Investigation of Emerging Contaminants in Environmental Waters

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Table of Contents

List of Tables

Table of Figures

Declaration of Originality

I hereby declare that the work contains herein is mine, and has not be submitted, in part or full, to any other university or institution for any award.

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Abbreviations and Acronyms

ACN	Acetonitrile
AUC	Area under curve/peak area
BCC	Bioaccumulative chemicals of concern
CID	Collision induced dissociation
DGF	Drying gas flow
DGT	Drying gas temperature
ECD	Electron capture detection
EIC	Extracted ion current
ESI	Electrospray ionisation
FID	Flame ionisation detection
GC	Gas chromatography
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
LPME	Liquid-phase microextraction
m/z	Mass (m) to charge (z) ratio
MeOH	Methanol
MRM	Multiple reaction monitoring
MS^E	Low and high collision-energy full scan acquisition acquired simultaneously
NI	Negative ESI
PBT	Persistent bioaccumulative toxic
PI	Positive ESI
POP	Persistent organic pollutants
ppb	Parts per billion ($\mu g\ L^{-1}$)
QbD	Quality by design
QQQ	Triple quadrupole
QTOF	Quadrupole time-of-flight
RSD	Relative standard deviation
RT	Retention time
SGF	Sheath gas flow
SGT	Sheath gas temperature
S/N	Signal(S) to noise (N) ratio
SIM	Selected ion monitoring
SPE	Solid phase extraction
SPME	Solid-phase microextraction
STP	Sewage treatment plant
TCEP	Tris(2-carboxyethyl)phosphine
TIC	Total ion current
TOF	Time-of-flight
UHPLC	Ultra-high performance liquid chromatography
VCap	Capillary voltage

Abstract

Emerging contaminants, such as pharmaceuticals, are a growing environmental concern. These contaminants have been detected in environmental waters (such as surface water, ground and wastewater flows) at concentrations ranging from μg L^{-1} to ng L^{-1} level. The majority are unregulated in the environment and as a result the risk assessment and ecotoxicological effect of the contaminants, their metabolites and/or transformation products are relatively unknown.

To meet the prerequisite of proper monitoring and risk evaluation of emerging contaminants, the development of a sensitive multi-residue screening analytical method for emerging contaminants in complex environmental matrices was undertaken. The first part of the study involved developing a UHPLC-QTOF-MS method for the analysis of a mixture of pharmaceuticals in water. The method was then expanded to include an on-line SPE pre-concentration step in the second part of the study.

The method has successfully analysed standards in Type I water, and was carried over with relative success to wastewater flows. Applications of this method to further environmental water studies could yield qualitative and quantitative information of emerging contaminants, and their transformation products.

Chapter 1: Introduction

1.1 Preamble

From the 1970s the focus of national water pollution control awareness and management programs were on priority pollutants[1]. The priority pollutants consisted of non-polar hazardous compounds such as persistent organic pollutants (POPs), persistent bioaccumulative toxic (PBT) and bioaccumulative chemicals of concern (BCCs) [1-2]. However the issue of priority pollutants has been made less relevant with the sharp reduction of emissions achieved through adoption of appropriate measures and elimination of the dominant sources of such pollutants[1-2]. Instead, there has been a shift of concern about a category of compounds referred to as emerging contaminants.

Emerging contaminants comprise primarily of products used in large quantities in modern life. These include human and veterinary pharmaceuticals, personal care products, pesticides and herbicides, plasticizers and various industrial additives[1, 3]. These contaminants have been usually detected in environmental waters (such as surface water, ground and wastewater flows) at concentrations ranging from the $\mu g\ L^{-1}$ to $ng\ L^{-1}$ level[4].

The majority of emerging contaminants are unregulated in the environment and as a result there is little environmental survey data, with the risk assessment and the ecotoxicological effects being relatively unknown[3, 5]. The lack of data available has made it difficult to predict the transport and fate of emerging contaminants in the environment[1]. Similarly, most existing toxicity data are based on tests performed for single compounds and for short term exposure, and there is a lack of information regarding the fate and effects of mixtures of compounds, and their metabolites and/or transformation products which may lead to more toxic and persistent contaminants[1, 6]. The absence of toxicity data of contaminants is in part due the lack of analytical methods for proper risk assessment and management of environmental water samples[5].

Emerging contaminants are also a greater concern in part due to the increasing reclamation and reuse of water such as the recycling of water for agriculture and human consumption[1, 3]. The presence of emerging contaminants and their metabolites and/or transformation products persisting in the water have potential for significant impact on human health and the environment[1, 3]. Additionally the behaviour of emerging contaminants during wastewater treatment and production of drinking water would need to be studied[1, 3].

The prerequisite for proper monitoring and risk evaluation of emerging contaminants is the availability of a sensitive multi-residue screening analytical method for emerging contaminants in complex environmental matrices[7].

1.2 Pharmaceuticals as an emerging contaminant

1.2.1 Sources

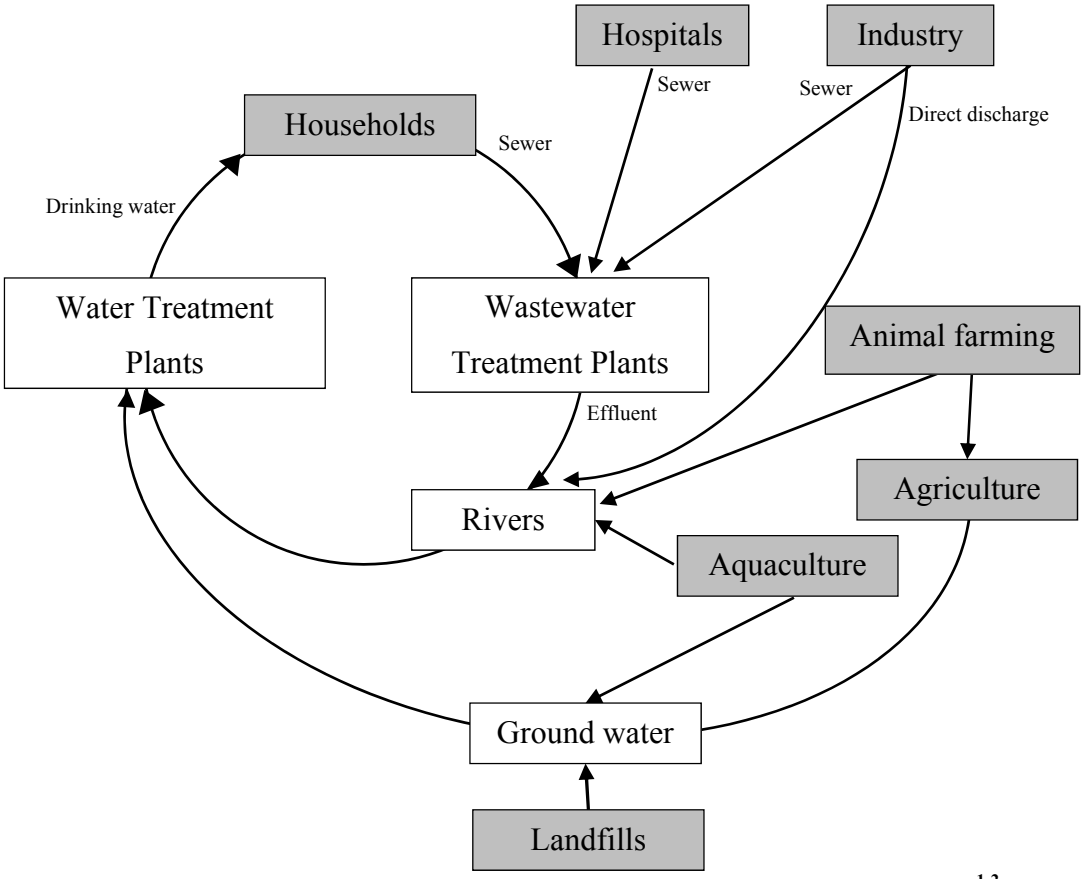


Figure 1.1 : Sources and transport of pharmaceuticals in a partially closed water cycle[1-2].

The main sources of pharmaceuticals in environmental waters include domestic households, hospitals and veterinaries, animal farming and agriculture, and aquaculture (Figure 1.1)[2].

The pharmaceuticals from domestic households are primarily as a result of excreta of consumed pharmaceuticals and disposal of expired medicines into the sewage system[2, 8]. Pharmaceuticals disposed into landfills may also leach to reach ground water.[4]

Hospitals are another major source of pharmaceuticals, primarily through the hospital sewage system for patients which can contain antibiotics and prescription drugs such as analgesics, antibiotics, blood-pressure regulating drugs, hormones and others, as well as veterinary hospitals which release veterinary waste to the domestic sewage system[2, 4]. Pharmaceuticals found in hospital effluent include methotrexate and methaqualone which have been detected at 1 $\mu g\ L^{-1}$ concentration[9].

Pharmaceuticals, such as human and veterinary drugs may also be released from industrial effluent as a result of the manufacturing process, which could pass through the sewage treatment system or pass directly into environmental waters[8]. The wastes containing

pharmaceuticals could end up in landfills which can then leach into groundwater, as well as storm runoffs that can carry powdered drugs[10]. The impact of industries as a source of pharmaceuticals in environmental waters has lessened due to more stringent control of the manufacture and disposal of waste pharmaceuticals[11]. However a study in 1995 by Holm et al. sampled groundwater downstream of a landfill and found sulfonamides at concentrations up to 5 mg L^{-1} that originated from pharmaceutical industrial waste[12].

Pharmaceuticals from humans, hospitals and industry which end up in the sewage system may not be completely removed during wastewater treatments[2, 13].[2, 132, 132, 13] The incomplete removal may be due to the high stability of the pharmaceutical or the stability of its metabolite[2, 13]. Treated sewage wastewater effluent has been found to contain a variety of pharmaceuticals[2, 13]. For example, diclofenac, ibuprofen, and propranolol have been detected in sewage treatment plant (STP) effluents at μg L^{-1} concentrations[2]. Additionally the transfer of sewage biosolids to land may result in the leaching of pharmaceuticals into environmental waters such as ground waters[2].

Hormones and antibiotics, such as tetracycline, sulphonamides and chloramphenicol can be found in the environment due to excreta from animals in animal farming and agriculture, as well as from the release from aquaculture of medicated feeds[5, 14]. These pharmaceuticals are not subject to the wastewater system and pass untreated into environmental waters[2]. Animal manure containing pharmaceuticals may also be used for agriculture[15].

1.2.2 General Effects

Despite pharmaceuticals being present at low concentrations in the environment (ranging from ng L^{-1} to μg L^{-1}) pharmaceuticals are classified as an emerging contaminant due to their continuous release into the environment[2, 5]. Additionally there is little information available regarding the long term risk of the continuous introduction of pharmaceuticals in the aquatic environment, or the effects of the metabolites which may have similar effects to the parent compound[16].

There are several major concerns regarding the presence of pharmaceuticals in environmental waters. These include the development of bacterial resistance, the uptake of pharmaceuticals by plants, and the exposure of aquatic organisms to pharmaceuticals[4]. The development of bacterial resistance could be promoted by the presence of antibiotics that end up in environmental waters[4-5]. However there is little evidence for the development of resistance due to the presence of antibiotics[14, 17].

Bioaccumulation of pharmaceuticals can affect plant development when treated wastewater is reused for irrigation, or when animal manure is applied to agricultural land[15].

Sulfadiomethoxine concentrations of 300 mg L^{-1} were found to affect root, stalk and leaf growth in species of barley, corn, millet and pea[17]. The bioaccumulation of pharmaceuticals in plants is also a concern due to contamination of food supplies and health risks associated with the consumption of resulting plant-based products[15].

The exposure of aquatic organisms to pharmaceuticals is a particular concern due to the constant introduction into surface waters, which means that aquatic ecosystems are continually exposed to the pharmaceuticals[5]. Additionally, the polarity and non-volatile nature of some drugs allows the drugs to remain in the aquatic environment[5]. Estrogenic responses, such as vitellogenesis and feminisation in fish species, may be induced if hormones such as estradiol and estrone are present even in low concentrations and these may result in adverse effects on aquatic and terrestrial organisms and humans[5]. Additionally, other types of hormones such as steroids would also have detrimental effects[5].

1.2.3 Transformation Products

Pharmaceuticals can form transformation products through a variety of pathways, including metabolism, prodrugs, and bio-transformation during wastewater treatment. These metabolites and transformation products may be more stable than the parent compound, and consequently avoid degradation during processes in sewage treatment plants[18]. Similarly, the conjugate form of drugs can be transformed back into the parent drug, through chemical processes such as hydrolysis[2, 5]. Additionally, once the pharmaceutical is in the environment, it can undergo transport and degradation processes including, but not limited to hydrolysis and photolysis[4].

1.2.4 Classes

Pharmaceuticals fall into several main classes, including antibiotics, non-steroidal anti-inflammatories, beta-adrenergic blockers, estrogenic hormones, androgens, and general pharmaceuticals (analgesics)[5]. Some of the most frequently used drug classes (such as antibiotics) are used in quantities similar to pesticides, and some pharmaceuticals can be sold without prescription[4]. The pharmaceutical classes and the target compounds in this study and the uses, potential effects, concentrations detected in environmental waters, and any known transformation products of the compounds are summarised below. The chemical formulas and structures of the target compounds are present in Appendix 1. The target compounds were selected according to information found in literature on their occurrence and ubiquity in environmental waters, as well as their high human use and consumption worldwide[8,24,28,32,42,43].

Antibiotics

Compound	Chloramphenicol		**Reference**
Uses	Antibiotic in veterinary and aquaculture practices.		[19]
Potential Effects	Inhibits a variety of aerobic and anaerobic microorganisms. Toxic to human bone marrow linked to blood disorders. Toxic to some algae species (*Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui*).		[19]
Concentrations detected	0.56 µg L^{-1}	Southern German sewage treatment plant effluent	[14]
	0.06 µg L^{-1}	Southern German small river	[14]
Transformations	Photocatalytic degradation to glycolic. The products (aldehyde, dichloroacetamide and 4-nitrobenzaldehyde) are resistant to further photooxidation.		[20]

Compound	Erythromycin		**Reference**
Uses	Antimicrobial agent in humans.		[21]
Potential Effects	Suspected antibiotic resistance induced in bacterial strains. However recent studies reveal no evidence for it yet, instead antibiotic resistance develop due to rare and random spontaneous mutation.		[14, 22]
Concentrations detected	2.5 µg L^{-1} (median) 6.0 µg L^{-1} (maximum)	German STP effluent	[14]
	0.15 µg L^{-1} (median) 1.7 µg L^{-1} (maximum)	German surface waters	[14]
	49 ng L^{-1} (maximum as Erythomycin-water)	Southern German ground waters	[23]
Transformations	Loss of water to form metabolite, Erythomycin-water (anhydro-erythromycin).		[24]

Compound	Sulfamethoxazole		**Reference**
Uses	Used in combination with trimethoprim as a prescribed combination antibiotic.		[25]
Potential Effects	Endocrine disrupting chemical affecting human and animal species.		[26]
Concentrations detected	0.15 µg L^{-1} (median) 1.9 µg L^{-1} (maximum)	U.S. streams	[27]
	0.40 µg L^{-1} (median) 2.00 µg L^{-1} (maximum)	German STP effluent	[14]
	0.03 µg L^{-1} (median) 0.14 µg L^{-1} (maximum)	German surface waters	[14]
Transformations	Ozonation to form toxic by-products.		[28]
	Up to nine different transformation products during photolysis.		[29]

Non-steroidal anti-inflammatories

Compound	Diclofenac		**Reference**
Uses	Non-steroidal anti-inflammatory drug.		[30]
Potential Effects	Affects liver and kidneys of fish (brown trout).		[10]
Concentrations detected	0.81 μg L^{-1} (median) 2.1 μg L^{-1} (max)	German STP effluent	[31]
	0.15 μg L^{-1} (median) 1.2 μg L^{-1} (max)	German rivers and streams	[31]
Transformations	Photolysis to form 2-[2-(chlorophenyl)amino]benzaldehyde, which has higher acute toxicity to *S. vacuolatus* than the parent compound.		[32]
	Photolysis to form 8-chloro-9H-carbazole-1yl-acetic acid.		[33]
	Photolysis to from the p-benzoquinone imine of 5-hydroxydiclofenac.		[30]

Compound	Ibuprofen		**Reference**
Uses	Non-steroidal anti-inflammatory drug.		[34]
Potential Effects	Chronic toxicity effects to aquatic organisms.		[10]
Concentrations detected	0.37 μg L^{-1} (median) 3.4 μg L^{-1} (max)	German STP effluent	[31]
	0.07 μg L^{-1} (median) 0.53 μg L^{-1} (max)	German Rivers and streams	[31]
Transformations	Metabolites hydroxy-ibuprofen and carboxy-ibuprofen.		[6, 35]

Compound	Ketoprofen		**Reference**
Uses	Non-steroidal anti-inflammatory drug.		[36]
Potential Effects	Potential toxicity effects to aquatic organisms.		[37]
Concentrations detected	0.20 μg L^{-1} (median) 0.39 μg L^{-1} (max)	German STP effluent	[31]
	0.12 μg L^{-1} (max)	German rivers and streams	[31]
Transformations	Photodegradation.		[36]
	Metabolites 3-(hydroxy-carboxymethyl)hydratopic acid, and 3-(keto-carboxymethyl)hydratopic acid		[30]

Compound	Naproxen		**Reference**
Uses	Non-steroidal anti-inflammatory drug.		[37]
Potential Effects	Potential toxicity effects to aquatic organisms.		[37]
Concentrations detected	0.30 μg L^{-1} (median) 0.52 μg L^{-1} (max)	German STP effluent	[31]
	0.15 μg L^{-1} (median) 0.39 μg L^{-1} (max)	German rivers and streams	[31]
Transformations	Phototransformation to form four photoproducts.		[38]
	Microbial degradation to O-Desmethyl-naproxen.		[30]

Beta blockers

Compound	Atenolol	**Reference**
Uses	Treatment of cardiovascular diseases.	[39]
Potential Effects	Low chronic toxicity to fish as some aquatic	[39]

	vertebrates have similar receptors that may interact with betablockers.		
Concentrations detected	Exceeded 40 μg L^{-1}	Spanish sewage treatment plant	[40]
	1197 ng L^{-1} (average)	Spanish STP influent	[41]
	1025 ng L^{-1} (average)	Spanish STP effluent	[41]
Transformations	Photocatalytical transformation		[42]
	Ozonation transformation.		[39]

Hormones

Compound	Beta-estradiol		**Reference**
Uses	Reproductive hormone.		[27]
Potential Effects	Vitellogenesis and feminization in fish.		[43]
Concentrations detected	0.16 μg L^{-1} (median) 0.20 μg L^{-1} (max)	USA surface waters	[27]
Transformations	Photogradation.		[44]
	Degradation to estrone.		[45]

Compound	Estriol		**Reference**
Uses	Reproductive hormone.		[27]
Potential Effects	Endrocrine disruptor compound.		[43]
Concentrations detected	0.019 μg L^{-1} (median) 0.051 μg L^{-1} (maximum)	USA surface waters	[27]
Transformations	-		

Compound	Estrone		**Reference**
Uses	Reproductive hormone.		[27]
Potential Effects	Vitellogenesis and feminization in fish.		[43]
Concentrations detected	0.027 μg L^{-1} (median) 0.112 μg L^{-1} (maximum)	USA surface waters	[27]
Transformations	-		

Compound	Ethinylestradiol		**Reference**
Uses	Oral contraceptive		[46]
Potential Effects	Endrocrine disruptor compound. Affects gene expression of freshwater fish, fathead minnows and juvenile salmon.		[10, 43]
Concentrations detected	0.073 μg L^{-1} (median) 0.831 μg L^{-1} (maximum)	USA surface waters	[27]
Transformations	Photochemical degradation.		[47]

Compound	Mestranol		**Reference**
Uses	Ovulation inhibitor		[27]
Potential Effects	Endrocrine disruptor compound.		[43]
Concentrations detected	0.074 μg L^{-1} (median) 0.407 μg L^{-1} (maximum)	USA surface waters	[27]
Transformations	-		

Antiepileptic

Compound	Carbamazepine		**Reference**
Uses	Anti-epileptic drugs		[48]
Potential Effects	Potential impact on aquatic organisms.		[49]
Concentrations detected	2.1 µg L^{-1} (median) 6.2 µg L^{-1} (max)	German STP effluents	[31]
	0.25 µg L^{-1} (median) 1.1 µg L^{-1} (max)	German STP surface waters (rivers and streams)	[31]
Transformations	Major metabolite 10, 11 epoxy-carbamazepine hydrolysed in vivo and excreted primarily as glucuronides. Inactivated glucuronide-conjugates, which can be cleaved in STP to release the carbamazepine, increasing the environmental concentrations.		[31]

Compound	Phenytoin		**Reference**
Uses	Anti-epileptic drugs		[48]
Potential Effects	Potential toxicity or effects to humans and aquatic organisms.		[50]
Concentrations detected	5.1 ng L^{-1} (median) 29 ng L^{-1} (max)	USA stream water	[51]
Transformations	-		

General Pharmaceuticals

Compound	Atorvastatin		**Reference**
Uses	Lipid regulator, prevention of cardiovascular events		[6, 52]
Potential Effects	Concern due to potential toxicological effects in the environment.		[53]
Concentrations detected	0.80 ng L^{-1} (median) 1.4 ng L^{-1} (max)	USA stream water	[51]
	0.022 µg L^{-1} (mean)	Canadian STP effluent	[54]
Transformations	Photochemical transformation to form a pyrrol-2(3H)-one system.		[52b]

Compound	Fluoxetine		**Reference**
Uses	Antidepressant.		[55]
Potential Effects	Potential aquatic exotoxicological effects.		[56]
Concentrations detected	0.012 µg L^{-1} (median) 1.2 µg L^{-1} (maximum)	US surface waters	[27]
Transformations	Photodegradation to form two O-dealkylated products.		[56]

Compound	Gemfibrozil	**Reference**
Uses	Lipid regulator.	[57]

Potential Effects	Impact on human health (toxicity) when treated wastewater used for crop irrigation.		[40]
Concentrations detected	0.40 μg L^{-1} (median) 1.5 μg L^{-1} (max)	German STP effluents	[31]
	0.052 μg L^{-1} (median) 0.51 μg L^{-1} (max)	German STP surface waters	[31]
Transformations	Phototransformation.		[4]

Miscellaneous Emerging Contaminants

These emerging contaminants serve as anthropogenic markers for wastewater contamination of surface waters.

