# Analysis of Compounds Related to the Synthesis of Amphetamine Type Stimulants in Wastewater and Their Stability

By

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# Declaration

“I certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another, except where due reference is made in the text”.

Russell S. Fuller

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# Summary

Illicit drug manufacture poses several health precautions to the general community as the chemicals used can be toxic and pose a risk of explosion during manufacture. This health risk is increased by the fact that the majority of clandestine laboratories are found in residential areas.

Amphetamine type stimulants (ATS) are the most common substances synthesised in clandestine laboratories both worldwide and in Australia. They can be synthesised via numerous methods including the: hypophosphorous, red phosphorous, Nazi/Birch and 1-phenylpropan-2-one method for synthesising methamphetamine, many of which produce route specific impurities. To try and avoid detection, clandestine waste is generally disposed of using domestic drainage, domestic garbage, burying or in landfill. Disposal of clandestine waste material into the municipal wastewater system may provide a valuable information source for law enforcement.

Currently wastewater has been analysed for illicit drug metabolites to gain estimates for the amount of illicit drug consumption. These estimates are in good agreement with population surveys and have the advantage of being time efficient and not relying on information from the consumers themselves, which may be misleading. With the recent development of new designer drugs, the consumer may be unaware or mislead about which drugs they are consuming making population surveys less reliable.

The motivation of this thesis was to fill this gap in knowledge by developing a method for the extraction and analysis of compounds relates to the production of common amphetamine type stimulants and to investigate the feasibility of wastewater analysis for their detection.

To achieve this goal compounds related to the synthesis of amphetamine synthesis namely: benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzladehyde, safrole, piperonal, ephedrine, pseudoephedrine, amphetamine and methamphetamine were used to develop a solid phase extraction method. A high recovery of each compound was achieved with the lowest recovery occurring for benzaldehyde (79 %). In addition, a liquid chromatography mass spectrometry (LCMS) method employing multiple reaction monitoring (MRM) was developed along with a gas chromatography mass spectrometry (GCMS) method operated in both scan and selected ion monitoring (SIM) modes for the analysis of the wastewater extracts.

In addition to investigating the presence of these targeted compounds in wastewater, the stability of: benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzaldehyde, safrole and piperonal in wastewater was tested at both room temperature and at storage conditions; pH = 2 adjusted at -

20 °C. This was achieved by spiking wastewater samples from several South Australian wastewater treatment plants (WWTP) with each compound prior to extraction and GCMS analysis.

The stability results showed that extreme care must be taken when analysing wastewater for these compounds particularly if not stored appropriately. Benzaldehyde was shown to be the least stable having a half-life of only 5.4 ± 0.2 days. The stability of all compounds increased significantly for the samples stored in the freezer with benzaldehyde again being the least stable having a half-life of 23 ± 1 days. In addition, some of the degradation products were identified by injection of a degraded wastewater sample which was spiked with a high concentration of the targeted compounds. This test showed the presence of benzyl alcohol, benzoic acid, 4-methoxybenzene methanol, piperonal alcohol, 4-methoxybenzoic acid, piperonylic acid and 1,3-diphenylpropan-2-one.

Wastewater samples were obtained from several Australian wastewater treatment plants (WWTP). These samples were filtered, pre-concentrated using SPE and were analysed using the developed GC-MS and LC-MS methods. In addition to the targeted compounds the presence of the degradation products identified in the stability experiments was also investigated. The only compounds routinely found in the wastewater extracts were benzaldehyde and benzoic acid, compounds which are expected to be present in wastewater from sources other than the illicit manufacture of amphetamine type stimulants. The non-detection of the other substances may be a result of sampling during periods where a dumping event may not have occurred or that the concentration of these substances was too low for the detection limits of the GCMS method. For this reason, the feasibility for the use of wastewater analysis for the detection of these compounds cannot be confirmed as the presence is highly dependent on the batch nature of illicit drug production.

Analysis of SPE samples via LCMS showed the ability of wastewater analysis for obtaining consumption estimates of ATS. A wastewater sample from Coombabah wastewater treatment plant was analysed and based on an external calibration method found to have methamphetamine, pseudoephedrine and Amphetamine present at levels of 175, 995 and 9.0 mg/day/1000 people respectively.

# Presentations

## Presentations resulting from this research:

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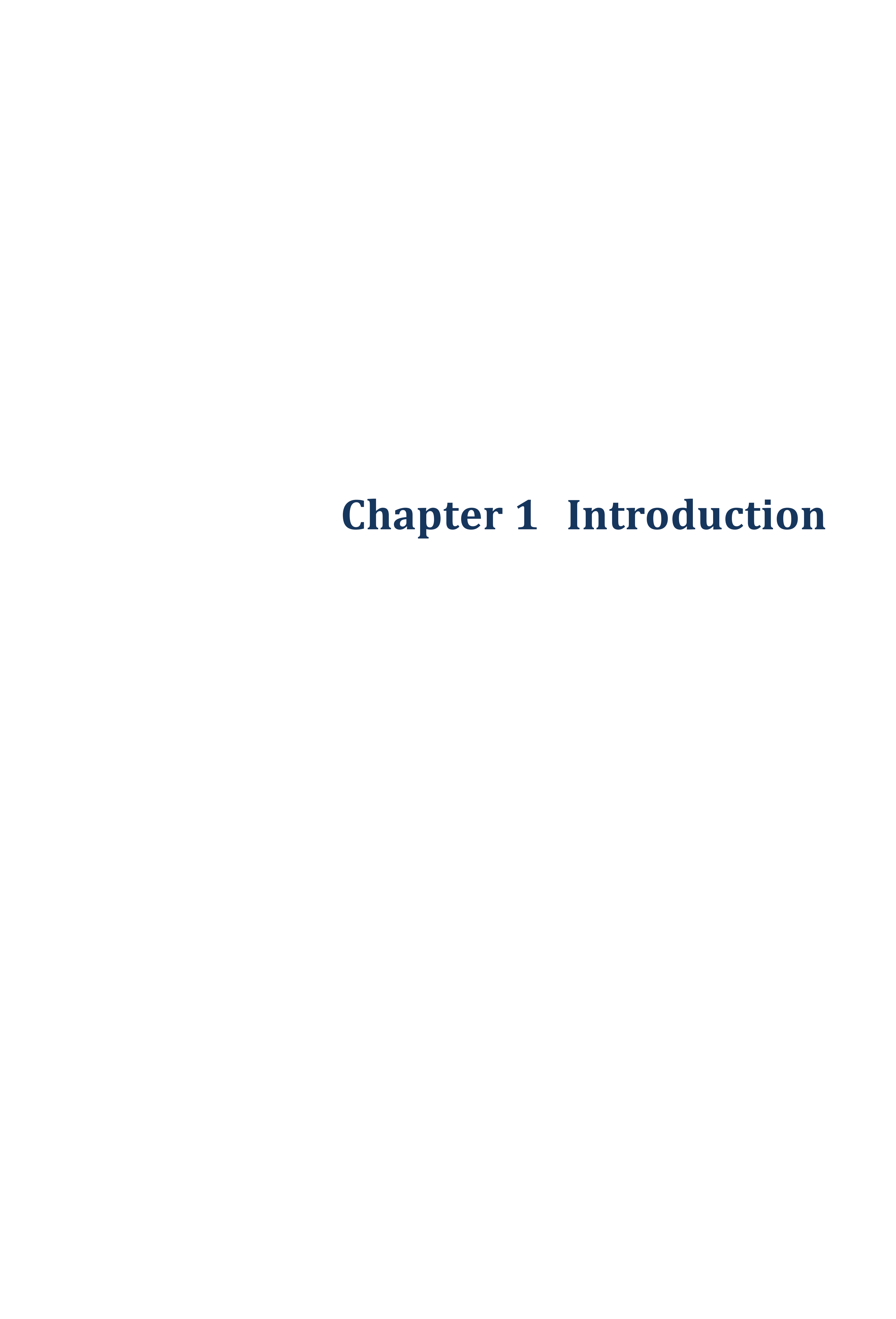
# Abbreviations

| | |
|---|---|
| **4-MBA** | 4-methoxybenzoic acid |
| **4-MBAl** | 4-methoxybenzaldehyde |
| **4-MBALC** | 4-methoxybenzyl alcohol |
| **5-HIAA** | 5-hydroxyindoleacetic acid |
| **ACC** | Australian Crime Commission |
| **ACIC** | Australian Criminal Intelligence Commission |
| **ADHD** | Attention deficit hyperactivity disorder |
| **AEC** | Anion Exchange Chromatography |
| **APAAN** | α-phenylacetoacetonitrile |
| **API** | Atmospheric pressure ionisation |
| **ASRS** | Anion Self-Regenerating Suppressor |
| **ATS** | Amphetamine Type Stimulants |
| **BA** | Benzoic acid |
| **BAl** | Benzaldehyde |
| **BALC** | Benzyl alcohol |
| **BOD** | Biological Oxygen Demand |
| **CAF** | Caffeine |
| **CBH** | Cellobiohydrolase |
| **CE** | Capillary Electrophoresis |
| **CEC** | Cation Exchange Chromatography |
| **ClF2AA** | Chlorodifluoroacetic anhydride |
| **CMP** | 1-(1’,4’-cyclohexadienyl)-2-methylaminopropane |
| **COD** | Chemical Oxygen Demand |
| **DAF** | Dissolved Air Flotation |
| **DC** | Direct current |
| **DCDMS** | Dichloro(dimethyl)silane |
| **DCM** | Dichloromethane |
| **DFT** | Density functional theory |
| **DoA** | Drugs of Abuse |
| **DTR** | Drug Target Residues |
| ***E*** | Electric Field Strength |
| **EF** | Enantiomeric fraction |
| **EI** | Electron Ionisation (or Electron Impact) |
| **EMCDDA** | European Monitoring Centre for Drugs and Drug Addiction |
| **ESI** | Electrospray Ionisation |
| **GC** | Gas Chromatography |
| **GCMS** | Gas Chromatograph Mass Spectrometry |
| ***HCl*** | Hydrogen Chloride |

| | |
|---|---|
| **HFBA** | Heptafluorobutyric anhydride |
| ***HI*** | Hydroiodic Acid |
| **HILIC** | Hydrophilic Interaction Chromatography |
| **HLB** | Hydrophilic lipophilic basic |
| **HOMO** | Highest occupied molecular orbital |
| **HPLC** | High Performance Liquid Chromatography |
| **HRMS** | High Resolution Mass Spectrometry |
| **IBU** | ibuprofen |
| **IC** | Ion Chromatography |
| **IS** | Internal Standard |
| **IT** | Ion trap |
| **L** | Litre |
| **LC** | Liquid Chromatography |
| **LCMS** | Liquid Chromatograph Mass Spectrometry |
| **LC-MS/MS** | liquid chromatography tandem mass spectrometry |
| **LHS** | Left hand side |
| **LINEST** | Line statistics |
| **LLE** | Liquid Liquid Extraction |
| **LLOD** | Lower limit of Detection |
| **LLOQ** | Lower limit of Quantification |
| ***m/z*** | Mass-to-charge ratio |
| **MCX** | Mixed mode cation exchange |
| **MDA** | 3, 4-Methylenedioxyamphetamine |
| **MDMA** | 3, 4-Methylenedioxymethamphetamine (Ecstasy) |
| **MDP2P** | 3,4-Methylenedioxyphenylpropan-2-one |
| **MDPBP** | 1(3,4-methylenedioxyphenyl)-2-bromopropane |
| **MeOH** | Methanol |
| **mg** | Milligram |
| **mL** | Millilitre |
| **ML** | Megalitre |
| **MRM** | Multiple Reaction Monitoring |
| **MS** | Mass Spectrometry |
| **MSD** | Mass spectrometry detector |
| **MTBSTFA** | N-methyl-N-t-butyldimethylsilyl trifluoroacetamide |
| ***NaOAc*** | Sodium Acetate |
| **N-formylMDA** | N-formyl-3,4-methylenedioxyamphetamine |
| **N-formylMDMA** | N-formyl-3,4-methylenedioxymethamphetamine |
| **N.D.** | Not detected |
| **ng** | Nanogram |
| ***NIST*** | National Institute of Standards and Technology |
| **nm** | nanometer |

| | |
|---|---|
| **NPLC** | Normal Phase Liquid Chromatography |
| **P2P** | 1-phenylpropan-2-one |
| **PAA** | Phenylacetic acid |
| **PAC** | Phenylacetylcarbinol |
| **PIP** | Piperonal |
| **PIPAC** | Piperonylic acid |
| **PIPOL** | Piperonyl alcohol |
| **PMA** | Paramethoxymethamphetamine |
| **PMK** | Piperonyl methyl ketone |
| **PMP2P** | Para-methoxyphenylpropan-2-one |
| **POCIS** | Polar organic chemical integrative sampler |
| **PPCP** | Pharmaceuticals and Personal Care Products |
| ***Py*** | Pyridine |
| **QqQ** | Triple quadrupole mass analyser |
| $R^2$ | Coefficient of determination |
| **RC** | Regenerated Cellulose |
| **RF** | Radio frequency |
| **RP** | Reverse Phase |
| **RPLC** | Reverse Phase Liquid Chromatography |
| **RT** | Room Temperature |
| **SA** | South Australia |
| **SAF** | Safrole |
| **SD** | Standard Deviation |
| **SIM** | Selected ion monitoring |
| **SPE** | Solid Phase Extraction |
| **S/N** | Signal to Noise Ratio |
| **SPM** | Solid Particulate Matter |
| **SRM** | Selected Reaction Monitoring |
| $T$ | Temperature |
| $t$ | Time |
| **TCI** | Tokyo Chemical Institute |
| **THC** | tetrahydrocannabinol |
| **THC-COOH** | 1-Hydroxy-6,6-dimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo[c]chromene-9-carboxylic acid |
| **Tof** | Time of Flight |
| $t_R$ | Retention time |
| **UNODC** | United Nations Office on Drugs and Crime |
| **UPLC** | Ultra Performance Liquid Chromatography |
| **USA** | United States of America |
| **UV** | Ultra Violet |
| **UV-VIS** | Ultra Violet Visible |
| **WA** | Western Australia |

| | |
|---|---|
| **WWTP** | Wastewater Treatment Plant |
| $x_m$ | Mean |
| $\sigma$ | Standard Deviation |
| **μg** | Microgram |
| **μL** | Microlitre |



# Chapter 1 Introduction

# 1.1 Amphetamine-type Stimulants (ATS)

Amphetamine-type stimulants (ATS) are a class of substances that contain a core structure derived from β-phenethylamine (Figure 1-1) [1]. The majority of amphetamine-type stimulants have an effect on the central nervous system (CNS) [2].

$NR_2$

β-phenethylamine

Figure 1-1: The structure of β-phenethylamine the core structure of amphetamine-type stimulants.

Currently amphetamine-type stimulants are the second most abused illicit drug type after cannabinoids both nationally and internationally [3, 4]. In 2010 it was reported that of the Australian population surveyed (n = 26,648) 3.0% had tried 3,4-methylenedioxymethamphetamine (MDMA) and 7 % had tried either methamphetamine or amphetamine [5]. Despite cannabis being the most commonly abused drug, the Australian Crime Commission (ACC) reported that the number of illicit seizures of ATS was higher than that of cannabis in South Australia during 2011-2012 [6].

Chemical modification of β-phenethylamine can produce a wide variety of amphetamine-type stimulants. The most commonly found ATS include: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), often called ecstasy, and 3,4-methylenedioxyamphetamine (MDA) [7].

## 1.1.1 Methamphetamine

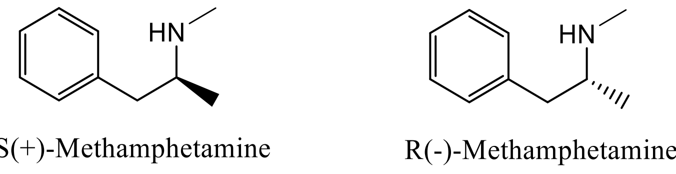



Figure 1-2: Structure of the S and R enantiomers of Methamphetamine

Methamphetamine or N-methyl-1-phenylpropan-2-amine (Figure 1-2) was first produced in 1893 and was first extensively used by military personnel in World War II [8]. In many countries methamphetamine was initially available legally without a prescription [9]. As the adverse effects of methamphetamine were noticed its removal from the pharmaceutical market resulted and its manufacture in clandestine laboratories began.

Most commonly methamphetamine is found in Australia in its hydrochloride salt form which is a powder usually administered orally or intranasally [10, 11]. Alternate, less common, routes of administration include injecting and smoking [12]. The crystalline form of methamphetamine is most commonly smoked.

In Asia and Australia, methamphetamine continues to be the main ATS used and synthesised, where in European countries amphetamine is significantly more readily available [13]. In February 2013, a major seizure of 585 kg of methamphetamine was made in Australia [14] and in December 2017 1.2 tonnes of methamphetamine (with an estimated street value of 1.04 billion $AU) was seized in Geraldton WA [15], these seizures help to emphasise the extent of the methamphetamine prevalence in Australia.

Methamphetamine exists as two different enantiomers (enantiomers are a pair of stereoisomers which are non-superposable mirror images of one another, whilst a stereoisomer is any isomer that differs only in the spatial arrangement of its atoms): S(+)-methamphetamine and R(-)-methamphetamine (Figure 1-2). The R and S enantiomers of methamphetamine have different effects on living organisms [16, 17]. S(+)-methamphetamine has stronger central nervous system stimulatory activity than R(-)-methamphetamine [18], and is described as being 3 -5 times more potent [19], however, there are different claims about the magnitude of physiological activities [20]. As the R(-) enantiomer has less of an effect on the central nervous system (CNS) and its effects are relatively short lived, the S(+) enantiomer is generally preferred [7]. In some countries R(-)-methamphetamine is used in some over the counter nasal inhalers [21].

Methamphetamine has a stronger effect on the central nervous system than amphetamine [6]. The neurotransmitters methamphetamine effects include the: dopamine, serotonin and noradrenaline neurotransmitters [22]. Dopamine influences body movement, memory, attention, problem solving, core body temperature, hunger, mood, and the pleasure centres of the brain; serotonin acts on the brain to influence mood, sleep, sexuality, and appetite whilst noradrenaline plays a large role in attention and the ability to concentrate [22]. Along with its effects on the central nervous system methamphetamine has been linked to the development of heart disease [23], depression, fatigue [24], and death.

#### *1.1.1.1 Methods for Manufacturing Methamphetamine*

There are several steps usually undertaken in the manufacture of amphetamine-type stimulants including: extraction, synthesis, conversion and tableting. Extraction is not only related to obtaining the illicit drug in a useable form but also includes removal of precursors such as pseudoephedrine from pharmaceuticals [25]. Synthesis involves utilising chemical reactions to create the final product. Once an illicit drug is synthesised it is commonly converted into a more usable form. The most common conversion utilised in the production of amphetamine-type stimulants is converting the free base of the drug, which is an oily liquid, into the hydrochloride salt and is commonly referred to as “salting out” [26]. This is generally done by addition of hydrochloric acid or bubbling hydrogen chloride gas (created by addition of table salt to sulphuric acid Eq. 1-1) through the solution of the free base [26]. Once the ATS is synthesised it is generally pressed with adulterants such as: dimethyl sulphone, sodium chloride, sodium thiosulphate, sodium glutamate and caffeine [27] into a tablet. In 2009 it was suggested that the importation of tablet presses into Australia should be prohibited and following this in 2010 tablet press was added to the amended customs (prohibited imports) regulations 1956 [28].

$$2\,NaCl_{(S)} + H_2SO_{4\,(l)} \rightarrow 2\,HCl_{(g)} + 2\,Na_2SO_{4\,(aq)} \qquad \text{Eq. 1-1}$$

Instead of tableting, the powder form of methamphetamine can be made more appealing to users by ‘icing’. This is done by dissolving the methamphetamine in acetone and cooling the solution in a refrigerator [29]. The only difference between the other forms of methamphetamine and “ice”, also referred to as crystal methamphetamine or just “crystal”, is that it has been further refined by removing impurities and typically has a purity of 90-100 %. It is typically referred to as ice because it has a crystalline structure and translucent appearance like shards of glass [30].

Numerous synthesis methods for methamphetamine have been detected in Australia and include: the P2P method, hypophosphorous method (commonly referred to as ‘hypo’ method), ‘HI/Red P’ method and the ‘Nazi/Birch’ method Figure 1-3 [31]. It should also be noted that in several clandestine laboratory discoveries more than one method was employed to produce methamphetamine, likely a result of limited availability of materials for one method. For this reason the total number of methods used for methamphetamine

synthesis in Figure 1-3 is greater than the number of detections for the corresponding year (554) [6].

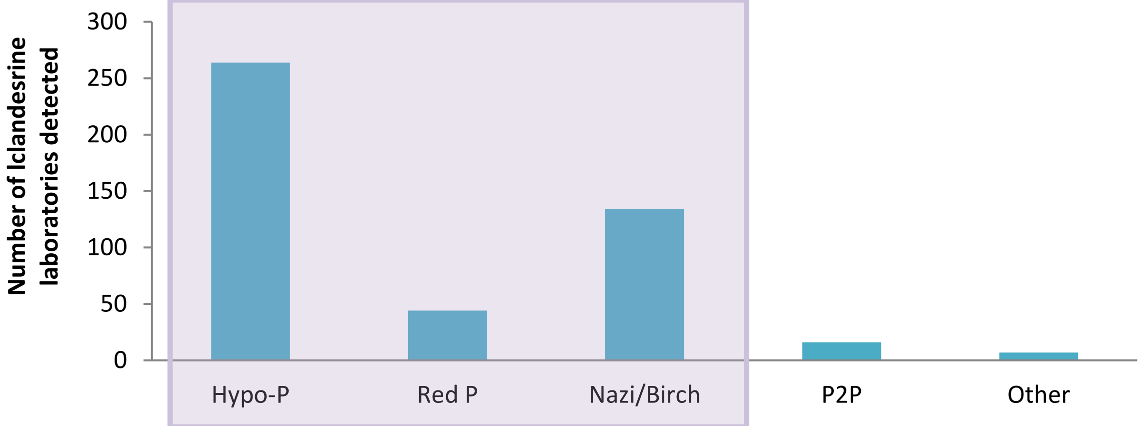



Figure 1-3: Various types of methamphetamine production methods found during 2011-12 in Australia note ‘other’ refers to clandestine labs producing illicit substances other than methamphetamine [6]. In addition, to the methods above there were 213 detections which were either awaiting analysis or were unable to be determined at the end of the reporting period.

Ephedrines, in particular 1R,2S(-)-ephedrine and 1S,2S(+)-pseudoephedrine (Figure 1-4), are methamphetamine cooks’ first choice when it comes to making methamphetamine in Australia. Profiling of methamphetamine seized in Australia between 2010 and 2013 suggests that over 70% of methamphetamine is synthesised from ephedrines [11]. This can be attributed to two factors, those being the ease of which it can be converted into methamphetamine and the product produced being the more desired, higher potency, S(+) enantiomer [18]. The only method mentioned above which does not utilise ephedrines as a precursor material is the P2P method.

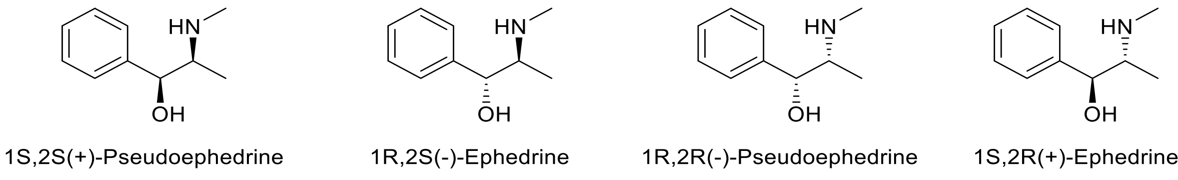



Figure 1-4: The stereoisomers of ephedrine.

## Pseudoephedrine/ephedrine Routes

### *Extraction of Pseudoephedrine from Tablets*

The pseudoephedrine used in methamphetamine production is a common ingredient in some over-the-counter (OTC) cold and flu medication. For this reason, pseudoephedrine is commonly extracted from cold and flu tablets. This is done by grinding the tablet (most commonly with a coffee grinder) and then adding the resultant powder to an organic solvent, generally methylated spirits [26]. The pseudoephedrine is soluble in the organic solvent whilst

the binder material, typically starch, is insoluble [32]. The solution is then decanted to a separate container where the solvent is evaporated off either at room temperature or with heating leaving pseudoephedrine behind [33]. Since cold and flu medications typically have other active ingredients such as paracetamol, impurities such as 4-aminophenol, produced from the amide hydrolysis of paracetamol, can be present in the final drug [34].

Paracetamol    4-aminophenol

Figure 1-5: Structures of N-acetyl-para-aminophenol (paracetamol) and its hydrolysis product 4-aminophenol which may be present in amphetamines produced from pseudoephedrine extracted from medications which also contain paracetamol.

### *Red Phosphorous methods*

The HI/Red P reduction method has been used in clandestine labs to produce S(+)-methamphetamine from 1R,2S(-)-ephedrine or 1S,2S(+)-pseudoephedrine since around 1982 [35]. However, this method has been used for many years prior by organic chemists to reduce carbonyl groups, alcohols and nitriles [36]. There are two common methods using red phosphorous to synthesise methamphetamine those being the Nagai and Moscow methods.

#### *Nagai Method*

The manufacture of S(+)-methamphetamine using the Nagai method is fairly simple. In summary, a mixture of pseudo/ephedrine, hydroiodic acid and red phosphorus (Red P) (generally obtained from the striking pads on match boxes which contain approx. 40 % Red P [37]) is heated for a couple of hours. Water is often added throughout the process to prevent boiling to dryness which results in phosphine gas being produced. An organic solvent (typically camping fuel, acetone or ether) and base (generally sodium hydroxide) is then added to the solution. This converts methamphetamine into its free base moves into the organic layer [35]. This free base form of methamphetamine is an insoluble oil, making it difficult to handle and unsuitable for several administration routes. To overcome this methamphetamine base is often converted to its hydrochloride salt [26]. The final solid, generally white or orange/brown in colour, is then filtered and dried. The yield obtained for methamphetamine production via the red P method in clandestine laboratories is usually a lot lower than the theoretical yield (50 %-75 % c.f. 92 % [35]).

Figure 1-6: Reaction schematic for production of methamphetamines via the HI/Red P method [35].

The main by-product of methamphetamine production via the Red P method is 1-phenylpropan-2-one, another common starting material for producing methamphetamine [38]. The presence of 1-phenylpropan-2-one in clandestine mixtures that were known to have been synthesised from ephedrine and pseudoephedrine puzzled forensic investigators for some time [39]. 1-phenylpropan-2-one can also undergo an aldol self-condensation reaction followed by dehydration, an internal aldol reaction and a second dehydration to give the by-products 1-benzyl-3-methylnaphthalene and 1,3-dimethyl-2-phenylnaphthalene as shown in Figure 1-6 [39].

Although the process is relatively easy to conduct, the regenerative role of the red phosphorous is extremely complicated. The catalytic regeneration of hydroiodic acid was described by Albouy et. al in both aqueous and anhydrous media. As can be seen from Figure 1-7 there are several pathways in which the hydroiodic acid can be recreated [40].

**Image removed due to copyright restriction. Please see reference for original image [40].**

*Moscow Method*

The Moscow method for synthesising methamphetamine is extremely similar to the Nagai method. The difference between the two methods is the source of the hydroiodic acid with iodine and water being used to create the hydroiodic acid in situ. The reaction schematic for the Moscow method is the same as above (Figure 1-6) and shows the same intermediates and main by-products of the reaction.

*Hypophosphorous Method*

The hypophosphorous method for the production of methamphetamine is the most common method employed by methamphetamine 'cooks' in Australia [31]. During 2011-12 approximately 11,000 kg of hypophosphorous acid was seized in Australia [6]. The hypophosphorous acid method or 'hypo' method is extremely similar to the red phosphorous method with the only difference being the source of phosphorous used in the reaction [41]. The Red P method uses red phosphorous and hydroiodic acid, whilst the hypophosphorous method uses hypophosphorous acid and iodine [42]. Both processes involve the production of hydroiodic acid in situ, in the hypophosphorous method it is the hypophosphorous acid which acts as a reducing agent to produce hydroiodic acid. There are many ways in which hydroiodic acid can be produced, alternatively, instead of using iodine and hypophosphorous or red phosphorous, potassium iodide and ortho-phosphoric acid can be used. Although low yielding, iodine crystals used can easily be produced by combining chemicals readily available to the public, iodine tincture with hydrogen peroxide [43].

Characterisation of phosphate, phosphite, and hypophosphite ions is essential to differentiate between the various phosphorus–iodine synthesis methods used in the manufacture of methamphetamine [44]. For this reason, if an amphetamine sample contains

phosphorous containing species from another source which phosphorous iodine method was used is unable to be determined.

### *Heterogeneous Catalytic Reduction / Emde Method*

Another common method used to produce methamphetamine is known as the Emde method. Also known as the catalytic reduction method, it is a two-step reduction in which chloroephedrine is utilised as a reaction intermediate [45]. This method is more predominant in Asia whereas other production methods including the hypophosphorous method are most common in Australia and America [46].

$SOCl_2$ or $PCl_5$

$Pd/BaSO_4$, HOAC, $H_2$
Pt, EtOH, $H_2$
CaH, Pd, HCl

1S,2S(+)-Pseudoephedrine — Chloropseudoephedrine — S(+)-Methamphetamine

Figure 1-8: Reaction schematic for the catalytic reduction method for manufacturing of methamphetamine [47] substituting $HClO_4$ into the first step is known as the Rosenmund method [27].

The ephedrine used in this process is dissolved in chloroform and then chlorinated using thionyl chloride or phosphorous pentachloride then diethyl ether is added to the chloropseudoephedrine until it precipitates out [48]. Upon precipitation the chloropseudoephedrine is filtered and dried before being reduced to methamphetamine.

Similar to the Red P method an impurity can form via a ring closure of the halo-ephedrine/pseudoephedrine intermediate to give 1,2-dimethyl-3-phenyl aziridines as depicted in Figure 1-6. These impurities are specific to methamphetamine synthesised from pseudoephedrine/ephedrine and are common in the Emde and Iodide/phosphorous routes [49]. However, it should be noted that 1-phenylpropan-2-one is not formed during the Emde method as it requires acidic conditions like in the iodide/phosphorous methods [39, 49].

### *Nazi/Birch Method*

According to the Australian crime commission In 2010-11 there was a 63 % increase in the number of laboratories producing methamphetamine by the Nazi/Birch method from the previous year [31]. Although hydrogen gas is produced as the metal dissolves it does not play a part in the reductive mechanism. The mechanism involves an electron transfer from the metal to pseudoephedrine forming a radical carbanion. This radical then abstracts hydrogen from the solvent or water absorbed from the atmosphere to complete the reduction (Figure

1-9) [48]. As anhydrous ammonia is used a lot in agriculture this method is commonly found in rural areas [50].

**Sensitive information removed. Please see references [113] and [114] for inform .**

The hydroxyl group of pseudo/ephedrine is preferentially reduced by the alkali metal, however, where there is an excess of the alkali metal and a proton source is present partial reduction of the methamphetamine can occur to produce the by-product 1-(1',4'-cyclohexadienyl)-2-methylaminopropane (CMP) Figure 1-10 [52].

Figure 1-10: Reaction schematic for Nazi/Birch method for manufacturing methamphetamine showing the primary by-product formed [58].

The most common alkali metal used in the Nazi/Birch method is lithium which is generally obtained from batteries. For this reason some stores in America limit the sale of lithium batteries [53]. This method has also been slightly adapted to be completed in less than one hour and is known as the 'shake and bake method' or the 'one pot method' which uses ammonium nitrate, generally obtained from fertilizer, instead of liquid ammonia [49].

It has also been reported that the methamphetamine can be purified by isolating it from the CMP via hydroxylation of the double bonds of CMP. This is conducted using potassium permanganate and an aqueous base to produce the tetra and dihydroxylated CMP which are soluble in aqueous solutions and hence can be separated from the methamphetamine [54].

## 1-phenylpropan-2-one (P2P) routes for manufacturing amphetamines

1-phenylpropan-2-one (P2P), Figure 1-11, also commonly referred to as benzyl methyl ketone (BMK) or phenylacteone is not only a by-product from some of the pseudoephedrine/ephedrine routes but can also be used to produce a variety of amphetamine type stimulants and is a relatively common method.

Figure 1-11: Structure of 1-phenylpropan-2-one a common starting material for the synthesis of amphetamine type stimulants.

### *Reductive amination method*

Figure 1-12 shows the reaction schematic for the P2P method and the intermediates formed. The 1-phenylpropan-2-one manufacturing method produces a racemate (an equal mixture of each stereoisomer) of methamphetamine and for this reason it is less desirable as the product is less potent. A racemate is produced due to the fact that the precursor utilised in the P2P method is achiral [35] unlike in the pseudoephedrine routes which are completely dependent on which stereoisomer of precursor is used.

This method is not only less desirable due to the mixture of enantiomers produced it is also extremely time consuming often involving specialised equipment and can take up to three days to complete [8].

1-Phenylpropan-2-one (P2P)
3,4-Methylenedioxyphenylpropan-2-one (MDP2P)
Methylamine (R = $CH_3$)
Ammonia (R = H)
Methamphetamine (R = $CH_3$)
Amphetamine (R = H)
3,4-Methylenedioxymethamphetamine (MDMA) (R = $CH_3$)
3,4-Methylenedioxyamphetamine (MDMA) (R = H)

Figure 1-12: Reaction mechanism for the production of amphetamine type stimulants via the P2P method created from [55] [56]. Alternate reduction methods, such as an aluminium mercury amalgam can be used in the final step instead of the catalytic hydrogenation to convert to methamphetamine.

The production of methamphetamine and amphetamine via this method decreased significantly when 1-phenylpropan-2-one was listed as a scheduled 2 controlled substance in 1980 [57, 58]. Since 1-phenylpropan-2-one has become harder to obtain the synthesis of 1-phenylpropan-2-one in clandestine labs has increased [59]. In addition to the difficulties obtaining 1-phenylpropan-2-one, the other main chemical used methylamine is also listed on the drug enforcement administration watch list [8].

As several different reductive amination reaction conditions can be used to create amphetamine type stimulants, a wide variety of impurities can be created in varying amounts.

### *Leuckart Method*

1-phenylpropan-2-one can also react via a Leuckart reaction to produce methamphetamine and amphetamine as discussed later in section 1.1.2.1.

### *Synthesis of 1-phenylpropan-2-one*

The most common way in which 1-phenylpropan-2-one is synthesised in clandestine laboratories is by reacting phenylacetic acid with acetic anhydride (Figure 1-13) [60]. Another relatively common method is the reduction of phenylacetic acid using lead (II) acetate Figure 1-14.

Phenylacetic acid — acetic anhydride, NaOAc or Py → Phenyl-2-propanone + Diphenyl-methanone

Figure 1-13: Synthesis of 1-phenylpropan-2-one from phenylacetic acid using acetic anhydride showing the diphenylmethanone by-product produced [60]

Phenylacetic acid — $Pb(OAc)_2$, - HOAc → … - $H^+$ → … - PbO → … + $H^+$, - $CO_2$ → 1-Phenylpropan-2-one

Figure 1-14: Proposed mechanism for the formation of 1-phenylpropan-2-one from the reduction of phenylacetic acid using lead (II) acetate [61].

As phenylacetic acid is also now listed as a controlled substance its use has fallen. One other possible method to produce 1-phenylpropan-2-one is by using benzyl cyanide as depicted in Figure 1-15.

Benzylcyanide + Ethyl acetate —Sodium ethoxide→ α-Phenylacetoacetonitrile (APAAN) —Sulphuric acid, Ethanoic acid→ Phenyl-2-propanone

Figure 1-15: Schematic for the conversion of benzyl cyanide to 1-phenylpropan-2-one [25]

This method first creates α-phenylacetoacetonitrile (APAAN) which can be easily converted to 1-phenylpropan-2-one. The synthesis of APAAN was rare in clandestine laboratories as APAAN was not covered by international precursor controls until May 2014 [62, 63] and as a result APAAN was typically imported directly from China [63]. It is estimated that over 95 % of all seized amphetamine laboratories are using 1-phenylpropan-2-one produced from APAAN [64].

### 1.1.2 Amphetamine

S(+)-amphetamine    R(-)-amphetamine

Figure 1-16: Enantiomers of amphetamine.

Amphetamine or 1-phenylpropan-2-amine (Figure 1-16) was first synthesised in 1887 by Edeleano who was interested in synthetic dyes [65, 66] however there was no pharmacological use for amphetamine until 1932 when the chemist Gordon Alles received a patent for the use of amphetamine in medicine [67]. This patent was later assigned to the Smith, Kline, and French (SKF) drug firm [65] who implemented amphetamine in over the counter inhalers [68]. It was first extensively used along with methamphetamine during World War II to prevent fatigue [8, 68]. After many years of abuse amphetamine was limited to prescription only and was used to combat obesity [69]. Amphetamine was listed as a strictly controlled substance in the 1970's. The first illicit amphetamine laboratory detection in Australia is believed to have occurred in NSW in 1976 [70].

Amphetamine is generally administered in powder form via oral, intranasal, injection and even anal insertion [6]. The S enantiomer of amphetamine is more potent than the R in terms of CNS effects whilst the R is slightly more potent when it comes to cardiovascular effects [71].

Despite methamphetamine being the most abundant amphetamine-type stimulant in Australia, amphetamine is more prevalent in the Netherlands and other European countries [72]. According to the European monitoring centre for drugs and drug addiction (EMCDDA) 1 ton of methamphetamine was seized compared to 5.9 ton of amphetamine in the European Union, Turkey and Norway in 2012 [13] However the availability and number of seizures of methamphetamine in Europe, despite still being relatively small, seem to be increasing, particularly in south east Europe [73].

#### 1.1.2.1 Methods for Manufacturing Amphetamine

There are several methods for synthesising amphetamine the most common one being via Leuckart method [74].

##### P2P methods

##### *Reductive amination*

Reductive amination of 1-phenylpropan-2-one as described in section 1.1.1.1 In addition to the synthesis of methamphetamine can also be utilised to create amphetamine. This is done by substituting methylamine with liquid ammonia.

##### *Leuckart Method*

The Leuckart reaction was first recorded by Rudolf Leuckart in 1885 [75]. In the early and mid 1970's, the Leuckart method was the most popular clandestine route to amphetamine and methamphetamine, despite the popularity of this method decreasing in the United States by the end on the 1970's it is still the method of choice for amphetamine synthesis in Europe [56, 76].

In the production of amphetamine using the Leuckart method, 1-phenylpropan-2-one is reacted with formamide in the presence of formic acid to form an intermediate. This intermediate is then extracted with diethyl ether and hydrolysed in the presence of acid into amphetamine [77]. The reaction scheme showing the Leuckart reaction for the synthesis of amphetamine, methamphetamine, MDA and MDMA is depicted in Figure 1-17 below. The Leuckart reaction is technically a reductive amination reaction, as it involves the conversion of a carbonyl group to an amine via an imine intermediate.

Figure 1-17: Leuckart method for the production of methamphetamine, amphetamine (black), 3,4 methylenedioxyamphetamine (MDMA) and 3,4-methylenedioxyamphetaine (MDA) (grey) [56].

The N-formyl intermediates have been used by forensic investigators for quite some time as being route specific impurities for illicit synthesis utilising the Leuckart method. However, the appearance of the N-formyl methamphetamine in batches of methamphetamine synthesised by the red phosphorous/Nagai method has since been observed [49, 78].

Along with the N-formyl-amphetamine intermediate, 4-methyl-5-phenylpyrimidine is also produced as a Leuckart specific impurity in the synthesis of amphetamine (Figure 1-18) [45].

Figure 1-18: Structure of 4-methyl-5-phenylpyimidine a common impurity in amphetamine synthesised via the Leukart route

## Norephedrine and Norpseudoephedrine methods

Despite being less common; in addition to the Leuckart method, amphetamine can also be synthesised utilising the manufacturing methods mentioned either involving ephedrine/pseudoephedrine (Red P, hypo method and Emde methods). These methods are adapted to produce amphetamine by substituting pseudo/ephedrine with norpseudoephedrine/norephedrine [79]. The structures of norpseudoephedrine and norephedrine are given in Figure 1-19.

Figure 1-19: Structures of: (1R,2R)-(-)-Norpseudoephedrine, (1S,2R)-(+)-Norpseudoephedrine, (1R,2S)-(-)-Norephedrine and (1S,2R)-(+)-Norephedrine. Note structures with 2R and 2S stereochemistry produce the R and S enantiomer of amphetamine respectively.

### *Nitrostyrene Reduction*

Another common method of synthesis of amphetamine is a Knoevenagel condensation reaction, also known as nitrostyrene reduction. This reaction utilises benzaldehyde and nitroethane to produce amphetamine via 1-phenyl-2-nitropropene which can easily be reduced to give amphetamine (Figure 1-20) [25].

