levels of ion suppression have also been reported for more complex matrices such as soil and sludge. Barron *et al.* reported low levels of suppression, <5% for most target analytes in soil extracts but much higher levels were noted for the analytes extracted from sludge samples. The average matrix induced suppression effect in sludge was 42 ± 20%, most likely due to the high organic load associated with sludge samples. However, the analytes that eluted late were most highly suppressed perhaps due to the high percentage of organic solvent in the mobile phase flushing out hydrophobic matrix components.[66] Stoob *et al.* also reported ion suppression of sulfonamides in soil samples as high as 63%. This method did not include a SPE clean-up step but the sample was filtered through a PVDF membrane with a 0.45 µm pore size.[139]

A number of operational techniques have been applied to the task of eliminating matrix effects in LC-MS/MS analysis. Improvement of chromatographic separation will generally reduce the signal suppression or enhancement.[88] SPE methods can be designed to selectively enrich only the analytes, leaving interfering compounds behind. However, exhaustive sample clean-up can be time-consuming, laborious and there is a risk of losing some target analyte and it is also challenging to develop preparation methods applicable to a wide range of PPCPs.[88] Size-exclusion techniques are a new method of removing high molecular weight interfering components from a sample. Restricted access materials (RAMs) and molecularly imprinted polymers (MIPs) may be the new generation of SPE sorbents for clean-up of environmental and biological matrices. Koeber *et al.* have applied these new clean-up materials to the extraction of triazines from riverwater with excellent elimination of matrix components.[158] This topic has also recently been reviewed.[159] Kloepfer *et al.* applied ultrafiltration to removal of organic interferents from riverwater with less successful results indicating that much of the matrix effect could be caused by small organic compounds.[117] In the same study, the flow of sample and mobile phase entering the interface was decreased using a column splitter. This technique reduced matrix effects very effectively and increased method sensitivity with signal intensity increasing up to nine-fold.[117] Dilution of samples has also been employed for a reduction in matrix effects.[100]

When quantifying traces of pharmaceuticals in the environment there are a number accepted approaches to overcoming the effect ion suppression and enhancement have on the accuracy of the results. External calibrations using matrix matched samples can be employed to obtain a calibration curve, however this technique requires an uncontaminated matrix which is not always easy to find.[134, 136] The conventional method of standard additions is still employed but does involve time and effort especially with multi-residue methods.[111] Addition of internal standards is now a widely used quantification technique in trace environmental analysis. Structurally similar analogues of the analyte can be added to standards to eliminate matrix effects to some extent. However, matrix effects are not constant throughout a chromatographic separation and so one internal standard is not sufficient. It can be difficult to source compounds that are structurally similar to all the analytes that will elute close to the analyte retention time and behave in a similar way in the ion source.[124] The most successful method of eliminating matrix effects is the use of isotopically labelled standards. These standards can be deuterated or contain C^{13}, however heavier deuterated standards will have slightly different retention times than the target analytes. However the commercial availability of these standards is limited and they can be quite expensive.[143] The different approaches to quantification have been compared and contrasted for aqueous environmental samples. Van de Steene *et al.* examined numerous methods to reduce signal suppression including structurally similar compounds as internal standards.[160] It was reported than this technique partially compensated for the matrix effects, however standard additions were still employed for quantification to overcome the problem of varying matrix effect between samples.[160] Gros *et al.* also investigated the ability of internal standards and matrix matched external calibration to reduce suppression effects in wastewater on 29 multi-class pharmaceuticals.[103] The study concluded that addition of isotopically labelled internal standards was the most efficient method of correcting loss in signal intensity without being labour intensive.[103] Internal standards have been applied in the analysis of more complex matrices such as sludge, biosolid and WWTP influent and effluent.[125, 138, 155]

The analytical techniques available for the determination of PPCPs in environmental samples have advanced immensely over the past decade. It is estimated that only approximately 5% of the approved medicinal compounds have been investigated in environmental samples.[93, 107] It is important to analyse all types of matrices for these traces in order to comprehend the impact on the environment. More sensitive, reliable and efficient methods of analysis are always needed in environmental analysis so that risk assessment data can be collected about these emerging pollutants. Further work is needed to solve the problems associated with the modern techniques such as matrix effects in LC-MS/MS and there is a need for more focus on the drug metabolites and not just the parent drugs entering our environment.

1.10 Analysis of PPCPs in Complex Biological Matrices:

1.10.1 Introduction:

The presence of pharmaceuticals particularly hormones and antibiotics in food products and the environment is a topic of growing concern. The exposure of healthcare and agricultural employees to harmful drugs is also receiving increased attention.[2, 4, 161] There is increasing concerns about the effects of occupational exposure to cytotoxic drugs and amended guidelines on the handling of these drugs have been introduced in many countries.[162] Antibiotic resistance is another pressing issue and is known to be spread by chronic exposure to antimicrobial agents.[163] The WHO has labelled the increasing rates of resistance to antibiotics as one of the most worrying health issues of the century.[164] Because of these environmental and healthcare issues, there is always a demand for analytical techniques suitable for the detection of low concentrations of pharmaceuticals in biological matrices.

There are many biological matrices that can be analysed for PPCPs and provide information about the fate and effects of PPCPs in the environment and in living organisms. These complex matrices include blood (includes plasma and serum), urine, saliva, hair and tissue and a wide range of analytical techniques have been used to investigate them. Biological samples are different to environmental matrices in their make-up but pose similar challenges for sample clean-up, sensitive analysis and elimination of matrix effects. Tissue is probably the least commonly used type of biological sample employed for drug analysis as it is highly-invasive to obtain. Recently fish tissues have been analysed using chromatographic techniques coupled to MS detection to investigate the bioaccumulation of PPCPs in living organisms. Similar extraction and analysis methods as discussed for environmental samples were applied to these biological matrices. Although matrix effects were problematic during the analysis, the studies indicated that bioaccumulation of drugs particularly anti-depressants in tissues of aquatic organisms does occur.[165, 166]

Biofluidic matrices such as urine and blood have been analysed using immunoassays[167], capillary electrophoresis[168], micellar electrokinetic chromatography (MEKC)[169] and more conventional chromatographic techniques such as GC and LC[170]. The advent of improved extraction procedures such as SPE and SPME along with easy access to selective MS detectors has resulted in increased use of GC-MS/MS and LC-MS/MS in the analysis of pharmaceuticals in biological matrices particularly hair and urine.[171, 172] Both hair and urine are non-invasive samples to collect and exposure to many different types of legal and illegal pharmaceutical compounds can monitored.[170, 171, 173] These sample types can also provide an indication of any chronic exposure to chemicals or harmful drugs in the environment and are useful for monitoring the use of veterinary drugs.[174, 175] This section aims to discuss the analytical techniques that are applied to the determination of PPCPs in biological matrices with particular focus on the use of chromatographic techniques for the analysis of hair and urine.

1.10.2 Analytical techniques for determination of PPCPs in hair:

Analysis of hair has received a lot of attention in the last three decades, since Baumgartner *et al.* used a radioimmunoassay (RIA) to detect opiates in hair strands at μg/g levels.[176] Prior to this hair had been analysed to evaluate exposure to toxic heavy metals such as arsenic, mercury and lead.[177] Baumgartner and co-workers demonstrated that the concentration of drug along the shaft of hair corresponded to the time of drug use.[176] The first chromatographic analysis of hair was conducted by Klug *et al.* in 1980 and led to a surge of research in the area of hair analysis over the next thirty years.[178] Today, standardised analysis techniques for hair testing have been published to provide recommendations in forensic hair analysis.[179, 180] In modern drug testing preliminary tests can be carried out for some drugs using immunoassys to determine any possible traces.[181, 182] GC-MS is the method of choice for confirmatory analysis,[183] however other hyphenated techniques such as LC-MS/MS are becoming popular.[184]

The main advantages of hair analysis over other matrices such as plasma, serum or urine is the longer window of analysis, (months or even years depending on hair length), the non-invasive nature of sample collection, segmented hair analysis can provide information of time and length of drug use and repetitive use can also be documented.[171] Applications of hair analysis include workplace drug testing, doping control in athletes, drug abuse history, exposure to environmental pollutants or food poisoning, post-mortem toxicology, drug-facilitated assaults and even determination of alcohol abuse.[171]

The anatomical and physiological properties of hair have been described in many publications, however the structure of hair and the incorporation of pharmaceuticals into the hair strand are still not fully understood.[185, 186,187] Hair is heterogenous in nature, it is composed of keratinised cells held together by a cell membrane structure (composed of proteins and lipids) and can be divided into three individual sections known as the cortex, cutical and medulla. The structure of a hair is shown in **Figure 1.6**.[171] Hair growth originates in the follicle below the surface of the skin. The growth process occurs in cycles composed of the anagen, catagen and telogen phases.[177] These phases last approximately 4-8 years, ~2 weeks and ~10 weeks, respectively. The anagen step involves rapid growth when the follicle is actively producing new hair cells at a rate of 0.6-1.4 cm/month. The catagen phase involves transition from active to a state of resting which is the telogen phase.[177] Axillary and pubic hair can also be employed for analysis as an alternative to head hair but obviously have differing growth rates. Studies have found that drug concentrations in pubic and axillary hair vary significantly from those found in head hair.[188-190] This variation could be explained by differing blood flow and the aforementioned differing growth rates.[191]

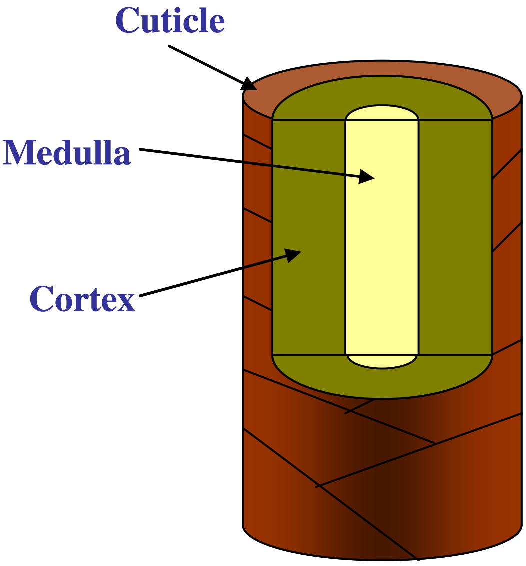

Figure 1.6: Structure of hair strand, adapted from ref. 171

Most models that describe the incorporation of drugs into hair propose external adsorption from the environment and passive diffusion from the blood during hair growth. However, studies have shown that substances can be integrated into the hair structure by other routes such as diffusions from sweat and sebum into the shaft and incorporation from skin cells.[171] A multi-compartment model such as this has been demonstrated by Henderson.[192] There are three main factors that affect the integration of drugs into hair: the amount of melanin in hair, the acidic, basic and lipophilic nature of the drug itself.[171] Hair samples with a lower melanin content or non-pigmented hair have been shown to incorporate a lower concentration of basic drugs than highly pigmented hair. There was however no difference in the concentrations of neutral drugs such as carbamazepine.[193] Drug molecules with a high degree of lipophilicity can easily pass through cell membranes. However for hydrophilic or ionised molecules, cell membranes are an impermeable barrier.[171] A study by van Erp *et al.* demonstrated that the intracellular pH of hair cells such as keratinocytes is acidic and in vitro studies by Claffey *et al.* confirmed that melanin has an affinity for basic drugs.[194, 195] The combination of these factors results in the accumulation of basic and neutral drugs in hair such as cocaine and acidic drugs such as Δ^9-tetrahydrocannabinol (THC).[196] Once incorporated into the hair, the drugs exhibit high levels of retention and stability and have even been detected up to three years after drug consumption, however this analysis depends on the length of the sample.[197]

As already mentioned, immunoassays are commonly used as a preliminary screening method which if returning positive results, will lead to a confirmatory chromatographic analysis.[182] Previously designed immunoassays for the analysis of urine were applied to detection of drugs in hair samples and suffered from lack of specificity and sensitivity.[171] More recently, enzyme-linked immunosorbent assays (ELISA) kits have been developed which employ coated well technology and have demonstrated suitable sensitivity for use in hair analysis.[198, 199] These ELISA test kits are also easily automated and are very useful for rapid and simple screening of hair samples in laboratories which handle many samples.[200] Despite these recent advances all positive results reported from an immunological test must be verified by chromatographic analysis.[171]

Hair from the test subject is usually collected from the vertex posterior due to relatively uniform rate of growth in hairs at this point. After collection, the hair should be stored at room temperature in dry, dark conditions preferably in a paper envelope,[171] however plastic containers have also been employed.[201] The former are preferred as plasticisers in the latter may damage the sample.[171] **Figure 1.7** below describes the steps involved in collection, extraction and analysis of hair samples.[171] There are sources of error associated with each step of the analytical procedure which will be discussed later in this Section.[202]

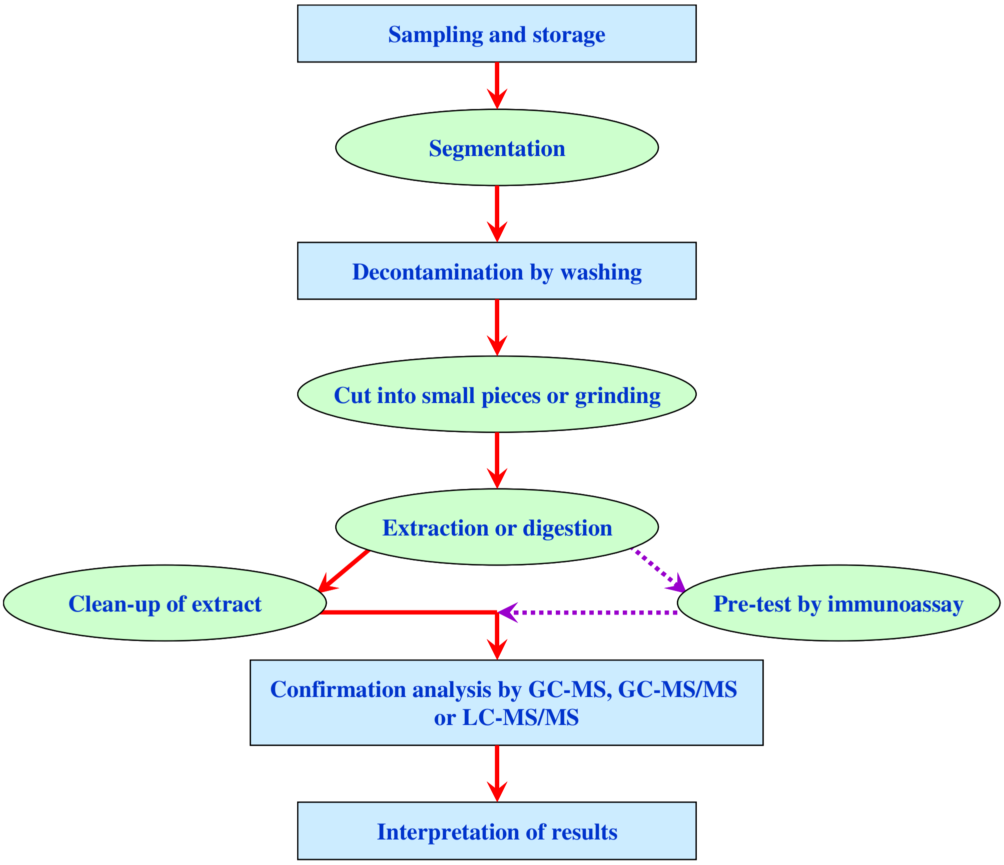

Figure 1.7: Flow diagram of the steps involved in hair analysis, adapted from ref. 171

Chromatographic techniques are the most commonly used methods of analysing hair samples but extensive extraction and clean-up procedures are needed to ensure the samples are compatible with the technique and to minimise matrix effects. **Table 1.2** below lists details of some of the chromatographic techniques that have been applied to the analysis of drugs in hair samples.

Table 1.2: List of chromatographic methods developed for the analysis of drugs in hair samples. (SFE: supercritical fluid extraction, HCl: hydrochloric acid, CH_2Cl_2: dichloromethane, NaOH: sodium hydroxide, MeOH: methanol, DIH_2O: distilled water)

Year	**Analytes**	**Preparation**	**Extraction**	**Clean-up**	**LOD**	**Recovery %**	**Analysis**	**Ref.**
1995	Opiates, cocaine, morphine, codeine, 6-MAM	CH_2Cl_2 + 50mg of pulverised hair	Incubated in 0.1 M HCl overnight at 56 ^oC	LLE and derivatised	ng/mg	68-86	GC-MS	203
1995	Amphetamines	CH_2Cl_2 + DIH_2O washing + 30-50 mg hair/sample	Incubated in 1 M NaOH at 95 ^oC for 10 min	LLE and derivatised	ng/mg	76-82	GC-MS	190
1995	Opiates	CH_2Cl_2 wash + 50 mg hair/sample	SFE – with pure CO_2 + modifier	Evaporated to dryness + derivatised	ng/mg	53-96	GC-MS	204
1995	Cocaine +BEG	MeOH wash + hair cut	Incubated in MeOH for 18 hrs at 40 ^oC	SPE and derivatised	ng/mg		GC-MS	205
1995	THC +THC-COOH	CH_2Cl_2 wash + 500 mg hair pulverised	Incubated in 1ml 1M NaOH 10min @ 95^oC	LLE and derivatised	ng/mg	75-80	GC-MS-SIM	189
1997	Benzodiazepines	CH_2Cl_2 wash and pulverised	Incubated in Sorensen buffer pH7.6 for 2 hrs at 40 ^oC	LLE and derivatised	pg/mg range	48-90	GC-NCI-MS	206
1997	Amphetamine, MDMA, MDA	CH_2Cl_2 wash + 30 mg hair pulverized	Incubated in 1 ml NaOH 10 mins @ 95 ^oC	LLE and derivatised	ng/mg		GC-MS	207

Table 1.2: Contd.

1998	Cocaine, BEG and Cocaethylene	CH_2Cl_2 and water washes + 100 mg of hair analysed	Incubated in 0.1 M HCl for 15 hrs @ 56 ^{o}C	SPE	ng/mg	88-92	LC-ESI-MS/MS	208
2000	Methadone and EDDP	DIH_2O and acetone washes and 50 mg of cut hair	DTT and Pronase extraction at 37 ^{o}C for 12 hrs	SPME	ng/mg	103-107	GC-MS	209
2000	Lipophilic organic drugs	DIH_2O + acetone	Alkaline hair digestion	HS-SPME	ng/mg		GC-MS	210
2001	Methadone + EDDP	DIH_2O, CH_2Cl_2 + MeOH	Incubated in 0.01 M HCl overnight at 60 °C	Automated SPE	ng/mg	80-86	GC-IT-MS	211
2002	Cannabinoids including THC	DIH_2O, petroleum ether +CH_2Cl_2	Alkaline hydrolysis	HS-SPME	ng/mg		GC-MS	212
2003	Opiates, cocaine and BEG	MeOH wash, pulverized + 20 mg analysed	Incubated in pH 5 phosphate buffer at 45 ^{o}C for 18 hrs	SPE and derivatised	ng/mg	81-90	GC-MS	213
2003	Tramadol	Sodium dodecylsulfate + MeOH	Incubated in 3 M HCl overnight at 60 °C	SPE	ng/mg	87-94	GC-MS	214
2004	Ethyl glucuronide + fatty acid ethyl esters	*n*-heptane	dimethyl sulfoxide/n-heptane	HS-SPME + SPE + derivatised	ng/mg		GC-MS	215

Table 2.1: Contd.

2004	Opiates + Cocaine	20 mg of hair pulverised	MeOH sonication @ 37 ^{o}C 3 hr	SPE	pg/mg	71-90	LC-APCI-MS/MS	216
2004	Zoplicone	CH_2Cl_2 wash, cut up + 20 mg analysed	Incubated in pH 8.4 phosphate buffer overnight	LLE	pg/mg	92	LC-ESI-MS/MS	217
2004	Bromazepam, clonazepam and metabolites	CH_2Cl_2 washes, powdered or cut up + 20 mg analysed	Incubated in pH 7.6 Sorensen buffer for 14 hrs @ 56 ^{o}C	LLE	pg/mg		LC-ESI-MS/MS	218
2005	Fentanyl, sufentanil + other opioids	CH_2Cl_2 wash + 50mg hair pulverised	Phosphate buffer pH 8.4 overnight	LLE and derivatised	pg/mg	>75	GC-MS/MS-SRM	219
2005	Benzodiazepines	CH_2Cl_2 wash, cut up + 20 mg analysed	Incubated in Sorensen buffer at 56 ^{o}C for 14 hrs	LLE	pg/mg		LC-ESI-MS/MS	220
2005	Benzodiazepines	CH_2Cl_2 wash, cut up + 20 mg analysed	Incubated in pH 8.4 phosphate buffer overnight	LLE	LOQ 0.5-5 pg/mg	32-76	LC-ESI-MS/MS	221
2006	Opiates, cocaine, BEG, methadone, EDDP and others	CH_2Cl_2 + petroleum ether, washings + hair cut	Incubated in methanol overnight at 40 ^{o}C or alkaline hydrolysis	SPE and HS-SPME	ng/mg		GC-MS	222
2007	Amphetamines + analogues	Ethanol + CH_2Cl_2	Incubated in 1 M NaOH for 15 min at 80 °C	LLE	pg/mg	71-99	LC-ESI-MS/MS	223

After sample collection, the next step of the analytical process is decontamination. This is a very important part of the analysis for a number of reasons. Firstly, if personal care products, sweat and dust are not removed prior to analysis they can enhance matrix effects. Secondly, external contamination of hair with drugs can occur without consumption of the compound.[171] Studies have shown that individuals exposed to a drug vapour in order to mimic an environment where a drug is being smoked, results in significant contamination to hair.[205] The ideal washing solvent should remove any interfering compounds completely without damaging the hair sample or extracting any drugs which maybe inside the matrix.[171] From **Table 1.2** there is a wide range of wash solvents employed for decontamination of hair samples. These include surfactants such as sodium dodecylsulfate, dichloromethane, methanol, acetone, petroleum ether and distilled water. Non-protic solvents are advantageous as they do not promote extraction of drugs during the wash procedure however methanol and phosphate buffer can swell the hair structure resulting in removal of compounds from the hair matrix. Choice of the incorrect solvent for decontamination could lead to false positives and inaccuracies [171]

As there is not yet a direct method available for the detection of drugs in hair, the matrix must be degraded to release any compounds from within. To aid in this process the sample is usually cut into small pieces or subjected to grinding or pulverizing.[218, 220] Before choosing an extraction method there are a number of factors to consider. The structure of the drug being extracted, the extraction solvent and the length of the process all have to be optimised. Incorrect extraction conditions could lead to insufficient extraction, drug decomposition or co-extracted impurities. From **Table 1.2** the most commonly used extraction solvents are methanol, aqueous buffers, and sodium hydroxide. Methanolic extraction can be used for almost all types of drugs. Extraction occurs due to swelling of the hair matrix and release of the drugs due to diffusion. This approach allows for the extraction of neutral and lipophilic compounds and does not result in as much hydrolysis of analytes during extraction as with other solvents.[213] Extraction with methanol can also allow for direct injection of the extract into GC-MS, however this is only suited to analytes that are at high concentrations as co-extraction of matrix components is inevitable and a clean-up step is almost always needed.[224] Aqueous buffer solutions such as phosphate buffers and dilute HCl extractions are particularly useful for the extraction of basic drug residues. The extracts are cleaner than those from methanol extraction however, hydrolysis of certain analytes such as cocaine and the marker of heroin usage, 6-monoacetylmorphine, (6-MAM) to benzoylecgonine and morphine respectively is a major disadvantage.[208, 211, 213] The most quantitative extraction method is sodium hydroxide extraction, which involves digestion of the hair matrix, however not all drugs are stable under such alkaline conditions, for example cocaine.[224] This extraction technique has shown particular use for the removal of amphetamine and its analogues,[207, 223] cannabinoids,[189, 212] and can also be used in conjunction with headspace solid phase micro-extraction (HS-SPME) for multi-residue analysis.[210] Enzymatic extractions have also been conducted using enzymes such as pronase which can hydrolyse the hair structure by reducing disulfide bonds.[209] Finally, supercritical fluid extraction (SFE) has been applied to extraction of opiates, amphetamines and other illicit drugs.[204, 225, 226] The supercritical fluid usually consists of CO_2 plus a modifier however this technique is not commonly used due to high expense.[171]

Direct injection of extracts is only possible after SFE or with methanolic extraction, more commonly a clean-up step is needed. LLE is still quite useful for elimination of matrix components as the samples are usually very small. The extraction is carried out under alkaline conditions and organic solvents used include diethyl ether and dichloromethane.[217-219] SPE is also a common method of sample enrichment as can be seen in **Table 1.2**. It has become more popular in recent years due to improved sorbents and is also easily automated.[171] Cartridges employed have generally been mixed-mode sorbents with hydrophobic and ion-exchange characteristics. Clauwert *et al.* used a mixed-mode C_8 and strong cation exchange functionalised silica for the extraction of basic drugs.[208] A similar sorbent manufactured by Varian (Bond Elut Certify) has also been used for extraction of opiates, cocaine and benzoylecgonine and Cleanscreen cartridges which employ reversed-phase and sulfonic acid retention mechanisms were used to enrich a similar group of analytes.[213, 216] However, more recently, SPME has received a lot of attention as an analyte enrichment technique with advantages such as miniaturization, automation, no organic solvent use and direct thermal desorption from the fibre into the GC instrument.[222, 227, 228] SPME can be used for direct extraction from the liquid extract which has previously described for methadone and its metabolite EDDP.[209] Another approach is to use the headspace version of the technique. HS-SPME has proven to be a simple and efficient method of enriching drug residues in hair extracts. It employs a silica fibre coated in a polymeric stationary phase and can be suitable for lipophilic substances and even those with relatively low volatility.[224] The analytes are adsorbed onto the fibre coating and can then be desorbed into the GC injection port directly.[224] Apart from pharmaceutical extraction, this clean-up technique has also been employed for the enrichment of markers of alcohol abuse such as fatty acid ethyl esters.[215] However if confronted with non-volatile solutes within a complex sample matrix, SPME may not be the most suitable extraction technique. In addition, the extraction procedure takes more time than

Analytical methods for determination of drugs in hair must be suitable for unequivocal identification and quantification. Difficulties in developing these methods are the small sample size and the low concentrations of drugs that are present.[171] For years, GC-MS has been the method of choice for hair analysis. The high resolution of the capillary separation coupled with the selectivity of MS detection results in specific and sensitive methods of analysis. The use of SIM mode measurements and deuterated standards to minimise matrix effects can improve the accuracy of the technique.[171] The limits of detection of recently developed GC-MS methods are in the low ng/mg or high pg/mg range.[209, 213] However, as is well known, GC requires thermally stable, volatile analytes which means that derivatisation is often required and many drugs do not possess these physiochemical characteristics. Particularly for multi-residue analysis in hair, this can be a problem as mixed derivatisation steps are needed for detection of all analytes.[171] Cirimele *et al.* used negative chemical ionization as an alternative ionization technique and achieved lower LODs in the range of 1-20 pg/mg for benzodiazepines.[206]

Recent advances in technology have made GC-MS/MS more available. This has resulted in the use of MRM mode where more than one fragment ion is monitored for each analyte. This technique has increased the sensitivity and specificity with LODs in the low pg/mg range, and is increasingly used in hair analysis.[219, 229-231]

LC-MS/MS analysis of hair almost eliminates issues such as derivatisation, volatility or thermal stability from the analytical procedure. LC-MS/MS has the ability to separate a diverse range of analytes often without derivatisation and even though it does not have the chromatographic resolution of GC, this is compensated for by the specificity of MS/MS detection. LC-MS/MS has been particularly useful for the analysis of a range of benzodiazepines and zoplicone in hair samples.[217, 221] However, the technique has also been applied to the analysis of medicinal drugs such as neuroleptics, psychotherapeutic drugs and markers of alcoholism.[232-234] LC-MS/MS analysis offers LODs, often <1 pg/mg which are as low if not lower than GC-MS/MS techniques.[217, 218] The most commonly used instrumental setups are ESI and APCI ionization coupled to QqQ or IT mass analysers operated in SRM or MRM modes. The advent of new q-TOF instruments has not gone unnoticed and may be very useful in the future for the identification of unknown compounds.[224] As already discussed, when analysing complex sample types using LC-MS/MS, the greatest challenge is the elimination of interfering components which can cause ion suppression or enhancement. Generally in hair analysis, deuterated standards or similarly structured analogues of the analytes are added as internal standards.[208, 220, 221] During the development of an analytical method for hair analysis, matrix effects must be documented.[235] Scheidweiler *et al.* quantified matrix effects observed during the analysis of opiates, cocaine and their metabolites. It was reported that benzoylecgonine, morphine and 6-acetylmorphine were matrix enhanced while cocaethylene demonstrated ion suppression.[216] Conversely Villain *et al.* reported no matrix effects from endogenous substances during the analysis of 16 benzodiazepines in hair after extraction in alkaline buffer and LLE clean-up.[221]

There are a vast number of applications for analysis of pharmaceuticals in hair and they have recently been comprehensively reviewed by Pragst.[171] Briefly, drug treatment programmes can use hair analysis as a tool to manage patients as it provides a window into their drug history. Consumption of prescribed drugs as well as any illicit compounds can be monitored.[222] Particularly in heroin treatment programmes, abuse of illicit heroin and consumption of prescribed pharmaceutical heroin can be distinguished.[236, 237] Hair analysis is very useful in the prosecution of drug-facilitated crimes (DFCs) as it provides a longer window for analysis to be carried out. Urine and blood analysis are useless unless analysed directly after an assault has taken place. Hair analysis overcomes this obstacle and can also provide information as to the time of the assault from segmented analysis.[224] A number of studies have been conducted to determine benzodiazepines and zolpidem in the hair of assault victims.[217, 218, 220] Other applications of hair analysis include: determination of fetal drug exposure,[238] monitoring drug abuse in the workplace,[219] and detection of alcohol abuse.[188, 215] To conclude, hair analysis is a valuable complementary technique to more conventional urine and blood testing. Sensitive and specific analysis and detection techniques are needed and MS/MS instrumentation is becoming a prerequisite.[224] Miniaturisation of the analytical process, application of more advanced instrumentation and further work in the area of segmented analysis should result in hair analysis becoming an even more valuable analytical technique.[171]

1.10.3 Analytical techniques for determination of PPCPs in urine:

Apart from blood, plasma and serum, urine is probably the most commonly analysed biological sample. The number of publications in this area is large and many are beyond the scope of this review. Therefore this Section will provide a brief review of sample preparation techniques, liquid chromatographic separation and mass selective detection of PPCPs in urine samples from recent years. Urine is the sample of choice for the analysis of many compounds because drugs and metabolites are present in relatively high concentrations, the window for analysis is longer than for blood, the sample is not very invasive to obtain and sample pre-treatment is not as complex as for some other biological matrices.[172] The limitations of urine analysis are the adulteration tactics, excretion of metabolites that must be targeted instead of the parent drug.[172] As with hair analysis, immunological techniques are commonly employed for initial screening of urine samples and if traces of a pharmaceutical compounds are reported, then chromatographic analysis is conducted.[172]

Certain analytes require a pre-treatment step prior to pre-concentration, e.g. Shima *et al.* added salts to precipitate protein from the sample matrix for the analysis of amphetamine and its conjugates in urine.[239, 240] The pH of the sample can also be adjusted prior to LLE and SPE to improve extraction efficiency and enrichment. Hirsch *et al.* and Tuerk *et al.*, demonstrated lyophilisation for enrichment of antibiotics in water samples prior to LC-ESI-MS analysis.[46, 173] However the same process is not applicable to biological matrices due to the complex sample matrix.[173] LLE has been the traditional pre-concentration method for biological matrices, however SPE and SPME techniques are currently more common particularly for multi-residue analyte enrichment.[170, 172] Over the years LLE has been applied to the selective preconcentration of a wide range of analytes including analgesics, anti-epileptics, benzodiazepines, antidepressants, illicits and anticoagulants.[170] Solvents employed in LLE for preconcentration of biological fluids include ethyl acetate,[241] acetone and chloroform,[242] dichloromethane and acetone,[243] toluene and ethyl acetate[243] and butyl acetate[244] to name a few. More recently LLE has been employed for the extraction of amphetamine-like 'designer drugs' from urine blood and post-mortem tissue.[245] Johansen *et al.* also employed butyl acetate in the LLE of hallucinogen, LSD and related compounds in forensic samples.[246]

In a review of analytical methods by Drummer in 1999, many different SPE cartridges were reported as suitable extraction sorbents for pharmaceutical residues in urine, plasma, serum and even liver cells.[170] However, mixed-mode sorbents particularly Bond Elut Certify,[242, 247] Chromabond,[248] and Cleanscreen[249] cartridges were reported as particularly useful for retaining all types of functional groups and demonstrating good recoveries.[170] More recently, both C_{18} and mixed-mode cartridges have remained popular for enrichment of drugs in urine with Chromabond C_{18} being applied to the extraction of both cannabinoids and basic drugs.[250, 251] Dams *et al.*

employed weak cation exchange sorbents for the preconcentration of opiates in urine with relative recoveries in the range of 61-116%.[252] Sixteen structurally different antibiotics were preconcentrated from urine using C_{18} SPE cartridges and reported recoveries >60 % in most cases at four different spiking concentrations.[173] A strong eluting solvent such as tetrahydrofuran was employed to ensure elution of the fluoroquinolone antibiotics which are known to adhere to C_{18} material.[253] For multi-residue analysis with analytes across several therapeutic classes and varying functional groups, mixed-mode sorbents are most common. Concheiro *et al.* employed the polymeric sorbent Oasis HLB for the extraction of opiates, amphetamines and cocaine simultaneously and reported all recoveries above 50%. Marquet *et al.* employed Oasis MCX, mixed-mode phase extraction cartridges which contain hydrophobic polymers and sulfonic acid functional groups for the preconcentration of compounds from eight different classes including antibiotics, benzodiazepines, pesticides and anti-coagulants in serum.[254] SPME and HS-SPME have also been employed for the extraction of drugs from biological fluids. Kurecková *et al.* used SPME to preconcentrate steroids from urine samples with promising results. HS-SPME has been employed for the extraction of amphetamines from urine with relative recoveries in the range of 50-104%.[255, 256]

A large number of GC-MS techniques for the determination of a diverse range of analytes were previously reviewed by Drummer.[170] In this review, Drummer acknowledged LC-MS and LC-MS/MS techniques as the future technique for multi-residue screening of pharmaceuticals and illicit drugs in biological samples.[170] This was mainly due to the elimination of derivatisation steps during sample preparation and coupling of LC to MS and MS/MS instruments. Additionally, LC methods allow for the detection of drug conjugates without the need for prior hydrolysis, a step that was required in many GC methods.[172] In the late 1990's, most LC methods applied to the analysis of drugs in urine employed DAD or UV detectors.[257, 258] However, with modern atmospheric pressure interfacing instrumentation and wider availability of MS detection, the older detectors are no longer popular.