Compound	Caffeine		**Reference**
Uses	Stimulant.		[27]
Potential Effects	-		
Concentrations detected	126 μg L^{-1}	STP effluent Norway	[35a]
	0.081 μg L^{-1} (median) 5.7 μg L^{-1} (maximum)	US surface waters	[27]
Transformations	Ozonation to form short chained carboxylic acids.		[58]

Compound	TCEP (tris(2-carboxyethyl)phosphine)		**Reference**
Uses	Flame-retardant and a plasticizer.		[59]
Potential Effects	Weakly cytotoxic. Neurotoxic and genotoxic agent.		[59-60]
Concentrations detected	986 ng L^{-1}	German STP influent	[59]
	352 ng L^{-1}	German STP effluent	[59]
Transformations	-		

Compound	Triclosan		**Reference**
Uses	Antimicrobial disinfectant.		[27]
Potential Effects	Aquatoxicity.		[61]
Concentrations detected	0.14 μg L^{-1} (median) 2.3 μg L^{-1} (maximum)	US surface waters	[27]
Transformations	Biological methylation to form methyl-triclosan.		[62]
	Photolysis.		[62]

1.3 Analysis

1.3.1 Pre-concentration

Pharmaceuticals have been detected at concentrations ranging from μg L^{-1} to ng L^{-1} [14, 27, 31, 39-43, 46]. Consequently any analytical methods for analysing pharmaceuticals requires methods that have measurement limits down to the low ng L^{-1} level[1].

The most common methods of pre-concentration involve solid phase extraction (SPE) to extract pharmaceuticals from a sample into a smaller volume of solvent. SPE also serves as a clean-up step by removing salts and other potentially interfering components from the matrix[4-5]. The most commonly used phases for pre-concentration, extraction or clean-up

include hydrophilic-lipophilic balanced polymers, strongly hydrophobic silica-based bonded phases, strong cation-exchanged mixed-mode polymeric sorbents, or modified polystyrene-divinylbenzene resins. The most common elution solvents are acetone, ethyl acetate and methanol[4].

SPE can be automated, which improves the accuracy and speed of analysis[4]. The main advantages of on-line SPE include improved precision and accuracy due to automatisation and minimal sample handling, reduced analysis time and high throughput, smaller sample volumes needed, and no loss of analytes due to evaporation steps. However on-line SPE is not without disadvantages with matrix effects such as ionic suppression and enhancement being more prominent in LC-MS methods, and the absence of extracts for verification and further analysis[5].

Other sample pre-concentration techniques include solid-phase microextraction (SPME) which is typically used in conjunction with gas chromatography (GC) methods, liquid-phase microextraction (LPME) and lyophilisation[4].

1.3.2 GC Methods

GC-MS and GC-MS/MS have been used for the analysis of pharmaceuticals at trace concentrations[13]. GC-MS was the most frequently used technique a decade ago, in part due to the availability of GC-MS systems in environmental laboratories[63], as well as the advantages that the GC afforded. These advantages included high sensitivity, high selectivity and resolution, good accuracy and precision, and a wide dynamic range[4]. Additionally, GC-MS is applicable for the identification of non-target compounds, due to the availability of commercially available or standardised spectral libraries[64].

Koutsouba et al. performed determination of pharmaceuticals using capillary GC-MS with selected ion monitoring (SIM) mode, with solid phase extraction (C18) and derivatisation with pentafluorobenyzl bromide[65]. Sewage influents and effluents were analysed for polar pharmaceutical residues. Mean recoveries of 67-90% in most cases. In full acquisition mode, the LOD were in the range of 36-340 ng L^{-1}, and were in 0.6-20 ng L^{-1} in SIM mode. The method allowed detection down to low ng L^{-1} levels concentrations[65].

However as GC is limited to the analysis of non-polar, semi-volatile and volatile compounds. However most pharmaceuticals are polar in nature and relatively non-volatile thus a derivatisation step is normally required for the analysis. Many derivatisation agents may be used including BF_3-MeOH[66], pentafluorobenzyl bromide[65], diazomethane[31]. However the accuracy of the method is affected by loss of analytes, through decomposition of thermolabile compounds during GC analysis or incomplete reactions during the derivatisation

step[1, 4, 67]. The derivatisation step is also laborious and time consuming[1]. Consequently there has been much effort on optimising the derivatisation step to improve GC-MS analysis of pharmaceuticals[4].

For example, Rodriguez et al. published a paper on the optimisation of a GC-MS method for acidic pharmaceuticals in sewage water, through derivatisation using a N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA). The study focused on the optimisation of the derivatisation step as that was the common weakest point of GC methods, with parameters altered based on factorial central composite design. Limit of quantitation ranged from 20 to 50 ng L^{-1}, with recoveries of 90-115% from spikes[63].

Other techniques that were commonly used in the past include GC being coupled to electron capture detection (ECD) and flame ionisation detection (FID). However GC methods have been mostly replaced by liquid chromatography (LC) methods after the introduction of atmospheric pressure ionization.[5]

1.3.3 LC Methods

1.3.3.1 LC-MS

The use of liquid chromatography has largely replaced GC methods for the analysis of emerging contaminants in aqueous environmental samples. An advantage LC presents over GC techniques is the identification of highly polar compounds without a derivatisation step, allowing for a quicker sample preparation time and reduction of errors associated with derivatisation[8, 65]. Additionally LC has a shorter analysis time, making it suitable for studies that involve monitoring[4].

An example of the application of LC-MS to the detection of pharmaceuticals in water is where Farre et al 2001. applied LC-ESI-MS for the determination of acidic and polar pharmaceuticals[66]. Sample enrichment was performed with SPE, to yield recoveries of 69-91% and detection limits of 15-56 ng L^{-1}. The samples were analysed in parallel with GC-MS methods with a BF_3-MeOH derivatisation step, with good agreement obtained between the methods.

However LC is susceptible to matrix effects, which reduce the accuracy, linearity, precision, and sensitivity of the method[4]. There is also a lack of commercially available or standardised mass-spectra libraries for LC-MS due to the absence of a normalised interface, making the identification of non-target compounds problematic and time consuming[4, 64]. The differences in results obtained due to the composition of the mobile phase or the cone voltage applied, and the differences in ionisation between existing surfaces makes it difficult to use standardised libraries. Instead home-made libraries have to be built to facilitate searching.

However without reference standards, interpretation of complicated fragmentation patterns in MS/MS is necessary for identification of unknown transformation products[4]. Similarly when home libraries are built, it should include as many contaminants as possible to minimise the risk of false negatives[64].

A major limitation of LC is the poor resolving power[68], which can be improved through the application of ultra-high performance liquid chromatography (UHPLC) or bypassed through the shift to tandem LC-MS/MS.

1.3.3.2 UHPLC

In recent years there has been an increased in the coupling of UHPLC systems to tandem LC-MS methods[69]. The use of UHPLC is to improve some of the limitations of LC such as the poor resolving power.

The importance of good chromatographic separation is to reduce ion suppression and isobaric interferences, which is a problem especially in trace level analysis. UHPLC results in better chromatographic resolution and peak capacity due to the elution of the sample in narrower more concentrated bands compared to high performance LC (HPLC)[8].

Lopez-Serna et al. described a multi-residue analytical method based on single-step SPE followed by UHPLC-MS/MS detection[70]. Simultaneous analysis of 74 multi-class pharmaceuticals in differential environmental samples was achieved. UHPLC afforded better chromatographic resolution and increased peak capacity, and a higher throughput. Detection limits of 0.01-50 ng L^{-1} were obtained for both environmental and wastewaters. However the method showed poor SPE recoveries, and ultimately the method is limited to targeted screening as it was performed on a triple quadrupole.

1.3.3.3 Tandem MS

There has been a shift to tandem LC MS/MS as the preferred technique for the qualitative and quantitative analysis of pharmaceuticals in complex matrices, to compensate for the limitations of GC-MS and LC-MS[4]. The most common types of tandem LC MS/MS include triple quadrupole (QQQ), quadrupole time-of-flight (QTOF), and Orbitrap, with the latter two enabling fast, sensitive and reliable qualitative and quantitative analysis of small compounds due to the high mass resolution and accuracy[4].

<u>QQQ-LCMS</u>

There have been a significant number of methods based on QQQ-LCMS for the detection of pharmaceuticals in water. The majority focus on a single method of analysis for various compound classes, as it results in the advantages of a shorter overall analysis time, reduced field sample and cost reduction[71]. Consequently due to the need for a multi-residue method,

the analysis should be sensitive, and this is achieved primarily by targeted analysis through the use of the QQQ in multiple reaction monitoring (MRM) mode.

Ternes et al. reported on the use of LC-ESI tandem MS for the analysis of pharmaceuticals in environmental samples[31]. Nine pharmaceuticals and metabolites were selected. Effective and time efficient preconcentration was achieved through SPE which allowed simultaneous enrichment of all selected pharmaceuticals. Recoveries from spikes exceeded 80% for the majority of the analytes in groundwater, with only 4-amino-antipyrine and caffeine at 64% and 66% respectively. In surface waters, some recoveries were slightly lower than the groundwater counterparts, although some compounds had considerably lower recoveries of 12% and 30% for oxyphenbutazone and phenylbutazone respectively. However in treated sewage effluent and raw sewage, the recoveries were substantially lowered for most analytes due to matrix impurities. The authors demonstrated how LC-ESI-MS-MS could be used in the environmental analysis of pharmaceuticals and their metabolites in environmental waters, with the MRM mode achieving sufficient sensitivity for analysis. However it was noted that the enrichment step is typically a difficult step in the analytical procedure.

Miao et al. similarly demonstrated the simultaneous analysis of nine acidic pharmaceutical drugs in sewage treatment plant effluents[18]. The authors used SPE and LC-ESI-MS-MS under multiple reaction monitoring (MRM) mode for the analysis of the underivatised acidic drugs, and the method was validated through analysis of STP effluents. Good chromatographic resolution and ion intensities were achieved during the run. The method also provided acceptable recoveries and limit of detections, with the mean recoveries from the spiked effluent samples ranged from 58.5% to 91.5%, with limit of detection from 5-20 ng mL^{-1} depending on the analyte. The suitability of LC-QQQ MS under MRM for targeted analysis was highlighted in this work as the method could be used to determine ng L^{-1} levels of acidic pharmaceuticals in aqueous samples.

QTOF

There have been a significant number of analytical methods developed for the analysis of different pharmaceutical classes. These are predominantly based on single and triple quadrupole LC-MS[8]. Some limitations of QQQ is that identification and quantification are separated steps, as MRM mode lacks the qualitative information needed to support structure elucidation, whilst full scan mode lacks sensitivity[8].

There has been growing interest in QTOF, due to the high mass accuracy and mass resolution obtained for both the parent and transition ions which allows for more accurate identification of target compounds as well as unknown compounds[13, 72, 13, 72, 13, 68, 13, 72].

Other advantages QTOF has over QQQ is that QTOF is able to distinguish between the ions of co-eluting compounds when similar masses are present in the sample unlike MRM[13]. This advantage allows for QTOF to potentially be used for post-targeted and non-targeted screening, unlike QQQ which is limited to targeted screening.

There are many methods published currently for the multi-residue screening of pharmaceuticals using QTOF LC-MS. Petrovic et al. described a method using a UPLC QTOF to screen and confirm for 29 different pharmaceuticals[8]. Identification was based on the accurate mass measurements of the molecular ions in time-of-flight (TOF) mode, then the application of collision induced dissociation (CID) in the QTOF to gain accurate mass measurements of the product ions. Quantitation was carried out in TOF mode, and the method applied to STP samples to obtain limits of detections of 10-500 ng L^{-1}.

Similarly, Farre et al. developed a rapid multi-residue method for the identification and quantification of a range of pharmaceutical classes[13]. Over 32 compounds were analysed that encompassed a wide range of pharmaceutical classes including anti-inflammatories, antibiotics, beta-blockers and phytoestrogens. Limit of quantitation in tap water ranged from 0.1-15 ng L^{-1} and in wastewater it was 2-300 ng L^{-1}. The results were compared to a triple quadrupole LC-MS with the QTOF providing more accurate mass information and mass accuracy, although the QQQ had lower limit of detections and a greater linear response.

The potential of QTOF for post-targeted screening is expanded upon by Ibanez et al., where acquisition can be acquired in MS^E mode, where low and high collision-energy full scan acquisitions are performed simultaneously[64]. The low energy mode provides information on the molecular ion, whilst the high energy yields fragmentation information which can be used for structure elucidation and confirmation of unknowns.

The potential of post-targeted analysis and non-targeted screening is a major contribution as this technique could potentially be used for the screening of environmental waters and the analysis of emerging contaminants and their unknown transformation products. Unlike QTOF, QQQ in MRM mode is not suitable for the analysis of unknown compounds. Consequently there is a need to further develop QTOF methods for the multi-residue analysis of emerging contaminants and their transformation products.

1.4 Method Development and Quality by Design

In the traditional approaches to development and validation of analytical methods, validation is a separate component performed after development[73]. However validation is often treated as a once-off event performed in a check box manner against ICH Q2(R1), with little consideration to ensuring focus on consistent method performance, or improvement in

quality and efficiency[74]. These and other issues give rise to a number of limitations including problems in routine use, low emphasis on method robustness and ruggedness, poor knowledge on critical parameters, and invested time is not significant. The shortcomings of the traditional approach have created a need to switch from traditional method validation requirements to a method validation approach that provides a high level of assurance of method reliability[75]. One concept gaining interest is quality by design (QbD).

QbD is defined as "a systematic approach to development that begins with predefined objectives and emphasises product and process understanding and process control, based on sound science and quality risk management"[76]. QbD was originally intended and used in the pharmaceutical product development to ensure a predefined product quality. However there have been recent moves to bring the principles of QbD to chemical analysis[77]. In contrast to traditional methods, QbD builds quality into the development process rather than checking for quality at the end of the development process[77]. Benefits include the development of a robust method, and sources of variability are well understood and are reduced or controlled.

Some of the principles of QbD will be incorporated into this research project.

1.5 Summary

Emerging contaminants, such as pharmaceuticals are a growing concern in recent years[69]. These contaminants have been usually detected in environmental waters (such as surface water, ground and wastewater flows) at concentrations from trace to μg L^{-1} levels[4].

The prerequisite for proper monitoring and risk evaluation of emerging contaminants is the availability of a sensitive multi-residue screening analytical method for emerging contaminants in complex environmental matrices[7]. More recently, tandem LC-MS has been identified as the technique of choice for the analysis of pharmaceuticals, with both QQQ and QTOF used[4]. There is interest in QTOF for the post-target analysis and non-targeted screening of pharmaceuticals in environmental waters.

1.6 Project Aims

The aims of the project are the development and validation of a sensitive multi-residue analytical method for the analysis of pharmaceuticals in environmental waters, using on-line SPE coupled to a UHPLC QTOF-MS.

Chapter 2: Experimental

2.1 Reagents

Chemicals

All pharmaceutical standards were analytical grade. 17-α-ethinylestradiol (ethinylestradiol), 17-β-estradiol (betaestradiol), atenolol, atorvastatin calcium salt trihydrate, caffeine, carbamazepine, chloramphenicol, diclofenac sodium, erythromycin, estriol, estrone, fluoxetine hydrochloride, gemfibrozil, ibuprofen sodium, irgasan (triclosan), ketoprofen, mestranol, naproxen sodium, phenytoin sodium, sulfamethoxazole, and tris(2-carboxyethyl)phosphine (TCEP), were purchased from Sigma-Aldrich (Australia). HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck. Other solvents including ammonium hydroxide and hydroxylamine were supplied by Sigma Aldrich (Australia) unless stated otherwise.

Pharmaceutical standards

Individual stock standard solutions were prepared on a weight basis in methanol, 0.2 μm filtered, and stored at -10 °C. Working standard solutions were prepared by appropriate dilution of the individual stock standards using Type I water obtained from Millipore Milli-Q® Integral 3 Water Purification System. All buffers were prepared using Type I water.

2.2 Samples

Environmental water samples were kindly provided by Melbourne University. Samples were from Victoria, Australia at sites up and down stream of an undisclosed secondary sewage treatment plant. Samples were kept frozen until analysis. Prior to analysis the samples were 0.2 μm filtered.

2.3 Instrumentation

SPE-UHPLC-QTOF

All instrumentation related to the on-line SPE coupled to UHPLC-QTOF system were from Agilent Australia. Mass spectrometry was performed using 6550 iFunnel Q-TOF LC/MS system. The UHPLC system was a 1290 Infinity Binary LC System. An on-line SPE system was later coupled to the UHPLC-QTOF-MS system using a 1260 Infinity Binary LC system. C-18 columns used included Poroshell 120 Column - EC-18 Analytical, 2.7μm, 5.6 x 50 mm, and 150 mm. SPE cartridges used were PLRP-s 15-20 μm 2.1 x 12.5 mm, in conjunction with a Reliance Guard-Column holder. All data acquisition and analysis was obtained using MassHunter Workstation Software, in particular the LC/MS Data Acquisition

for 6200 series TOF.6500 series QTOF, Qualitative Analysis, and Quantitative Analysis for TOF and QTOF.

Off-line SPE

The cartridges used for off-line SPE were Oasis HLB (60 mg, 3 mL) from Waters. The cartridges were first conditioned with 5 mL of methanol, followed by 5 mL of Type I water under gravity. After conditioning, the water samples were percolated through the cartridges. The cartridges were rinsed with 2 x 5 mL of Type I water and the cartridge allowed to dry to remove excess water. Elution was performed with 5 mL of 80% acetonitrile. The extract was concentrated under nitrogen gas to 0.5 mL and diluted to 1 mL with water.