Figure 1-20: Production of amphetamine and 3,4-methylenedioxyamphetamine from benzaldehyde [25] and piperonal [80] respectively. This method can also be employed to manufacture 1-phenylpropan-2-one (P2P) and 1-(3,4-methylenedioxyphenyl)propan-2-one (MDP2P) by substitution elemental iron and hydrochloric acid into the final step [81].

## 1.1.3 3,4-Methylenedioxymethamphetamine (MDMA)

Figure 1-21: Enantiomers of MDMA commonly referred to as ecstasy.

3,4-Methylenedioxymethamphetamine (MDMA) formally known by N-methyl-1-(3,4-methylenedioxyphenyl)propan-2-amine (Figure 1-21), is typically referred to as ecstasy even though nowadays tablets sold as ecstasy don't necessarily contain MDMA [6]. MDMA does not occur naturally and is often incorrectly referred to being designer drug which was created to try and circumvent the legal restrictions around 3,4-methylenedioxyamphetamine (MDA) [82]. This however is not the case as it was first synthesised in 1912 before controls on MDA were introduced in the 1970's [83]. MDMA has been reported to have been initially presented as an appetite suppressant [84, 85] however this has been disputed [83, 86] as Its synthesis

was first presented in a patent by the chemical company Merck where it was produced via safrole hydrobromination and used as a precursor to an anti-haemorrhagic compound [86]. Despite being synthesised at around the same time as MDA, MDMA was listed as a schedule 1 substance in 1985 [87] approximately a decade after MDA was controlled.

As with the majority of ATS, MDMA can exist as two enantiomers both with differing neurotoxicity and effects. S(+)-MDMA is believed to have a more potent serotonergic neurotoxicity than the R(-) enantiomer [88]. The effects of each enantiomer are also different with the R(-) enantiomer behaving like a hallucinogen and S(+)-MDMA more like a stimulant [89].

Whilst MDMA is commonly referred to as ecstasy the term is frequently used for several mixtures of illicit substances both including and excluding MDMA [90, 91]. This is believed to be as a result of a decline in the availability of safrole [92], a precursor in the production of MDMA. This shortage is also believed to be linked to the increase in the availability of designer drugs [93]. In Europe during 2010 only three MDMA laboratories were dismantled c.f. 50 in 2002 [94]. In addition, in 2009 only three countries in Europe reported that MDMA-like substances were present in a large proportion of the ecstasy tablets analysed [95], however the ecstasy market seems to be recovering with the number of tablets containing MDMA increasing in recent times [96] with the number of European countries reporting MDMA-like substances increasing to 11 in 2011 [73]. This increase in MDMA has not only been observed as a result of an increase in the content of MDMA in ecstasy pills but also the number of tablets seen [14]. MDMA is commonly taken in powder or tablet form orally, but can also be insufflated (commonly referred to as snorted), smoked or injected [73]. Despite the number of detections of MDMA being relatively low in recent times, its appearance in Australia still occurs. An example of this was the finding of 117 kg of MDMA concealed in olive oil bottles in March 2013 [14].

#### 1.1.3.1 Methods for Manufacturing 3,4-Methylenedioxymethamphetamine (MDMA)

There are 3 common methods for producing MDMA including: safrole bromination, reductive amination and the Leuckart reaction [81]. All of these reaction methods produce a racemate of MDMA as the reagents utilised are achiral.

Świst et al. investigated the by-products produced via the various methods of manufacturing MDMA [81]. It was found that for each manufacturing method a by-product was present

which wasn't produced in the other MDMA procedures Figure 1-22. This paper showed that the synthesis route used could be determined by looking for the method specific impurities which have important forensic applications.

**Image removed due to copyright restriction. Please see reference for original image [81].**

## Safrole Bromination Method

Safrole is typically obtained via extraction of the fruit or bark of sassafras plants [97]. It is a colourless to slightly yellow oily liquid which is generally extracted in the form of sassafras oil [97]. The use of safrole as a food additive is now prohibited [98] and as a result commercial sassafras oil generally has the safrole removed. This aids in preventing the deviation of the use of safrole to clandestine manufacture of MDMA [99].

Safrole is converted to MDMA using hydrobromination followed by nucleophilic substitution of the bromine as depicted in Figure 1-23. Although safrole can be converted directly into MDMA via 1-(3,4-methylenedioxyphenyl)-2-bromopropane (MDPBP) it can also be used to create 3,4-methylenedioxyphenylpropan-2-one (MDP2P) sometimes referred to as piperonyl methyl ketone (PMK) another common starting material for producing MDMA [100].

Safrole → HBr → 1-(3,4-methylenedioxyphenyl)-2-bromopropane (MDPBP) → $CH_3NH_2$ → MDMA

Figure 1-23: Hydrobromination of safrole followed by methylamine substitution to produce MDMA [101].

### MDP2P methods

MDP2P seems to be the favoured starting material among clandestine manufacturers for the synthesis of MDMA. It is used as a precursor for several methods of creating MDMA including: the Leuckart method and reductive amination method described earlier in section 1.1.2.1.

#### *Reductive Amination Methods*

There are several substances used to produce MDMA via the reductive amination method. All of these methods react MDP2P and methylamine together analogous to the P2P method for methamphetamine shown in Figure 1-12. The most common reducing material to perform the reduction is hydrogenation using a platinum catalyst but other common methods include an aluminium mercury amalgam, sodium borohydride or sodium cyanoborohydride [102].

#### *Leuckart Method*

The Leuckart method is the most common method for manufacturing MDMA. The Leuckart synthesis of MDMA is shown in Figure 1-17 in section 1.1.2.1.

#### *Production of 3,4-Methylenedioxyphenylpropan-2-one (MDP2P)*

MDP2P is not commercially available but is commonly produced synthetically from several precursors including isosafrole and piperonal. Isosafrole is a structural isomer of safrole and is commonly produced from safrole. This isomerism can be completed using several catalysts, including potassium hydroxide [103], phase transfer catalysts and transition metal catalysts [104]. A reaction scheme is given in Figure 1-24 below showing some common methods in which safrole is converted to isosafrole.

Safrole —HBr, aniline; KOH/$C_2H_5OH$→ *trans*-Isosafrole + *cis*-Isosafrole

Figure 1-24: Schematic for the Isomerism of safrole to isosafrole [99].

The isomerisation of safrole gives a mixture of the cis- and trans-isomers of isosafrole. Since the trans-isomer is more thermodynamically stable than the cis isomer, increasing the reaction time results in an increase in the ratio of the trans-isomer with respect to the cis [99].

This isosafrole can then be converted to MDP2P which can subsequently be transformed into MDMA. This can be done via oxidation using performic acid, $HCO_3H$ (Figure 1-25) or using Wacker oxidation [101]. Similarly this method can be applied to produce para-

methoxyphenylpropan-2-one (PMP2P), a precursor to para-methoxyamphetamine (PMA), as described later [105].



Figure 1-25: MDP2P synthesis by oxidation of isosafrole using performic acid [81].

One other method which can be used to synthesise MDP2P which doesn't involve safrole or isosafrole is via piperonal [106]. This method is outlined earlier in section 1.1.2.1. Piperonal has many legitimate uses including its use in perfumes, cherry and vanilla flavourings and the manufacture of mosquito repellents [107]. The impurities associated with synthesis of MDMA from piperonal created from oxidation of piperine extracted from black pepper has also been investigated [108]. This study found that alongside piperonal, 6-chloropiperonal was also produced. This reacted analogously to piperonal and as a result the equivalent chlorinated intermediates, by-products and MDMA formed, these were originally believed to be adulterants added to MDMA and may be route specific impurities [108].

### 1.1.4 3,4-methylenedioxyamphetamine (MDA)



Figure 1-26: Enantiomers of 3,4-methylenedioxyamphetamine (MDA).

3,4-Methylenedioxyamphetamine (MDA) or (1-(3,4-methylenedioxyphenyl)propan-2-amine), Figure 1-26, is a synthetic drug as it does not occur naturally. MDA was first synthesised in 1910 by Mannish and Jacobson [109]. MDA had been trialled for several medical uses including its use as a cough suppressant and for the treatment for depression and Parkinson's disease [110]. MDA was not controlled until the 70's. Today there is no accepted medical use for MDA [105].

MDA has been linked to several deaths [111] with the main cause being as a result of cardiac effects and subsequent stroke [112].

#### 1.1.4.1 Methods for Manufacturing MDA

MDA can be synthesised similarly to MDMA via the methods listed in section 1.1.3.1 with minor adjustments.

**Sensitive information removed. Please see references [113] and [114] for information.**

### 1.1.5 Para-methoxyamphetamine (PMA) and Para-methoxymethamphetamine (PMMA)

S(+)-PMA R(-)-PMA S(+)-PMMA R(-)-PMMA

Figure 1-28: Enantiomers of para-methoxyamphetamine (PMA) and para-methoxymethamphetamine (PMMA).

1-(4-methoxyphenyl)propan-2-amine or para-methoxyamphetamine is a 'designer drug' which was first reportedly available in May 1970. PMA has been associated with numerous user deaths [116, 117] and was prevalent in Australia during the 1990's commonly being sold as "ecstasy"

PMA can be found naturally as it is suggested to be found in trace amounts in a few plant species including *acacia* and *browningia candelaris* [118, 119]. PMA is usually illicitly

produced from either anethole (1-methoxy-4-(1-propenyl)benzene) or anisaldehyde (4-methoxybenzadehyde). Due to its presence in star anise, anise and fennel essential oils, anethole is more commonly used then anisaldehyde [120].

#### 1.1.5.1 Methods for Manufacturing Para-Methoxyamphetamine (PMA) and Para-Methoxymethamphetamine (PMMA)

PMA is commonly believed to be a route specific impurity for the production of MDMA from isosafrole due to the fact that anethole, the main precursor to PMA, can be present in sassafras oil [101]. The anethole present in sassafras oil would, following peracid oxidation to the diol and a pinacol-type rearrangement under acidic conditions, be converted to PMP2P as depicted in Figure 1-29 [101]. This PMP2P can then be converted to PMA and PMMA analogous to the Leuckart production of MDMA from isosafrole via MDP2P [121].



Figure 1-29: Production of PMP2P via peracid oxidation of anethole.

It has been reported by Waumans et al. that anethole can undergo oxidative cleavage of the propenyl double bond to yield 4-methoxybenzaldehyde which can then undergo a Baeyer-Villiger rearrangement with the peracid to produce O-formyl-4-methoxyphenol which can be hydrolysed to yield 4-methoxyphenol as depicted in Figure 1-30 below [122]. It is believed that 4-methoxyphenol is a route specific impurity from the peroxide oxidation of anethole to PMP2P [122].



Figure 1-30: Formation of methoxyphenol from the Baeyer-Villiger rearrangement of anisaldehyde with performic acid [121].

Alternatively, PMA can be synthesised from 4-methoxybenzaldehyde as depicted in Figure 1-31 below. In addition 4-methoxybenzaldehyde can also be converted to PMP2P via reduction of the intermediate, 1-methoxy-4-(2-nitroprop-1-enyl)benzene, by elemental iron [123] analogous to the nitrostyrene synthesis mentioned earlier in section 1.1.2.1 Nitrostyrene reduction.

Figure 1-31: Production of PMA via 4-methoxybenzaldehyde. Note: LiAlH4 can be substituted for hydrogenation on a nickel or palladium catalyst to create para-methoxyamphetamine (PMA).

### 1.1.6 Differentiating Between Manufacturing Methods

Differentiation between the various forms of illicit drug manufacturing methods and precursors used is extremely important in providing information as to whether several samples of an illicit drug came from the same source. Several methods are generally employed in analysing samples found in clandestine laboratories. These methods are typically required to give fast and accurate results so that the health of investigators entering clandestine laboratories is not affected [124]. If an illicit substance and/or its precursors are found, further analysis and confirmation is generally conducted using other techniques. The most common methods employed include: liquid chromatography mass spectrometry (LC-MS), gas chromatography mass spectrometry (GC-MS) and capillary electrophoresis (CE).

Impurity profiling is also an extremely important area concerning differentiation of illicit drug samples. Unlike pharmaceuticals illicit drugs produced in clandestine laboratories contain a significant amount of impurities and contaminants. These impurities include, pre-cursors, intermediates, by-products, degradation products and adulterants and are often more toxic then the illicit drug being produced [125]. In some cases, the impurities can be present in a higher concentration than the illicit drug itself. Adulterants are often added to either enhance the effects of the illicit substance or to make the drug more profitable by acting as diluting agent [126, 127]. Impurity profiling has been employed to differentiate between manufacturing methods and sources [98, 128].

#### *1.1.6.1 Liquid Chromatography Mass Spectrometry (LC-MS)*

LC-MS is commonly used to analyse substances from clandestine seizures for impurity profiling [104]. Additionally toxicological samples including plasma [129] and urine [130] have been investigated. However, other techniques including GC-MS are used much more commonly for analysing clandestine laboratory reaction mixtures. See section 4.2 for the theory behind liquid chromatography mass spectrometry.

#### 1.1.6.2 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS is the most common confirmative technique used when investigating suspect chemicals found during clandestine laboratory raids [97, 131]. It has also been readily used in analysing toxicology samples including extracts from urine [132], hair [133] and plasma [134]. In addition to looking at methamphetamine concentrations, GC-MS has regularly been employed to determine reaction by-products in the synthesis of illicit substances to aid in impurity profiling [98, 102]. GC-MS has an advantage over LC-MS due to the fact that it does not suffer from matrix ionisation effects [135].

A disadvantage of GC-MS in analysis of ATS is that artefacts can be created, due to the high temperatures in the injection port and column. One example is methamphetamine which has been found when analysing urine samples which contain excessive amounts of pseudoephedrine and ephedrine and for this reason care must be taken when interpreting results in which these substances are present [136]. See section 4.2 for the theory behind gas chromatography mass spectrometry.

## 1.2 Clandestine Laboratories

Many illicit drugs, including amphetamine type stimulants (ATS) (section 1.1), were initially readily available through legal channels [8]. One example is amphetamine which was widely prescribed in the mid 1900's for depression and to help with obesity [69]. Nowadays illicit drugs and precursors to these illicit substances are produced in clandestine drug laboratories [60].

In addition to the health risks associated with the synthesis of ATS, an increase in crime rate could potentially be seen due to the fact that a high level of illicit drug use has been linked with those having contact with the criminal justice system [137] (According to the Australian institute of health and welfare (AIHW) approximately 70 % of the Australian prison entrants surveyed in 2012 (n = 794) reported a history of illicit drug use in the past 12 months a proportion which is significantly higher than the general population [138]).

These laboratories vary greatly and can be anywhere from crude small batch operations, generally ran by users themselves, to large highly sophisticated setups [139]. Independent to the size of the clandestine laboratory, the substances used and produced in clandestine laboratories have significant health risks to the public [31, 140]. As well as being an explosive risk, many of the chemicals used are toxic and volatile and can contaminate the surrounding water, soil and air [41]. These health risks are heightened as an increased number of people

are potentially being exposed to the chemicals due to the fact that the majority of clandestine laboratories are consistently found in residential areas [31] (68.5 % in both 2010-11 and 2015-2016 [31, 141]). Although this number fluctuates, it seems to remain reasonably steady with residential areas being the most common location of detected clandestine laboratories with 67.8 % of clandestine laboratories in 2013-2014 being located in residential areas [139].

**Image removed due to copyright restriction. Please see reference for original image [141].**

Transportable clandestine laboratories have also been discovered. These labs were created to try and avoid detection as the smell given off from the process can easily be identified in residential areas [45]. As an indication of the extent of mobile laboratories in Australia during 2011-12 8.5 % of clandestine laboratory detections were located in a vehicle [6]. This number also appears to be increasing with 12.5 % of clandestine detections occurring in vehicles throughout 2016-17 [142]. Transportable clan labs can readily be moved and unpacked to synthesise illicit substances and are also occasionally set up to manufacture the drug while the lab is travelling [143]. In addition, the toxic residue can be disposed of on the roadside limiting detection and resulting in the likelihood of it entering storm water drains.

Amphetamine-type stimulants are the most common illicit substances synthesised in clandestine laboratories worldwide [144]. The majority of chemicals required for the synthesis or refinement of ATS have legitimate industrial uses and for this reason it can be difficult for policing personnel in control of stopping the deviation from legal use of these substances to illicit drug synthesis [145].

In relation to methamphetamine synthesis, many of the required chemicals and apparatus can be simply acquired through legal sources including: cold and flu medications, lithium batteries, battery acid, pool acid, drain cleaner, distress flares, iodine tincture, matches, lighter fluid, paint thinners and propane [146]. In some cases, due to limitations in their availability, restricted precursor materials are also manufactured in illegal laboratories [63].

Regulating illicit substance precursor availability has been a key strategy to reducing clandestine manufacture in the United States, Australia and Europe [147]. In Australia, 'Project STOP' directed at minimising the illegal use of pseudoephedrine containing pharmaceutical products in the synthesis of methamphetamine, was introduced in August 2007 [148]. As of 30 June 2012, 79.2 % of pharmacies had registered with project STOP [6] however this percentage decreased to 75.4 % by the end of the 2014-15 financial year. These restrictions have influenced clandestine manufacturers to find alternate methods of production and precursors [31]. The success of precursor regulation for methamphetamine has been investigated by McKetin et al. who conducted a systematic review of studies which looked at the effect of precursor restrictions on the supply and use of methamphetamine [149]. Unsurprisingly, this study confirmed that regulations on precursor materials can reduce supply of methamphetamine, shown by indicators such as an increase in price and decrease in both detections and use shown by a decline in the number of arrests, hospital admissions and toxicological reports.

The relatively low cost of producing amphetamine-type stimulants compared to their resale value makes illicit synthesis an attractive business proposition for many users and suppliers [150]. It has been estimated that AU$1000 worth of methamphetamine costs approximately AU $100 to manufacture [151]. In addition, to their relatively low-cost amphetamine-type stimulants can often be synthesised rapidly with minimal supplies and equipment, making illicit drug production alluring to untrained chemists. The majority of clandestine drug manufacturers are self-educated with less than 5 % of 'cooks' believed to have formal training in synthetic chemistry [26]. Clandestine manufacturers seem to gain knowledge about new sources of precursors and recipes of manufacturing both precursor materials and illicit drugs from the plethora of sources available on the internet, including some underground literature by people such as "Uncle Fester" and "Strike"[152]. According to Wright et al. who interviewed 21 individuals in Australia who were convicted with manufacturing ATS the most

common method for gaining information on clandestine synthesis is via direct teaching processes present within these networks [153] [154].

The 2011 World Drug Report, reported a global increase in clandestine laboratory detections over the previous two decades [144]. Not only has a global increase occurred, a significant rise in the findings of clandestine laboratories has also eventuated in Australia [6]. McKetin et al. reported in 2004 that there had been an increase in both the supply and consumption of methamphetamine between 1997 – 2002 [137]. The number of detections of clandestine laboratories increased rapidly in 2010-11 with the Australian Crime Commission (ACC) reporting a rise of 221 % in the number of detections since the 2001-02 financial year [31]. However, after peaking in 2011-12 this number has now appeared to have reduced Figure 1-33.

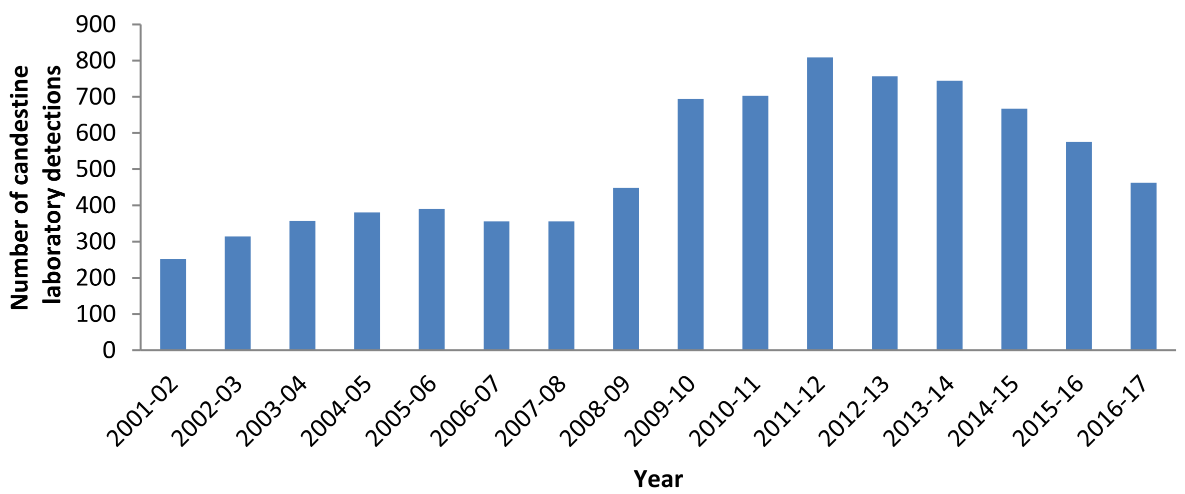



Figure 1-33: Number of clandestine lab detections in Australia 2001-02 – 2016-2017 adapted from [6, 11, 31, 139, 141, 142, 155]

According to the United Nations Office on Drug and Crime (UNODC) approximately 90% of ATS seized in Australia is locally produced, however it is believed that for crystal methamphetamine a higher portion is imported [14, 156]. On the other hand, border seizures suggest that methamphetamine manufactures in South East Asia may be competing with local methamphetamine 'cooks' [14]. During 2011–12, the number and weight of amphetamine-type stimulant precursor detections at the Australian border increased, however this is also when the number of clandestine laboratory detections in Australia was at its peak. In 2010-11 to 2011–12 it was reported that the number of detections of ATS increased by 27.8 % (from 733 to 937 [6]). Not only were the number of detections increasing, but the size of the detections also significantly increased. According to the ACC a substantial increase in mass of

124 % was seen in 2011-12 compared to the previous year (from 781 kg to 1745 kg) [6]. Although the amount of MDMA related detections seems to be relatively low compared to other ATS there seems to be significant fluctuations in the number of border detections and a significant decrease of ~ 90 % of MDMA precursor material was reported in 2011-2012 (240 L) compared to the previous year (2570 L) [6].

### 1.2.1 Clandestine Waste

Not only are the chemicals used in clandestine synthesis toxic, several of the by-products are also toxic. One example from the synthesis of methamphetamine using the red phosphorous method is the production of phosphine gas. Phosphine has been reported to have caused fatalities and injuries from exposure to clandestine labs [157].

Even after detection and removal of a clandestine laboratory the site remains to be a threat to health due to residual hazardous chemicals. These chemical residues are deposited on and around the site in which synthesis, storage and disposal of clandestine chemicals and wastes occurred. The remediation cost of clandestine laboratories is often more expensive than the properties themselves [26].

It is estimated that for every gram of amphetamine-type stimulants manufactured, approximately 6 grams of waste material is produced [158, 159]. This waste is generally discarded in an attempt to limit detection. Due to the several adverse health effects associated with chemicals involved in clandestine synthesis the improper disposal of clandestine waste can have a devastating impact on the environment. There are several ways in which clandestine waste is disposed of including: burying, pouring down a drain, in landfill, along roadside and into rivers and lakes.

According to interview data by Wright et al., who interviewed 21 individuals who were convicted of the manufacture of ATS (14 from SA and 7 from WA) focusing on the type of drug manufactured, method used, location of cook and disposal of waste, the most common waste disposal method was found to be via drainage, followed by via the toilet and dumping. It has been noted however, that the interview data may be skewed as those known to be associated with larger criminal organisations declined to participate. For comparison, 15 police members or forensic investigators involved with ATS drug laboratories were asked about the observed locations of waste disposal. From their responses, the most common disposal method was in the ground or in a pit, however it was noticed that the next most common method was down

a drain outside and that it could be difficult to determine if waste had been disposed of via drainage and would only be noted if specific evidence was present at the drain [154]. The disposal of clandestine waste via drainage or down a toilet is likely to result in its appearance in our municipal wastewater system.

## 1.3 Wastewater Analysis

The presence of substances in wastewater as a result from industry and agriculture has been a key focus of environmental scientists for quite some time. These studies generally focus on the risks of the substances to the human population, this is of particular interest when the treated wastewater is being reused. Despite interest in other compounds, research on the presence and fate on licit and illicit drugs in wastewater has been minimal until recent times. The first discovery of an anthropogenic drug in wastewater is believed to have occurred soon after Garrison et al. reported the presence of clofibric acid, a bio-active metabolite of numerous serum triglyceride lowering drugs, in groundwater which was replenished with treated wastewater in 1976 [160]. Later in 1977 Hignite et al. reported finding salicylic acid, a metabolite of aspirin, in the effluent of a Missouri wastewater treatment plant [161]. Following this the number of research papers reporting the presence of compounds in wastewater as a result of human consumption was limited for around a decade.

### 1.3.1 Analysis of Wastewater for Illicit Drug Consumption Estimates

Illicit drugs are emerging pollutants in the aquatic environment and several studies have found illicit substances as well as pharmaceuticals to be present in waste and surface water [125, 162-164]. There are various routes in which illicit drugs can be introduced into wastewater, some possible ways include: dumping of illicit substances, washing of equipment in contact with illicit substances and excretion as a result of drug use [165].

The use of wastewater to gain consumption estimates was first suggested by Daughton et al. in 2001 [166, 167] who believed that pooled samples of urine provide a good representation of the population served by a wastewater treatment facility. The first report in peer reviewed literature relating to the presence of illicit substances in treated wastewater is believed to have occurred in the United States in 2004 [168]. This study conducted by Jones-Lepp et al. detected methamphetamine and MDMA at low ng/L levels in the effluent of wastewater treatment plants in Nevada and South Carolina [169].

The use of wastewater as a means to gather consumption estimates of illicit substances was first reported in 2005 by Zuccato et. al. [170]. This study attempted to approximate the

amount of cocaine consumption in Italy by analysing the amount of cocaine and its primary metabolite benzoylecgonine in wastewater [170]. Since then several studies have looked at analysing wastewater to approximate the amount of illicit drug consumption in various parts of the world [165, 171-175].

Wastewater analysis is gaining recognition both internationally and in Australia as being the most effective and only objective means of reliably measuring the level of use of a number of illicit substances. This is one of the reasons as to why a 3 year national pilot wastewater analysis program to be undertaken by Australian Criminal Intelligence Commission (ACIC) has received funding from proceeds of crime with the main focus being on methamphetamine [176].

#### *1.3.1.1 Illicit Drug Consumption Estimates from Wastewater Analysis*

To gain consumption estimates for illicit substances in wastewater the mass loads of each substance needs to be known. This mass load is generally obtained by utilising liquid chromatography tandem mass spectrometry (LC-MS/MS). As wastewater is a complicated matrix, prior to analysis a lot of sample preparation needs to be performed. This sample preparation includes: filtration, removal of impurities and pre-concentration.

Once a wastewater sample is collected a preservative is generally added to prevent degradation of the analytes. Filtration is also necessary to remove suspended particulate matter from the wastewater. This filtration step has been shown by Chen et al. not to affect the recovery of some illicit substances including amphetamine-type stimulants [177], however this study only investigated one type of filter (Whatman GF/A glass microfiber 1.2 µm).

In addition to removal of solids, a pre-concentration step is generally employed due to the fact that most illicit drugs and their metabolites are present at extremely low levels in wastewater (ng/L range) [178]. The most common pre-concentration method employed is solid phase extraction (SPE) [179]. This extraction method can be performed on-line where the SPE is automated before the chromatography run, [180, 181] or off-line, where the SPE is conducted manually [179, 182, 183]. This pre-concentration step may not be required depending on the analysis technique used and drug under investigation. One study which omitted this pre-concentration step was conducted by Lai et al. [184]. This study utilised ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

Along with increasing the concentration of the analytes, SPE also assists in removing some matrix components which can compete with the target analytes in the ionisation process in LC–MS analysis. This removal of matrix components helps lower the limit of detection [164]. The most common sorbents in the literature for pre-concentration of illicit substances in wastewater are: Oasis HLB and Oasis MCX [178, 185-188].

After extraction/pre-concentration the method employed for analysis seems to be standardised in the fact that LC-MS with electrospray ionisation (ESI) is used (occasional study utilises GC-MS [135] however due to the longer sample preparation time for derivatisation LC-MS is preferred). The type of column employed can vary from a reverse phase (RP) to a hydrophilic interaction (HILIC) column depending on the drugs under investigation [168, 179, 182, 183, 188-191]. When analysing for amphetamine-type stimulants a HILIC column is preferred due to the poor retention on a RP column [183, 188-191]. One study conducted by Kasprzyk-Hordern et al. looked at the use of chiral cellobiohydrolase (CBH) UPLC column to separate enantiomers and diastereomers of drugs of abuse (DoA) in wastewater [192]. This study not only looked at the enantiomeric purity/ diastereomeric ratio of the drugs of abuse in water to gain information about their source but also their fate throughout the treatment process.

The ionisation mode employed for the majority of illicit drugs analysed tends to be operating in the positive ESI mode, however some illicit metabolites such as cannabinoids utilise a negative ESI [178]. It has been noted that due to strong matrix effects, which can lead to suppression and enhancement of the ionisation signal, there is a need for using deuterated standards for quantification to account for any matrix suppression or enhancement [182]. For this reason, deuterated internal standards are generally added to the wastewater sample prior to filtration and extraction.

The most common mass analyser utilised in wastewater analysis seems to be a triple quadruple (QqQ) mass analyser. A triple quadrupole system is preferred for analysing complex matrices such as wastewater due to its high sensitivity and selectivity [193]. The use of high-resolution mass spectrometry (HRMS) has in recent times gained interest in wastewater analysis, as unlike multiple reaction monitoring (MRM) used in QqQ, HRMS has the ability to be used as a screening tool due to being a non-targeted analysis. The use of a linear ion trap orbitrap mass analyser for quantitative analysis of illicit substances in

wastewater was investigated for the first time by Bijlsma et al. in 2013 [194]. The results showed that the analyser had comparable sensitivity to a triple quadruple mass analyser however HRMS such as orbitrap instruments are capable of performing both targeted and non-targeted analysis making it more alluring. The ability to perform a non-targeted screen along with a targeted approach is preferred as with targeted analysis you only detect what you are looking for, whereas, non-targeted approach you will detect other chemicals present. With the use of full scan experiments the confirmation becomes difficult particularly when there are a series of possible candidates with the same m/z. For this case a reference standard would be required, for confirmation, however, an issue can arise due to the lack of reference standard availability. The use of predicted retention times ($t_R$) for confirmation, along with a semi-targeted approach for illicit substances, pharmaceuticals and personal care products (PPCPs) in both river and wastewater has been conducted by Munro et al. using HRMS [195]. This work used an artificial neural network along with the: pKa, partition and distribution coefficient, number of oxygen atoms, rings, double and triple bonds within the structure to predict the retention times. These retention times along with the mass-to-charge ratio of the parent and fragments can be used for confirmation. One drawback by not using a targeted approach is that a significant amount of time is needed to process the extremely large datasets produced.

#### 1.3.1.2 Consumption estimate back calculations

Estimating drug consumption from wastewater is conducted by either: analysing wastewater for the parent drug itself [168] or for its primary metabolites [165, 183, 184, 196, 197]. These processes utilise knowledge of the flow rate of the wastewater treatment plant, the percentage of the drug or metabolite excreted in urine and their molecular masses to gain an accurate estimation for the amount of drug consumed. The equation that is most commonly employed when the drug target residue (DTR) is the primary metabolite is given below (Eq. 1-2) [178, 179]:

$$Drug\ load\left(\frac{ng}{day}\right) = \frac{M_{(Drug)} \times C_{(primary\ metabolite)}\left(\frac{ng}{L}\right) \times Flowrate\left(\frac{L}{day}\right)}{M_{(metabolite)} \times Excretion\ ratio_{ave}} \qquad \text{Eq. 1-2}$$

The equation above can be altered to give the version utilised when the drug target residue is the primary drug itself by removing the molecular mass ratio. The drug target residue used depends on several factors which include the: excretion ratio, stability, amount present and limit of quantification. In all published work to date the drug target residue employed for the

consumption estimates of ATS is the primary drug itself. It has been shown that analysing the concentration of the parent drug may lead to overestimates for the amount consumed [178]. This overestimate arises from alternate routes of entry into wastewater primarily being as a result of dumping of an illicit drug for this reason, where appropriate, analysis of metabolites is the preferred method employed. In addition, results obtained from this method seem to be in good agreement with results from population surveys [173, 198]. Analysis of wastewater has the advantage over population surveys as it can gather information on population drug in a timely and inexpensive manner, as well as minimising the chance of bias as it is not reliant on information obtained by the consumers themselves. With the recent, constant development of new designer drugs the consumer may be unaware or mislead about the drug they are consuming making population surveys less reliable. In addition, there is less ethical issues involved as the samples are all pooled in the wastewater treatment plant and as a result the identity of the consumers are kept anonymous [199]. However, care must still be taken in reporting results particularly when the population served by the wastewater treatment plant is low [199].

The original equation for drug load in wastewater first proposed by Zuccato, has since been altered to account for the stability and sorption of the drug target residue [200, 201]. This alteration was first reported by Baker et al. in 2013 and is provided below in Eq. 1-3, where *stability* is the percentage change in the concentration of the drug target residue and *sorption* is the percentage of the DTR adsorbed to the suspended particulate matter [200, 201].

$$Drug\ load_{refined}\left(\frac{ng}{day}\right) = Drug\ load\left(\frac{ng}{day}\right) \times \left(\frac{100}{100 + stability}\right) \times \left(\frac{100}{100 - sorption}\right) \quad \text{Eq. 1-3}$$

The amount of drug consumed by the inhabitants of the area served by the wastewater treatment plant is generally reported as the number of doses per day per 1000 inhabitants. This is done by dividing the mass load of the drug by the average dose and the population served by the wastewater treatment facility in thousands Eq. 1-4 [165]. This normalisation of results to population is required for comparison of estimates from differing locations with differing populations.

$$consumption\left(\frac{doses}{day.1000\ inhibitants}\right) = \frac{Drug\ load\left(\frac{ng}{day}\right)}{m(dose)_{ave.} \times population\ served} \qquad \text{Eq. 1-4}$$

#### 1.3.1.3 Uncertainties Associated with Wastewater Analysis

Care must be taken when approximating the amount of illicit substances consumed using the equations mentioned above. Uncertainties can arise from various sources including the sample method employed and variation in the: flow rate of the wastewater treatment facility, excretion ratio of the illicit substance, contributing population served and the average dosage size.

The sampling method employed is crucial to limit uncertainties in results. The most accepted and representative method is using a peristaltic sample pump that collects samples proportional to the flow rate of the wastewater treatment plant [179, 202]. This sampling method helps limit the effect of the variability in the flow rate.

The flow rate of the wastewater treatment facility is typically non-uniform hour-to-hour and day-to-day [203] as is the number of inhabitants served by the wastewater treatment facility. This change in population can occur both as a result of local population growth in developing areas and as a result of events. One such event where the population changes significantly in a given area is schoolies, in which a significant increase in population of areas such as Victor Harbour and the Gold Coast is observed (In 2013 40,000 schoolies or school leavers were in the Gold Coast during the event).

Variation in population served by the wastewater treatment plant can be corrected for by use of biomarkers present in the wastewater. One attempt to correct for population variance was conducted by Van Nuijs et. al which looked at the level of nutrients in wastewater. This study monitored the concentration of phosphorous, nitrogen and the chemical and biological oxygen demand. The concentrations were then used to back calculate estimates for the population served based on the information that one inhabitant accounts for approximately 1.7 g/day of phosphorous, 12.5 g/day nitrogen, 59 g/day biological oxygen demand (BOD) and 128 g/day of chemical oxygen demand (COD) [183]. The use of COD, BOD, nitrogen and phosphorous is undesired as they are not human specific and can be altered by industry and food waste [204] as well as requiring additional analytical analysis and suffering from seasonal variation.

Most commonly the use of pharmaceuticals, licit drugs and their metabolites are used as biomarkers as they are more specific and a result of human activity [179, 205-207]. However, the concentrations of these biomarkers are likely to vary significantly in differing regions and in some cases may not be present at all. For this reason the use of biomarkers from human endogenous biochemical processes has also emerged. The use of creatinine, a breakdown substance of creatine, a compound found in muscle tissue, as a biomarker was investigated by Brewer et al. [205]. Creatinine was selected due to the large information known about the concentration excreted in urine and its variance with ethnicity and age. The use of creatinine to normalise the mass loads of illicit substances resulted in a significant difference in the trends observed, which shows the importance of these population biomarkers. The viability of creatinine as a population biomarker was investigated along with other potential biomarkers by Chen et al in 2013 [206]. Creatinine, despite being useful in fresh urine samples, was found to be of limited use as it is unstable in untreated wastewater and degraded almost completely within 24 hours. Chen et al. also suggested the use of 5-hydroxyindoleacetic acid (5-HIAA), the primary metabolite of serotonin, as a population biomarker viable for international comparison. The use of single-substance approach to estimating de facto population can have limitations and variability, for this reason a multi-substance approach was developed by O'Brein et al. which used census data along with 14 chemicals and Bayesian inference to validate estimates for population size [208]. Data from mobile cellular network signals has also been used to account for population activity [209]. This study showed that population estimates can account for uncertainties of up to 55 % of the per capita normalised drug load when static population is used as opposed to a dynamic population. The uncertainty in the population served by the wastewater treatment facility is believed to be the largest source of uncertainty in reported data.

The excretion ratio can vary from person to person and as a result may not be representative of a typical drug user as the study group is generally a small sample of healthy volunteers [210]. In addition, as these studies are generally relatively old it can be extremely difficult to obtain values for new and emerging illicit substances. The excretion ratio not only varies from person to person but is also highly dependent on the route of administration of the illicit substance. To account for this variance weighted averages are commonly employed to gain more reliable estimates [211]. The equation used to calculate the weighted excretion ratio is

given below (Eq. 1-5) where $n$ is the number of routes in which an illicit substance is administered.

$$Excretion\ Ratio_{ave} = \sum_{i=1}^{n} \%\ adminstered\ via\ route_i\ \times Excretion\ ratio_i \quad \text{Eq. 1-5}$$

The measured concentration of the targeted drug can be affected by release or uptake of water to the wastewater system. This can result from damage to the wastewater network along with the porosity of the pipes used. This is particularly noticeable during times of high rainfall when the flowrate of the wastewater treatment facility is much higher.

Reporting the amount of illicit substances consumed in number of doses compared to mass loads can also increase the uncertainty as the purity of the substance can differ widely between batches and as a result the average dose can vary significantly with time.

The analyte selection is extremely important as several factors need to be considered which include the stability of the metabolite in wastewater and potential alternative sources (some licit drugs produce the same metabolites as illicit drugs [179, 212]). This stability can be affected by sample treatment, transport and storage conditions.

One common method used to limit biodegradation is to use hydrochloric acid to lower the pH of wastewater samples to, most commonly to pH 2 , to kill bacteria and prevent degradation of compounds [170, 183, 211, 213, 214]. One drawback to this however is that it can also assist in the degradation of compounds which undergo acid catalysed hydrolysis as mentioned later. Alternatively the addition of preservatives such as sodium metabisulphite has been used to prevent degradation [93].

Appropriate transportation and storage are essential to ensure that transformation/degradation of the target analytes is minimal. It has been reported that for a large majority of illicit substances including amphetamine-type stimulants storage of samples at 4 °C is sufficient (however storage at -19 °C is preferred) to stabilise the analytes for at least 2 weeks, and refrigeration is not required for sample transportation if less than 3 days [177].

Adsorption of the analytes to solid particulate matter (SPM) is likely to result in the concentration in the filtered wastewater samples being lower than that present in the wastewater [215]. Since SPM can be incinerated or introduced to the environment by being

spread on agricultural land the content of illicit substances in SPM is important to understand. However, it has been reported by Chen et al. that the binding of amphetamine-type stimulants to suspended solids in wastewater was minimal. This was conducted by comparing the analyte recoveries of filtered and non-filtered wastewater samples [177]. The percentage adsorbed onto solid particulate matter was also reported for amphetamine-type stimulants by Baker et al. and was reported not to be an important factor affecting the uncertainty (Methamphetamine 2.3 %, MDMA 1.6 % and amphetamine 3.1 %) [214-216]. Chiral analysis of SPM along with the wastewater is not generally undertaken and was first investigated by Evans et al. [217]. The results of this study showed that the enantiomeric fraction of chiral drug target residues in wastewater was different to that of the SPM, for this reason great care must be taken particularly when using the ratio of enantiomers in wastewater to predict possible dumping events of illicit substances.

#### 1.3.1.4 Disadvantages with wastewater analysis

Along with the uncertainties described above in section 1.3.1.3, wastewater analysis cannot provide all the information available through population surveys, crime statistics and hospital admissions. This includes information about the individual consumers of illicit substances such as the age, sex, and ethnicity of the drug user along with details about dosage taken, whether taken alone or combined with other drugs and frequency of which a drug is taken.

Some information may be given about the route of administration of a limited number of illicit substances, due to differing routes providing differing metabolites [191, 200, 218]. For this reason, the combination of wastewater analysis with population surveys and other sources of information such as hospital admissions and crime statistics is encouraged.