The most commonly used stationary phases employed in the analysis of urine are reversed-phase C_{18} and C_8 columns.[173, 259] Synergi^{TM} reversed phase columns have been employed for multi-residue analysis in conjunction with a phenyl-hexyl stationary phase for separation of basic drugs and cannabinoids respectively, with detection limits in the low ng/mL range for all analytes.[250] Alternatively, pentafluorophenylpropyl (PFPP) bonded silica column was employed by Needham *et al.* for the separation of cocaine and its metabolites. The method demonstrated good retention and peak shape while only a 1/10 dilution of the urine sample was required as sample preparation.[260] Mobile phases similar to those employed for hair and environmental analysis are employed for urine analysis. Methanol and acetonitrile with additives such as ammonium acetate, formic acid and ammonium formate have been employed in most cases.[173, 251, 259, 261] UPLC analysis of amphetamines and ketamine in blood was conducted by Apollonio *et al.* Nine forensically relevant compounds could be separated in 3 minutes indicating that this technique may have use for rapid screening of many compounds in one sample.[262]

MS/MS detection is now becoming a requirement in analytical methods in order to avoid false positive results. Identification of compounds in biological and environmental samples must now be based on agreement of retention times, molecular ions and more than one product ion transition.[172] SRM mode is still employed for biological analysis but there is no denying the advantages of MRM with tandem MS which has demonstrated much lower detection limits.[173, 239] A trend is appearing towards the development of multi-residue analytical methods for pharmaceutical traces in biological and environmental samples.[172] Bogusz has developed an analytical method applicable to different types of biological samples for quantification of many different groups of drugs.[263] The method involved the use of two separation columns and two mobile phase of slightly different composition. LC-APCI-MS-SIM was employed for quantification with deuterated standards to overcome matrix effects and LODs for all compounds were in the low ng/mL range.[263] Dams *et al.* demonstrated direct injection of urine after only centrifuging to remove suspended material for determination 25 target analytes including opiates and cocaine. The analysis was carried out on an IT mass analyser with deuterated standards added for quantification and no matrix effects were reported despite the lack of sample preparation.[264] Finally, Mueller *et al.* have recently developed a screening technique for 301 pharmaceutical residues in blood and urine samples using a hybrid quadrupole linear ion tap mass analyser (q-LIT).[261] Information dependant acquisition (IDA) experiments were carried out using MRM and enhanced product ion (EPI) scans. Identification of compounds is carried out using an MS/MS library.[261]

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Chapter 2.0: Analysis of Hair Samples for Illicit Drug Residues

2.1 Introduction:

The analysis of hair samples has received increasing attention in recent years due to an increasing number of applications, more sensitive analysis and detection techniques. Determination of pharmaceuticals in hair is now an important process for workplace testing, determining history of drug abuse, postmortem toxicology, gestational drug exposure and application to drug facilitated crimes (DFCs).[1] Several recent publications have dealt with development of a method with application to actual evidential samples from an assault case, monitoring of doping in athletes and workplace testing of medical care professionals.[2-6] Many of the applications of hair analysis are in the area of forensics and there is great focus on the illicit drugs as target analytes.

The advantages of hair as a sample for forensic analysis are a longer time period for drug detection, segmented analysis which can allow for the time of drug intake to be estimated and the non-invasive manner of sample collection. Another advantage is that hair analysis can allow for the detection of illicit drugs in hair after a single dose which is often the case with a DFC. Urine and blood analysis may not result in the detection of the drug particularly if the victim only reports the crime some days after consumption.[3]

Hair samples are generally obtained from volunteers by shaving an area or cutting several strands from the posterior vertex of the head. Samples are stored at room temperature in plastic containers[10] or paper envelopes.[11] Numerous methods have been developed for the determination of illicit drugs in hair samples however most of them follow a similar process. The first step involves decontamination usually with an organic solvent to remove oils, fats and any possible drug contamination from the outside of the hair strands. This step is generally followed by extraction, sample clean-up, chromatographic separation and mass selective detection. The analytical methods developed for the extraction and analysis of drugs in hair samples have been reviewed in Chapter 1.

GC-MS was previously the method of choice for analysis of hair samples but LC-MS and LC-MS/MS have grown in popularity in recent years. LC methods allow for the determination of large numbers of underivatised analytes from a range of pharmaceutical classes with excellent specificity and sensitivity. For these reasons, LC-MS/MS is particularly applicable to new multi-residue methods.[7] These multi-analyte techniques allow for screening of a large number of analytes in one run and are possible due to newly introduced SPE sorbents with mixed-mode retention characteristics and advances in LC-MS technologies. A multi-residue method was recently developed by Villain *et al.* for 16 benzodiazepines and hypnotics in hair using LC-MS/MS with quantification limits in the low pg/mg range.[8]

This work presents a quick, multi-residue analytical method for the determination of twelve illicit drug residues in hair samples. The analytes chosen are from a number of different pharmaceutical families including amphetamines, cocainics, benzodiazepines, opiates and hallucinogens. A methanolic extraction was chosen for removal of drug residues from hair samples due to its applicability for different types of analytes and low levels of analyte hydrolysis.[1, 9] A number of SPE sorbents were evaluated as preconcentration media for the analytes and the extracts were submitted for LC-MS/MS analysis using SRM mode for identification and quantification. The validated method was then applied to the analysis of hair sample from a recreational drug user.

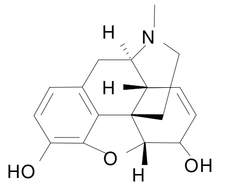
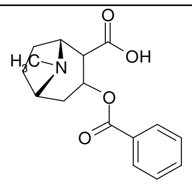
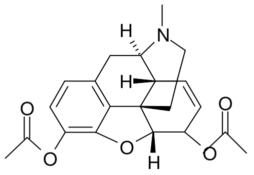
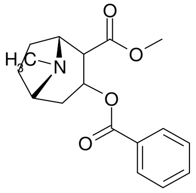
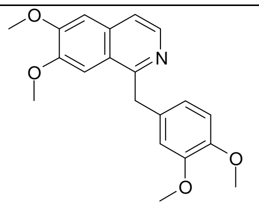
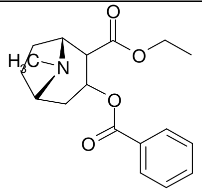
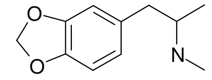
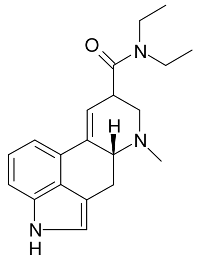
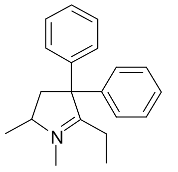
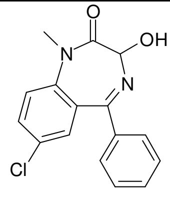
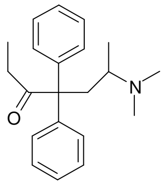
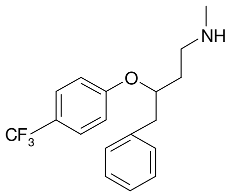
2.2 Experimental:

2.2.1 Reagents and Preparation of glassware and standards:

Analytical grade (all purity ≥ 97%) benzoylecgonine hydrate, cocaethylene, temazepam, diazepam, 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). Cocaine hydrochloride, morphine sulfate salt pentahydrate, methadone hydrochloride, heroin and lysergic acid diethylamide (LSD), were purchased under license from Sigma-Aldrich (St. Louis, MO, USA). The chemical structure of each drug is provided in **Table 2.1**.

All solvents used were HPLC grade or better. Isopropanol, acetonitrile and methanol were obtained from Labscan (Dublin, Ireland). Acetone, dichloromethane, dichlorodimethylsilane and ethyl acetate were purchased from Aldrich (Gillingham, UK) as was the mobile phase additive ammonium acetate. Ammonium hydroxide (LC-MS additive grade) was purchased from Fluka (Steinheim, Germany). Analytical reagent grade glacial acetic acid was received from BDH Chemicals (Poole, UK). Ultra-pure water was obtained from a Millipore Milli-Q water purification unit (Millipore, Bedford, MA, USA) with specific resistance of 18.2 MΩ/cm or better. All glassware utilised for storage of illicit drug stocks and standards were silanised prior to the preparation to prevent the drugs from adhering to glass surfaces. This was carried out by rinsing the glassware with 10 % dichlorodimethylsilane in dichloromethane. This was followed by rinsing with dichloromethane and rinsing with methanol twice each. Stock solutions of the illicit drugs were prepared in methanol to a concentration of 1000 mg/L and were stored in a refrigerator at 4 ^{o}C. Working standards were prepared freshly before analysis in ultra pure water.

Table 2.1: The chemical structure of each illicit drug

Drug	Structure	Drug	Structure
Morphine		Benzoylecgonine	
Heroin		Cocaine	
Papaverine		Cocaethylene	
MDMA		LSD	
EDDP		Diazepam	
Methadone		Temazepam	

2.2.2 Extraction of hair samples:

The extraction procedure is summarised in **Figure 2.1**. Hair samples were obtained from volunteers by shaving an area or cutting several strands from the posterior vertex of the head and were stored in plastic containers at room temperature until analysis time. Before analysis, the hair was vortexed twice in 5 mL of dichloromethane to remove any external residues. The hair was dried by pressing between two sheets of glass fibre filter paper. The sample was then ground up using a mortar and pestle for approximately 5 minutes and 25-50 mg of crushed hair was weighed out depending on the amount of sample available. The samples were placed in silanised glass jars. A volume of illicit drug standard prepared in milli-Q water was added to the samples at this point where required and samples were allowed to stand for 20 minutes at room temperature. To each of the jars, 2.5 mL of methanol was added, the jars were capped and incubated in a water bath for 3 hours at 37 ^{o}C and shaken manually at regular intervals. After extraction, the content of each jar was emptied into a 50 mL centrifuge tube and the jar was rinsed with 7.5 mL of ultra pure water. The water rinsing was also added to the centrifuge tube. The sample was then centrifuged at 6000 rpm for 15 minutes, the supernatant was removed into a silanised beaker and made up to 50 mL using ultra pure water. This was done to reduce the methanol concentration in the sample to 5 % or less. The pH of samples was adjusted to pH 3.5 using acetic acid or ammonium hydroxide solutions. SPE was carried out on Oasis MCX cartridges using 6 mL of methanol followed by 6 mL of water to pre-condition the sorbent. After sample loading the cartridge was washed with 1 mL of 2% acetic acid and allowed to dry under vacuum for 30 minutes. The samples were eluted in 5 mL of 5% ammonium hydroxide in methanol, dried down under nitrogen at 50 ^{o}C and reconstituted in 100 µL of 90 : 10 v/v 10 mM ammonium acetate in H_2O : acetonitrile.

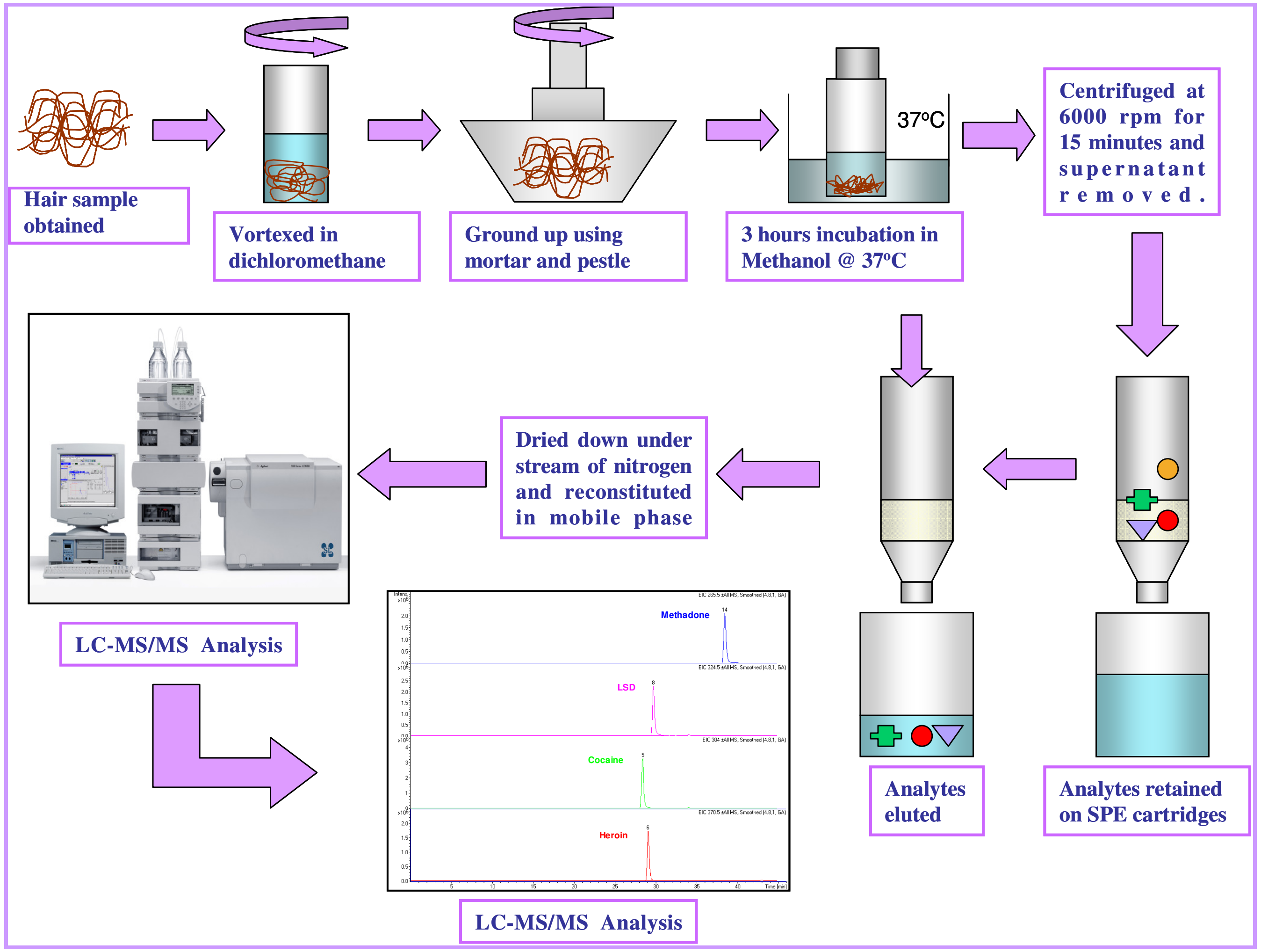


Figure 2.1: Schematic diagram of hair extraction procedure

2.2.3 Liquid chromatography –mass spectrometry:

Chromatographic separations were performed on an Agilent 1100 series high performance liquid chromatograph with a vacuum degasser, binary pump, ALS autosampler, and diode array detector. This LC was coupled to a Bruker Daltonics Esquire~LC ESI-ion trap mass spectrometer. Agilent Chemstation version A.09.03 (Agilent Technologies, USA) and Bruker Daltonics esquire control version 4.0 (Bruker Daltonics, UK) were employed to control the system and data analysis was performed using Bruker Daltonics Data Analysis 3.0 (Bruker Daltonics, UK).

Separations were performed using a Waters Sunfire 150 x 2.1 mm i.d. 3.5 µm column. A multi-step gradient was employed with mobile phases of (A) 90 : 10 and (B) 20 : 80 10 mM ammonium acetate : acetonitrile. Gradient conditions were 100% mobile phase A for 5 minutes, then an increase to 50% mobile phase B over 23 minutes followed by another increase to 100% B over the next 7 minutes. This was held constant for 10 minutes and a re-equilibration of 15 minutes was applied giving an overall runtime of 1 hour. Flow rate of 0.2 mL/min and injection volume of 10 µL were employed. A longer multi-step gradient was also employed.

In order to tune the mass spectrometer and determine the parent and precursor ions for each drug, individual 10 mg/L solutions of each drug made up in 100% methanol were infused directly into the mass spectrometer. This was carried out using a Cole-Palmer syringe pump and a Hamilton micro-syringe. Once the molecular ion was identified, it was fragmented to obtain the product ion for each drug in tandem MS mode. During analysis definitive identification of drug residues was obtained by identifying the individual transitions between parent and product ions in tandem MS mode. Quantitative measurements were achieved by integrating the most intense peak exhibited for each illicit drug. The final MS parameters are given in **Table 2.2**.

Table 2.2: ESI-MS parameters employed for analysis.

Parameter	Positive Mode
Capillary Voltage	4500
End Plate Offset (V)	-561
Skimmer 1 (V)	28.1
Skimmer 2 (V)	6.7
Cap. Exit Offset (V)	63.7
Octopole (V)	2.51
Octopole RF (Vpp)	155.3
Octopole Delta (V)	1.98
Lens 1	-2.4; -37.8
Trap Drive	34.7
Dry Gas Flow (N_2; L/min)	8
Nebulizer Pressure (psi)	50
Dry Gas Temp. (^oC)	300

2.2.4 Method Validation:

Recovery data was obtained by spiking n=3 blank hair samples with illicit drugs to a concentration of 400 pg/mg and extracting under optimised conditions. The spiked extracts were then dried down under nitrogen and reconstituted in 100 µL of 90 : 10 10 mM ammonium acetate : acetonitrile giving a final 100% recovery concentration of 0.2 mg/L. The spiked extracts were then compared to a blank hair extract which had been dried down under nitrogen and reconstituted in 100 µL of a 0.2 mg/L standard solution of illicits in Milli-Q water. A blank hair sample was also analysed with the recovery samples and background subtraction was performed were necessary. Method precision data was obtained by calculating the standard deviation of peak height values for each drug in the three spiked hair extracts. Suppression of analytes in hair was also investigated by comparing the samples spiked post extraction with a 0.2 mg/L standard in Milli-Q water. Percentage suppression was calculated as loss of analyte recovery e.g. if the analyte had 60% recovery when compared to the standard then it was 40% suppressed.

Linearity samples were prepared by spiking blank hair samples with concentrations of illicit drugs ranging from 50-1000 pg/mg. The samples were extracted as described and reconstituted in 100 μL of 90 : 10 10 mM ammonium acetate : acetonitrile. Acceptable linearity was taken for correlation coefficients ≥0.95 for curves containing at least five data points.

The concentrations at which the signal-to-noise ratio was closest to 10 : 1 and 3 : 1 were calculated as the LOQ and LOD, respectively. Instrumental retention time precision was determined by n=6 repeat injections of a 1 mg/L standard of illicits in milli-Q water and this data was also employed for the calculation of peak height precision.

2.3 Results and Discussion:

2.3.1 MS/MS detection of illicit drugs:

Table 3.3: Precursor and product ions recorded for MS/MS monitoring of illicit drugs

Analyte	**m/z**	**Precursor ion**	**m/z**	**Product ion (m/z)**
Morphine	286	$[M + H]^+$	268	$[M + H]^+ - [H_2O]$
Benzoylecgonine	290	$[M + H]^+$	168	$[M + H]^+ - [C_6H_5COOH]$
MDMA	194	$[M + H]^+$	163	$[M + H]^+ - [NH_2CH_3]$
Cocaine	304	$[M + H]^+$	182	$[M + H]^+ - [C_6H_5COOH]$
Heroin	370	$[M + H]^+$	268	$[M + H]^+ -[CH_3CO]-[CH_3CO_2]$
LSD	324	$[M + H]^+$	223	$[M + H]^+ - [CO\text{-}N(C_2H_5)_2]$
Cocaethylene	318	$[M + H]^+$	196	$[M + H]^+ - [C_6H_5COOH]$
Papaverine	340	$[M + H]^+$	202	$[M + H]^+ -[C_6H_4(OCH_3)_2]$
Temazepam	301	$[M + H]^+$	283	$[M + H]^+ - [H_2O]$
EDDP	278	$[M + H]^+$	249	$[M + H]^+ - [C_2H_5]$
Diazepam	285	$[M + H]^+$	257	$[M + H]^+ - [CO]$
Methadone	310	$[M + H]^+$	265	$[M + H]^+ - [NH(CH_3)_2]$

Simple molecular ion spectra were recorded for the illicit drugs as they all produced an easily interpreted protonated molecular ion [$M+H^+$]. **Table 2.3** summarises the parent and product ion transitions that were recorded for each of the drugs.

The tandem MS signals for each analyte were generally due to loss of distinct groups from each structure. The parent molecules of both morphine and temazepam undergo loss of a water moiety, (18 mass units), resulting in product ions of m/z 268 and m/z 283, respectively. The molecular ion of EDDP loses an ethyl group or 29 mass units to form a product ion at m/z 249. In the case of MDMA and methadone the product ions 163 and 265 are formed by loss of a methylamine and dimethylamine, respectively. The heptagonal ring of diazepam undergoes a contraction to hexagonal, losing the carbonyl group and 28 mass units producing a product ion at m/z 257. Cocaine and its two metabolites, benzoylecgonine and cocaethylene undergo a similar transition by losing a benzoic acid group for their structures. The product ion of LSD is formed when the $CON(C_2H_5)_2$ branch of the molecule is removed during fragmentation yielding m/z 223 in the tandem MS spectrum. A dimethoxyphenyl moiety is lost from the structure of papaverine and corresponds to a product ion at m/z 202, 138 mass units less than the precursor. Finally, heroin loses 102 mass units, which is thought to be due to loss of both acetyl groups (CH_3O) and a subsequent dehydration reaction. Due to the nature of the analytes of choice, analysis could be carried out in positive MS mode only. In some cases the fragment ion appeared at a higher intensity than the parent ion in single MS mode. This occurred in particular for methadone and MDMA where peaks at 265 and 163 respectively, were integrated under single MS conditions instead of the parent ion in order to achieve the greatest sensitivity. This phenomenon can be explained due to collisionally induced fragmentation of the parent ions in single MS mode.

2.3.2 Liquid chromatography of illicit drugs:

The gradient and set of chromatographic conditions described above were previously applied to the analysis of pharmaceutical residues in aqueous and solid environmental matrices with successful results.[12] Consequently this seemed a suitable starting point for the separation of the twelve illicit drugs. Ammonium acetate was added to the mobile phase mixture to aid in the ionisation process. The system was then applied to the separation of the thirteen illicit drugs. Extracted ion chromatograms illustrating the separation of a 1 mg/L standard solution of the twelve target analytes are shown in **Figure 2.2**.

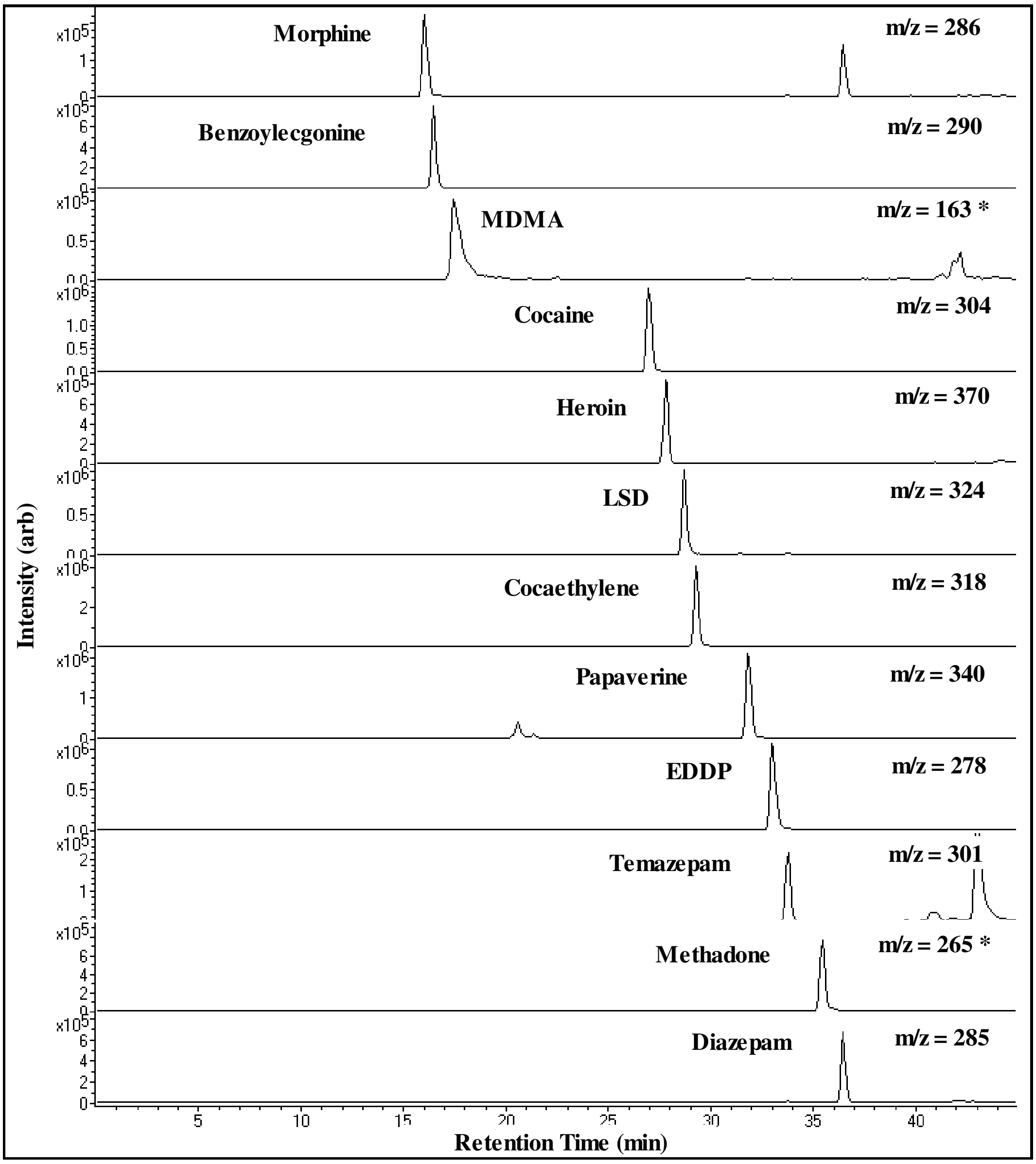


Figure 2.2: Separation of a 1 mg/L standard solution of 12 illicit drugs in milli-Q water. Chromatograms marked with * denotes analytes represented by their fragment ion in single MS mode.

To assess the performance of the separation, resolution data was calculated for each of the analytes. Resolution was calculated using Eqn. 2.1 and the results are shown in **Table 2.4**.

$$R_S = \frac{2(t_{R(2)} - t_{R(1)})}{W_1 + W_2} \qquad \text{(Eqn. 2.1)}$$

All values were 1.5 and above which indicates complete separation of analytes and is the desired result according to the literature.[13] An overall run time of one hour for the entire separation and re-equilibration was deemed satisfactory.

Table 2.4: Resolution data for twelve separated drugs of abuse.

Drug	Retention time (min)	Resolution
Morphine/Benzoylecgonine	16.0/16.4	2.7
Benzoylecgonine/MDMA	16.4/17.4	2.2
MDMA/Cocaine	17.4/26.9	16.2
Cocaine/Heroin	26.9/27.7	2.7
Heroin/LSD	27.7/28.7	2.5
LSD/Cocaethylene	28.7/29.2	2.1
Cocaethylene/Papaverine	29.2/31.8	5.8
Papaverine/EDDP	31.8/32.9	2.5
EDDP/Temazepam	32.9/33.7	3.9
Temazepam/Methadone	33.7/35.4	2.4
Methadone/Diazepam	35.4/36.4	3.8

2.3.3 Solid phase extraction (SPE):

A study of four commercially available SPE cartridges was carried out to assess the affinity of the illicit drugs for a range of sorbents. The sorbents chosen for the study were Varian HF Bond Elut certify II 3 mL cartridges, Phenomenex Strata X 3 mL cartridges, Phenomenex Strata XC 1 mL cartridges and finally Waters Oasis MCX 6 mL cartridges. The Strata X tubes contained a purely reversed-phase polymeric sorbent of modified styrene divinylbenzene for the extraction of polar and aromatic analytes due to π-π interactions and hydrogen bonding. The Strata XC tubes consist of a mixed mode phase for extraction of basic compounds consisting of the polymeric sorbent described above surface modified with sulfonic acid groups for cation exchange. This type of phase was chosen as most of the illicit drugs are basic in nature. Similarly, the Oasis MCX phase facilitates cation exchange with a hydrophilic-hydrophobic balanced polymer backbone and sulfonic acid groups present to provide dual mode extraction. Finally, the Bond Elut Certify II tubes also contain a mixed mode sorbent, consisting of a non-polar C_8 silica backbone bonded to octyl and quaternary amine groups suited to anion exchange. Details on the size and format of the sorbents as well as the procedures used to carry out the extractions are given in **Table 2.5**.

The SPE optimisation experiments were carried out using 50 mL of ultra pure water spiked to a concentration of 1 µg/L of illicits and set to the required pH using dilute glacial acetic acid or ammonium hydroxide and were performed in duplicate. After loading and elution, the samples were dried down under N_2 and reconstituted in 100 µL of 90 : 10 10 mM ammonium acetate : acetonitrile. The SPE method preconcentration factor was 500 resulting in a final concentration of drugs in the extracts of 0.5 mg/L. The resulting peak heights were compared to those of a 1 mg/L standard solution of the analytes to calculate recoveries from each type of cartridge. The results of the SPE study are presented in **Table 2.6**.

Table 2.5: Specifications and procedures employed for 5 SPE cartridges.

Name	**Barrel Volume**	**Sorbent mass**	**Functional group**	**Sample pH**	**Conditioning solvent**	**Wash solvent**	**Elution solvent**
Oasis HLB	6 mL	200 mg	Hydrophilic-lipophilic, reversed-phase sorbent	pH 5.5	6 mL methanol 6 mL ultra pure water	1 mL ultra pure water	50/50 ethyl acetate/acetone
Oasis MCX	6 mL	150 mg	Mixed-mode: cation-exchange and reversed-phase sorbent	pH 3.5	6 mL methanol 6 mL ultra pure water	1 mL 2% acetic acid	5% ammonium hydroxide in methanol
Strata X	3 mL	200 mg	Polymeric sorbent, 33 µm	pH 9.5	3 mL methanol 3 mL ultra pure water	1 mL 5% methanol in ultra pure water	50/50 ethyl acetate/acetone
Strata XC	1 mL	30 mg	Mixed-mode: polymeric sorbent, 33 µm bonded to strong cation exchanger	pH 3.5	1 mL methanol 1 mL 2% acetic acid	1 mL 2% acetic acid	5% ammonium hydroxide in methanol
Bond Elut Certify II	3 mL	200 mg	Mixed-mode: octyl and quaternary amine	pH 3.5	3 mL methanol 3 mL 2% acetic acid	1 mL 2% acetic acid	78/20/2 CH_2Cl_2/ isopropanol/ammonium hydroxide

Table 2.5: Relative recoveries of illicit drugs using four different SPE sorbents for the extraction of twelve illicit drugs and compared to a 1 mg/L standard in ultra pure water.

Drug	**pK_a**	**Bond Elut**	**Strata X**	**Strata XC**	**Oasis MCX**
Morphine		27 ± 4	111 ± 19	110 ± 18	97 ± 11
Benzoylecgonine	11.20[14]	79 ± 2	120 ± 12	110 ± 19	116 ± 18
MDMA	9.90[15]	69 ± 2	44 ± 8	58 ± 6	41 ± 4
Cocaine	8.60[16]	64 ± 9	36 ± 15	85 ± 15	76 ± 41
Heroin		36 ± 13	30 ± 4	23 ± 2	5 ± 1
LSD		91 ± 7	78 ± 2	15 ± 5	32 ± 1
Cocaethylene		65 ± 10	59 ± 12	80 ± 8	60 ± 2
Papaverine	8.07[17]	59 ± 11	78 ± 1	87 ± 23	63 ± 1
Temazepam	1.60[18]	119 ± 8	108	91 ± 7	93 ± 1
EDDP		39 ± 11	6 ± 2	50 ± 21	22 ± 2
Diazepam	3.46[19]	71 ± 3	92 ± 4	110 ± 41	99 ± 26
Methadone	9.10[20]	50 ± 10	ND*	58 ± 36	39 ± 21

Analytes were most likely retained due to hydrophobic interactions with the polymeric sorbents and ion exchange in the case of the mixed-mode sorbents. In general the mixed-mode sorbents, the Bond Elut, Strata XC and Oasis MCX gave the most consistently high recoveries with seven of the target analytes out of the twelve having relative recoveries greater or equal to 60%. Due to the cationic nature of the ion exchanging groups in the Bond Elut sorbent which were not suited to the mainly basic illicits, the main retention mechanism was thought to be non-polar interactions. Recoveries of heroin were low in all cases. The Strata XC cartridges demonstrated good affinity for the illicits, however the 1 mL barrel volume resulted in extended loading times that lengthened the preconcentration process. The Bond Elut and Oasis MCX cartridges were deemed suitable for preconcentration of illicit drug traces however Oasis MCX were chosen for further analysis due to the mixed mode cation exchange reversed-phase sorbent being most suited to the basic nature of the illicits.