Chapter 3: Results and Discussion

A method for the analysis of pharmaceuticals in water by LC-QTOF-MS cannot be easily developed *a priori* and used without refinement. Thus to develop the final method, it was necessary to develop many systems along the way, with the development and optimisation of many parameters. Each subsequent system is a refinement of the previous, building on the knowledge obtained. A great deal of preliminary work was necessary to develop the final system.

Some principles of QbD were also incorporated into the method development in order to build quality into the development process rather than checking for quality at the end of the development process. Towards the goal of QbD, an Ishikawa diagram (a causal diagram) was created to identify potentially critical factors that can impact on the end method (Figure 3.1) and will be referred to in subsequent sections of method development.

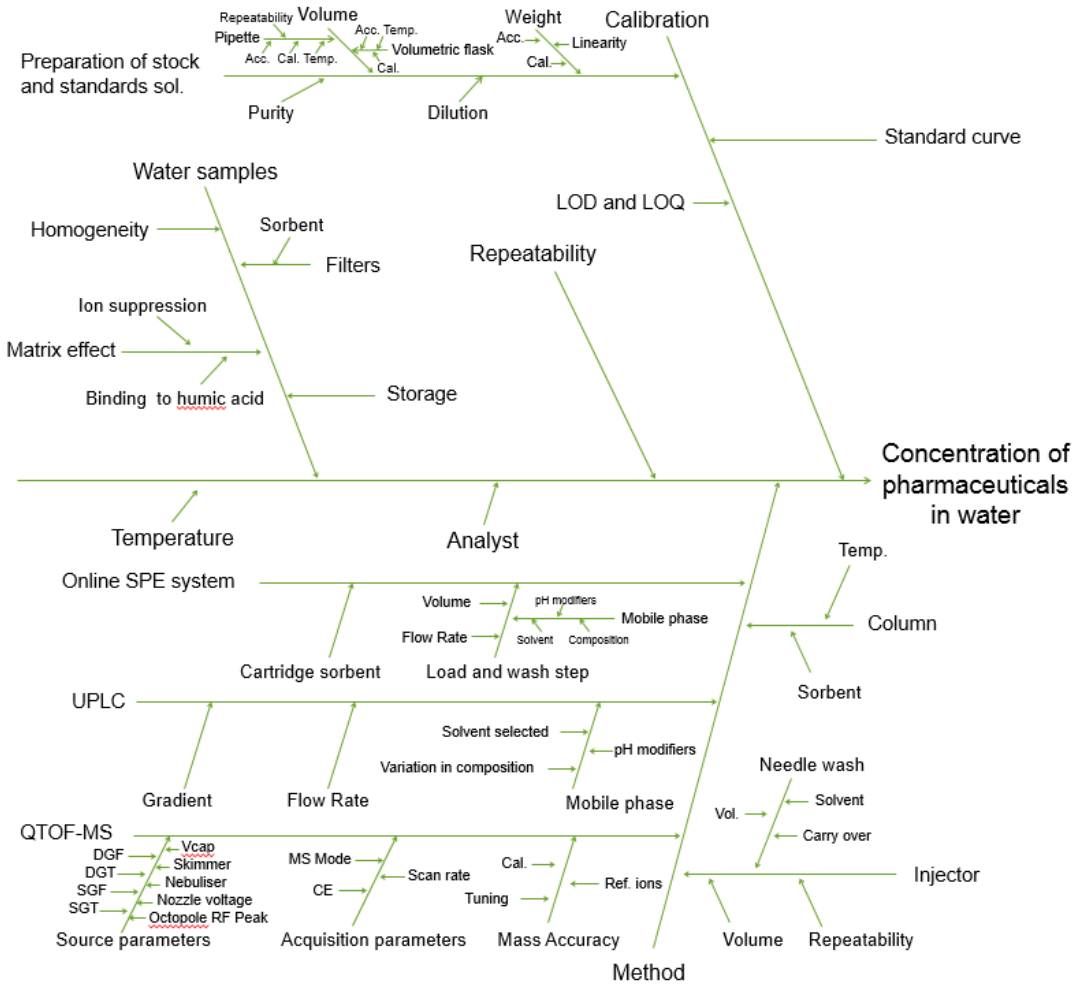


Figure 3.1 : Ishikawa diagram identifying causal factors that contribute to the desired outcome of a method for determining the concentrations of pharmaceuticals in water

3.1 UHPLC-QTOF-MS Method

The first stage of method development was to establish a working method for direct multi-residue analysis using the UHPLC-QTOF-MS system without a pre-concentrated step.

3.1.1 Selection of mobile phases and ESI modes

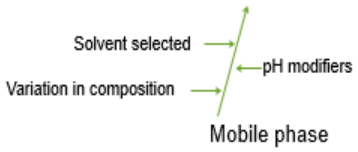


Figure 3.2 : Sub-diagram of factors from Ishikawa diagram relevant to this section.

The first set of experiments was to establish the MS-detectability of the pharmaceutical standards under study. Detectability of compounds would depend primarily on the mobile phase used as well as the ESI run mode. Different mobile modifiers were trialled in a water-ACN mix encompassing a range of different pHs levels and these are summarised in Table 3.1. The ESI source was run in both positive (PI) and negative (NI) ionisation modes. As part of experimental design, only the mobile phases and ESI modes were varied whilst the QTOF source parameters were kept constant (Table 3.2). Two schematics of the LC set up was used, including a direct injection mode detailed in Figure 3.3.a, and a C18-column set up given in Figure 3.3.b with different binary timetables (Table 3.3).

Initially, the individual pharmaceutical standard solutions (1 mg L^{-1}) were introduced into the MS by flow injection (Figure 3.3 a.) with ACN/0.1% formic acid carrier (Table 3.1 b.). Data were acquired in both positive and negative ESI modes.

Under these conditions it was found that many of the study compounds were MS-detectable, however some compounds, in particular the hormones, had little to no MS-detectability in either ESI modes. This lack of MS-detectability is understandable given that for a compound to be ionisable via ESI, it needs to have functional groups that can be either protonated by H_3O^+ or de-protonated by OH^-. For compounds that have functional groups with pKa's higher than water, such as estrone which has only a single hydroxyl group, only a small fraction would be charged when it enters the MS. Consequently, the choice of chromatographic buffers and pH is critical for compounds to be in a charged state.

It has been reported in literature that optimal determination of estrogenic hormones occurs using a mixture of water and acetonitrile without the addition of any modifiers[78], although pH adjustment with ammonium hydroxide was also recommended[79]. Adjustment of pH with ammonium hydroxide was trialled with the results discussed in Section 3.3.2.

Table 3.1 : Conditions of the four mobile phases trialled (a-d)

#	Description	A - Water	B – 95% ACN in water
a	No modifiers	No modifiers	No modifiers
b	Formic acid	0.1% formic acid (pH ≈ 3)	0.1% formic acid
c	Formic acid and ammonium formate	0.1% formic acid and 15 mM ammonium formate (pH ≈ 5)	0.1% formic acid and 15 mM ammonium formate
d	Ammonium formate	15 mM ammonium formate (pH ≈ 7)	15 mM ammonium formate

Table 3.2 : Initial QTOF source parameters

Drying gas temperature (DGT) /°C:	125	Capillary voltage (VCap) /V:	2500
Drying gas flow (DGF) /L min^{-1}:	16	Nebuliser pressure /psig:	20
Sheath gas temperature (SGT) /°C:	350	Nozzle voltage /V:	1500
Sheath gas flow (SGF) /L min^{-1}:	12		

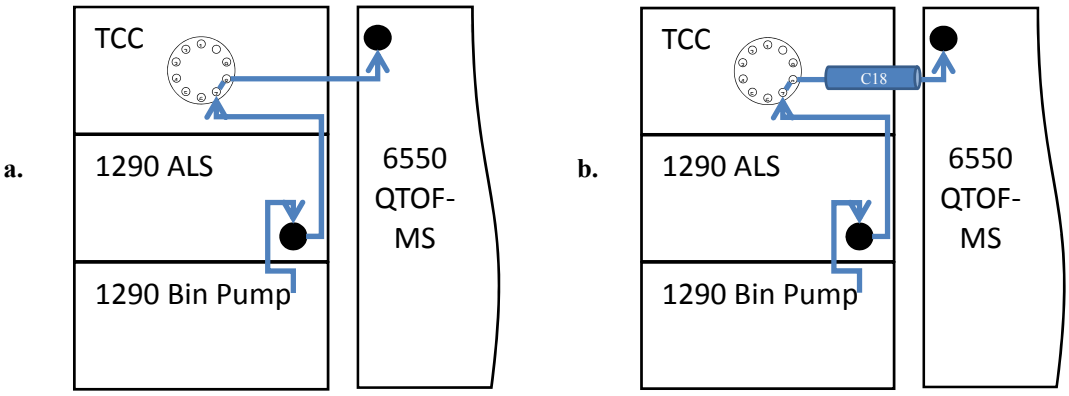


Figure 3.3 : UHPLC-QTOF-MS schematic for a. flow injection with column bypass and; b. chromatographic analysis with C-18 column.

Table 3.3 : 1290 Binary timetable for a. flow injection with column bypass; b. chromatographic analysis with C-18 column.

a.		
Time /min	**A /%**	**B /%**
0	50	50
Flow rate: 0.6 mL min^{-1} Stop time: 0.5 min Post time: 0.5 min		

b.		
Time /min	**A /%**	**B /%**
0	90	10
5	90	10
20	0	100
Flow rate: 0.5 mL min^{-1} Stop time: 22 min Post time: 2 min		

Having established appropriate ionisation conditions via flow injection, the MS was reconfigured for reverse phase chromatographic analysis (Figure 3.3 b.). In this configuration, the four mobile phase combinations detailed in Table 3.1 and using the gradient program detailed in Table 3.3 b., were evaluated using a test mixture, containing 100 µg L^{-1} all of the study pharmaceuticals. For each condition, the test mixture was run in triplicate with 2 µL injections, and with the system equilibration between chromatographic systems achieved through running multiple double blanks (no injection).

The average areas for the standards are summarised in Appendix 2 with a graphic representation shown in Figure 3.4. For positive ESI mode, chromatographic system B (ACN/0.1% formic acid) is most ideal. The selection was based on the compounds which had weaker responses including ketoprofen, mestranol, naproxen and TCEP, which favoured the ACN/formic acid carrier. The majority of compounds, however, preferred chromatographic system A (ACN/water) although ACN/0.1% formic acid had relatively high responses regardless. The ideal chromatographic buffer for negative ESI mode on the other hand was the ACN/water carrier. The majority of compounds preferred the ACN/water carrier with the exception of erythromycin and ethinylestradiol which preferred the formic acid carrier. Based on the above results, the ACN/water and ACN/0.1% formic acid carrier system were selected as most appropriate.

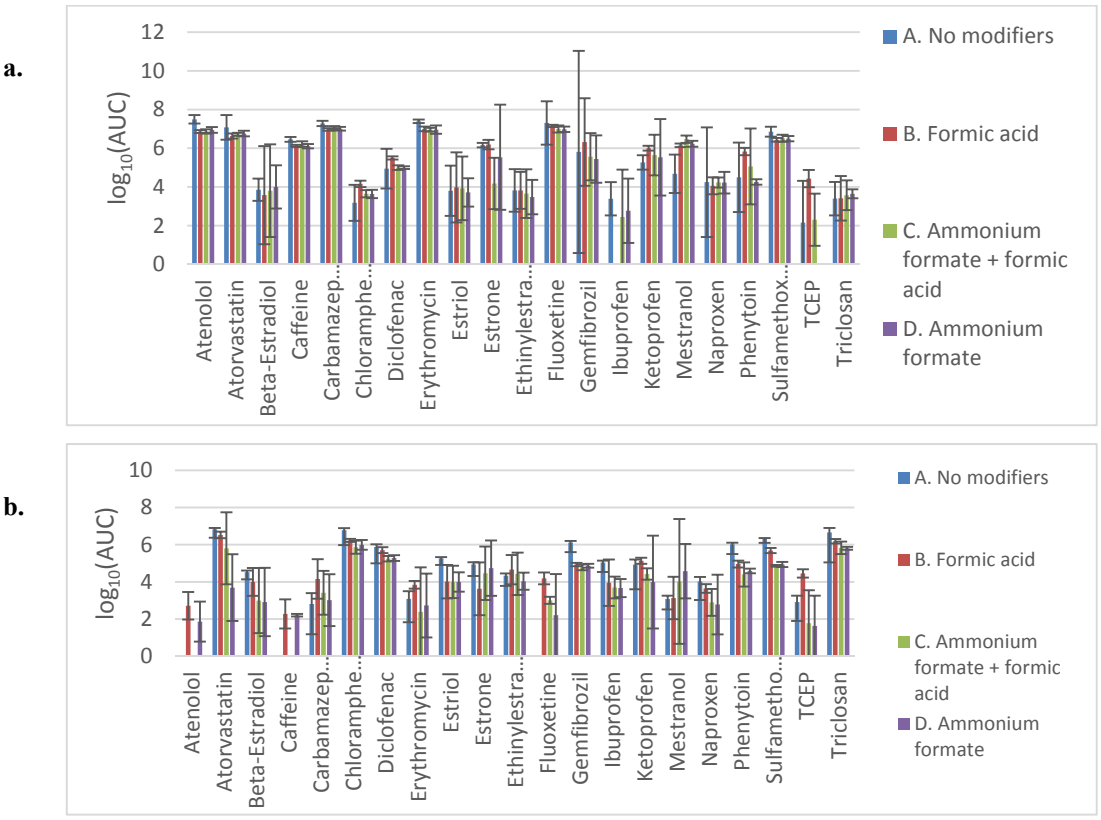


Figure 3.4 : Comparison of the log_{10}(AUC) of each compound against the four chromatographic buffer conditions for a. positive ESI mode and; b. negative ESI mode.

3.1.2 Establishment of a pharmaceutical MS/MS database

Targeted MS/MS data acquisition was set up based on predicted m/z for the $[M+H]^+$ and $[M-H]^-$ adducts for positive and negative ESI modes respectively. The QTOF source parameters were initially set according to Table 3.2. Triplicate injections of 0.5 µL of 1 mg L^{-1} individual standards were introduced into the MS by flow injection (Figure 3.3 a.) with a ACN/water carrier (Table 3.1 a.). The MS/MS spectra obtained were curated to remove ions less than 5% of total abundance and were then added to a database on MassHunter PCDL.

The initial MS/MS spectra obtained with the parameters in Table 3.2 were later updated with spectra obtained with optimised source parameters (Section 3.1.3).

3.1.3 Optimisation of QTOF source parameters

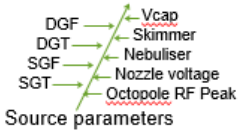


Figure 3.5 : Sub-diagram of factors from Ishikawa diagram relevant to this section

For the following optimisation experiment, data acquisition was obtained in All-Ions mode which is equivalent to MS^E mode. In the MS^E mode, high and low collision energy (20 eV and 0 eV) settings are alternated. Consequently, both molecular ion and nonselective MS/MS fragment ion data are obtained for the detected compounds.

Variation of the source parameters in the QTOF was known to affect the responses of the standards[80]. Several parameters were altered and optimised through modification in a sequential manner according to the order of greatest effect on the ionisation process. Initially the nebuliser pressure and nozzle voltages were optimised, followed by sheath gas temperature (SGT) and sheath gas flow (SGF), then drying gas temperature (DGT) and drying gas flow (DGF), and finally capillary voltage(VCap).

i. Nebuliser pressure and nozzle voltage optimisation

Different combinations of nebuliser pressure (20, 30, 40 psig) and nozzle (0, 500, 1000, 1500, 2000 V) voltages were trialled in an ACN/water carrier (Table 3.1 a.), with the remaining parameters kept constant (Table 3.4). The total AUC for the compounds were extracted and used to generate a surface plot (Figure 3.6). Based on the responses, for positive ESI mode, the optimised nebuliser and nozzle voltage were found to be 20 psig and 2000 V respectively and for negative ESI mode, 20 psig and 1500 V respectively.

Table 3.4 : Optimisation conditions trialled for nebuliser and nozzle voltages. Grey shading indicates the modified values for optimisation.

DGT /°C:	125	**Capillary voltage /V:**	2500
DGF /L min^{-1}:	16	**Nebuliser /psig:**	20, 30, 40
SGT /°C:	350	**Nozzle voltage /V:**	0, 500, 1000, 1500, 2000
SGF /L min^{-1}:	11		

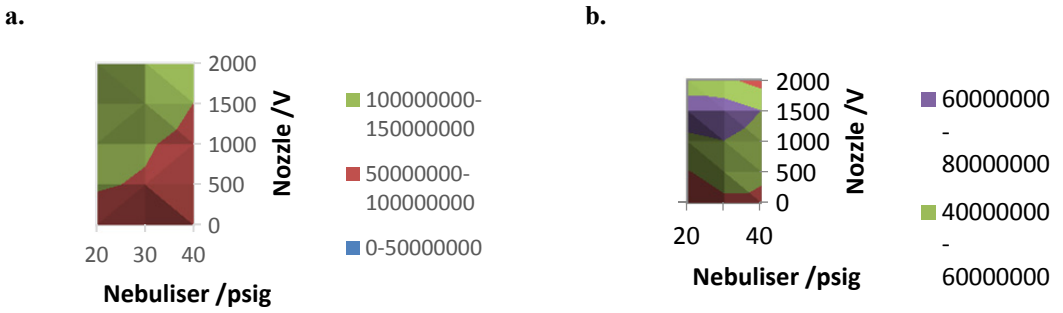


Figure 3.6 : Surface charts of responses from different combinations of nebuliser and nozzle values acquired in ACN/water mobile phase in a. positive ESI mode and; b. negative ESI mode. The different shades represent the total response of the compounds.

ii. Sheath gas flow and temperature optimisation

Different combinations of SGF (10, 11, 12 L min^{-1}) and SGT (200, 250, 300, 350, 400 °C) were trialled, with the other parameters kept constant, with the updated optimised nebuliser and nozzle values. The results are summarised in Figure 3.7. Based on the responses, for positive ESI mode, the optimised SGF and SGT were found to be 10 L min^{-1} and 400 °C respectively and for negative ESI mode, and 12 L min^{-1} and 400 °C respectively.

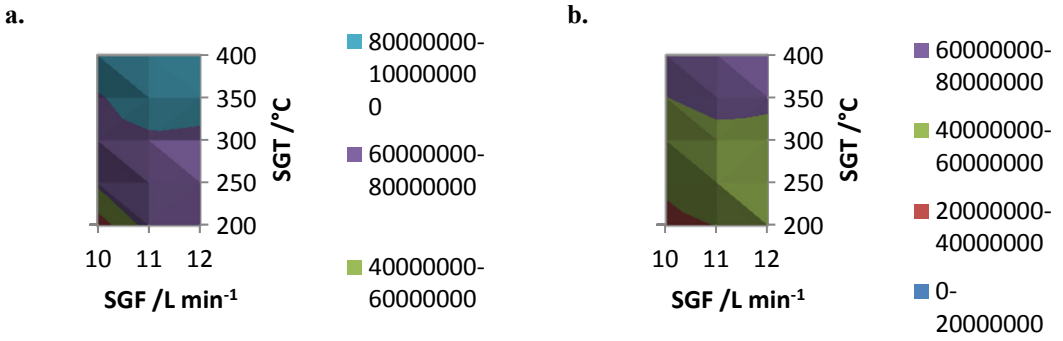


Figure 3.7 : Surface charts of responses from different combinations of SGF and SGT values acquired in ACN/water mobile phase in a. positive ESI mode and; b. negative ESI mode. The different shades represent the total response of the compounds.

iii. Drying gas temperature and flow optimisation

Similarly, combinations of DGF (12, 14, 16, 18 L min-1) and DGT (125, 150, 175, 200, 225 °C) were trialled, and the results are summarised in Figure 3.8. Based on the responses, for positive ESI mode, the optimised parameters for DGF and DGT were 16 L min^{-1} and 125 °C respectively and for negative ESI mode, and 18 L min^{-1} and 125 °C respectively.