#### 1.3.1.5 Current results from wastewater analysis

The current results from studies utilising wastewater for consumption estimates of amphetamine-type stimulants show that there is generally a higher amount consumed over the weekend compared to weekdays [162, 165, 172, 173, 196]. This may suggest that the majority of users in fact are not regular and chronic users but recreational users. Intra daily trends have also been analysed by collecting samples over a shorter time period [211]. These results indicate that there seems to be peak drug entry into the municipal waste system during the morning (generally 7 am-9 am, but varies on illicit substance [205]) and at night (7 pm-12 am) [211].

The verification of enantiomeric fractions of chiral drugs including amphetamine, methamphetamine and MDMA in wastewater has also been conducted as it provides important information regarding patterns of drug usage and can assist with the differentiation between their legal and illegal use [219]. In addition, the use of enantiomeric fractions can be used to determine which illicit substance had been consumed. One example is the presence of MDA in wastewater which can be a result of the metabolism of MDMA or MDA consumption [220]. Since MDMA is metabolised primarily to S(+)-MDA and consumption of MDA results in higher amounts of the R(-) enantiomer, due to preferential metabolism of the S(+) enantiomer, the enrichment of the S(+) enantiomer would indicate the consumption of MDMA while the enrichment of the R(-) enantiomer would indicate the consumption of MDA [88]. Enantiomeric fractions have also been utilised to link the sources of methamphetamine in wastewater to its synthesis method [221] and to attempt to determine possible dumping events. One such study conducted by Emke et al looked at the enantiomeric fraction of MDMA Dutch wastewater treatment plants [222]. In this study, there was a couple of instances where the enantiomeric fraction was almost racemic (EF = 0.51) which is significantly lower than the mean (EF = 0.68 ± 0.4) which was associated to direct disposal of MDMA.

An alternate method for determining possible dumping events was first conducted by Lai et al. This study utilised the variation in the ratio of the parent drug and metabolite and comparing this ratio to the excretion ratio. This dumping can also be a result of washing implements used during the handling the illicit material [205, 211].

The spatial distribution of drugs of abuse has also been investigated by wastewater analysis. One such study conducted by Banta-Green et al. investigated the spatial distribution of illicit drug use by comparing the loads of different illicit substances from 96 wastewater treatment facilities of differing sizes in Oregon [223]. The results of this study found that the mass index loads of methamphetamine had little to no difference with urbanicity, whilst ecstasy was present in significantly higher levels in urban areas. This spatial distribution of illicit substances has also been found in Australia, one study conducted by Davey et al. in 2014 looked at the prevalence of methamphetamine, cannabis and MDMA in road side drug testing in Queensland and observed strong regional variations [224].

Wastewater analysis has not only been conducted in large populations, but has also been used to obtain information on drug use in colleges/universities [7, 160, 225], hospitals [62,

160], prisons[226, 227], sporting events [228] and even fitness centres [229, 230]. One study conducted by Boles et al. looked at the use of a polar organic chemical integrative sampler (POCIS) upstream from the wastewater treatment plant [231]. This study looked at placing POCIS's at the junctions between private and city owned sewer lines of three buildings suspected to harbour illegal drug activities. Despite being unable to determine quantitatively the methamphetamine concentration present (due to several unknown variables) this work provided proof of concept of the use of POCIS as sampling devices by qualitatively detecting methamphetamine and shows the potential of POCIS's to be used as a forensic tool in law enforcement.

Wastewater analysis has also been investigated to obtain the removal efficiencies of illicit substances for differing wastewater treatment processes [196]. Little is known about the aquatic ecotoxicity of illicit substances and their environmental fate and release into the aquatic environment may provide long-term effects due to exposure. The efficiency of wastewater treatment facilities is typically calculated by monitoring the drug concentrations at both the influent ($C_I$) and effluent ($C_E$) of the wastewater treatment plant and taking into account the residence time ($t_r$) in the wastewater treatment plant [232] and is calculated using Eq. 1-6 below, where $t$ is the time of collection of the influent wastewater. One study conducted by Bijlsma et al. investigated the removal efficiency of several sewage treatment facilities in Spain at a time of high drug usage (during the time of a pop/rock concert) [233]. This study found that the removal efficiency at the time of the festival was significantly lower than that during a 'regular' week this was believed to be a result in the increase in population and consumption of illicit material.

$$E = \left(1 - \left(\frac{C_{E_{(t+t_r)}}}{C_{I_{(t)}}}\right)\right) \times 100\ \% \qquad \text{Eq. 1-6}$$

It has also been found that depending on the removal efficiency of the wastewater treatment plant these illicit drugs can still be present in the effluent [234, 235] and as a result can reach and contaminate surface water [234, 236]. If these toxic samples contaminate surface water their appearance in drinking water could result. For this reason illicit substances are emerging pollutants in our water systems and have the ability to bio-accumulate, persist in the environment, and due to their toxicity can negatively affect the quality of water [237]. The accumulation of ATS in water can have a significant impact on health as currently there are

no regulations in place regarding the occurrence of these chemicals in treated wastewater and receiving waters [238].

### 1.3.2 Analysis of Wastewater for Clandestine Drug Precursors and By-products

To date there is no published research linking the differentiation of clandestine methods to wastewater analysis. As clandestine waste is typically disposed of via drainage the detection of starting materials, route specific impurities, intermediates or by-products in wastewater may have the potential to provide "real-time" knowledge about the synthesis techniques employed in a given area.

Apart from the amphetamine type stimulants and pseudo/ephedrine, which are drugs of abuse in themselves, there seem to be little to no analysis of wastewater for other chemicals likely to be present from disposal of ATS synthesis in published literature. One possible reasoning to this lack of knowledge may be the fact that the detection of these substances is likely to be highly dependent on the batch nature of clandestine synthesis and on the sampling location and time. As there is no continual entry of these compounds into the wastewater system, they are expected to be present at much lower levels than the amphetamines and as a result the sampling method utilised could also significantly impact the detection of these compounds occurring because of the dilution of composite samples.

In addition, there is little to no information is known about the stability of these samples in wastewater. This research aims for the first time to analyse wastewater for compounds related to ATS synthesis, and investigate their stability in wastewater.

### 1.3.3 Selection of Analytes

In addition to the amphetamine-type stimulants under investigation, this study will analyse wastewater for several species involved in the clandestine manufacture of these illicit compounds. This includes precursors, intermediates and by-products of the manufacture of amphetamine-type stimulants. The clandestine substances were selected depending on their likelihood of being present in clandestine waste and whether their presence may have some indication as to which method was used to produce the ATS.

The occurrence of 1-phenylpropan-2-one will also be investigated however this will not provide information as to which method was being employed to manufacture

meth/amphetamine as 1-phenylpropan-2-one is not only a precursor to meth/amphetamine but also a by-product of its production via several of the ephedrine phosphorous routes.

Along with the analytes mentioned above, the occurrence of other impurities and starting materials in wastewater will be explored (Table 1-1). This could give information as to which methods are commonly used to create these amphetamine-type stimulants and possible trends in the methods used based on availability of the precursors and location.

Table 1-1: Targeted compounds for wastewater analysis their structure and use.

| Compound Name | Structure | Use |
|---|---|---|
| Pseudoephedrine | OH, HN | Methamphetamine Precursor |
| Ephedrine | OH, HN | Methamphetamine Precursor |
| Phenylpropan-2-one (P2P) | O | Meth/Amphetamine Precursor |
| Safrole | O, O | Precursor MDMA and MDP2P |
| Isosafrole | O, O | Precursor MDMA and MDP2P |
| Piperonal | O, O, O | Precursor MDP2P |
| Benzaldehyde | O, H | Amphetamine precursor |
| 4-methoxybenzaldehyde (Anisaldehyde) | O, O | Precursor para-methoxyamphetamine |

### 1.3.4 Uncertainties Associated with Using Wastewater for Information on Clandestine Manufacturing

Along with uncertainties seen in all wastewater analysis to date including uncertainties associated with sampling, recoveries and the methods used, several other uncertainties arise when comparing clandestine synthesis data from differing wastewater treatment plants. These additional uncertainties include: reagents may have been added in varying amounts and as a result a higher mass loads will not necessarily be representative of a higher amount

of illicit drug production (methamphetamine 'cooks' may not add reagents in stoichiometric amounts as the majority are not trained chemists), background levels of analytes of interest need to be known, bi-products produced vary greatly with reaction conditions both in amount and identity, waste from industries could potentially affect results, alternate disposal methods and different manufacturing methods including new and unknown methods cannot be accounted for.

In addition, events including music festivals and other proceedings, that may have their own on-site wastewater treatment plant cannot be accounted for and, whereas, consumption may be occurring, illicit production is probably less likely to occur at these events [190].

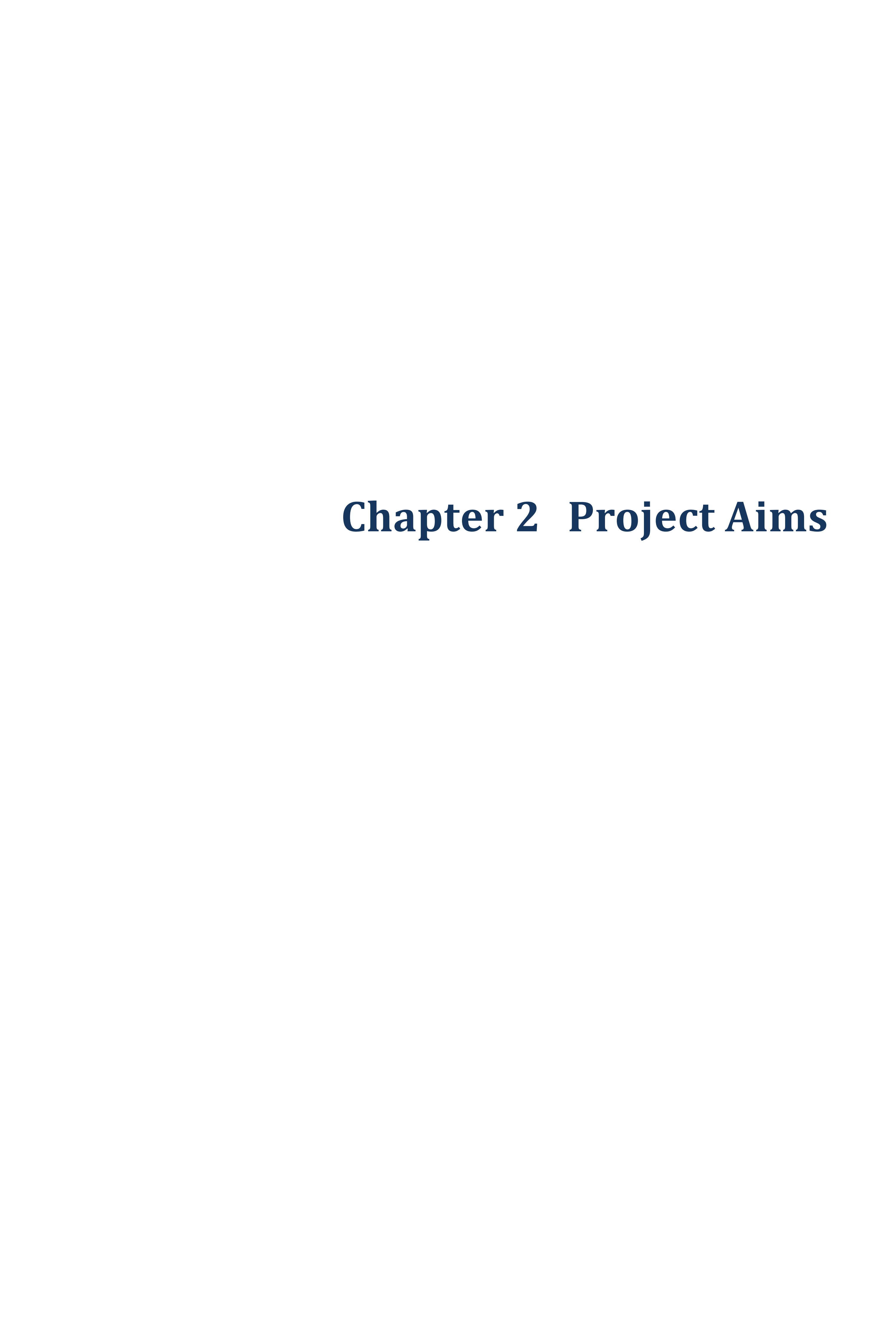
### 1.3.5 Background Levels of Analytes

As mentioned earlier in order to obtain accurate mass loads of the illicit drugs, precursors and by-products under investigation, it is important to know the background levels and various sources of the analytes. The background levels arise from alternate pathways of the chemical reaching the wastewater and include sources such as: waste from industry, excretion from the human diet and run off from land.

Some of the amphetamine type stimulants may be excreted into the wastewater system as a result of consumption of licit pharmaceuticals [239]. One example is the use of selegiline, a chemical used for the treatment of Parkinson's disease, dementia and depression, which metabolises to methamphetamine and amphetamine [111]. For this reason, the presence of amphetamine in wastewater does not imply illicit usage has occurred. In addition, substances such as dexamphetamine are prescribed in Australia to treat attention deficit hyperactive disorder (ADHD), narcolepsy and in rare cases depression and obesity [240]. In order to compensate for this knowledge of the amount prescribed is required. However, as commonly reported it is believed that the contribution to the wastewater system via this route will be negligible compared to the amount released as a result from other methods such as dumping and from illicit consumption [241].

Several of the clandestine synthesis methods utilise chemicals which have legitimate uses and are widely available, some of which are extremely common in the everyday household. For this reason, it is not only important to know the various methods of entry of the illicit substances into wastewater treatment plant, but also the precursors to and by-products from clandestine manufacture. For example, ephedrine and pseudoephedrine are expected to be

present in wastewater not only from their use in clandestine manufacture but also as a result of their use in pharmaceuticals.



# Chapter 2 Project Aims

Since 2005 several studies have emerged which utilise wastewater analysis for gaining consumption estimates for various pharmaceuticals, personal care products and illicit substances including amphetamine type stimulants. However, there has been little to no research conducted investigating the presence of compounds related to the synthesis of amphetamines besides pseudoephedrine in wastewater.

The research presented in this thesis investigates the presence of compounds rated to the production of amphetamine type stimulants in wastewater. The study utilised solid phase extraction along with liquid chromatography mass spectrometry and gas chromatography mass spectrometry to analyse wastewater samples obtained from several Australian wastewater treatment facilities.

As little work has been conducted on the presence of these chemicals in wastewater, their stability in wastewater for the first time was also investigated. The stability was investigated by spiking wastewater samples which were left at two different conditions: at room temperature (T ~ 25 °C) and under storage conditions (pH = 2 HCl adjusted T = - 20 °C). These samples were then extracted and analysed over the period of 35 days and kinetic analysis performed on the degradation of each compound. From this the half-lives were calculated. Adsorption and entrapment of targeted compounds was investigated using humic acid.

The main aim of this project is to investigate the feasibility of wastewater analysis to provide information to forensic investigators in order to help identify which areas and chemicals may be “high risk” and warrant additional surveillance.

The aims of this thesis will be achieved by the following project components:

1. Developing a SPE method for the extraction of: benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzaldehyde, safrole, piperonal, ephedrine, pseudoephedrine, amphetamine and methamphetamine from wastewater.
2. Developing a GCMS and LCMS method for the analysis of wastewater extracts
3. Investigate the stability of benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzaldehyde, safrole and piperonal, both in wastewater at room temperature (~ 25 °C) and storage conditions (pH = 2 HCl adjusted stored at -20 °C).
4. Investigate the loss of compounds due to adsorption and entrapment of humic substances.

5. Investigate the presence of targeted compounds in wastewater obtained from several Australian wastewater treatment facilities.

# Chapter 3 Standards and Samples

## 3.1 Chemicals and Samples

Unless otherwise specified all water besides wastewater samples was produced by a Labconco WaterPro PS water purification system (18.2 MΩ).

All chemicals used are listed in Table 3-1 and the drug standards are listed in Table 3-2

Table 3-1: Chemicals used, supplier and purity.

| Reagent | Supplier | Grade/Purity |
|---|---|---|
| Methanol Supragradient HPLC grade | Chem Supply (Scharlau) | > 99.8 % |
| Acetonitrile Supragradient HPLC grade | Chem Supply (Scharlau) | > 99.8 % |
| Acetic Acid HPLC grade | Chem Supply (Scharlau) | ≥ 99.8 % |
| Formic Acid | Chem-Supply | ≥ 99 % |
| 2 M Ammonia in Methanol Solution | Sigma Aldrich | Not Specified |
| Chloroform | ACI Labscan | ≥ 99.8 % |
| Dichloromethane | Chem-Supply | ≥ 99.8 % |
| Extra dry toluene (< 30ppm water) | Acros Organics (Chem supply) | Not Specified |
| ~ 5 % dichloro(dimethyl)silane DCDMS in toluene, for GC derivatisation | Supelco (Sigma Aldrich) | Analytical Reagent |

### 3.1.1 Silanisation of Glassware

Unless mentioned within text, glassware was silanised prior to use in a similar method previously reported by Baker et al. [216]. In brief the glassware was rinsed with 5 % dichloro(dimethyl)silane in toluene for 30 s, followed by rinsing in extra dry toluene (thrice) and finally methanol (thrice).

### 3.1.2 Working Drug Standards

Working drug solutions (10 µg/mL) were prepared by dilution of the certified standard materials in methanol (Table 3-2). The drugs used and concentrations are noted in the text. These solutions were used for drug spikes and producing calibration curves.

Table 3-2: Internal standards, supplier and purity.

| Reagent | Supplier | Concentration | Grade/Purity |
|---|---|---|---|
| (±)-Methamphetamine in methanol | Novachem | 1 mg/mL | > 99 % |
| (±)-Amphetamine in methanol | Novachem | 1 mg/mL | > 99 % |
| 1R,2S-Ephedrine HCl in methanol | Novachem | 1 mg/mL | > 99 % |
| 1S,2S-Pseudophedrine in methanol | Novachem | 1 mg/mL | > 99 % |

| | | | |
|---|---|---|---|
| 1-phenylpropan-2-one (P2P) | Halewood Chemicals Ltd. | > 99% | Neat |
| Benzaldehyde | Sigma-Aldrich | > 99 % | > 99 % |
| 4-Methoxybenzaldehyde p-Anisaldehyde | Chem-Supply Pty Ltd.- Tokyo Chemical Institute (TCI) | > 99 % | > 99 % |
| Piperonal | Forensic Science South Australia | Neat | Neat |
| Safrole | Forensic Science South Australia | Neat | Neat |
| Phenylacetic acid | Forensic Science South Australia | Neat | Neat |
| (±) methamphetamine-$D_5$ in methanol | Forensic Science South Australia | 100 µg/mL | > 99 % |
| (±) 3,4 methelenedioxymethamphetamine-$D_5$ in methanol | Forensic Science South Australia | 100 µg/mL | > 99 % |
| (±) amphetamine-$D_5$ in methanol | Forensic Science South Australia | 100 µg/mL | > 99 % |

### 3.1.3 Internal Drug Standards

Internal standard (IS) solutions were prepared by spiking wastewater samples with 10 ng of each amphetamine and pseudo/ephedrine and 1 µg of the other targeted compounds using the working drug standard unless noted otherwise in text. Drugs used as an internal standard are noted throughout the text, along with the diluted concentration.

### 3.1.4 Wastewater Sampling

Wastewater samples were collected from metropolitan South Australian wastewater treatment facilities. Table 3-3 below lists the locations for each wastewater samples and the day of collection.

#### *3.1.4.1 Composite samples*

All composite samples were collected over a 24-hour period from approximately 7 am until 7 am the following day unless stated otherwise. Wastewater samples were collected at volumes proportional to the flow rate of the wastewater treatment plant using a peristaltic pump. These samples were immediately preserved at pH = 2 using 2 M hydrochloric acid, stored in polyethylene containers and stored in a refrigerator (4 °C) until collection. The wastewater

samples were transported on ice to the laboratory where they were filtered and extracted prior to analysis. Table 3-3 summarises the location and dates of the composite wastewater samples. The area served by each wastewater treatment facility is outlined below in Figure 3-1.

**Table 3-3: Metrolpolitin Wastewater samples time and location obtained Note after the date the day of the week in which the sample was taken is given in brackets.**

| Wastewater treatment plant location | Date/s collected (day) | Influent / effluent | Approx. Population Served (2011) | Average flow rate (ML/day) | Sample type |
|---|---|---|---|---|---|
| **South Australian Wastewater Treatment Plants** | | | | | |
| Bolivar | 9-15/04/2017 (Sun-Sat) | Influent | 830 000 | 136.6 | 24 hr composite |
| | 2-8/05/2017 (Tue-Mon) | | | 163.9 | |
| | 17-23/02/2018 (Sat-Fri) | | | 163.2 | |
| Bolivar High Salinity (Port Adelaide Region) | 9-15/04/2017 (Sun-Sat) | Influent | 75 000 | 22.6 | 24 hr composite |
| | 2-8/05/2017 (Tue-Mon) | | | 23.1 | |
| | 17-23/02/2018 (Sat-Fri) | | | 22.6 | |
| Christies Beach | 9-15/04/2017 (Sun-Sat) | Influent | 155 000 | 32.1 | 24 hr composite |
| | 2-8/05/2017 (Tue-Mon) | | | 33.9 | |
| | 17-23/02/2018 (Sat-Fri) | | | 31.3 | |
| Glenelg | 9-15/04/2017 (Sun-Sat) | Influent | 205 000 | 48.9 | 24 hr composite |
| | 2-8/05/2017 (Tue-Mon) | | | 49.6 | |
| | 17-23/02/2018 (Sat-Fri) | | | 48.7 | |
| **Queensland Wastewater Treatment Plant** | | | | | |
| Coombabah | 07/05/14 (Wed) | Influent | 230 000 | 68.7 | 24 hr composite |

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#### *3.1.4.2 Grab samples*

Grab wastewater samples were collected from Christies Beach wastewater treatment plant on Monday 22$^{nd}$ of January 2018. Samples were collected after the removal of large solids prior to the activated sludge treatment process. grab samples were collected every two hours from 7:30 am until 3:30 pm for a total of five samples. Samples were acidified to pH = 2 immediately upon collection with 37 % HCl and stored in the refrigerator until transportation on ice to the laboratory.

# Chapter 4 Commonly used Instrumentation and Techniques

## 4.1 Solid Phase Extraction (SPE)

### 4.1.1 Principles Solid Phase Extraction

Solid phase extraction (SPE) is an extremely valuable sample preparation technique that purifies and concentrates an analyte from a sample solution. It is the most popular sample preparation technique today [243]. SPE has many advantages over liquid-liquid extraction (LLE) as it is more efficient, can be automated, uses a much smaller volume of solvents and produces quantitative extractions [244]. SPE is employed to isolate the compounds of interest from impurities [245]. Three parameters must be considered when performing SPE those being selection of: the SPE sorbent, the washing solvent and the elution solvent.

Choosing the right SPE cartridge is important, and knowledge about the properties of both the sample and analytes is important. The SPE sorbent should bind either the analytes or impurities in the sample. The sorbent is most commonly an *n*-alkylsilica (C8, C18) [246] however nowadays there are more and more sorbent materials available including: mixed-mode sorbents (sorbents having: hydrophilic, lipophilic and charged sites [247]), graphitised carbon and highly cross-linked copolymers [243]. The selected components are retained on the SPE sorbent, and either the absorbed analytes are collected through elution, or the cartridge containing the extracted impurities is discarded. The washing solvent is required to be strong enough to remove impurities from the sorbent but weak enough to retain the analytes [245]. The elution solvent must be able to elute the analytes from the cartridge and leave the more strongly retained impurities.

In general, SPE contains 4 steps those being (1) conditioning of the sorbent, (2) sample introduction, (3) removal of sample impurities and finally (4) removal of the analytes.

To condition the sorbent a solvent is passed through the cartage which wets the packing materials, removes any air present and fills the void volumes in the cartage with solvent, allowing water to wet the silica surface efficiently. Generally, methanol is used as the conditioning solvent, followed by the same volume of water or an aqueous buffer to activate the cartridge. The sample solution is then introduced to the cartridge. The mechanism for the retention of an analyte on the sorbent is highly dependent on the SPE cartridge used and the structure of the analyte. Some retention mechanisms include: hydrogen bonding, dipole-dipole interactions, size exclusion, and ion exchange [243]. The use of a low flow rate is recommended as it significantly improves the recovery and is

generally achieved with a peristaltic pump. A wash solvent, typically an aqueous buffer or water–organic solvent mixture, is commonly employed to remove sample impurities from the sorbent, whilst still retaining the analytes. Finally, the analytes are eluted from the cartridge by using a solvent which disrupts the analyte-sorbent interaction. The SPE process is outlined in Figure 4-1.

**Image removed due to copyright restriction. Please see reference for original image [248].**

### 4.1.2 Solid Phase Extraction

Figure 4-2: Structure of the mixed cation exchange (MCX) solid phase extraction (SPE) sorbent.

The Solid Phase extraction was performed using Oasis® MCX cartridges. The Oasis® MCX is a Mixed-mode SPE cartridge which means that it contains both lipophilic and charged sites. The sorbent contains a sulphonic acid moiety (Figure 4-2) which is negatively charged and therefore is a strong cation exchanger. For this reason, it is commonly employed for compounds which contain a positive charge and has a working pKa range of 2-10 [249]. All the amphetamine-type stimulants, due to their amine functionally, are basic and under

acidic conditions become protonated and charged. When the pH of the wastewater samples is adjusted via the addition of hydrochloric acid not only is the stability of the drug improved, but it also assists with the sample clean-up/extraction. Once the sample is loaded the drugs can then be eluted using a basic solution which would return the drug to its uncharged state which would no longer be strongly retained by the sulphonic acid moiety.

$+ \; H_3O^+ \rightleftharpoons \; + \; H_2O$

Figure 4-3: Equilibria between methamphetamine and its ionised form.

## 4.2 Liquid Chromatography Mass Spectrometry (LC-MS)

LC-MS combines high performance liquid chromatography (HPLC) which separates analytes depending on their interaction with a stationary phase with mass spectrometry (MS) which separates ions based on their mass-to-charge ratio. Liquid chromatography (LC) can be used to separate a wide variety of organic compounds of varying sizes from small-molecules such as drug metabolites to large proteins. Unlike gas chromatography (GC), which cannot be used for non-volatile and thermally unstable compounds, the sample is in a liquid phase and high temperatures can be avoided. When the compounds being analysed are thermally unstable, LC is preferred over GC due to the reduced temperature which is employed.

The combination of HPLC and MS has numerous advantages, compounds such as isomers which have the same mass and cannot normally be differentiated by a mass detector. If it is possible to separate isomers through chromatography beforehand, differentiation can be achieved. In addition, HPLC only separates compounds but gives little to no information as to its identification or purity. With the addition of MS, information can be gained about the mass of each of the chemicals present in the peak, and is an excellent method to check for co-elution and peak purity, the same argument holds for GC-MS

### 4.2.1 Principles of Liquid Chromatography Mass Spectrometry

#### 4.2.1.1 Liquid Chromatography Separation

*Hydrophilic Interaction Chromatography (HILIC)*

Hydrophilic interaction chromatography is a variant of normal phase chromatography. HILIC differs from normal phase liquid chromatography (NPLC) in mobile phase composition and mechanism of separation. In NPLC water immiscible organic mobile phases are normally employed where in HILIC the organic mobile phases are generally water miscible [250]. In NPLC the separation mechanism is absorptive whilst in HILIC the separation is believed to occur primarily as a result of liquid-liquid partitioning created by the water layer produced on the surface of the column packing [251]. For this reason the analytes elute in order of increasing polarity and degree of solvation [252]. Liquid-liquid partitioning is not the only separation mechanism which can occur in a HILIC system other effects include hydrogen donor interactions and electrostatic separation mechanisms. The pH of the mobile phase has a far greater affect in retention and selectivity in HILIC than in RP separations.

The use of HILIC chromatography has been advantageous in which the drug target residues are metabolites. This is due to the fact that as the body metabolises lipophilic substances, they become more polar and hence water soluble and are more readily excreted in urine. As these compounds become more polar their retention on a RP column is reduced making it more difficult to obtain adequate separation.

Reverse Phase Liquid Chromatography (RPLC)

Reverse phase chromatography is the most common type utilised in LC applications. In RP chromatography the station phase has a non-polar coating. The retention time of a chemical in RP chromatography is dependent on phase equilibria i.e. the time the compound spends in the mobile phase, compared to the stationary phase, which is dependent on the interaction of the compound with both phases

#### 4.2.1.2 Ion Generation

Since mass spectroscopy separates analytes based on their mass-to-charge ratio, the analytes must first be converted in to ions. There are several means in which to ionise a sample the most common methods in LCMS applications being: electrospray ionisation

(ESI) and chemical ionisation (CI) whilst GCMS methods commonly use electron ionisation (EI).

### Electrospray Ionisation (ESI)

In electrospray ionisation analytes are sprayed out of a nebulising needle (generally a metal capillary), which is positioned into a metal shell. A large potential difference is applied across the needle and the shell which separates the ions present in the sample and expels highly charged droplets from the nebuliser [253]. The ions, due to electrostatic repulsions, reside on the outside of the droplet. The droplets are exposed to a vacuum and a counter flow gas which assists with solvent evaporation. Once the Rayleigh limit is reached, i.e. the electrostatic repulsion forces exceed the surface tension of the droplet, the droplet separates into smaller droplets. This continues until all of the solvent is removed and the ions remain is in the gaseous phase. There are two types of mechanisms in which the solvent is removed from the sample ions as depicted in Figure 4-4, field evaporation and Coulombic explosion, however the processes of ionisation in ESI are not well understood. As opposed to Electron ionisation ESI is considered to be a soft ionisation technique and as a result the molecular ion is nearly always observed as less fragmentation occurs.

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### Chemical Ionisation (CI)

Chemical ionisation creates ions by colliding analytes with ions of a reagent gas. The reagent gas is ionised in an ion source which uses an electromagnetic field to charge the gas molecules. This form of ionisation is a soft ionisation technique, meaning that the energy of the parent ion formed is relatively low: this leads to less fragmentation then

other ionisation techniques, such as electron impact (EI) ionisation. There are several mechanisms in which the product ion form including: protonation, abstraction, adduct formation, and charge exchange.

### Electron Ionisation (EI)

Electron ionisation previously referred to as electron impact is a hard ionisation technique commonly used in gas chromatography. In electron ionisation an analyte under vacuum is exposed to a beam of electrons produced via thermionic emission, i.e. by heating a filament (cathode). These electrons are then accelerated towards an anode and their close proximity to the gaseous analyte molecules cause local electromagnetic field fluctuations which causes a radical cation formation as a result of the loss of an electron from the highest occupied molecular orbital (HOMO) (Figure 4-5). The electrons typical have an energy of approximately 70 eV which is significantly higher than that required to ionise the majority of analytes. For this reason, the radical cation produced is in a highly energetic state and a large proportion of the energy provided to the analyte is dissipated via vibrational and electronic excitation states. In addition, this excess energy also assists in the fragmentation and rearrangement of the radical cation to a more stable state. As this ionisation technique provides a large amount of excess energy and results in a large proportion of fragmentation the molecular ion is sometimes not observed. Electron ionisation is considered a hard ionisation technique compared to several of the other softer ionisation methods such as electrospray ionisation and chemical ionisation.

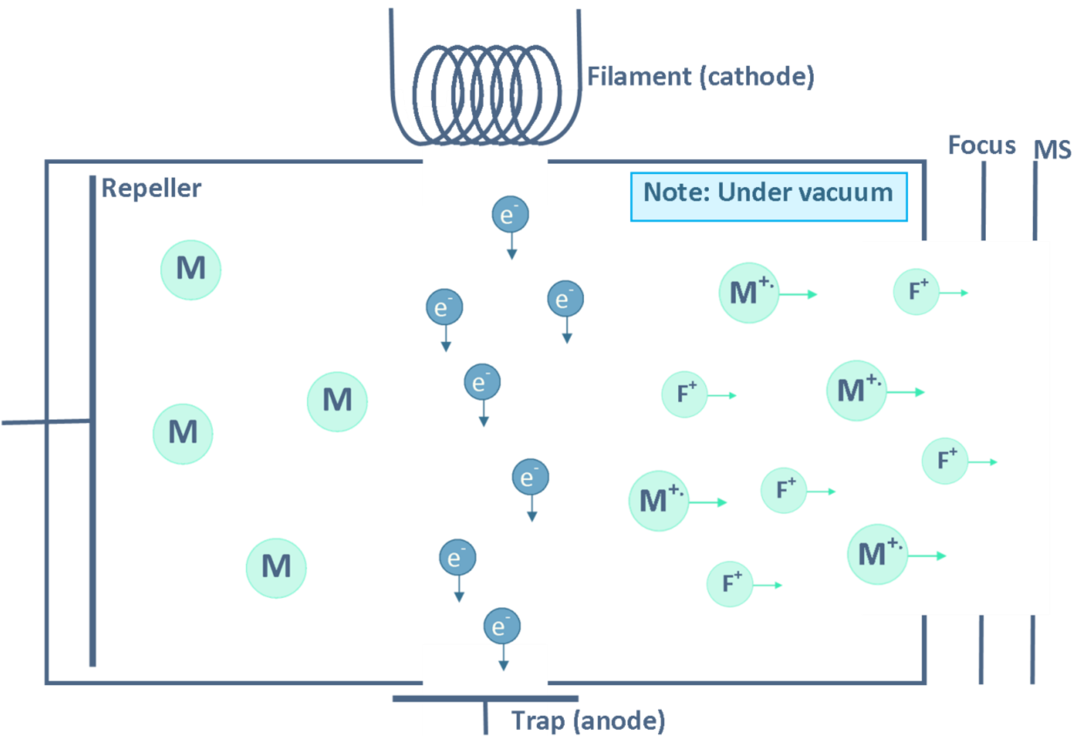


Figure 4-5: Schematic showing production of an electron beam via thermionic emission and electron ionisation of a molecule.

#### *4.2.1.3 Mass analysers*

Once a sample is ionised the sample enters a mass analyser where the ions are separated before entering the detector. There are many types of mass analysers used in mass spectrometry including linear quadrupole, ion trap and time of flight. All mass analysers are based on the separation of charged particles in an electric or magnetic field based on their mass-to-charge ratio.

### Triple Quadrupole Mass Analyser (QqQ)

Quadrupole mass analysers consist of 4 symmetrically arranged parallel rods in which an oscillating electric field is generated (Figure 4-6) by applying DC voltage and radiofrequency (RF). The voltage and RF applied to the rods affects the trajectory of the ions travelling down the centre of the quadrupole. As a result the selectivity of the quadrupole mass filter can be changed by altering the voltage applied to the quadrupole rods [255] . Stable oscillations are only achieved by ions which have a certain mass-to-charge ratio (m/z) range. These ions will travel in a "corkscrew" trajectory and reach the detector, whilst ions with an incorrect m/z will undergo an unstable oscillation, where by the amplitude of the oscillation will continue to increase until the ion strikes one of the

rods [256]. The window of allowed mass ranges can be relatively large or small (down to one mass-to-charge ratio).

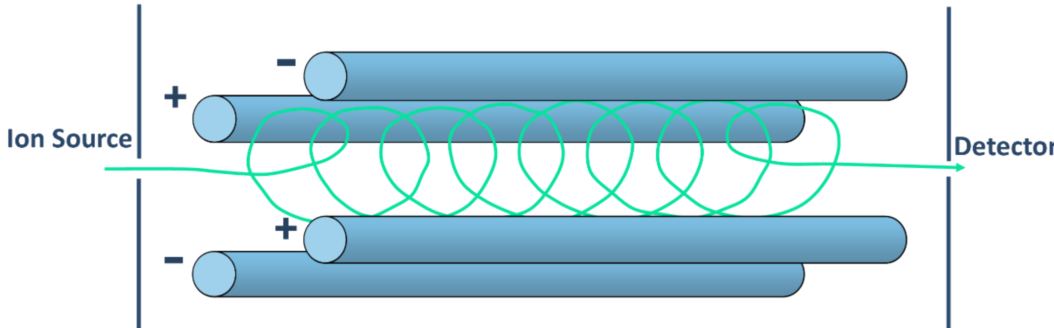


Figure 4-6: Schematic of a quadrupole mass analyser.

A variant on the quadrupole mass analyser uses three consecutive quadrupoles and is known as a triple quadrupole mass spectrometer. In this set up the first quadrupole acts as a mass filter as described above, whilst the second quadrupole acts as a collision chamber which fragments ions by colliding them with an inert gas (typically nitrogen or argon), and the final quadrupole is a second mass filter.

## Ion trap (IT)

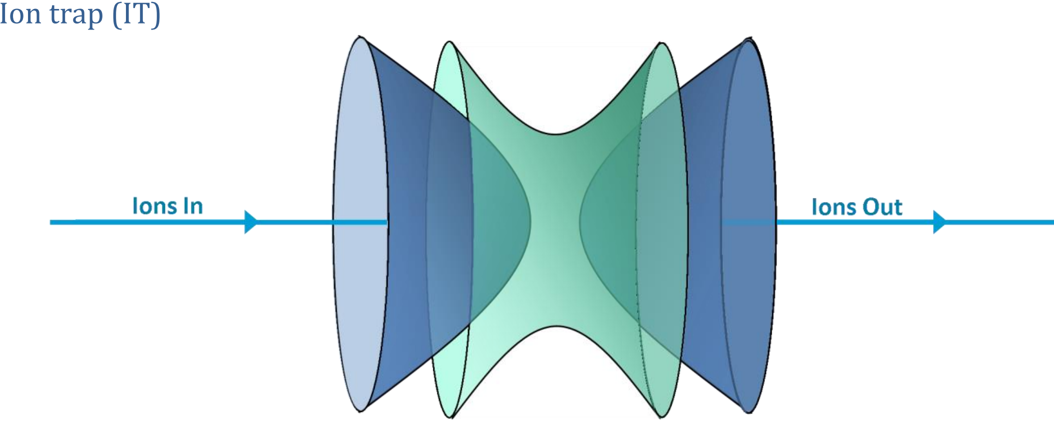


Figure 4-7: Schematic of an ion trap mass filter.

An ion trap mass analyser typically consists of three electrodes, two end cap electrodes (in blue) and one ring electrode (in green), as depicted in Figure 4-7. Various voltages are applied to these electrodes which results in a cavity in which the ions are trapped. The ion trap is filled with a buffer gas, typically helium, which collides with the ions and dampens their kinetic energy focusing them towards the centre of the trap [257]. An oscillating potential is applied across the ring electrode which also helps to focus the ions at the centre of the trap. The trajectory of an ion inside the ion trap and whether it is stable or not depends on several factors including: its mass-to-charge ratio, the size of the ion trap,

and the oscillating frequency and the voltage of the ring electrode. The potentials on the electrodes are then altered to destabilise the motion of ions, causing the ions to be ejected from the trap via the endcap. These ions are then focused onto a detector to produce the mass spectrum.

#### *4.2.1.4 Detector*

Upon exiting the mass spectrometer, ions enter a detector. Since the number of ions leaving the mass analyser is small an electron multiplier is generally used to amplify the signal. As an ion hits the first dynode of an electron multiplier, multiple electrons are emitted which can then hit another dynode and multiply further creating a cascade of electrons until a measurable pulse is produced.

## 4.3 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS combines gas chromatography GC which separates analytes depending on their volatility and polarity with mass spectrometry (MS) which separates compounds on their mass-to-charge ratio. The compounds analysed via GC must be volatile and thermally stable.

One drawback for the analysis of ATS using GC is that more sample preparation time is often required due to the need to derivatise the compound. As ATS contain polar functional groups such as amines and occasionally hydroxyls derivatisation not only has the benefit of making the ATS less polar and more volatile but also generally improves their separation, peak shape, detectability and stability [135]. The most common derivatisation agents used for amphetamine-type stimulants include: pentafluropropionic anhydride (PFPA), heptafluorobutyric anhydride (HFBA) and trifluroacetic anhydride (TFFA) [258, 259].

Another drawback in the use of GC is that the high temperature used to vaporise the sample in the inlet can cause the formation of artefacts as a result of decomposition, rearrangement as well as bimolecular reactions.

### 4.3.1 Principles of Gas Chromatography Mass Spectrometry Separation

#### *4.3.1.1 Gas Chromatography (GC) separation*

As with all chromatography gas liquid chromatography, often referred to as gas chromatography, separates compounds based on their partitioning between different

phases of materials. Unlike in liquid chromatography the mobile phase does not interact with the sample molecules and is used to sweep the sample through the column.

In GC the mobile phase also known as the carrier gas is comprised of an inert gas (most commonly helium, hydrogen) whilst the stationary phase is often a high boiling point waxy polymer absorbed onto a solid. The main types of GC columns include: packed columns in which a waxy polymer stationary phase is bound to a porous support and capillary columns which have the polymer bonded to the inner surface of the column. The diameter of a GC column varies greatly with type and generally ranges for approximately 0.3 mm – 4 mm and can vary in length from approximately 3 m to 150 m.