The entire extraction method including the optimised SPE procedure was then applied to the separation of twelve illicit drugs. Amphetamine was discarded as a target analyte as it was undetected from the SPE study. A complete separation of the twelve target analytes in a spiked hair sample (600 pg/mg) extracted under optimised conditions is shown in **Figure 2.3**.

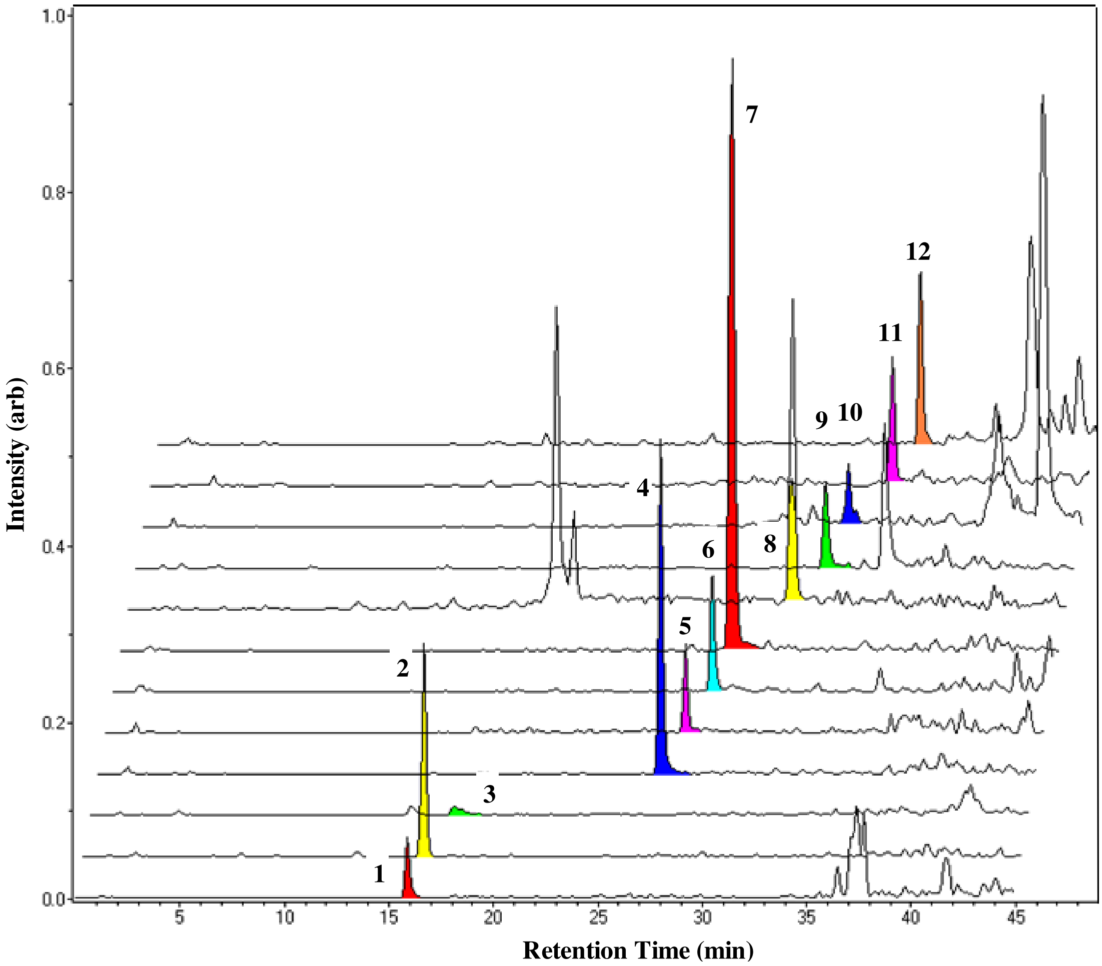


Figure 2.3: Separation of a hair sample spiked with 12 illicit drugs to a concentration of 600 pg/mg, extracted and separated using the 45 minute gradient on Waters Sunfire column at a flow rate of 0.2 mL/min with ESI-MS detection. The analytes are in order of retention time and are as follows: morphine, benzoylecgonine, MDMA, cocaine, heroin, LSD, cocaethylene, papaverine, EDDP, temazepam, methadone and diazepam.

2.3.4 Method validation:

The method validation data for all twelve illicit drugs are listed in **Table 2.6**. Standard curves were plotted for spiked hair samples in the range of 50-1000 pg/mg. Curves contained at least n=5 data points, a trend line was fitted and linearity was examined based on correlation coefficients (R^2). Acceptable results ($R^2 \geq 0.95$) were achieved in all cases except for MDMA which had an R^2 value of 0.9280. Correlation coefficients of 0.9737 and 0.9762 for cocaine and diazepam respectively were also disappointing. Overall benzoylecgonine, LSD, temazepam, papaverine, methadone and cocaethlyene exhibited the best linear responses of $R^2 \geq 0.99$ over $n \geq 6$ data points.

Limits of quantification and detection were calculated at four different low to mid range concentrations. All analytes reported LODs of <30 pg/mg except for MDMA and temazepam. Cocaine, benzoylecgonine and cocaethylene all reported very low LODs <10 pg/mg. These values for cocaine and benzoylecgonine were quite similar to those obtained in a study by Scheidweiler *et al.* in 2003.[9] Detection limits for methadone and its metabolite EDDP were much lower than those reported by Lucas *et al.* in 2000 using an SPME clean-up step with GC-MS.[21] One study in 2004 which involved the analysis of a range of drugs such as benzodiazepines and illicit stimulants reported excellent LODs for cocaine and benzoylecgonine and amphetamine, demonstrating that it is possible to sensitively detect amphetamine along with other illicits. The method employed an 18 hour incubation in a methanol/acetonitrile extraction medium.[22]

Absolute recoveries of the drugs from hair were acceptable with all values ≥60%. Method precision was acceptable with almost all recoveries varying by less than 20% for n=2/n=3 replicates of the complete extraction method. Recoveries greater than 100% were reported for both morphine and papaverine with relatively low variation between samples. This indicates enhancement from interfering compounds in the hair matrix, because a blank extract was analysed so that any traces of drugs in the sample could be corrected for. Scheidweiler *et al.* also reported enhancement of morphine signals in their publication which employed a similar analysis procedure.[9] These results indicate that perhaps different strand and sections of hair contain varying amounts of interfering matrix components and could lead to false positive results. False positives and interferences due to methanolic extraction have previously been reported during hair analysis.[23-25]

An investigation of instrument precision showed a variation in peak height of <6% in all but two cases, (temazepam and morphine). Finally percentage ion suppression values were calculated for each of the illicits. The values reported were surprisingly high particularly for morphine, MDMA, EDDP and LSD. The suppression values were calculated for three replicate sample extractions and the level of variation between samples was quite high, almost 30% variation between samples in three cases. This could be explained by different matrix components being extracted from each of the samples or by the fact that the samples were not agitated while being extracted leading to non-uniform extraction. In future a method of agitation, such as sonication, during incubation to ensure reproducible extraction of each sample may be useful. In all cases ion suppression was reported. Ion suppression was reported by Scheidweiler *et al.* for cocaine and cocaethylene using a similar extraction method in 2004, however other drugs such as benzoylecgonine and morphine demonstrated signal enhancement.[9]

Table 2.7: Method validation data for twelve illicit drugs in hair.

Analyte	Instrument precision (% RSD)	Correlation coefficient (R^2)	LOD in hair (pg/mg)	LOQ in hair (pg/mg)	Absolute % recovery in hair	% MS ion suppression in hair
Morphine	6.5	0.9951	26	87	129 ± 2	56 ± 7
Benzoylecgonine	2.3	0.9957	7	23	91 ± 3	13 ± 6
MDMA	5.3	<0.9500	58	193	61 ± 1	67 ± 7
Cocaine	4.6	0.9737	3	9	101 ± 18	36 ± 29
Heroin	5.6	0.9876	29	98	76 ± 16	34 ± 29
LSD	5.2	0.9925	22	73	85 ± 2	56 ± 12
Cocaethylene	2.8	0.991	3	10	89 ± 10	40 ± 28
Papaverine	3.9	0.9944	20	68	129 ± 7	63 ± 7
EDDP	1.3	0.9868	23	76	60 ± 7	54 ± 16
Temazepam	7.9	0.9989	35	115	92 ± 13	21 ± 10
Methadone	5.6	0.9958	26	85	81 ± 21	50 ± 9
Diazepam	4.7	0.9762	10	34	86 ± 16	20 ± 21

2.3.5 Application of optimised method to analysis of possibly contaminated hair:

A hair sample from a recreational drug user was obtained and extracted using the optimised extraction and clean-up method. The sample was analysed by LC-MS/MS. Traces of cocaine were identified in the hair sample and its presence was confirmed monitoring the product ion of cocaine in MS^2 mode, see **Figure 2.4**. The correlation between the cocaine mass spectrum in the contaminated sample and the mass spectrum of a 1 mg/L standard is illustrated in **Figures 2.4 and 2.5**. The fragment ion of the cocaine at m/z 182 can be clearly observed in the MS^2 spectrum of both figures and is formed due to loss of 122 mass units from both the cocaine molecule. The amount of cocaine present was determined to be 128 pg/mg from the cocaine calibration curve.

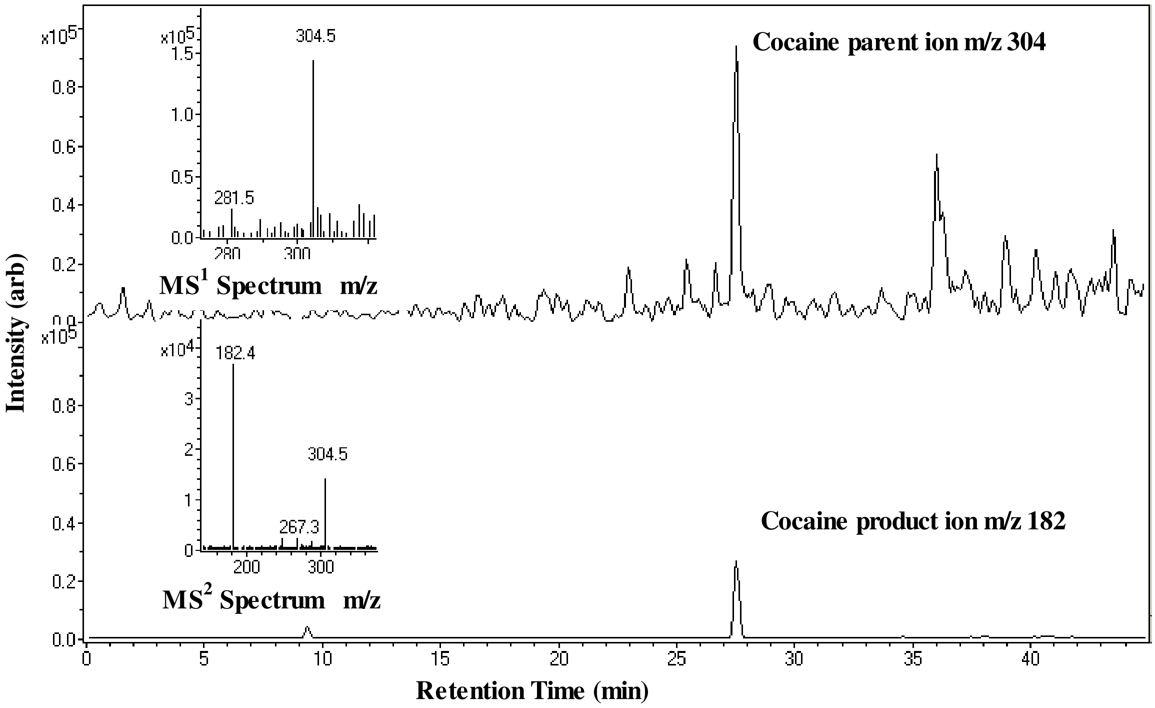
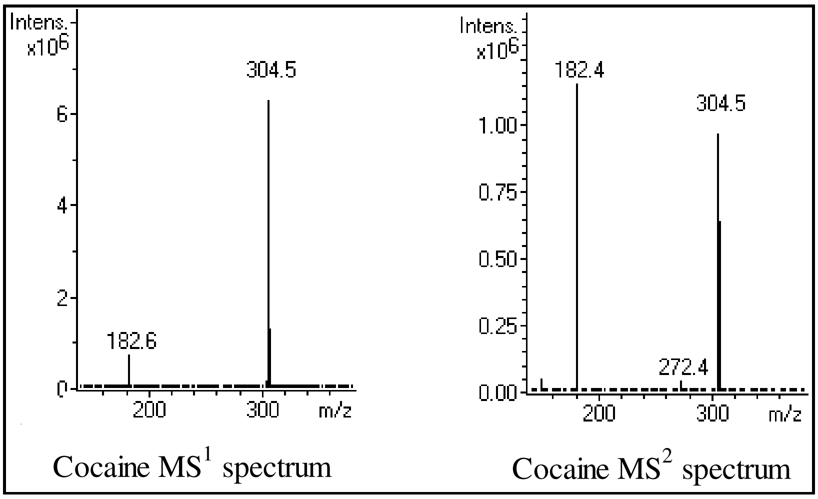


Figure 2.4: Extracted ion chromatogram of cocaine in hair at m/z = 304 and its fragment ion at m/z = 182 with corresponding mass spectra in MS^1 and MS^2 modes for confirmation of result.



Cocaine MS^1 spectrum Cocaine MS^2 spectrum

Figure 2.5: Single and tandem mode mass spectra for injection of a 1 mg/L standard of cocaine in ultra pure water, taken from peak apex.

2.4 Conclusions:

A multi-residue method for the separation and detection of twelve illicit drugs in hair samples was developed. The twelve drugs were separated in 45 minutes with excellent resolution between peaks. A number of commercially available SPE sorbents were investigated as preconcentration techniques for the illicit analytes. A mixed-mode sorbent was chosen as the optimum and was applied to the preconcentration of the illicits from hair extracts. Method validation data was compiled and limits of detection were comparable to those in other publications. A hair sample from a known drug user was analysed using the optimised method and traces of cocaine in the sample were quantified. Future work would involve examining a larger number of analytes using the optimised method. An experiment should be conducted to determine if 100 % of the analytes were extracted during methanol incubation and more hair samples are needed for further quantification of illicits. The purchase of deuterated standards can reduced the matrix effect offer a more accurate approach to quantification.

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Chapter 3.0: Characterisation of a Half-metre Monolithic Column as a High Efficiency Stationary Phase for HPLC

3.1 Introduction:

The demand for more efficient separations in recent years has seen the rise of a new generation of LC. With UPLC, reducing particle size has been the primary approach to shortening analysis time while maintaining column performance in recent years despite the expensive equipment and extremely high backpressures.[1] More recently, the combination of 2 µm particles with high flow rates and elevated column temperatures, as high as 90 °C, has been demonstrated. This technique is known as high-temperature UPLC (HT-UPLC). The viscosity of the mobile phase is reduced due to the elevated temperatures therefore allowing high flow rates to be employed without loss of efficiency.[2, 3] However, elevated backpressures of up to 1000 bar are observed with UPLC techniques even with the use of high temperatures.[3]

Monolithic silica columns can provide efficient separations at high flow rates due to there bimodal pore structure of macropores and mesopores. The structure of the skeleton and pore sizes in monolithic silica rods can be controlled and optimised during manufacture and the macroporous structure results in a highly permeable separation bed which is advantageous in fast HPLC.[4] The smaller mesopore structure supplies the surface area (300 m^2/g) required for kinetic processes of the separation to occur and results in faster mass transfer rates which maintains high efficiency at high flow rates.[5] With particle packed columns this is not possible as the smaller particles employed to achieve improved mass transfer result in higher backpressures.[4] In 2000, Merck released the first commercially available monolithic column which had macro-pores of approximately 2 µm and meso-pores of approximately 13 nm in diameter.[5] Silica monoliths have been primarily employed in fast HPLC separations due to their total porosity being up to 20 % greater than particulate columns and their efficiency of separation comparable to that of a 3.5 µm particulate column.[6]

The trend towards minimising particle diameter and shortening column length concentrates on reducing analysis time. However, complex sample types may require a lengthier and, as a result of high generated pressures, a more permeable stationary phase bed with high theoretical plate numbers in order to resolve all components of the mixture.[5] A new approach to maintaining high plate numbers is to increase the length of the column by manufacturing or by coupling columns in series based on high porosity monolithic columns. In 2001, it was suggested that monolithic columns were the ideal candidates to be connected in series for high efficiency separations of complex mixtures.[5] Several papers demonstrating high efficiency separations using coupled Chromolith RP-18e monolithic columns (10 cm x 0.46 cm) have been published since. The number of coupled columns ranged from six to fourteen with plate numbers over 100,000 reported for a wide range of samples separated such as drugs and complex isomeric mixtures.[7, 8, 6] Pellicular particles and traditional 5 µm particle packed columns have also been investigated in long column format resulting in very high plate counts of 180,000 at elevated temperatures.[9, 10] The performance of these long columns has been assessed in a number of ways. Ikegami *et al.* evaluated the performance of different numbers of coupled monoliths based on band broadening and extra-column effects.[11] Gray *et al.* investigated multiple monoliths in series and in parallel as a method of improving peak capacity and loading capacity.[12]

Since 2000, monolithic columns have been characterised using van Deemter plots as with particulate columns despite the obvious differences in internal structure.[4] Both the A and C terms in the van Deemter equation rely heavily on particle size, however this problem has been addressed by assigning a particle size to monoliths by comparing macropore diameter to inter-particle spaces in conventional columns.[13] The van Deemter plots reported for long monoliths have emphasised their excellent efficiency at high linear velocities and illustrated their advantages over particle-packed beds.[6, 14] Bones *et al.* demonstrated the benefits of even two coupled Phenomenex Onyx monoliths (100 x 3.0 mm) over a 1.8 µm particulate column using van Deemter and kinetic plots. Smaller plate height was observed for the particle-packed column, however the 20 cm monolith excelled in plate number at considerably lower backpressure and was therefore better suited to separation of a complex mixture of drugs.[14] More recently, peak capacity values were calculated for ten monoliths in series (one metre total length) with excellent results of up to 244 peaks per hour and 90,000 plates.[15] The study was carried out at pressures associated with a conventional LC separation and both studies by this group employed mass spectrometric detection with ESI as an interface.[14, 15]

In this work a half metre monolithic column composed of five 10 cm monolithic rods connected in series is introduced as a highly efficient separation phase for complex environmental samples. The monolithic column was characterised using van Deemter plot experiments and compared to a conventional particulate column to illustrate the advantages of high plate number and low plate height at reduced backpressures. The performance of the coupled monolith was investigated at a range of temperatures and by calculation of peak capacity values. The increased loading capacity of coupled monoliths was also demonstrated.

3.2 Experimental:

3.2.1 Reagents and preparation of glassware and standards:

All solvents used including acetonitrile and methanol were HPLC grade or better. Acetonitrile and methanol were obtained from Labscan (Dublin, Ireland). Acetone, dichloromethane, dichlorodimethylsilane and ethyl acetate were purchased from Aldrich (Gillingham, UK) as was the mobile phase additive ammonium acetate. Ammonium hydroxide (LC-MS additive grade) was purchased from Fluka (Steinheim, Germany). Reagent water was obtained from a Millipore Milli-Q water purification unit (Millipore, Bedford, MA, USA) and was 18.2 MΩ/cm or better.

All pharmaceuticals purchased were ≥97% purity. Paracetamol, salicylic acid, propranolol hydrochloride, clofibric acid, ketoprofen, diclofenac sodium salt, bezafibrate, warfarin, flurbiprofen, indomethacin, ibuprofen sodium salt, meclofenamic acid sodium salt, atenolol, salbutamol, sulfamethoxazole, sulfapyridine, sulfamethazine sodium salt, carbamazepine, nimesulide, (±)-metoprolol (±)-tartrate salt, mefenamic acid, salbutamol, erythromycin, doxazosin mesylate, phenazone, nifedipine, budesonide, simvastatin, tamoxifen, sertraline hydrochloride, citalopram hydrobromide, 3,4,4_-trichlorocarbanilide, amitriptyline hydrochloride, nortriptyline hydrochloride, cimetidine, ranitidine hydrochloride, tramadol hydrochloride, chloramphenicol, and clotrimazole were all obtained from Sigma-Aldrich (Steinheim, Germany). Trimethoprim, caffeine, S(±)-2-(6-methoxy-2-naphthyl)-propionic acid and irgasan were ordered from Fluka (Buchs, Switzerland). Cocaine hydrochloride, morphine sulfate salt pentahydrate, methadone hydrochloride and heroin were purchased under license from Sigma-Aldrich (St. Louis, MO, USA). Benzoylecgonine hydrate, cocaethylene, D-amphetamine sulfate salt, temazepam, diazepam, papaverine hydrochloride, 3,4-methylenedioxymethamphetamine hydrochloride (MDMA) and 2-ethylidine-1,5dimethyl-3,3-diphenylpyrrolidine perchlorate were purchased under license from Sigma-Aldrich (Poole, UK).

All glassware utilised in the preparation of pharmaceutical and illicit drug stocks and standards were all silanised prior to the preparation to prevent the drugs from adhering to glass surfaces. This was carried out by rinsing the glassware with 10% dichlorodimethylsilane in dichloromethane. This was followed by rinsing with dichloromethane and rinsing with methanol twice each. Stock solutions of pharmaceuticals were prepared in methanol to a concentration of 1000 mg/L and were stored in a fridge at 4 ^{o}C. Working standards were prepared freshly before analysis in ultra pure water.

3.2.2 Liquid chromatography-UV detection:

For column characterisation and van Deemter plot experiments, liquid chromatography was carried out on two systems. The first was an Agilent 1200 series high performance liquid chromatograph with a vacuum degasser, quaternary pump, ALS autosampler, thermostatted column compartment and variable wavelength detector. Data analysis was performed using Agilent Chemstation programme, version B.02.01 (Agilent Technologies, USA). The second system was an Agilent 1100 series high performance liquid chromatograph with a vacuum degasser, binary pump, ALS autosampler, and diode array detector. Agilent chemstation version A.09.03 (Agilent Technologies, USA) was employed for data analysis and system control. For the temperature study a SparkHolland MISTRAL heater was employed to ensure that a precise column temperature was reached before analysis.

Separations were performed using a Waters Sunfire 150 x 2.1 mm i.d. 3.5 μm column and five Phenomenex Onyx Monolithic 100 x 3.0 mm i.d. columns. The five monolithic columns were coupled in sequence using peek tubing and ferules to give a half-metre column. For characterisation experiments, an isocratic mobile phase was employed of 70 : 30 10mM ammonium acetate acetonitrile for separation of 10 mg/L solutions of diclofenac, cocaine and metoprolol. For the separation of mixed drug standards, linear and multi-step gradients were employed for both columns with mobile phases of (A) 90 : 10 and (B) 20 : 80 10 mM ammonium acetate acetonitrile. A flow rate of 0.2 mL/min and injection volume of 10 µL were utilised.

3.3 Results and Discussion:

3.3.1 Characterisation of half-metre monolith and comparison to 3.5 µm particulate column:

The efficiency of the columns was characterised using van Deemter curves. Diclofenac, a common anti-inflammatory and analgesic, was chosen as a test analyte for the study. The separations were carried out isocratically as already described with UV detection at 225 nm. A 10 mg/L working standard of the drug was prepared from a 1000 ppm stock solution. Injection volume was 10 µL and flow rates from 0.4 mL/min to 0.01 mL/min were employed throughout the study.

The first experiment compared the separation efficiency of a single monolith with that of five monoliths in series and the Waters Sunfire particle column. The experiments were all carried out at 20 °C and a 10 µL volume of the diclofenac standard was injected at a range of flow rates. The retention time, peak width, dead volume and pressure were recorded at each flow rate. Each measurement was carried out in duplicate and average values were calculated. Flow rates up to only 0.26 mL/min were applicable with the 3.5 µm Sunfire column due to excessive backpressures of over 3200 psi. The monolithic columns were investigated up to 0.4 mL/min, however further increases in flow rate could have been investigated due to much lower backpressures of 525 psi on one monolith and 1710 psi on the coupled monoliths at 0.4 mL/min.

Figure 3.1 and 3.2 below depict the van Deemter curves obtained for the three columns investigated in terms of plate height (HETP), plate number per column (N/column) and linear velocity (υ). From **Figure 3.1**, the Sunfire column produced higher linear velocities overall, these were accompanied by an increase in plate height due to poor mass transfer rates. A higher optimum linear velocity was observed for the the monolithic columns than the Sunfire column at much lower backpressures. A plate height of 26.19 µm at a linear velocity of 0.306 was recorded for the Sunfire. The five coupled monoliths were slightly better with HETP = 25.90 at υ = 0.316 while a single monolith gave the most efficient result with HETP = 19.68 µm at υ = 0.689. This result was achieved at a flow rate of 0.24 mL/min, six times higher than the flow rate on the Sunfire of 0.04 mL/min. This illustrates the obvious advantage monoliths have over particle columns of equal if not improved efficiency at higher flow rates. Higher flow rates should result in decreased mass transfer; however the porous structure of the monolith allows for faster rates of mass transfer and therefore provides excellent efficiency despite the higher flow.

Figure 3.2 illustrates the beneficial effect of coupling monoliths in series. The 0.5 m monolith produced considerably higher numbers of theoretical plates (19304 plates) than both the single monolith (5081 plates) and Sunfire column (5728 plates). The single monolith produced much the same number of plates as the Sunfire which corresponds to earlier statements made that the efficiency of monoliths is about that of a 3.5 µm particle-packed column.[6]

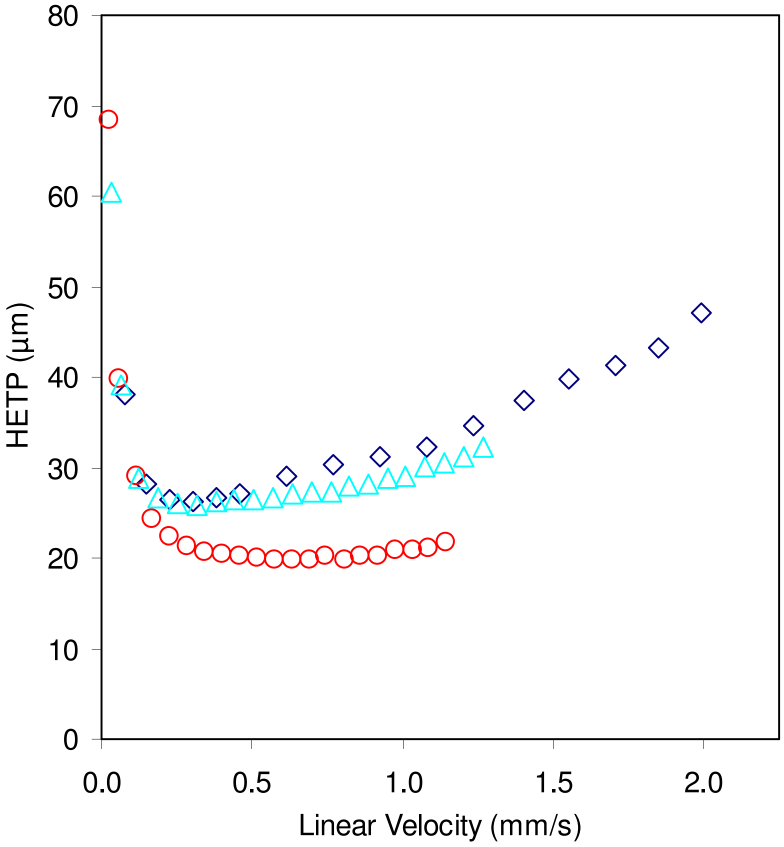

Figure 3.1: Graph of plate height (HETP) against linear velocity (υ) for the single monolith (○), the five coupled monoliths (△), and the Sunfire column (□).

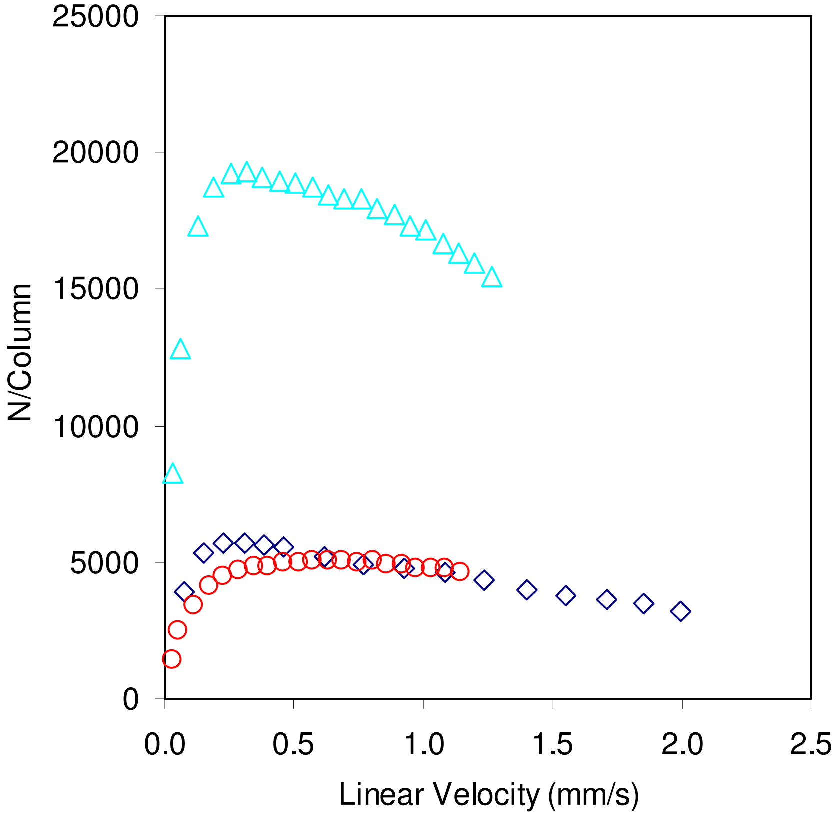

Figure 3.2: Graph of number of theoretical plates per column (N/column) against linear velocity (υ) for the single monolith (○), the five coupled monoliths (△), and the Sunfire column (□).

Figure 3.3 highlights the low operating backpressures generated by monolithic columns. The five monoliths in series generated 1710 psi while the Sunfire column generated almost double, 3278 psi. In addition, the long monolith produced more than three times the number of theoretical plates per column than the Sunfire at half the operating pressure. The single monolith as expected exhibited the lowest backpressures but was comparable to the Sunfire in the number of plates provided. These plots demonstrate that the main advantage of long coupled monoliths over standard particulate columns, are the low operating pressures at high flow rates with excellent numbers of theoretical plates and hence improved separation efficiency.

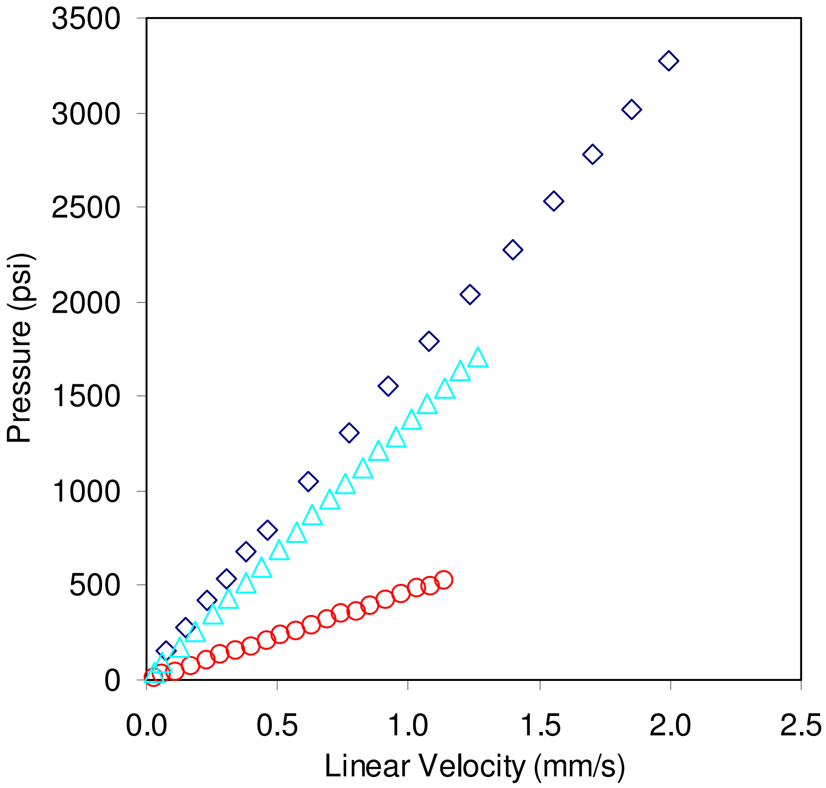

Figure 3.3: Graph of number of backpressure (psi) against linear velocity (υ) for the single monolith (○), the five coupled monoliths (△), and the Sunfire column (□).

3.3.2 Investigation of effect of temperature on efficiency of 0.5 m coupled monolithic column:

Recently increasing temperature has been employed in UPLC as a way of increasing efficiency.[2, 3] With fast mass transfer rates already a characteristic of monolithic separations, in theory increased temperatures could result in even better efficiency. Increased temperatures would reduce the mobile phase viscosity allowing for faster interactions between mobile and stationary phase hence reduces C_m.

Experiments were carried out using the five coupled monoliths over a range of temperatures with an identical isocratic system as employed before. From the product information, the monolith was stable up to 45 ^{o}C so experiments were conducted at 10, 15, 20 and 45 °C. Linear velocities were plotted against plate height (H), plate number per column and backpressure (psi) to illustrate the improved performance of the half metre column at elevated temperatures. **Figure 3.4, 3.5 and 3.6** illustrate the advantages of employing high temperature separations over those at ambient conditions. The number of theoretical plates for the 0.5 m monolithic column increased from 14522 at 10 °C to 23486 at 45 °C with a decrease in plate height of approximately 13 μm and a reduction in backpressure of over 1000 psi. The slopes of the van Deemter plots indicate that there was only marginal loss of efficiency at higher linear velocities with a plate count of almost 24,000. Particularly in **Figure 3.4**, as the linear velocity increased and hence the flow rate increased, the expected increase in plate height did not occur to a great extent. Therefore this column shows promise for application to very complex sample types such as soil or sludge.