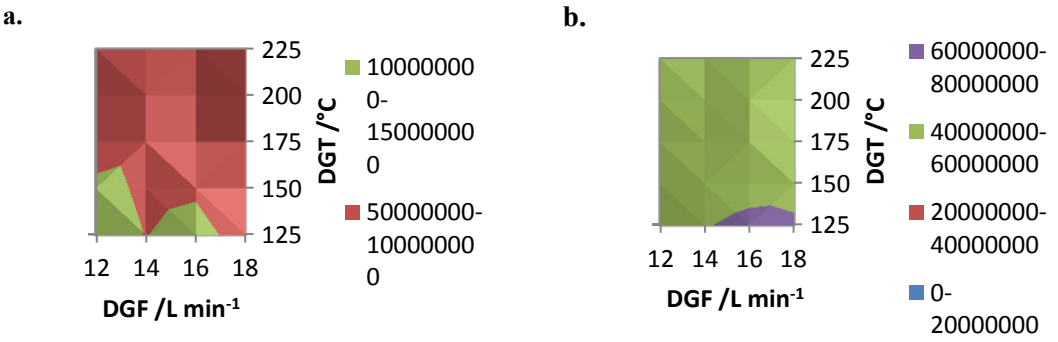


Figure 3.8 : Surface charts of responses from different combinations of DGF and DGT values acquired in ACN/water mobile phase in a. positive ESI mode and; b. negative ESI mode. The different shades represent the total response of the compounds.

iv. Capillary voltage optimisation

Finally the capillary voltages were modified (1500, 2000, 2500, 3000, 3500, 4000, 4500 V). Based on the responses (Figure 3.9), for positive ESI mode the optimal values were 1500 V for positive ESI mode as it favoured the weaker responding compounds, and for negative ESI mode, 2000 V.

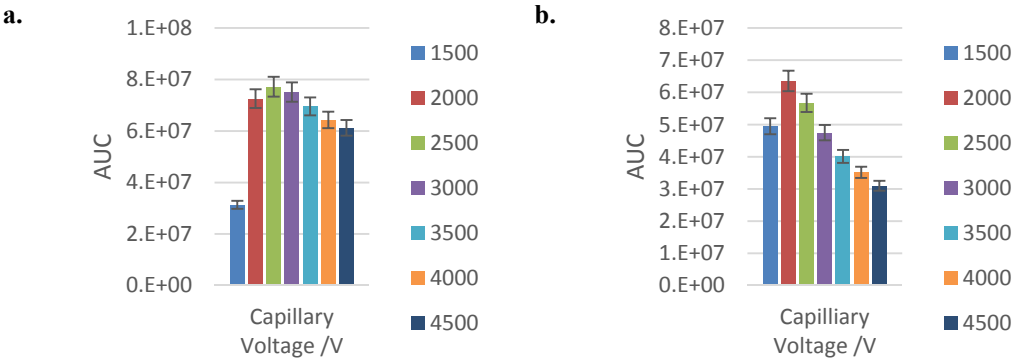


Figure 3.9 : Bar graphs comparing the responses of each compound or the total response at different flow rates acquired in ACN/water mobile phase in a. positive ESI mode and; b. negative ESI mode. The legend represent the capillary voltages.

Summary

The optimal conditions for the source parameters obtained with the ACN/water carrier (Table 3.1a.) are tabulated in Table 3.5, and these parameter values were used for subsequent experiments. Similar experiments were carried out with the ACN/0.1% formic acid carrier (Table 3.1 a.) with the results summarised in Table 3.6 (Appendix 7).

Table 3.5 : Optimised source parameters for ACN/water mobile phase; positive (PI) and negative (NI) ESI mode.

DGT /°C:	125		**Capillary voltage /V:**	PI: 1500	NI: 2000
DGF /L min^{-1}:	PI: 16	NI: 18	**Nebuliser pressure /psig:**	20	
SGT /°C:	400		**Nozzle voltage /V:**	PI: 2000	NI: 1500
SGF /L min^{-1}:	PI: 10	NI: 12			

Table 3.6 : Optimised source parameters for ACN/0.1% formic acid mobile phase; positive (PI) and negative (NI) ESI mode

DGT /°C:	125		**Capillary voltage /V:**	2500
DGF /L min^{-1}:	16		**Nebuliser pressure /psig:**	20
SGT /°C:	PI: 400	NI: 350	**Nozzle voltage /V:**	500
SGF /L min^{-1}:	PI: 11	NI: 10		

In line with principles of QbD, it would have been preferable to perform optimisation experiments using factorial design rather than sequential and systematic modification of parameters. Factorial design would have examined multivariate interactions between the critical factors, and also test for the robustness and ruggedness of the method. Unfortunately time constraints did not allow these experiments to be performed.

3.1.4 Selection of Ideal LC conditions

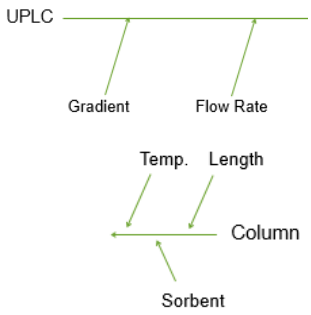


Figure 3.10 : Sub-diagrams of factors from Ishikawa diagram relevant to this section

Numerous LC conditions were trialled, with timings adjusted depending on the C18 column attached. The column was selected based according to information in literature as a C18 column is conventionally used in LC studies on pharmaceuticals[81]. Initially a Poroshell 120 Column - EC-18 Analytical, 2.7µm, 5.6 x 150mm (Agilent P.N 693975-302) was used. However the column experienced high back pressure which made it unsuitable for future applications with the on-line SPE as the SPE cartridge holder used has a maximum pressure limit of 300 bar which limited the system as the C-18 column could be used up to 600 bar. A shorter column Poroshell 120 Column - EC-18 Analytical, 2.7µm, 5.6 x 50mm (Agilent P.N 699975-302) was substituted and used.

A binary gradient was initially selected based on an existing internal method for pesticide screening. The timetable was then adjusted to account for the difference in column length. Slight adjustments to the timetable were made based on running standard mixes until well resolved chromatographic peaks were observed (Table 3.7).

Table 3.7 : Final LC conditions for chromatographic analysis with a C-18 column

Time/min	A /%	B/%	Flow Rate /mL min^{-1}
0	95	5	0.4
0.4	95	5	0.4
3	50	50	0.4
10	5	95	0.4
12	5	95	0.4
Post time = 1 minute			

3.1.5 Determination of LODs, LOQs and linearity

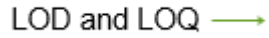


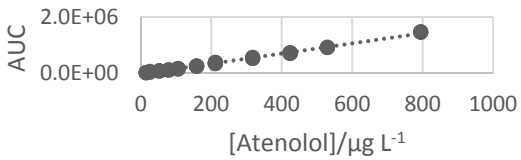
Figure 3.11 : Sub-diagram of factors from Ishikawa diagram relevant to this section

The linearity, limit of detection (LOD), and limit of quantitation (LOQ) were determined through a series of three replicate injections of twelve standards. A pharmaceutical standard mix was prepared containing the 21 compounds of interest, and serially diluted in Type I water to form standards whose concentrations spanned from approximately 15 µg L^{-1} to 1000 µg L^{-1}. The samples were run in positive and negative ESI modes under both chromatographic conditions, with the LC method according to Table 3.7.

The area under the curve (AUC) for the compounds of interest were taken as the response signal, and were plotted against the concentration. The limit of detection was defined as the concentration which corresponded to a response three times the residual standard deviation of the regression line of the calibration curve[82]. Similarly, the limit of quantitation was the concentration which corresponded to a response ten times the residual standard deviation. Statistical tests were also performed to determine if the set of calibration data was linear as the residuals should be randomly distributed around zero. The randomness of the residuals can be determined using a runs test. Additionally it is expected for the linear regression to have an intercept of zero as there should be no signal when there is no concentration. This was statistically tested by checking that the value of the intercept, with its known confidence interval, fell within zero. If the regression did not pass through zero, the calibration plot can still be used however it would result in an inflated LOD and LOQ. An example calculation to determine the LOD and LOQ, runs test, and intercept check is shown below, with the results summarised in Table 3.8.

An example calculation for LOD, LOQ, and evaluation of linearity

For the responses of atenolol under no modifier carrier in positive ESI mode, the AUC was plotted against the concentration to generate regression data.



Correlation Coefficient =	*0.9984*
Slope (b) /µg L^{-1} =	*1811.24*
Intercept (a) =	*-21643.88804*
Residual StdDev ($S_{y/x}$) =	*23406.78472*

Determining LOD and LOQ

The values were calculated as such:

$$y_{LOD} = 3 \times S_{y/x} = 3 \times 23406.78472 \ = 70220.35$$

$$x_{LOD} = \frac{y_{LOD} - a}{b} = \frac{70220.35 + (-21643.88804)}{1811.24\ \mu g^{-1}\ L} = 51\ \mu g\ L^{-1}$$

$$y_{LOQ} = 10 \times S_{y/x} = 10 \times 23406.78472\ \mu g\ L^{-1} = 234067.8472$$

$$x_{LOQ} = \frac{y_{LOQ} - a}{b} = \frac{234067.8472 + (-21643)}{1811.24\ \mu g^{-1}\ L} = 141\ \mu g\ L^{-1}$$

Checking for random residuals

The linearity was evaluated by performing a runs test on the residuals. The residuals were calculated for each data point, where it is the difference between the experimentally obtained responses versus the calculated response based on the regression plot equation. For example:

$$Residual_{15.90 \mu g\ L^{-1}} = 20932 - \left(1811.24\ \mu g^{-1}\ L \times 15.90\ \mu g\ L^{-1} + (-21643.88804)\right)$$
$$= 13777.2$$

The standardised Z-score was calculated for each residual and if the value was greater than 2 it was considered an outlier. Outliers were removed, and the binary values were taken for each average residual at each concentration level where each data point was assigned 1 if it was a positive value, or 0 if it was negative. The sequence of the runs is then:

1, 1, 1, 0, 0, 0, 1, 0, 0, 0, 1

From this data set the following values were obtained.

Observed number of runs, R =	*5*
Count of negative residuals, n_0 =	*6*
Count of positive residuals, n_1 =	*5*
Total number of residuals, n =	*11*
Expected number of runs, $E(R) = 1 + (2n_0n_1)/n)$=	*6.455*
Variance, Var = $2n_0n_1(2 * n_0 * n_1 - n)/(n^2 * (n-1))$=	*2.430*
Standard deviation, StDev(R)=	*1.559*
Test statistic, $\|Z\| = \|(R - E(R))/StDev(R)\|$	*0.933*
Z-critical based on 2-sided t-test =	*1.960*

From these values, the sequence is produced randomly if the absolute test statistic less than the Z-critical value. For atenolol, the test statistic. 0.933 is less than the Z-critical value of 1.960 so the residuals are produced randomly. However this approach relies on the assumption that there is a population of data, so ideally 20 samples should be acquired for the assumption to confidently be made.

Checking intercept

When AUC is plotted against the concentration, it would be expected that the y-intercept of the calibration plot should pass through zero as there should be no AUC if the compound of interest is not present. Failure of the calibration plot to pass through zero is indicative of some form of systematic error or poor regression fit. Thus the confidence interval of the regression y-intercept was evaluated

to determine if the calibration plot passes through zero:

$$SD_{intercept} = S_{y/x}\sqrt{S_{x2}/(n \cdot S_{xx})} = 6152$$

$$CI_{interept} = t \cdot SD = 12601 \; at \; the \; 95\% \; confidence \; level$$

The intercept is -21644 ± 12601 *which does not pass through zero.*

Table 3.8 : Summary of results (LOD and LOQ, results of a runs test for random residuals, and whether the calibration plot passes through zero (Y-intercept)) from statistical analysis of calibration standards obtained for ACN/water and ACN/0.1% formic acid mobile phase in both positive and negative ESI mode.

Run conditions: ACN/water mobile phase; positive ESI mode				
Compound	**LOD /µg L^{-1}**	**LOQ /µg L^{-1}**	**Random residuals**	**Y-intercept**
Atenolol	34	115	True	False
Caffeine	68	225	False	True
Carbamazepine	89	296	False	False
Erythromycin	165	548	False	False
Sulfamethoxazole	97	324	True	True
Run conditions: ACN/water mobile phase; negative ESI mode				
Compound	**LOD /µg L^{-1}**	**LOQ /µg L^{-1}**	**Random residuals**	**Y-Intercept**
Atorvastatin	48	160	False	False
Betaestradiol	84	279	True	True
Chloramphenicol	56	185	True	True
Diclofenac	55	183	False	True
Estriol	80	268	True	True
Estrone	74	246	False	False
Ethinylestradiol	305	1016	True	True
Gemfibrozil	32	207	False	False
Ibuprofen	60	200	True	True
Ketoprofen	58	195	True	True
Naproxen	362	1208	True	False
Phenytoin	85	284	True	False
Sulfamethoxazole	79	263	True	True
TCEP	265	884	True	True
Triclosan	73	243	True	True
Run conditions: ACN/0.1% Formic acid mobile phase; positive ESI mode				
Compound	**LOD /µg L^{-1}**	**LOQ /µg L^{-1}**	**Random residuals**	**Y-Intercept**
Atenolol	39	129	True	False
Atorvastatin	109	363	False	False
Caffeine	57	189	False	True
Carbamazepine	51	169	True	True
Diclofenac	48	159	True	True
Erythryomycin	145	485	False	False
Fluoxetine	204	679	True	True
Ketoprofen	124	415	True	False
Phenytoin	166	554	True	True
Sulfamethoxazole	68	228	False	False
Run Conditions: ACN/0.1% formic acid mobile phase; negative ESI mode				

Compound	LOD /µg L^{-1}	LOQ /µg L^{-1}	Random residuals	Y-Intercept
Chloramphenicol	98	326	True	True
Ketoprofen	494	1648	True	True
Sulfamethoxazole	73	242	True	True

Based on the results in Table 3.8 the limit of detections of the current method are not at a low enough level to detect emerging contaminants in water (which are typically in the ng L^{-1} range[1]). In order to meet the project's aims of having a method for the screening of emerging contaminants in environmental samples, a pre-concentration step will be necessary.

It is worth noting that the statistical means of calculating LOD and LOQ errs on the side of caution and has a higher value than actual working limits. In chromatography, a common approach to obtain the LOD is to determine the analyte concentration that is required to produce a signal greater than three times the noise level. In practice this is measured by analysing 7 or more standards at the estimated LOD then calculating the standard deviation from the measured concentrations of those standards. The LOD being three times the standard deviation, whilst LOQ would be 10-20 times the standard deviation. This approach is not as suitable for MS based chromatography as the noise levels are dependent on the software settings.

However as a comparison, the LODs for the no modifier carrier in NI mode were determined by finding the minimum concentration level where the compound had a S/N greater than three (Table 3.9). As expected for the majority of the compounds, a lower LOD was obtained with the S/N approach. Although the LODs from the S/N approach were lower than expected this was not subject to further study.

Table 3.9 : Comparison of LOD based on S/N approach ($LOD_{S/N}$), and LOD based on statistical regression (LOD_{Reg})

Compound	$LOD_{S/N}$ /µg L^{-1}	LOD_{reg} /µg L^{-1}
Atorvastatin	15	48
Beta-Estradiol	75	84
Chloramphenicol	15	56
Diclofenac	15	55
Estriol	15	80
Estrone	75	74
Ethinylestradiol	100	305
Ibuprofen	50	60
Ketoprofen	300	58
Naproxen	1000	362
Phenytoin	25	85
Sulfamethoxazole	50	79
TCEP	1000	265
Triclosan	25	73

Some compounds did not have a random distribution of the residuals, which suggests there is not a true linear relationship between the response and the concentration. This may be due to the non-linearity of the area as initial concentrations would lift the concentration too high whilst at higher concentrations it may plateau out. However this is not a concern as long as the responses of the unknown samples are within the known working linear range of the calibration plots.

Variations in peak responses for replicate injections was observed for some compounds including, but not limited to, atorvastatin, diclofenac, and erythromycin under ACN/water carrier; positive ESI mode run conditions. These variations were due to the peaks obtained for the compounds which often had two peaks at close retention times, resulting in the incorrect peak being automatically integrated. Other issues with the peak area were due to the peak shape being substantially non-gaussian for some compounds, which lead to variation in the obtained responses between replicates, and possibly a higher peak area than the true value.

The lack of reproducibility may be due to insufficient sampling of data points across a peak resulting in a loss of detail. Ideally at least twenty data points across a peak should be obtained for a more detailed fit to peak. Similarly, in the cases where the peak is non-gaussian, having a more accurate image of the peak shape is important as it improves reproducibility and quantification. The sampling rate can be increased through increasing the scan rate. However it is important to not have too high a scan rate as then noise can be introduced[83]. At present, some compounds had insufficient sampling across a peak (less than ten) whilst others had appropriate sampling.

3.1.6 Selecting the ideal run conditions

The responses of compounds in the two mobile phases and two ESI modes are compared in Table 3.10 with a checkmark representing a linear correlation between AUC and concentration based on the results presented in Section 3.1.5. Although some compounds (e.g. Sulfamethoxazole) displayed a good response in several run modes, ultimately only two run modes were selected to reduce the run time of the end method.

A mobile phase of ACN/water (Table 3.1 a.) under negative ESI mode was the only mode which favoured the estrogenic hormones as well as naproxen, TCEP and Triclosan. ACN/0.1% formic acid (Table 3.1 b.) under positive ESI mode was similarly chosen as it favoured atenolol, caffeine, diclofenac, and fluoxetine. Unfortunately mestranol did not have a linear relationship in any of the four modes. The MS-detectability and linearity of mestranol could be improved by trialling different mobile phases, or possibly through derivatisation, although further studies were not carried out at this point.

Table 3.10 : Comparison of the two mobile phases and two ESI modes under study with respect to linear correlation of AUC and concentration. A checkmark represents a linear correlation between AUC and concentration.

	ACN/water		ACN/0.1% formic acid	
Compound	**Positive ESI**	**Negative ESI**	**Positive ESI**	**Negative ESI**
Atenolol	✓		✓	
Atorvastatin		✓	✓	
Beta-estradiol		✓		
Caffeine	✓		✓	
Carbamazepine			✓	
Chloramphenicol		✓		✓
Diclofenac		✓	✓	
Erthyromycin	✓		✓	
Estriol		✓		
Estrone		✓		
Ethinylestradiol		✓		
Fluoxetine			✓	
Gemfibrozil		✓		
Ibuprofen		✓		
Ketoprofen		✓	✓	✓
Mestranol				
Naproxen		✓		
Phenytoin		✓	✓	
Sulfamethoxazole	✓	✓	✓	✓
TCEP		✓		
Triclosan		✓		

3.1.7 Determination of method precision and trueness

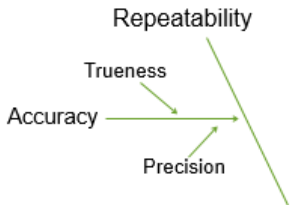


Figure 3.12 : Sub-diagram of factors from Ishikawa diagram relevant to this section

Accuracy (precision and trueness) was studied through seven determinations at three concentration levels. Concentration levels were selected to encompass the low range of linearity, the middle, and near the high end of linearity based on the calibration plots (i.e. 250, 500 and 750 µg L^{-1}).

Standards were spiked into tap water (which had been previously boiled to remove chlorine and filtered). A calibration plot was generated for the compounds of interest by running duplicate injections of standards in Type I water at four concentration levels (15, 500, 750, 1000 µg L^{-1}). Further concentrations levels were not taken as the working linearity of the standards was previously established in Section 3.1.5. The compounds in the spiked samples were identified based on the m/z of the molecular ion and retention time (RT) similarity to the

known standards. For the spiked samples, some compounds produced multiple peaks which corresponded to the compound. In those cases the AUC was taken for the peak at the RT that was known to have a linear relationship even if that peak was not the strongest peak.