In GC a substance partitions itself between the liquid stationary phase and the carrier gas. The retention time of a compound in GC is dependent on several parameters including: the boiling point of the compound, the temperature of the column, solubility in the liquid phase, the flow rate of the carrier gas and the length of the column.

For principles behind mass spectrometry see section 4.2.1.

# Chapter 5 Method Development

## 5.1 Chapter Overview

This chapter details the development of a method for both the extraction and analysis of compounds related to amphetamine type stimulants that may be present in wastewater.

GCMS and LCMS methods were optimised and validated via injection of standards of the targeted compounds. The effect of derivatisation of 1-phenyl-2-propanone on the detection limit of the LCMS method was investigated. This derivatisation was performed by converting the P2P to the oxime using hydroxylamine hydrochloride. The kinetics and stability of the derivatisation was investigated ensuring that complete conversion occurred and its suitability for analysis.

The extraction was performed using solid phase extraction. Each step of the solid phase extraction process including the: flow rate, percentage of organic modifier in the wash solvent, sample volume, elution volume, evaporation temperature and wash volume was optimized in order to obtain the optimal limit of detection for the targeted compounds.

The methods created in this chapter were used for determining the stability of the targeted compounds (chapter 7) and the analysis of real wastewater samples collected from the 4 major South Australian treatment facilities (Chapter 8) in order to test the feasibility of wastewater for the analysis of amphetamine precursors.

## 5.2 Liquid Chromatography Mass Spectrometry

### 5.2.1 Preliminary HPLC Work and Method Development

Method development was initially conducted on a HPLC (Agilent 1200 Series) system with UV detection. An initial attempt to gain adequate separation was conducted using a HILIC column (Phenomenex Luna 5 µm, 200 Å, 150 mm x 3.0 mm) equipped with a guard column of the same material, utilising both an ammonium acetate and ammonium formate buffer.

An example chromatogram of the separation utilising the HILIC column is given in Appendix Figure 4. Retention of the amphetamine type stimulations was lower than anticipated on the HILIC column and as the majority of the precursors and by-products targeted are less polar than the amphetamine type stimulants, even poorer retention was expected for these compounds. The use of a RP column (C18 Zorbax eclipse XDB18 I.D. 4.6 mm, length 150 mm, 5 µm) surprisingly provided better retention of the amphetamine type stimulants than the HILIC column (Appendix Figure 5). The conditions used with the RP column are outlined in Appendix Table 2. As the RP column provided better separation of the amphetamine type stimulants than the HILIC column, and was predicted to have greater retention for the other target precursors and by-products, the decision was made to use reverse phase chromatography for all analyses.

In addition, the detection limits of the UV detector were not low enough for the targeted compounds so the decision was made to use a much more sensitive and selective mass spectrometry detector. The mass spectrometer was operated in multiple reaction monitoring mode for details regarding the optimised instrumental parameters for each mass transitions selected see section 6.4.1.

### 5.2.2 UPLC

The method was transferred to a Waters Aquity UHPLC utilising a Phenomenex Kinetex C18 core-shell column (2.6 µm; 2.1 mm × 100 mm), which was used for all subsequent LC separations with the final optimised method outlined in section 6.4.1.

An example overlayed chromatogram of each of the mass transitions for amphetamine, methamphetamine, pseudoephedrine and ephedrine is given in Figure 5-1. Complete separation of ephedrine and pseudoephedrine was not obtained. This could be expected as being diastereomeric compounds their structures are extremely similar, and they were not strongly retained on the reverse phase column. However, this was not anticipated to be an

issue as the concentration of ephedrine in wastewater was expected to be much lower than that of pseudoephedrine.

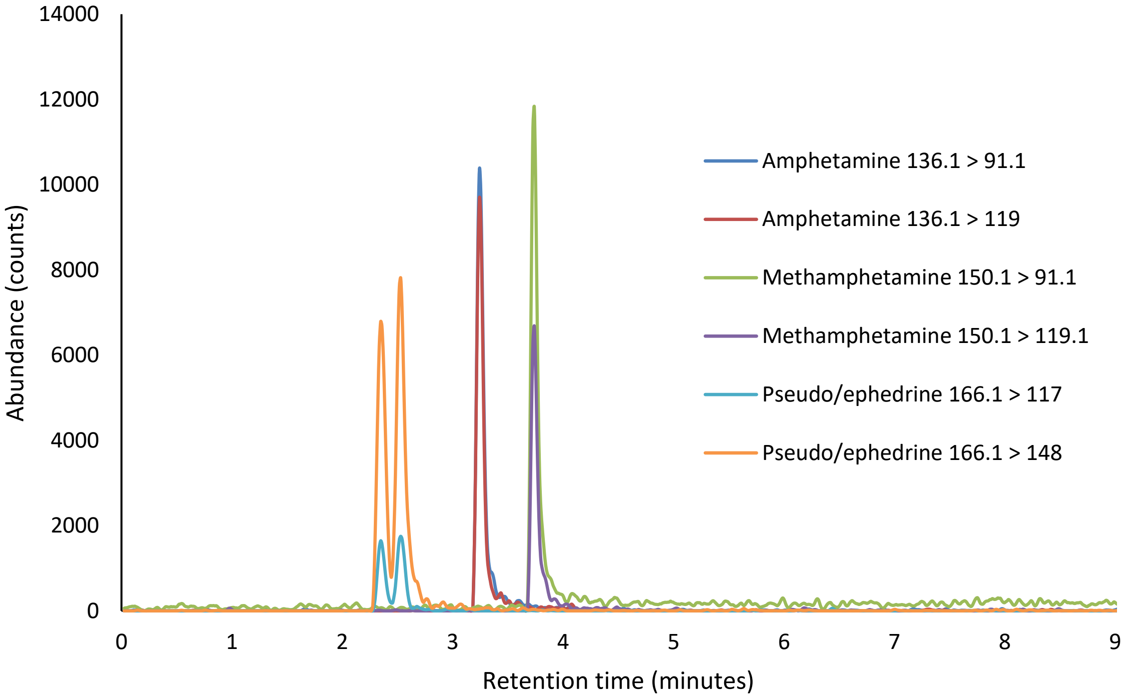


Figure 5-1: Chromatogram of a 10 ng/mL standard containing methamphetamine ($t_R$= 3.74 minutes), amphetamine ($t_R$ = 3.24 minutes) ephedrine ($t_R$ = 2.36 minutes) and pseudoephedrine ($t_R$ =2.53 minutes), showing the selected mass transitions.

Mixed drug standards made in the initial mobile phase buffer with concentrations ranging from 0.2 µg/L – 500 µg/L were then used to create a calibration curve for each target analyte in order to determine the limits of detection of the LC-MS/MS system. An example external calibration curve for methamphetamine is given below in Figure 5-2 for calibration plots related to the other mass transitions and compounds of interest see Appendix Figure 6 - Appendix Figure 12.

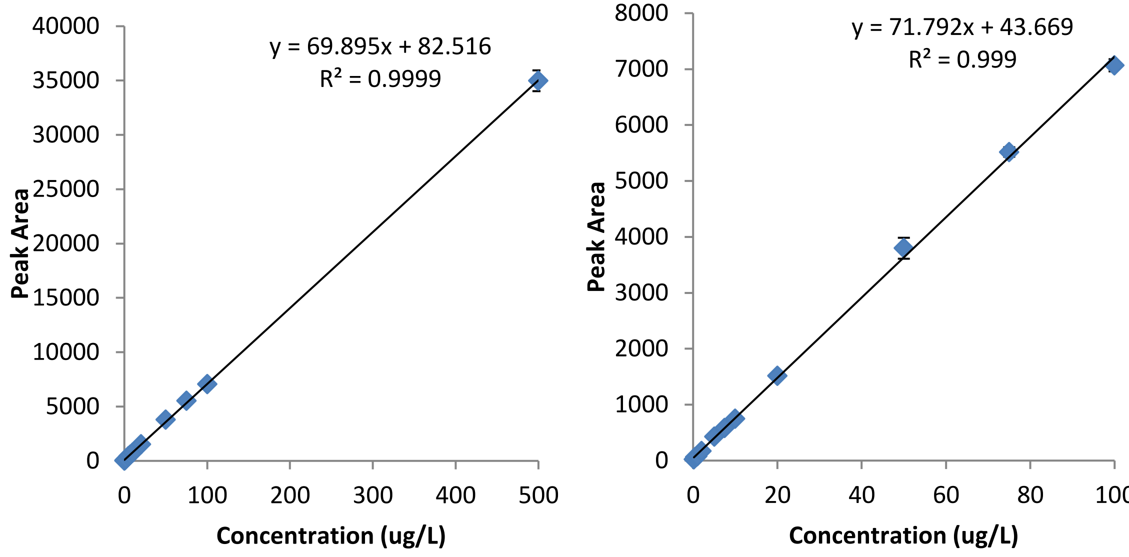


Figure 5-2: Example external calibration curve showing the linearity in the response for the methamphetamine 150.1 > 91.1 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows a concentration range of 0.2 µg/L – 100 µg/L (n = 3).

Method validation was performed for the developed LCMS method by investigating the linearity and limit of quantification through injection of samples with decreasingly lower concentration levels. The calibration range was linear from 2 µg/L to 500 µg/L with all calibration curves having coefficient of variation of at least 0.99 (Table 5 1). In order to determine the limit of quantification a signal to noise ratio of greater or equal to ten was chosen (S/N ≥ 10). An additional requirement for determining the limit of quantification was that the response of the sample at the concentration of the LLOQ needed to be within 15 % (± 15 %) of the value predicated from the line of best fit.

Table 5-1: Method validation data for the LCMS method: Linear range in µg/L, number of calibration points,coefficeient of determination and lower limit of quantification for selected comounds (n = 3). LLOQ was determined using two critera: S/N ≥ 10 and the peak area response had to be within ± 15 % of that predicted by the line of best fit.

| Target Analyte | Retention Time (minutes) | Linear range (µg/L) | # Calibration Points | Coefficient of determination $R^2$ | LLOQ (µg/L) |
|---|---|---|---|---|---|
| **Methamphetamine** | 3.74 | 2 - 500 | 9 | 0.9999 | 2 |
| **Amphetamine** | 3.24 | 2 - 500 | 9 | 0.9995 | 2 |
| **Pseudoephedrine** | 2.53 | 2 - 500 | 9 | 0.9977 | 2 |
| **Ephedrine** | 2.36 | 2 - 500 | 9 | 0.9994 | 2 |
| **1-phenylpropan-2-one** | 7.20 | | | | ~ 750 |

### 5.2.3 1-phenylpropan-2-one in UPLC-MS

The peak corresponding to 1-phenylpropan-2-one was extremely small even at concentrations well above the other target analytes. This was at first to thought to be a result of 1-phenylpropan-2-one irreversibly binding to the column, however after injecting the same sample into a union, and directly injecting the sample into the mass spectrometer it was determined that the signal response was extremely low. This low response is likely to be a result of poor ionisation efficiency of the ketone group leading to poor detectability of 1-phenylpropan-2-one. Due to the low ionisation efficiency of 1-phenylpropan-2-one using ESI the decision was made to derivatise 1-phenylpropan-2-one by addition of a more readily ionisable functional group.

#### *5.2.3.1 Derivatisation of 1-phenylpropan-2-one*

In an attempt to achieve better detection of 1-phenylpropan-2-one, hydroxylamine hydrochloride was used to convert the ketone group on 1-phenylpropan-2-one into the readily ionisable oxime moiety. The reaction scheme for the derivatisation of 1-phenylpropan-2-one is outlined below in Figure 5-3.

Figure 5-3: Reaction mechanism for the derivatisation of 1-phenylpropan-2-one using hydroxylamine.

It should also be noted that the oxime formed from the derivatisation of 1-phenylpropan-2-one with hydroxylamine hydrochloride can undergo both a Beckman rearrangement and acid catalysed hydrolysis. The hydrolysis of the oxime is the reverse reaction of the derivatisation shown in Figure 5-3 and results in the reformation of 1-phenylpropan-2-one. A Beckman rearrangement can also occur under acidic conditions leading to the formation of an amide as outlined below (Figure 5-4).

Figure 5-4: Beckmann rearrangement of the oxime produced from the derivatisation of 1-phenylpropan-2-one.

### Kinetics of Derivatisation Reaction

The effect of temperature on the kinetics of the derivatisation was analysed by comparing the reaction at room temperature (~ 23 °C) and 40 °C. This was conducted keeping the concentration of the hydroxylamine hydrochloride and 1-phenylpropan-2-one constant at 4 mg/mL (0.06 M) and 100 µg/mL (7.5 x $10^{-4}$ M) respectively. All reactions were performed in methanol with the samples sonicated for 1 minute prior to analysis on a Varian Saturn GC-MS. The instrument parameters utilised for monitoring the derivatisation reaction are outlined in section 6.3.2.

Initially three peaks with retention times of 5.137 min, 5.711 min and 5.837 min were observed in the gas chromatogram (Figure 5-5). The identity of the peak eluting at 5.137 min was confirmed to be 1-phenylpropan-2-one by analysis of the mass spectra along with direct injection of a 1-phenylpropan-2-one in methanol standard. The identity of the remaining peaks eluting at 5.471 and 5.84 minutes needed to be confirmed with one of them expected to be the oxime of P2P.

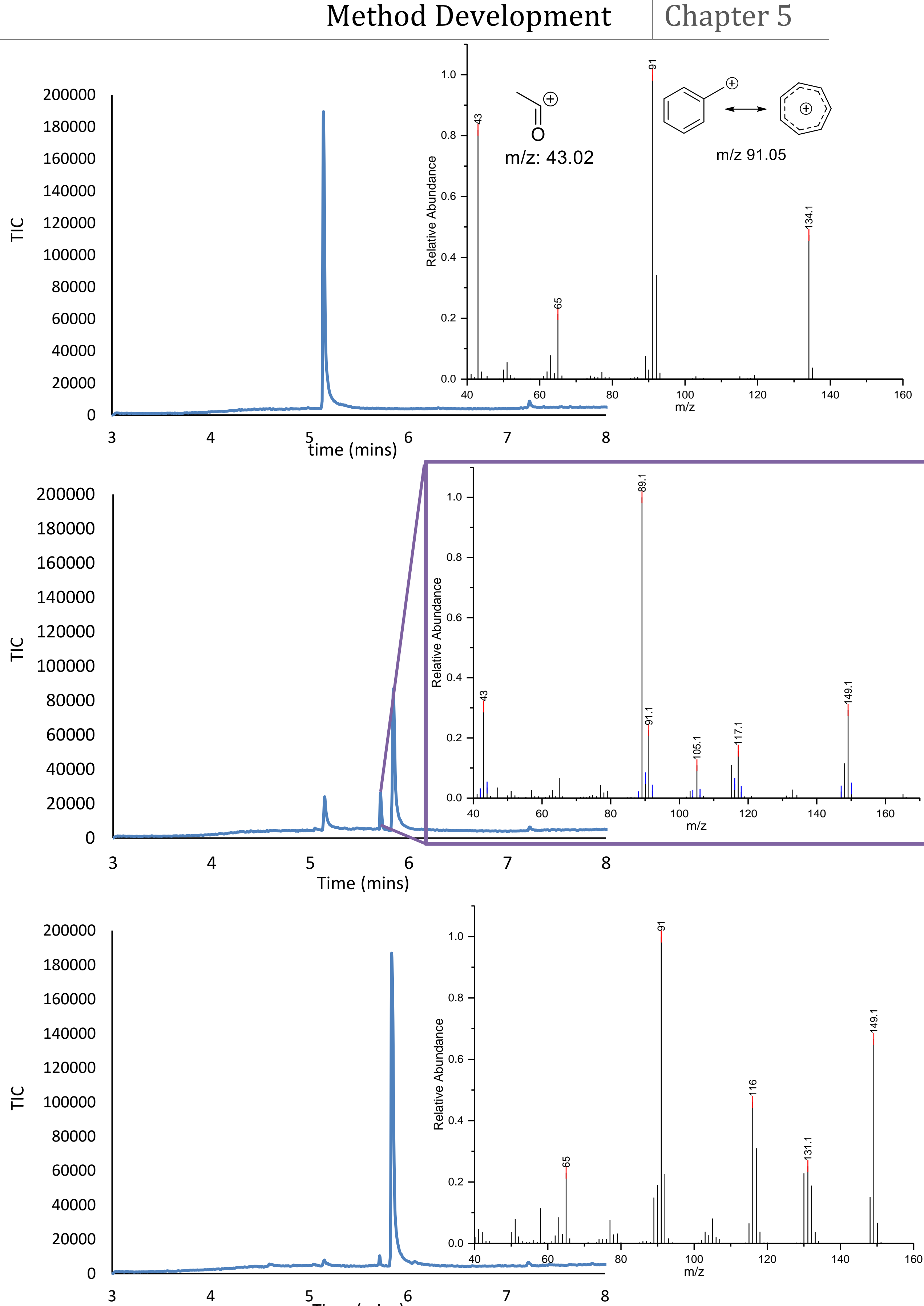


Figure 5-5: GC-MS Chromatograms showing mass fragmentation patterns for the species involved with the derivatisation of 1-phenylpropan-2-one. Showing P2P in methanol (top), t = 45 minutes P2P derivatisation at room temperature ( ~23 °C) (middle) and P2P oxime (T ~23 °C) derivatisation at t = 15 hours (bottom)

As the sample was reinjected continually over several hours the peak area for each compound could be used in order to obtain an idea about the kinetics of the derivatisation reaction and hence an indication of whether the derivatisation was quantitative and how long it takes for the reaction to reach completion. A plot of the relative peak area versus time at both ~ 23 °C and 40 °C is given in Figure 5-6 below.

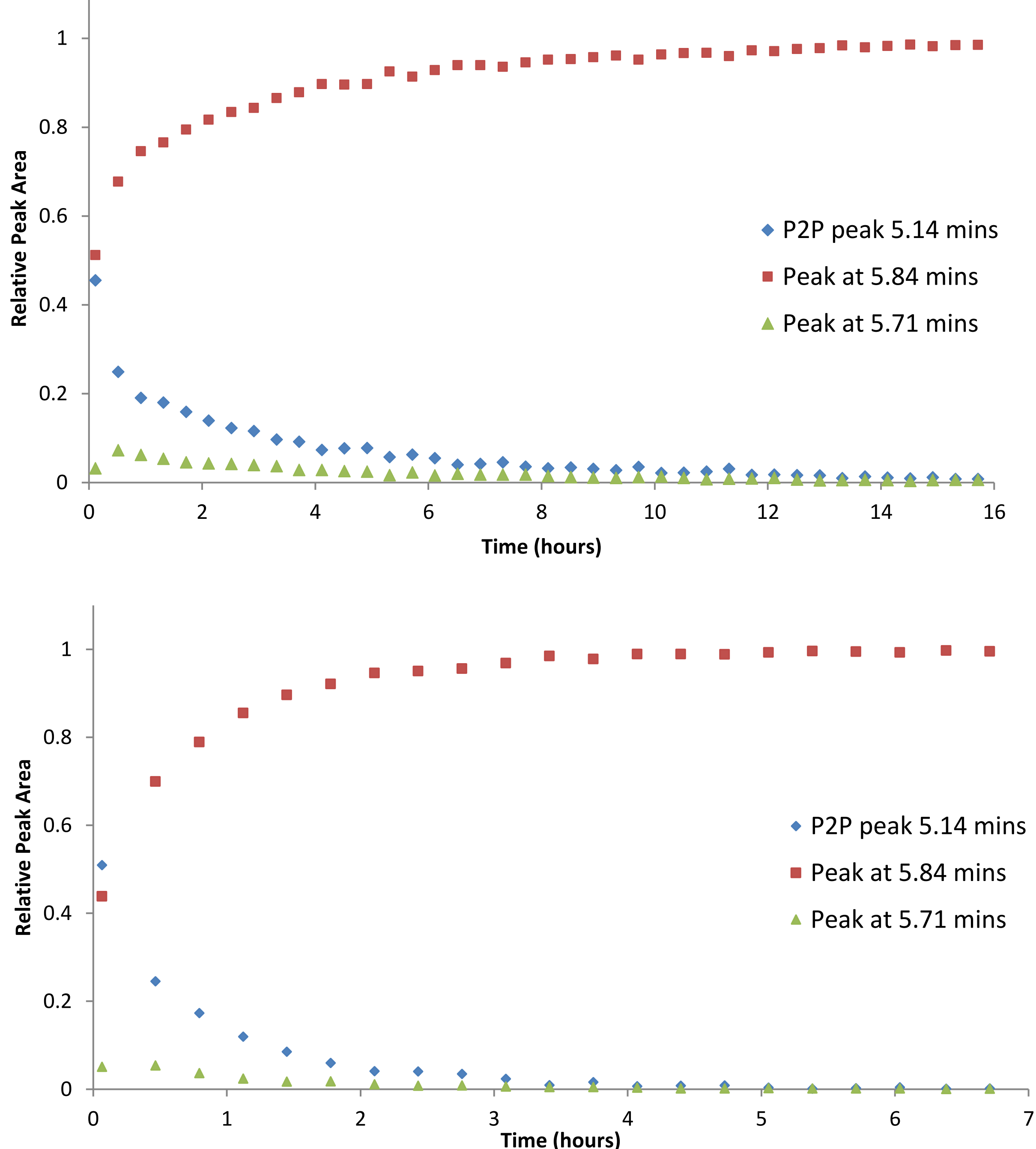


Figure 5-6: Relative peak area of the three peaks observed in the GC-MS spectra for the derivatisation of 1-phenylpropan-2-one (0.0030 M) to 1-phenylpropan-2-one oxime (Phenylacetone ketoxime) using hydroxylamine hydrochloride (0.060 M) at room temperature (uncontrolled but was measured at t = 0 to be 23 °C) and 40 °C (bottom).

The mass fragmentation observed for the peak eluting at 5.84 minutes matched closely to the expected fragmentation of P2P oxime. Whilst the peak eluting at 5.71 minutes despite having a fragment with the same mass-to-charge ratio as the molecular ion of the oxime (149.1 m/z) the fragmentation pattern didn't match that expected.

Based on an increase of the peak eluting at 5.84 minutes over time and the corresponding mass fragmentation pattern the identity of this peak was believed to be P2P oxime. However, the identity of the remaining peak was difficult to determine. Figure 5-6 suggest that the unknown compound produced could be an intermediate of the derivatisation reaction, as it appears initially and by the end of the derivatisation is consumed. However, the mass-to-charge ratio doesn't match any of the expected intermediates in the reaction mechanism. One possible reason why this may occur is that the compound readily fragments and the molecular ion is not visible.

Some possibilities for the identity of the peak eluting at 5.84 minutes included: the nitroso form of the oxime (similar to the enol form of ketones) and a Beckmann rearrangement product. However, both of these possibilities seem unlikely for several reasons including; the lack of the loss of water fragmentation peak (m/z = 18), a decrease of the relative peak area overtime, and tautomerism typically occurs so fast making them difficult to separate. It is believed that in both of these cases the corresponding peak would either remain of a similar peak area in the case of the nitroso tautomer or increase overtime in the case of the Beckmann rearrangement (Figure 5-4).

N OH
Oxime Form
N O
Nitroso Form

Figure 5-7: Structures of the oxime and nitroso form of 1-phenypropan-2-one oxime

The presence of both the E and Z isomers of 1-phenylpropan-2-one oxime could also be expected. If this was the case, the disappearance of the peak eluting at approximately 5.7 minutes may be explained by the E isomer being more thermodynamically stable than the Z and conversion of the Z to the E isomer of 1-phenylpropan-2-one oxime occurring over time. However, if the peak at 5.84 minutes was in fact the other stereoisomer of P2P oxime the mass fragmentation pattern is expected to be indistinguishable from the other stereoisomer, however the mass fragmentation pattern observed was clearly different.

(E)-P2P Oxime (Z)-P2P Oxime

Figure 5-8: E and Z isomers of 1-phenylpropan-2-one oxime

To help support that idea that the identity of the additional peak was not the other geometric isomer of the oxime, the difference in energy between the E and Z form of 1-phenylpropan-2-one oxime was determined using Gaussian09. Section 6.5.1 outlines the Gaussian calculation parameters used. The E isomer was calculated to have an energy which was approximately 4.9 kJ/mol lower than that of the Z isomer (Appendix Table 1). This lower energy was expected as it is the isomer with the most sterically hindered groups trans to each other, however this small difference in energy is unlikely to lead to a large preference of one isomer over the other.

The most likely identity of the unknown peak eluting at 5.84 minutes occurs as a result of an injection port reaction artefact between 1-phenylpropan-2-one and another species present within the reaction mixture. One such artefact, which is likely to give a peak with the same mass-to-charge ratio as the oxime, can arise from the formation of the hemi-ketal of 1-phenylpropan-2-one and subsequent loss of a hydroxyl group. This hemi-ketal artefact is formed in the GC inlet from the reaction of 1-phenylpropan-2-one with the methanol solvent (Figure 5-9). It would be expected that this peak should be at its highest concentration at the beginning of the derivatisation when 1-phenylpropan-2-one is the predominant species present in the sample. The presence of this peak in a 1-phenylpropan-2-one in methanol standard wasn't observed. However, a peak with the same retention time and mass spectrum was observed in solid phase extraction samples where methanol was used as an elution solvent. this is likely a result of the lower pH of these extracts and supports the idea that the additional peak is in fact the hemiketal as the reaction is assisted at low pH. This helps support the idea that the peak is unrelated to 1-phenylpropan-2-one oxime and is in fact a result of P2P being present in the sample.

P2P ROH / $H^+$ Hemi-ketal 1-phenyl-2-methoxypropan-2-ol

Figure 5-9: Formation of the hemi-ketal of 1-phenylpropan-2-one as a result of an injection port reaction of 1-phenylpropan-2-one with the methanol solvent.

### Rate of derivatisation

The rate of the consumption of P2P in the derivatisation reaction was examined as follows:

$$rate = -\frac{d[P2P]}{dt} = k[P2P][Hydroxylamine] \qquad \text{Eq. 5-1}$$

Once the rate equation above (Eq. 5-1) is integrated the following equation (Eq. 5-2) results. The full step by step derivation is shown in Appendix Derivation 1.

$$\frac{1}{[P2P]_0 - [Hydroxyl]_0} \ln\left(\frac{[Hydroxyl]_0[P2P]_t}{[P2P]_0[Hydroxyl]_t}\right) = kt \qquad \text{Eq. 5-2}$$

The bimolecular, second order integrated rate law above (Eq. 5-2) can be simplified when one of the reagents in used a large excess. In this case when $[Hydroxyl] \gg [P2P]$, the assumptions $[Hydroxyl]_0 \approx [Hydroxyl]_t$ and $[P2P]_0 - [Hydroxyl]_0 = -[Hydroxyl]_0$ can be made which simplifies the equation to Eq. 5-3:

$$-\frac{1}{[Hydroxyl]_0} \ln\left(\frac{[P2P]_t}{[P2P]_0}\right) = kt \qquad \text{Eq. 5-3}$$

This equation is known as a pseudo first order equation as when one of the reagents is used in large excess the equation appears first order. Rearrangement of the integrated pseudo first order equation (Eq. 5-3) results in the following:

$$\ln[P2P]_t = -kt[Hydroxyl]_0 + \ln[P2P]_0 \qquad \text{Eq. 5-4}$$

$$\ln[P2P]_t = -k't + \ln[P2P]_0$$

It can clearly be seen that the equation above (Eq. 5-4) is in the form of a straight line, $y = mx + c$. Therefore, if the natural logarithm of 1-phenylpropan-2-one concentration is plotted against time, the result is a linear relationship with slope of $-k'$ ($k' = k[Hydroxyl]_0$) and intercept of $ln[P2P]_0$. When utilising the peak area instead of the concentration the line of best fit is shifted such that the intercept becomes the natural logarithm of the initial peak area but the slope remains unchanged.

An example of the plot of the natural logarithm of the relative peak area of 1-phenylpropan-2-one versus time is given below in Figure 5-10.

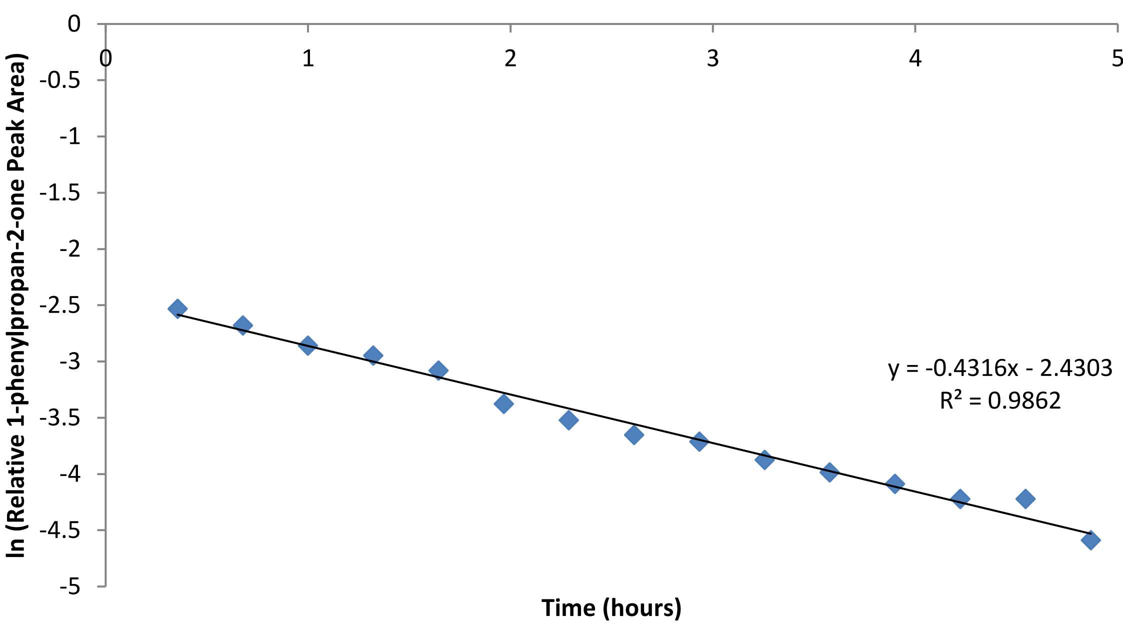


Figure 5-10: Pseudo first order kinetics plot for the derivatisation of 1-phenylpropan-2-one (0.0037 M) to 1-phenylpropan-2-one oxime with hydroxylamine hydrochloride (0.0725 M) in methanol at room temperature (~ 23 °C).

The observed rate constant, k', can easily be extracted by looking at the slope of the line of best fit. A summary of the observed rate constants, k', for each condition tested is summarised in Table 5-2 below.

Table 5-2: Observed rate constants for the derivatisation reaction of 1-phenylpropan-2-one to 1-phenylpropan-2-one oxime using hydroxylamine hydrochloride under various conditions.

| Ratio of hydroxylamine : 1-phenylpropan-2-one concentrations | Concentration of hydroxylamine hydrochloride (M) | Concentration of 1-phenylpropan-2-one. (M) | Temperature (°C) | Observed Rate Constant, k' ($hr^{-1}$) | Standard error in the observed rate constant, k' ($hr^{-1}$) |
|---|---|---|---|---|---|
| 1.02 | 0.0038 | **0.0037** | 23 °C | 0.0185 | 0.0011 |
| 2.05 | 0.0076 | **0.0037** | 23 °C | 0.0254 | 0.0011 |
| 3.89 | 0.0145 | **0.0037** | 23 °C | 0.0633 | 0.0038 |
| 9.72 | 0.0362 | **0.0037** | 23 °C | 0.1175 | 0.0029 |
| 19.45 | 0.0715 | **0.0037** | 23 °C | 0.2725 | 0.0133 |
| 29.81 | 0.1111 | **0.0037** | 23 °C | 0.4316 | 0.0141 |
| 40.17 | 0.1497 | **0.0037** | 23 °C | 0.5464 | 0.0619 |
| 10.04 | **0.0599** | 0.0060 | 23 °C | 0.1988 | 0.0175 |
| 11.48 | **0.0599** | 0.0052 | 23 °C | 0.2366 | 0.0100 |
| 13.39 | **0.0599** | 0.0045 | 23 °C | 0.2073 | 0.0160 |
| 16.07 | **0.0599** | 0.0037 | 23 °C | 0.2533 | 0.0150 |
| 20.09 | **0.0599** | 0.0030 | 23 °C | 0.2249 | 0.0112 |
| 26.78 | **0.0599** | 0.0022 | 23 °C | 0.2231 | 0.0374 |
| **80.3** | 0.0599 | 0.00075 | 23 °C | 0.2407 | 0.0081 |
| **80.3** | 0.0599 | 0.00075 | 40 °C | 0.8630 | 0.0545 |

When hydroxylamine hydrochloride was used largely in excess it was clear that the concentration of 1-phenylpropan-2-one had no effect on the observed rate constant. This is shown by the plot of the observed rate constant versus the concentration of 1-phenylpropan-2-one (Figure 5-11).

Conversely, the dependency of the observed rate constant, k', on the concentration of hydroxylamine hydrochloride was observed as apparent by the non-zero slope of the plot of rate constant versus hydroxylamine concentration when a constant 1-phenylpropan-2-one concentration was used, Figure 5-12.

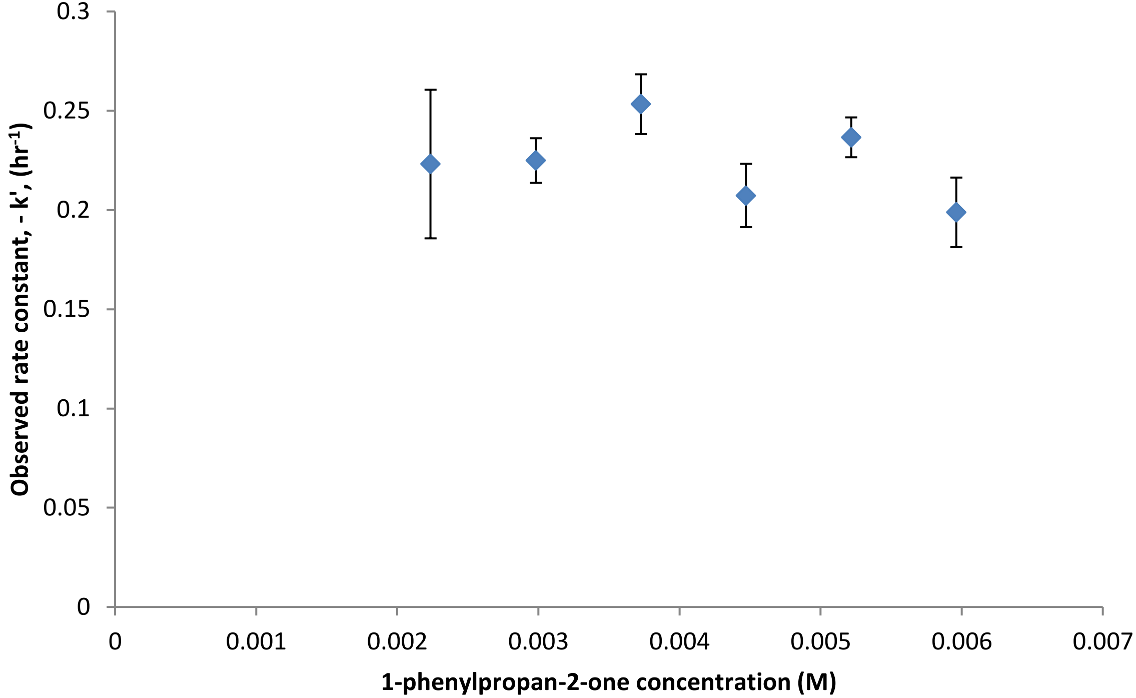


Figure 5-11: Plot of the observed rate constant, k', (determined from the slope of the natural logarithm of 1-phenylpropan-2-one GCMS peak area versus time) versus the concentration of 1-phenylpropan-2-one for the formation of 1-phenylpropan-2-one oxime derivatisation with a constant concentration of hydroxylamine hydrochloride (0.060 M) at room temperature (~ 23 °C). Error bars are equal to the standard error in the slope determined from the LINEST function in Microsoft Excel.

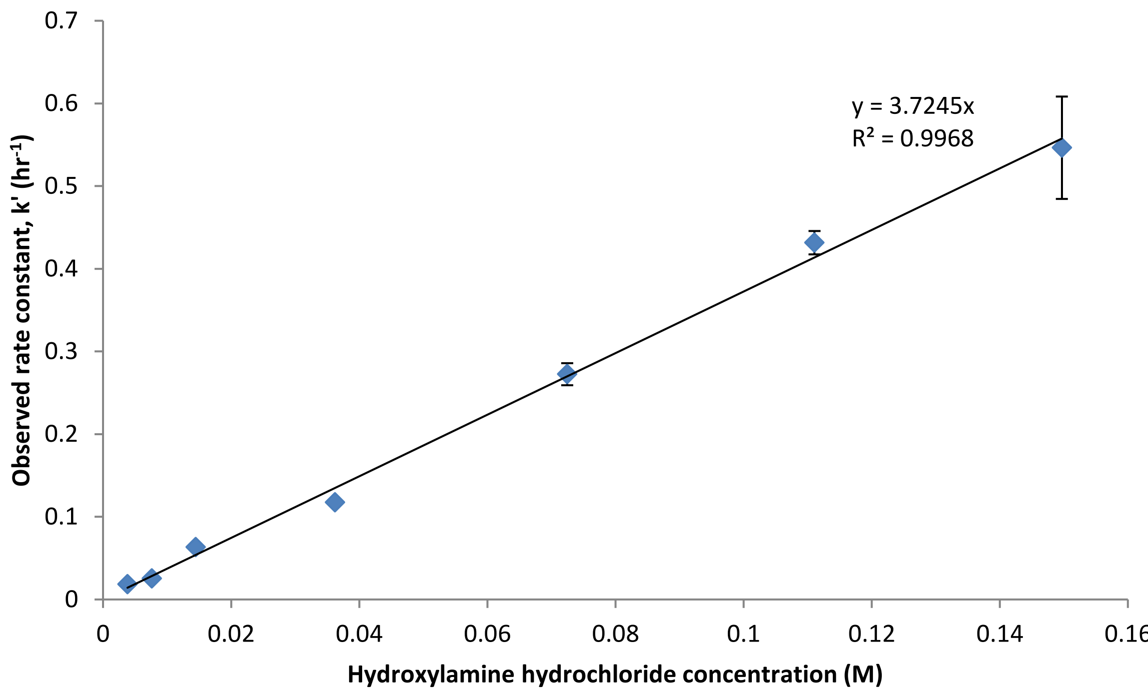


Figure 5-12: Plot of the observed rate constant, k', (determined from the slope of the natural logarithm of 1-phenylpropan-2-one GCMS peak area versus time) versus the concentration of hydroxylamine for the formation of 1-phenylpropan-2-one oxime derivatisation with a constant concentration of 1-phenyl-2-propanol (0.0037 M) at room temperature (~ 23 °C). Error bars are equal to the standard error in the slope determined from the LINEST function in Microsoft excel.

The plot of rate constant vs hydroxylamine gives the observed rate constant for the derivatisation of P2P to P2P-oxime. This plot can be used to ensure that enough time is given for the derivatisation prior to analysis to ensure that the reaction has gone close to full conversion and will be quantitative.

## Stability of 1-phenylpropan-2-one Oxime

As 1-phenylpropan-2-one oxime has the potential to undergo acid catalysed hydrolysis and the mobile phase used in LC is an aqueous formic acid solution the stability of the oxime in the mobile phase needs to be investigated.

### *Stability of 1-phenylpropan-2-one oxime via UV-Vis Analysis*

The stability was initially tested using UV-Vis spectroscopy. 100 µg/mL samples of 1-phenylpropan-2-one oxime, 1-phenylpropan-2-one and a methanol blank were prepared alongside a blank immediately prior to analysis by diluting 1 mg/mL samples in methanol by a factor 10 in the initial HPLC mobile phase composition (95:5 0.1 % formic acid:95 % acetonitrile).

**Image removed due to copyright restriction. Please see reference for original image [260].**

A Varian Cary 50 Scan UV-Vis spectrophotometer (Figure 5-13) (Varian, Palo Alto, California, USA) was used with the scan program (software version 3.10 (222)), to obtain ultraviolet spectra from 200 nm to 400 nm, acquiring data with the "slowest" speed setting (60 nm/min). Prior to analysis Solutions were analysed in 6Q quartz cuvette (Starna) and zero and baseline correction performed using the blank. Following analysis of a 1-phenylpropan-2-one sample, the scan program was set to cycle every 5 minutes and record a spectrum for 1-phenylpropan-2-one oxime for a total of 65 hours. The data were exported as ".csv" files and analysed using Microsoft® Excel 2010.