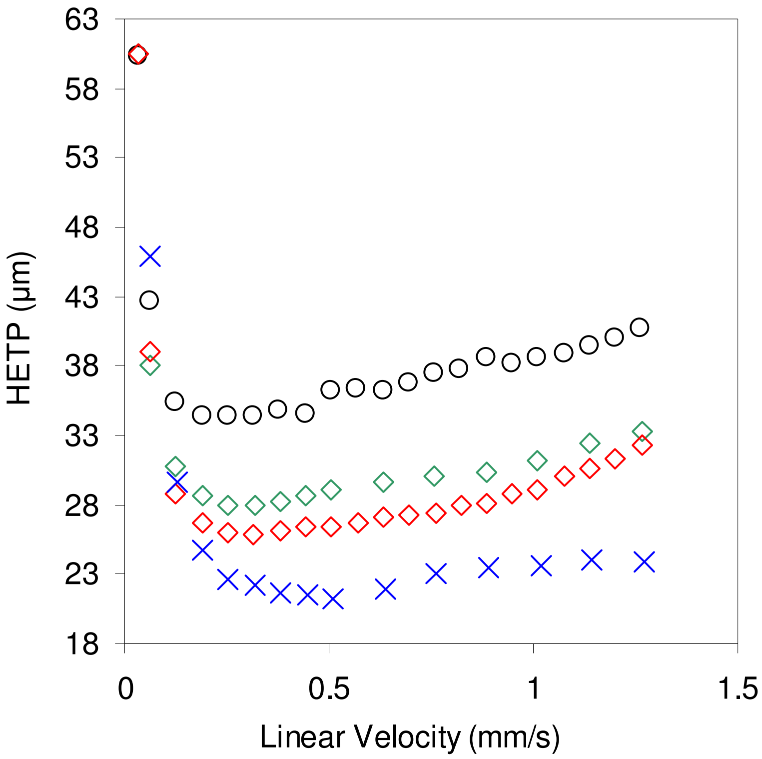

Figure 3.4: Plot of linear velocity (υ) versus plate height (HETP) generated on five coupled monoliths at 10 °C (○), 15 °C (□), 20 °C (□) and 45 °C (x).

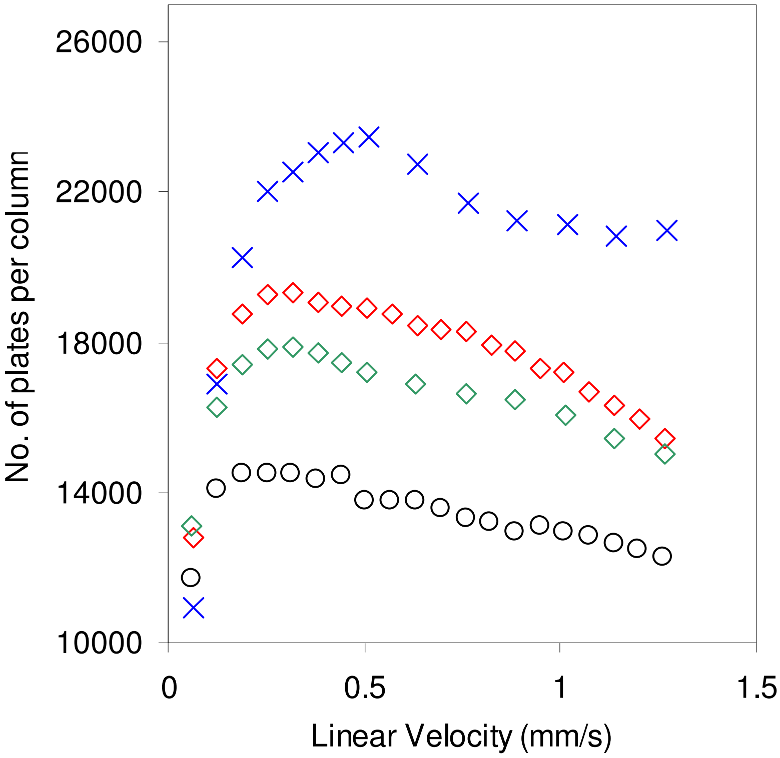

Figure 3.5: Plot of linear velocity (υ) versus number of theoretical plates per column (N/column) generated on five coupled monoliths at 10 °C (○), 15 °C (□), 20 °C (□) and 45 °C (x).

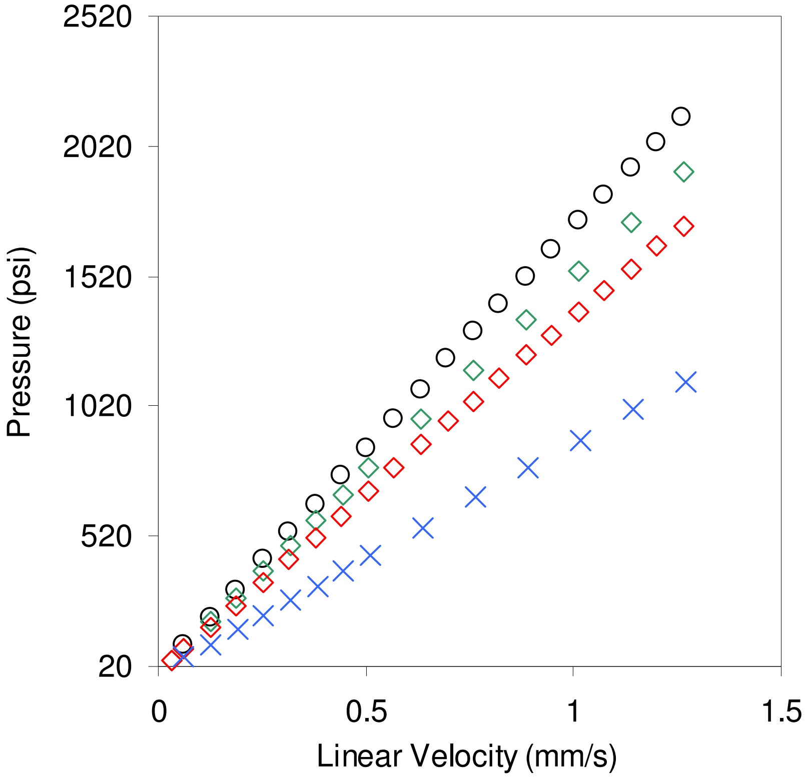

Figure 3.6: Plot of linear velocity (υ) versus backpressure (psi) generated on five coupled monoliths at 10 °C (○), 15 °C (□), 20 °C (□) and 45 °C (x).

When a comparison was made by plotting the monolith data at 45 °C against the Sunfire data at ambient temperatures, the advantages of coupled monoliths were obvious as illustrated in **Figure 3.7, 3.8 and 3.9**. The Sunfire column reached its optimum efficiency at a plate height of 26.19 μm and then steadily loses efficiency as linear velocity increases. This optimum plate height was at a flow rate of only 0.04 mL/min which is impractical due to long analysis times. Therefore, to increase analysis time efficiency has to be sacrificed. The monolith however, reached a minimum plate height of 21.29 at a flow rate of 0.16 mL/min, four times that of the Sunfire. Also the plate height did not increase as steeply after this minimum as it does for the Sunfire. This illustrates that higher flow rates can be used without a significant loss of efficiency.

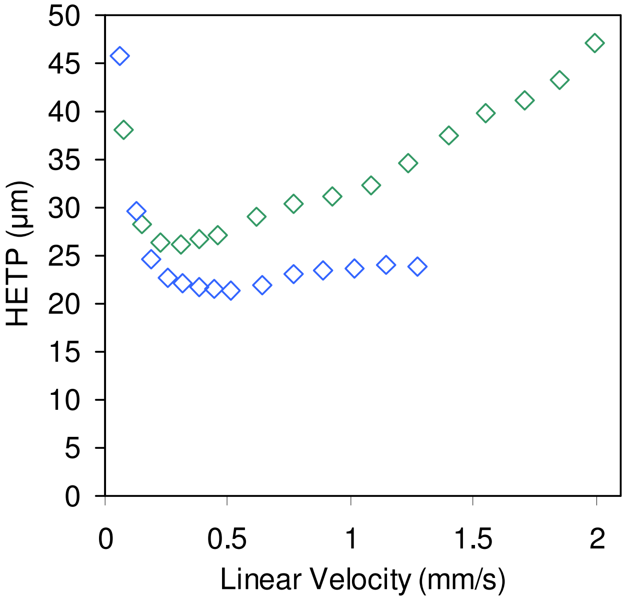

Figure 3.7: Plot of linear velocity (υ) versus plate height (HETP) for the five coupled monoliths at 45 ^{o}C (□) and the Sunfire particulate column at ambient temperature (□).

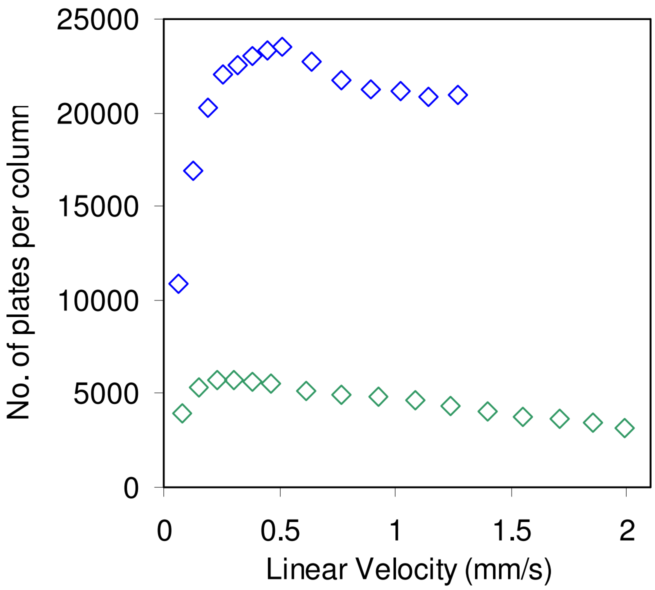

Figure 3.8: Plot of linear velocity (υ) versus number of theoretical plates per column (N/column) for the five coupled monoliths at 45 ^{o}C (□) and the Sunfire particulate column at ambient temperature (□).

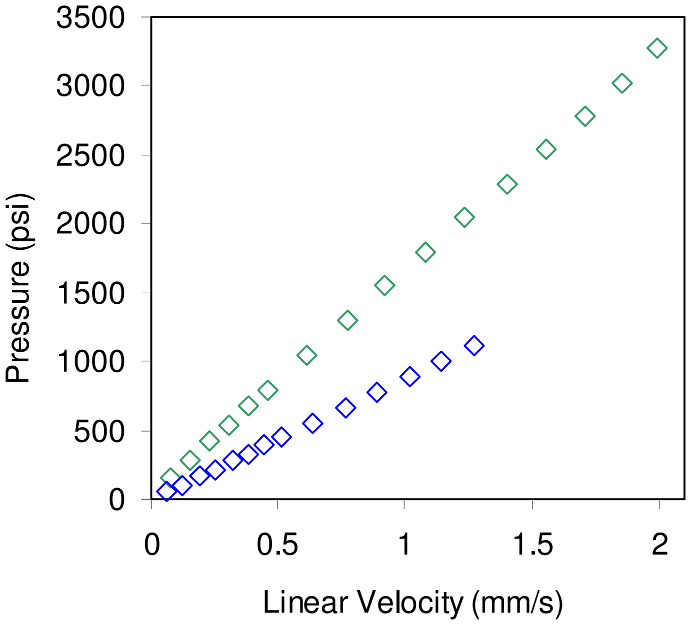

Figure 3.9: Plot of linear velocity (υ) versus backpressures (psi) for the five coupled monoliths at 45 °C (□) and the Sunfire particulate column at ambient temperature (□).

3.3.3Peak capacity study:

Calculating the number of peaks that can be separated in a specific time period is an excellent way of assessing the performance of a column. Giddings first proposed a formula for calculating peak capacity.[16] Neue *et al.* developed an equation that could be applied to linear gradient chromatography and it is this equation (shown below) that was used to calculate peak capacity in this study.[17] Peak capacity has previously been used to determine the performance of monolithic columns with good results when compared to particle columns and at high flow rates.[15]

$$P_c = 1 + \frac{t_g}{W} \qquad \text{(Eqn. 3.1)}$$

A working standard containing approximately fifty PPCPs in ultra pure water was injected at a volume of 20 μL and detected at a wavelength of 230 nm. An identical gradient was employed for separations with both the long monolith and the Sunfire columns. The gradient run time was ninety minutes over which the concentration of mobile phase B (20 : 80 10 mM ammonium acetate : acetonitrile) increased from 0 to 100%. From the resulting chromatograms twenty peaks were selected and divided into the gradient run time, the average of these figures was then taken and the peak capacity per hour was also reported. **Table 3.1** contains the data obtained from the peak capacity study.

Table 3.1: Peak capacity values calculated for half-metre monolith and Sunfire columns

	Flow Rate[a]		**Backpressure**[b]		**Deadtime**[c]		**Linear Velocity**[d]	
	P_c	**P_c/hr**	**P_c**	**P_c/hr**	**P_c**	**P_c/hr**	**P_c**	**P_c/hr**
Monolith	104	69	86	57	108	72	132	88
Sunfire	103	69	50	33	50	33	93	62

[a] Flow rate was 0.2 mL/min for both columns with backpressures of 34 bar for the monolith and 146 bar for the Sunfire.

[b] Flow rates were 0.18 mL/min and 0.04 mL/min for the monolith and Sunfire columns respectively resulting in backpressures of ~ 30-31 bar (570 psi).

[c] Flow rates were 0.28 mL/min and 0.04 mL/min for the monolith and Sunfire columns respectively resulting in dead times of 9.94 and 9.73 minutes respectively.

[d] Flow rates were 0.36 mL/min and 0.16 mL/min for the monolith and Sunfire columns respectively resulting in linear velocities of 1.07 and 1.04 mm/s respectively.

Peak capacity (P_c) studies have been carried out on monolithic capillary columns with impressive results.[18] Luo *et al.* reported peak capacities greater than 1000 at 345 bar on a 70 cm monolithic capillary column. More recently, Bones *et al.* investigated ten coupled monolithic columns (100.0 x 3.0 mm I.D.) for the separation of protein digests. The one metre monolith reported excellent peak capacity values per hour, which increased with increasing flow rate.[15]

In this study peak capacity values per chromatographic run and per hour were compared for the half-metre monolith and the Sunfire particulate columns. Four experiments were carried out each under a different set of chromatographic conditions.

At identical flow rate the columns reported identical peak capacities, however when backpressures were matched the monolith was operated a much higher backpressure which allowed almost twice the number of peaks to be separated within the run time. A similar result was obtained when the void volumes of the columns were correlated. For the t_0 value of the Sunfire column to match that of the monolith, the flow rate had to be lowered to only 0.04 mL/min. This resulted in only half the number of peaks being separated in 90 minutes than those generated on the monolithic column. Finally linear velocities of mobile phase were matched and the peak capacities were again evaluated. Once more the monolithic column reported higher peak capacity at lower backpressure of only 61 bar. Bones *et al.* calculated a ratio of peak capacity/hour : backpressure as an indication of the column providing highest efficiency separation per hour at the lowest pressure drop.[15] This ratio was calculated for the half-metre monolithic and Sunfire columns at identical linear velocities, the results were 1.44 and 0.42 respectively. The higher value demonstrated for the monolithic column indicates that it is an excellent choice for high efficiency separations at backpressures that can be achieved using standard HPLC instrumentation.

3.3.4 Investigation of loading capacity:

The volume of sample loaded onto columns in recent years has decreased due to shorter columns with smaller particles which can be easily saturated with sample. In the case of trace analysis this becomes a significant problem. A highly efficient phase which has the capacity to receive a wide range of injection volumes is desirable. Due to the increased length and increased surface area of the monolith available for separation of analytes, it was expected that the long column would have an increased capacity for large sample volumes. An earlier study by Gray *et al.* determined that increasing the length of a monolith increased the loading capacity while improving resolution.[12]

Three test compounds metoprolol, cocaine and diclofenac were chosen for the experiment. A mixed working standard at a concentration of 10 mg/L was prepared and was injected at volumes of 10 to 900 µL, (the maximum syringe volume on the instrument). The separations were carried out isocratically with mobile phase 70 : 30 10 mM ammonium acetate : acetonitrile over a thirty minute runtime. Peak height, width at half height and asymmetry were plotted against increasing injection volume to monitor changes in peak shape and the ability of the column to manage increased loading. These results are illustrated in **Figures 3.10, 3.11 and 3.12**.

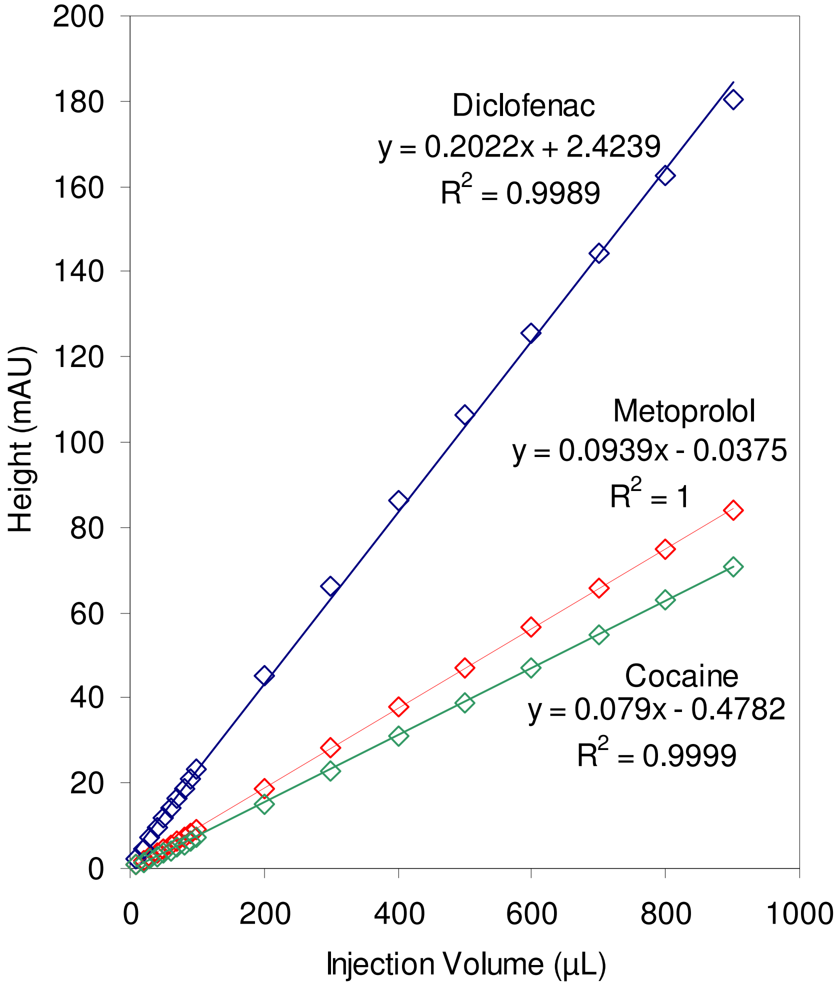

Figure 3.10: Plot of peak height (mAU) versus injection volume (µL) for (□) = metoprolol, (□) = diclofenac and (□) = cocaine.

From **Figure 3.10**, peak height increased linearly with injection volume up to 900 µL for all three test compounds. Correlation coefficients of $r^2 = 1.000$, 0.9989 and 0.999 were obtained from graphs of peak height versus injection volume for metoprolol, diclofenac and cocaine respectively. The 0.5 m monolith was capable of accepting almost 1 mL of sample.

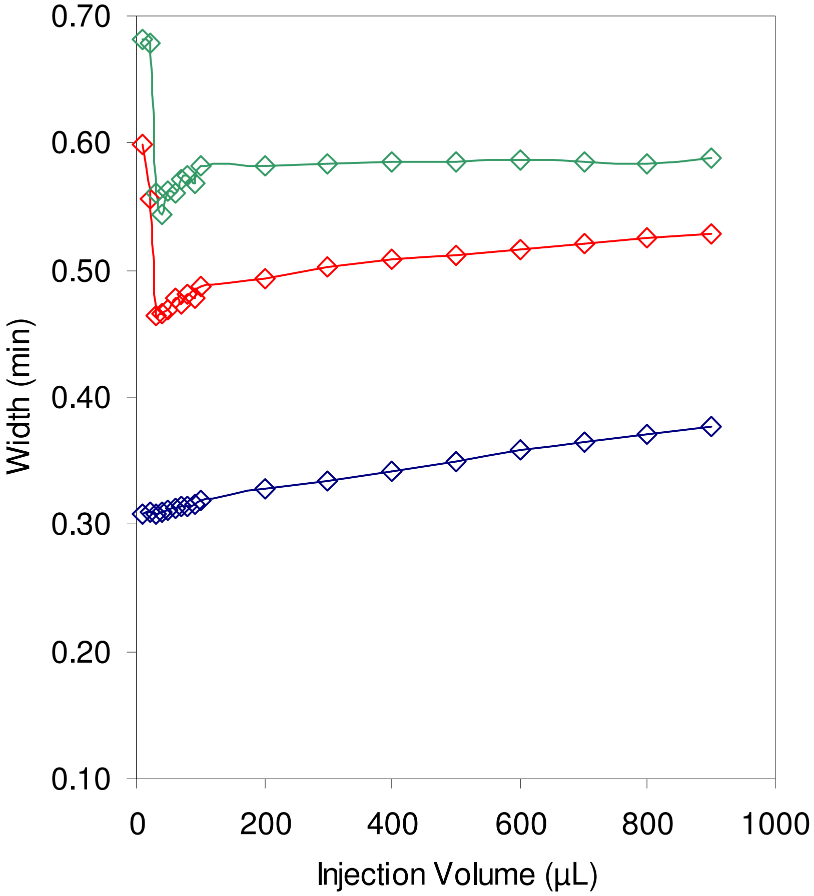

Figure 3.11: Plot of peak width at half maximum (min) versus injection volume (µL) for (□) = metoprolol, (□) = diclofenac and (□) = cocaine.

Peak width increased from 30 µL up to 900 µL for the test compounds as expected, (**Figure 3.11**). The peak widths recorded were width at half maximum peak height. Although metoprolol and cocaine exhibited some deviations at low injection volumes of 10, 20 and 30 µL, the increase in width throughout the study was not large. From **Figure 3.11** width increased only minimally for metoprolol and diclofenac and remained constant or even slightly decreased for cocaine. This result illustrates that despite the increasing injection volume, sharp peaks were being recorded. The surface area and capacity of the monoliths was sufficient for the concentration of solutes within the injected volumes and because of this the sample plug did not disperse significantly. If the column were overloaded, some solutes would be eluted more quickly due to saturation of stationary phase adsorption sites. The small variation in peak width corresponds to the earlier linearity result, as the injection volume increased the intensity or peak height increased but the peak width remained relatively constant. Overall the width at half maximum increased from a minimum of 0.4638 (30 µL) to a maximum of 0.5279 min (900µL) for metaprolol corresponding to an increase of ~ 4 seconds. The same increase applied for diclofenac with a minimum at 0.3075 min (10 µL) and maximum at 0.3769 min (900 µL). Cocaine had an increase of ~ 2.5 seconds overall with minimum peak width of 0.5445 min (40 µL) and a maximum at 0.588 min (900 µL).

Asymmtery is calculated by bisecting a peak perpendicularly and dividing the right half width into the left half width. Asymmetry due to tailing is caused by alternative retention mechanisms taking place within the column leading to the tail of peak being drawn out. Tailing results in an asymmetry factor greater than one. Fronting is the second type of asymmetry which is more relevant to this study. It is mainly caused by overloading the column with sample. Fronting results in an asymmetry value less than unity.[19] **Figure 3.12** depicts the graph of peak asymmetry against injection volume for the three test compounds. Diclofenac maintained a high level of symmetry up to 100 µL with a fronting value of 0.883, after which the symmetry of the peaks decreased with increasing injection volume. Metoprolol and cocaine both exhibited signs of asymmetry at low injection volumes and these values decreased rapidly until after 100 µL when they began to even out. According to the graph minimal peak tailing was occurring throughout the separation.

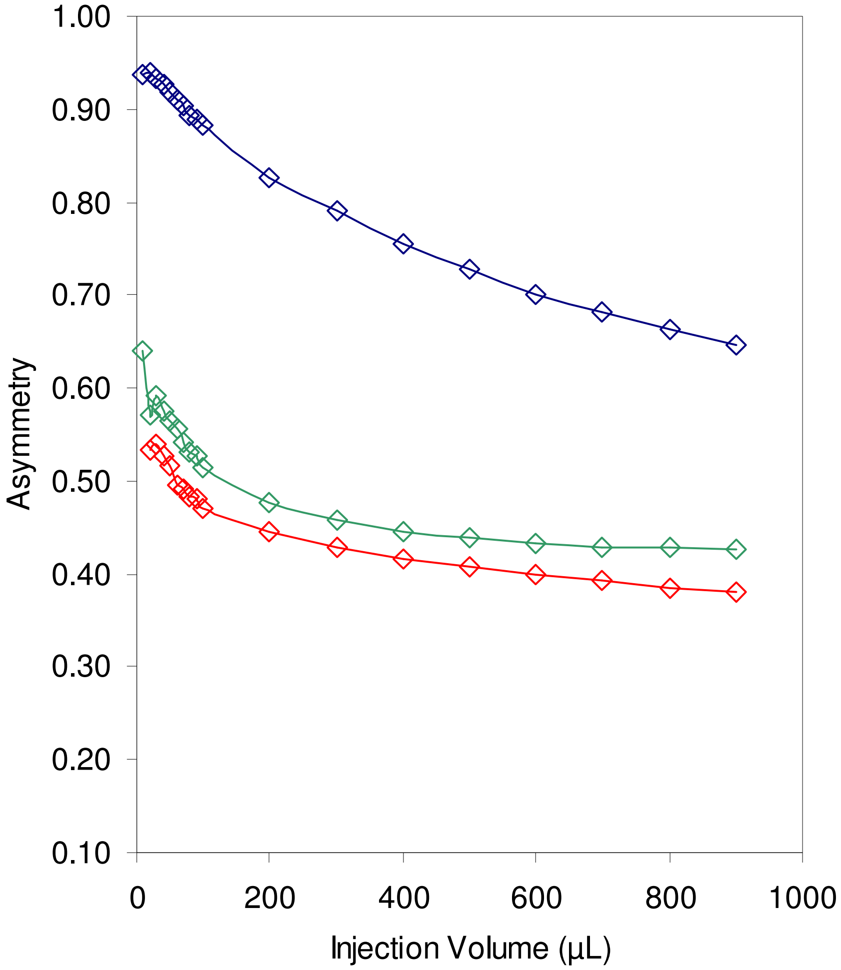

Figure 3.12: Plot of peak asymmetry versus injection volume (µL) for (□) = metoprolol, (□) = diclofenac and (□) = cocaine.

To determine the effect of increased sample loading on the efficiency of the column, the number of theoretical plates per column was calculated for each analyte using Eqn. 3.2 and plotted against injection volume.

$$N = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \qquad \text{(Eqn. 3.2)}$$

Figure 3.13 demonstrates that only about a 10 % loss in efficiency was observed for each analyte at an injection volume of 200 µL. This result is mirrored very well by the peak width plot in Figure 3.11. The peak widths for metoprolol and diclofenac increased by approximately 6 % between sample volumes of 10 and 200 µL while the peak widths for cocaine only increased by approximately 3 % over the same range. Separation efficiency for diclofenac and metoprolol demonstrated quite a linear decrease with respect to increased sample volumes. The number of theoretical plates decreased initially for cocaine before levelling off and remaining quite linear. This result demonstrates the excellent ability of the monolithic stationary phase to effectively separate large volumes of solutes and emphasises the advantages in applying these phases to trace analysis and separation of complex sample types.

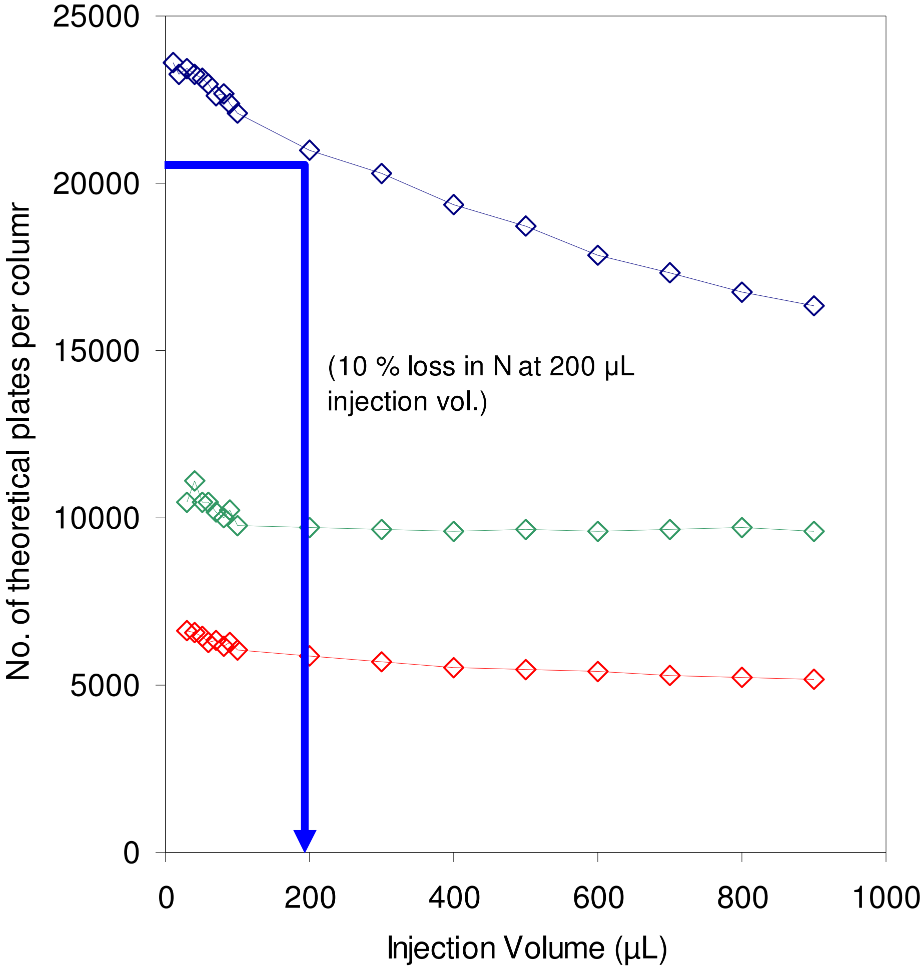

Figure 3.13: Plot of number of theoretical plates (N) versus injection volume (µL) for (□) = metoprolol, (□) = diclofenac and (□) = cocaine.

The long monolith was then applied to the separation of increasing volumes of a mixed standard containing up to 50 pharmaceuticals and illicit drugs in ultra pure water. This study was carried out to demonstrate the superior loading capacity of the 0.5 m monolith when separating a complex mixture of analytes and also to optimise gradient conditions for the separation of numerous target analytes. Injections of the 0.33 mg/L standard were made at volumes of 10, 20, 50 and 100 µL and separated over a 110 min run time at 0.2

mL/min, a column temperature of 45 ^{o}C and detected at a wavelength of 230 nm. A gradient was employed, mobile phase B (20 : 80 10 mM ammonium acetate : acetonitrile) was increased from 0 – 100% over 90 minutes and held for 20 minutes and a re-equilibration time back to 100% mobile phase A (90 : 10 10 mM ammonium acetate : acetonitrile) of 30 minutes. Chromatograms obtained from the experiments are shown in **Figures 3.14 and 3.15** below.

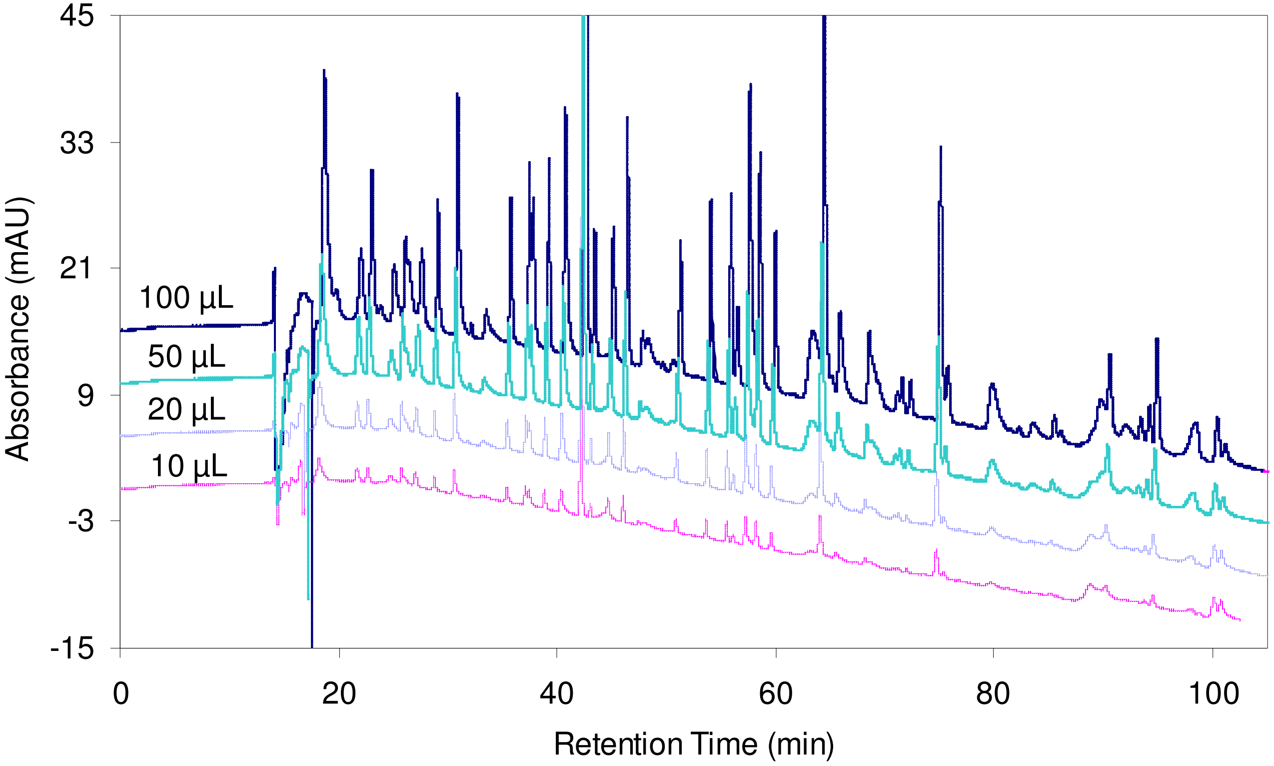

Figure 3.14: Overlaid chromatograms obtained after 10 µL (----), 20 µL (----), 50 µL (----) and 100 µL (----) injections of a 0.33 mg/L standard of 51 pharmaceuticals and illicit drugs in ultra pure water.