The precision was calculated as the relative standard deviation (RSD) of the seven replicates at each concentration level, through calculating the percentage ratio of the sample standard deviation, s, to the sample mean $\bar{x}$.

$$RSD = s/\bar{x} \times 100\%$$

Trueness was calculated as the percentage deviation of the nominal and experimental concentration.

$$Recovery = \left(\frac{Experimental}{Nominal}\right) \times 100\%$$

The results for the spikes are summarised in Table 3.11 and Table 3.12. The precision at each concentration level did not exceed a RSD of 15%, which is the recommended limit[82]. On the other hand, recovery should be within 15% of the actual value (85%-115%) which was not the case for some compounds. This may be due to variations in the spiked sample matrix compared to the standards.

It would be of practical interest to have several internal standards (representing different pharmaceutical categories) to normalise peak areas.

Table 3.11 : Summary of results for experimentally determined concentration (C_{Exp}), the relative standard deviation (RSD) and the recovery (Rec) at three concentration levels for ACN/water; negative ESI mode. Grey shading indicates samples where the recoveries were outside the recommended limit of 15% variation.

Concentration Level	**250 µg L^{-1}**			**500 µg L^{-1}**			**750 µg L^{-1}**		
Compound	**C_{Exp} /µg L^{-1}**	**RSD /%**	**Rec /%**	**C_{Exp} /µg L^{-1}**	**RSD /%**	**Rec /%**	**C_{Exp} /µg L^{-1}**	**RSD /%**	**Rec /%**
Atorvastatin	174	3.6	66	604	4.9	114	1186	3.6	149
Beta-estradiol	234	12	94	784	13	157	1161	12	155
Chloramphenicol	324	8.0	122	734	1.9	139	937	0.8	118
Dicloclofenac	201	4.7	74	535	2.5	99	846	2.4	104
Estriol	224	11	88	648	4.0	127	947	4.0	124
Estrone	195	9.3	67	646	8.7	111	1045	6.4	120
Ethinylestradiol	217	14	72	705	11	118	1086	8.8	121
Gemfibrozil	221	5.0	80	587	1.6	107	924	3.4	112
Ibuprofen	204	4.5	77	525	2.8	99	759	2.4	95
Naproxen	212	11	74	556	4.8	97	948	7.0	111
Phenytoin	265	4.3	95	600	1.8	107	848	2.3	101
Sulfamethoxazole	270	5.5	97	666	3.0	119	970	2.5	116
TCEP	286	9.7	98	777	4.9	134	1145	4.2	132
Triclosan	297	6.0	99	860	3.8	143	1428	3.2	159

Table 3.12 : Summary of results for experimentally determined concentration (C_{Exp}), the relative standard deviation (RSD) and the recovery (Rec) at three concentration levels for ACN/0.1% formic acid; positive ESI mode. Grey shading indicates samples where the recoveries were outside the recommended limit of 15% variation.

Concentration level	250 µg L^{-1}			500 µg L^{-1}			750 µg L^{-1}		
Compound	C_{Exp} /µg L^{-1}	RSD /%	Rec /%	C_{Exp} /µg L^{-1}	RSD /%	Rec /%	C_{Exp} /µg L^{-1}	RSD /%	Rec /%
Atenolol	382	2.6	144	693	1.0	131	870	0.90	109
Atorvastatin	216	3.2	73	659	2.3	112	1137	2.2	128
Caffeine	274	5.0	100	652	0.94	118	922	1.3	112
Carbamazepine	377	3.0	135	734	1.2	131	936	1.0	111
Chloramphenicol	233	4.9	88	582	3.3	110	844	4.0	106
Diclofenac	194	6.1	72	536	3.2	99	853	2.6	105
Erythromycin	195	12	72	628	3.4	116	916	0.84	113
Fluoxetine	207	6.7	71	593	2.7	102	923	2.9	106
Ketoprofen	185	13	63	611	6.9	103	1038	2.6	117
Naproxen	181	4.7	70	547	1.7	105	837	1.8	107
Phenytoin	227	3.7	80	610	2.7	107	932	3.2	109
Sulfamethoxazole	221	5.4	79	564	2.9	101	882	1.6	105

3.1.8 Analysis of water samples

The samples from Melbourne were filtered and analysed in the ACN/water carrier; negative ESI mode, and in the ACN/0.1% formic acid carrier; positive ESI mode. None of the analytes under study were detectable above noise levels. These results were expected due to the LODs of the UHPLC-QTOF-MS method.

3.1.9 Summary of UHPLC-QTOF-MS method development

A working UHPLC-QTOF-MS method was established with optimised source parameters (Table 3.5 and Table 3.6) for the two chromatographic buffer systems A and B (Table 3.1) with an established gradient (Table 3.7). The method displays good precision although accuracy has room for improvement. The main limitations of this system are the high LODs making it unsuited to analyse real water samples where the expected concentration of compounds are in the ng L^{-1} range.

3.2 SPE-UHPLC-QTOF-MS Method

The second stage of method development was to expand on the working UHPLC-QTOF-MS method from Section 3.1 through the addition of a pre-concentration step. Ultimately an on-line SPE-UHPLC-QTOF-MS method is developed.

3.2.1 Off-line SPE

A concentrated three compound standard mix was prepared consisting of an early, middle and late eluting compound (10 µg L^{-1} sulfamethoxazole, 20 µg L^{-1} caffeine, and 50 µg L^{-1} ketoprofen) in methanol and water, such that the end concentration of methanol was 8.0%. The concentrated standard mix was then diluted in water (1:50) such that the end methanol content was 0.16%. This was necessary as earlier experiments carried out in higher methanol content had poor recoveries with SPE. Two replicates of samples were subject to the SPE procedure and were each made up to the same volume as the initial concentrated sample.

All samples, including the initial concentrated standard mix were analysed by direct injection on the QTOF (Figure 3.3 a.) in a 50% ACN/water carrier (Table 3.3. a.). Ideally there should be no differences in response between the concentrated standard and the diluted standard mix after SPE. The recoveries were calculated based on the ratio of the AUC of the sample compared to the undiluted standard (Table 3.13).

Table 3.13 : AUC and recoveries from off-line SPE

	Average AUC			Recovery /%	
Compound	**No SPE**	**Sample1**	**Sample2**	**Sample1**	**Sample2**
Caffeine	187833	130673	106969	70	57
Ketoprofen	631506	394507	287911	62	46
Sulfamethoxazole	164567	113873	127384	69	77

From the results in Table 3.13 it can be seen that recoveries were generally poor, and also that there are large variations between replicates. The poor recoveries may be due to the choice of SPE sorbent material (which is a m-divinylbenzene and N-vinylpyrrolidone copolymer), and the variations between replicates may be due to manual handling[4]. Additionally SPE extraction recovery could also be improved by adjusting the pH of the sample. For example, pharmaceuticals containing acidic groups are typically ionised at neutral pH so acidification of water samples is necessary[84], however this is problematic in the case of this method which simultaneously determines several classes of pharmaceuticals.

These preliminary results suggested that Oasis HLB sorbents, although recommended for the isolation of both polar and non-polar compounds simultaneously from aqueous media[85],

may not be suitable for this application and that poor reproducibility was due to the need for manual handling. This result justified the use of on-line SPE.

3.2.2 Compound retention onto the on-line SPE cartridge

PLRP-s columns (a macroporous polystyrene and divinylbenzene copolymer) were selected as the on-line SPE cartridge type. It was selected as it was reported to have good retention of polar compounds making it suitable for this application[86]. An on-line SPE system was used based on advantages previously outlined in Section 1.3.1.

Different iterations of the on-line SPE-LC-QTOF system were used throughout the establishment of an SPE method. For completeness the final SPE system is described in Section 3.2.5. Problems encountered included retention of compounds onto the SPE cartridge, the low pressure limitation of the SPE holder, and the cartridges used initially.

The first set of SPE cartridges used had poor lateral fit into the corresponding holder, and the cartridge had to be tightened beyond the recommended limit to prevent leakages. This resulted in splitting of the cartridge, and the column packing passing through the rest of the system and resulting in high pressure in the column. Upon checking with the supplier, the first set of cartridges had been phased out and instead a new type was supplied. The new set of SPE cartridges had better fit into the holder, and all subsequent results from Section 3.2.4 were obtained with using these newer cartridges.

The first series of tests were to ensure that compounds of interest were retained onto the SPE cartridge. A simplified three compound standard mix was used, and consisted of an early, middle and late eluting compound (caffeine, sulfamethoxazole, ketoprofen) during a normal chromatographic run. It was essential that the overall content of methanol in the standard did not exceed 5% as it would result in earlier elution of compounds due to the higher organic modifier content compared to the initial mobile phase.

The instrument connection was set so that BinaryPump2 delivered to the SPE cartridge, and then directly into the QTOF for analysis (Appendix 8). Initial experiments were carried out with a mobile phase of 5% ACN:water followed by a clean-up step of 95% ACN at the end of the run. However there was poor retention of the compounds onto the SPE cartridge, with elution of all three compounds at 5% ACN:water (Figure 3.13). Pure water was tested, with the early eluter (caffeine) retaining on the cartridge (Figure 3.14) – however it was not preferably to have pure water as a wash step since it might not remove proteins and other potential contaminants.

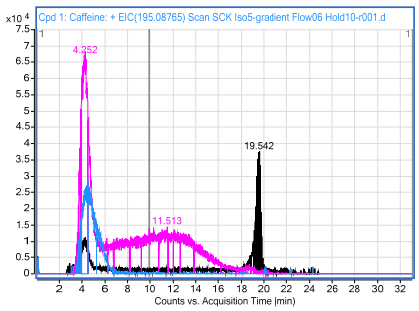


Figure 3.13 : Overlay of the EICs where an isocratic 5% ACN:water is applied from 0-18 minutes, followed by an ACN clean up. Caffeine (blue) and sulfamethoxazole (pink) have eluted at isocratic conditions, whilst ketoprofen (black) only elutes when a high ACN content is applied.

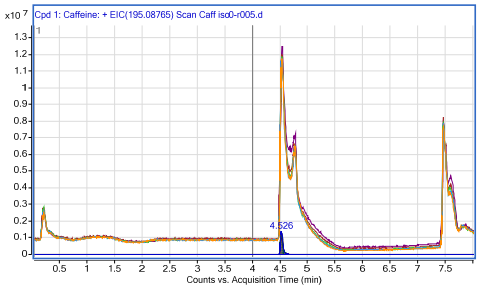


Figure 3.14 : Overlay of five replicates of caffeine, where pure water is applied from 0-4.5 minutes, followed by an ACN clean up. The EIC caffeine is represented by the dark blue small at 4.525 min, whilst the overlay TICs of the chromatographic runs has good repeatability between the runs.

Instead methanol with ammonium formate was trialled as the organic modifier, as methanol has a lower solvation power than acetonitrile in the case of caffeine[86]. The binary timetable was held at isocratic 5% B, before a gradient was applied to clean the SPE cartridge.

The resulting chromatogram is shown in Figure 3.15 where the elution of all three compounds occur with the gradient, and do not elute earlier, indicating good retention onto the column. The improvement in retention could either be due to methanol having lower solvation power, or could also be due to the ion pairing effect contributed by ammonium formate. These propositions will be explored further in the next section (Section 3.2.3).

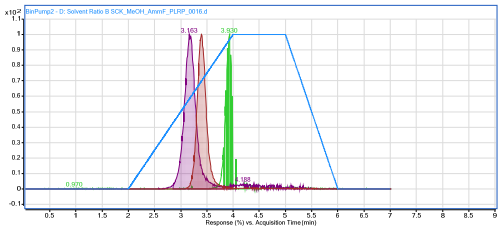


Figure 3.15 : Chromatogram of the three compound mix, with caffeine at 3.163 min (purple), sulfamethoxazole at 3.4 min (brown), and ketoprofen at 3.930 min (green). The binary gradient is represented by the blue line, where it starts at 5% and steps up to 95% below returning to starting conditions.

3.2.3 Selection of load mobile phases

Different loading mobile phase conditions were trialled, with different modifiers and organic solvents used (Table 3.14). A pharmaceutical mix was analysed

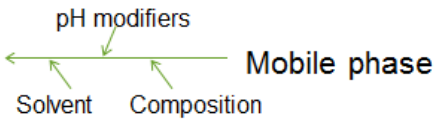


Figure 3.16 : Sub-diagram of factors from Ishikawa diagram relevant to this section

in ACN/water; positive ESI mode only, and the optimal mode was selected based on the chromatographic peak shapes. The instrument schematic is outlined in Appendix 9.

Table 3.14 : Mobile phases conditions trialled for the load step onto the SPE cartridge

#	Description	A - Water	B – 95% organic solvent in water
i.	MeOH - no modifiers	No modifiers	No modifiers
ii.	MeOH - ammonium formate	5 mM ammonium formate	5 mM ammonium formate
iii.	MeOH - formic acid and ammonium formate	0.1% formic acid and 5mM ammonium formate	0.1% formic acid and 5mM ammonium formate
iv.	MeOH - formic acid	0.1% formic acid	0.1% formic acid
v.	ACN – no modifiers	No modifiers	No modifiers
vi.	ACN – ammonium formate	5 mM ammonium formate	5 mM ammonium formate

Some of the chromatograms are illustrated below in Figure 3.17. The no modifier in methanol load buffer was selected as the most appropriate load buffer as for the majority of the compounds it resulted in a good response and peak shape. There were some compounds for which the presence of modifiers improved the chromatogram, for example ketoprofen. However the end aim was a multi-residue method, thus the condition that suited the majority of the compounds was selected.

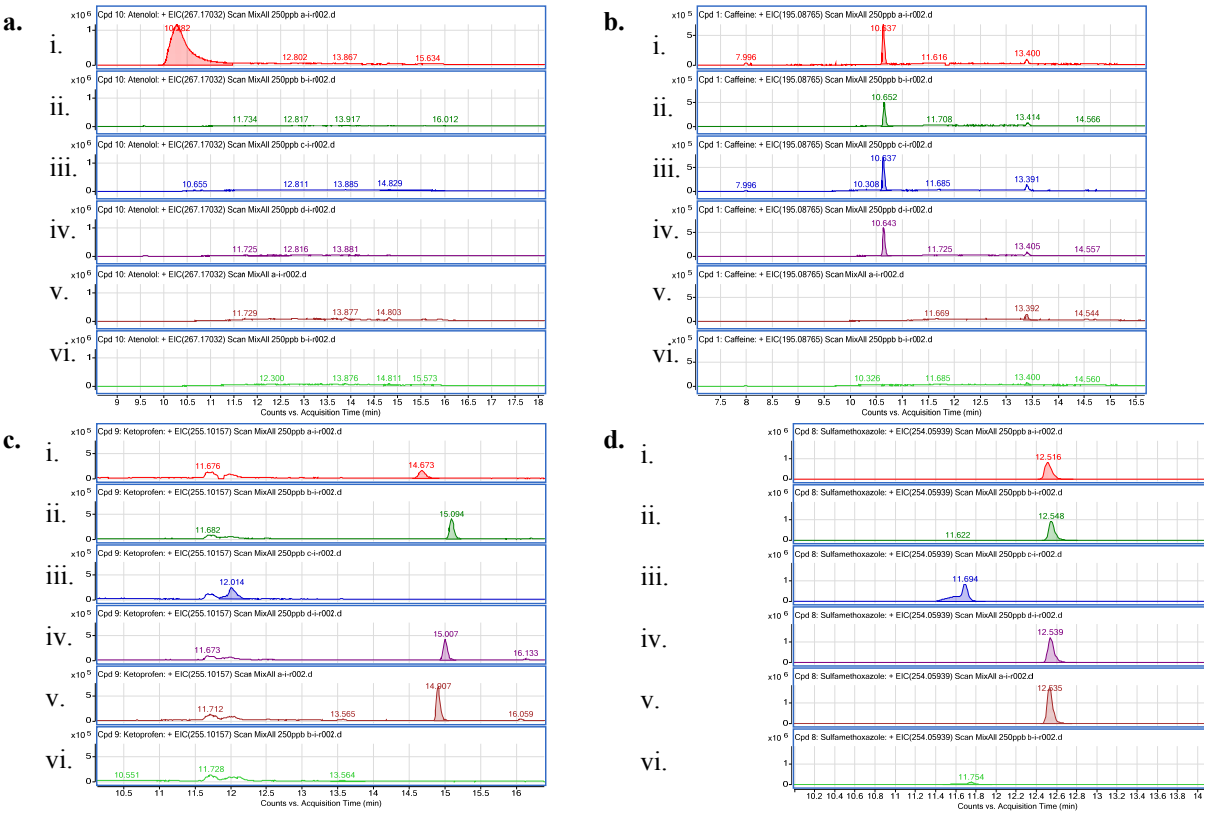


Figure 3.17 : EICs of compounds at different load conditions (i-vi corresponding to conditions in Table 3.14) for a. Atenolol; b. Caffeine; c. Ketoprofen and d; Sulfamethoxazole

3.2.4 Optimisation of load flow rates and load volumes

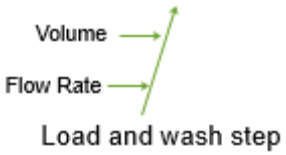


Figure 3.18 : Sub-diagram of relevant factors from Ishikawa diagram

Experiments to determine the optimal flow rate were performed through adjustment of the existing method such that flow rate was systematically altered whilst the overall load volume was kept constant through changing the load time (Table 3.15).

Table 3.15 : Summary of different flow rates trialled, with the appropriate load times such that the total volume is constant

Flow Rate /mL min^{-1}	Load time /min	Load volume /mL
1	6	6
1.5	4	6
2	3	6
2.5	2.4	6
3	2	6

The responses obtained are summarised in Appendix 3 and Appendix 4 and the relative response compared to the peak area at flow rate 1 mL min^{-1} was used as the criteria for judging which parameter was optimal (Figure 3.19).

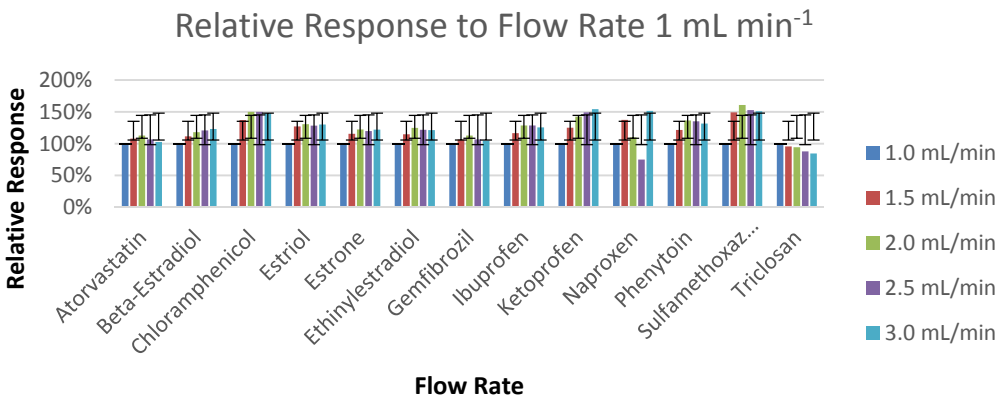


Figure 3.19 : Relative responses compared to the responses at flow rate of 1 mL min^{-1} under run conditions ACN/water carrier; negative ESI mode. Error is estimated from precision experiments (Section 3.1.7)

The optimal load flow rate was found to be 2 mL min^{-1} as it had the highest relative response for the majority of the compounds. The experiment was repeated for ACN/0.1% formic acid mobile phase; positive ESI mode, with the optimal flow rate also found to be 2 mL min^{-1} (Figure 3.20) although the results were not as consistent as the ACN/water mobile phase; negative ESI mode.