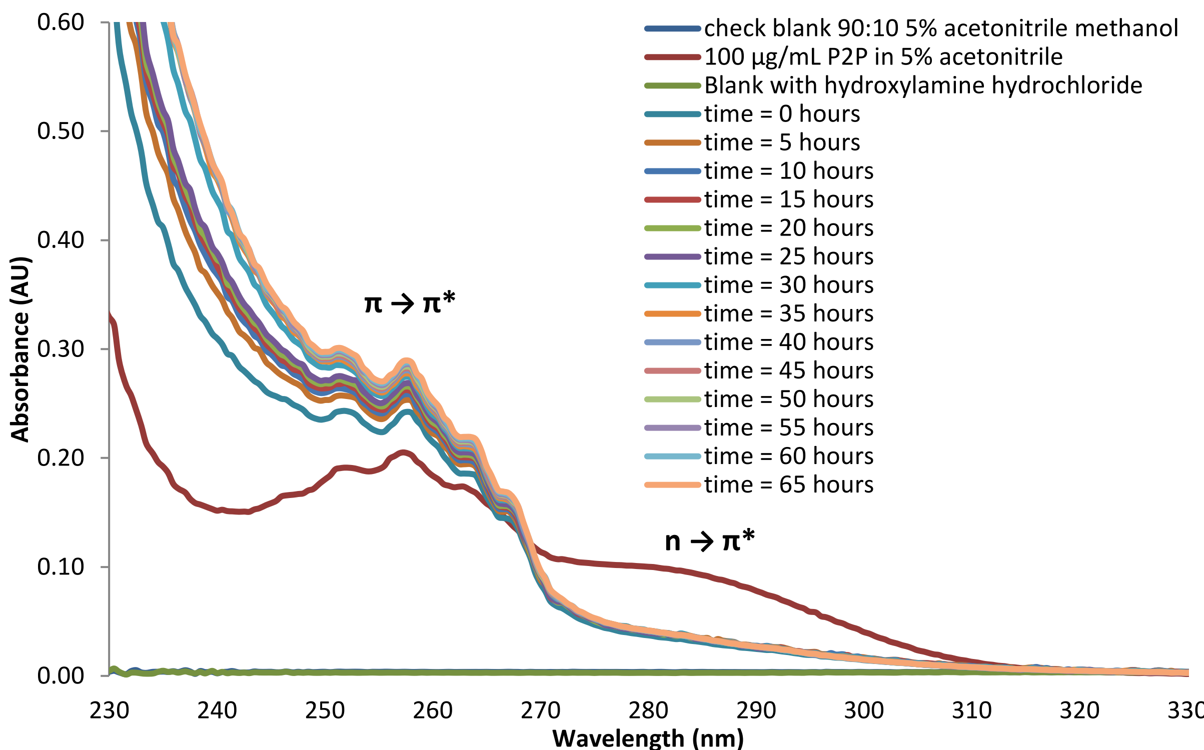


Figure 5-14: UV-Vis absorption spectra of 1-phenylpropan-2-one and 1-phenylpropan-2-one oxime in 0.1% formic acid 5% acetonitrile.

As can be seen from Figure 5-14 above little to no change in the spectra was observed over 65 hours. This indicates that the 1-phenylpropan-2-one oxime is stable for at least 65 hours, with only minor changes observed in the spectra likely to be as a result of evaporation of the solvent over time. There are two main peak regions occurring in the spectra of 1-phenylpropan-2-one; those being at approximately 258 nm as a result of the aromatic pi bond electrons being transferred to the pi anti bonding orbital (π → π*), and at around 283 nm likely due to the transfer of a non-bonding electron of the oxygen atom to the pi anti-bonding orbital (n → π*). The transition of the non-bonding electron to the anti-bonding pi orbital occurs with a lower molar extinction co-efficient than that of the aromatic π → π* (typically 3 orders of magnitude smaller) which could potentially mean that the stability of 1-phenylpropan-2-one oxime cannot be effectively determined using UV-Vis spectroscopy alone. In addition as the 1-phenylpropan-2-one oxime sample used contains unreacted hydroxylamine hydrochloride any 1-phenylpropan-2-one produced via degradation can be converted back to 1-phenylpropan-2-one oxime by residual hydroxylamine hydrochloride.

As UV-Vis is unable to effectively determine the stability of the 1-phenylpropan-2-one oxime in the mobile phase the stability was checked utilising LC-MS/MS. LCMS has the advantage over UV-Vis analysis for investigating the stability as it separates the components in the

derivatisation mixture. Monitoring the peak area of P2P oxime overtime can then be used to investigate its stability in the mobile phase.

*Stability of 1-phenylpropan-2-one oxime via HPLC Analysis*

Samples of 1-phenylpropan-2-one oxime were prepared to check its stability using UPLC. A sample of 1-phenylpropan-2-one oxime (9 µg/mL) was created in aqueous 5% acetonitrile solutions both with and without 0.1% formic acid.

Samples were analysed using LC-MS/MS with the MS run in scan mode according to the following parameters Table 5-3.

Table 5-3: Mass spec scan parameters used for the LCMS analysis of P2P oxime

| | |
|---|---|
| **Start mass** | 80 m/z |
| **End mass** | 300 m/z |
| **Scan time** | 0.3 s |
| **Inter scan delay** | 0.05 s |
| **Cone Voltage** | 20 V |

An extracted ion chromatogram showing the presence of the 150 m/z ion was obtained from the LCMS analysis P2P oxime and is depicted in Figure 5-15 below. The mass fragmentation pattern of the peak eluting at 5.97 minutes is shown in Figure 5-16.

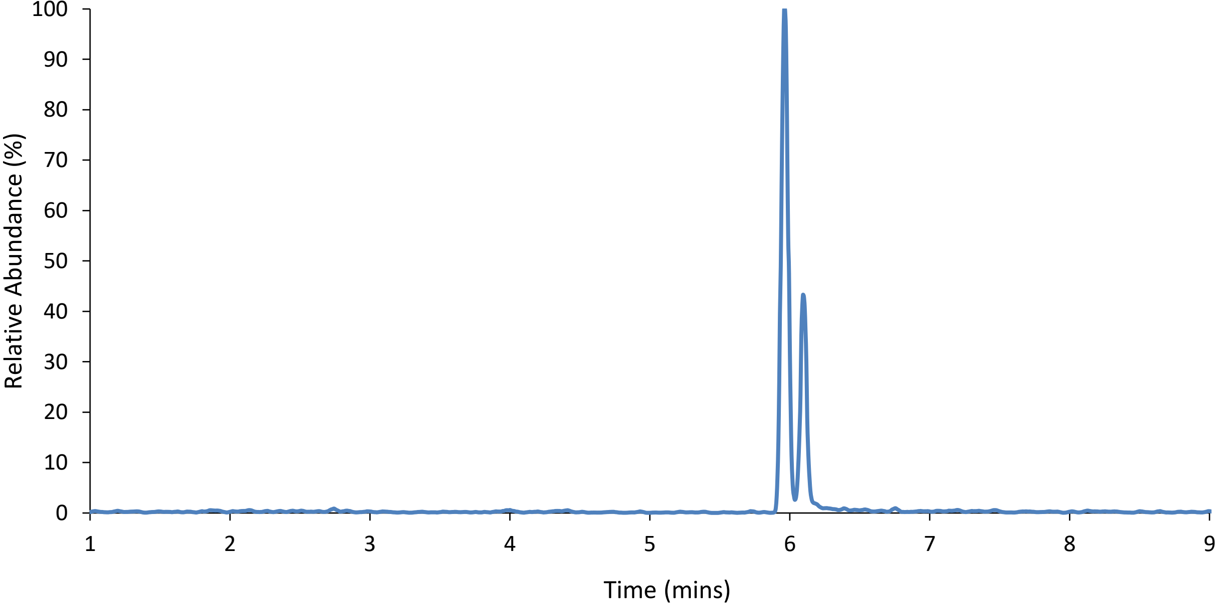


Figure 5-15: 150 m/z extracted ion +ESI LCMS chromatogram showing the 2 peaks observed in the P2P oxime sample derivatised from P2P using hydroxylamine hydrochloride

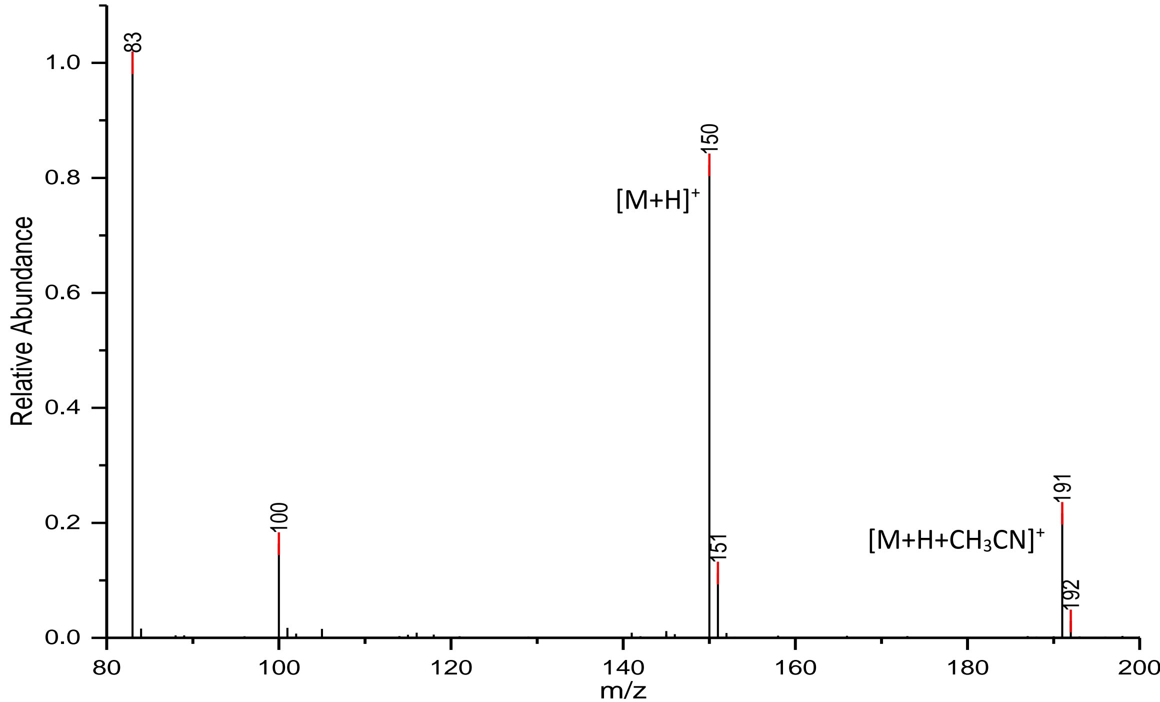


Figure 5-16: Mass fragmentation pattern of the peak eluting at 5.97 minutes expected to be P2P-oxime. It should be noted that the same mass fragmentation pattern of the peak eluting at 6.10 minutes was identical with the exception of an increase in the background m/z peak (m/z =100)

It can be seen that in the 1-phenylpropan-2-one oxime sample that 2 peaks were present at 5.97 min and 6.10 min (Figure 5-15) believed to be a result of the E/Z isomers. It should also be noted that in addition to the molecular ion $[M+H]^+$ occurring at 150 m/z., a mass fragment at m/z 191 was also present as a result of an acetonitrile adduct $[M+H+CH_3CN]^+$. The mass spectrum of the peak eluting at 6.1 min looked identical to that eluting at 5.97 minutes (Figure 5-16) with the exception of the 83 and 100 m/z mass fragments having a higher relative intensity. These fragments however were present throughout the LC run and were present on either side of both peaks which suggests that they are from another source and as a result don't appear to be from the fragmentation of the oxime. The presence of the peak at 83 m/z is believed to be a result of the acetonitrile solvent $[2M+H]^+$. As these peaks are not from the fragmentation of the oxime an increase in the relative abundance of these peaks is expected for the second peak as the peak eluting at 6.10 minutes was smaller than that at 5.97 minutes.

In an attempt to overcome this acetonitrile adduct formation the cone voltage was further increased to 35 V, however the acetonitrile adduct was still present. The samples were injected several times to see if any change in peak area in the selected ion chromatograms occurred. A large variation in peak area was observed with both the peak area and relative peak area (relative to a quinine internal standard) changing randomly with no trend observed.

Despite not being fully investigated the lower limit of detection of 1-phenylpropan-2-one oxime was observed to be significantly lower than that of the ketone. This was shown by detection of the oxime utilising a less sensitive scan method at 450 µg/L a concentration at which the P2P was not detected using a more sensitive multiple reaction monitoring (MRM) method.

Despite one derivatisation product being preferred for an optimal detection limit, the presence of two peaks helps confirm that the oxime is in fact present in the sample and limits misidentification. Other possible derivatisation reagents were going to be trialled to see if one could be found which would impart a more readily ionisable moiety, produce only one product and not form an acetonitrile adduct in the LCMS. However, the mass spectrometer which was coupled to the liquid chromatography system developed an electrical fault following a power outage and became unusable. For this reason, the decision was made to use GC-MS for 1-phenylpropan-2-one and LC-MS/MS for the other analytes.

## 5.3 Gas Chromatography Mass Spectrometry

### 5.3.1 GC-MS Method development

Due to the poor ionisation efficiency of 1-phenylpropan-2-one in ESI, the variability of the 1-phenylpropan-2-one oxime signal and lack of access to an LCMS system the decision was made to analyse the first fraction of the solid phase extraction via GC-MS.

A spit-less method is preferred as it allows the highest amount on column without the need of having a high concentration of analyte in the sample. A GCMS method was developed by adapting the method used for the derivatisation studies (section 5.2.3) which utilised a 20:1 split. It is advised for a split-less injection that the starting column temperature should be approximately 20 °C below the boiling point of the solvent. This ensures that the sample condenses on the front of the column and reduces the chance of broad asymmetrical peaks.

As 1-phenylpropan-2-one is the main compound of interest the decision was made to operate the mass spectrometer in selected ion monitoring (SIM) mode to obtain a lower limit of detection. This SIM method was set up targeting the molecular ion along with two most abundant ions in the mass spectrum (m/z 134, 91 and 43). The SIM mode was as expected shown to be more sensitive than the scan mode. This is due to the fact that the scan mode operates by scanning across a wide mass range whilst the SIM mode cycles through the selected ions and as a result allows more ions through the mass analyser to the mass

spectrometer detector. The SIM mode also has the benefit of reducing the background of the chromatogram as only the ions with the selected mass-to-charge ratio pass through the mass analyser to the detector. Example chromatograms showing the difference in the SIM method compared to the typical scan method in Appendix Figure 15.

This increase in sensitivity can be seen by comparing the lower limit of detection (LLOD) of the GC-MS operating in the scan mode to the SIM mode. The SIM mode was an order of magnitude more sensitive than that of the scan mode having a lower limit of detection of approximately 5 µg/L compared to 50 µg/L.

The downside to using the SIM mode is that it is a targeted approach and only counts the ions with the selected mass-to-charge ratio and hence cannot be used as a survey for non-targeted compounds, for this reason wastewater extracts were analysed with both the scan and SIM method to ensure that the data could be re-analysed if any compounds of interest were added to the targeted list.

A mixed drug standard containing 1-phenyl-propan-2-one, benzaldehyde, 4-methoxy benzaldehyde, piperonal and safrole in chloroform was then injected to ensure that separation of each compound was achieved. Figure 5-17 below shows a chromatogram for the injection of a mixed standard.

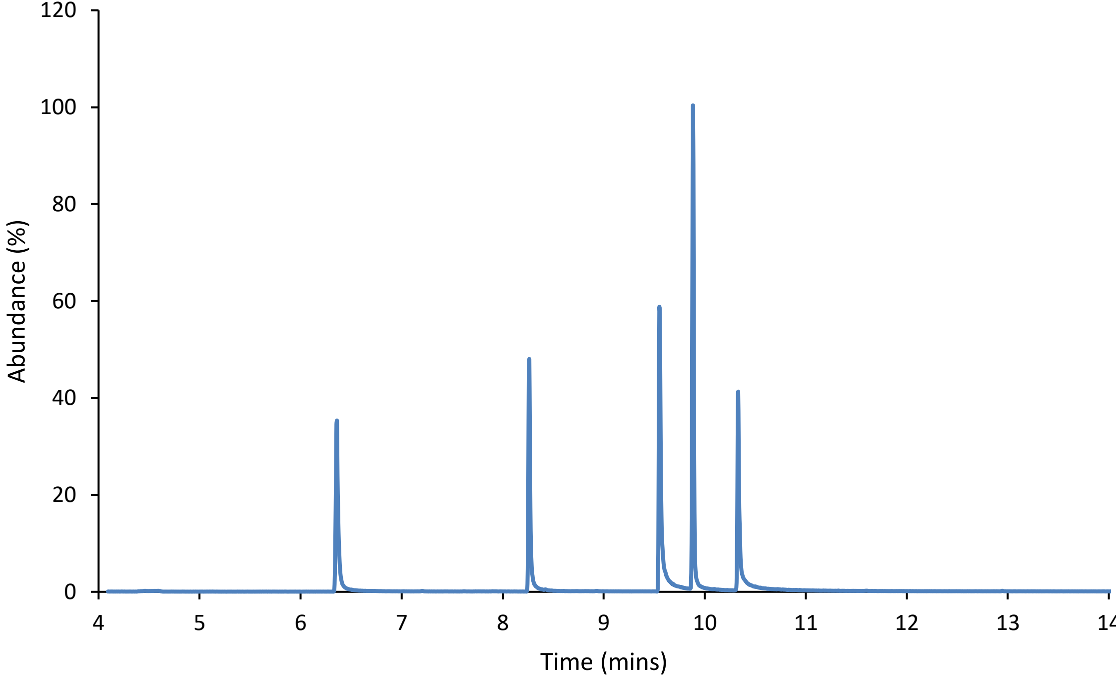


**Figure 5-17:** GCMS Chromatogram obtained from the separation of benzaldehyde (6.36 mins), 1-phenyl-propan-2-one (8.26 mins), 4-methoxybenzaldehyde (9.56 mins), safrole (9.89 mins) and piperonal (10.33 mins)

The order of elution of the standards was benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzaldehyde, safrole, piperonal which had retention times of 6.36, 8.26, 9.56, 9.89 and 10.33 mins respectively. The order of elution of each compound was in the same order as their boiling points with the exception of safrole and 4-methoxybenzaldehyde. The safrole however is less polar than the 4-methoxybenxaldehyde and as a result has a stronger interaction with the non-polar column increasing its retention time.

Method validation was performed for the developed GCMS method by investigating the linearity and limit of quantification by injection of samples with decreasingly lower concentration levels. In order to determine the limit of quantification a signal to noise ratio of greater or equal to ten was chosen (S/N ≥ 10). A summary of the method validation data for the compounds of interest along with their retention time and their corresponding mass fragmentation peaks is outlined in Table 5-4 below. For the full mass spectra and how the mass fragments are likely formed see Appendix Figure 18 – 9.

Table 5-4: Method validation data for the GCMS method along with the structures of targeted compounds their retention times, linear range in µg/L, number of calibration points, and lower limit of quantification for selected comounds (n = 3) and electron ionisation mass fragments. LLOQ was determined using two critera: S/N > 10 and the peak area response had to be within ± 15 % of that predicted by the line of best fit.

| Compound Name | Structure | Retention Time (min) | Linear Range (µg/L) | # Calibration Points | LLOQ (µg/L) | Mass Fragments (Base Peak) |
|---|---|---|---|---|---|---|
| Benzaldehyde | | 6.36 | 100-2000 | 5 | 100 | 106, 105, 77, 74, 51 |
| 1-phenylpropan-2-one (P2P) | | 8.26 | 20-2000 | 7 | 20 | 134, 91, 65, 43 |
| 4-methoxybenzaldehyde | | 9.56 | 100-2000 | 5 | 100 | 136, 135, 107, 92, 77, 51 |
| Safrole | | 9.89 | 50-2000 | 6 | 50 | 162, 131, 104, 77, 51 |
| Piperonal | | 10.32 | 100-2000 | 5 | 100 | 150, 149, 121, 91, 63 |

## 5.4 Extraction and Pre-Concentration Recovery

The recovery of each step of the sample preparation was investigated. This was done to ensure that no significant loss of analytes was occurring and that each step of the sample preparation could be optimised.

### 5.4.1 Solid Phase Extraction Analyte Recovery

Analysis of the concentrations of the internal drug standards spiked both before and after the solid phase extraction process provides information as to the percentage recovery of the analytes. Figure 5-18 shows the percentage of analyte recovered for each of the targeted compounds spiked in milli Q water.

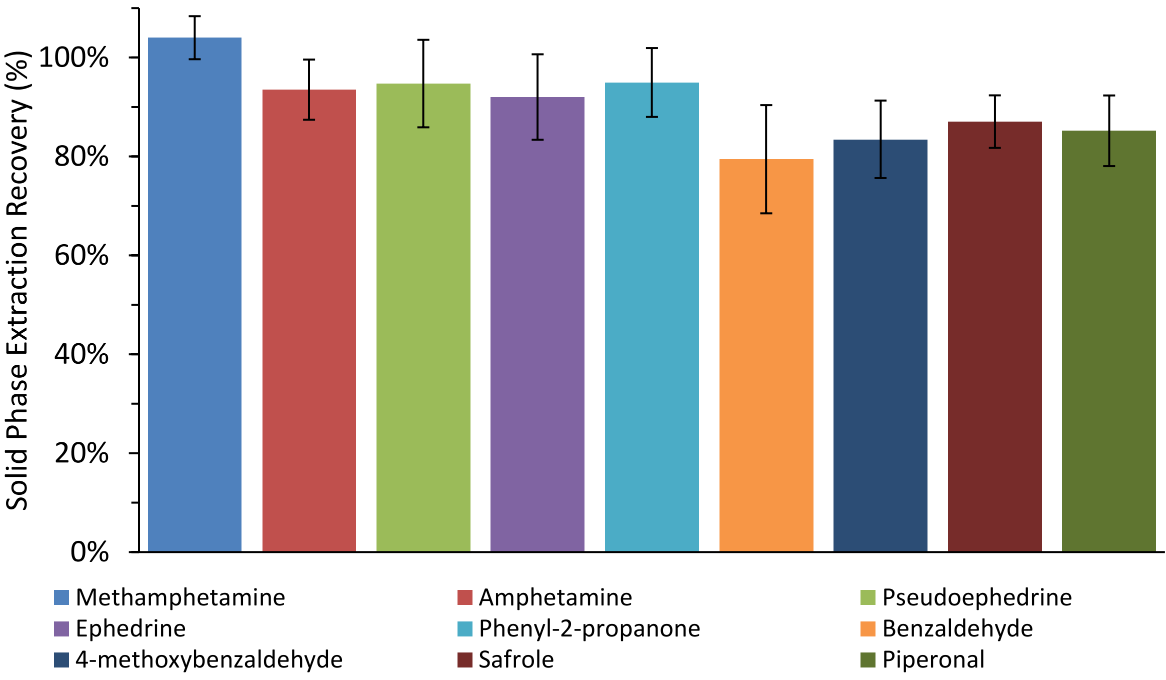


Figure 5-18: Extraction recovery of the drug target residues passed through the mixed cation exchange (MCX) cartridge error bars are 1 std (n = 3).

As can be seen from Figure 5-18 above high recovery for all of the target analytes throughout the SPE process was achieved with the majority of compounds having a recovery between 80 % and 120 %, The only analyte with a recovery slightly outside of this range was benzaldehyde at 79 %.

It is worth noting that the solid phase extraction process utilises two separate elution solvents. The first fraction was collected via elution with chloroform (1 mL) and the second with 1 M (~ 4.3 % w/w) ammonium hydroxide in methanol (4 mL). The protonated amines, namely: methamphetamine, amphetamine, ephedrine and pseudoephedrine were eluted

with the methanolic ammonium hydroxide whilst the remaining compounds were eluted in the chloroform. The presence of the compounds in each elution was expected as the chloroform is strong enough to disrupt the weak interactions retaining the neutral compounds (phenyl-2-propanone, benzaldehyde, 4-methoxybenzaldehyde, safrole and piperonal), whilst not strong enough to disrupt the stronger ionic interactions retaining the protonated amines (methamphetamine, amphetamine, ephedrine and pseudoephedrine). When the methanolic ammonium hydroxide is used the amines are deprotonated and converted back to their free base form. When this occurs, the ionic interactions retaining these compounds no longer exists and as a result the amines can be eluted from the SPE cartridge.

#### *5.4.1.1 Effect of SPE flow rate*

The effect of the flow rate through the solid phase extraction cartridge on the recovery of targeted compounds was investigated. The recovery of each compound at the flow rates tested is summarised below in Table 5-5.

Table 5-5: Effect of solid phase extraction flow rate on the recovery of targeted compounds. Samples were milli Q water samples spiked at 20 µg/L for compounds in elution 1 and 200 ng/L for those in elution 2. The filtration step was omitted and wash solvent pH = 2 HCl adjusted milli Q water.

| Compound | Approximate Flow Rate | | | |
|---|---|---|---|---|
| | 0.5 mL/min | 1 mL/min | 2 mL/min | 5 mL/min |
| | Recovery ± 1 SD (%) (n = 3) | | | |
| 1-phenylpropan-2-one | 91 ± 7.8 | 97 ± 5.8 | 82 ± 8.3 | 71 ± 7.9 |
| Benzaldehyde | 78 ± 7.6 | 78 ± 7.3 | 74 ± 7.8 | 60 ± 6.8 |
| 4-methoxybenzaldehyde | 88 ± 8.1 | 85 ± 7.2 | 77 ± 7.3 | 67 ± 7.4 |
| Safrole | 93 ± 7.4 | 96 ± 7.2 | 80 ± 6.6 | 67 ± 8.3 |
| Piperonal | 91 ± 7.5 | 88 ± 9.1 | 84 ± 6.8 | 71 ± 6.3 |
| Methamphetamine | 101± 6.8 | 99 ± 7.0 | 96 ± 7.3 | 80 ± 7.0 |
| Amphetamine | 96 ± 5.5 | 97 ± 6.5 | 95 ± 5.7 | 79 ± 5.0 |
| Pseudoephedrine | 96 ± 8.1 | 95 ± 8.3 | 89 ± 8.2 | 77 ± 7.6 |
| Ephedrine | 95 ± 7.3 | 96 ± 7.4 | 88 ± 7.8 | 79 ± 7.5 |

As can be seen from Table 5-5 little to no difference was observed in the recovery of target compounds when the loading flow rate was approximately 0.5 mL/min and 1 mL/min. Increasing the flow rate to 2 mL/min resulted in a significant decrease in a recovery of the

majority of the targeted compounds. This could be expected as when the flow rate is increased the compounds have less time to interact with the binding sites on the solid phase extraction sorbent. This can result in both break-through of the targeted compounds and the compounds eluting in a wider band. Further increasing the flow rate of the loading step resulted in unacceptable loss of all target compounds. Considering the recoveries of the targeted compounds at the different flow rates the optimal loading rate was determined to be 1 mL/min.

#### *5.4.1.2 Effect of wash solvent on SPE recovery*

The retention of the acidic and neutral compounds in the SPE extraction process is based on interactions such as Van Der Waals dispersion forces, including pi-pi stacking. These forces are not as strong as the ionic interaction retaining the protonated basic compounds. For this reason, a wash which utilises a high organic percentage can negatively impact their recovery. The wash however may still be beneficial as it can help to clean the sample by removing some of the interfering compounds and yield better detection limits. To test the optimal wash conditions several pH = 2 (HCl adjusted) aqueous methanol solutions were prepared with differing percentages of methanol. Solid phase extraction was then performed on samples spiked with 1 µg of each compound eluting in the first fraction and 10 ng of each of the amphetamines. The recovery of each target compound is summarised in Table 5-6 below.

Table 5-6: Recovery of targeted compounds using wash solvents with a different percentage of methanol as an organic modifier.

| Compound | Percentage of methanol in wash solvent | | | | | |
|---|---|---|---|---|---|---|
| | 0% | 10 % | 25 % | 50 % | 75% | 100 % |
| | Recovery ± 1 SD (%) (n = 3) | | | | | |
| 1-phenylpropan-2-one | 97 ± 5.8 | 98 ± 8.2 | 85 ± 10.8 | 60 ± 8 | 34 ± 7.1 | 0 (<LOD) |
| Benzaldehyde | 78 ± 7.3 | 79 ± 6.5 | 60 ± 8.3 | 46 ± 7.6 | 18 ± 8 | 0 (<LOD) |
| 4-methoxybenzaldehyde | 85 ± 7.2 | 89 ± 7.1 | 66 ± 6.4 | 44 ± 7.8 | 24 ± 6.1 | 0 (<LOD) |
| Safrole | 96 ± 7.2 | 95 ± 5.6 | 88 ± 8.3 | 74 ± 10.7 | 39 ± 6.8 | 8 ± 10.8 |
| Piperonal | 88 ± 9.1 | 87 ± 9 | 65 ± 8.8 | 51 ± 10 | 27 ± 6.5 | 0 (<LOD) |
| Methamphetamine | 99 ± 7.0 | Not Tested | | | | 101 ± 6.8 |
| Amphetamine | 97 ± 6.5 | | | | | 94 ± 6.8 |
| Pseudoephedrine | 95 ± 8.3 | | | | | 95 ± 8.5 |
| Ephedrine | 96 ± 7.4 | | | | | 95 ± 7.6 |

It can be seen from Table 5-6 that when the percentage of organic modifier in the SPE wash solvent was increased to above 10 % the recovery of all compounds in the first solid phase extraction elution decreased. For this reason, 10 % methanol was chosen as a wash solvent for all SPE as it achieved good recovery for all targeted compounds and having a higher amount of methanol would help to clean the samples to help remove potentially interfering compounds.

#### *5.4.1.3 Effect of sample volume loaded onto the cartridge*

With any pre-concentration method it is always desired to get a high pre-concentration factor. With solid phase extraction there are two ways to achieve this; those being: increasing the amount of sample loaded onto the cartridge and limiting the volume of the extract. However, when pre-concentration is employed the matrix can also be pre-concentrated leading to a higher chance for more significant matrix effects.

The recovery of the target compounds was investigated by spiking 25 mL and 50 mL with 10 ng of amphetamine, methamphetamine and pseudoephedrine and extracting the samples. The recovery of each analyte was consistent even when using a larger volume which suggests that a LLOD may be increased by simply increasing the sample volume loaded onto the cartridge.

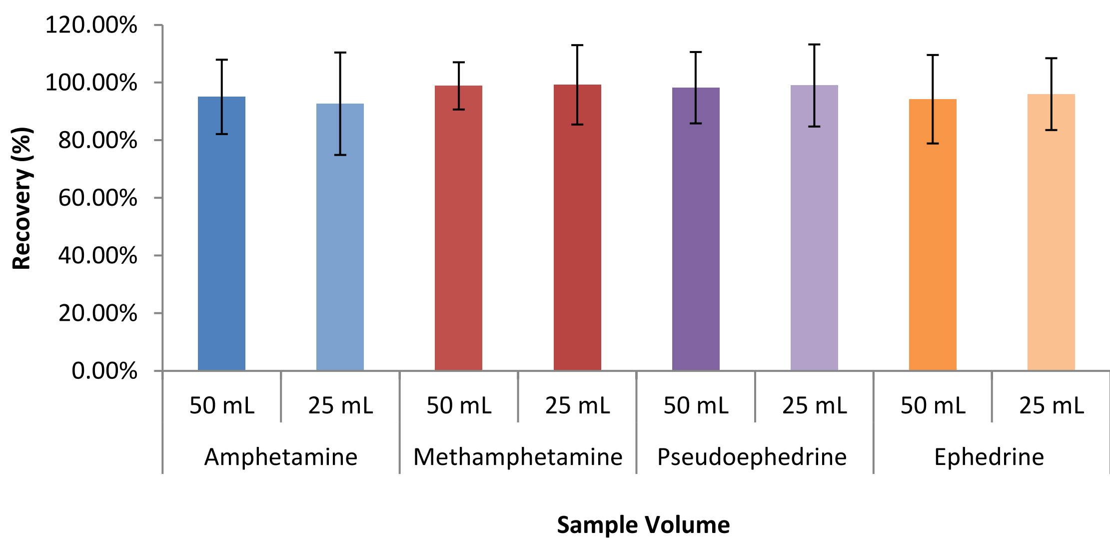


Figure 5-19: Effect of sample volume loaded onto the MCX solid phase extraction column on the recovery of amphetamine, methamphetamine, pseudoephedrine and ephedrine. Error bars shown are 1 std. (n = 3)

The sample volume was not increased beyond 50 mL as the detection in the GCMS method is not as selective as the MRM method used for the LCMS and at 50 mL some of the peaks in the wastewater extracts were approaching the cut-off point of the mass detector.

### *5.4.1.4 Effect of elution volume on SPE recovery*

The effect of elution volume was first trialled with 1-phenylpropan-2-one in methanol by loading a 50 mL sample containing 100 µg of 1-phenylpropan-2-one on the SPE cartridge, drying the cartridge under vacuum and eluting the 1-phenylpropan-2-one in several fractions of differing volumes. The elution volumes tested were 250 µL, 500 µL, 750 µL and 1 mL all of which were passed through a separate SPE cartridge which was then dried under vacuum before 2 additional elutions on the same volume. All samples were made up to the same volume (1 mL by mass) prior to analysis. It should be noted that not all of the solvent volume was recovered as some of the solvent was retained with the sorbent, due to wetting of the SPE sorbent and as a result with the 250 µL elution little solvent was collected. All samples were then analysed using the Varian GC-MS method (described in section 6.3.2) to obtain the minimum volume needed to elute all of the 1-phenylpropan-2-one.

Table 5-7 Recovery of 1-phenyl-propan-2-one eluted with differing volumes of methanol

| Elution volume (µL) | Elution number | Recovery (%) |
|---|---|---|
| 250 | 1 | 8.0 |
| | 2 | 53.9 |
| | 3 | 30.0 |
| 500 | 1 | 86.7 |
| | 2 | 10.1 |
| | 3 | 0 (< LLOD) |
| 750 | 1 | 90.31 |
| | 2 | 4.12 |
| | 3 | 0 (< LLOD) |
| 1000 | 1 | 100.8 |
| | 2 | 0 (< LLOD) |
| | 3 | 0 (< LLOD) |

It could be seen from Table 5-7 that the only set of elutions which did not contain 1-phenylpropan-2-one in the second and third elutes was 1 mL with only a very small peak corresponding to P2P present in the second 750 µL elution volume. This indicated that the optimum elution volume for 1-phenylpropan-2-one was 1 mL.

An extraction of a spiked mill Q sample was then performed in two 1 mL fractions with Chloroform as the elution solvent in duplicate. This was to ensure that the 1-phenylpropan-

2-one would elute in 1 mL of chloroform and that the chloroform was compatible with the SPE sorbent. This was conducted as chloroform is a preferred solvent over methanol for GC-MS analysis as it is less likely to flood the GC column due to its lower polarity, resulting in better peak shape and a higher amount can be injected due to its lower expansion volume allowing a higher amount on column.

By comparing the peak areas of the 1-phenylpropan-2-one peak in the GC trace of the extraction, to a 1-phenylpropan-2-one standard the extraction efficiency was good (98.96 ± 0.55 %). It was also seen that there were no additional peaks present in the sample from eluting with chloroform compared with methanol (Appendix Figure 16). This suggests that chloroform is compatible with the SPE cartridge and sorbent.

**Table 5-8: Solid phase extraction recovery of P2P eluted with chloroform determined by GCMS peak area. Uncertainty shown is 1 std. dev. (n = 2)**

| Elution volume (mL) | Elution number | Recovery ± 1 SD (%) |
|---|---|---|
| 1 | 1 | 98.96 ± 0.55 |
| | 2 | 0 (< LOD) |

### 5.4.2 Evaporation and Reconstitution Analyte Recovery

#### *5.4.2.1 Effect of Temperature*

The effect of the evaporation temperature on the recovery of each analyte was investigated for each fraction. This was observed by allowing each fraction to evaporate to dryness under a stream of nitrogen at different temperatures.

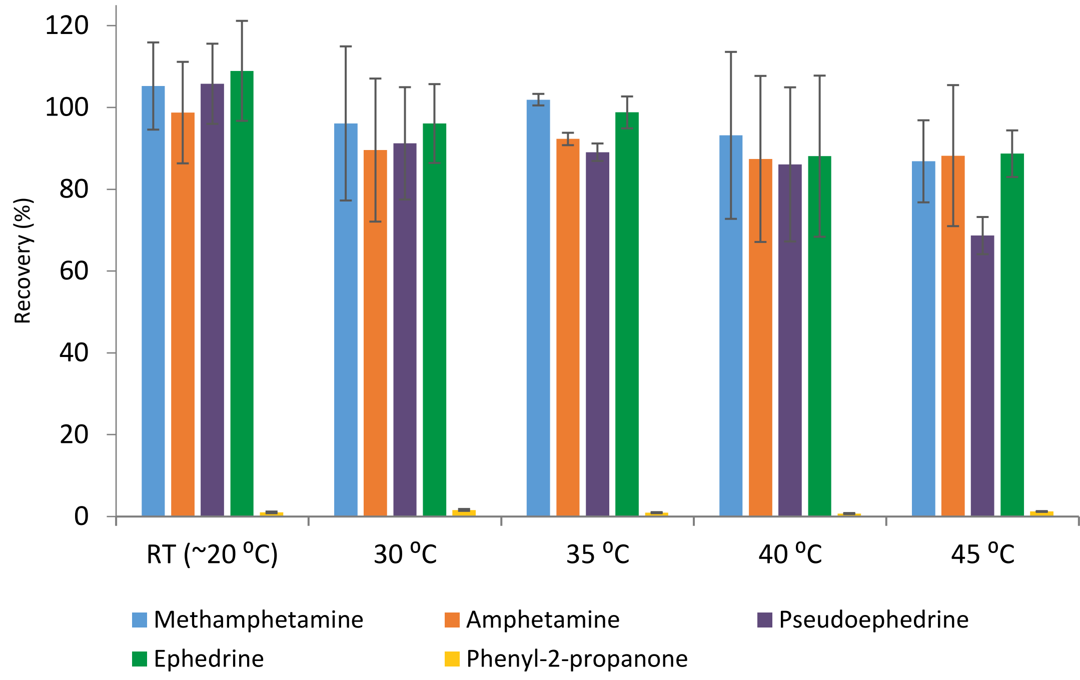


Figure 5-20: Effect of evaporation temperature on the recovery of the drug target analyte. Note 1-phenylpropan-2-one recovery was determined via GC-MS whilst recovery of other substances was determined using LC-MS. The error bars shown are 1 std. dev (n = 2).

As could be expected, the recovery of the analytes seemed to decrease for most compounds as the drying temperature increased as can be seen in Figure 5-20. A compromise must also be taken as the time it takes for the evaporation of the extract to occur increases as the drying temperature decreases lowering the throughput of the method (for methanol extracts it took approximately 50 minutes to evaporate to dryness at 20 °C compared with 20 minutes at 45 °C). For this reason, a trade-off needs to be made in terms of time taken for extracts to dry and recovery of analyte. The temperature chosen to be undertaken for further experiments was 35 °C as the recovery for all analytes tested beside 1-phenylpropan-2-one was acceptable with the recovery ranging from 89 % to 101.9 %.

As 1-phenylpropan-2-one was lost with evaporation to dryness the decision was made to alter the evaporation method to try and account for this loss of recovery. Firstly, an attempt was made to change the solvent to a more volatile solvent such as DCM and chloroform, however significant losses of 1-phenylpropan-2-one still occurred.

As solvent evaporation could not be used to concentrate the samples, the compounds were eluted in minimal solvent, as this ensures that the concentration of 1-phenylpropan-2-one will be as high as possible and hence make 1-phenylpropan-2-one more likely to be detected.

This optimisation was performed by loading a 50 mL milli Q water sample spiked with 1-phenylpropan-2-one and eluting fraction 1 see section 5.4.1.4

### 5.4.2.2 Effect on Addition of Acid to Basic Extract

As the second fraction collected via elution of the SPE extraction contained the basic analytes such as the amphetamine type stimulants themselves, the decision was made to acidify the extract prior to evaporation and reconstitution. This was performed as it was believed that the drugs when protonated and in their hydrochloride salt form would be less volatile and as a result their loss due to evaporation would be limited. As can be seen the recovery of the drugs improved slightly with the addition of hydrochloric acid.

Table 5-9: Effect of recovery of basic compounds in the evaporation at 35 °C of 1 M ammonia in methanol relative to samples evaporated with the addition of 200 µL HCl in methanol (0.1 M).

| Compound | Recovery of non-acidified extracts relative to acidified (% ± 1 SD n = 3) |
|---|---|
| Methamphetamine | 91 ± 8.6 |
| Amphetamine | 87 ± 10.1 |
| Pseudoephedrine | 92 ± 7.5 |
| Ephedrine | 96 ± 9.5 |

Table 5-9 shows the relative recovery of samples evaporated in 1 M ammonia in methanol without the addition of HCl, compared to with (200 µL of pH = 1 HCl adjusted MeOH). In general, there appeared to be a slight increase in recovery of all compounds tested with the addition of acid then without with the recovery of amphetamine benefiting the most with the addition of acid.