The chromatogram in **Figure 3.14** illustrates the excellent separation power of the coupled monoliths. Increases in peak height relative to the increase in injection volume can be seen from the overlay of the signals. An expansion of a section of the four chromatograms and identification of some pharmaceuticals can be seen in **Figure 3.15**.

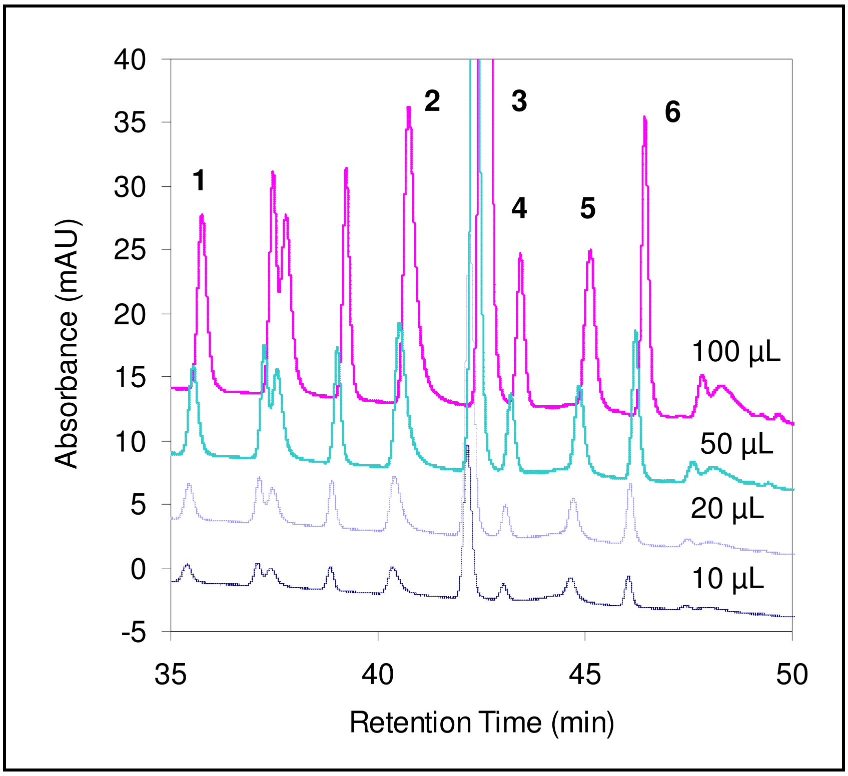

Figure 3.15: Expansion of overlaid chromatograms in figure. Peak 1 = sulfamethazine, 2 = trimethoprim, 3 = naproxen, 4 = ketoprofen, 5 = methadone and 6 = bezafibrate.

Alternative gradient conditions were investigated for the separation of 51 pharmaceuticals on the half-metre monolith in order to optimise the separation conditions before application to any real samples. These gradients included shorter linear gradients and stepwise gradients over 75-140 minute time periods. A flow rate of 0.3 mL/min was also employed with these condensed gradients in an effort to shorten the run time. However the shorter linear gradients resulted in unresolved peaks and stepwise gradients did not improve the separation significantly. The original linear gradient over 110 minutes at 0.2 mL/min was chosen for further work.

3.4 Conclusion:

Five monolithic columns were coupled in series and characterised using van Deemter experiments. The exceptional numbers of theoretical plates and low plate heights were illustrated by comparison of the long monolithic column to a conventional particulate stationary phase. Column efficiency was investigated at a range of temperatures and it was found that an 18% increase in theoretical plates could be achieved at 45 ^{o}C while operating at even lower backpressures. Peak and loading capacity of the half-metre monolith were also investigated. The monolith demonstrated excellent loading capacity, with only 10% loss of efficiency with a 200 µL injection. The optimised method was then successfully applied to the separation of 51 pharmaceuticals. High efficiency separations were possible at flow rates that are applicable to MS detection. With coupling to a mass selective detector, this column could be applied to screening for a large number of forensically and environmentally relevant analytes.

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Chapter 4.0: Application of a Half-metre Monolithic Column to the LC-MS/MS Analysis of Complex Environmental and Biological Matrices

4.1 Introduction:

Analysis of environmental and biological matrices is of utmost importance today in order for scientists to better understand the occurrence, fate and effects of PPCPs in our environment. LC-MS/MS has grown as a tool for biological and environmental monitoring in recent years. Atmospheric pressure interface technologies such as ESI are perfectly suited to the analysis of polar compounds e.g. pharmaceuticals. Matrix effects are the pitfall of LC-MS/MS analysis and reduce linearity, accuracy and precision of the analytical method.[1] Ion suppression or enhancement in MS detection can be caused by co-elution of matrix components which have similar m/z values to analytes or, and more commonly, signal suppression or enhancement occurs due to interactions between matrix components and analytes within the ion source.[2]

In the case of pharmaceuticals in aqueous environmental samples, signal suppression has been thoroughly investigated and it is now common practice to report matrix effects along with other method validation data.[3-7] Matrix effects have also been quantified for biological matrices such as urine and a number of different preparation techniques have been investigated.[8-10] A limited number of publications have described matrix effects in the extracts of solid environmental matrices, however it is a well known fact that the inhomogeneity and high load of organic material in solid samples leads to increased matrix effects despite exhaustive clean-up procedures.[11-13] Pfeifer *et al.* demonstrated that antibiotics analysed using ESI was more sensitive to matrix effects than when detected using APCI.[11] Barron *et al.* demonstrated higher suppression effects for a range of pharmaceuticals in sludge than in soil extracts, while Stoob *et al.* reported very high levels of suppression for sulphonamides in soil.[12, 13]

There are several generally accepted techniques for the improvement of matrix effects in LC-MS/MS analysis. The traditional method of standard addition is laborious and time consuming, as is preparing external calibration standards in a suitable replacement matrices.[2, 14-16] New and improved sample separation techniques such as IP-LC using volatile ion-pairing agents have also been devised as a solution to the suppression problem with good results.[17] A study in 2005 found that reducing flow rates greatly reduced matrix effects in LC-MS of trace organics in wastewater.[6] However, the most common method of quantifying analytes affected by matrix components in both environmental and biological samples, is the addition of structurally similar compound or isotopically labelled compounds to act as internal standards.[9, 18-22] A number of studies have compared the different matrix correction techniques and while isotopically labelled standards are expensive, they are considered to provide the most accurate solution to ion suppression and enhancement.[23, 24] As is obvious from this discussion, analytical techniques for the determination of PPCPs in environmental and biological samples generally involve time-consuming sample preparation and method development prior to analysis.

As mentioned above, thorough optimisation of the chromatographic separation can help to reduce matrix effects.[2, 25] An important aspect of separation development is the selection of a suitable column length and stationary phase. Monolithic stationary phases have previously been applied to high efficiency separations of protein digests in capillary column format[26], determination of pharmaceuticals in wastewater[27] and analysis of antibiotics in bodily fluids.[28] Samanidou *et al.* analysed urine samples which had been simply precipitated and filtered before direct injection onto the monolithic column. When compared to the performance of a conventional particulate column, the efficiency of the monolith was excellent with almost twice the number of theoretical plates at the same linear velocity.[28] Direct injection of urine samples was also carried out by Dams *et al.* for the detection of opiates and cocaine. This technique reported little matrix effects and shortened analysis time considerably due to lack of sample clean-up step.[9]

In this work, matrix effects in LC-MS/MS analysis of the solid environmental matrices soil and sludge were evaluated using a half-metre monolithic column and a conventional particulate column. Alternative sample preparation techniques were employed for each sample type. It is theorised that the longer stationary phase of the monolith serves to more efficiently separate matrix components and analytes within the column and therefore reduce matrix effects. The long monolith was also applied to the analysis of urine by direct injection after only minimal sample pre-treatment and suppression effects were also quantified. Finally, a screening method for approximately 40 PPCPs was applied to urine samples and a number of compounds were detected.

4.2 Experimental:

4.2.1 Reagents and preparation of glassware and standards:

All solvents used including acetonitrile and methanol were HPLC grade or better. Acetonitrile and methanol were obtained from Labscan (Dublin, Ireland). Acetone, dichloromethane, dichlorodimethylsilane and ethyl acetate were purchased from Aldrich (Gillingham, UK) as was the mobile phase additive ammonium acetate. Ammonium hydroxide (LC-MS additive grade) was purchased from Fluka (Steinheim, Germany). Reagent water was obtained from a Millipore Milli-Q water purification unit (Millipore, Bedford, MA, USA) and was 18.2 MΩ/cm or better.

All pharmaceuticals purchased were ≥97% purity. Propranolol hydrochloride, ketoprofen, bezafibrate, indomethacin, atenolol, salbutamol, sulfamethoxazole, sulfapyridine, sulfamethazine sodium salt, carbamazepine, (±)-metoprolol (±)-tartrate salt, erythromycin, doxazosin mesylate, phenazone, nifedipine, budesonide, simvastatin, tamoxifen, sertraline hydrochloride, citalopram hydrobromide, amitriptyline hydrochloride, nortriptyline hydrochloride, cimetidine, ranitidine hydrochloride, tramadol hydrochloride, chloramphenicol, metformin, and clotrimazole were all obtained from Sigma-Aldrich (Steinheim, Germany). Trimethoprim, caffeine and ciprofloxacin were ordered from Fluka (Buchs, Switzerland). Cocaine hydrochloride, morphine sulfate salt pentahydrate, methadone hydrochloride and heroin were purchased under license from Sigma-Aldrich (St. Louis, MO, USA). Benzoylecgonine hydrate, cocaethylene, temazepam, diazepam, papaverine hydrochloride, 3,4-methylenedioxymethamphetamine hydrochloride (MDMA), ketamine hydrochloride, lysergic acid diethylamide (LSD) and 2-ethylidine-1,5dimethyl-3,3-diphenylpyrrolidine perchlorate (EDDP) were purchased under license from Sigma-Aldrich (Poole, UK).

All glassware utilised in the preparation of pharmaceutical and illicit drug stocks and standards was silanised prior to the preparation to prevent the drugs from adhering to glass surfaces. This was carried out by rinsing the glassware with 10% dichlorodimethylsilane in dichloromethane. This was followed by rinsing with dichloromethane and rinsing with methanol twice each. Stock solutions of pharmaceuticals were prepared in methanol to a concentration of 1000 mg/L and were stored in a fridge at 4 ^{o}C. Working standards were prepared freshly before analysis in ultra pure water. Structural information of each pharmaceutical is given in **Table 4.1**, the illicit drugs are not included as they have previously been described.

4.2.2 Sample collection and preparation:

Samples of digested sludge were taken from two different wastewater treatment facilities. After collection these samples were frozen until required for analysis. The sampling site from which the sludge was obtained serves a population of approximately 1.7 million and had a wastewater throughput of 5 x 10^8 L per day. The sample was a thermally treated granular sludge with $\geq$90% dried solid matter and is one of two biosolid fertilisers employed at the plant. The sludge sample was freeze dried to remove excess moisture and ground up with a mortar and pestle resulting in a low density, flocculent sample.

Soil grab samples were obtained from farmland in Ireland. The samples were allowed to air dry in a fridge for a week before being ground up in a mortar and pestle. The resulting mass was passed through a 100 μm sieve to ensure a homogenous particle size and comprehensive extraction of the organic sample matrix.

Urine samples were collected from volunteers and stored at 4 ^{o}C in the fridge until analysis. For the peptide study a MassPREP digestion standard of bovine serum albumin was obtained from Waters Corporation (Dublin, Ireland). The standard had been prepared by digesting bovine serum albumin using trypsin. The standard was stored at room temperature in a sealed packet until ready for analysis as specified.

Table 4.1: Structural information for each target pharmaceutical

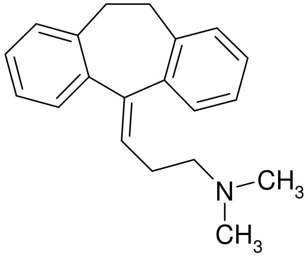
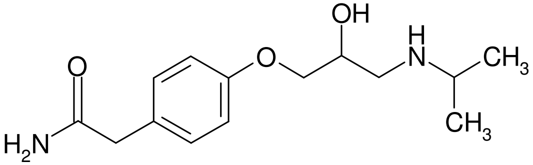
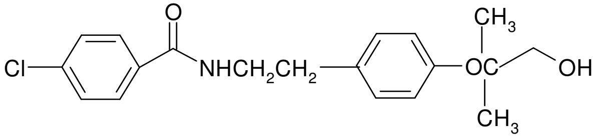
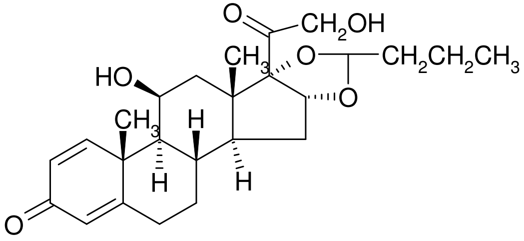
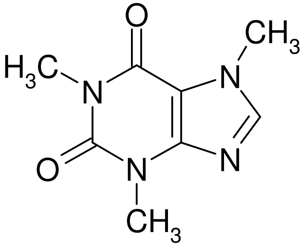
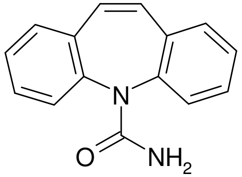
Name	Structure	Name	Structure
Amitriptyline		Atenolol	
Bezafibrate		Budesonide	
Caffeine		Carbamazepine	

Table 4.1 Contd.

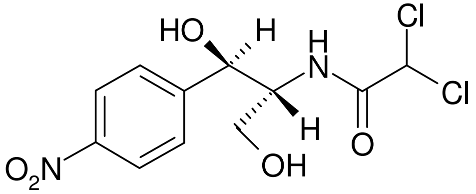
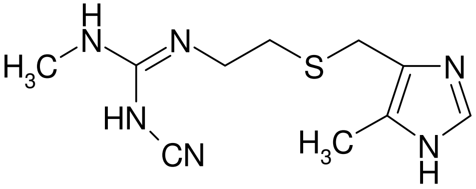
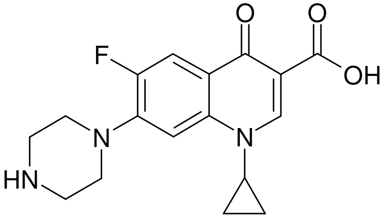
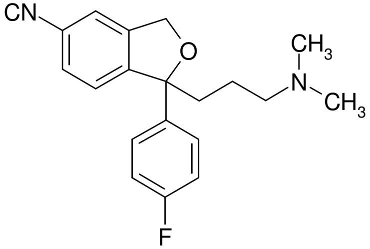
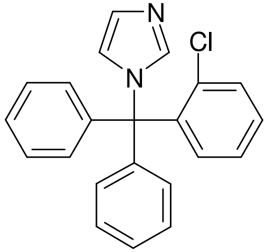
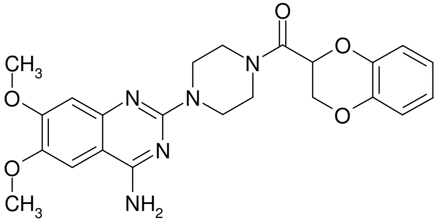
Chloramphenicol		Cimetidine	
Ciprofloxacin		Citalopram	
Clotrimazole		Doxazosin	

Table 4.1 Contd.

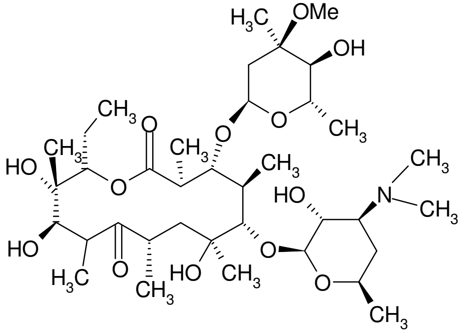
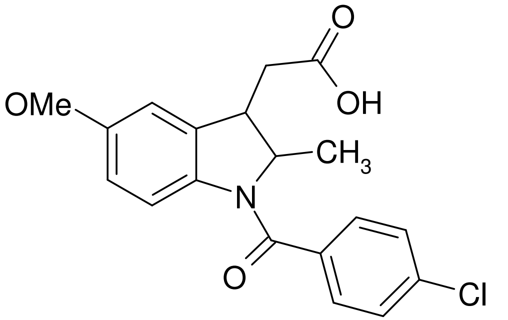
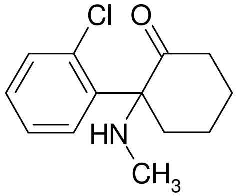
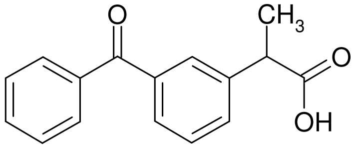
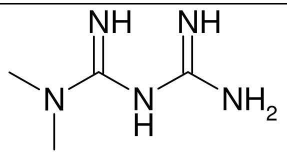
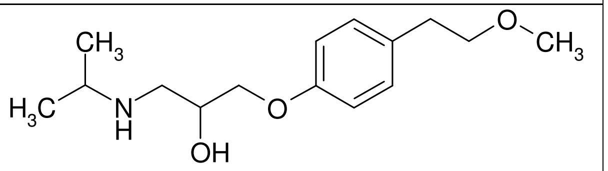
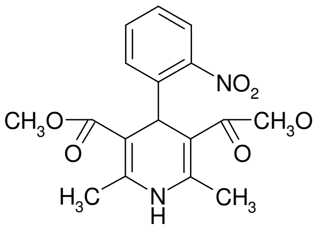
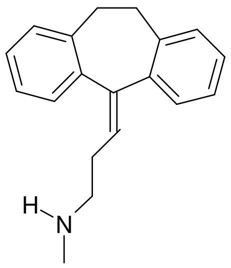
Erythromycin		Indomethacin	
Ketamine		Ketoprofen	
Metformin		Metoprolol	
Nifedipine		Nortriptyline	

Table 4.1 Contd.

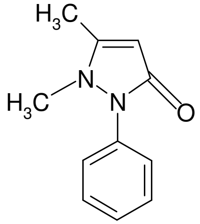
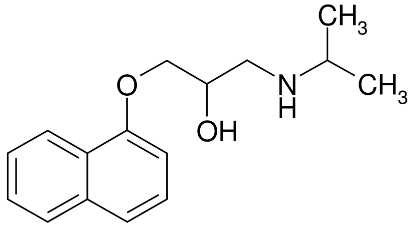
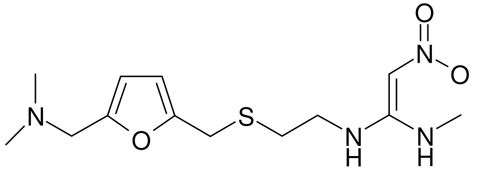
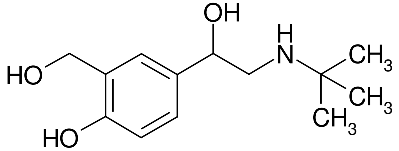
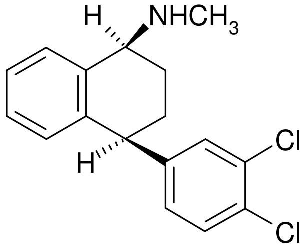
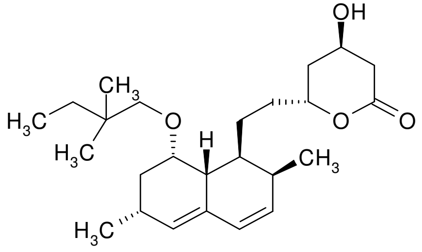
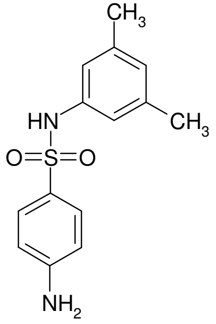
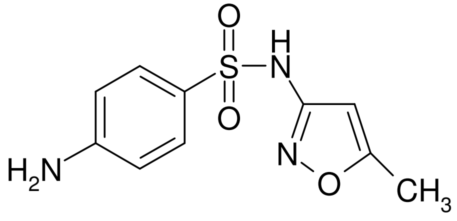
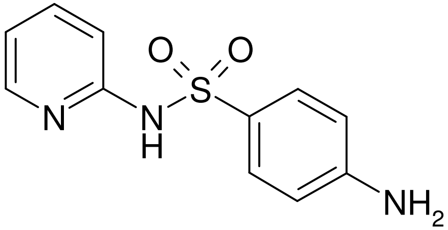
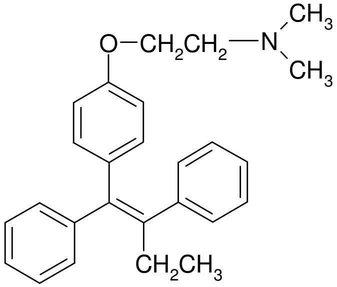
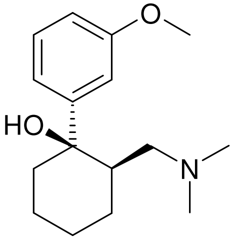
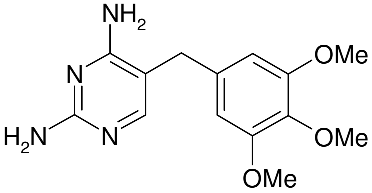
Phenazone		Propranolol	
Ranitidine		Salbutamol	
Sertraline		Simvastatin	
Sulfamethazine		Sulfamethoxazole	

Table 4.1 Contd.

Sulfapyridine		Tamoxifen	
Tramadol		Trimethoprim	

4.2.3 Extraction procedures:

In the case of the soil and sludge extraction, pressurised liquid extraction (PLE) was employed. Depending on the type of experiment, 2.7 g or 5 g of soil or 1 g of sludge were weighed out and mixed with white quartz sand (analytical grade) and transferred directly to the stainless steel extraction cell (33 cm^3). The sample was spiked with a working standard of pharmaceuticals in milli-Q water at this time if required. Any remaining empty space in the cell was filled with sand and the cell was tightly sealed. Extraction was carried out in the Dionex ASE-200 (Dionex Corporation, Sunnyvale, CA, USA). The extraction solvent was 50 : 50 v/v methanol : milli-Q water and the resulting extract of approximately 50 mL was collected in brown glass collection vials. Extraction method parameters were previously optimised.[12] Operating temperature and pressure were 60 ^oC and 1,500 p.s.i. respectively, heating and static periods were five minutes each and the flush volume was 33 cm^3 over one minute.[12]

After extraction, soil samples were simply exaporated to dryness under a nitrogen stream at 50 ^oC and reconstituted in 1 mL of 90 : 10 v/v 10 mM ammonium acetate in water : MeCN or a 1 mg/L standard solution of pharmaceuticals in ultra pure water where required. Sludge extracts, however, due to a higher organic load, required a clean-up/preconcentration step. The extracts were diluted to a 1 L using milli-Q water and the sample solutions were adjusted to approximately pH5. This was done using 2% acetic acid solution. Solid phase extraction was performed on Waters Oasis HLB 6 mL cartridges (Waters Ireland, Dublin, Ireland). The cartridges were conditioned with 6 mL of methanol and 6 mL of milli-Q water prior to loading. The samples were then left to extract on the cartridges overnight. The sorbents were washed with 1 mL milli-Q water and allowed to dry for 30 minutes after loading. The target analytes were eluted in 10 mL of 50 : 50 ethylacetate : acetone and collected in 12 mL glass vials. The eluates were then dried down under nitrogen at 50 ^oC and reconstituted in 1 mL of 90 : 10 v/v 10 mM ammonium acetate in water : MeCN or a 1 mg/L standard solution of pharmaceuticals in milli-Q water where required. For study of suppression effects in ESI-MS detection of pharmaceuticals in soil and sludge, an unspiked sample was extracted using the described method, the extract was evaporated to dryness and reconstituted in 1 mL of a 1 mg/L standard solution of pharmaceuticals in milli-Q water.

Urine samples were collected from volunteers in Sterilin 50 mL centrifuge tubes (Bibby Sterilin Ltd, Stone, Staffs, UK) and were immediately stored in the fridge (≤4 °C) until analysis. 1 – 1.5 mL aliquots of sample were transferred to Sterilin 1.5 mL centrifuge tubes (Bibby Sterilin Ltd, Stone, Staffs, UK) and centrifuged at 10,000 rpm for 10 minutes to remove any precipitated matter. The pH of the sample was then measured to ensure it was in the range pH 2-7 so as not to damage the monolithic column. If needed the pH was adjusted using acetic acid and ammonia solutions. Urine samples were then analysed neat or were spiked by evaporating 1 mL of a 1 mg/L standard solution of pharmaceuticals to dryness under nitrogen and reconstituting the residue in 1 mL of neat urine sample. No clean-up or preconcentration procedure was required prior to analysis of the urine samples.

The peptide sample was reconstituted in 100 µL of 0.1% formic acid in milli-Q water and vortexed. A 10 µL injection of the protein digest was analysed on the half-metre monolith using UV detection at 214 nm tandem.

4.2.4 Liquid chromatography-mass spectrometry:

Liquid chromatography was carried out using five Phenomenex Onyx Monolithic 100 x 3.0 mm i.d. columns. The five monolithic columns were coupled in sequence using peek tubing and ferules resulting in a total column length of approximately 50 cm or 0.5 m. Previously optimized gradient conditions were 100 % 90 : 10 10 mM ammonium acetate acetonitrile linearly increasing organic content to 100 % 20 : 80 10 mM ammonium acetate acetonitrile over 90 minutes and held constant over 20 minutes. Re-equilibration time was 20 minutes. Separations were carried out at a flow rate of 0.2 mL/min using an injection volume of 10 µL at an optimised column temperature of 45 °C. For suppression studies the monolithic column was compared to a Waters Sunfire 150 x 2.1 mm i.d. 3.5 µm column. Separations were carried out on the Sunfire using an identical method to the monolithic column except for column temperature which was ambient.

LC-UV, LC-MS and LC-MS/MS analysis was carried out using an Agilent 1100 series high performance liquid chromatograph with a vacuum degasser, binary pump, ALS autosampler, and diode array detector. This LC was coupled to a Bruker Daltonics Esquire~LC ESI-ion trap mass spectrometer. Detection was carried out in single and tandem MS modes. Agilent chemstation version A.09.03 (Agilent Technologies, USA) and Bruker Daltonics esquire control version 4.0 (Bruker Daltonics, UK) were employed to control the system and data analysis was performed using Bruker Daltonics Data Analysis 3.0 (Bruker Daltonics, UK).

In order to tune the mass spectrometer and determine the parent and precursor ions for each drug, individual 10 mg/L solutions of each drug made up in 100% methanol were infused directly into the mass spectrometer. This was carried out using a Cole-Palmer syringe pump and a Hamilton micro-syringe. Once the molecular ion was identified, it was fragmented to obtain the product ion for each drug in tandem MS mode. During analysis definitive identification of drug residues was obtained by identifying the individual transitions between parent and product ions in tandem MS mode. Quantitative measurements were achieved by integrating the most intense peak exhibited for each illicit drug. The optimised MS conditions are listed in **Table 4.2**.

Table 4.2: ESI-MS parameters employed for analysis.

Parameter	**Positive Mode**
Capillary Voltage	4500
End Plate Offset (V)	-561
Skimmer 1 (V)	28.1
Skimmer 2 (V)	6.7
Cap. Exit Offset (V)	63.7
Octopole (V)	2.51
Octopole RF (Vpp)	155.3
Octopole Delta (V)	1.98
Lens 1	-2.4; -37.8
Trap Drive	34.7
Dry Gas Flow (N_2; L/min)	8
Nebulizer Pressure (psi)	50
Dry Gas Temp. (^oC)	300

4.3 Results and Discussion:

4.3.1 Mass spectromety and tandem mass spectrometry detection:

Standard solutions containing ~1 mg/L of all analytes in methanol were infused directly into the electrospray source at a flow rate ~300 µL/hr in positive ionisation mode to determine precursor and product ions. A large proportion of the target analytes listed in **Table 4.3** were previously investigated in our laboratory.[12, 29, 30] In agreement with these studies, simple pseudo molecular ion $[M + H]^+$ spectra were recorded for the majority of pharmaceuticals in single MS mode, e.g. atenolol, caffeine, trimethoprim and metoprolol. Clotrimazole pseudo molecular ions however displayed no signal of significant intensity. An intense signal at m/z 277 indicated fragmentation and the loss of an imidazole group under MS^1 conditions which most likely arose from collisionally induced dissociation (CID).

Table 4.3: Proposed precursor and product ions for target analytes in positive ion mode MS.

Analyte	m/z	Precursor ion	m/z	Product ion
Amitriptyline	278	$[M + H]^+$	233	$[M + H]^+ - [NH(CH_3)_2]$
Atenolol	267	$[M + H]^+$	190	$[M + H]^+ - [H_2O]\text{-}[C_3H_7]\text{-}[NH_2]$
Benzoylecgonine	290	$[M + H]^+$	168	$[M + H]^+ - [C_6H_5COOH]$
Bezafibrate	362	$[M + H]^+$	316	$[M + H]^+ - [HCOOH]$
Budesonide	431	$[M + H]^+$	413	$[M + H]^+ - [H_2O]$
Caffeine	195	$[M + H]^+$	138	$[M + H]^+ - [CONCH_3]$
Carbamazepine	237	$[M + H]^+$	194	$[M + H]^+ - [CONH]$
Chloramphenicol	345	$[M + Na]^+$	275	$[M + Na]^+ - [NO_2H]$
Cimetidine	253 275	$[M + H]^+$ $[M + Na]^+$	211	$[M + H]^+ - [NH_2\text{-}CN]$
Ciprofloxacin	332	$[M + H]^+$	314 288	$[M + H]^+ - [H_2O]$ $[M + H]^+ - [CO_2]$
Citalopram	325	$[M + H]^+$	262	$[M + H]^+ - [F]\text{-}[NH(CH_3)_2]$
Clotrimazole	277	$[M - C_3H_3N_2H]^+$	165	$[M - C_3H_3N_2H] - [C_6H_5Cl]$
Cocaethlyene	318	$[M + H]^+$	196	$[M + H]^+ - [C_6H_5COOH]$
Cocaine	304	$[M + H]^+$	182	$[M + H]^+ - [C_6H_5COOH]$
Diazepam	285	$[M + H]^+$	257	$[M + H]^+ - [CO]$
Doxazosin	452	$[M + H]^+$	344	$[M + H]^+ - [C_6H_4O_2]$
EDDP	278	$[M + H]^+$	249	$[M + H]^+ - [C_2H_5]$
Erythromycin	734	$[M + H]^+$	576	$[M + H]^+ - [C_5H_4O(CH_3)_2(OH)(OCH_3)]$
Heroin	370	$[M + H]^+$	268	$[M + H]^+ - -[CH_3CO]\text{-}[CH_3CO_2]$
Indomethacin	358	$[M + H]^+$	174	$[M + H]^+ - [C_6H_4Cl\text{-}CONHCH_2CH_3]$
Ketamine	238	$[M + H]^+$	220	$[M + H]^+ - [H_2O]$

Table 4.3 Contd.