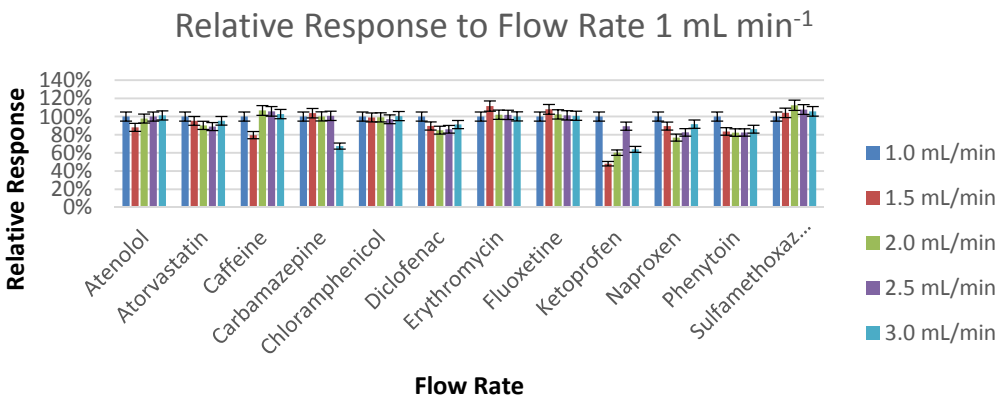


Figure 3.20 : Relative responses compared to the responses at flow rate of 1 mL min^{-1} under run conditions ACN/0.1% formic acid carrier; positive ESI mode. Error is estimated from precision experiments (Section 3.1.7)

The load volume was also optimised, with the volume of solution passing through the SPE cartridge during the load stage systematically varied (2-8 mL) whilst maintaining the flow rate at the optimal rate of 2 mL min^{-1}. The responses are detailed in Appendix 5 and 6.

The relative responses to load volume 2 mL are compared in Figure 3.21 and Figure 3.22 for the two chromatographic run conditions. For the ACN/water carrier; negative ESI mode, the optimal flow rate was determined to be 5 mL, whilst for ACN/0.1% formic acid there were good responses at volumes greater or equal to 5 mL but ultimately 5 mL was selected to keep the two methods as consistent as possible. The differences between 5 mL and 6 mL for ACN/0.1% formic acid carrier; positive ESI mode may have also been due to the instrument automatically stopping between the two runs due the volume of mobile phase reaching below the minimum limit.

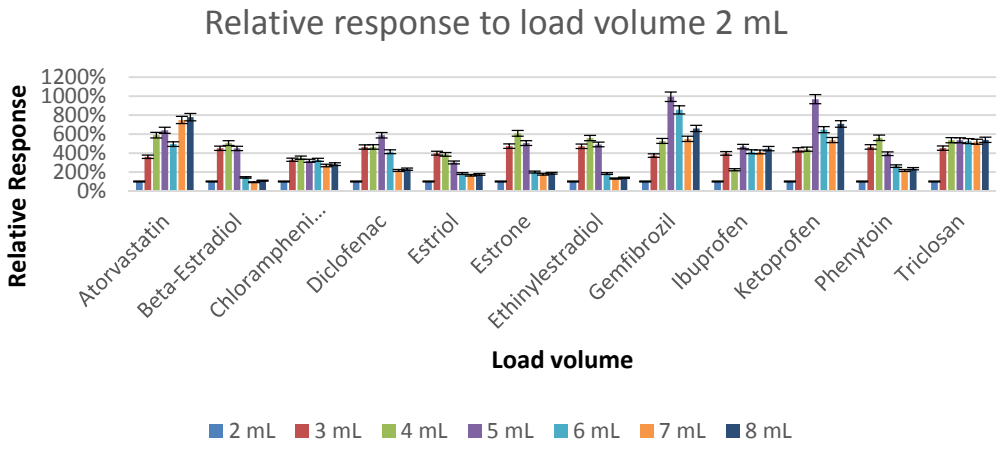


Figure 3.21 : Relative responses compared to responses at 2 mL min^{-1} obtained under run conditions ACN/water carrier; negative ESI mode. Error is estimated from precision experiments (Section 3.1.7)

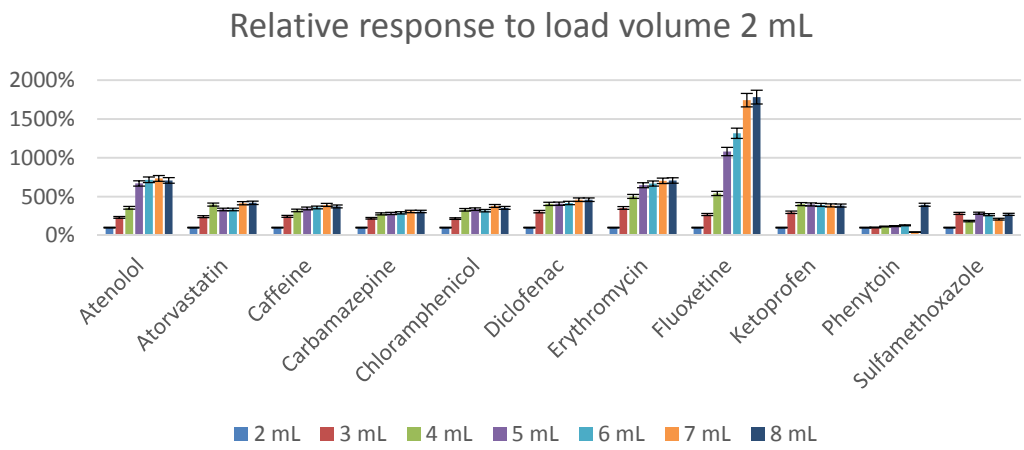


Figure 3.22 : Relative responses compared to responses at 2 mL/min obtained under run conditions ACN/0.1% formic acid carrier. Error is estimated from precision experiments (Section 3.1.7).

3.2.5 LC conditions and comparison to non-concentrated method

An on-line SPE system (Agilent 1260 binary pump) was connected to the existing UHPLC-QTOF-MS system (Agilent 1290 Binary Pump and 6550 QTOF). The system was set up such that one TCC position allowed for loading of the SPE cartridge, whilst the salt and protein contamination goes into waste, whilst the other position allowed for elution of the SPE cartridge into the C18 column and onto the QTOF (Appendix 9). The elution conditions for the method were kept identical to the method established in the Section 3.1.

Table 3.16 : Final LC conditions for the SPE-UHPLC-QTOF-MS method.

BinPump1		
Time/min	**B/%**	**Flow**
0	5	2
2.5	5	2
3	100	2
3.5	100	2
4	5	2
6.49	5	2
6.5	5	0
13.5	5	0
13.51	5	2
14	5	2
14.5	100	2
16.5	100	2
17	5	2
18.5	5	2

BinPump2	
Time/min	**B/%**
0	5
2.9	5
5.5	50
12.5	95
16.5	95
16.51	5

Sampler
Injection volume = 1800 µL
Overlap injection at 5.5 minutes.

Column and QTOF		
Time/min	**Column Position**	**QTOF**
0	1->2	Don't record
2.5	1->10	Record
14.5	1->2	Don't record.

During the first 2.5 minutes of the run sequence, the 1260 Bin Pump is loading the sample onto the SPE cartridge and washing with 5% MeOH (Schematic Appendix 9 a.). The QTOF is not recording at this time, whilst the 1290 Bin Pump is equilibrating the C18 column with 5% Buffer Bottle B (ACN).

At 2.5 minutes, the TCC switches position (Schematic Appendix 9 b.). The mobile gradient from 1290 Bin Pump passes through the SPE cartridge to elute the compounds onto the C18 column where it separates and enters the QTOF, whilst the QTOF is recording the data. Meanwhile the 1260 Bin Pump is cleaning the sample loop by applying a gradient to a high MeOH content before switching back to 5% MeOH as the sampler prepares for overlapped injection at 9 minutes. At 6.5 minutes the 1260 Bin Pump switches the flow off to conserve solvent since there is nothing currently occurring at this point.

At 14.5 minutes, the TCC switches back to load configuration (Appendix 9 a.). The 1290 Bin Pump cleans the C18 column with 95% Buffer B (ACN) before returning back to the starting conditions of 5% Buffer B. The 1260 pump meanwhile preconditions the SPE cartridge with 4 mL of methanol and then with 5% MeOH.

As the elution conditions for the compounds through the C18 column are kept identical to the method established in Section 3.1, it is expected that the compounds will have the same elution order, and the same relative retention time to the start of elution. The average retention times were extracted from standards run for a calibration plot, and are shown below in Table 3.17 and Table 3.18. Unexpectedly, the elution order was different between the two methods, and it would be interesting to investigate the cause of this discrepancy as the SPE process should not affect the elution order.

Table 3.17 : Comparison of the average retention times of compounds at run conditions ACN/water carrier; negative ESI mode for the method with and without a SPE pre-concentration step.

Without SPE		**With SPE**	
Compound	**RT /min**	**Compound**	**RT /min**
TCEP	0.74	Ketoprofen	5.61
Estriol	3.25	Naproxen	5.72
Chloramphenicol	3.40	Estriol	5.90
Ketoprofen	3.84	TCEP	6.06
Phenytoin	3.88	Chloramphenicol	6.06
Naproxen	3.95	Diclofenac	6.38
Diclofenac	4.23	Phenytoin	6.52
Beta-Estradiol	4.42	Ibuprofen	6.83
Atorvastatin	4.68	Atorvastatin	6.96
Ethinylestradiol	4.77	Beta-Estradiol	7.09
Ibuprofen	4.83	Ethinylestradiol	7.44
Estrone	4.94	Estrone	7.62
Gemfibrozil	6.30	Gemfibrozil	8.75
Triclosan	7.31	Triclosan	10.13

Table 3.18 : Comparison of the average retention times of compounds at run conditions ACN/0.1% formic acid carrier; positive ESI mode for the method with and without a SPE pre-concentration step.

Without SPE		**With SPE**	
Compound	**RT /min**	**Compound**	**RT /min**
Chloramphenicol	0.80	Atenolol	4.54
Atenolol	1.91	Caffeine	4.96
Caffeine	2.32	Erythromycin	5.99
Sulfamethoxazole	3.31	Chloramphenicol	6.02
Erythromycin	3.42	Carbamazepine	6.41
Carbamazepine	3.83	Phenytoin	6.47
Phenytoin	3.88	Fluoxetine	6.53
Fluoxetine	4.01	Ketoprofen	7.19
Ketoprofen	4.64	Atorvastatin	7.99
Atorvastatin	5.55	Sulfamethoxazole	8.24
Diclofenac	5.59	Diclofenac	8.29

The effect on the efficiency of the chromatographic system as a result of the SPE step was evaluated by examining the change in the number of theoretical plates (N) with and without SPE. The retention time from the method without SPE was used as is, whilst the relative retention from the start from signal reaching the detector was used for the method with SPE.

$$N = 5.54\left(\frac{{t_R}'}{w_{1/2}}\right)^2 \qquad \begin{array}{rl} where\ t_R' = t_R - 0\,min & for\ without\ SPE\ method \\ or\ t_R' = t_R - 2.6\,min & for\ with\ SPE\ method \end{array}$$

Ideally a dead time, where the time for a non-interacting species to pass through the column, should have been determined in order to have more accurate relative retention times. However for simple comparison purposes, the manner in which the relative RT was calculated is sufficient. The retention times, widths and the efficiencies of the method with and without SPE pre-concentration were extracted from existing calibration data and are tabulated in Table 3.19.

For the majority of the compounds (excluding TCEP and chloramphenicol), a higher theoretical plate count was observed with the QTOF method. This was to be expected as although the SPE cartridge does concentrate the compounds, there would be a degree of band broadening caused by the SPE cartridge compared to a sample loop resulting in increased peak widths.

Table 3.19 : Comparison of the efficiencies of the peak shapes (N) from the method without and with a SPE pre-concentration step.

Negative ESI mode	**Without SPE**			**With SPE**		
Compound	t_R	$w_{1/2}$	**N**	t_R	$w_{1/2}$	**N**
Atorvastatin	4.68	0.407	731	6.96	0.467	483
Beta-Estradiol	4.42	0.119	7644	7.09	0.283	1395
Chloramphenicol	3.40	0.207	1492	6.06	0.394	428
Diclofenac	4.23	0.382	681	6.38	0.568	246
Estriol	3.25	0.175	1907	5.90	0.269	832
Estrone	4.94	0.119	9631	7.62	0.295	1605
Ethinylestradiol	4.77	0.114	9704	7.44	0.292	1524
Gemfibrozil	6.30	0.243	3739	8.75	0.481	907
Ibuprofen	4.83	0.389	854	6.83	0.498	400
Ketoprofen	3.84	0.176	2647	5.61	0.389	331
Naproxen	3.95	0.086	11695	5.72	0.322	521
Phenytoin	3.88	0.163	3124	6.52	0.278	1098
TCEP	0.74	0.190	85	6.06	0.188	1883
Triclosan	7.31	0.314	3008	10.13	0.477	1383
Positive ESI mode	**Without SPE**			**With SPE**		
Compound	t_R	$w_{1/2}$	**N**	t_R	$w_{1/2}$	**N**
Atenolol	1.91	0.305	218	4.54	0.388	138
Atorvastatin	5.55	0.151	7522	4.96	0.363	234
Caffeine	2.32	0.197	769	5.99	0.338	558
Carbamazepine	3.83	0.123	5408	6.02	0.268	899
Chloramphenicol	0.80	0.213	78	6.41	0.323	772
Diclofenac	5.59	0.299	1943	6.47	0.258	1252
Erythromycin	3.42	0.199	1635	6.53	0.429	465
Fluoxetine	4.01	0.142	4441	7.19	0.500	467
Ketoprofen	4.64	0.150	5279	7.99	0.411	952
Phenytoin	3.88	0.109	7092	8.24	0.319	1733
Sulfamethoxazole	3.31	0.210	1384	8.29	0.410	1065

3.2.6 Determination of LODs, LOQs and linearity

The LOD, LOQ, and linearity were determined in the same manner as Section 3.1.5. Pharmaceutical mixes were prepared at concentrations ranging from 5 ng L^{-1} to 1 µg L^{-1}. For the majority of the compounds there was linearity observed between 5 ng L^{-1} and 1000 ng L^{-1} however at higher concentrations the response would drop off (Figure 3.23). Consequently standards above 1000 ng L^{-1} were not included in the linear regression plots.

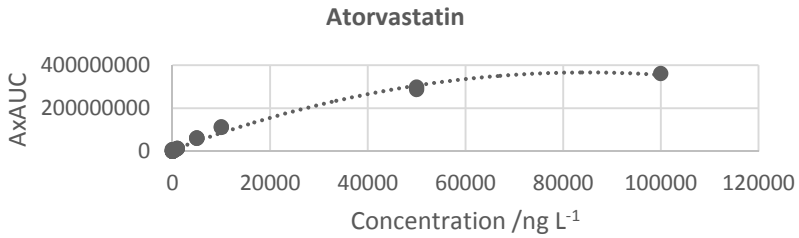


Figure 3.23 : Calibration plot of atorvastatin, with response plateauing at higher concentrations.

The results of LOD, LOQ and linearity are summarised in Table 3.20. The majority of compounds have suitable limit of detections in the ng L^{-1} range, although the calibration plot not passes through zero suggests a bias despite the random residuals.

Table 3.20 : Summary of results (LOD and LOQ, results of a runs test for random residuals, and whether the calibration plot passes through zero (Y-intercept)) from statistical analysis of calibration standards obtained under ACN/water; negative ESI mode and ACN/0.1% formic acid; positive ESI mode

Run Conditions: ACN/water; negative ESI mode				
Compound	**LOD /ng L^{-1}**	**LOQ /ng L^{-1}**	**Random residual**	**Y-Intercept**
Atorvastatin	516	1719	False	True
Beta-estradiol	72	240	True	True
Chloramphenicol	268	894	True	False
Diclofenac	221	736	True	False
Estriol	128	425	True	False
Estrone	44	148	True	False
Ethinylestradiol	63	210	True	False
Gemfibrozil	42	139	True	True
Ibuprofen	118	393	True	True
Naproxen	74	247	True	True
Phenytoin	134	448	True	False
TCEP	4261	15404	True	False
Triclosan	212	707	True	False
Run Conditions: ACN/0.1% formic acid; positive ESI mode				
Compound	**LOD /ng L^{-1}**	**LOQ /ng L^{-1}**	**Random residual**	**Y-Intercept**
Atenolol	216	719	True	False
Atorvastatin	121	405	True	False
Caffeine	135	449	True	True
Carbamazepine	329	1098	True	False
Chloramphenicol	76	252	True	False
Diclofenac	76	252	True	False
Erythromycin	282	940	True	True
Fluoxetine	536	1787	True	True
Ketoprofen	56	186	True	True
Phenytoin	124	415	True	True

As described in Section 3.1.5, the LODs were determined by finding the minimum concentration level where the compounds had a S/N greater than three. The S/N of all

compounds at the lowest concentration level analysed (1 ng L^{-1}) is summarised in Table 3.21. At 1 ng L^{-1}, ibuprofen, ketoprofen and naproxen are at their LOD, whilst the measured concentration level greatly exceeds the LOQ for the remainder. These results contrast to the LOD and LOQ based on the statistical approaches (Table 3.21) which were significantly higher in value.

Table 3.21 : S/N at 1 ng L^{-1} for all compounds, where LOD should be greater than 3 and LOQ > 10-20

ACN/water; negative ESI mode	
Compound	**S/N**
Atorvastatin	56
Beta-Estradiol	110
Chloramphenicol	265
Diclofenac	56
Estriol	153
Estrone	100
Ethinylestradiol	65
Gemfibrozil	20
Ibuprofen	4
Ketoprofen	5
Naproxen	3
Phenytoin	302
Triclosan	59

ACN/0.1% formic acid; positive ESI mode	
Compound	**S/N**
Atenolol	1751
Atorvastatin	656
Caffeine	864
Carbamazepine	1534
Chloramphenicol	145
Diclofenac	960
Erythromycin	919
Fluoxetine	283
Ketoprofen	336
Phenytoin	482
Sulfamethoxazole	21

3.2.7 Determination of method precision and trueness

Precision and trueness was determined as described earlier (Section 3.1.5). The three concentration levels covered the low, middle and high end of linearity (50 ng L^{-1}, 250 ng L^{-1} and 750 ng L^{-1}). The results are summarised in Table 3.22 and Table 3.23.

Table 3.22 : Summary of results for experimentally determined concentration (C_{Exp}), the relative standard deviation (RSD) and the recovery (Rec) at three concentration levels for the ACN/water carrier; negative ESI mode with SPE pre-concentration. Grey shading indicates samples where the recoveries were outside the recommended limit of 15% variation.

Concentration level	**50 ng L^{-1}**			**250 ng L^{-1}**			**750 ng L^{-1}**		
Compound	**C_{Exp} /ng L^{-1}**	**RSD /%**	**Rec /%**	**C_{Exp} /ng L^{-1}**	**RSD /%**	**Rec /%**	**C_{Exp} /ng L^{-1}**	**RSD /%**	**Rec /%**
Beta-estradiol	53	11	106	257	2.1	103	696	1.6	93
Chloramphenicol	25	7.6	47	99	2.9	37	238	2.2	30
Dicloclofenac	18	7.6	33	91	11	33	226	8.6	28
Estriol	10	7.4	20	50	4.8	20	150	3.2	20
Estrone	49	12	85	297	2.4	102	838	2.4	96
Ethinylestradiol	30	10	52	232	3.5	80	766	5.4	88
Ibuprofen	173	5.6	326	232	3.7	87	517	3.4	65
Phenytoin	17	15	30	103	6.2	37	315	1.8	37
Triclosan	83	2.7	137	348	1.8	116	741	1.7	82

Table 3.23 : Summary of results for experimentally determined concentration (C_{Exp}), the relative standard deviation (RSD) and the recovery (Rec) at three concentration levels for the ACN/0.1% formic acid carrier; positive ESI mode with SPE pre-concentration. Grey shading indicates samples where the recoveries were outside the recommended limit of 15% variation.