Acidification of the solution prior to evaporation resulted in the formation of ammonium chloride salt which is evidenced by the large amount of white solid on the edges of the test tube (Figure 5-21). This white solid along with the residue was reconstituted in the 95 % acetonitrile 5 % aqueous formic acid solution prior to analysis.

**Figure 5-21: Comparison of ammonia in methanol extract evaporation with (left) and without (right) the addition of hydrochloric acid.**

The formation of this ammonium chloride was not an issue with the samples analysed via LCMS as the solid dissolved during the reconstitution step, however when the method was later altered so that the fraction could be analysed via GCMS the issue arose where the ammonium chloride was not soluble in the organic solvents used for direct injection into the GCMS, as a result the acid couldn't be used to achieve a higher recovery for the basic compounds.

#### *5.4.2.3 Effect of silanisation of glassware on analyte recovery*

It has been shown that loss due to absorption can be a major issue for basic compounds such as amines. This is particularly when low level analysis is being conducted. For this reason it is necessary to deactivate or silanise the surface of the glassware [261]. This is typically done by derivatising the glass by covalently binding a silicone layer on the surface most commonly with dichloro(dimethyl)silane (DCDMS). This silanisation makes the surface of the glassware more hydrophobic which has been shown to prevent loss of polar compounds due to adsorption. As can be seen from Figure 5-22 below the dichloro(dimethyl)silane (DCDMS) can react with either one or two silanol (Si-OH) groups. After rinsing with toluene to remove any remaining DCDMS the glassware was rinsed with methanol to convert the residual chlorine to methoxy groups. If the surface is not rinsed with methanol immediately after silanisation the moisture from the air can covert the residual chlorine to silanol groups.

Si–OH
Si–OH
Si–OH
Dichlrodimethysilane (DCDMS)
- HCl
$CH_3OH$
- HCl

Figure 5-22: Silanisation of the glass surface using dichloro(dimethyl)silane (DCDMS).

Figure 5-23 is an image of milli Q water on the surface of both silanised glass and unmodified glass. Despite the contact angle of the glass not being measured due to the curved surface of the vessel a clear increase in water contact angle was observed. This was expected as the surface becomes more hydrophobic during the silanisation as the silanol groups on the surface are removed, resulting in less interaction between the polar water and hydrophobic surface. This change in properties of the glass is what limits the loss due to adsorption of polar molecules to the surface of the vessel.

Figure 5-23: Top down view of water droplet showing the effect of silanisation on the hydrophobicity of the surface of the glass. The test tubes in the image are in order from top to bottom: 10 µL milli Q water on silanised glass tube, 10 µL milli Q water on unmodified glass tube, 100 µL milli Q water on silanised glass tube and 100 µL milli Q water on unmodified glass tube. For side view of droplets see Appendix Figure 17

The effect on silanisation of the glass surface was investigated by evaporating spiked 1 M ammonia in methanol solution (the elution solvent for the second fraction) in both silanised and unmodified glass tubes. It is important to note that the recoveries shown are comparing the amount recovered of the unmodified samples relative to those evaporated down in silanised vials.

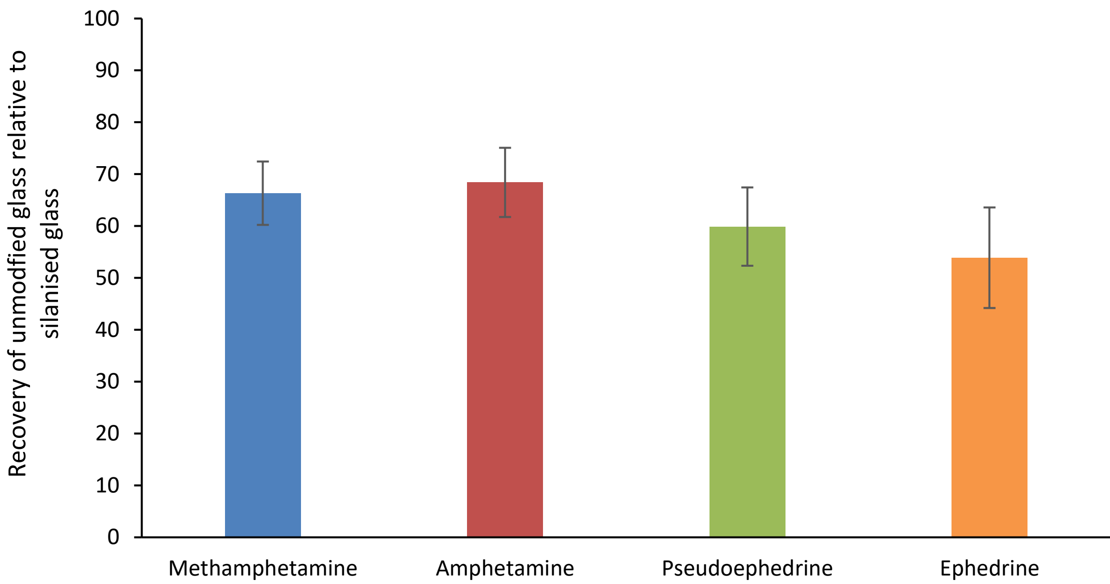


Figure 5-24: Effect of silanisation on the recovery of compounds present in the second elution of solid phase extraction process (n = 3).

As can be seen from Figure 5-24 the recovery of all compounds was significantly lowered when samples were evaporated in unmodified glass tubes compared to those in the silanised test tubes. For this reason, all glassware was silanised before use.

### 5.4.3 Effect of Filtration

As some substances have a high affinity for the solid particulate matter an increase in recovery may be seen if the samples are not filtered prior to analysis. However, when filtering isn't implemented prior to solid phase extraction it becomes difficult to achieve a constant flowrate through the SPE cartridge due to build-up of solids on the surface of the cartridge. Filtering of the wastewater samples was conducted using several different stages, to increase the throughput and limit blocking of the filtration membrane. Firstly, the wastewater was filtered through a wire mesh to remove the majority of the large solids, (believed to be majorly fibrous paper based on appearance), following this samples were filtered through a 1.6 µm and subsequently a 0.45 µm nylon membrane. Figure 5-25 below shows wastewater samples at various stages of filtering.

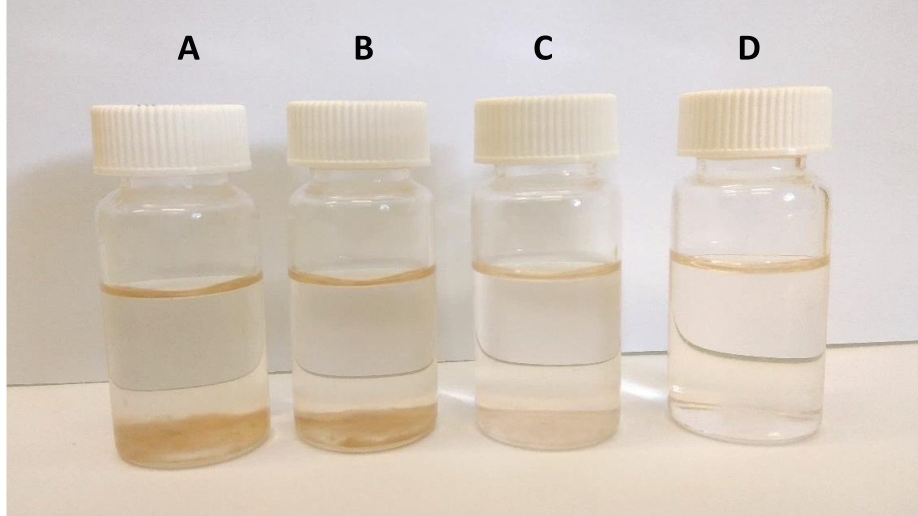


Figure 5-25: Image showing wastewater from Christies Beach wastewater treatment plant at different stages of filtering. A. Raw wastewater, B. mesh filtered wastewater, C. 1.6 µm nylon membrane filtered wastewater D. 0.45 µm nylon filtered wastewater.

Unsurprisingly, it can be seen that when the pore size reduced with each subsequent filtration the number of solids decreased leading to an increase in clarity of the solutions. Wastewater samples were analysed with and without filtering to see the effect filtering has on the wastewater. Figure 5-26 below shows chromatograms for extracted samples which have and have not been filtered.

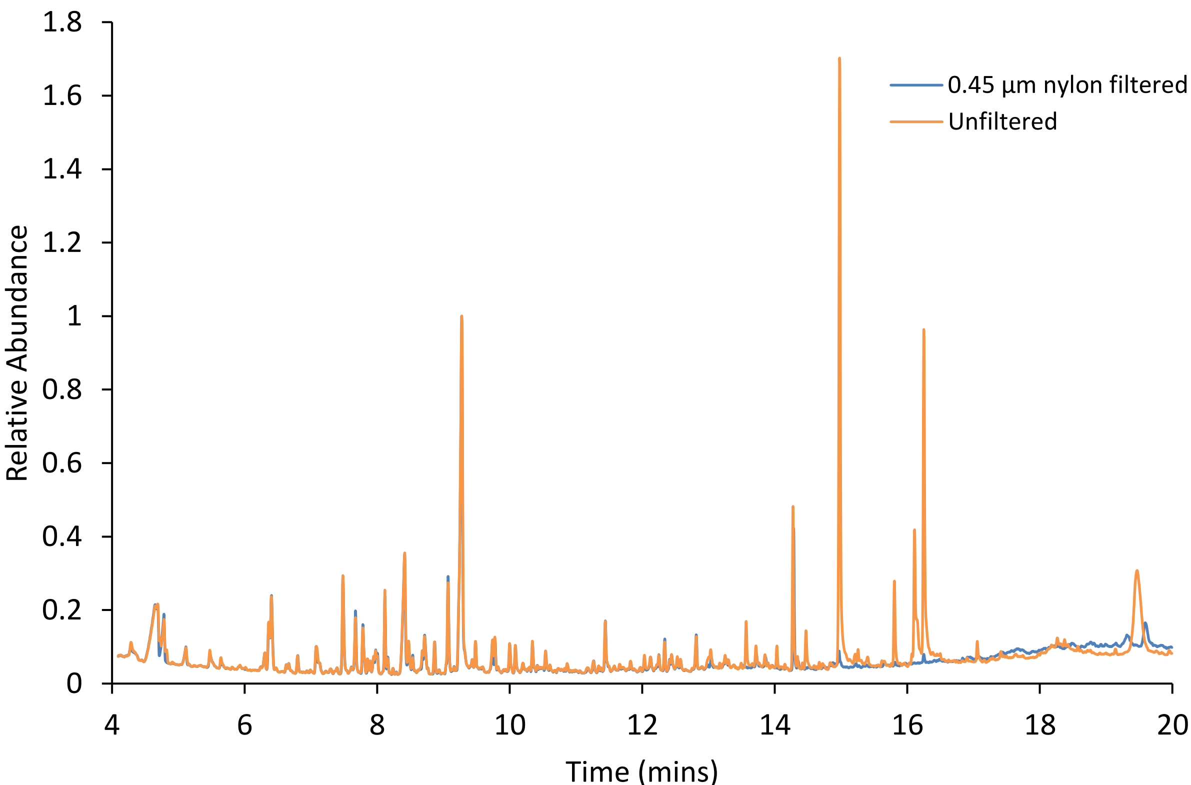


**Figure 5-26: Comparison of chromatograms obtained from the analysis of non-filtered and 0.45 um nylon filtered wastewater sample. The Wastewater sample used in both cases was a collected from Christies Beach on 17/02/2018.**

The flow rate of unfiltered samples through the solid phase extraction process was difficult to control with a reduction in flow rate occurring over time. This reduction was the result of solid build up on the SPE cartridge. As a result, the extraction had to be monitored throughout and in order to maintain the flow rate the vacuum pressure was continually adjusted.

As can be seen from Figure 5-26 above the main difference between the filtered and unfiltered samples is the absence of the peaks at 14.98, 15.81, 16.11 and 16.25 mins. These peaks have been identified as being long chain fatty acids. As these compounds are extremely hydrophobic in nature, at any given time a significant proportion is expected to exist in the solid phase compared to the aqueous phase and as a result these substances are removed when the sample is filtered.

In addition, the reduction in the peak heights and areas of some peaks was also observed. However, whether this is a result of the sorption to solid particulate matter or interaction with the 0.45 um nylon filter is unknown. In order to confirm that the reduction in these substances was in fact a result of their association with the solid particulate matter as opposed to interaction with the nylon membrane. The filtered solids were added to a small volume of chloroform and sonicated in an ultrasonic bath for 5 minutes. The chloroform extract was then analysed via GCMS (Figure 5-27).

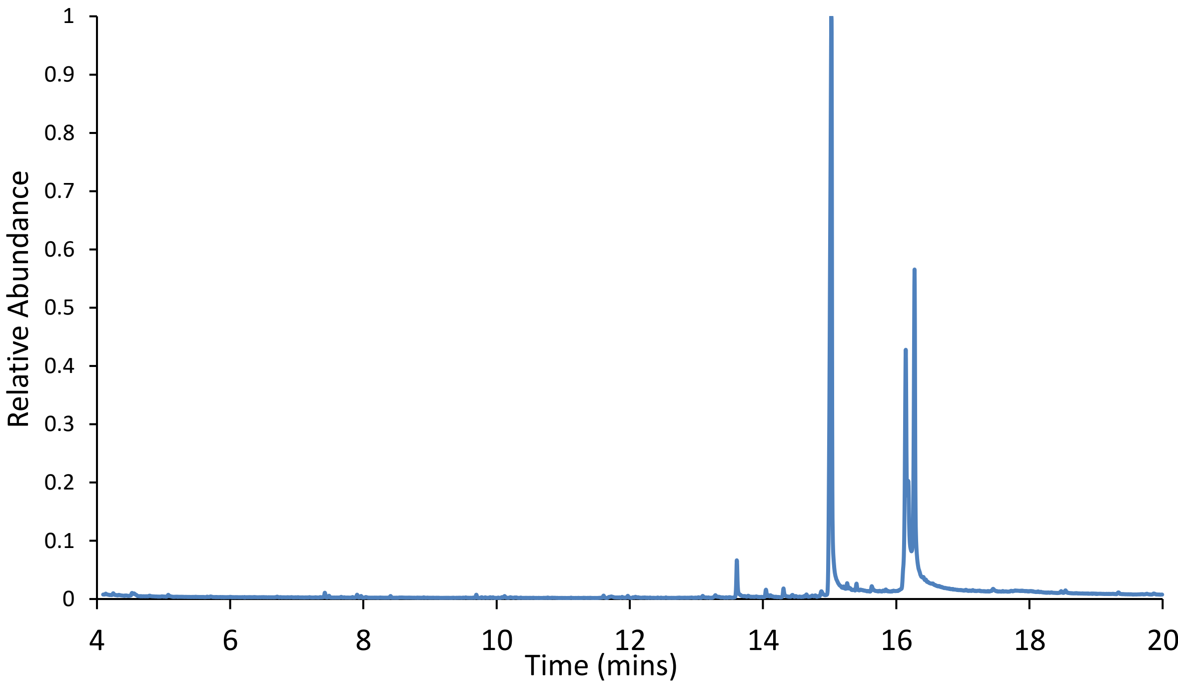


**Figure 5-27: Chromatogram obtained from a chloroform extract of filtered solids obtained from filtration of a Christies Beach wastewater sample.**

As could be predicted, Figure 5-27 above confirms that the reduction in the peak area in the filtered samples compared to the non-filtered samples was a result of their high partition coefficient (octanol/water) leading to their high adsorption to the solid particulate matter.

One interesting observation was the difference in appearance of the bolivar high salinity wastewater samples compared to the samples from all of the other wastewater treatment facilities. The bolivar high salinity samples appeared dark grey in colour whist the samples from the other treatment plants had a pale brown colour. This difference in appearance was obvious when looking at the filtered solids (Figure 5-28). One possible reason for this difference in colour is that the bacterial composition of the sewage may be different.

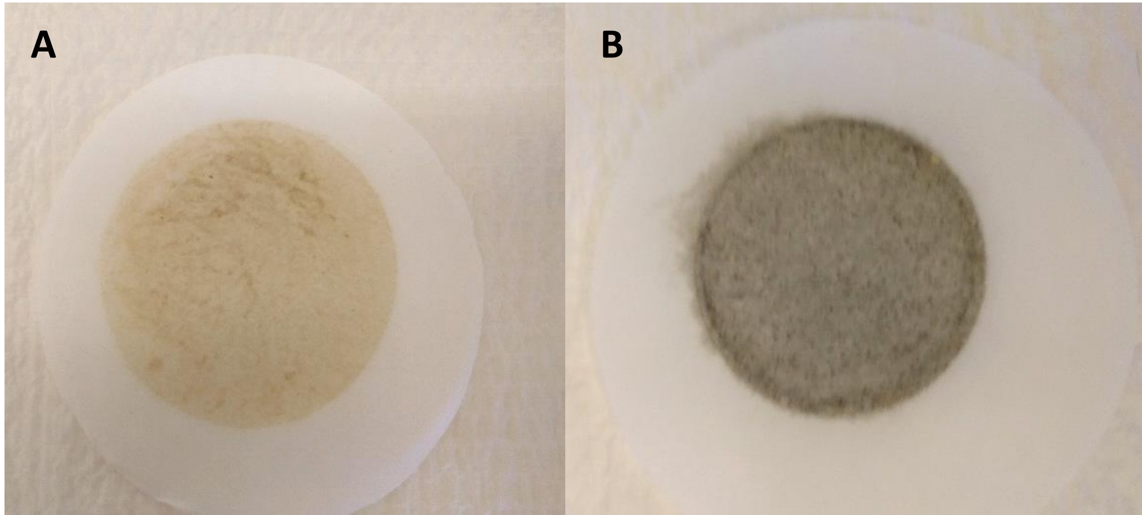


Figure 5-28: Image showing the difference in the appearance of the filtered solids from the bolivar wastewater treatment facility (A) compared to the Bolivar high salinity wastewater treatment facility (B)

### Filtering of spiked milli Q water samples

To determine effect of filtration on the analyte recovery, filtered and unfiltered milli Q water samples were spiked with the target analytes (10 ng for the amines and 1 µg for other compounds) in duplicate. The peak areas of the compounds of interest in both the filtered and unfiltered samples were compared in order to calculate the recovery throughout the filtration process Table 5-10.

Table 5-10: Recovery of targeted compounds throughout the filtration process.

| Compound | Recovery through filtration process (% ± 1 SD n = 2) |
|---|---|
| Methamphetamine | 98 ± 5.1 |
| Amphetamine | 98 ± 6.8 |
| Pseudoephedrine | 97 ± 6.5 |
| Ephedrine | 101 ± 5.9 |
| Phenylpropan-2-one | 97 ± 9.5 |
| Benzaldehyde | 94 ± 9.6 |
| 4-methoxybenzaldehyde | 95 ± 8.4 |
| Safrole | 94 ± 10.1 |
| Piperonal | 97 ± 8.0 |

The recovery for all targeted compounds was high indicating that loss due to adsorption to the nylon membrane is minimal. However, Minimal loss due to adsorption to the membrane does not indicate that a loss in recovery of targeted compounds would not occur as a result of filtration as sorption to and entrapment within suspended solids may occur. This is much harder to test for and was investigated using humic acids as described later in section 7.3.

## 5.5 Overall Process Limit of Detection and Quantification

The limit of quantification of the first fraction collected during the solid phase extraction process was not as low as the compounds in the second fraction. This was due to both the pre-concentration factor of the SPE method being lower for the first fraction than the second fraction (50 c.f. 500) and the sensitivity of the LCMS method compared to the GCMS method. The limit of quantification of the neutral compounds ranged from approximately 20 - 100 µg/L. This corresponds to an original sample concentration of 0.4 – 2 µg/L.

The lower limit of quantification for each of the amines in the final SPE extract was 2 µg/L. This concentration corresponds to an original sample concentration of approximately 4 ng/L. The limits of detection and quantification is the same order of magnitude as other similar studies involving the analysis of amphetamines in wastewater [180, 237].

**Table 5-11: Limit of detection quantification for the overall extraction and analysis process developed. The values reported are based on the LCMS analysis method for the methamphetamine, amphetamine, ephedrine, pseudoephedrine, GCMS analysis using the SIM method for phenyl-2-propanone and he scan method for 4-methoxybenzaldehyde, safrole and piperonal. The limit of detection was chosen to have a signal to noise ratio greater than three whist for the limit of quantification a signal to noise ratio of ten was chosen.**

| Compound | Lower limit of detection (ng/L) | Lower limit of quantification (ng/L) |
| --- | --- | --- |
| Methamphetamine | 1.5 | 4 |
| Amphetamine | 1.5 | 4 |
| Pseudoephedrine | 1.5 | 4 |
| Ephedrine | 1.5 | 4 |
| Phenylpropan-2-one | 150 | 400 |
| 4-methoxybenzaldehyde | 700 | $2 \times 10^{-3}$ |
| Safrole | 350 | $1 \times 10^{-3}$ |
| Piperonal | 700 | $2 \times 10^{-3}$ |

## 5.6 Conclusion

GCMS and LCMS methods were optimised and validated. Derivatisation of P2P showed an improvement in its detection limits when analysed using LCMS. This method couldn't be rigorously investigated however, due to instrument failure, and instead the use of GCMS was implemented. Despite GCMS being used for the majority of the analysis, the LCMS method was significantly more selective and sensitive.

A solid phase extraction method was created for the extraction of several compounds of interest from wastewater. A flowrate of 1 mL/min for the extraction process was determined to be optimum due to it being the highest rate tested which didn't result in a significant loss of the targeted analytes. The percentage of methanol used in the wash process seemed to significantly affect the analyte recovery with the highest percentage of organic modifier suitable being 10 %. The sample loaded onto the column didn't seem to influence the recoveries of the targeted compounds for the volumes tested. However, one drawback to increasing the sample volume loaded was the increase in other compounds. This was particularly an issue with the less selective GCMS method and for this reason the optimal sample volume selected was 50 mL. In order to get the highest concentration of the targeted compounds as possible the elution volume for the fraction analysed via GCMS (elution 1) was

selected to be 1 mL, the minimum volume in which the targeted compounds were eluted from the column. The elution volume needed to be optimized for the first fraction of the extraction process as significant loss of these compounds occurred when evaporation and reconstitution was performed. For the fraction containing the amines (elution 2) an elution volume of 4 mL was chosen as the samples could be evaporated at 30 °C and reconstituted without significant loss of recovery. Filtration of the samples prior to extraction did not affect the targeted compounds as no significant loss occurred.

The effect of silanisation on the recovery of the targeted compounds was investigated. This showed that there was a significant improvement in the recovery of the amines tested when the glassware was silanised. The optimised method showed good recovery for all compounds of interest, with the recovery of the previously reported compounds (amines) comparable to those reported in literature.

# Chapter 6 Final Methods Employed

## 6.1 Filtration of Wastewater Samples

Unless otherwise stated, all wastewater samples were filtered in three stages. The first filtration was through a wire mesh to remove large solids, followed by filtration through a 1.3 µm and subsequently 0.45 µm Nylon filter membrane.

## 6.2 Solid Phase Extraction of Wastewater Samples

The solid phase extraction was performed on an Oasis® MCX cartridge (3 mL, 6 mg, 30 µm) similar to the method conducted by Van Nujis et al. [183]. The cartridge was preconditioned with methanol (8 mL), Milli-Q water (6 mL) and pH = 2 milli-Q water (6 mL) (pH adjusted with 37% hydrochloric acid). After loading the sample onto the cartridge at a flow rate of ~ 1 mL/min, the cartridges were washed with 4 mL of 10 % aqueous methanol pH = 2 (HCl adjusted). Cartridges were left to dry on the SPE vacuum manifold with the vacuum pump left on for approximately 45 minutes. Analytes were collected in two fractions. The first fraction was eluted with chloroform (1 mL) and the second with 1 M (~ 4.3 % w/w) ammonium hydroxide in methanol (4 mL) into glass vials and silanised glass tubes respectively.

To the second fraction 200 µL of HCl acidified methanol (0.1 M) was then added prior to evaporation of the solvent over a stream of high purity nitrogen at 30 °C. This fraction was then reconstituted in an aqueous solution of 5% acetonitrile and 0.1% formic acid (100 µL).

## 6.3 GC-MS Analysis

Unless noted within text all analysis of the first SPE fractions were analysed using an Agilent Technologies 7890A GC system coupled to an Agilent 5975C mass spec detector fitted with an Agilent 7693 autosampler. Data was acquired and analysed using the MSD ChemStation software version E.02.02.1431

### 6.3.1 Agilent GC-MS Instrument Parameters

Table 6-1: Agilent Gas Chromatography Mass Spectrometry Instrumental Parameters:

| | |
|---|---|
| **Column:** | DB-5MS Ultra Inert, 30 m x 0.25 mm 0.25 µm |
| **Column Temperature Gradient:** | 40 °C held for 2 minutes<br>Heat rate 45 °C/min<br>Final temperature 280 °C |
| **Carrier gas** | Helium |
| **Flow Rate:** | 1.2 mL/min |
| **Split ratio** | Split-less |
| **Injection Volume** | 0.5 µL |

### 6.3.2 Varian GC-MS Instrument Parameters

Unless otherwise specified all kinetic studies involving 1-phenylpropan-2-one were conducted using a Varian CP 3800 GC coupled to a Varian Saturn 2200 ion trap mass spec detector. Data was collected and analysed using the MS Data review software version 6.5 service pack 1 (Varian, CA).

Table 6-2: Varian Gas Chromatography Mass Spectrometry Instrumental Parameters:

| | |
|---|---|
| **Column:** | DB-5MS Ultra Inert, 30 m x 0.25 mm 0.25 µm |
| **Column Temperature Gradient:** | 60 °C<br>45 °C/min<br>280 °C |
| **Carrier gas** | Helium |
| **Flow Rate:** | 1.2 mL/min |
| **Split ratio** | 20:1 |
| **Injection Volume** | 1 µL |

## 6.4 LC-MS/MS Analysis

After pre-concentration the samples were analysed along with calibration standards using liquid chromatography (Waters Aquity fitted with fitted with a Phenomenex Kinetex C18, 2.6 µm; 2.1 mm × 100 mm LC column) coupled with tandem mass spectrometry (Waters Micromass Quattro micro) according to the instrumental parameters outlined in section 6.4.1.

### 6.4.1 Liquid Chromatography Mass Spectrometry Instrument Parameters

All LC-MS/MS was conducted using a Waters Aquity UPLC system (Waters, UK) consisting of an Acquity UPLC binary solvent manager coupled to a Micromass Quattro micro atmospheric pressure ionisation (API) tandem quadrupole system with a hexapole gas collision cell (Waters). The UPLC was fitted with a Phenomenex Kinetex C18 (2.6 µm; 2.1 mm × 100 mm) LC column, and guard column comprised of the same material. Samples were injected into the HPLC using an auto sampler (Acquity UPLC sample manager) and the injection needle was washed with acetonitrile between injections.

Binary gradient elution was performed according to the method as follows: 0 – 2 min 5 % solvent B which was ramped to 50 % by 10 min and further increased to 90 % by 14 minutes and held for 1.5 minutes before returning to the initial conditions (95 % A) by 19 minutes and

held for a further 5 min to allow the column to equilibrate before the subsequent run. For instrumental parameters see Table 6-3 below.

Table 6-3: Water Aquity Ultra Performance Liquid Chromatography Instrumental Parameters:

| | | | |
|---|---|---|---|
| **Column Temperature:** | 30 °C | | |
| **Column** | Phenomenex Kinetex C18 (2.6 µm; 2.1 mm × 100 mm) | | |
| **Mobile Phase Composition:** | A: 0.1% formic acid. | | |
| | B: 0.1% formic acid in Acetonitrile | | |
| **Gradient** | **Time (mins)** | **% A** | **% B** |
| | 0 - 2 | 95 | 5 |
| | 10 | 50 | 50 |
| | 14 -15.5 | 10 | 90 |
| | 19 -24 | 95 | 5 |
| **Flow Rate:** | 0.4 mL/min | | |
| **Injection Volume** | 10 µL | | |

Specific mass spectrometry parameters such as fragmentation voltage, collision energy and ionisation mode were optimised separately for each compound via direct injection. The decision was made to only use one mass transition for the deuterated standards as the samples were spiked the presence of the deuterated standards was known and confirmation was achieved with the use of retention time. The optimised conditions and mass transitions used for each compound and the selected MRM transitions are detailed in Table 6-4.

Table 6-4: Optimised mass spectrometer parameters and MRM transitions used for the targeted compounds and the deuterated internal standards

| Compound Name | Transition | Dwell Time (s) | Collision Energy (eV) | Cone Voltage (V) | Quantification / Qualification |
|---|---|---|---|---|---|
| **Methamphetamine** | 150.1 > 119.1 | 0.05 | 6 | 13 | Qual. |
| | 150.1 > 91.1 | 0.05 | 15 | 13 | Quant. |
| **Methamphetamine-D5** | 155.1 > 121.1 | 0.05 | 15 | 13 | Quant |
| **Amphetamine** | 136.1 > 119.1 | 0.05 | 6 | 11 | Qual. |
| | 136.1 > 91.1 | 0.05 | 13 | 11 | Quant. |
| **Amphetamine-D5** | 141.1 > 93.1 | 0.05 | 13 | 11 | Quant |
| **Pseudo/ephedrine** | 166.1 > 148 | 0.05 | 9 | 12 | Qual. |
| | 166.1 > 117 | 0.05 | 15 | 13 | Quant. |

Mass spectra were recorded using positive electron spray ionisation (ESI), with a source gas temperature of 350 °C with a gas flow of 500 L/hr and the source set to 80 °C. The capillary voltage set at 2.5 kV (Table 6-5) and argon was used as the collision gas for all MS/MS experiments unless stated otherwise.

MassLynx (version 4.1) (Waters, U.K.) software was used to acquire and analyse the obtained data.

Table 6-5: Mass Spectrometry Instrumental Parameters:

| | |
|---|---|
| **Ion Mode** | Positive electrospray ionisation (ESI) |
| **Capillary Voltage** | 2.5 kV |
| **Extractor Voltage** | 4 V |
| **RF lens Voltage** | 0.4 V |
| **Source Temperature** | 80 °C |
| **Desolvation Temperature** | 350 °C |
| **Desolvation Gas (Nitrogen) Flow Rate** | 500 L/hr |

## 6.5 Molecular Modelling - Gauss View Calculations

### 6.5.1 Energy Optimisation Calculations

In order to calculate the energy difference between E and Z stereoisomers calculations were performed using Gaussian09. The energy of each structure was optimised using density functional theory (DFT) with the B3LYP functional and the 6-31G(d) basis. All calculations were performed in vacuum at 298.15 K with the parameters outline in Table 6-6 below.

Table 6-6: Guassian calculation setup for the energy optimisation of the E and Z isomers of 1-phenylpropan-2-one oxime

| | |
|---|---|
| **Job Type** | Optimisation |
| **Method** | Ground state<br>Density Functional Theory (DFT)<br>B3LYP Hybrid functional<br>Default Spin |
| **Basis Set** | 6-31G(d) |

### 6.5.2 UV -Vis gaussian Calculation

Expected UV-Vis spectra were calculated by performing an energy calculation on the DFT optimised molecules. This was done by calculating the energy using density functional theory using the time dependent-SCF functional and the 6311G (s,p) basis level.

## 6.6 Data Analysis

Software used for further analysis of instrument data was Microsoft® Excel (Office 2013). This software package was used on personal computers with Windows® Seven operating systems.

The main data analysis involved calculating the mean ($x_m$) of the data as per Eq. 6-1 (where $x_i$ is the $i$th data point and $n$ is the number of data points) and the standard deviation (σ) as per Eq. 6-2.

$$x_m = \sum_{i=1}^{n} \frac{x_i}{n} \qquad \text{Eq. 6-1}$$

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - x_m)^2}{n-1}} \qquad \text{Eq. 6-2}$$

# Chapter 7 Stability of Compounds Related to the Synthesis of Amphetamines Type Stimulants

## 7.1 Chapter Overview

Knowledge on the fate of some precursors and by-products from the synthesis of ATS via some clandestine disposal methods such as in soil has been reported previously by other research groups [264, 265]. However, despite being believed to be the most common method of disposal, there is very little to no knowledge on their stability in wastewater.

This chapter details the stability of: 1-phenyl-2-propanone, 4-methoxybenzaldehyde, benzaldehyde, safrole and piperonal in wastewater stored at room temperature (≈ 25 °C) and in the freezer at -20 °C.

The potential identity of degradation products produced from the targeted compounds was investigated by analysing a degraded spiked wastewater sample. Knowledge on degradation products provides useful information as it can identify other possible compounds of interest. In addition, it can also help determine the extent of degradation of an old sample.

As precipitation of humic acids may occur when water samples are acidified, loss of analyte molecules may occur due to entrapment. This potential entrapment was investigated by spiking water samples with both the targeted substances and humic acids at levels higher than those expected in wastewater.

As targeted compounds are required to be stable and not suffer from significant loss in wastewater, the results presented in this chapter help to ensure that the compounds tested are appropriate candidates for use as targeted compounds and may have the potential to be used in the future to determine the extent of clandestine synthesis in a given area.

## 7.2 Stability in Wastewater

The majority of studies on the stability of drug target residues in wastewater have looked at the conditions and pH in which a collected sample is stored at not the conditions experienced within the sewer network.

A study mimicking a typical sewer system was performed by Thai et. al. in conditions which involved the use of both aerobic and anaerobic biofilms/sediments on several laboratory scale sewage reactors [213]. This research used an elaborate setup with reactors that mimicked the conditions likely to be found in different segments and included a control sewer containing no biofilms along with a rising main (anaerobic biofilm), and gravity sewer (aerobic and anaerobic biofilm). Despite having enhanced degradation of several compounds, the results for amphetamine type stimulants seemed to agree with other studies and showed MDMA and methamphetamine were relatively stable for the 12 hrs investigated under all sewage conditions. In addition those which utilise conditions likely experienced when the drug target residue enters the sewer network generally only involved sampling at intervals of around 12 hrs [261] which is significantly longer than typical residence times, which is normally just a couple of hours. The stability of illicit substances in unaltered wastewater samples was first conducted at appropriate intervals (every hour for 12 hours) by van Nuijs [262] and later by Senta in 2012 [263].

Despite a lot of information on the stability of the amphetamines themselves, there is very little to no information in the published literature investigating the stability of precursors in both unaltered and pH adjusted wastewater.

The stability of the chemicals of interest was investigated in wastewater to determine appropriate storage times and percentage lost due to biodegradation/transformation. Wastewater samples were spiked with internal standards (100 ng/mL) at levels well above their limit of detection and stored under aerobic conditions at room temperature (≈ 25 °C) and in the freezer at (-20 °C). Samples were then analysed in duplicate at regular intervals over the course of 35 days using GCMS as per the method described in section 6.3.2.

### 7.2.1 Creation of Wastewater Sample

A wastewater sample representative of all four of the major wastewater treatment plants in SA was created by mixing wastewater samples from 8-16/04/2017 in ratios proportional to

the average weekly flowrate of the treatment plant. As it is also known that there is day-to-day variation in the contents of wastewater the volumes used from each wastewater plant were selected to all be divisible by 7 and equal volumes from each week day were used to create a sample which was approximately 4 L (4.06 L) (Table 7-1).

**Table 7-1: Volume of wastewater used from each wastewater treatment facility to create a pooled sample for analysing the stability of benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzaldehyde, safrole and piperonal.**

| Wastewater Treatment Plant | Volume of Wastewater Used (mL) | Volume used per Day (mL) |
|---|---|---|
| Bolivar | 2450 | 350 |
| Bolivar High Salinity | 350 | 50 |
| Christies Beach | 490 | 70 |
| Glenelg | 770 | 110 |

As opposed to comparing the peak area of the extraction at t = 0 hrs to the subsequent extractions, the decision was made run a sample spiked directly before each set of extractions as a control. This was done so that not only any changes in the matrix and hence matrix effects but also inter-day variation with the extraction could be accounted for.

In order to achieve this, the combined wastewater sample was split into two 2 L aliquots. One of which was spiked initially (t = 0 hrs) with 200 µg of each targeted compound (200 µL of 1 mg/mL mixed drug standard in acetonitrile).

At regular time intervals a 50 mL aliquot from each of the combined wastewater samples was collected in duplicate and 5 µg of the mixed drug standard (50 µL of 100 µg/mL mixed drug standard in acetonitrile) was added to the unspiked 50 mL aliquots immediately prior to solid phase extraction. A summary of this process is given in Figure 7-1.

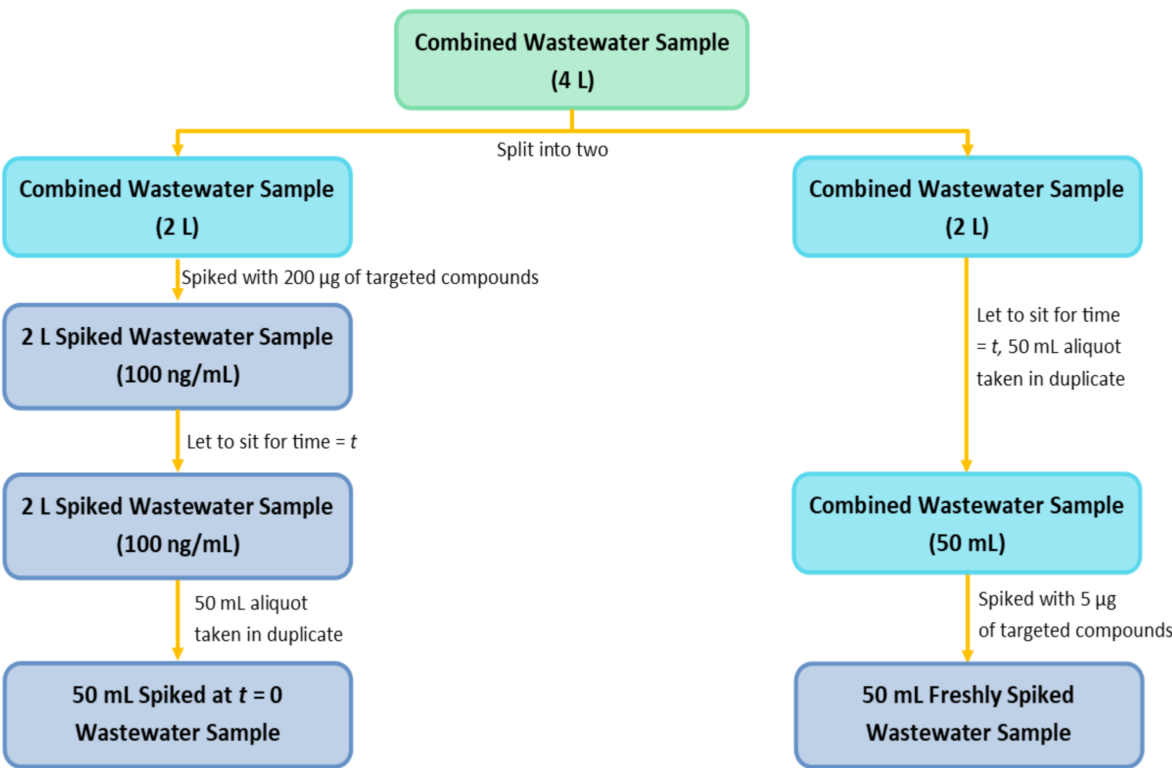


Figure 7-1: Summary of the process used for testing the stability of benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzladehyde, safrole and piperonal.

### 7.2.2 Room Temperature (≈ 25 ℃) Stability

The stability of targeted compounds in wastewater was assessed by extraction of the spiked wastewater samples followed by analysis via GC-MS.

In order to determine the stability of the compounds of interest, the peak area of wastewater samples spiked at t = 0 were compared to that of the control samples spiked immediately prior to the extraction. The relative peak area for each targeted compound was plotted against time and fitted with a trendline (Figure 7-2-Figure 7-6). The fitted trendline was then used to calculate the rate constant and the half-life of the target compounds. These plots didn’t match any typical order kinetics, likely due to the fact that the degradation of the targeted species is complicated and may involve many different degradation pathways, and the “food” source of any bacteria changing overtime as compounds in the wastewater are biodegraded.

Despite the kinetics of the degradation of the drug target residues imitating neither first nor second order kinetics, the fit more closely resembled that of first order kinetics. For this reason, first order kinetics was assumed and an exponential trendline was utilised with a y intercept of 100 %.