Ketoprofen	255	$[M + H]^+$	209	$[M + H]^+ – [HCOOH]$
LSD	324	$[M + H]^+$	223	$[M + H]^+ – [CO\text{-}N(C_2H_5)_2]$
MDMA	194	$[M + H]^+$	163	$[M + H]^+ – [NH_2CH_3]$
Metformin	130	$[M + H]^+$	–	
Methadone	310	$[M + H]^+$	265	$[M + H]^+ – [NH(CH_3)_2]$
Metoprolol	268	$[M + H]^+$	116	$[M + H]^+ – [C_8H_8OHOCH_3]$
Morphine	286	$[M + H]^+$	268	$[M + H]^+ – [H_2O]$
Nifedipine	345	$[M + H]^+$	–	
Nortriptyline	264	$[M + H]^+$	233	$[M + H]^+ – [NH_2CH_3]$
Papaverine	340	$[M + H]^+$	202	$[M + H]^+ –[C_6H_4(OCH_3)_2]$
Phenazone	189	$[M + H]^+$	–	
Propranolol	260	$[M + H]^+$	116	$[M + H]^+ – [C_6H_4\text{-}C_4H_3\text{-}OH]$
Ranitidine	315	$[M + H]^+$	270	$[M + H]^+ – [NH(CH_3)_2]$
Salbutamol	240	$[M + H]^+$	166	$[M + H]^+ – [NH_2\text{-}C(CH_3)_3]$
Sertraline	275	$[M – NH_2CH_3]^+$	–	
Simvastatin	441	$[M + Na]^+$	–	
Sulfamethazine	279	$[M + H]^+$	156	$[M + H]^+ – [NH_2\text{-}C_4HN_2\text{-}(CH_3)_2]$
Sulfamethoxazole	254	$[M + H]^+$	156	$[M + H]^+ – [NH_2\text{-}C_3HNO\text{-}CH_3]$
Sulfapyridine	250	$[M + H]^+$	–	
Tamoxifen	372	$[M + H]^+$	327	$[M + H]^+ – [NH\text{-}(CH_3)_2]$
Temazepam	301	$[M + H]^+$	283	$[M + H]^+ – [H_2O]$
Tramadol	264	$[M + H]^+$	–	
Trimethoprim	291	$[M + H]^+$	123	$[M + H]^+ – [C_6H_3\text{-}(OCH_3)_3]$

Other more recently obtained pharmaceuticals were also infused and their base peak and fragment ions elucidated. Again, in most cases, the pseudo molecular ion was observed, e.g. doxazosin, tamoxifen and ranitidine, however some other base peak ions were also observed. For example, the sodiated adducts $[M + Na]^+$ of chloramphenicol, cimetidine and simvastatin were recorded at m/z 345, 275 and 441, respectively. Sodiated or potassiated adducts generally arise due to impurities in solvents used for mobile phase preparation. Sertraline fragmented under MS^1 conditions, losing a methylamine group $[M – NH_2CH_3]^+$ to give a base peak at m/z 275. For simvastatin and chloramphenicol, the sodiated ion exhibited an intense signal, whilst for cimetidine a pseudo molecular ion was also present at a higher intensity and was the primary ion used in detection. Generally the ionisation mode used for monitoring an analyte can be predicted by its acidic or basic nature. It is unusual therefore that acidic drugs e.g. bezafibrate, ketoprofen, nifedipine, phenazone and temazepam, which have pK_a values of 3.6, 4.4, 1.0, 1.45 and 1.6, respectively can be successfully monitored in positive mode.[12]

The majority of the MS^2 mode transitions involved the loss of easily identifiable groups from the pseudo molecular ion. One of the most common moieties to be lost was those containing amine, methyl amine and dimethyl amines e.g. amphetamine, atenolol, ranitidine, salbutamol and tamoxifen. Substituted benzene rings are also often cleaved from the molecular ion during fragmentation e.g. trimethoprim, propranolol, doxazosin and clotrimazole. Some similarities in fragmentation patterns were observed for related drug molecules. The antidepressants amitriptyline and nortriptyline both undergo cleavage of an amine side-chain resulting in a shared fragment ion at m/z 233. As previously discussed, cocaine and its two metabolites, benzoylecgonine and cocaethylene all underwent a cleavage of a benzoic acid group from the 7-membered ring. Metoprolol and propranolol also exhibited similar fragmentation pathways which lead to a shared product ion at m/z 116. The sulfonamide antibiotics sulfamethazine and sulfamethoxazole both underwent fragmentation leading to the formation of a common product ion at m/z 156. A third sulfonamide sulfapyridine, despite having structural features in common with sulfamethazine and sulfamethoxazole e.g. sulphur dioxide group, had no fragment ion and was monitored wholly in single MS mode. As can be seen in **Table 4.3** fragment ions were not observed for all analytes e.g. tramadol, simvastatin, sertraline, sulfapyridine and phenazone. For this reason the sensitivity of the method for these analytes was limited to single mode MS monitoring using molecular ions. For calculations of analyte recovery and percentage suppression of pharmaceuticals, the most intense signal observed for each of the target analytes was integrated. The molecular ion was predominantly chosen, however in some cases collisionally induced dissociation of molecular ions to a fragment ion led to a more intense peak being observed for the fragment ion in single MS mode. This phenomenon occurred in particular for the illicit drugs such as MDMA and methadone whose fragment ions were observed as the base peak in single mode MS, not their molecular ions. In these cases the fragment ion was integrated instead of the molecular ion.

4.3.2 Liquid chromatography-mass spectrometry of PPCPs in soil and sludge:

A soil sample was prepared by extracting 5 g spiked with 1 mL of a 1 mg/L standard of pharmaceuticals in milli-Q water using 50 : 50 v/v methanol : milli-Q water as described above. The extracts which had a final spiking concentration of 0.2 mg/Kg were analysed by LC-MS using the half-metre monolithic column. The extraction procedure was very simple with no clean-up or preconcentration step required. A list of single mode MS extracted ion chromatograms for each of the target analytes are shown in **Figure 4.1**. A number of the pharmaceuticals were not detected in the spiked soil sample e.g. amphetamine, doxazosin, budesonide, citalopram, sertraline, papaverine and ranitidine. This could be due to formation of adducts between the compound and a matrix component resulting in a signal at a different mass being detected. Bezafibrate appeared to elute very close to a large peak most likely a background signal and so exhibited a very weak signal in single MS mode and is not displayed in **Figure 4.1**.

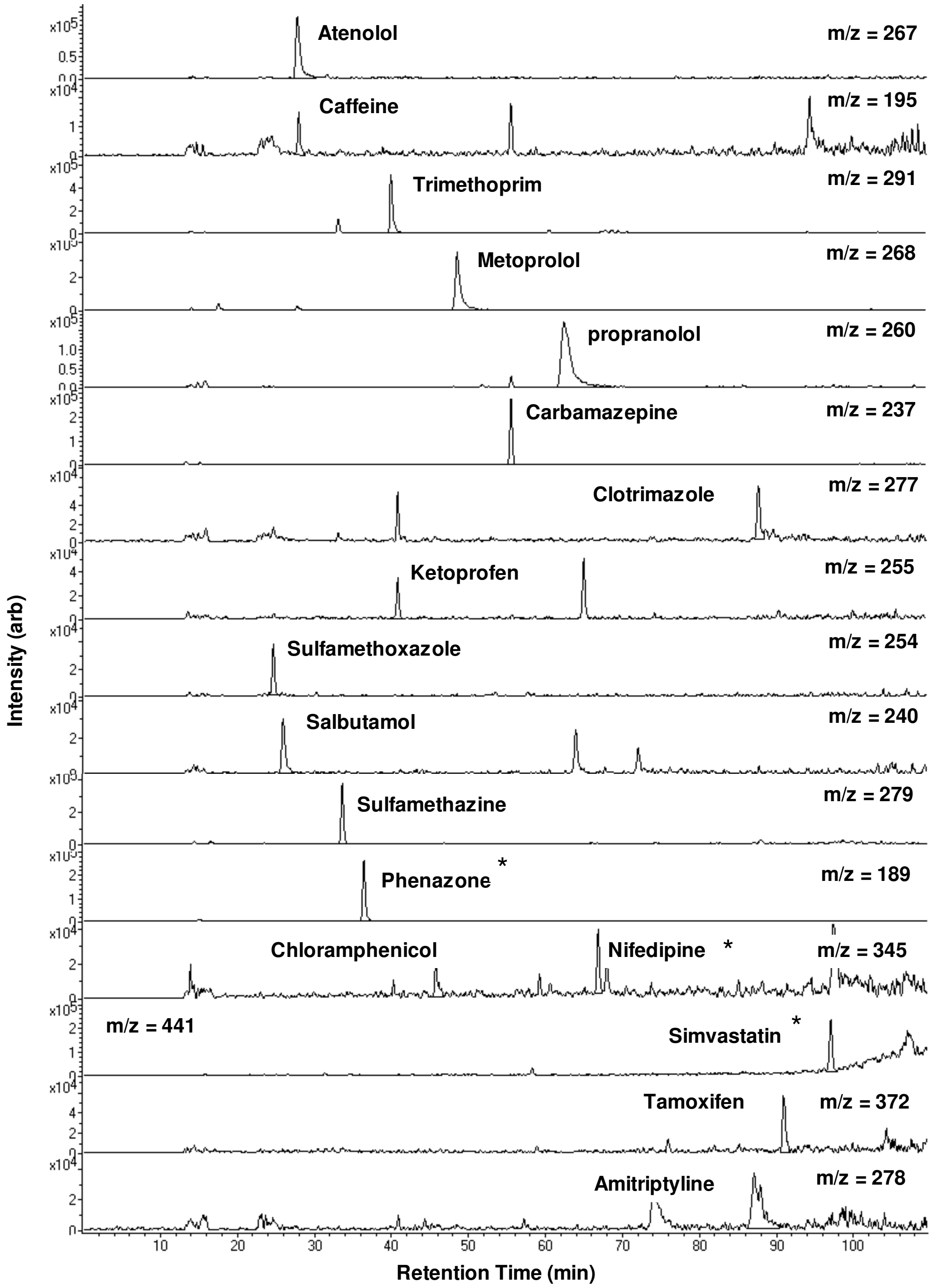


Figure 4.1: Extracted ion chromatograms for 34 pharmaceuticals spiked into soil sample at a concentration of 0.2 mg/Kg. Chromatograms marked with * not subject to tandem MS fragmentation.

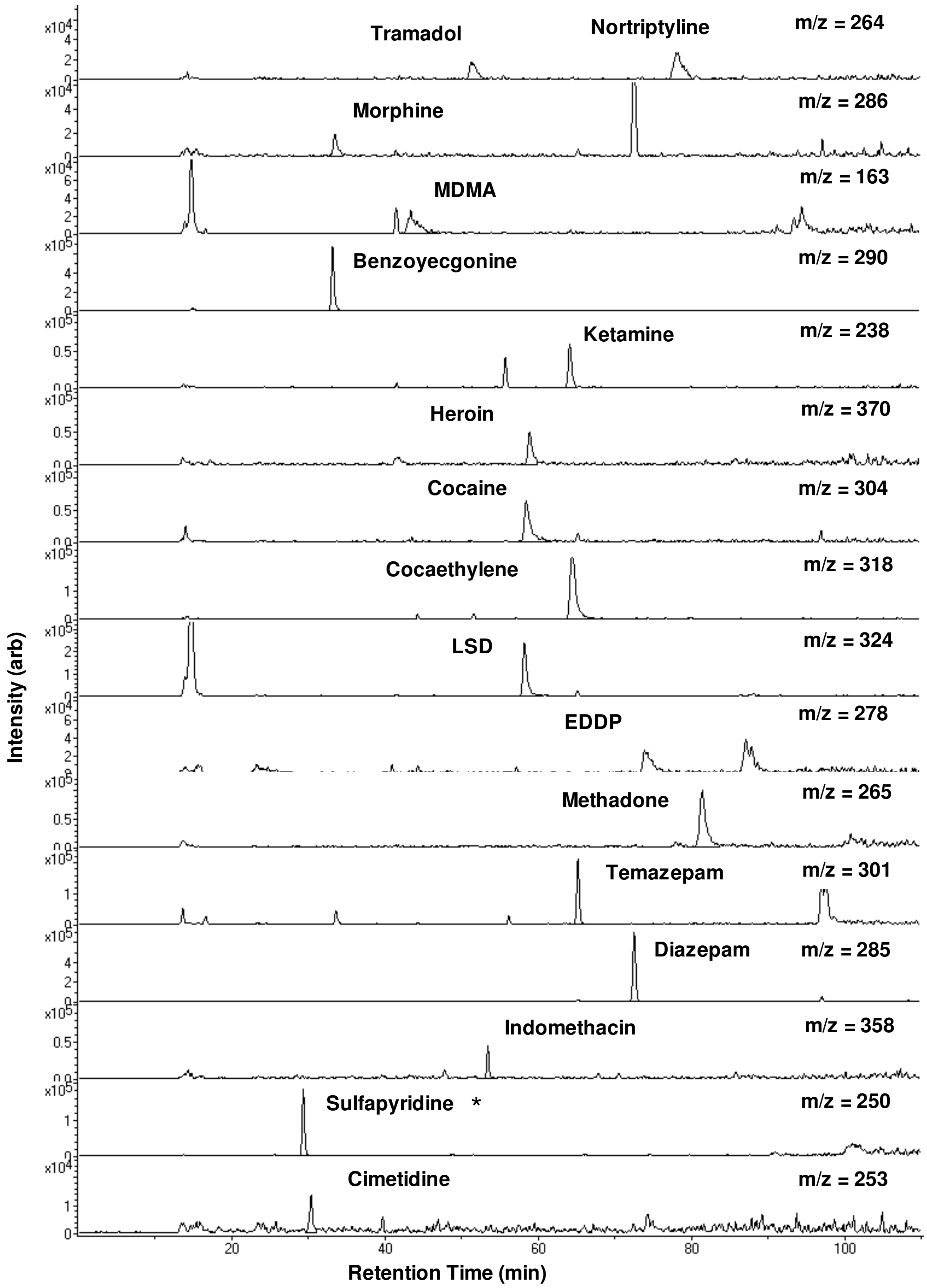


Figure 4.1 Contd.: Extracted ion chromatograms for 34 pharmaceuticals spiked into soil sample at a concentration of 0.2 mg/Kg. Chromatograms marked with * not subject to tandem MS fragmentation.

Table 4.4: Chromatographic data calculated for each of the analytes in **Figure 4.1**.

Name	**Retention time (min)**	**Width (min)**	**Resolution (R_s)**	**Capacity factor (k)**
Sulfamethoxazole	24.6	0.8		0.6
Salbutamol	25.8	1.8	0.9	0.7
Atenolol	27.8	2.9	0.9	0.8
caffeine	27.9	1.4	0.0	0.8
Sulfapyridine	29.3	1.25	1.1	0.9
Cimetidine	30.2	1.3	0.7	0.9
Benzoyecgonine	33	1.8	1.8	1.1
Morphine	33.4	2	0.2	1.1
Sulfamethazine	33.5	1.6	0.1	1.1
Phenzone	36.4	1.6	1.8	1.3
Trimethoprim	40	2	2.0	1.5
Ketoprofen	40.8	1.5	0.5	1.5
MDMA	43.3	3.6	1.0	1.7
Chloramphenicol	45.7	1.6	0.9	1.8
Metoprolol	48.6	3.8	1.1	2.0
Tramadol	51.1	2.6	0.8	2.2
Indomethacin	53.4	1.5	1.1	2.3
Carbamazepine	55.7	1.4	1.6	2.4
LSD	58.1	3	1.1	2.6
Cocaine	58.4	2.9	0.1	2.6
Heroin	58.8	1.8	0.2	2.6
Propranolol	62.5	5	1.1	2.8
Ketamine	64	1.6	0.5	2.9
Cocaethylene	64.3	4	0.1	2.9
Temazepam	65.1	1.5	0.3	3.0
Nifedipine	66.8	0.9	1.4	3.1
Diazepam	72.5	1.5	4.8	3.4
EDDP	73.9	3.1	0.6	3.5
Nortriptyline	78.2	3.2	1.4	3.7
Methadone	81.3	3.4	0.9	3.9
Amitriptyline	87.1	4.3	1.5	4.3
Clotrimazole	88	1.2	0.3	4.3
Tamoxifen	90.9	1.2	2.4	4.5
Simvastatin	97	1.4	4.7	4.9

Table 4.4 displays chromatographic information describing the separation of the 34 analytes listed in **Figure 4.1**. Resolution and capacity factor (α) were calculated using Eqn. 2.1 and Eqn. 4.1, respectively.

$$\alpha = \frac{(t_R)_B - t_0}{(t_R)_A - t_0} \qquad \text{(Eqn. 4.1)}$$

From **Table 4.4** it is obvious that not all pharmaceuticals were completely resolved from each other particularly in the case of caffeine and atenolol, morphine and benzoylecgonine and cocaine and heroin. Analytes that are not adequately separated from one another and elute at the same retention time can result in suppression of signals during ESI-MS detection. However, many of the target analytes were adequately resolved with values reported greater than 1.0 e.g. indomethacin and carbamazepine, amitriptyline and methadone and clotrimazole and tamoxifen. The low resolution values were mimicked by low relative retention for these groups of analytes also indicating incomplete separation. Capacity factors (k) of 2-10 are desirable,[31] however a number of the analytes showed little or no affinity for the monolithic stationary phase e.g. sulfamethoxazole, salbutamol, cimetidine and sulfapyridine, reporting k values of less than one. Due to the length of the stationary phase even the low affinity analytes appeared at retention times of between 24 and 45 minutes and were in many cases well resolved from each other. Later eluting compounds were well retained by the monolithic bed but not so much so as to over extend retention times and broaden peaks. As gradient and mobile phase conditions had been optimised previously (Chapter two) as well as column efficiency, this separation was deemed adequate taking into account the number of analytes and the selectivity of the detection method.

4.3.3 Matrix effects in soil and sludge:

A UV chromatogram of the separation blank sludge extract on the half-metre monolith is depicted in **Figure 4.2**. This figure illustrates the complexity and abundance of the matrix components present in the sample and highlights the excellent ability of the long column to separate them.

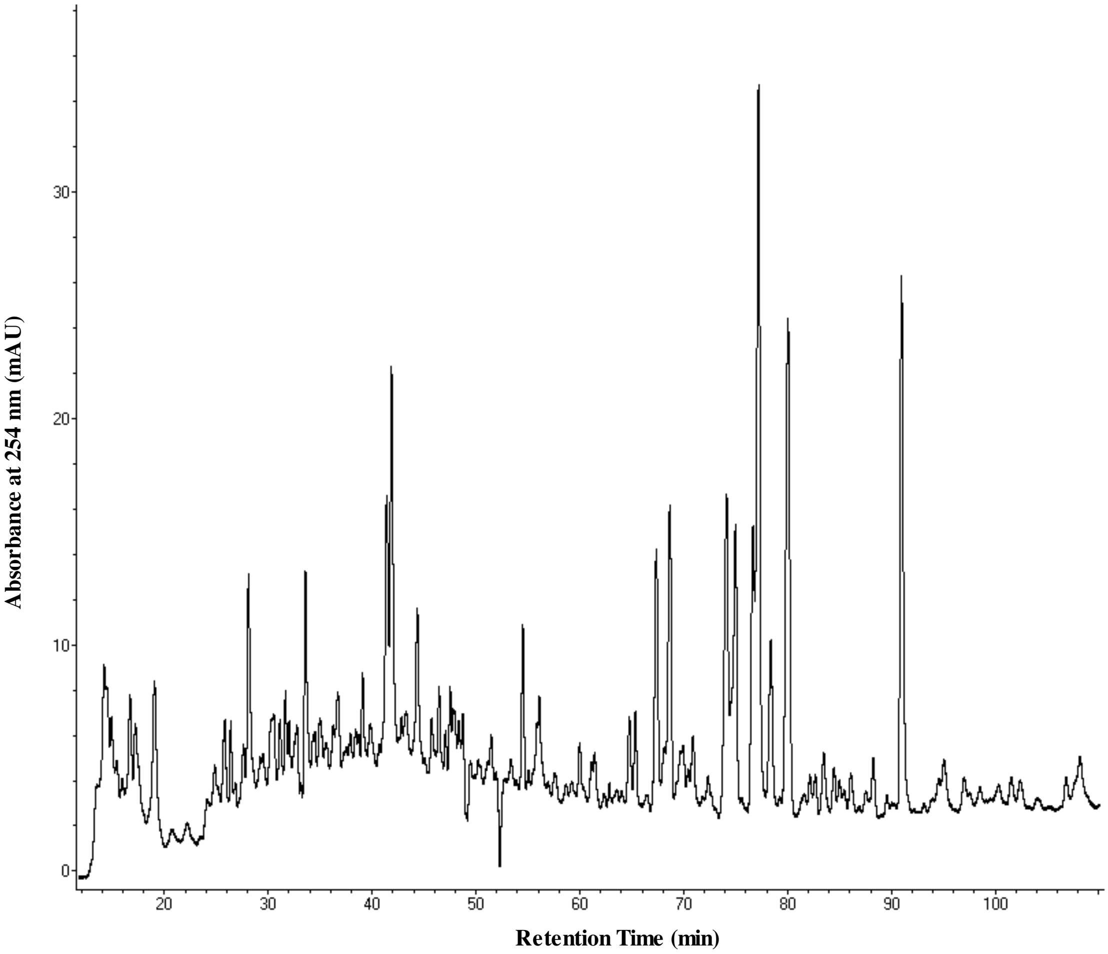


Figure 4.2: Separation of blank sludge extract on 0.5 m monolithic column at 254 nm. Time axis offset by 10 minutes.

Two blank sample extracts were prepared as described using 2.7 g of soil and 1 g of sludge. Post extraction, the samples were spiked with 1 mL of a 1 mg/L standard of pharmaceuticals in milli-Q water. The degree of analyte signal suppression in both soil and sludge was determined by comparing these sample extracts spiked with post extraction with a 1 mg/L standard as previously described.[12] The pharmaceuticals spiked into the sample post extraction underwent none of the extraction procedures and so should report 100% recovery. Any reduction in signal intensity must therefore be due to matrix components within the sample or coeluting pharmaceuticals which cause analyte suppression. To further demonstrate the applicability of the monolithic column to the analysis of complex samples, the extracts were analysed on a Waters Sunfire 150 x 2.1 mm i.d. 3.5 µm column under the same chromatographic and gradient conditions. Suppression is calculated as the percentage loss of analyte recovery. Relative suppression of all analytes on both columns was calculated as a ratio of their respective absolute suppression percentages with a relative suppression of less than one indicating lower levels of suppression on the monolithic column. The results of the suppression studies in soil are presented in **Table 4.5** and the results of the suppression study in digested sludges are presented in **Table 4.6**.

Table 4.5: Percentage recovery and suppression for 41 pharmaceuticals in soil. Calculated by comparing a soil extract spiked post extraction with a 1 mg/L standard solution in milli-Q water. The shaded cells indicate those that exhibited less suppression on the monolithic column.

	% MS suppression in soil		**Relative Suppression**
Name	**Sunfire**	**Monolith**	
Metformin	53 ± 24	4 ± 14	0.1
Salbutamol	-14 ± 2	-2 ± 9	0.1
Sulfamethoxazole	-2 ± 1	8 ± 24	4.5
Atenolol	4 ± 2	5 ± 3	1.4
Caffeine	10 ± 8	19 ± 19	1.9
Sulfapyridine	10 ± 1	8 ± 9	0.9
Cimetidine	15 ± 1	3 ± 7	0.2
Sulfamethazine	12 ± 2	11 ± 5	0.9

Table 4.5 Contd.			
Morphine	17 ± 0	-1 ± 2	0.1
Benzoylecgonine	-9 ± 3	-5 ± 7	0.5
Ranitidine	26 ± 2	11 ± 4	0.4
Phenazone	10 ± 3	1 ± 6	0.1
MDMA	2 ± 3	-33 ± 28	17.8
Trimethoprim	12 ± 1	1 ± 7	0.1
Metoprolol	9 ± 5	6 ± 4	0.7
Ketoprofen	16 ± 4	14 ± 9	0.8
Tramadol	1 ± 9	1 ± 15	
Chloramphenicol	-70 ± 108	8 ± 16	0.1
Bezafibrate	35 ± 5	18 ± 15	0.5
Cocaine	31 ± 7	24 ± 0	0.8
Heroin	38 ± 4	19 ± 0	0.5
Propranolol	20 ± 1	15 ± 8	0.7
LSD	11 ± 2	-1 ± 3	0.1
Indomethacin	15 ± 6	6 ± 8	0.4
Carbamazepine	14 ± 3	7 ± 7	0.5
Cocaethylene	17 ± 9	7 ± 0	0.4
Ketamine	13 ± 5	-3 ± 1	0.2
Citalopram	25 ± 1	23 ± 1	0.9
Papaverine	17 ± 1	12 ± 2	0.7
EDDP	11 ± 0	6 ± 2	0.5
Doxazosin	34 ± 4	38 ± 2	1.1
Temazepam	18 ± 3	17 ± 3	1.0
Nortriptyline	42 ± 4	43 ± 0	1.0
Nifedipine	3 ± 31	14 ± 4	5.5
Budesonide	20 ± 20		
Methadone	19 ± 3	17 ± 7	0.9
Amitriptyline	40 ± 1	41 ± 2	1.0
Diazepam	12 ± 9	9 ± 4	0.8
Sertraline	66 ± 2	63 ± 2	0.9
Clotrimazole	73 ± 0	67 ± 2	0.9
Tamoxifen	72 ± 1	56 ± 6	0.8
Simvastatin	62 ± 0	58 ± 4	0.9

Overall the monolith was by far the better phase for separating complex samples with an average suppression value for the monolith being 15% and 19% for the sunfire. In general the effect of the soil matrix was quite low for the monolithic column with percentage suppression equal to or below 10% for half the target analytes. This was true for only nine of the pharmaceuticals when separated on the Sunfire. Of the 41 drugs compared in the study levels of suppression were lower for 32 drugs on the monolith, six analytes exhibited lower levels of ion suppression on the Sunfire column and 3 of the analytes were equally affected in both experiments. Erythromycin, ciprofloxacin and amphetamine were not detected in the study perhaps due to complete suppression of the signal, however erythromycin was not present during the analysis of a drug standard suggesting that it was not included in preparation or had degraded. Amphetamine was visible in the separation of a standard solution of the pharmaceuticals but exhibited a very weak signal. This was not uncommon for the stimulant which was detected with difficulty during the hair study carried out in Chapter one. The Sunfire reported reasonable recovery of budesonide, whilst no data was recorded for the drug when using the monolith. This corresponded to the absence of budesonide from the extracted ion chromatograms of the spiked soil sample separated on the monolith presented in **Figure 4.1**.

High levels of suppression were spread throughout the separation though most of the highly affected analytes eluted near the end of the run time e.g. amitriptyline, sertraline, clotrimazole, tamoxifen and simvastatin. This phenomenon could be explained by the high levels of organic matrix components eluting simultaneously due to higher organic content in the mobile phase.[12] Suppression values reported as negatives indicate some enhancement of signals and in these cases the superior result was determined as being that closest to 0% suppression. This is exemplified by benzoylecgonine with % suppression of 109% and 105% on the Sunfire and monolith, respectively. The monolith was determined to be superior as the result was closest to 0% suppression. Results were in some cases completely dissimilar. On the monolith a high level of error (± 28) was reported for MDMA and the suppression result indicated huge enhancement of the peak. The Sunfire however, reported excellent results for this particular analyte with % suppression of only 2% and standard deviation of the same. Chloramphenicol exhibited enhancement on the Sunfire while low levels of suppression were reported by the monolith. Conversely, many results were quite consistent between columns with suppression values for the monolith considerably lower, e.g. sulfapyridine, cimetidine, phenazone, trimethoprim, ketoprofen.

Table 4.6: Percentage recovery and suppression for 37 pharmaceuticals in digested sludge. Calculated by comparing a digested sludge extract spiked post extraction with a 1 mg/L standard solution in milli-Q water. Cells marked n/a (not applicable) indicate that values could not be calculated. The cell marked * indicates that no error could be calculated and the value is based on one analysis alone. The shaded cells indicate those that exhibited less suppression on the monolithic column.

	% MS Suppression in sludge		**Relative Suppression**
Name	**Sunfire**	**Monolith**	
Metformin	73 ± 4	81 ± 3	1.1
Salbutamol	8 ± 4	19 ± 2	2.3
Sulfamethoxazole	47 ± 1	53 ± 2	1.1
Atenolol	12 ± 2	19 ± 1	1.6
Caffeine	7 ± 1	21 ± 18	2.9
Sulfapyridine	55 ± 2	54 ± 3	1.0
Cimetidine	42 ± 1	40 ± 2	1.0
Sulfamethazine	70 ± 1	54 ± 2	0.8
Morphine	68 ± 2	27 ± 10	0.4
Benzoylecgonine	19 ± 4	24 ± 5	1.3
Ranitidine	n/a	n/a	n/a
Phenazone	16 ± 3	15 ± 7	1.0
MDMA	63 ± 3	37 ± 7	0.6
Trimethoprim	45 ± 2	44 ± 5	1.0
Metoprolol	63 ± 1	53 ± 7	0.8
Ketoprofen	63 ± 3	44 ± 0	0.7
Tramadol	42 ± 1	31 ± 2	0.7

Table 4.6 Contd.

Chloramphenicol	n/a	n/a	n/a
Bezafibrate	57 ± 4	33 ± 15	0.6
Cocaine	47 ± 1	53 ± 1	1.1
Heroin	39 ± 1	56 ± 6	1.4
Propranolol	58 ± 1	47 ± 3	0.8
LSD	67 ± 1	52 ± 2	0.8
Indomethacin	60*	60 ± 13	1.0
Carbamazepine	59 ± 8	54 ± 14	0.9
Cocaethylene	50 ± 2	51 ± 3	1.0
Ketamine	33 ± 3	33 ± 3	1.0
Citalopram	73 ± 1	53 ± 1	0.7
Papaverine	54 ± 1	49 ± 2	0.9
EDDP	58 ± 1	50 ± 1	0.9
Doxazosin	46 ± 2	42 ± 3	0.9
Temazepam	58 ± 5	54 ± 7	0.9
Nortriptyline	60 ± <1	47 ± 2	0.8
Nifedipine	n/a	n/a	n/a
Budesonide	23 ± 15	n/a	n/a
Methadone	68 ± <1	42 ± 1	0.6
Amitriptyline	56 ± 1	47 ± 13	0.8
Diazepam	50 ± 4	49 ± 3	1.0
Sertraline	n/a	13 ± 4	n/a
Clotrimazole	78 ± 1	57 ± 8	0.7
Tamoxifen	39 ± <1	46 ± 2	1.2
Simvastatin	69 ± 2	45 ± 20	0.7

Ion suppression was much more significant for pharmaceuticals in sludge extracts. The average level of matrix induced ion suppression for the target analytes on the monolith and Sunfire columns were 43% ± 14 and 50% ± 19 respectively. Only 37 pharmaceuticals were compared in the sludge suppression study as ranitidine, chloramphenicol and nifedipine were undetected by both columns whilst budesonide and sertraline were undetected by the monolith and Sunfire columns, respectively. Signals corresponding to chloramphenicol and nifedipine were low intensity peaks, a fact that is illustrated in **Figure 4.1**. Ranitidine, which was also a weak responder, was undetected in the spiked soil sample. It is most likely that the complexity of the sludge matrix is the reason for the loss of these signals.

Overall the monolith reported lower levels of ion suppression for a total of 25 pharmaceuticals out of 37 with two drugs being equally quenched by matrix components on both columns. As with suppression levels in soil, ion suppression in sludge increased as the separation progressed perhaps due to higher acetonitrile content eluting much of the digested sludge organic matter. However, high levels of suppression were present throughout the separation, with metformin, sulfamethazine, sulfamethoxazole and morphine reporting very low recoveries in the early stages. Polar matrix components with little or no affinity for the hydrophobic stationary phases could be the cause of such results. The sludge matrix obviously contains a wide range of organic compounds ranging from polar to non-polar eluting throughout the run-time and affecting all target analytes. The superior performance of the monolithic column is particularly prevalent when noting the number of analytes with percentage suppression greater than 60%. Only two analytes separated on the monolithic phase were suppressed to this extent whilst 13 analytes were on the Sunfire column. The ability of the half-metre monolith to reduce suppression effects caused by complex matrices such as soil and sludge can be attributed to its increased capacity to separate all sample components (including matrix species) over a greater temporal concentration. This was previously optimised in Chapter two. The sheer length of the stationary bed may result in more efficient separation of the organic matrix leading to less interferents co-eluting or interacting with the pharmaceuticals.

4.3.4 Analysis of neat urine on half-metre monolith by LC-UV-MS/MS:

The half-metre monolith was applied to the direct analysis of PPCPs in undiluted urine samples using a greatly simplified sample clean-up procedure with no need for preconcentration. Initially a preconcentration step was included in sample preparation; however this resulted in not only concentration of any drug residues within the sample but also preconcentration of the matrix components and hence resulted in a much higher background signal and a less sensitive method.

Neat urine samples were prepared as described earlier, by centrifuging and adjusting the pH. A direct injection of 10 μL was made onto the half-metre monolithic column and analysed under identical chromatographic conditions as the soil and digested sludge. The separation of a neat urine sample on the 0.5 M monolith using UV detection at 230 nm is depicted in **Figure 4.3**. From the UV chromatogram, it is clear that a large number of unretained species are eluted near the start of the separation which are unresolved. The inset of **Figure 4.3** shows an expansion of a later section of the separation which illustrates the excellent ability of the monolith to separate complex sample matrix components.

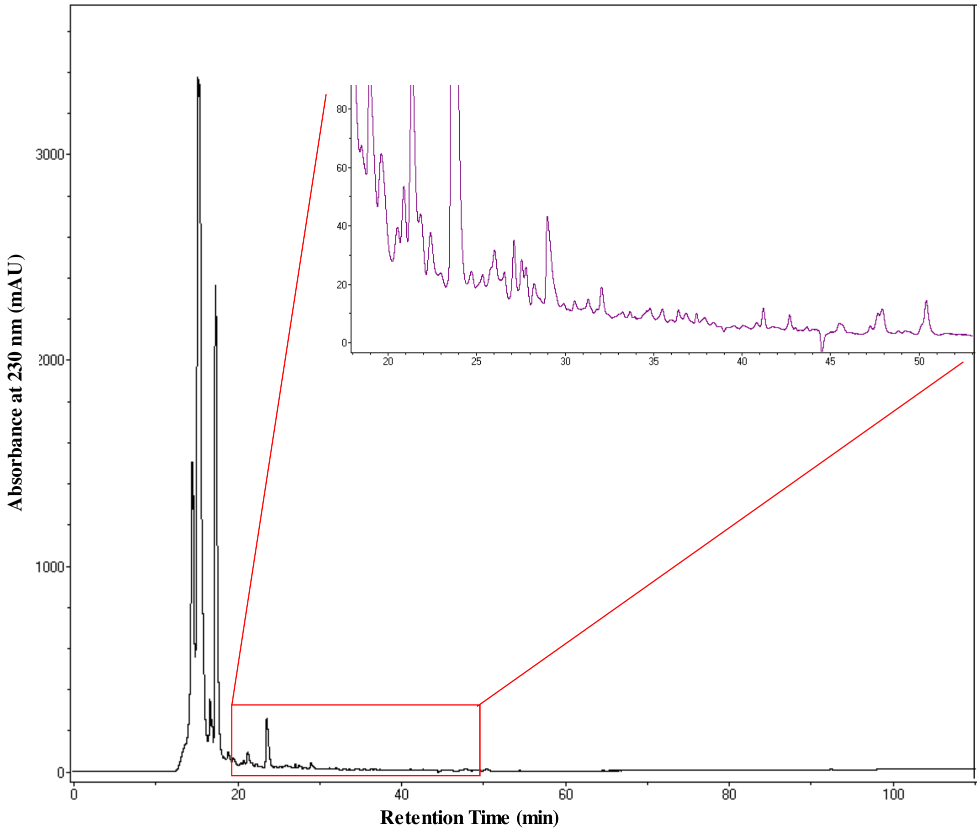


Figure 4.3: UV chromatogram of neat urine sample separated on the half-metre monolith at 230 nm. Inset: Expansion of 30 minute period of the separation, illustrating individual matrix components separated on long monolith.