Concentration level	50 ng L^{-1}			250 ng L^{-1}			750 ng L^{-1}		
Compound	**C_{Exp} /ng L^{-1}**	**RSD /%**	**Rec /%**	**C_{Exp} /ng L^{-1}**	**RSD /%**	**Rec /%**	**C_{Exp} /ng L^{-1}**	**RSD /%**	**Rec /%**
Atenolol	80	5.4	150	355	6.4	134	779	3.5	98
Atorvastatin	40	7.4	68	220	6.5	74	681	6.1	77
Caffeine	72	8.0	130	107	6.6	39	289	2.2	35
Carbamazepine	30	4.0	53	139	2.3	50	377	0.34	45
Chloramphenicol	36	39	68	127	15	48	317	7.0	40
Diclofenac	38	2.9	70	195	4.8	72	587	5.1	72
Erythromycin	53	5.6	98	318	9.5	118	944	0.81	116
Fluoxetine	119	7.1	201	773	15	261	2521	5.1	284
Ketoprofen	30	3.6	57	148	3.4	57	443	2.6	57
Naproxen	262	14	92	105	23	184	552	5.6	64
Phenytoin	28	22	48	128	10	45	390	2.6	45

The precision for most compounds is less than 10% which is within acceptable limits, however the accuracy is poorer than the QTOF method. The poor recoveries are likely due to the compounds not being fully retained by the SPE cartridge thus being lost during the load step. This decreased retention may be the result of interaction between the acidic and basic compounds of the pharmaceutical mixture resulting in charged species and hence increased hydrophilicity of those species. However as the method has good precision, stable isotopically labelled analogues can be used to correct analyte response in order to achieve more accurate quantification[87]. Additionally, tap water is not necessary a similar matrix to environmental water samples, so it would be of interest to carry out spiking experiments in an environmental matrix.

Within-day (intra-day) and between-day (inter-day) precision was also determined through measuring a pharmaceutical mix of 500 ng L^{-1}. For within-day, the sample was measured at the start, middle and end of a working day. For between-day, diluted samples were prepared and the instrument calibrated daily, until seven replicates were obtained. The within day precision (RSD of the response) is tabulated in Table 3.24, and between day in Table 3.25. In some cases, only six days of data were used since there were no matches found to particular compounds.

Table 3.24 : Within day RSD of a standard mix analysed in ACN/water; negative ESI mode and ACN/0.1% formic acid; positive ESI mode.

ACN/water; negative ESI mode	
Compound	**RSD /%**
Atorvastatin	32
Beta-Estradiol	13
Chloramphenicol	3.3
Diclofenac	14
Estriol	5.0
Estrone	16
Ethinylestradiol	19
Gemfibrozil	24
Ibuprofen	13
Ketoprofen	33
Naproxen	30
Phenytoin	16
Sulfamethoxazole	27

ACN/0.1% formic acid; positive ESI mode	
Compound	**RSD /%**
Atenolol	6.4
Atorvastatin	13
Caffeine	9.5
Carbamazepine	5.4
Diclofenac	8.6
Erythromycin	11
Fluoxetine	47
Gemfibrozil	6.2
Ketoprofen	3.5
Naproxen	13
Phenytoin	3.9

Table 3.25 : Between day RSD of n-days of a standard mix analysed in ACN/water; negative ESI mode and; ACN/0.1% formic acid; positive ESI mode.

ACN/water; negative ESI mode		
Compound	**n**	**RSD /%**
Atorvastatin	7	20
Beta-Estradiol	7	37
Chloramphenicol	7	3.5
Diclofenac	7	10
Estriol	7	37
Estrone	7	27
Ethinylestradiol	7	35
Gemfibrozil	7	6.4
Ibuprofen	7	16
Ketoprofen	7	22
Phenytoin	7	19
Sulfamethoxazole	7	9.5
Triclosan	7	27

ACN/0.1% formic acid; positive ESI mode		
Compound	**n**	**RSD /%**
Atenolol	7	12
Atorvastatin	7	25
Caffeine	6	8.4
Carbamazepine	7	5.7
Diclofenac	7	11
Erythromycin	7	9.3
Fluoxetine	7	28
Ketoprofen	7	10
Naproxen	6	26
Phenytoin	7	11
Sulfamethoxazole	7	7.3

The within-day precision for ACN/0.1% formic acid; positive ESI mode is less than 15% RSD for all the compounds excluding fluoxetine. ACN/water in negative ESI mode on the other hand had a RSD exceeding 15% for the majority of compounds.

Between-day precision had approximately half of the compounds with higher variation than the expected 15%. However the RSDs of within-day and between-day should not be directly compared as within-day only had 3 replicates whilst between-day had 6-7 replicates. These preliminary results still indicate a large variation in batch precision which can be reduced through normalisation with internal standards.

3.2.8 Analysis of water samples

Triplicate injections of 1.8 mL of each of the Melbourne Water samples listed in Table 3.26 were analysed, with matches based on m/z and retention time to standards. The responses of the samples were interpolated via a calibration plot of standards to yield the concentrations in ng L^{-1}, and the RSD of each sample was calculated (Table 3.27 and Table 3.28).

Table 3.26 : Melbourne Water Samples with ID numbers and sample description

Sample ID	Sample Description
5793	50 m upstream of STP
5794	At STP
5795	30 m downstream of STP
5796	10 km downstream of STP and upstream of tributary
5797	Tributary 10 km downstream (not influenced by STP)
5798	10 km downstream of STP and downstream of tributary
5799	20 km downstream of STP and downstream of tributary

Positive ESI mode resulted in good matches for some compounds including atenolol and erythromycin, as the matches had close mass accuracy, similar retention times to standards, and had fragments ions matching to MS/MS data in the compound library.

Unfortunately the method for negative ESI mode did not have any fragment ions matching to the MS/MS library although the compounds had close mass accuracy and similar retention times to standards. The trend for the pharmaceuticals in the water samples is somewhat expected as the samples at the STP (5794) tend to have a higher concentration of pharmaceuticals than upstream, and the further downstream the lower the concentration of pharmaceuticals, which is either due to dilution in the water source or degradation of the pharmaceuticals. The concentrations of pharmaceuticals found is below the LOD based on the statistical regression line for the majority of the compounds, however it is above the LOD based on S/N.

Table 3.27 : Summary of results of the analysis of Melbourne water samples(5793-5799), including the average concentration (C_{Avg}) and RSD of three replicate injections under ACN/0.1% formic acid carrier; positive ESI mode. Samples with high mass accuracy, and ion intensity and similar retention times to standards are indicated by green shading. Samples with qualifying fragment ions are indicated by purple shading, and samples with both high mass accuracy, ion intensity, similar retention times to standards, and qualifying fragments are highlighted in blue.

Compound	Sample:	5793	5794	5795	5796	5797	5798	5799
Atenolol	C_{Avg} /ng L^{-1}	41	168	373	26	6	6	6
	RSD /%	18	5	4	5	14	25	8
Atorvastatin	C_{Avg} /ng L^{-1}	0	0	2	4	0	0	2
	RSD /%	0	0	11	87	173	0	163
Caffeine	C_{Avg} /ng L^{-1}	6	14	14	3	5	7	3
	RSD /%	52	14	14	19	38	22	27
Carbamazepine	C_{Avg} /ng L^{-1}	2	78	7	10	1	9	8
	RSD /%	58	4	15	12	36	9	35
Chloramphenicol	C_{Avg} /ng L^{-1}	0	0	0	0	0	0	0
	RSD /%	0	0	0	0	0	0	0
Diclofenac	C_{Avg} /ng L^{-1}	1	31	8	2	1	2	3
	RSD /%	44	5	7	10	1	26	7
Erythromycin	C_{Avg} /ng L^{-1}	8	2	59	7	1	2	2
	RSD /%	25	51	8	10	15	9	10
Fluoxetine	C_{Avg} /ng L^{-1}	20	14	17	16	15	7	14
	RSD /%	39	11	20	23	14	59	79
Ketoprofen	C_{Avg} /ng L^{-1}	9	7	20	5	0	2	6
	RSD /%	7	91	8	33	0	103	58
Naproxen	C_{Avg} /ng L^{-1}	26	109	102	37	0	0	26
	RSD /%	97	40	102	173	0	0	173
Phenytoin	C_{Avg} /ng L^{-1}	5	100	270	17	0	0	0
	RSD /%	45	77	159	55	0	0	0

Table 3.28 : Summary of results of the analysis of Melbourne water samples (5793-5799), including the average concentration (C_{Avg}) and RSD of three replicate injections under ACN/water carrier; negative ESI mode. Samples with high mass accuracy, and ion intensity and similar retention times to standards are indicated by green shading.

Compound	Sample:	5793	5794	5795	5796	5797	5798	5799
Beta-estradiol	C_{Avg} /ng L^{-1}	27	30	25	19	18	7	16
	RSD /%	25	8	75	77	72	101	42
Chloramphenicol	C_{Avg} /ng L^{-1}	0	1	2	1	1	0	0
	RSD /%	96	34	3	12	13	18	31
Diclofenac	C_{Avg} /ng L^{-1}	0	1	1	0	0	0	1
	RSD /%	51	91	112	91	157	87	101
Estriol	C_{Avg} /ng L^{-1}	0	2	2	0	1	0	0
	RSD /%	87	96	14	173	135	0	0
Estrone	C_{Avg} /ng L^{-1}	6	7	9	3	2	3	2
	RSD /%	20	58	2	27	14	59	45
Ethinylestradiol	C_{Avg} /ng L^{-1}	0	16	8	4	6	4	8
	RSD /%	0	89	89	116	87	119	105
Fluoxetine	C_{Avg} /ng L^{-1}	502	830	974	1476	483	1423	994
	RSD /%	6	24	93	54	93	28	31
Gemfibrozil	C_{Avg} /ng L^{-1}	0	18	0	0	0	0	0
	RSD /%	0	10	173	0	0	0	0
Ibuprofen	C_{Avg} /ng L^{-1}	36	178	17	36	37	12	39
	RSD /%	12	31	38	9	11	10	43
Phenytoin	C_{Avg} /ng L^{-1}	5	20	8	6	3	3	3
	RSD /%	136	27	31	57	74	20	33
Triclosan	C_{Avg} /ng L^{-1}	11	10	45	6	6	6	6
	RSD /%	36	14	12	39	31	20	6

The RSD for the replicates was unexpectedly high which may be due to matrix effects in the water samples, where co-elution of other compounds can lead to signal suppression or enhancement[88]. The efficiency of the analyte ionisation is affected by matrix compounds entering the mass spectrometer at the same time, with the matrix effect affecting reproducibility and accuracy of the method. The variation is present in the difference in the peak shapes of the compounds which were not as well resolved and consistent as they were for the standards.

Possible methods to account for the matrix effect including optimising chromatography and sample preparation, but a more robust solution would be to use isotopically labelled internal standards or structural analogues(surrogates) [89]. However, structural analogues need to be carefully selected as the analogue should not be present in environmental samples [90], so other pharmaceuticals and emerging contaminants should be avoided.

The concentration levels obtained may also vary significantly based on when the samples are analysed. Some compounds, in particular caffeine is readily degradable in environmental water either by UV or microbial processes [91], and consequently the compounds in the samples could have degraded from when they were initially sampled. Steps were taken to minimise degradation by freezing the samples in dark bottles until analysis however such degradation cannot be discounted.

3.2.9 Summary of SPE-UHPLC-QTOF-MS method

An on-line SPE UHPLC-QTOF-MS was established based on the method in Section 3.1, with optimised load conditions (Table 3.16). The method displays a suitable limit of detections with respect to this project's aim, being able to detect down to 1 ng L^{-1} and displaying good precision, although accuracy has room for improvement. Although the method was developed using standards in Type I water, the method is also applicable for real water samples which met the end goal. However further work will have to be carried out to improve matrix effects, accuracy and MS-detectability of compounds such that the method can be used for environmental water samples. Additionally isotopically labelled internal standards should be used to account for matrix effects and improve reproducibility and accuracy.

3.3 Exploratory Experiments

This section contains exploratory experiments to expand on the capabilities of the method.

3.3.1 Derivatisation and pH modification

Some of the problems encountered during analysis were related to the MS-detectability of the compounds. Currently the method developed has a limitation in that the two buffer systems used are different, which means that column equilibration is necessary between switching methods over (alternatively, a column switcher and a second column could be used).

The estrogenic hormones displayed poor to no detectability under formic acid buffers for example, whilst mestranol displayed poor detectability in either method. Some experiments were performed to explore the possibility of derivatisation to improve ionisation of the compounds. A set of experiments were performed where the autosampler was programmed to mix 0.5 µL of estrogenic sample with ammonium hydroxide (10%) for derivatisation before injection in a formic acid modifier mix. Different ratios of sample to ammonium hydroxide were trialled and the results are tabulated below in Table 3.29. For estriol, estrone and ethinylestradiol a higher response was obtained as the volume of derivatisation agent was increased. These preliminary results are confirmation of how adjustment of pH can be used to improve detectability of compounds. However care has to be taken with the amount of acidic and basic modifiers used as damage to the column could occur.

Table 3.29 : Responses of estrogenic compounds based on the volume of ammonium hydroxide (NH_4OH) introduced. A dash indicates that the compound was not MS-detectable.

	AUC			
$Volume_{NH4OH}$ /µL	**Beta-estradiol**	**Estriol**	**Estrone**	**Ethinylestradiol**
0.0	-	-	-	-
0.1	-	-	63579	-
0.2	-	-	109162	66043
0.3	-	56621	129551	88028
0.4	-	55283	163526	96138
0.5	-	60467	172426	84692
1.0	-	26191	207227	119838

Similarly, derivatisation experiments were explored targeting the derivatisation of ketones with hydroxylamine to form oximes. Oximes are much more readily ionised by ESI compared the parent ketone. The derivatisation was performed in a similar manner to above mixing via Autosampler of sample and 10% hydroxylamine in 1:1 MeOH/water. Some success was observed with estrone producing a weak signal although further experiments

were not carried out. Other derivatisation strategies for LC-MS are summarised in a review by Qi et al. and these strategies could be explored in further work.[92]

3.3.2 Post target analysis

The data obtained for the Melbourne Water samples was subject to post target analysis. The masses of potential transformation products of the pharmaceuticals detected in the samples were added to a separate library. The run data from the analysis of water samples was searched against the database to find any potential matches (Table 3.30).

Table 3.30 : Potential transformation products and their chemical formula

Pharmaceutical	Potential transformation Product	Formula
Chloramphenicol	Dichloroacetamide	$C_2H_3Cl_2NO$
	4-nitrobenzaldehyde	$C_7H_5NO_3$
	Glycolaldehyde	C_2H_4O
Ibuprofen	Hydroxy-ibuprofen	$C_{13}H_{18}O_3$
	Carboxy-ibuprofen	$C_{13}H_{16}O_4$
Diclofenac	(4 or 5)-hydroxy-diclofenac	$C_{14}H_{11}Cl_2NO_3$
	p-benzoquinone imine of 5-hydroxydiclofenac	$C_{13}H_9Cl_2NO_4$
Erythromycin	Erythromycin-water	$C_{37}H_{65}NO_{12}$
Ketoprofen	3-(hydroxy-carboxymethyl)hydratopic acid	$C_{11}H_{12}O_5$
	3-(keto-carboxymethyl)hydratopic acid	$C_{11}H_{10}O_5$

The criteria for a potential match was based on having a score of greater than 75, where the accurate mass and ion intensity contribute to the score. Potential transformation products identified are tabulated in Table 3.31.

Table 3.31 : Potential transformation products identified in water samples

Sample	Compounds identified
5793	Carboxy-ibuprofen
	Hydroxy-ibuprofen
5794	Hydroxy-ibuprofen
5795	Erythromycin-water
	Hydroxy-ibuprofen
	3-(hydroxy-carboxymethyl)hydratopic acid
5796	Hydroxy-ibuprofen
5797	Carboxy-ibuprofen
5798	Hydroxy-ibuprofen
5799	3-(hydroxy-carboxymethyl)hydratopic acid
	Hydroxy-ibuprofen

An interesting observation is the presence of hydroxyl-ibuprofen in the majority of the samples, although it is not present in 5797 which is the tributary. The tributary is not influenced by the STP stream which could explain the absence of the transformation product.

However the presence of some transformation products carboxy and hydroxyl-ibuprofen upstream of the STP suggests their introduction was through other sources and not through the STP.

Confirmation of identification could have been achieved by running a targeted MS/MS scan with the m/z of the potential transformation products. The resultant fragmentation information could be used for confirmation as the structure of the transformation products are known. Unfortunately time constraints did not allow this confirmation to be performed.

3.3.3 Summary of exploratory experiments

The preliminary results from the exploratory experiments show promise to improve the method. The pH modification with ammonium hydroxide was able to improve the MS detectability of the estrogenic compounds, whilst the derivatisation strategy could be further explored as a future direction.

The results from the post targeted screening also shows great promise for the QTOF method to be used for non-targeted screening of environmental waters.

Chapter 4: Conclusion

A working on-line SPE UHPLC-QTOF-MS method has been developed and validated for the sensitive multi-residue analytical method for the analysis of pharmaceuticals in environmental waters. It has been demonstrated that the method is applicable to a wide range of different pharmaceutical classes, however certain compounds (such as mestranol) in the chosen list had poor MS detectability. Different mobile phases, pHs and derivatisation strategies were also explored in an effort to improve detectability.

The method is able to achieve limit of detections down to 1 ng L^{-1} making it suited for the analysis of trace emerging contaminants in waters. Whilst the method is currently limited by the low recovery after SPE, it was not considered an obstacle for reliable determination, as the sensitivity and reproducibility of the method is good. Accurate quantification can instead be achieved through normalisation with isotopically labelled internal standards. Furthermore, the method developed is inherently adjustable, allowing different pH ranges and gradients to be used. It has also been shown that the method has great promise for post-target analysis of water samples.

While this method has similar LODs (low ng L^{-1}) to existing methods in literature (some summarised in Section 1.3.3.3), a major advantage is that the analysis is run in a non-targeted MS^E mode allowing for post-target analysis to be performed. Existing data can be analysed for new target compounds suspected to be present, with both MS (molecular ion) and MS/MS (fragmentation information) data available. This is a major contribution of the method, as it opens up opportunities for future research in the study of emerging contaminants and their transformation products.