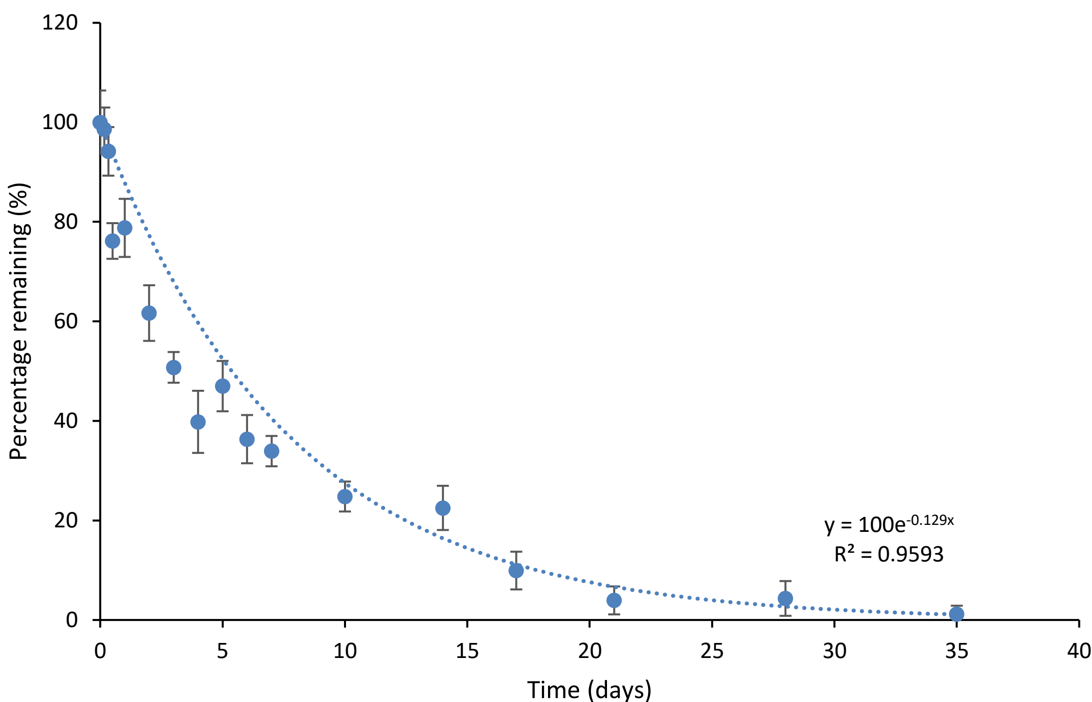


Figure 7-2: Percentage of benzaldehyde remaining in a spiked wastewater sample left to degrade at RT (~ 25 °C). Percentage remaining was determined from the GCMS peak area of benzaldehyde (6.29 mins) in the 106 m/z extracted ion chromatograms over 35 days. Error bars are ± 1 SD (n = 2).

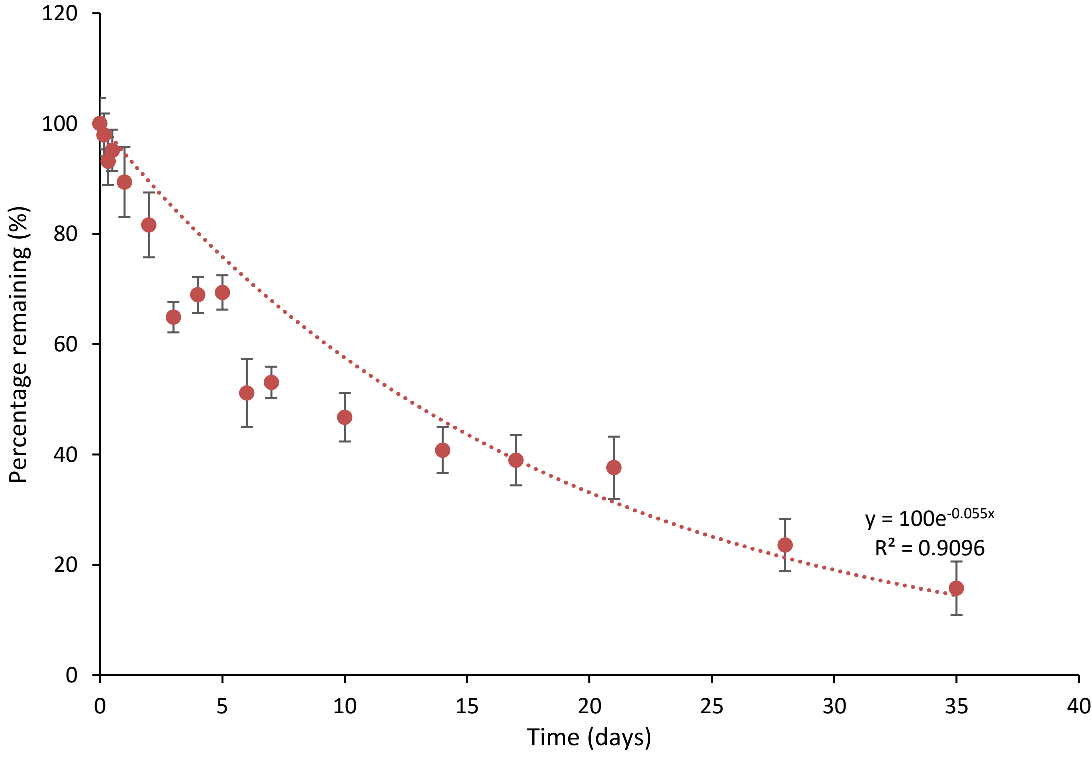


Figure 7-3: Percentage of 1-phenylpropan-2-one remaining in a spiked wastewater sample left to degrade at RT (~ 25 °C). Percentage remaining was determined from the GCMS peak area of 1-

phenylpropan-2-one (8.23 mins) in the 91 m/z extracted ion chromatograms over 35 days. Error bars are ± 1 SD (n = 2).

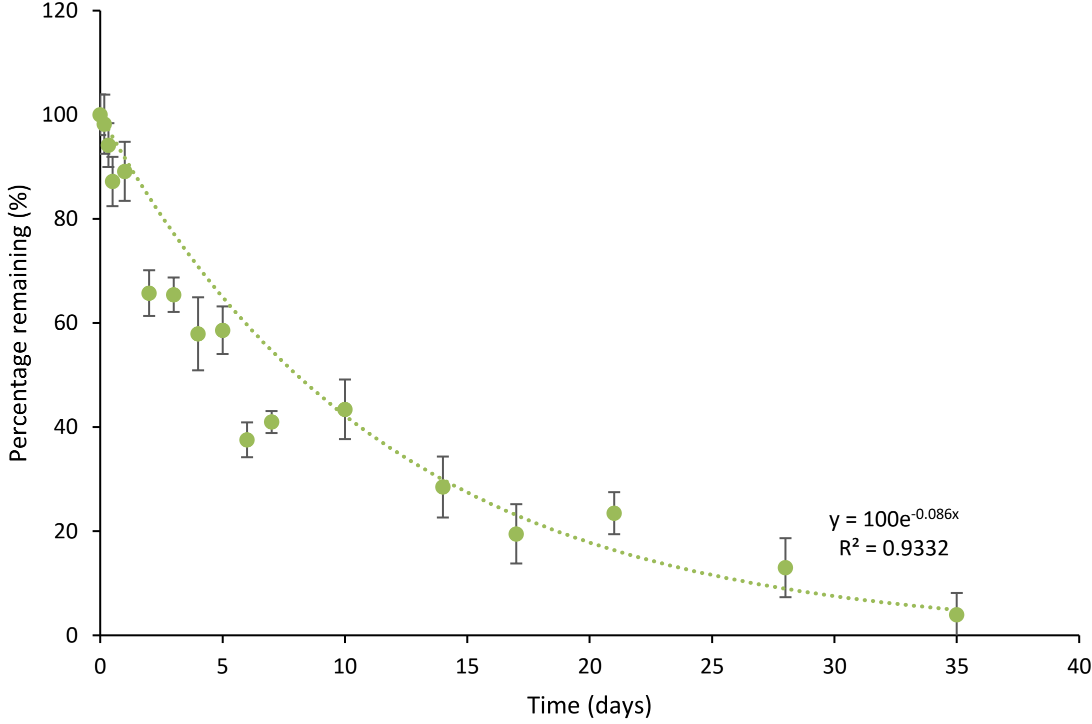


Figure 7-4: Percentage of 4-methoxybenzaldehyde remaining in a spiked wastewater sample left to degrade at RT (~ 25 °C). Percentage remaining was determined from the 4-methoxybenzaldehyde GCMS peak area from the 135 m/z extracted ion chromatogram (9.53 mins) over 35 days. Error bars are ± 1 SD (n = 2).

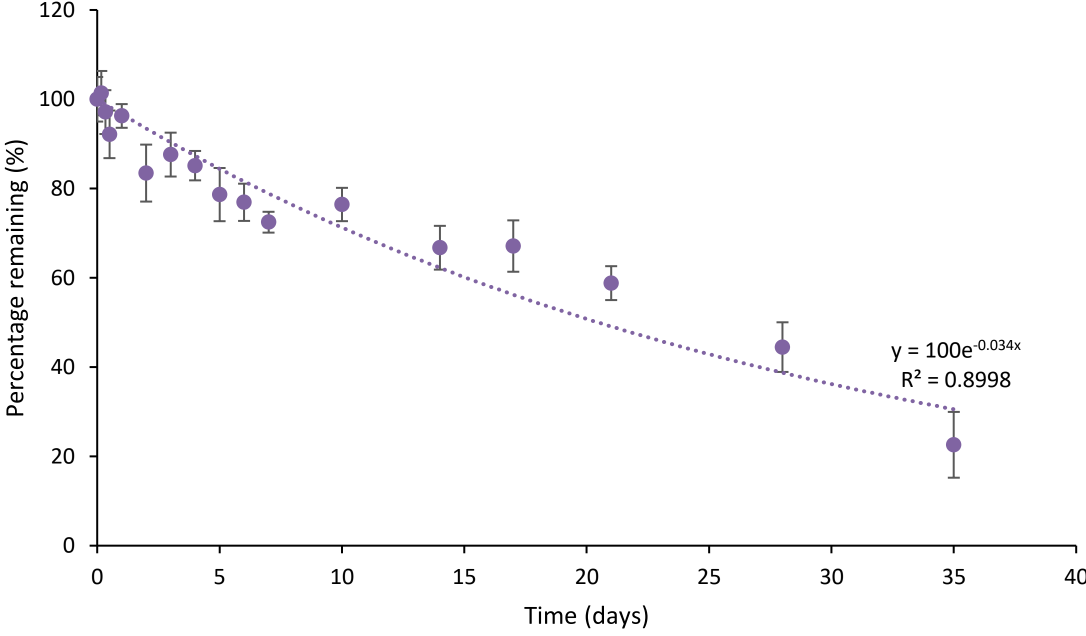


Figure 7-5: Percentage of safrole remaining in a spiked wastewater sample left to degrade at RT (~ 25 °C). Percentage remaining was determined from the GCMS peak area of safrole in the 162 m/z extracted ion chromatogram (9.86 mins) over 35 days. Error bars are ± 1 SD (n = 2).

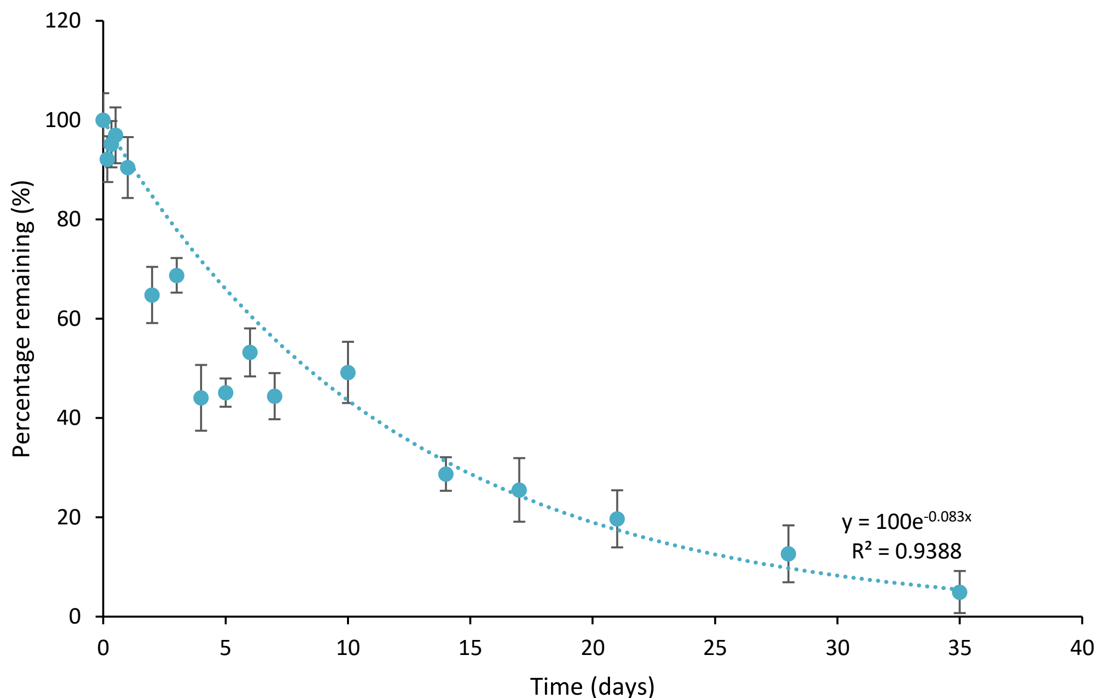


Figure 7-6: Percentage of piperonal remaining in a spiked wastewater sample left to degrade at RT (~ 25 °C). Percentage remaining was determined from the GCMS peak area of piperonal in the 149 m/z extracted ion chromatogram (10.30 mins) over 35 days. Error bars are ± 1 SD (n = 2).

#### *7.2.2.1 Determining half-life of targeted compounds in wastewater*

The exponential fits have the form:

$$y = Ae^{-kx} \qquad \text{Eq. 7-1}$$

Where $y$ is the percentage remaining, $A$ is the y-intercept, $k$ is the rate constant and $x$ is the time in days. The half-life was can be determined by rearranging the line of best fit:

$$\frac{\ln\left(\frac{A}{y}\right)}{k} = x \qquad \text{Eq. 7-2}$$

When the amount remaining y is half of the original ($\frac{A}{y}$ = 2) the half-life can be calculated.

$$\frac{\ln(2)}{k} = t_{1/2} \qquad \text{Eq. 7-3}$$

The uncertainty in the half-lives was determined from the standard error in the rate constant, k. The standard error in the slope, -k, was determined by using the LINEST

function in excel for the linear equation $ln(y) = -kx + ln(A)$. The half-lives of the target compounds are listed in Table 7-2 below.

Table 7-2: Half-lives of benzaldehyde, 4-methozybenzaldehyde, piperonal, 1-phenylpropan-2-one and safrole in wastewater stored at room temperature (~ 25 °C) determined by GCMS peak area.

| Compound Name | Rate constant, k ($day^{-1}$) | Half-life (days) |
|---|---|---|
| Benzaldehyde | 0.129 ± 0.0046 | 5.37 ± 0.19 |
| 4-methoxybenzaldehyde | 0.086 ± 0.0040 | 8.04 ± 0.37 |
| Piperonal | 0.083 ± 0.0036 | 8.34 ± 0.36 |
| 1-phenylpropan-2-one | 0.055 ± 0.0028 | 12.55 ± 0.64 |
| Safrole | 0.034 ± 0.0021 | 20.47 ± 1.25 |

As can be seen from Table 7-2 above the half-lives of the aldehydes were lower than the rest of the target compounds. This was expected as the aldehyde moiety is quite reactive and can undergo a series of chemical reactions.

Despite all of the half-lives being significantly longer than the residence time in the sewer network (typically a couple of hours) the half-lives could have a significant effect when analysing 24-hour composite samples, the most common sample technique utilised for wastewater analysis and sewage epidemiology.

Although the kinetics of the degradation products could also be investigated this was decided against as analysis of degradation products could lead to misleading results as: multiple degradation products could be produced from one compound, the degradation product could itself undergo further degradation and wastewater contains several other compounds which may also degrade creating one of the targeted compounds making it difficult to know for certain that the targeted peak was in fact due to the degradation of the desired compound.

#### *7.2.2.2 Potential identity of the degradation products*

The identity of some of the degradation products was obtained by spiking a 50 mL wastewater with a large amount of each compound (20 µg) leaving the sample for 14 days and analysing an extract of the wastewater for compounds not present in the original spike mix.

A chromatogram of the extracted wastewater sample is shown in Appendix Figure 23, with mass spectrums for peaks of interest along with their retention times are given in Appendix

Figure 24-20. A summary of the tentatively identified compounds resulting from the degradation of the target analytes in wastewater is given in Table 7-3 below.

Table 7-3: Tentatively identified compounds appearing throughout the degradation of benzaldehyde, 4-methoxybenzaldehyde, 1-phenylpropan-2-one, safrole and piperonal along with their GC retention time and mass fragmentation peaks. Expected fragmentation path ways of each compound along with the full mass spectrum of each peak is displayed in Appendix Figure 24-20.

| Compound Name | Structure | Retention Time (min) | Mass Fragments (Base peak) |
|---|---|---|---|
| Benzyl alcohol (Phenylmethanol) | | 7.20 | 108, 107, 91, 79, 77, 65, 63 51 |
| Benzoic acid | | 8.71 | 122,105, 77, 51 |
| 4-methozybenzene methanol | | 9.81 | 138, 137, 121, 109, 107, 94, 77, 51 |
| Piperonal alcohol 3,4-(methylenedioxy)benzyl alcohol | | 10.70 | 152,151, 135, 123, 121, 93, 65 |
| 4-methoxy benzoic acid | | 11.19 | 152, 135, 107, 91, 77 |
| Piperonylic acid 3,4-(methylenedioxy)benzoic acid | | 11.85 | 166, 165, 149, 121, 119, 91, 63 |
| 1,3 diphenylpropan-2-one | | 13.89 | 210, 119, 91, 65 |

Surprisingly it can be seen that products from both reduction to the alcohol and oxidation to the carboxylic acid was observed for the aldehydes tested. This suggests that competing degradation pathways for the aldehydes exists.

The presence of a by-product formed from either the addition of water (to form the alcohol) or hydrochloric acid across the double bond of safrole could be expected, as acidification of the wastewater samples prior to extraction would be expected to facilitate these reactions, however these products were not observed.

In addition, the presence of 1,3- diphenylpropan-2-one was observed. This compound however, is not believed to have formed due to degradation within the wastewater, but instead is more likely a result of the high concentration of P2P used promoting an injection port reaction resulting from 1-phenylpropan-2-one reacting with another P2P molecule in a bimolecular reaction.

### 7.2.3 Frozen Stability (- 20 °C) pH = 2 HCl Adjusted Wastewater

The stability of targeted compounds was also investigated in the storage conditions used. In order to determine the stability under the storage conditions the process in Figure 7-1 was repeated with the exception that the samples were stored at - 20 °C as opposed to at room temperature (~ 25 °C). As the samples were frozen, prior to the extraction the samples needed be thawed. In order to achieve this samples were placed in to the refrigerator at 4 °C on the night prior to the extraction. For this reason, only one sample was analysed in during the first 24 hours.

It was observed that a phase separation seemed to appear in the frozen samples with a yellow-brown oil like substance appearing on top of the wastewater. To overcome this, after samples were thawed, they were mixed thoroughly to obtain a homogenised solution prior to extraction.

The percentage of each spiked compound remaining for the different length of storage times are summarised in Figure 7-7 - Figure 7-11 below.

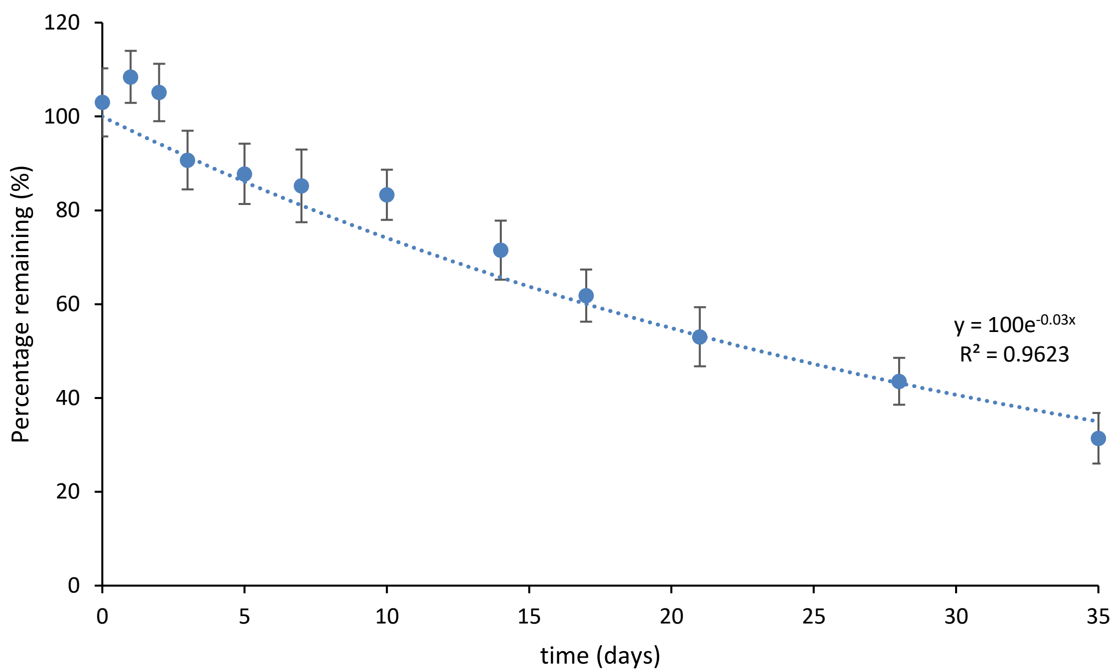


**Figure 7-7:** Percentage of Benzaldehyde remaining in pH=2 acidified wastewater stored at –20 °C determined from the GCMS peak area of benzaldehyde in the 106 m/z extracted ion chromatogram (9.86 mins) over 35 days. Error bars are ± 1 SD (n = 2).

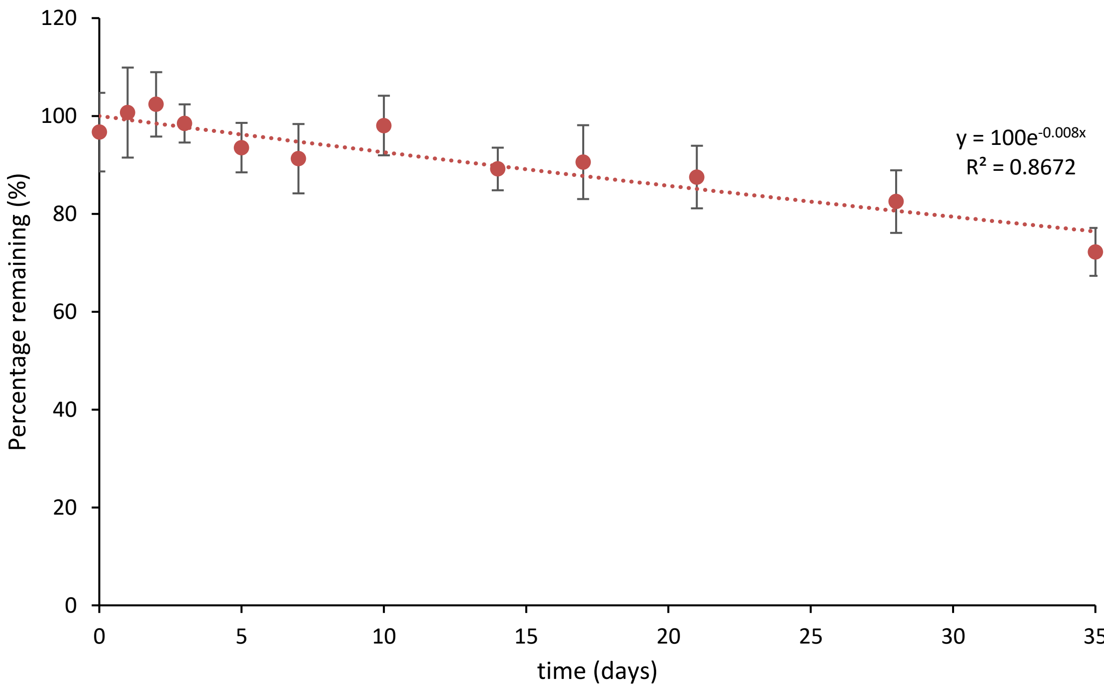


**Figure 7-8:** Percentage of 1-phenylpropan-2-one remaining in pH=2 acidified wastewater stored at –20 °C determined from the GCMS peak area of 1-phenylpropan-2-one in the 91 m/z extracted ion chromatogram (8.23 mins) over 35 days. Error bars are ± 1 SD (n = 2).

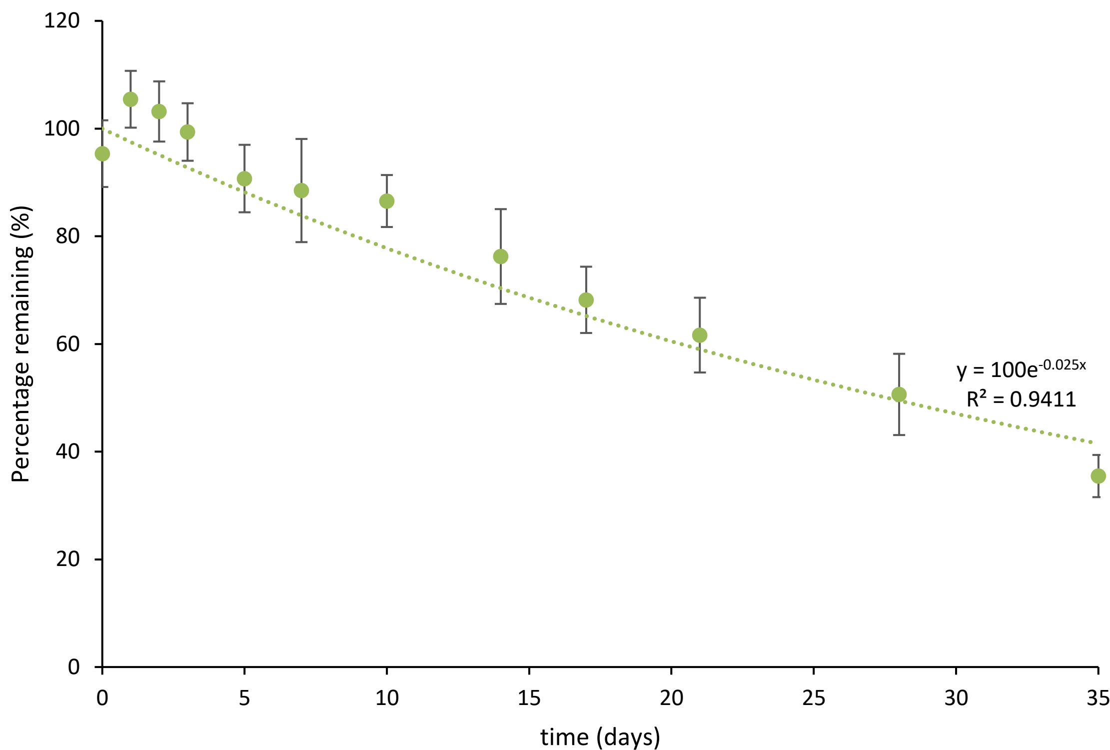


**Figure 7-9:** Percentage of 4-methoxybenzaldehyde remaining in pH=2 acidified wastewater stored at –20 °C determined from the GCMS peak area of 4-methoxybenzaldehyde in the 135 m/z extracted ion chromatogram (9.53 mins) over 35 days. Error bars are ± 1 SD (n = 2).

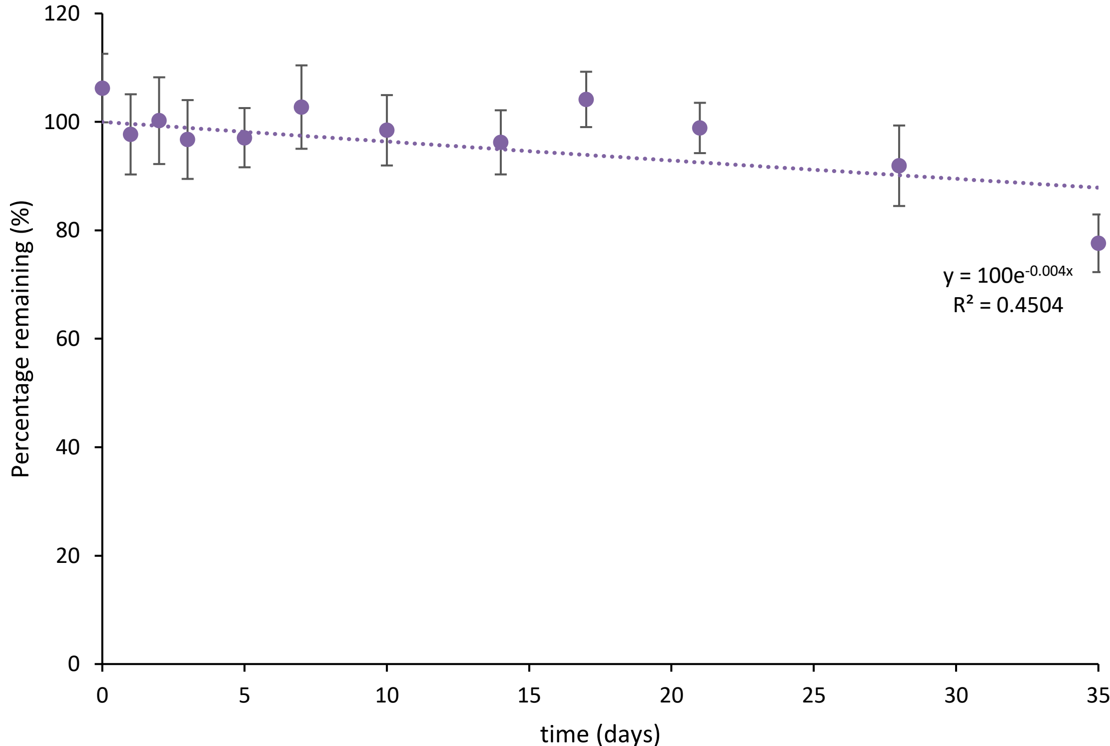


**Figure 7-10:** Percentage of safrole remaining in pH = 2 acidified wastewater stored at –20 °C determined from the GCMS peak area of safrole in the 162 m/z extracted ion chromatogram (9.86 mins) over 35 days. Error bars are ± 1 SD (n = 2).

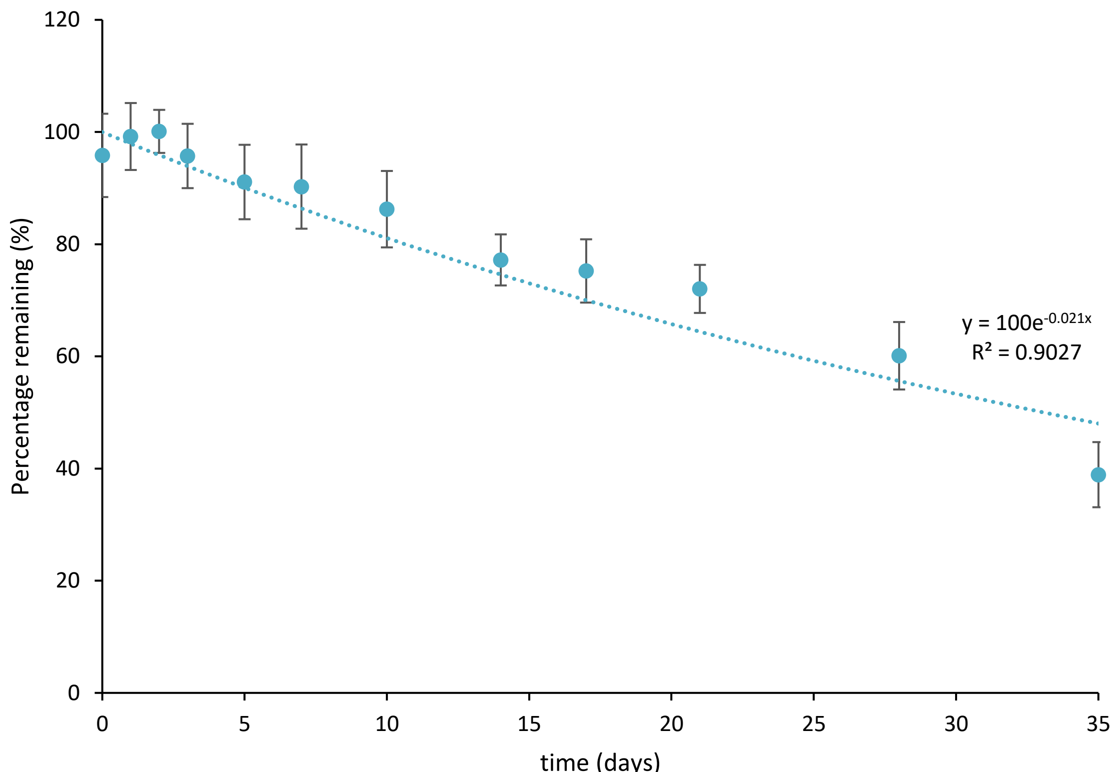


**Figure 7-11:** Percentage of piperonal remaining in pH=2 acidified wastewater stored at –20 °C determined from the GCMS peak area of piperonal in the 149 m/z extracted ion chromatogram (10.30 mins) over 35 days. Error bars are ± 1 SD (n = 2).

Despite an exponential trendline not accurately representing a close fit for all of the compounds, in particular safrole ($R^2$ = 0.45), for direct comparison to the room temperature samples each plot was fitted using an exponential curve. In order to obtain the rate constant and the standard error in the rate constant, the natural logarithm of the percentage remaining was plotted against time. The rate constant was then used to calculate the half-life for each of the target compounds (Table 7-2).

Table 7-4: Half-lives of benzaldehyde, 4-methozybenzaldehyde, piperonal, 1-phenylpropan-2-one and safrole in wastewater stored in the freezer at –20 °C determined by GCMS peak area.

| Compound Name | Rate constant, k ($day^{-1}$) | Half-life (days) |
|---|---|---|
| Benzaldehyde | 0.030 ± 0.0013 | 23.10 ± 1.0 |
| 1-phenylpropan-2-one | 0.008 ± 0.0006 | 90.13 ± 7.5 |
| 4-methoxybenzaldehyde | 0.025 ± 0.0014 | 27.58 ± 1.6 |
| Safrole | 0.004 ± 0.0011 | 187.5 ± 54.0 |
| Piperonal | 0.021 ± 0.0015 | 33.08 ± 2.4 |

By comparing the half-lives obtained in the room temperature experiment to that of the frozen experiment an increase in stability for each of the targeted compounds was observed. The half-life of benzaldehyde was the shortest of those tested with 50 % of the sample degraded after 23 days. This suggest that care must be taken when analysing samples stored in the freezer as significant losses may occur.

Safrole and 1-phenylpropan-2-one appeared to be relatively stable over the 35 days with approximately 78 % and 72 % remaining after 35 days respectively. The standard error in the half-life of safrole was extremely large in comparison to the other compounds as the exponential curve didn't fit the data well. The stability of the targeted compounds may be overestimated slightly as there is a higher potential for adsorption to the edges of the vessel in the spiked sample due to the higher surface area of the container (container V = 2 L) compared to the samples spiked immediately before extraction (container volume = 200 mL).

## 7.3 Sorption to Solids - Humic Acid Experiments

As the wastewater samples were acidified upon collection the decision was made to check for the effect of humic acids on the recovery of the target residues. Humic acids are a complex mixture of a wide variety of substances which are produced by the biodegradation of organic matter such as plant material.

A large majority of humic acids are known to be insoluble at low pH. For this reason, when the pH of the wastewater is lowered upon collection it is likely that that the precipitation of humic acids could occur. This precipitation could lead to the entrapment of the target analytes lowering their apparent concentration in the original wastewater sample. There is also potential for absorption of the analytes to the surface of the humic acids to occur leading to a reduction in their recovery. Despite being formed via biodegradation, humic substances themselves have the benefit that they are very resistant to further biodegradation.

The effect of humic substances on the recovery of benzaldehyde, 1-phenylpropane-2-one, 4-methoxybenzaldehyde and piperonal was investigated by creating two 0.1 mg/L humic acid solutions in tap water. One of these solutions was acidified to pH = 2 using 2 M hydrochloric acid. To each of these solutions along with two controls (tap water with no humic acid, acidified and non-acidified) 5 µg of each drug standard was added (100 µL of 50 µg/mL standard). The sample was mixed thoroughly and was inverted and shaken every 15 minutes

for 6 hours. Following this each sample was adjusted to pH = 2 with HCl and extracted using SPE as per the method outlined in section 6.2. Following extraction, the samples were analysed using GCMS according to the method described in section 6.3.1. It is worth noting that the effect of humic substances on the recovery of safrole was not investigated due to its availability at the time of the experiment.

Table 7-5: Effect of humic substances (0.1 mg/mL) on the recovery of target compounds in tap water, acidified to pH=2 before and after the addition of targeted compounds determined relative to samples without humic substances added GCMS peak area.

| Compound Name | Relative recovery acidified before targeted compounds added (%) | Relative recovery acidified after targeted compounds added (%) |
|---|---|---|
| Benzaldehyde | 104 ± 3.4 | 94.9 ± 4.5 |
| 4-methoxybenzaldehyde | 102 ± 5.0 | 97.2 ± 4.8 |
| Piperonal | 104 ± 4.3 | 93.2 ± 6.3 |
| 1-phenylpropan-2-one | 103 ± 3.6 | 95.1 ± 4.2 |

Table 7-5 above shows that the humic acids, despite being at a relatively high concentration compared to that typically found in waterways, had very little effect on the recovery of all the analytes tested. The recovery of the samples acidified after addition of humic substances all had a lower recovery than that which were acidified before. Despite it being possible that this lower recovery could potentially be as a result of the targeted compounds reacting with the extremely low pH solution that is created where the hydrochloric acid comes into contact with the solution (until the concentration gradient is removed by mixing thoroughly), this is unlikely as the control used for comparison would also have had this similar concentration gradient. This suggests that the small difference in recovery is likely a result of agglomeration of the humic acids and potential entrapment of target compounds. The loss in recovery observed was considered to be insignificant compared to other losses that occur including bio-degradation.

## 7.4 Conclusion

Of the compounds tested benzaldehyde was shown to be the least stable while safrole was the most stable in wastewater stored at room temperature having half-lives of 5.4 and 20.5 days respectively.

The stability of all compounds of interest increased significantly when they were acidified to pH=2 and stored in the freezer (- 20 °C) with the half-life of benzaldehyde almost quadrupling to 23.1 days. These results show that care must be taken during transport and that samples should be acidified once obtained and transported on ice to limit loss due to bio-degradation.

The potential identity of degradation products produced from the degradation of the target compounds was investigated in wastewater. Products related to both the oxidation and reduction were observed showing that degradation of these compounds is relatively complicated and that competing degradation pathways exist.

Entrapment of target molecules may occur with precipitation of humic acids when water samples are acidified. This potential entrapment was investigated by comparing water samples containing humic acids which were acidified both before and after spiking with targeted compounds. This study showed that compared to other losses such as biodegradation the loss due to entrapment was negligible.

These results show that if held under the appropriate storage conditions the samples are relatively stable, do not need to be analysed immediately and can be stored for a short period of time. However, if samples are left for an extended period of time prior to analysis the degradation product could potentially be targeted instead. Further investigation into the stability of these substances along with any alternate sources in wastewater would need to occur prior to ensure its feasibility. The stability along with the lack of entrapment in humic substances help to ensure that the compounds tested are suitable for use as targeted compounds as it shows that they may have the potential to be used in the future by forensic investigators in order to determine the extent of clandestine synthesis in a given area.

# Chapter 8 Analysis of Wastewater Extractions

## 8.1 Chapter Overview

This chapter details the analysis of wastewater samples using both gas chromatography mass spectrometry and liquid chromatography mass spectrometry. The solid phase extraction method detailed in chapter 6 eluted the compounds in two fractions; the first consisting of the neutral and acidic compounds and the second the basic compounds.

The first fraction consisting of the amines was analysed via LCMS and GCMS. The LC method was showed to be significantly more sensitive and selective than the GCMS method making it more suited to the analysis of wastewater extracts than GCMS. Due to instrument constraints wastewater extracts from only a couple of samples was investigated using LCMS.

The second fraction of the SPE was analysed using GCMS only. The presence of the targeted compounds was investigated along with the presence of products produced from their degradation identified in chapter 7.

The presence of targeted compounds in real-world wastewater samples was used to determine the feasibility of this approach as acting as an information source for forensic investigators.

## 8.2 Analysis of First SPE Elution – Neutral and Acidic Compounds

### 8.2.1 GC-MS Results from Wastewater Analysis

Extracted ion chromatograms for the molecular ion along with the two most abundant peaks were created for each targeted analyte along with compounds identified in the stability experiments (Table 7-3) for each wastewater sample analysed. If a peak was present in each of the three extracted ion chromatograms at the correct retention time, the mass fragmentation pattern was observed. If the ratio of the mass fragments matched that of the targeted compound its presence was recorded. The presence of peaks detected in the first elution of solid phase extracted wastewater samples is summarised below (Table 8-1 - Table 8-4).

The appearance of a small peak in the SIM chromatograms at 8.3 minutes was observed. This peak despite having the three most abundant mass fragments for P2P, the ratio of the mass fragments was not as expected as the intensity of the m/z = 43 ion was too abundant. For this reason, the peak cannot be conclusively identified as being due to the presence of P2P in the sample.