The unretained species would most likely be polar and ionic species with a high affinity for the polar mobile phase. Uric acid is formed due to the breakdown of food in the body and is excreted in our urine. It has a molecular mass of 168 g/mol and has previously been detected in a range of biological samples by LC-MS using predominantly negative ion mode at m/z 167.[32] It has been reported that the purine metabolite is usually determined in negative ion mode due to better sensitivity; however a molecular ion is also present in positive mode at m/z 169 and a sodiated adduct at m/z 191.[32] A large peak was present at m/z 169 in three of the neat urine samples analysed (**Figure 4.4**) with smaller peaks present at m/z 191. A fragment ion of uric acid due to the loss of 43 mass units (m/z 126) has previously been used in its identification using negative ion mode.[33] However, a peak at m/z 127 was present in positive ion mode analysis of the three neat urine samples which could be due to loss of the same moiety. The presence of the fragment ion in the single mode mass spectrum could be a result of collisionally induced dissociation of the parent ion in single MS mode. This is illustrated by the signal at m/z 126/127 that can be seen in the mass spectrum of uric acid in **Figure 4.4**.

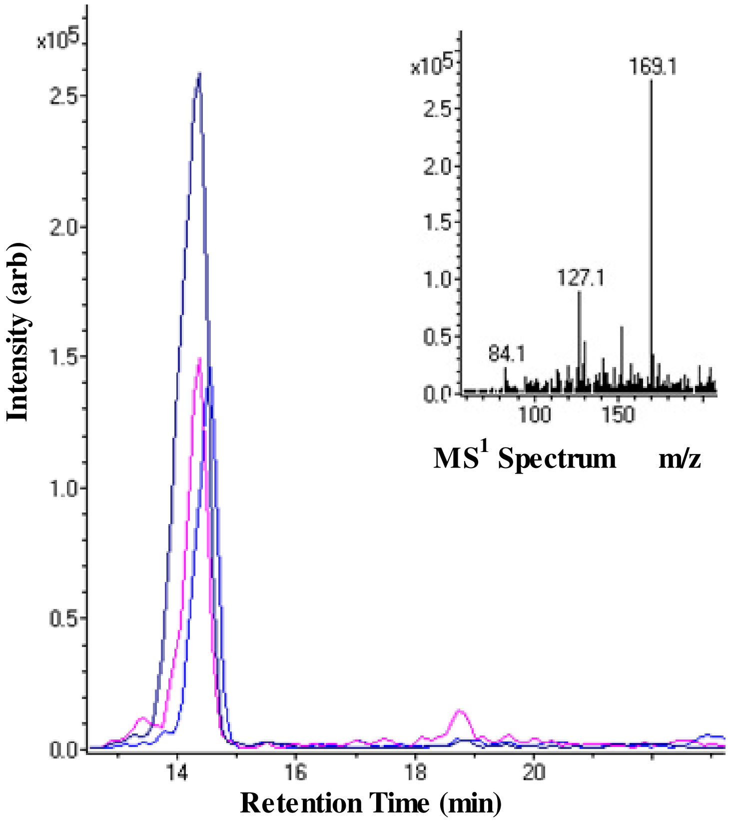


Figure 4.4: Overlaid peaks at m/z 169 thought to be uric acid in three neat urine samples. Inset: MS^1 spectrum at m/z 169.

Two other common components of urine, creatine and creatinine which are broken down in the body to form uric acid have been observed at m/z 132 and m/z 114.[34] Signals were present at these masses in all three neat urine samples analysed and could theoretically represent creatine and its metabolite creatinine (**Figures 4.5 and 4.6**).

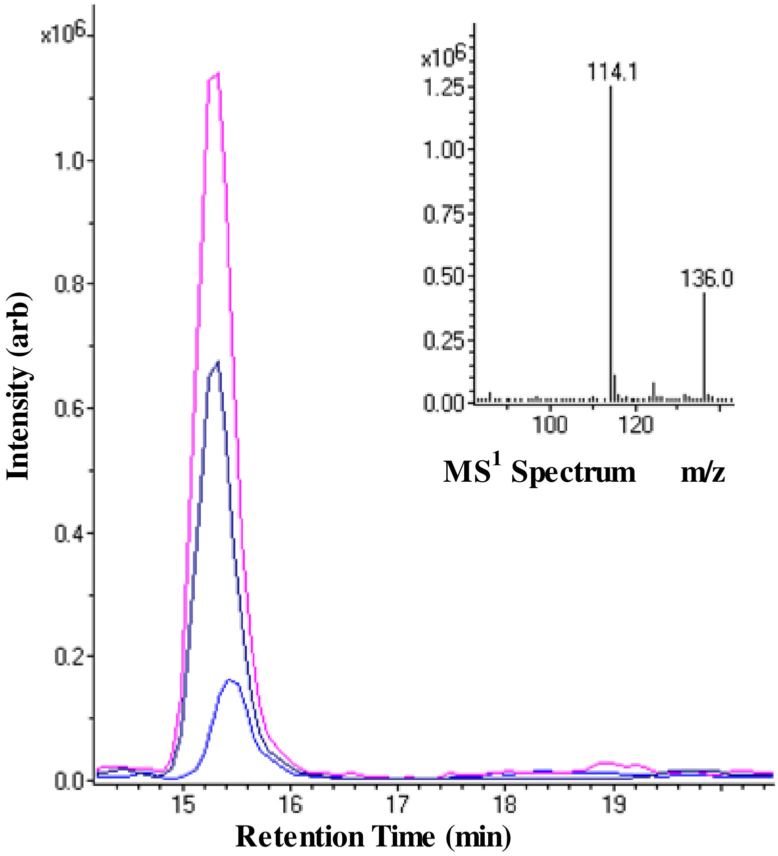


Figure 4.5: Overlaid peaks at m/z 114 thought to be creatinine in three neat urine samples. Inset: MS^1 spectrum at m/z 114.

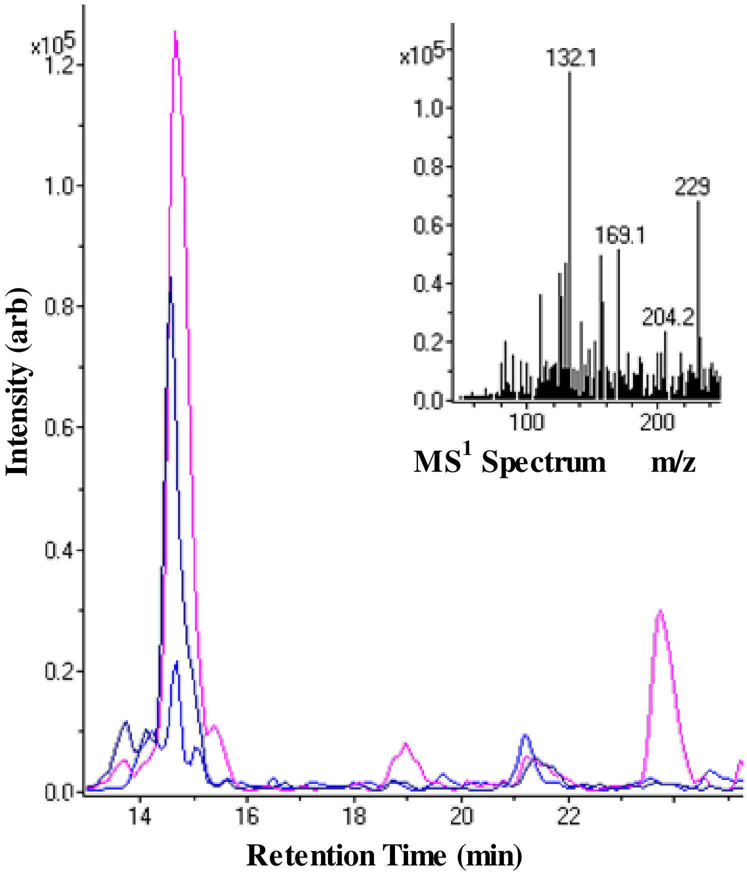


Figure 4.6: Overlaid peaks at m/z 132 thought to be creatine in three neat urine samples. Inset: MS^1 spectrum at m/z 132.

Creatinine, creatine and uric acid are all absorbent in the UV range and had similar retention times to the large signal at the beginning of the UV chromatogram in **Figure 4.3** indicating they could be elements of the weakly retained urine matrix. These substances are just a few of the many which could be eluted quickly from the monolithic column. Other unretained components could include urea, carbohydartes or ions such as chloride, potassium and calcium.

Figure 4.7 presents the base peak chromatogram of second neat urine sample separated on the monolithic column. This diagram illustrates the high efficiency separation of all ionisable groups present in the urine sample.

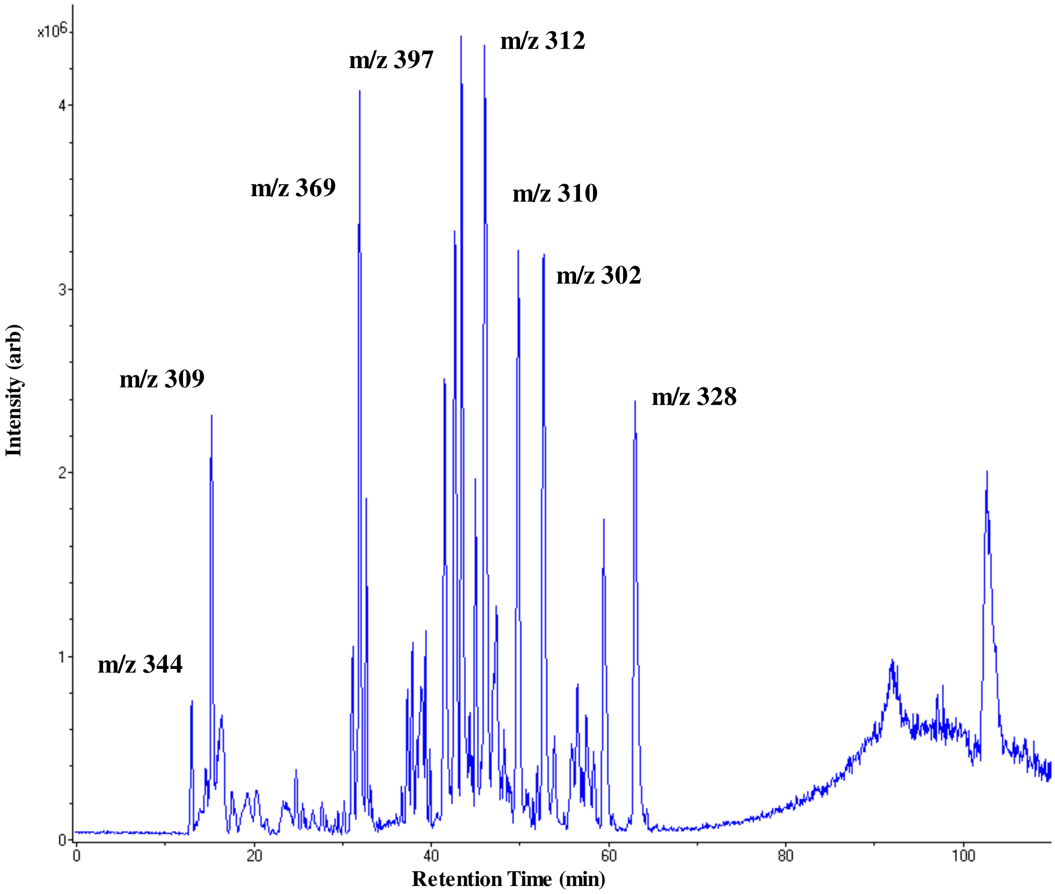


Figure 4.7: Base peak chromatogram of neat urine sample analysed on the half –metre monolith.

4.3.5 Liquid chromatography-mass spectrometry of PPCPs in urine and ion suppression study:

Spiked urine samples were prepared by drying down 1 mL of a 1 mg/L standard solution of PPCP's in milli-Q water and reconstituting in 1 mL of neat urine sample. The complete separation of 38 illicit drugs and PPCP's spiked into urine is shown in **Figure 4.8**.

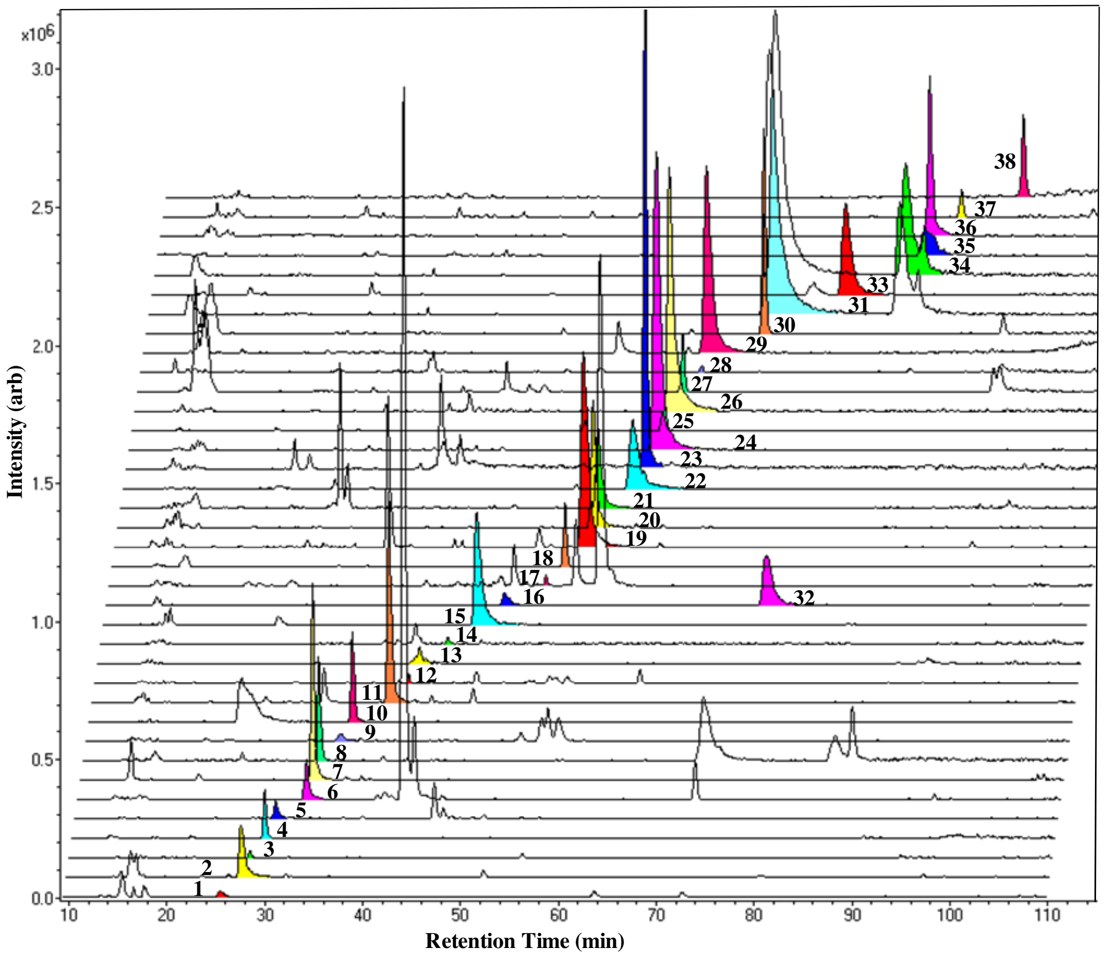


Figure 4.8: Extracted ion chromatograms of 38 illicit drugs and PPCP's spiked into urine at a concentration of 1 µg/mL. The axis is offset by 4 minutes. The analytes are in order of retention time and are as follows: salbutamol, atenolol, caffeine, sulfapyridine, cimetidine, morphine, benzoylecgonine, sulfamethazine, ranitidine, phenazone, trimethoprim, ketoprofen, MDMA, bezafibrate, metoprolol, tramadol, indomethacin, carbamazepine, cocaine, LSD, heroin, propranolol, papaverine, cocaethylene, ketamine, doxazosin, temazepam, nifedipine, citalopram, diazepam, EDDP, nortriptyline, methadone, amitriptyline, sertraline, clotrimazole, tamoxifen and simvastatin.

As with the soil and sludge samples, budesonide, erythromycin and ciprofloxacin were undetected during the separation of spiked urine. Chloramphenicol, which also exhibited weak signals in spiked soil samples and disappeared completely in the sludge matrix, was also absent from the spiked urine sample. More unusual was the absence of metformin and sulfamethoxazole were easily identified in previous complex samples. Both of these drugs elute at the beginning of the separation with retention times in a 1 mg/L standard of 21.5 and 23.6 minutes respectively. Due to their short residence time on the column they could have been subject to complete ion suppression by the abundance of weakly retained matrix compounds. Due to the high concentration of sodium and potassium ions present on urine, sodiated and potassiated adducts of these two drugs were investigated as possible ions for their detection. However, no ions were present for either species at the $[M + Na]^+$ or $[M + K]^+$ mass.

The effects of ion suppression in urine were studied by analysing a spiked urine sample already described and comparing the resultant peak heights to those in a 1 mg/L standard of the 38 illicit drugs and PPCP's. The results of the suppression study are presented in **Table 4.7**.

Table 4.7: Percentage relative recovery and percentage suppression values for 38 illicit drugs and PPCP's spiked into undiluted urine at concentration of 1 µg/mL. Values calculated by comparing spiked urine sample with a 1 mg/L standard solution in milli-Q water.

Name	**% Suppression of pharmaceuticals in urine**	**Name**	**% Suppression of pharmaceuticals in urine**
Salbutamol	27 ± 5	LSD	1 ± 5
Atenolol	26± 5	Heroin	8 ± 5
Caffeine	46 ± 8	Propranolol	-7 ± 1
Sulfapyridine	28 ± 4	Papaverine	-8 ± 2
Cimetidine	42 ± 2	Cocaethylene	-3 ± 5
Benzoylecgonine	16 ± 1	Ketamine	-11 ± 19
Sulfamethazine	26 ± 1	Doxazsoin	7 ± <1
Morphine	11 ± 2	Temazepam	12 ± 3
Ranitidine	9 ± 3	Nifedipine	-18 ± 24
Phenazone	7 ± <1	Citalopram	-7 ± 6
Trimethoprim	10 ± 2	Diazepam	1 ± 3
Ketoprofen	4 ± 19	EDDP	-7 ± 5
MDMA	-18 ± 40	Nortriptyline	1 ± <1
Bezafibrate	36 ± <1	Methadone	-2 ± 1
Metoprolol	8 ± 5	Amitriptyline	-3 ± 12
Tramadol	-3 ± 2	Sertraline	8 ± 5
Indomethacin	33 ± 1	Clotrimazole	4 ± 13
Carbamazepine	10 ± 2	Tamoxifen	48 ± 5
Cocaine	<1 ± 4	Simvastatin	3 ± 17

Overall the suppression effects in undiluted urine were quite low with 28 out of 38 target analytes reporting recoveries ≥80%. However, some analytes were strongly suppressed e.g. caffeine, cimetidine and tamoxifen reported ion suppression of 46, 42 and 48 %, respectively. Suppression in urine was generally less than that in soil and sludge with an average ion suppression value of 11% ± 22. The lower suppression value in urine may be due to less organic matter eluting late in the separation. These compounds are thought to be responsible for much of the signal interferences in environmental samples such as soil and sludge. Conversely the higher suppression values in urine were noted at the beginning of the separation due to unretained polar and ionic species. The second half of the separation was relatively suppression free with only two analytes reporting recoveries <90% from 55 minutes onwards. The retention time of the analytes was also monitored during the suppression study in order to highlight any retention shifts that occurred due to endogenous compounds. All target analytes reported changes in retention time compared to retention time in a standard of ≤0.2 minutes except for 5 analytes. Consequently these included some of the most severely suppressed analytes e.g. cimetidine and atenolol.

4.3.6 Application of half-metre monolith to screening of drugs in urine:

Samples were injected as described before with no dilution or preparation apart from centrifugation. The urine was centrifuged to remove any precipitants and the pH was checked to ensure it adhered to the operating guidelines of the monolith. 1 mL aliquots of neat urine were transferred to HPLC vials and 10 µL injections were made for LC-MS analysis. Three different urine samples were analysed in this way, one urine sample assumed to be blank containing no pharmaceutical residues, one from a recreational drug user and one from the sufferer of a kidney infection. The blank sample contained no therapeutic or illicit drugs but traces of caffeine were detected in the sample despite high levels of ion suppression being reported for caffeine in the earlier study. The extracted ion chromatograms of the parent and product ions for caffeine are shown in **Figure 4.9**. The signal for the fragment ion of caffeine at m/z 138 was quite weak but the MS^2 spectrum confirmed its presence. It should be noted that only 10 µL of urine was injected for this analysis. However, as discussed in Chapter 3 of this work, the half-metre monolithic column had a high sample loading capacity with only 10 % loss of efficiency at an injection volume of 200 µL. The urine sample matrix would obviously decrease the loading capacity of the column however; it would be possible to increase the injection volume to a certain extent if better sensitivity was required.

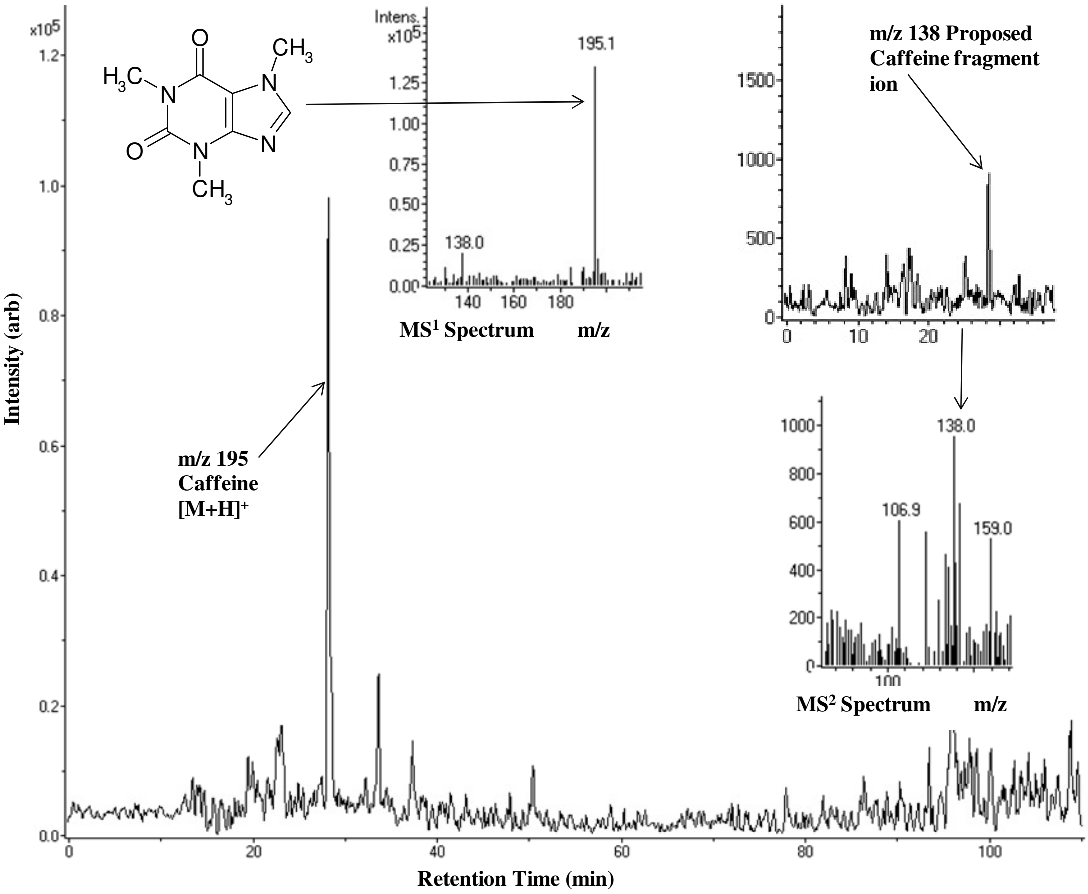


Figure 4.9: Detection of caffeine in undiluted urine sample using half-metre monolith. Inset illustrates the proposed fragment ion at m/z 138 and shows MS^1 and MS^2 spectra for both the parent and product ions.

The second urine sample contained traces of the cocaine metabolite benzoylecgonine. There were no traces of cocaine itself or the product of cocaine and ethanol consumption cocaethylene which was also included in the study. However, this was not thought to be unusual as studies have shown that cocaine has a very short half-life and is rapidly converted to benzoylecgonine and a range of seven other metabolites once ingested.[35] In studies of urinary excretion levels, benzoylecgonine was shown to be present at the highest concentration and was present in urine samples collected several days after consumption of the cocaine. Conversely the majority of cocaine was excreted within the first two hours due to its extremely short half-life within the body.[36] The urine sample in question was collected up to 24 hours or more after consumption of cocaine; therefore the detection of benzoylecgonine is not surprising. The presence of the benzoylecgonine in the urine is illustrated in **Figure 4.10**. This result demonstrates that benzoylecgonine is a good indicator of cocaine usage that allows for wider collection period for the specimen.

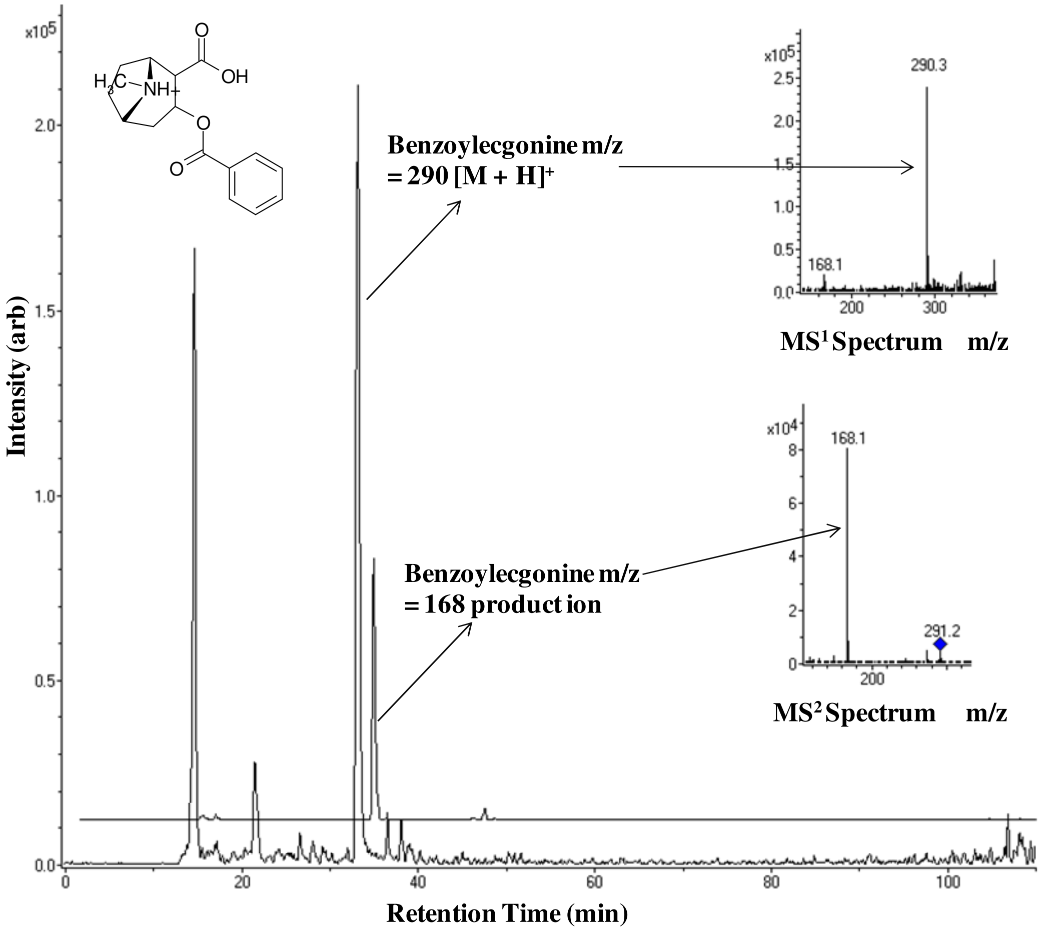


Figure 4.10: Detection of benzoylecgonine in undiluted urine sample using the half-metre monolith. Inset illustrates the proposed fragment ion at m/z 168 and shows MS^1 and MS^2 spectra for both the parent and product ions. Time offset for better view of fragment ion.

Lastly, the urine of a volunteer who had suffered a kidney infection was analysed and two antibiotics used to treat the condition were detected. Trimethoprim and ciprofloxacin were both found in the urine sample and the results of the study are shown in **Figures 4.11 and 4.12** respectively. The signals for both analytes were very intense, in the 10^6 – 10^7 intensity range.

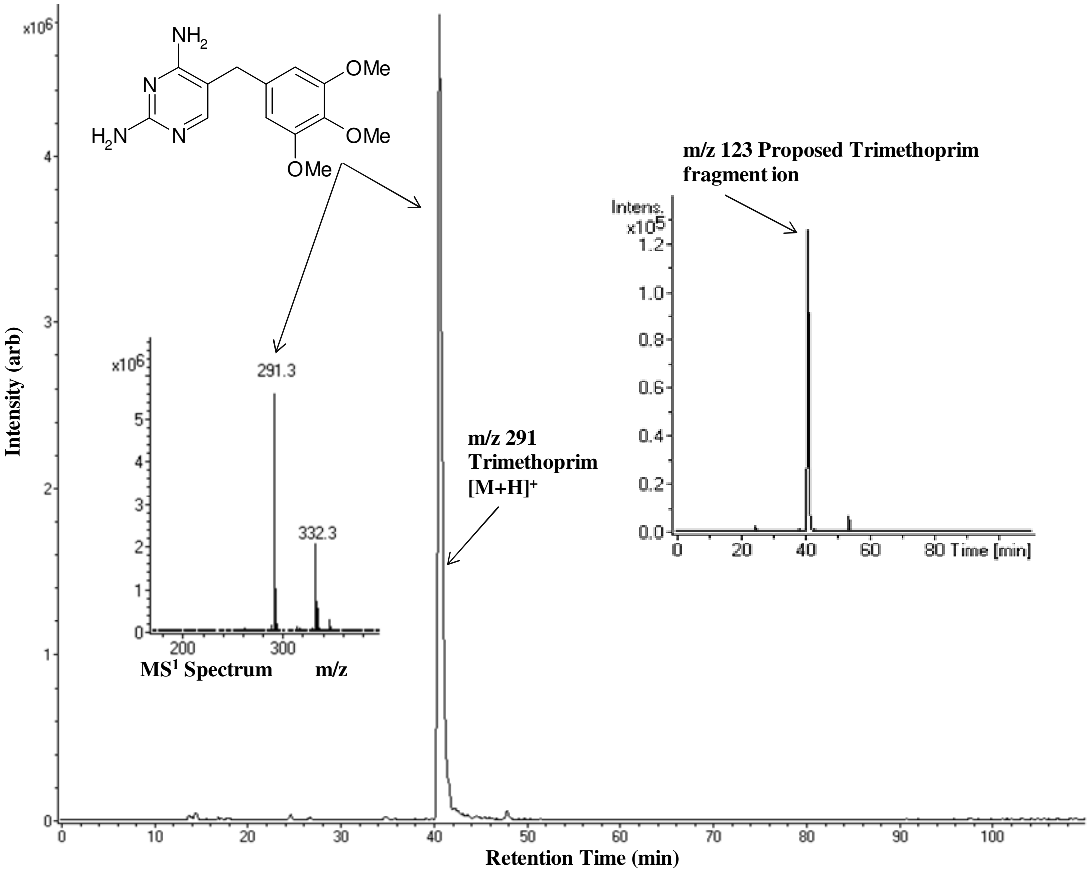


Figure 4.11: Detection of trimethoprim in undiluted urine sample using the half-metre monolith. Inset illustrates the proposed fragment ion at m/z 123 and shows MS^1 and MS^2 spectra for both the parent and product ions.