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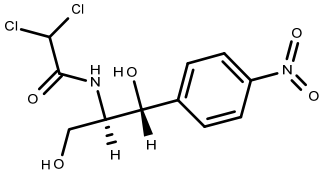
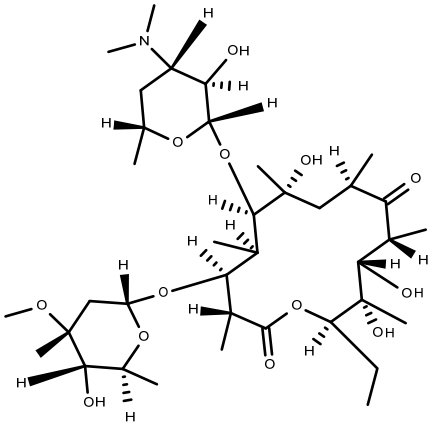
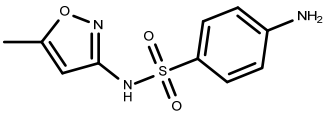
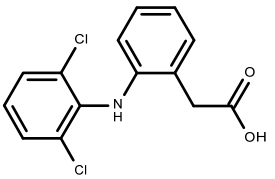
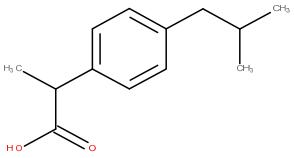
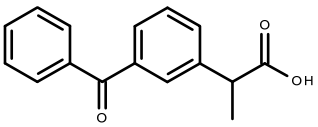
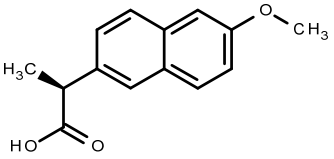
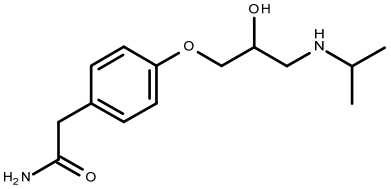
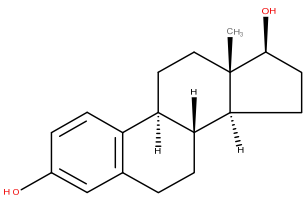
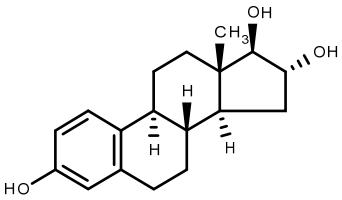
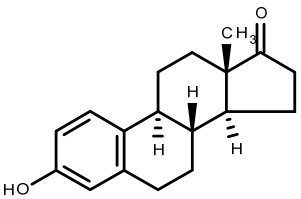
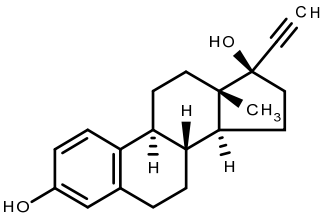
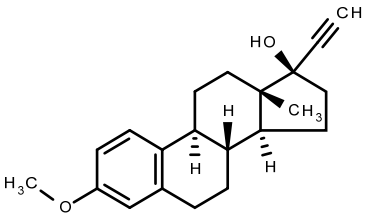
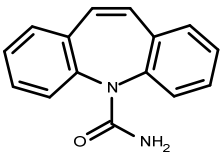
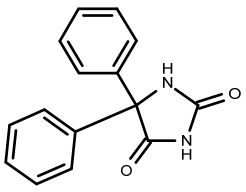
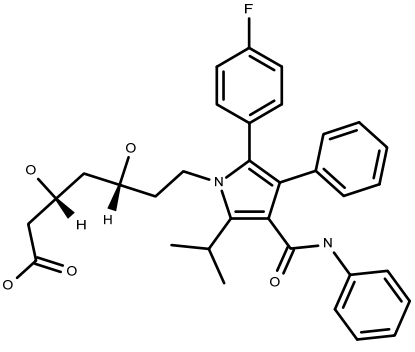
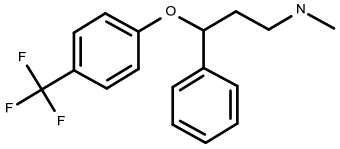
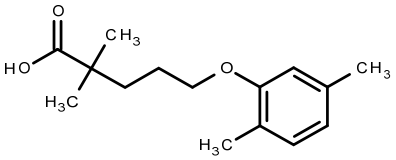
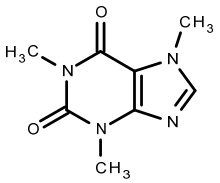
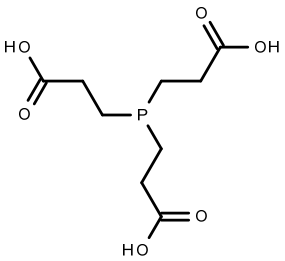
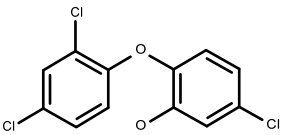
Appendices

Appendix 1 : List of target compounds, elemental formulas and chemical structure

Class of compounds	Target compound and elemental formula	Chemical Structure
Antibiotics	Chloramphenicol $C_{11}H_{12}Cl_2N_2O_5$	
	Erythromycin $C_{37}H_{67}NO_{13}$	
	Sulfamethoxazole $C_{10}H_{11}N_3O_3S$	
Anti-inflammatories	Diclofenac $C_{14}H_{11}Cl_2NO_2$	
	Ibuprofen $C_{13}H_{18}O_2$	
	Ketoprofen $C_{16}H_{14}O_3$	
	Naproxen $C_{14}H_{14}O_3$	
Beta blockers	Atenolol $C_{14}H_{22}N_2O_3$	

Hormones	17β-Estradiol (E2) $C_{18}H_{24}O_2$	
	Estriol (E3) $C_{18}H_{24}O_3$	
	Estrone (E1) $C_{18}H_{22}O_2$	
	Ethinylestradiol (EE2) $C_{20}H_{24}O_2$	
	Mestranol $C_{21}H_{26}O_2$	
Anti-epileptic	Carbamazepine $C_{15}H_{12}N_2O$	
	Phenytoin $C_{15}H_{12}N_2O_2$	
General pharmaceuticals	Atorvastatin $C_{33}H_{35}FN_2O_5$	

	Fluoxetine $C_{17}H_{18}F_3NO$	
	Gemfibrozil $C_{15}H_{22}O_3$	
Miscellaneous (Non-pharmaceutical emerging contaminants)	Caffeine $C_8H_{10}N_4O_2$	
	TCEP (tris(2-carboxyethyl)phosphine) $C_9H_{15}O_6P$	
	Triclosan $C_{12}H_7Cl_3O_2$	

Appendix 2 : Responses (AUC) of compounds under the four chromatographic conditions in both ESI modes; a-d corresponding to conditions in Table 3.1.

	Negative ESI mode				Positive ESI mode			
Compound	**a**	**b**	**c**	**d**	**a**	**b**	**c**	**d**
Atenolol	0.E+00	0.E+00	0.E+00	0.E+00	3.E+07	7.E+06	7.E+06	9.E+06
Atorvastatin	7.E+06	3.E+06	6.E+05	5.E+03	1.E+07	4.E+06	5.E+06	6.E+06
Beta-Estradiol	3.E+04	1.E+04	2.E+03	2.E+03	5.E+03	1.E+03	1.E+03	1.E+04
Caffeine	0.E+00	0.E+00	0.E+00	0.E+00	3.E+06	1.E+06	2.E+06	1.E+06
Carbamazepine	1.E+03	1.E+04	3.E+03	1.E+03	2.E+07	9.E+06	1.E+07	1.E+07
Chloramphenicol	6.E+06	2.E+06	7.E+05	1.E+06	2.E+03	1.E+04	4.E+03	4.E+03
Diclofenac	8.E+05	5.E+05	2.E+05	2.E+05	1.E+05	3.E+05	1.E+05	1.E+05
Erythromycin	2.E+03	7.E+03	1.E+03	1.E+03	2.E+07	9.E+06	9.E+06	9.E+06
Estriol	2.E+05	1.E+04	1.E+04	1.E+04	1.E+03	2.E+04	2.E+03	5.E+03
Estrone	9.E+04	4.E+03	3.E+04	6.E+04	2.E+06	1.E+06	2.E+04	3.E+05
Ethinylestradiol	2.E+04	5.E+04	3.E+04	1.E+04	9.E+03	1.E+04	2.E+03	3.E+03
Fluoxetine	0.E+00	2.E+04	1.E+03	0.E+00	2.E+07	1.E+07	1.E+07	1.E+07
Gemfibrozil	1.E+06	9.E+04	6.E+04	7.E+04	5.E+04	3.E+06	1.E+05	3.E+05
Ibuprofen	1.E+05	9.E+03	5.E+03	5.E+03	0.E+00	0.E+00	0.E+00	0.E+00
Ketoprofen	9.E+04	1.E+05	3.E+04	1.E+04	1.E+05	1.E+06	5.E+05	3.E+05
Mestranol	1.E+03	1.E+03	1.E+04	4.E+04	5.E+04	1.E+06	3.E+06	2.E+06
Naproxen	1.E+04	4.E+03	8.E+02	1.E+03	2.E+03	1.E+04	1.E+04	2.E+04
Phenytoin	1.E+06	9.E+04	3.E+04	4.E+04	5.E+03	7.E+05	2.E+05	2.E+04
Sulfamethoxazole	2.E+06	5.E+05	7.E+04	9.E+04	7.E+06	3.E+06	3.E+06	3.E+06
TCEP	1.E+03	3.E+04	0.E+00	0.E+00	0.E+00	3.E+04	0.E+00	0.E+00
Triclosan	5.E+06	2.E+06	7.E+05	6.E+05	1.E+03	4.E+03	2.E+03	4.E+03

Appendix 3 : Average AUC at different load flow rates for ACN/water carrier; negative ESI mode

	Average AUC at load flow rate /mL min^{-1}				
Compound	**1**	**1.5**	**2**	**2.5**	**3**
Atenolol	8.5E+05	8.9E+05	1.2E+06	9.2E+05	1.3E+06
Atorvastatin	2.2E+06	2.4E+06	2.5E+06	2.4E+06	2.3E+06
Beta-Estradiol	3.4E+05	3.8E+05	4.0E+05	4.1E+05	4.2E+05
Caffeine	1.1E+05	1.3E+05	1.7E+05	2.2E+05	1.7E+05
Chloramphenicol	1.3E+07	1.8E+07	1.9E+07	1.9E+07	1.9E+07
Diclofenac	0	0	5.1E+04	6.6E+04	1.0E+05
Estriol	1.5E+06	1.9E+06	1.9E+06	1.9E+06	1.9E+06
Estrone	4.5E+05	5.2E+05	5.5E+05	5.4E+05	5.5E+05
Ethinylestradiol	3.4E+05	3.9E+05	4.3E+05	4.2E+05	4.2E+05
Gemfibrozil	5.1E+06	5.4E+06	5.7E+06	5.4E+06	5.3E+06
Ibuprofen	2.6E+05	3.1E+05	3.4E+05	3.4E+05	3.3E+05
Ketoprofen	1.9E+05	2.4E+05	2.7E+05	2.9E+05	3.0E+05
Mestranol	3.2E+05	2.9E+05	1.4E+05	1.5E+05	1.7E+05
Naproxen	1.2E+05	1.7E+05	1.3E+05	9.0E+04	1.8E+05
Phenytoin	1.7E+06	2.1E+06	2.3E+06	2.3E+06	2.2E+06
Sulfamethoxazole	2.4E+06	3.6E+06	3.9E+06	3.7E+06	3.7E+06
Triclosan	7.2E+06	6.9E+06	6.8E+06	6.3E+06	6.1E+06

Appendix 4 : Average AUC for different load flow rates for ACN/0.1% formic acid; positive ESI mode

	Average AUC at load flow rate /mL min^{-1}				
Compound	**1**	**1.5**	**2**	**2.5**	**3**
Atenolol	1.7E+07	1.5E+07	1.7E+07	1.7E+07	1.7E+07
Atorvastatin	4.4E+06	4.2E+06	3.9E+06	3.9E+06	4.2E+06
Caffeine	2.7E+06	2.2E+06	2.9E+06	2.9E+06	2.8E+06
Carbamazepine	2.4E+07	2.4E+07	2.4E+07	2.4E+07	1.6E+07
Chloramphenicol	6.8E+04	6.8E+04	6.8E+04	6.6E+04	6.9E+04
Diclofenac	1.4E+06	1.3E+06	1.2E+06	1.2E+06	1.3E+06
Erythromycin	2.2E+06	2.4E+06	2.2E+06	2.2E+06	2.2E+06
Fluoxetine	2.0E+07	2.1E+07	2.0E+07	2.0E+07	2.0E+07
Ketoprofen	2.9E+06	1.4E+06	1.8E+06	2.6E+06	1.9E+06
Naproxen	1.3E+05	1.2E+05	1.0E+05	1.1E+05	1.2E+05
Phenytoin	4.9E+05	4.1E+05	4.0E+05	4.0E+05	4.2E+05
Sulfamethoxazole	4.8E+06	5.0E+06	5.4E+06	5.2E+06	5.1E+06

Appendix 5 : Average AUC at different flow volumes for ACN/water; negative ESI mode

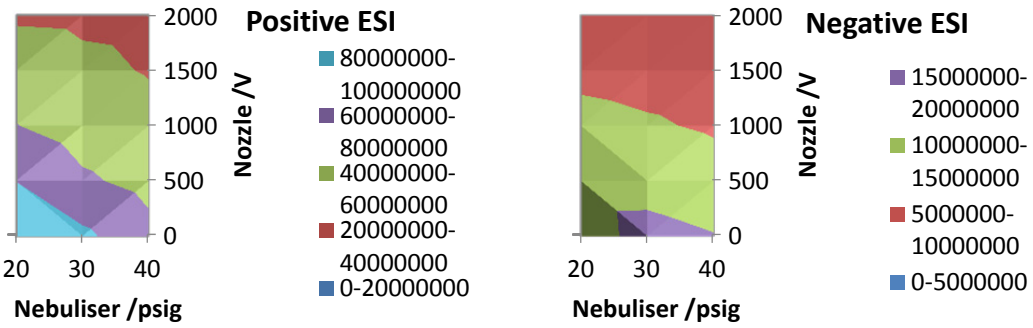
	Average AUC at load flow volume /mL						
Compound	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Atorvastatin	8E+05	3E+06	5E+06	5E+06	4E+06	6E+06	6E+06
Beta-Estradiol	1E+06	6E+06	7E+06	6E+06	2E+06	1E+06	1E+06
Chloramphenicol	2E+07	7E+07	7E+07	6E+07	7E+07	5E+07	6E+07
Diclofenac	2E+06	9E+06	9E+06	1E+07	8E+06	4E+06	5E+06
Estriol	5E+06	2E+07	2E+07	2E+07	9E+06	8E+06	9E+06
Estrone	1E+06	7E+06	8E+06	7E+06	3E+06	3E+06	3E+06
Ethinylestradiol	1E+06	6E+06	7E+06	6E+06	2E+06	2E+06	2E+06
Gemfibrozil	3E+06	1E+07	2E+07	3E+07	3E+07	2E+07	2E+07
Ibuprofen	3E+05	1E+06	7E+05	1E+06	1E+06	1E+06	1E+06
Ketoprofen	1E+05	6E+05	6E+05	1E+06	8E+05	7E+05	9E+05
Phenytoin	2E+06	1E+07	1E+07	9E+06	6E+06	5E+06	6E+06
Triclosan	5E+06	2E+07	3E+07	3E+07	3E+07	3E+07	3E+07

Appendix 6 : Average AUC at different load volumes for ACN/0.1% formic acid; positive ESI mode

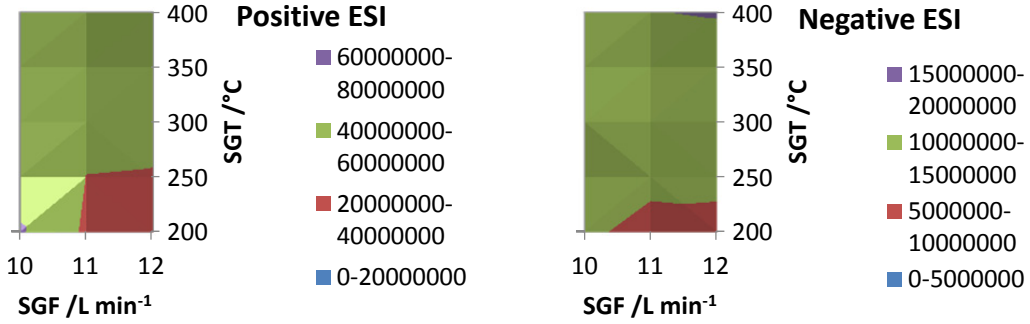
	Average AUC at load flow volume /mL						
Compound	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Atenolol	2E+06	5E+06	8E+06	2E+07	2E+07	2E+07	2E+07
Atorvastatin	1E+06	3E+06	5E+06	4E+06	4E+06	5E+06	5E+06
Caffeine	1E+06	3E+06	4E+06	4E+06	4E+06	5E+06	5E+06
Carbamazepine	1E+07	2E+07	3E+07	3E+07	3E+07	3E+07	3E+07
Chloramphenicol	6E+04	1E+05	2E+05	2E+05	2E+05	2E+05	2E+05
Diclofenac	6E+05	2E+06	2E+06	2E+06	2E+06	3E+06	3E+06
Erythromycin	3E+06	1E+07	2E+07	2E+07	2E+07	2E+07	2E+07
Fluoxetine	8E+05	2E+06	5E+06	9E+06	1E+07	2E+07	2E+07
Ketoprofen	7E+05	2E+06	3E+06	3E+06	3E+06	3E+06	3E+06
Phenytoin	2E+05	2E+05	2E+05	2E+05	2E+05	7E+04	7E+05
Sulfamethoxazole	9E+04	3E+05	2E+05	3E+05	2E+05	2E+05	2E+05

Appendix 7 : Surface area charts for source parameter optimisation for ACN/0.1% formic acid carrier in both positive and negative ESI mode. Where a = nebuliser and nozzle optimisation; b = SGF and SGT optimisation; c = DGF and DGT optimisation and; d = capillary voltage optimisation.

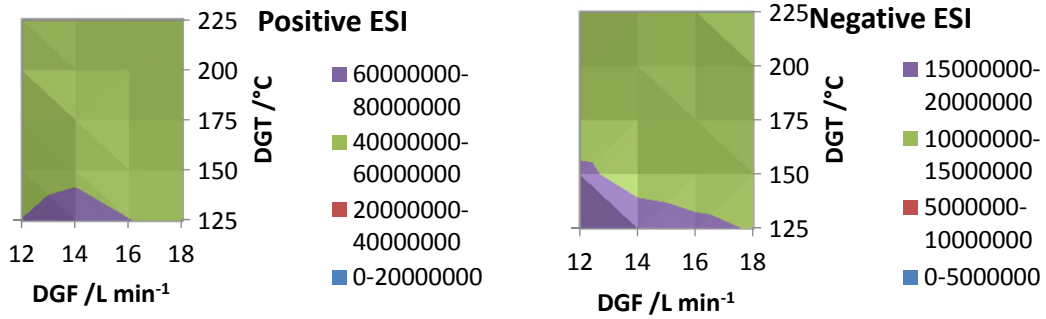
a.



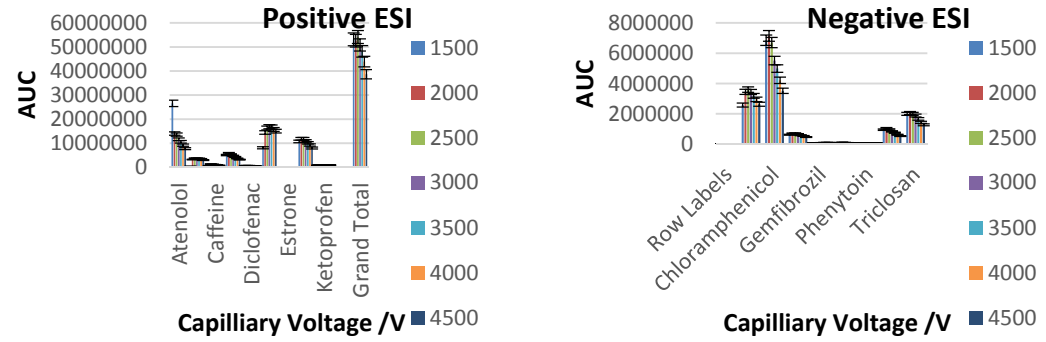
b.



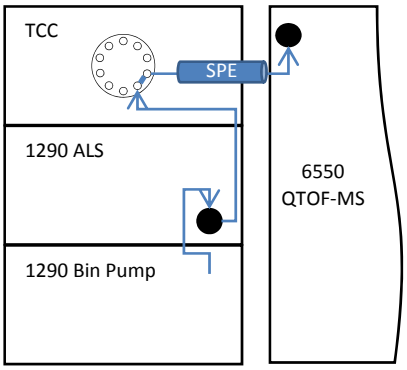
c.



d.

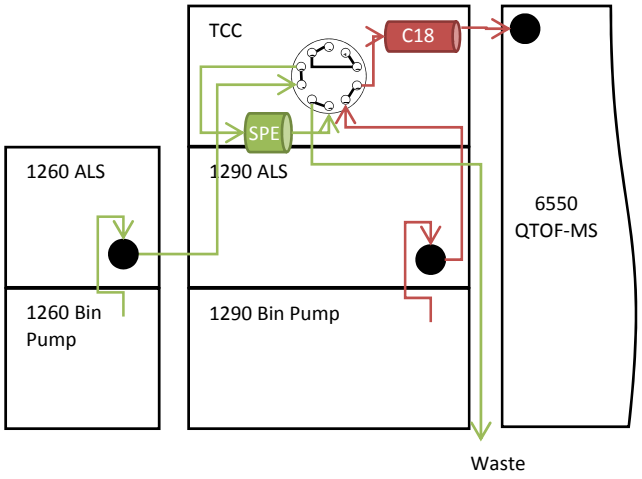


Appendix 8 : Schematic for direct SPE analysis using BinaryPump2



Appendix 9 : Schematic for SPE-UHPLC-QTOF with a. load position and; b. elute position

a.



b.