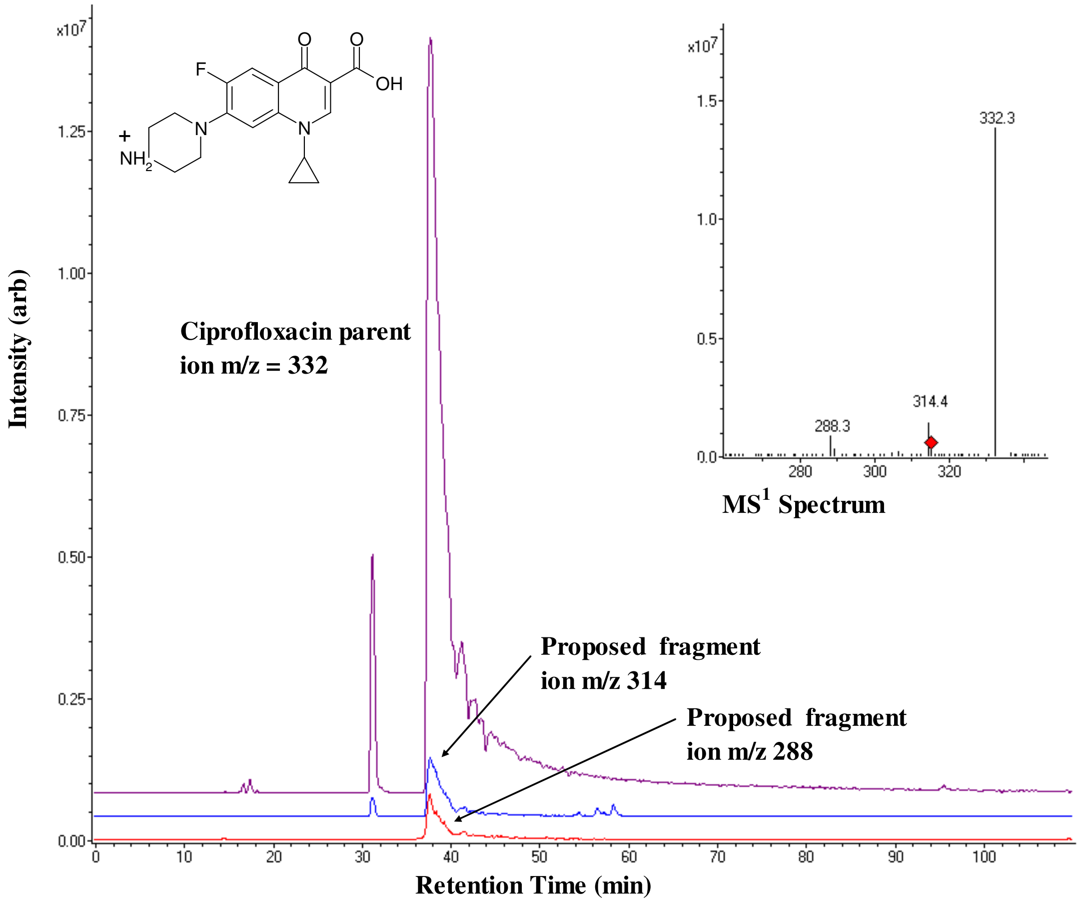


Figure 4.12: Detection of ciprofloxacin in undiluted urine sample using the half-metre monolith. Inset illustrates the proposed fragment ions at m/z 314 and m/z 288 in the MS^1 spectrum due to CID.

Ciprofloxacin was undetected by LC-MS/MS when separating a mixed 1 mg/L standard solution of all the illicit drugs and PPCP’s. It was also absent during analysis of the spiked urine sample. When preparing the ciprofloxacin stock solution in methanol at a concentration of 1000 ppm, there were issues with solubility of the antibiotic. This may have caused its absence and could have been due to the fact that pure ciprofloxacin had been purchased from Sigma Aldrich and not ciprofloxacin hydrochloride. The analyte may have precipitated out of solution or may have been suppressed due to the sheer number of other pharmaceuticals present in solution.

Therefore there was no standard retention time available to match with the peak found in the real sample of urine. To remedy this, two fragment ions of ciprofloxacin instead of just one were monitored in tandem MS mode. Also a 10 mg/L standard solution of ciprofloxacin in milli-Q water was analysed by LC-UV under optimized conditions at 280 nm[37] to confirm the detection of ciprofloxacin at 37.7 minutes.

The fragment ions of ciprofloxacin that were monitored were at masses of m/z 314 and 288. These ions were present at a much higher intensity in single MS mode than in tandem MS mode due to collisionally induced dissociation (CID). In **Figure 4.12** only the single mode mass spectrum is presented as it clearly illustrates the presence of both product ions. The average retention time recorded for the ciprofloxacin peak was 38.84 minutes. This result confirmed the presence of ciprofloxacin in the urine sample and distinguished it as the second larger peak in the spectrum (**Figure 4.12**) and not the smaller sharper peak present at approximately 30 minutes. The slight variation in retention times between the standard and urine analysis can most likely be explained by the instrument variation as the retention time test was carried out on an alternative HPLC system to other analyses or could be an effect of matrix components.

Ciprofloxacin and trimethoprim were also detected in the UV spectrum during the analysis of the urine sample. The ciprofloxacin and trimethoprim peaks can be clearly seen in the UV spectrum overlaid with the extracted ion chromatograms from mass spectrometric detection in **Figure 4.13**.

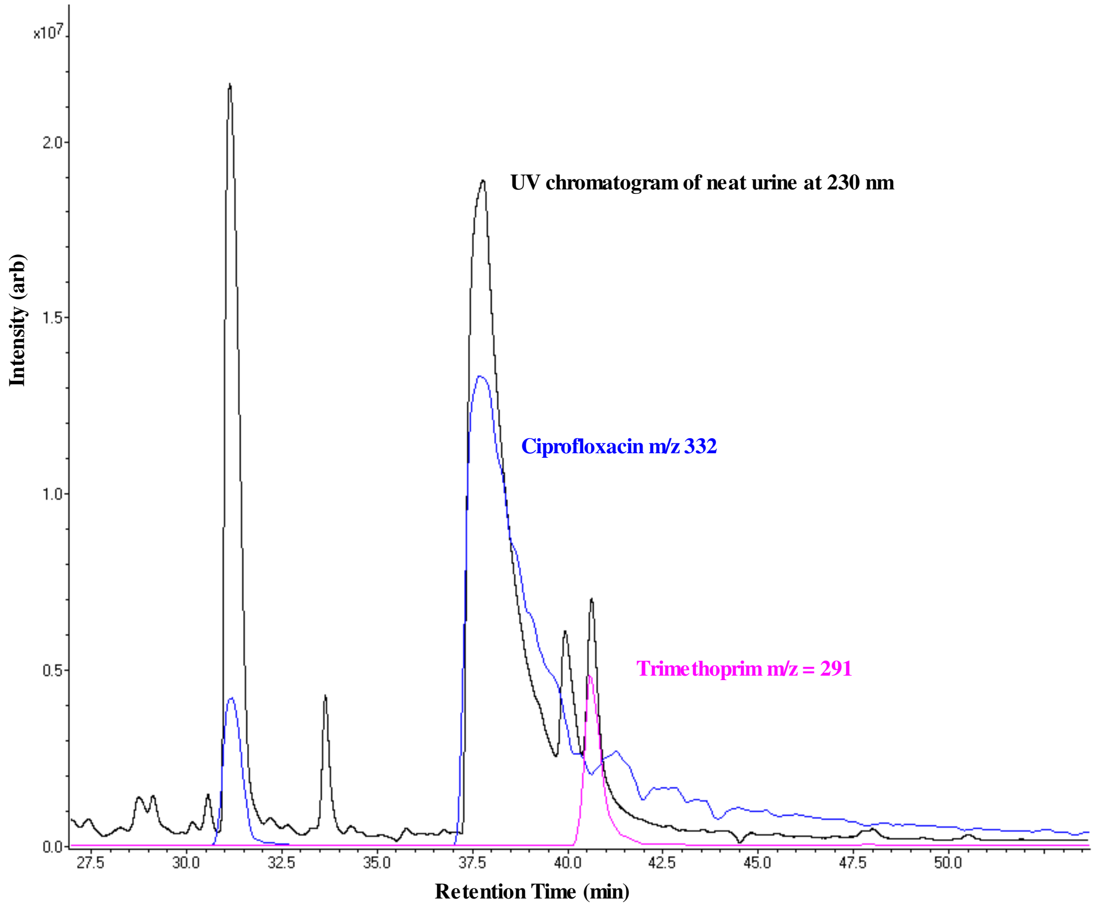


Figure 4.13: UV chromatogram of neat urine at 230 nm overlaid with extracted ion chromatograms for ciprofloxacin and trimethoprim at m/z 332 and m/z 291, respectively.

In previous studies of antibiotics in urine, a substantial amount of preparation has been required including SPE.[38] In some cases UV detection could not be used despite the sample clean-up. However, in these studies, only trace amounts of the pharmaceuticals were present in the samples. The concentrations of analytes separated in these studies are quite high and so future work might include validation of the separation method and calculation of detection limits.

4.4 Conclusions:

This work has demonstrated that long monolithic columns are an efficient stationary phases for application to LC-MS/MS analysis of PPCPs in environmental and biological matrices. Matrix effects, though not eliminated were considerably lower for analytes separated on the monolith than on a more conventional particle HPLC phase. Coupled monoliths could also reduce the amount of time spent on sample pretreatment by allowing direct injection of biological samples. Urine samples were screened for a large number of pharmaceutical analytes and several were identified. Future work may include validation of the multi-residue method for quantification of a large number of environmentally and forensically relevant compounds without sample preparation.

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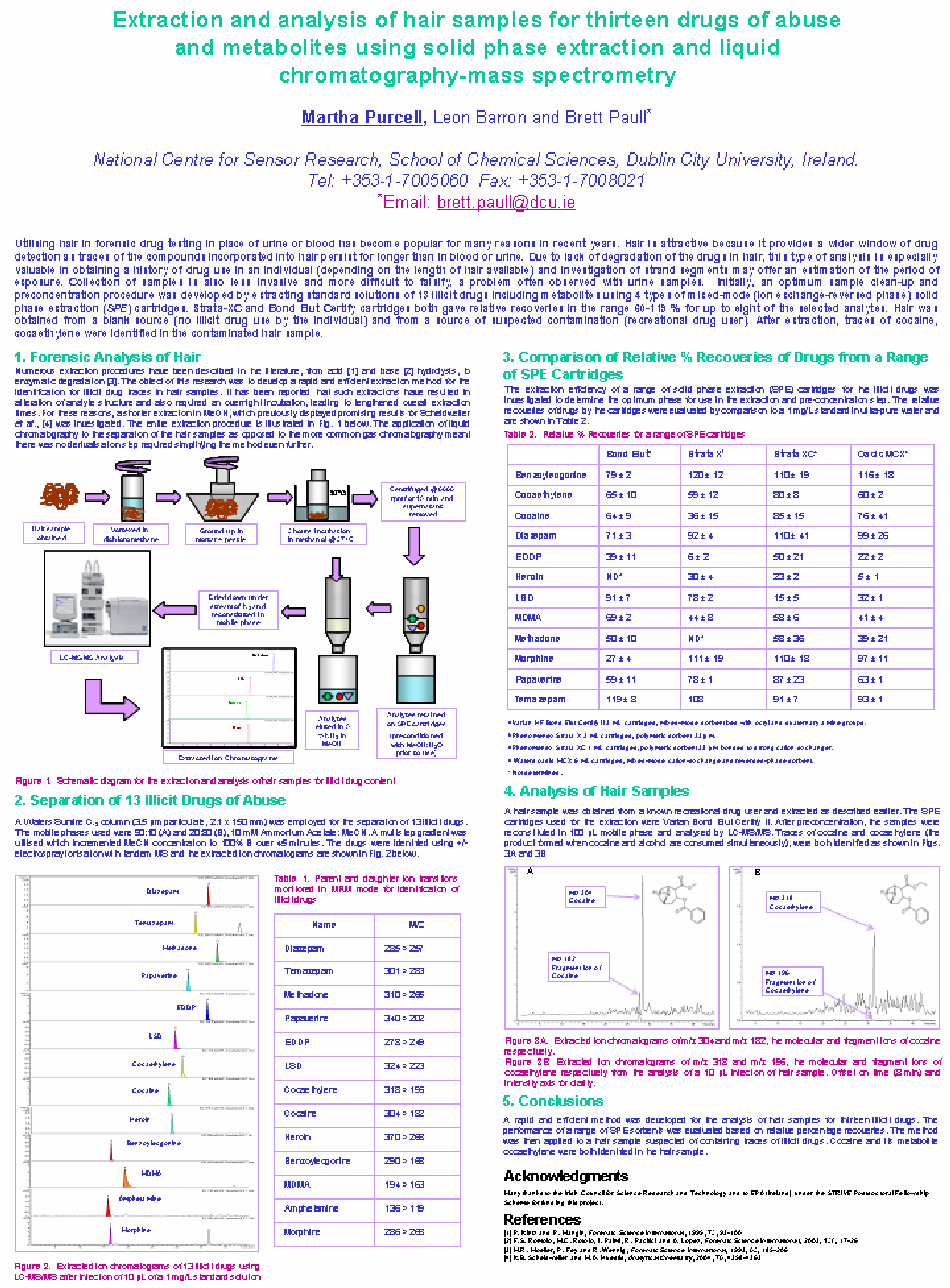
Final Conclusions:

It is clear from this study that PPCPs are present in quite substantial concentrations in many environmental matrices. Further research is needed to determine other pharmaceutical residues that have not yet been investigated. Although not covered under this work, techniques for removal of PPCPs from WWTPs and drinking water are also receiving a lot of attention. These techniques need to be thoroughly monitored to ensure that the relevant compounds are being eliminated. Persistence of pharmaceutical compounds in the environment is a major issue and more focus is needed in this area to determine the long term effects of non-degrading medicinal drugs. Biological analysis is important to determine the effects of these pollutants on animal and human life and to monitor the metabolites also being excreted into the environment. LC-MS/MS is the technique of choice, however matrix effects and laborious sample preparation processes continue to limit its applicability to all samples.

Monolithic stationary phases have been characterised as highly efficient separation media for HPLC analysis. Multi-residue analytical techniques are becoming more popular due to the comprehensive approach and determination of several therapeutic families of compounds in one run. Monolithic stationary phases have yet to be applied to multi-residue analysis of PPCPs in solid environmental and biological matrices. The reduction in ion suppression illustrated in this study may allow for less time spent on sample preparation.

Poster Presentations:

Conference Poster Prize Winner, 5th Biennial Conference on Analytical Science in Ireland, Waterford Institute of Technology, May 2008.



Extraction and analysis of hair samples for thirteen drugs of abuse and metabolites using solid phase extraction and liquid chromatography-mass spectrometry

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Utilising hair in forensic drug testing in place of urine or blood has become popular for many reasons in recent years. Hair is attractive because it provides a wider window of drug detection as traces of the compounds incorporated into hair persist for longer than in blood or urine. Due to lack of degradation of the drugs in hair, this type of analysis is especially valuable in obtaining a history of drug use in an individual (depending on the length of hair available) and investigation of strand segments may offer an estimation of the period of exposure. Collection of samples is also less invasive and more difficult to falsify, a problem often observed with urine samples. Initially, an optimum sample clean-up and preconcentration procedure was developed by extracting standard solutions of 13 illicit drugs including metabolites using 4 types of mixed-mode (ion exchange-reversed phase) solid phase extraction (SPE) cartridges. Strata-XC and Bond Elut Certify cartridges both gave relative recoveries in the range 60-119 % for up to eight of the selected analytes. Hair was obtained from a blank source (no illicit drug use by the individual) and from a source of suspected contamination (recreational drug user). After extraction, traces of cocaine, cocaethylene were identified in the contaminated hair sample.

1. Forensic Analysis of Hair

Numerous extraction procedures have been described in the literature, from acid [1] and base [2] hydrolysis, to enzymatic degradation [3]. The object of this research was to develop a rapid and efficient extraction method for the identification for illicit drug traces in hair samples. It has been reported that such extractions have resulted in alteration of analyte structure and also required an overnight incubation, leading to lengthened overall extraction times. For these reasons, a shorter extraction in MeOH, which previously displayed promising results for Scheidweiler et al., [4] was investigated. The entire extraction procedure is illustrated in Fig. 1 below. The application of liquid chromatography to the separation of the hair samples as opposed to the more common gas chromatography meant there was no derivatisation step required simplifying the method even further.

Figure 1. Schematic diagram for the extraction and analysis of hair samples for illicit drug content

2. Separation of 13 Illicit Drugs of Abuse

A Waters Sunfire C_{18} column (3.5 µm particulate, 2.1 x 150 mm) was employed for the separation of 13 illicit drugs. The mobile phases used were 90:10 (A) and 20:80 (B), 10 mM Ammonium Acetate : MeCN. A multistep gradient was utilised which incremented MeCN concentration to 100% B over 45 minutes. The drugs were identified using +/- electrospray ionisation with tandem MS and the extracted ion chromatograms are shown in Fig. 2 below.

Figure 2. Extracted ion chromatograms of 13 illicit drugs using LC-MS/MS after injection of 10 µL of a 1 mg/L standard solution

Table 1. Parent and daughter ion transitions monitored in MRM mode for identification of illicit drugs

Name	M/Z
Diazepam	285 > 257
Temazepam	301 > 283
Methadone	310 > 265
Papaverine	340 > 202
EDDP	278 > 249
LSD	324 > 223
Cocaethylene	318 > 196
Cocaine	304 > 182
Heroin	370 > 268
Benzoylecgonine	290 > 168
MDMA	194 > 163
Amphetamine	136 > 119
Morphine	286 > 268

3. Comparison of Relative % Recoveries of Drugs from a Range of SPE Cartridges

The extraction efficiency of a range of solid phase extraction (SPE) cartridges for the illicit drugs was investigated to determine the optimum phase for use in the extraction and pre-concentration step. The relative recoveries of drugs by the cartridges were evaluated by comparison to a 1 mg/L standard in ultrapure water and are shown in Table 2.

Table 2. Relative % Recoveries for a range of SPE cartridges

	Bond Elut^a	Strata X^b	Strata XC^c	Oasis MCX^d
Benzoylecgonine	79 ± 2	120 ± 12	110 ± 19	116 ± 18
Cocaethylene	65 ± 10	59 ± 12	80 ± 8	60 ± 2
Cocaine	64 ± 9	36 ± 15	85 ± 15	76 ± 41
Diazepam	71 ± 3	92 ± 4	110 ± 41	59 ± 26
EDDP	39 ± 11	6 ± 2	50 ± 21	22 ± 2
Heroin	ND^e	30 ± 4	23 ± 2	5 ± 1
LSD	91 ± 7	78 ± 2	15 ± 5	32 ± 1
MDMA	69 ± 2	44 ± 8	58 ± 6	41 ± 4
Methadone	50 ± 10	ND^e	58 ± 36	39 ± 21
Morphine	27 ± 4	111 ± 19	110 ± 18	97 ± 11
Papaverine	59 ± 11	78 ± 1	87 ± 23	63 ± 1
Temazepam	119 ± 8	108	91 ± 7	93 ± 1

a Varian HF Bond Elut Certify II 3 mL cartridges, mixed-mode sorbent bed with [illegible] quaternary amine groups.
b Phenomenex Strata X 3 mL cartridges, polymeric sorbent 33 µm.
c Phenomenex Strata XC 3 mL cartridges, polymeric sorbent 33 µm bonded to a strong cation exchanger.
d Waters Oasis MCX 6 mL cartridges, mixed-mode cation-exchange and reversed-phase sorbent.
e Not determined.

4. Analysis of Hair Samples

A hair sample was obtained from a known recreational drug user and extracted as described earlier. The SPE cartridges used for the extraction were Varian Bond Elut Certify II. After preconcentration, the samples were reconstituted in 100 µL mobile phase and analysed by LC-MS/MS. Traces of cocaine and cocaethylene (the product formed when cocaine and alcohol are consumed simultaneously), were both identified as shown in Figs. 3A and 3B

Figure 3A. Extracted ion chromatograms of m/z 304 and m/z 182, the molecular and fragment ions of cocaine respectively.
Figure 3B. Extracted ion chromatograms of m/z 318 and m/z 196, the molecular and fragment ions of cocaethylene respectively from the analysis of a 10 µL injection of hair sample. Offset on time (3 min) and intensity axis for clarity.

5. Conclusions

A rapid and efficient method was developed for the analysis of hair samples for thirteen illicit drugs. The performance of a range of SPE sorbents was evaluated based on relative percentage recoveries. The method was then applied to a hair sample suspected of containing traces of illicit drugs. Cocaine and its metabolite cocaethylene were both identified in the hair sample.

Acknowledgments

Many thanks to the Irish Council for Science Research and Technology and to EPA Ireland under the STRIVE Postdoctoral Fellowship Scheme for funding this project.

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[2] F.S. Romolo, M.C. Rotolo, I. Palmi, R. Pacifici and A. Lopez, Forensic Science International, 2003, 138, 17-26
[3] H.R. [illegible], P. Fey and R. Wennig, Forensic Science International, 1993, 63, 185-206
[4] K.B. Scheidweiler and M.A. Huestis, Analytical Chemistry, 2004, 76, 4358-4363

Half-metre monolithic columns coupled to electrospray ion trap mass spectrometry for ultra high efficiency separations of pharmaceutical and personal care products

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Analytical separations employing coupled monolithic silica columns in series have many advantages including low operating back pressures, flow rates compatible with mass spectrometry and excellent separation efficiency. In this work, a 0.5 m monolithic column was constructed using five 100 x 3 mm i.d. reversed-phase monoliths in series. The efficiency of the long column was characterised using traditional Van Deemter curve experiments using diclofenac as the test compound. Furthermore, these were conducted at 10, 15, 20 and 45 °C in order to determine the optimum separating temperature. Highest efficiency was observed at 45 °C with 23,500 theoretical plates (*N*) and operating with a relatively low back pressure of bar. Efficiency was compared to a 0.15 m particulate Sunfire column characterised using the same approach. The particulate column produced *N* and *H* values in the region of 6,000 and 26 µm respectively, indicating lower efficiency while operating at much higher back pressures. The effect of sample loading on to the extended column was also evaluated using three test compounds; diclofenac, metoprolol and cocaine. Due to the high capacity of the long column, peak height remained linear up to an injection volume of 900 µL for the three drugs, with minimal increase in peak width, however a decrease in peak symmetry was observed. The 0.5 m monolithic column was developed for application in the analysis of pharmaceuticals and personal care products

1. Characterisation of Monolith and Comparison to Sunfire Column using Traditional Van Deemter Curve Experiments

The efficiency of the long monolithic column was characterised using van Deemter curves. The experiments were carried out over a range of temperatures with 45°C being chosen as the optimum. At this temperature, the number of theoretical plates was as high as 24,000 (Fig. 1 below). The slopes of the mass transfer term of van Deemter curves indicated only marginal loss of efficiency at higher linear velocities. Therefore this column shows promise for application to very complex sample types such as soil or sludge. Fig. 2 illustrates the increased efficiency of the monolith compared to a 3 µm particulate column and Fig. 3 compares the backpressures generated by each column.

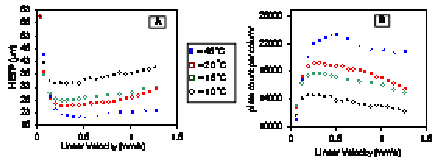

Figure 1. Comparison of plate height (A), number of theoretical plates (B) for the 0.5 m monolithic column over a range of operating temperatures.

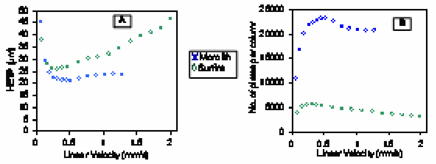

Figure 2. Comparison of plate height (A), number of theoretical plates (B) for the 0.5m monolith and Waters Sunfire C18 particulate column (3.5 µm, 2.1 x 150mm).

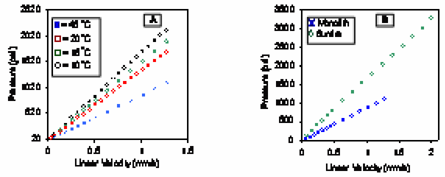

Figure 3. Comparison of backpressures generated on 0.5 m monolith over range of temperatures (A) and monolith backpressure compared to that of Sunfire (B).

2. Peak Capacity

Peak capacity values were calculated for the Sunfire and long monolithic column using identical gradients, injection volumes (10 µL) and column temperatures (45 °C). Conditions of flow rate, backpressure, linear velocity and dead time and their effect on the peak capacity of each column was investigated. The results obtained from the study are shown in Table 1 and clearly indicate the superior performance of the 0.5 m monolithic column over the particulate column. Almost identical values were obtained when separations were carried out at one flow rate but when the other conditions were taken into account, up to double the number of peaks could theoretically be separated on the long monolith.

Table 1. Peak capacity (P_c) values for monolith and Sunfire columns

	Flow Rate[a]		Backpressure[b]		Linear Velocity[c]		Deadtime[d]	
	P_c	P_c/hr	P_c	P_c/hr	P_c	P_c/hr	P_c	P_c/hr
0.5 m Monolith	104	69	86	57	132	88	108	72
0.15 m Sunfire	103	69	50	33	93	62	50	33

[a] Flow rate was 0.2 mL/min for both columns with backpressures of [illegible] bar for the monolith and [illegible] bar for the Sunfire.

[b] Flow rates were 0.[illegible] mL/min and 0.2[illegible] mL/min for the monolith and Sunfire columns respectively resulting in backpressures of ~[illegible] bar ([illegible] psi).

[c] Flow rates were 0.[illegible] mL/min and 0.1[illegible] mL/min for the monolith and Sunfire columns respectively resulting in linear velocities of 1.[illegible] and [illegible] mm/s respectively.

[d] Flow rates were 0.[illegible] mL/min and 0.2[illegible] mL/min for the monolith and Sunfire columns respectively resulting in dead times of [illegible] and [illegible] mins respectively.

3. Sample Loading Capacity

The effect of increased sample size on the performance of the coupled monolith was also investigated. Sample volumes of 10 – 900 µL were injected to identify the maximum loading capacity possible without loss of efficiency. Fig. 4 and 5 below illustrates the ability of the monolith to separate large sample volumes while maintaining efficiency up to at least 100 µL.

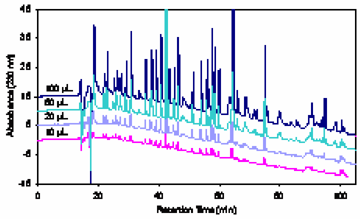

Figure 4. Separation of a solution of 0.33 mg/L of 51 pharmaceuticals and illicit drugs in ultra pure water.

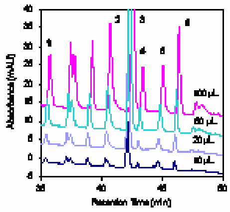

Figure 5. A selection of analyte responses at increasing injection volume. 1 = Sulfamethazine, 2 = Trimethoprim, 3 = Naproxen, 4 = Ketoprofen, 5 = Methadone and 6 = Bezafibrate.

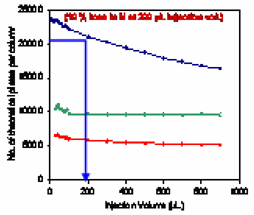

Figure 6. Loss of only 10% in the number of theoretical plates/column after a 200 µL injection of 🟦 Diclofenac, 🟩 Cocaine and 🟥 Metoprolol.

4. Conclusions

The half-metre monolith exhibited superior separation power when compared to a 3 µm particulate column indicating its possible application to separations of complex samples by demonstrating better peak capacity in all but one of the experiments conducted. The sample loading capacity of the long column was also higher with only marginal loss in efficiency up to 200 µL injection volumes making it ideal for trace analysis.

5. Future Work

Work to date has indicated the excellent capabilities of coupled monoliths as a stationary phase. In future work, the column will be applied to the separation of a large range of pharmaceutical residues in complex samples such as sludge or soil. Incomplete separations of such large component mixtures has often resulted in MS ion suppression effects, reducing overall sensitivity at trace levels. It is hoped that coupled monoliths may reduce this effect.

Acknowledgements

Many thanks to the Irish Council for Science Research and Technology and to the EPA (Ireland) under the STRIVE Postdoctoral Fellowship Scheme for funding this project.

Ultra high efficiency liquid chromatographic separations of trace pharmaceuticals and personal care products in digested sludge using half metre columns

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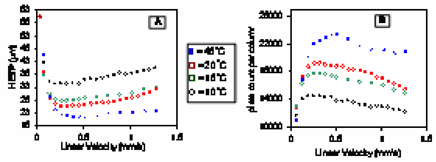
Analytical separations employing coupled monolithic silica columns have many advantages including low operating back pressures, mass spectrometry (MS) compatible flow rates and enhanced separation efficiency. In this work, a 0.5 m monolithic column was constructed using five 100 x 3 mm i.d. reversed-phase monoliths in series. The efficiency of the long column was characterised by constructing van Deemter curves using diclofenac as the test compound. Elevated temperatures of 45 °C (for n=4 temperatures studied) were used to optimise *N* at ~23,500 plates, operating with a relatively low back pressure of 30 bar at 0.16 mL/min. This column offered an approximate 4-fold improvement in *N* with markedly reduced backpressure in comparison to a 150 x 2.1 mm, 3 µm particulate phase. The effect of sample loading on to the extended column was also evaluated using three test compounds; diclofenac, metoprolol and cocaine. Due to the increased loading capacity of the long column, only a 10 % loss in *N* was observed by injecting up to 100 µL and only 30 % loss up to 900 µL volumes. The long monolith was applied to the determination of approximately 50 pharmaceutical and personal care product residues (PPCPs) in digested sludge and waste water taken from the Ringsend facility in Dublin City. Improved separation power was investigated as a possible means to reduce the loss in MS sensitivity due to associated ion suppression effects typically resulting from co-eluting components within such complex sample types. Therefore, a comprehensive and quantitative study on observed suppression effects was carried out using both the particulate and monolithic phases to observe any improvements.

1. Characterisation of Monolith and Comparison to Sunfire Column using Traditional Van Deemter Curve Experiments

The efficiency of the long monolithic column was characterised using van Deemter curves. The experiments were carried out over a range of temperatures with 45°C being chosen as the optimum. At this temperature, the number of theoretical plates was as high as 24,000 (Fig. 1 below). The slopes of the mass transfer term of van Deemter curves indicated only marginal loss of efficiency at higher linear velocities. Therefore this column shows promise for application to very complex sample types such as soil or sludge. Fig. 2 illustrates the increased efficiency of the monolith compared to a 3 µm particulate column and Fig. 3 compares the backpressures generated by each column.



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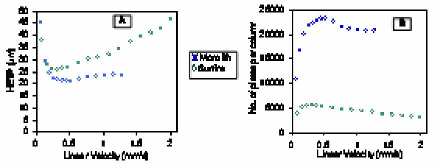

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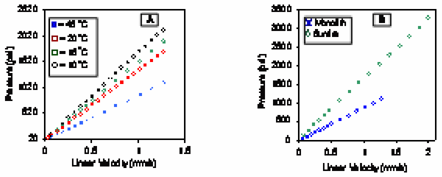

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Table 1. Peak capacity (P_c) values for monolith and Sunfire columns

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^a Flow rate was 0.2 mL/min for both columns with backpressures of [illegible] bar for the monolith and [illegible] bar for the Sunfire.

^b Flow rates were [illegible] mL/min and [illegible] mL/min for the monolith and Sunfire columns respectively resulting in backpressures of [illegible] bar/[illegible] psi.

^c Flow rates were [illegible] mL/min and [illegible] mL/min for the monolith and Sunfire columns respectively resulting in linear velocities of [illegible] and [illegible] mm/s respectively.

^d Flow rates were [illegible] mL/min and [illegible] mL/min for the monolith and Sunfire columns respectively resulting in dead times of [illegible] and [illegible] min respectively.

3. Sample Loading Capacity

The effect of increased sample size on the performance of the coupled monolith was also investigated. Sample volumes of 10 – 900 µL were injected to identify the maximum loading capacity possible without loss of efficiency. Fig. 4, 5 and 6 below illustrates the ability of the monolith to separate large sample volumes while maintaining efficiency up to at least 100 µL.

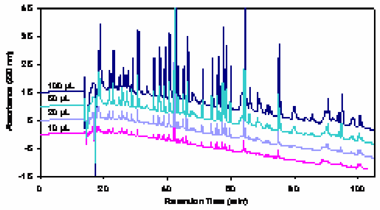

Figure 4. Separation of a solution of 0.33mg/L of 51 pharmaceuticals and illicit drugs in ultra pure water.

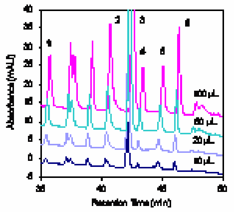

Figure 6. A selection of analyte responses at increasing injection volume. 1 = Sulfamethazine, 2 = Trimethoprim, 3 = Naproxen, 4 = Ketoprofen, 5 = Methadone and 6 = Bezafibrate.

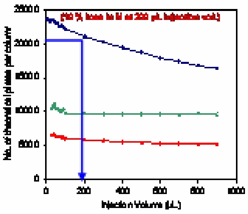

Figure 8. Loss of only 10% in the number of theoretical plates/column after a 200 µL injection of ▫ Diclofenac, ▫ Cocaine and ▫ Metoprolol.

4. Suppression Study

The half metre monolith was applied to the separation of a large range of pharmaceutical residues in extracted sludges. Incomplete separations of such large component mixtures has often resulted in MS ion suppression effects, reducing overall sensitivity at trace levels. It was hoped that coupled monoliths may reduce this effect. Table 2 below compares suppression data obtained for the long monolith with previously published data for the Sunfire column.[1] A blank sludge sample was also analysed on the monolith and traces of triclosan and erythromycin were identified. The EIC's are shown in Fig. 7 A and B below.

Table 2. Percentage MS ion suppression values for half metre monolith compared to previously calculated values for Sunfire column.[1]

Drug	Monolith	Sunfire
Warfarin	20	61
Sulfamethazine	35	30
Trimethoprim	25	52
Ketoprofen	44	41
Bezafibrate	41	45
Flurbiprofen	<5	49
Diclofenac	<5	56
Nimesulide	9	50

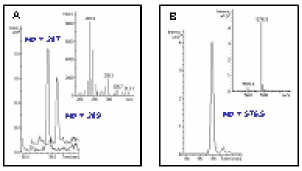

Figure 7. Extracted ion chromatograms and corresponding mass spectra for the identification of (A) triclosan and (B) erythromycin in digested sludge.

5. Conclusions and Future Work

The half metre monolith exhibited enhanced separation power when compared to a 3 µm particulate column indicating its possible application to separations of complex samples by demonstrating higher peak capacity under a range of conditions. The sample loading capacity of the long column was also higher with only marginal loss in efficiency up to 200 µL injection volumes making it ideal for trace analysis. Lower levels of MS ion suppression were observed when analysing PPCP's in a complex sample matrix on the long monolithic column. Further investigation of suppression effects are needed for a wider range of analytes and perhaps alternative matrices such as urine.

Acknowledgements and References
Many thanks to the Irish Council for Science Research and Technology and to the EPA (Ireland) under the STRIVE Postdoctoral Fellowship Scheme for funding this project.

[1] L. Barron, J. Tobin and B. Paull, *Journal of Environmental Monitoring*, 2008, 10, 353-361