Some possible reasons why the ratio of the mass fragments may not match that of P2P include both: coelution with another compound which produces at least one of the mass fragments, or the result of another compound eluting at the same retention time which produces fragments with the same mass-to-charge ratio. The ions produced from the fragmentation of P2P are extremely common and as a result the GCMS method is not very selective making identification extremely difficult. If this peak was in fact due to P2P, despite using the more sensitive SIM method, the peak was extremely small and below the limit of quantification for the GCMS method in all wastewater samples analysed.

In addition, it is known that in Australia methamphetamine is generally produced by methods which utilise pseudoephedrine. This is evidenced through data from the Australian crime commission illicit drug report which shows that there were only 48 and 45 ATS clandestine laboratory detections in South Australia for 2016-17 and 2017-18 respectively [142, 264]. Amongst these detections only 5 were involving P2P [142, 264]. Considering that the majority of the clandestine laboratories detected are considered to be either addict based or small scale the number and size of disposal events is expected to be small emphasising the increased difficulty of detecting the precursors over the parent drugs themselves. As the entry

of the targeted compounds into the wastewater system is likely to be much more sporadic and not continual and the amounts likely disposed of are not expected to be large the detection of 1-phenylpropan-2-one may be highly dependent on the sampling date, method and time.

Table 8-1: Detection of phenylacetic acid (PAA), 1-phenylpropan-2-one (P2P), 1,3 diphenylpropan-2-one (P2P dimer), benzoic acid (BA), benzaldehyde (BAL), 4-methoxybenzoic acid (4-MBA), 4-methoxybenzaldehyde (4-MBAL), piperonal (PIP), safrole (SAF), caffeine (CAF), Benzyl alcohol (BALC), 4-methoxybenzyl alcohol (4-MBALC), piperonyl alcohol (PIPOL), piperonylic acid (PIPAC),and ibuprofen (IBU) in extracted wastewater samples analysed via GCMS.

√ = detected in sample at the correct $t_R$

N.D.= not detected in sample

? = mass fragments present, however the ratio of the mass fragments was different to that expected or at a different $t_R$

| WWTP | Date | PAA | P2P | P2P dimer | BA | BAL | 4-MBA | 4-MBAL | PIP | SAF | CAF | BALC | 4-MBALC | PIPOL | PIPAC | IBU |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Christies Beach | 9/04/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | ? | ? | √ |
| | 10/04/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | ? | ? | √ |
| | 11/04/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | ? | ? | √ |
| | 12/04/2017 | √ | ? | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | ? | ? | √ |
| | 13/04/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | ? | ? | √ |
| | 14/04/2017 | √ | ? | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | ? | N.D. | √ |
| | 15/04/2017 | √ | ? | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 2/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 3/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 4/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 5/05/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 6/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 7/05/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 8/05/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | ? | N.D. | √ |
| | 17/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 18/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 19/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 20/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 21/02/2018 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 22/02/2018 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 23/02/2018 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |

Table 8-2: Detection of phenylacetic acid (PAA), 1-phenylpropan-2-one (P2P), 1,3 diphenylpropan-2-one (P2P dimer), benzoic acid (BA), benzaldehyde (BAL), 4-methoxybenzoic acid (4-MBA), 4-methoxybenzaldehyde (4-MBAL), piperonal (PIP), safrole (SAF), caffeine (CAF), Benzyl alcohol (BALC), 4-methoxybenzyl alcohol (4-MBALC), piperonyl alcohol (PIPOL), piperonylic acid (PIPAC),and ibuprofen (IBU) in extracted Glenelg wastewater samples analysed via GCMS.

√ = detected in sample at the correct $t_R$

N.D. = not detected in sample

? = mass fragments present, however the ratio of the mass fragments was different to that expected or at a different $t_R$

| WWTP | Date | PAA | P2P | P2P dimer | BA | BAL | 4-MBA | 4-MBAL | PIP | SAF | CAF | BALC | 4-MBALC | PIPOL | PIPAC | IBU |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9/04/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 10/04/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 11/04/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 12/04/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 13/04/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 14/04/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 15/04/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 2/05/2017 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 3/05/2017 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 4/05/2017 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| Glenelg | 5/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 6/05/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 7/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 8/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 17/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 18/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 19/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 20/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 21/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 22/02/2018 | N.D. | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 23/02/2018 | √ | ? | N.D. | √ | ? | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |

**Table 8-3: Detection of phenylacetic acid (PAA), 1-phenylpropan-2-one (P2P), 1,3 diphenylpropan-2-one (P2P dimer), benzoic acid (BA), benzaldehyde (BAL), 4-methoxybenzoic acid (4-MBA), 4-methoxybenzaldehyde (4-MBAL), piperonal (PIP), safrole (SAF), caffeine (CAF), Benzyl alcohol (BALC), 4-methoxybenzyl alcohol (4-MBALC), piperonyl alcohol (PIPOL), piperonylic acid (PIPAC),and ibuprofen (IBU) in extracted Bolivar high salinity wastewater samples analysed via GCMS.**

√ = detected in sample at the correct $T_R$

N.D. = not detected in sample

? = mass fragments present, however the ratio of the mass fragments was different to that expected or at a different $t_R$

| WWTP | Date | PAA | P2P | P2P dimer | BA | BAL | 4-MBA | 4-MBAL | PIP | SAF | CAF | BALC | 4-MBALC | PIPOL | PIPAC | IBU |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bolivar high salinity | 9/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 10/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 11/04/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 12/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 13/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 14/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 15/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 2/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 3/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 4/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 5/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 6/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 7/05/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 8/05/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 17/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 18/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 19/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 20/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 21/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 22/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 23/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |

**Table 8-4: Detection of phenylacetic acid (PAA), 1-phenylpropan-2-one (P2P), 1,3 diphenylpropan-2-one (P2P dimer), benzoic acid (BA), benzaldehyde (BAL), 4-methoxybenzoic acid (4-MBA), 4-methoxybenzaldehyde (4-MBAL), piperonal (PIP), safrole (SAF), caffeine (CAF), Benzyl alcohol (BALC), 4-methoxybenzyl alcohol (4-MBALC), piperonyl alcohol (PIPOL), piperonylic acid (PIPAC),and ibuprofen (IBU) in extracted Bolivar wastewater samples analysed via GCMS.**

√ = detected in sample at the correct $t_R$ N.D. = not detected in sample ? = mass fragments present, however the ratio of the mass fragments was different to that expected or at a different $t_R$

| WWTP | Date | PAA | P2P | P2P dimer | BA | BAL | 4-MBA | 4-MBAL | PIP | SAF | CAF | BALC | 4-MBALC | PIPOL | PIPAC | IBU |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bolivar | 9/04/2017 | √ | ? | N.D. | √ | N.D. | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 10/04/2017 | √ | ? | N.D. | √ | N.D. | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 11/04/2017 | √ | ? | N.D. | √ | N.D. | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 12/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 13/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 14/04/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 15/04/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 2/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 3/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 4/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 5/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 6/05/2017 | √ | ? | N.D. | √ | √ | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 7/05/2017 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 8/05/2017 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | ? | √ |
| | 17/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 18/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 19/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 20/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | ? | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 21/02/2018 | √ | ? | N.D. | √ | √ | N.D. | N.D. | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 22/02/2018 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |
| | 23/02/2018 | √ | ? | N.D. | √ | ? | N.D. | ? | N.D. | N.D. | √ | N.D. | N.D. | N.D. | N.D. | √ |

Despite 1-phenylpropan-2-one not being detected at significant levels in any of the SA wastewater samples the presence of a large peak at 9.47 minutes was observed in all samples analysed using the SIM method. The other fragmentation peaks associated with the compound eluting at 9.47 minutes was investigated by looking at the same sample run with the scan method. Following interpretation of the mass spectrum, the peak was tentatively identified to be phenylacetic acid, a possible precursor to 1-phenylpropan-2-one. The corresponding mass fragmentation pattern along with the expected fragments for phenylacetic acid is given below in Figure 8-1

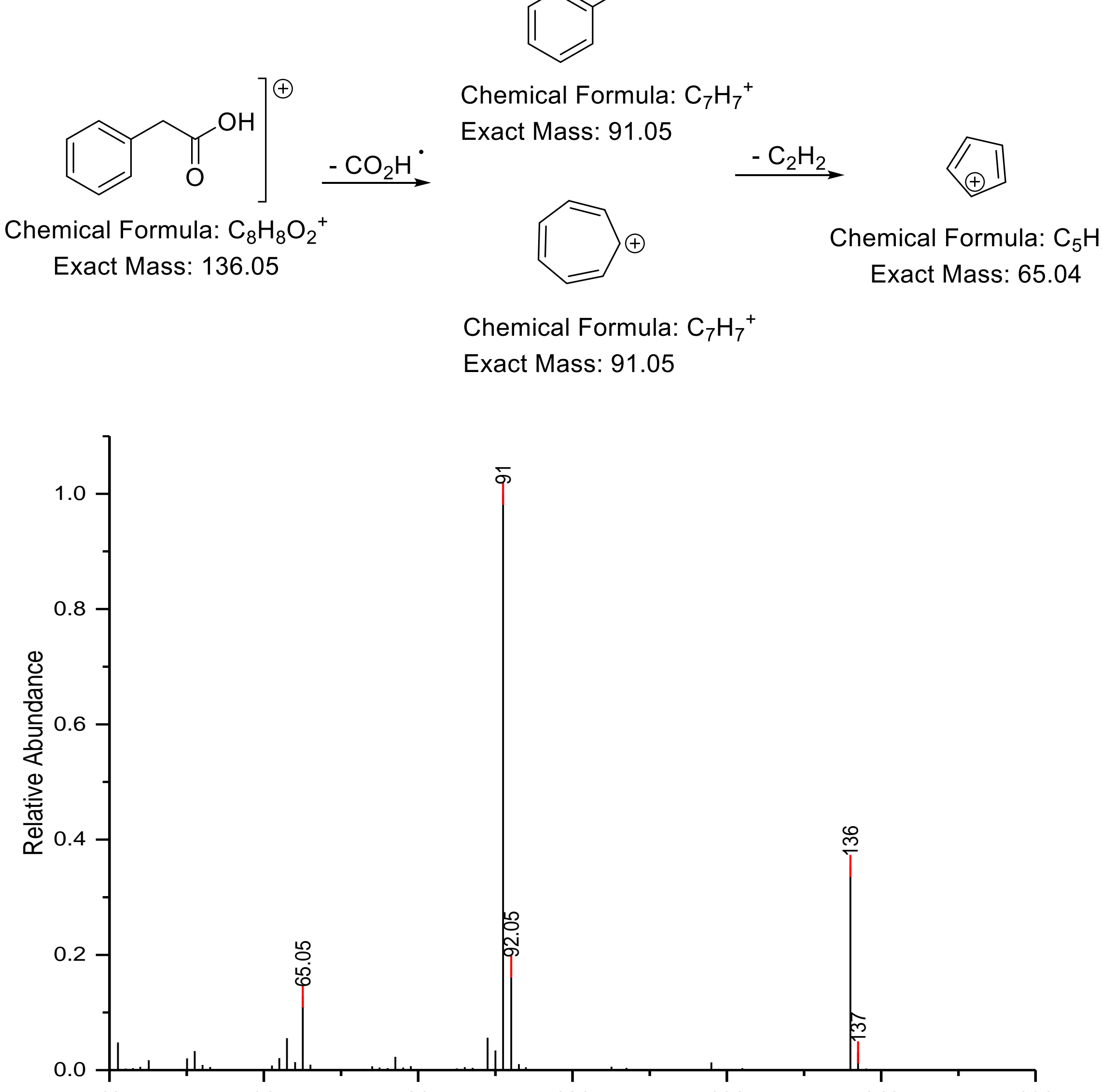


Figure 8-1: Mass spectrum of the peak eluting at 9.47 min believed to be a result of phenylacetic acid

The identity was further supported by injection of a standard and confirmation of retention time (Appendix Figure 13). Although phenylacetic acid can be used to make 1-phenylpropan-

2-one and hence meth/amphetamine, it can also be present in some fruits, perfumes and is produced by many species of ants. If the phenylacetic acid peak was a result of any of the aforementioned reasons its presence in the GC trace would be expected to occur in the majority if not all of the wastewater samples, which was the case for all wastewater samples analysed. For this reason, the presence of phenylacetic acid in wastewater cannot be associated with the illicit manufacture of amphetamine type stimulants.

Despite not having a standard, the presence of the N-formyl intermediates of the production of ATS along with napthalenes and aziridines was investigated by analysis of extracted ion chromatograms. These substances were not found to be present in any of the samples analysed. This was expected as they were predicted to be either less stable (the N-formyl-intermediates have been shown to degrade via biotic process in soil [265]) than the other targeted compounds or in extremely low concentrations.

The only compounds that were consistently found in the extracted wastewater samples other than phenylacetic acid were: benzoic acid, benzaldehyde, caffeine and ibuprofen. Caffeine and ibuprofen despite not being related to the synthesis of amphetamine type stimulants were used to ensure that nothing went wrong with the extraction as their presence in relatively large amounts was expected.

The occurrence of benzaldehyde could be expected in wastewater due to its use in cooking as it used to impart an almond flavour. Benzaldehyde can also be produced from the metabolism of amygdalin which is present in many fruits and nuts [266]. The presence of benzoic acid could be produced in wastewater from the oxidation of benzaldehyde, as seen in its appearance in the stability experiments. One other likely source for the presence of benzoic acid is the use of sodium benzoate, the basic sodium salt of benzoic acid, in many personal care products such as shampoos.

### 8.2.2 Analysis of Grab Samples

The composition of wastewater can vary throughout the day. When wastewater epidemiology is used 24-hour composite samples are preferred as they give an indication as to how much of a particular substance has been consumed in a given 24 hr period.

However, as this research involves the analysis of illicit drugs as well as their corresponding by-products and precursors related to the synthesis of these substances, there is not a continual supply of these substances into the wastewater system. For this reason, grab

samples may have an advantage over composite samples. If these substances are being introduced into the wastewater sporadically, from events such as disposal via drainage or washing of equipment, then the analysis of a composite sample may not be feasible as the further dilution of the composite may make the targeted compound much more difficult to detect.

The downside with grab samples is that it is unknown when these disposal events may occur and as a result the time at which a grab sample is taken will be extremely important as to whether a sample may contain by-products or precursors to amphetamine type stimulants.

Grab wastewater samples were collected from Christies Beach wastewater treatment facility every 2 hours during their typical business hours (7 am – 4 pm). These samples were filtered and extracted as per the method discussed in section 6.2. As can be seen from the GCMS chromatograms obtained for the grab samples (Appendix Figure 33) there was no significant difference observed in the composition of wastewater at each time tested.

## 8.3 Analysis of Second SPE Elution -Basic Compounds

### 8.3.1 LC-MS for Analysis

As mentioned earlier, the triple quadrupole mass spectrometer developed an electrical fault following a power outage. For this reason, only a limited number of wastewater extractions were able to be analysed using LCMS. All wastewater extractions analysed via LCMS utilised an external calibration instead of the preferred isotope dilution method using a deuterated internal standard. Below is the chromatogram for an extracted wastewater sample which was obtained from Coombabah wastewater treatment facility in Queensland on 07/05/2014 (Figure 8-2).

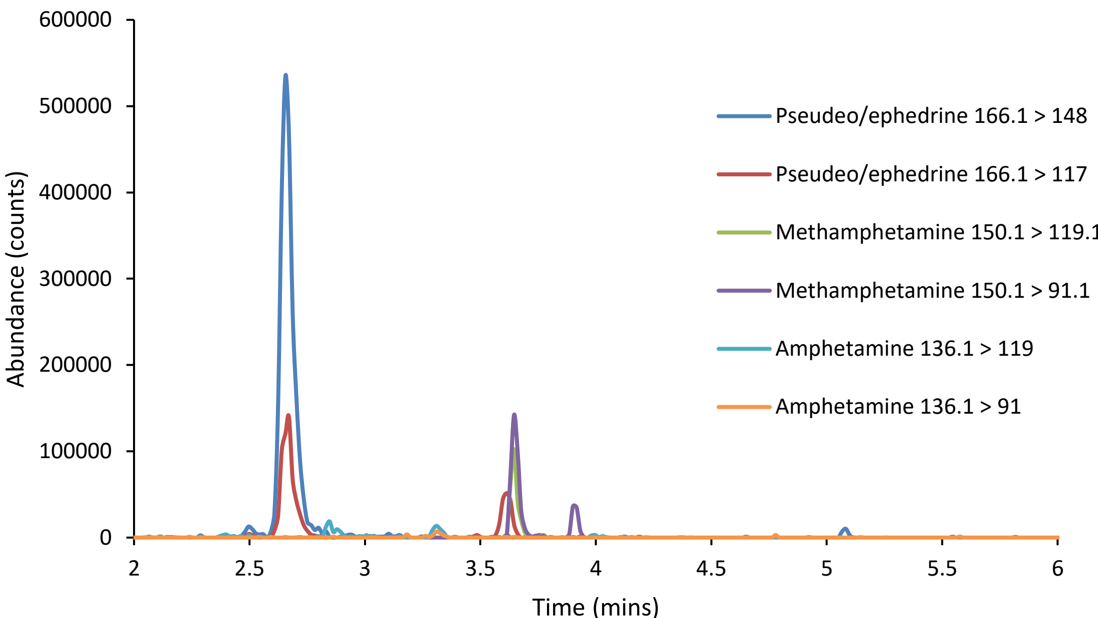


**Figure 8-2:** Example LCMS chromatogram showing the selected mass transitions for each amphetamine type stimulant under investigation. Chromatogram shown is of a solid phase extracted Coombabah (Qld.) wastewater sample collected 07/05/2014

By looking at Figure 8-2 it can be seen that both transitions at the correct retention times were observed for pseudoephedrine, methamphetamine and amphetamine. From this the peak area can be used along with the external calibration to obtain the concentration of each species in the sample. It should be noted that care must be taken when interpreting this result as the concertation obtained using this method does not take into account matrix effects and as a result any suppression or enhancement of the signal is unaccounted for.

After determining the concentration of each amphetamine type stimulant in the extract, the concentration of each compound in the wastewater can be back calculated. This can then be used to calculate the amount of substance per capita for the area served by the wastewater treatment facility. An example calculation is given in Appendix Example Calculation 1.

The concentration of each substance present in the second fraction of the Coombabah wastewater sample is summarised in Appendix Table 3.

### 8.3.2 GC-MS for Analysis

Gas Chromatography was trialled for the analysis of the second fraction of the solid phase extraction. This was done by removing the addition of acid to the extract prior to evaporation and changing the reconstitution solvent to chloroform (1 mL). These samples were analysed using the GCMS method used for the first fraction (section 6.3.1).

The detection limits of the GCMS method were much higher than that of the LCMS method. This is likely a result of the high polarity of the amine moiety which leads to adsorption and decomposition of amines on the GC column causing reduction in detector sensitivity and broad tailing peaks [267, 268].

The broadening and tailing of the peaks corresponding to the amphetamine type stimulants was apparent particularly with the injection of low concentration standards. Appendix Figure 34 is an example chromatogram showing the broad tailing peak obtained from the injection of a 4 μg/mL methamphetamine standard. This broadening negatively impacted on the detection limits of the amphetamine type-stimulants with the LLOD of methamphetamine seen to be approximately 2 μg/mL for the standard, and expected to be even higher in wastewater extracts. This concentration is 3 orders of magnitude lower than the LLOQ for the LCMS method.

In addition to the poor detection limits, mass fragments produced from the electron ionisation of amphetamine type stimulants were either in low relative abundance compared to other mass fragments or relatively common ions, e.g. m/z = 91 from the tropylium ion. For this reason, a SIM method could not be used to significantly improve their limit of detection. As an adequate detection limit was not achieved using GCMS for the amphetamine type stimulants, their detection in wastewater extracts was not observed. In order to achieve better detection of these compounds the use of a derivatising agent would be recommended to improve their chromatography along with the use of a more selective detection method similar to the MRM method used for the LCMS analysis.

## 8.4 Conclusion

The amount of methamphetamine, amphetamine and pseudoephedrine was determined in a Coombabah wastewater sample to be 175, 9.0 and 995 mg/day/1000 people respectively. The presence of these levels is not outside the ordinary and is consistent with typical levels reported elsewhere.

The presence of phenylacetic acid, benzoic acid and benzaldehyde was observed in the large majority of samples analysed. This suggests that there is an alternate entry source of these substances into the wastewater system and that their use as potential targets to provide information on clandestine synthesis in a given area is limited.

Piperonal, 4-methoxybenzoic acid, phenyl-2-propanone and safrole were not found in the wastewater extracts. This non-detection does not necessarily rule out their potential for use in providing valuable information to forensic investigators and may have been a result of sampling location and time, due to the batch nature of clandestine synthesis.

# Chapter 9 Conclusions and Further Research

This research outlines the development of a method for the extraction of compounds related to the synthesis of amphetamine type stimulants namely; 1-phenypropan-2-one, pseudoephedrine, ephedrine, benzaldehyde, 4-methoxybenzaldeyde, piperonal and safrole from wastewater along with the amphetamine type stimulants methamphetamine and amphetamine. The recovery of each targeted compound was investigated throughout the various steps during the extraction procedure.

Silanisation of glassware was extremely important for the amino containing targeted compounds and was shown to be required as significant losses believed to be due to adsorption of the basic compounds to the surface of the glassware occurred.

Evaporation and reconstitution could not be used as a way to increase the concentration of the substances in the first SPE extract as significant loss of P2P occurred during the evaporation process. Instead to increase their concentration and to improve detection limits the elution was performed with an optimised elution volume of 1 mL.

The use of humic substance showed that no significant loss of all targeted compounds was seen due to adsorption, however a small reduction in recovery was observed upon acidification believed to be a result of entrapment due to the agglomeration of humic substances at the lower pH.

In addition to developing an extraction method the stability of benzaldehyde, 1-phenylpropane-2-one, 4-methoxybenzaldehyde, safrole and piperonal in wastewater was investigated for the first time. This was done by extracting spiked wastewater over the course of 35 days at both stored conditions (pH = 2 adjusted samples stored at (- 20 °C) and left at room temperature (~ 25 °C). First order kinetics was used to fit the data and the half-life for each compound calculated. A significant increase in stability for all compounds was observed in the frozen samples compared to those at room temperature. At room temperature benzaldehyde, 1-phenylpropane-2-one, 4-methoxybenzaldehyde, safrole and piperonal were found to have half-lives of 5.4 ± 0.2, 8.0 ± 0.4, 8.3 ± 0.4, 12.6 ± 0.6 and 20.5 ± 1.3 days respectively which increased significantly to 23.1 ± 1.0, 90.1 ± 7.5, 27.6 ± 1.6, 188 ± 54.0 and 33.1 ± 2.4 days respectively for the frozen samples.

The identity of some of the degradation products produced from benzaldehyde, 1-phenylpropan-2-one, 4-methoxybenzaldehyde, safrole and piperonal was determined via analysis of a wastewater sample spiked with a large amount of the target compounds and

allowed to degrade. The presence of: benzyl alcohol, benzoic acid, 4-methoxylbenzene methanol, piperonyl alcohol, 4-methoxybenzoic acid, piperonylic acid and 1,3-diphenylpropan-2-one was observed in the degraded sample.

Derivatisation of 1-phenylpropane-2-one was undergone to improve its ionisation and hence its LCMS detection limits. This was undertaken using hydroxylamine hydrochloride to convert the carbonyl moiety to an oxime. The kinetics of the derivatisation was investigated using GCMS to ensure that near complete conversion occurred and that the derivatisation would be quantitative. The oxime appeared stable via UV-Vis analysis however when checking the stability via LCMS the response from the mass spectrometer was variable. This variability was believed to be a result of inconsistent formation of an acetonitrile adduct. In addition, the presence of two peaks was observed, due to the presence of both the E and the Z stereoisomer. Despite observing an improvement in detection limit of the oxime compared to that of the ketone (even with both stereoisomer products formed), the development of a method for the analysis of the first SPE elution could not be completed due to an electrical fault developing in the mass spectrometer. As the detection of the oxime seemed promising, future research investigating the derivatisation of 1-phenylpropan-2-one along with the other compounds eluting in the first SPE fraction should be investigated as it could lead to a lower detection limit.

Analysis of the second SPE elution showed the presence of methamphetamine, amphetamine and pseudoephedrine in a Coombabah wastewater extract at levels of 175, 9.0 and 995 mg/day/1000 people determined via external calibration using the developed LCMS method. Further research could be contacted with the analysis of these samples using an isotope dilution method to account for any ion suppression or enhancement that occur in the wastewater matrix.

A GCMS method was also developed for the analysis of wastewater extracts. The GCMS was operated in selected ion monitoring (SIM) mode for the detection of P2P as a significant improvement in detection limit was observed (5 µg/L compared to 50 µg/L). Wastewater samples were analysed for targeted compounds along with the degradation products found in the stability experiment via GCMS.

The only compounds related to the synthesis of amphetamines regularly found in wastewater samples were benzaldehyde, benzoic acid and phenylacetic acid. As these are used in other

applications their presence in wastewater could not be related to the synthesis of amphetamine type stimulants. For this reason, the feasibility of obtaining useful information for forensic investigators in regards to determining potential amphetamine synthesis from the detection of benzaldehyde, benzoic acid and phenylacetic acid is low. Despite not being detected, it is likely that the analysis of the other targeted compounds could have potential use for forensic investigators as they were stable and not consistently present in wastewater from alternate sources. The application of wastewater analysis for monitoring these compounds in the real-world may not be feasible at this time as routine wastewater analysis would require extra sample preparation such as an additional solid phase extraction elution. In addition, unless either a large or numerous ATS waste dumping events occurs within one day 24-hr composite samples may be inadequate. Whilst not typically performed routinely, it is not economically feasible to use grab samples as the sampling frequency required would need to be high in order to capture dumping events.

As some of the compounds analysed are expected to have a high partition coefficient future work analysing sewage sludge could be undertaken (an oil like layer typically removed by skimming the surface of the wastewater during treatment). Wastewater samples from areas where the P2P method is more common than the ephedrine routes could also be performed.

An adequate detection limit was not achieved for analysis of the second SPE fraction via GCMS for the amphetamine type stimulants, as a result their detection in solid phase extracted wastewater samples was not observed. The poor detection limit was associated with the polar amine functional group on the ATS leading to broad tailing peaks. Future work would look at developing a GCMS method for the detection of amphetamines in wastewater extracts. One possible way to achieve this could be via derivatisation of the amino group to an amide moiety via acylation. Some typical acylating include: heptafluorobutyric anhydride (HFBA), chlorodifluoroacetic anhydride (ClF2AA) and N-methyl-N-t-butyldimethylsilyl trifluoroacetamide (MTBSTFA). However, even with derivatisation, GCMS is expected to be less selective and less sensitive method than an LCMS method.

In addition to the additional work listed above further work should focus on the detection of additional compounds related to the synthesis of both amphetamine type stimulants and other illicit substances in wastewater samples of differing urbanicities.

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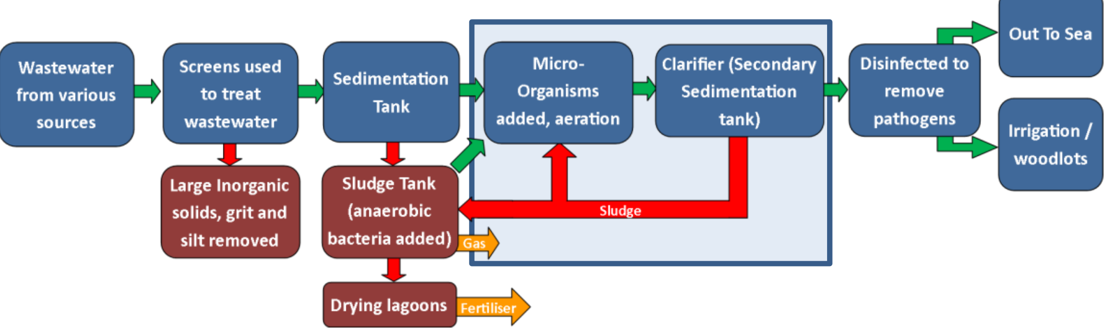
# Chapter 11 Appendices

### 11.1.1 Treatment of Wastewater

The treatment of wastewater consists of several steps to get the water to a quality which can be reused. Although wastewater treatment methods can vary from plant to plant the most common method utilised in Australia and throughout the world is known as the activated sludge process [269, 270]. A general schematic for this treatment method is shown below Appendix Figure 1.




Appendix Figure 1: Overview of the most common wastewater treatment plant method. Adapted from SA water [271].

The first stage of treatment is the removal of large inorganic material such as; rags, plastic and paper. The elimination of grit and silt then follows so that there is no interference or damage affecting the operation of the plant equipment [272].

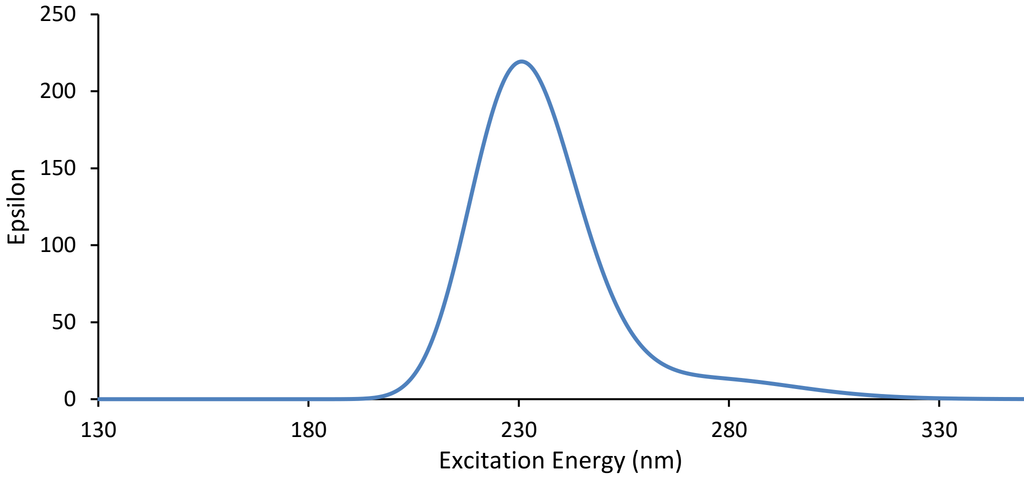
Wastewater is then passed through a sedimentation tank where settleable suspended solid particles are allowed to settle under gravity [273]. This accounts for approximately 65 % of all the suspended solids [272]. The resultant sludge is collected at the centre of the tank where it is removed and taken additional treatment [274]. In addition, the surface of the wastewater is skimmed to remove oil and grease.

The next stage known as the activated sludge process uses micro-organisms to decompose suspended solids and dissolved matter [275]. There are several types of aerobic biological treatment systems, all of which have the same aim and basic mode of operation [272]. This aim is to provide a suitable environment in which a diverse population of micro-organisms (mainly bacteria referred to as the 'biomass') may be introduced to the wastewater for a long enough period to remove the organic content and use it for growth of new biomass. The 'biomass' uses the dissolved oxygen and consumes organic contaminants and nutrients in the wastewater to reproduce and grow. For this reason, the wastewater is aerated and mixed by blowing oxygen through [271].

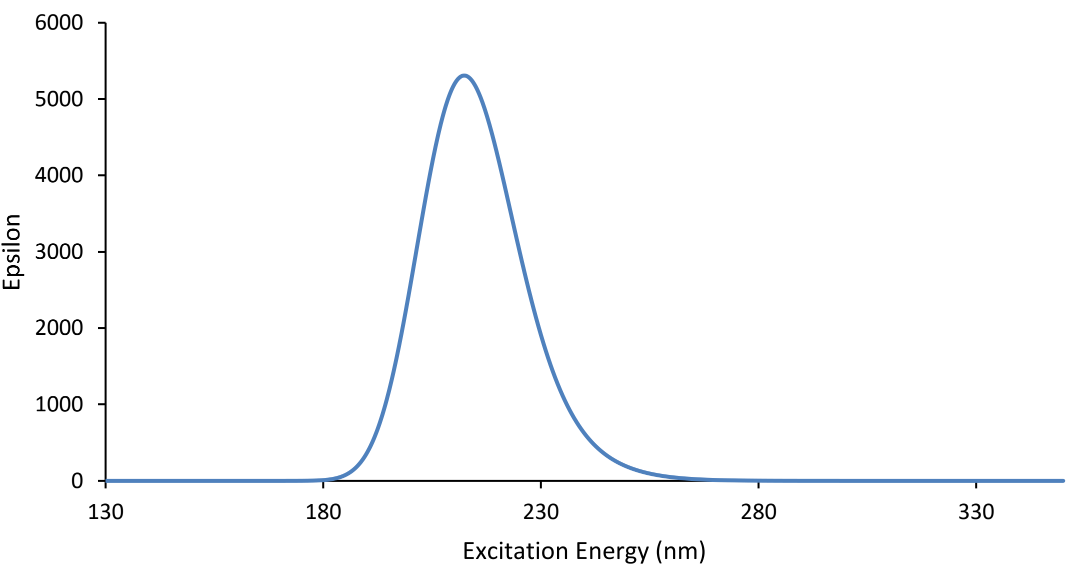
The wastewater and micro-organisms subsequently pass into a clarifier (a second sedimentation tank) where the suspended biomass settles, due to gravity, and is concentrated as sludge. This sludge is later used for two processes, the first is to maintain the biomass in the aeration tank and the second involves pumping the remainder to the anaerobic digesters for further treatment [273].

The clarified wastewater is then filtered through granular media filters before being disinfected to reduce the amount of pathogens. This is generally done by addition of chlorine, and ultra violet light but can also be completed in large lagoons where sunlight and micro-organisms reduce the amount of pathogens [276]. If the water is going to be reused for irrigation or any activity that is likely to have human contact dissolved air flotation (DAF) is generally used [277]. DAF is used for further treatment for removing suspended material and oils. This is done by applying pressure to the wastewater and dissolving air, when the pressure is returned to atmospheric pressure tiny bubbles form which adhere to this matter which can then be removed using a skimmer [278].

The sludge is treated with anaerobic bacteria. The treatment of sludge occurs in sealed digesters which don't allow oxygen to permeate; the digester is also heated to 35℃ so that the anaerobic bacteria can thrive [271]. In addition, gas is produced during the breakdown of the sludge which contains a high amount of methane. This methane is often used as fuel to heat the sludge or alternatively for the production of electricity [271]. Upon treatment with micro-organisms the water is either removed by evaporation or centrifuging. The solids remaining are generally used as a conditioner for fertilisers whilst the remaining liquid is pumped for further treatment to the aeration tank [275].



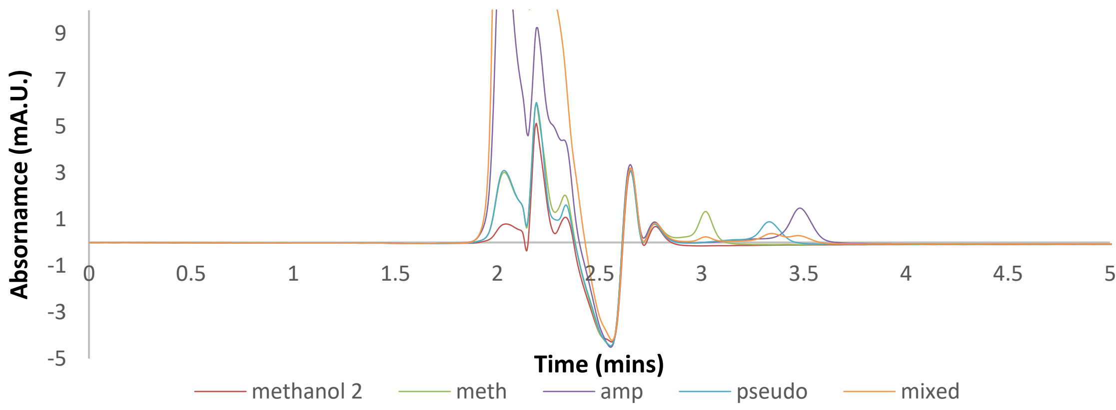

**Appendix Figure 2:** Calculated UV-Vis spectrum from Gaussian 09. for 1-phenylpropan-2-oxime.




**Appendix Figure 3:** Calculated UV-Vis spectrum from Gaussian 09. for 1-Penylpropan-2-oxime.

**Appendix Table 1:** Gaussian results for the energy optimisation of both the E and Z stereoisomers of 1-phenykpropan-2-oxime.

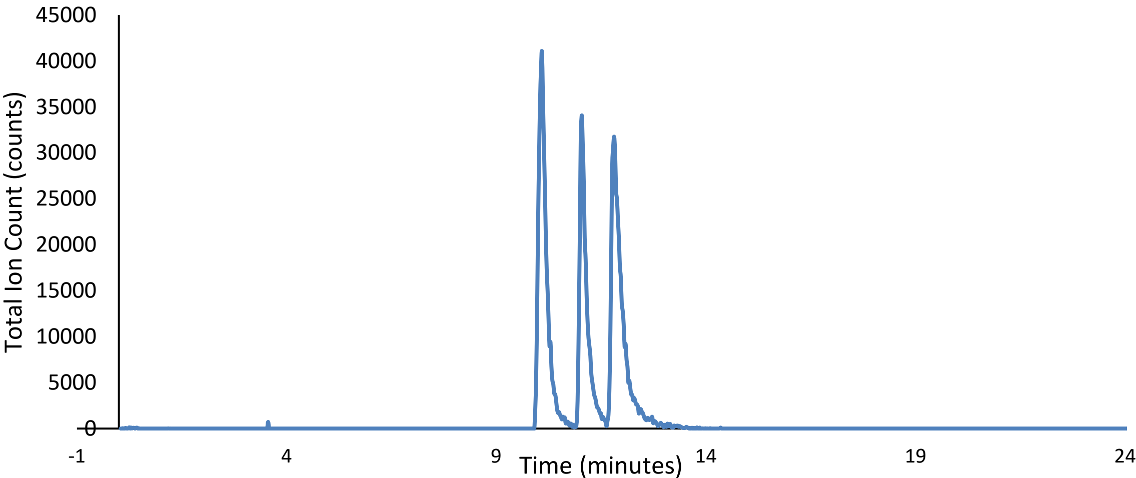
| | |
|---|---|
| **Z-P2P-oxime calculated energy (a.u.)** | -479.50044150 |
| **E-P2P-oxime calculated energy (a.u.)** | -479.50231254 |
| **Energy difference (a.u.)** | -0.001871 |
| **Energy difference (kJ/mol)** | -4.912 |



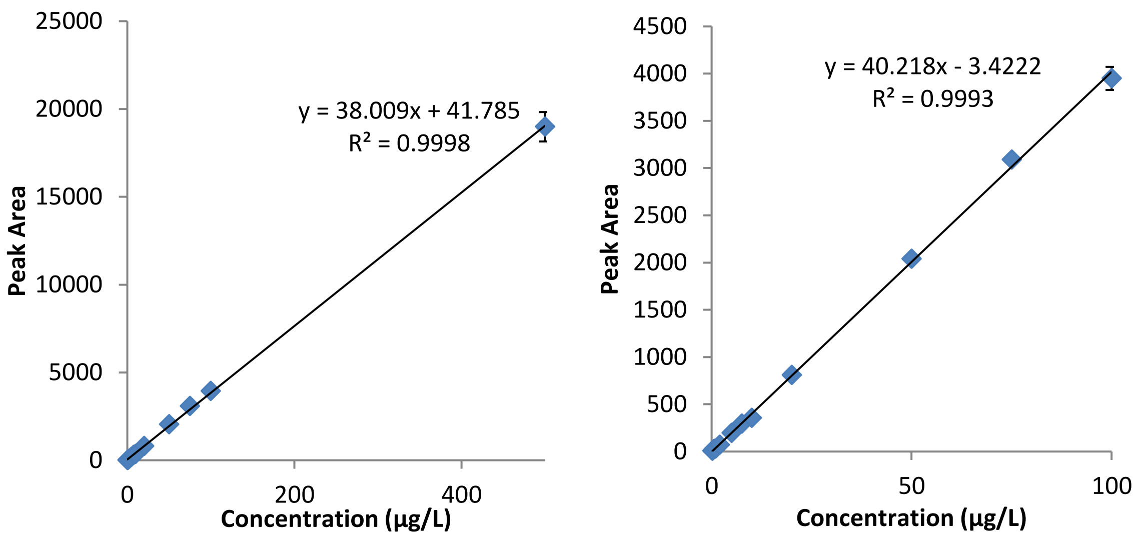

Appendix Figure 4: Separation of a 10 µg/mL mixture of methamphetamine, amphetamine and pseudoephedrine methamphetamine, amphetamine, and pseudoephedrine on a Phenomenex Luna HILIC column with a flow rate of 0.4 mL/min

Appendix Table 2: Agilent 1200 Series Liquid Chromatography Instrumental Parameters C18 Zorbax Eclipse Column:

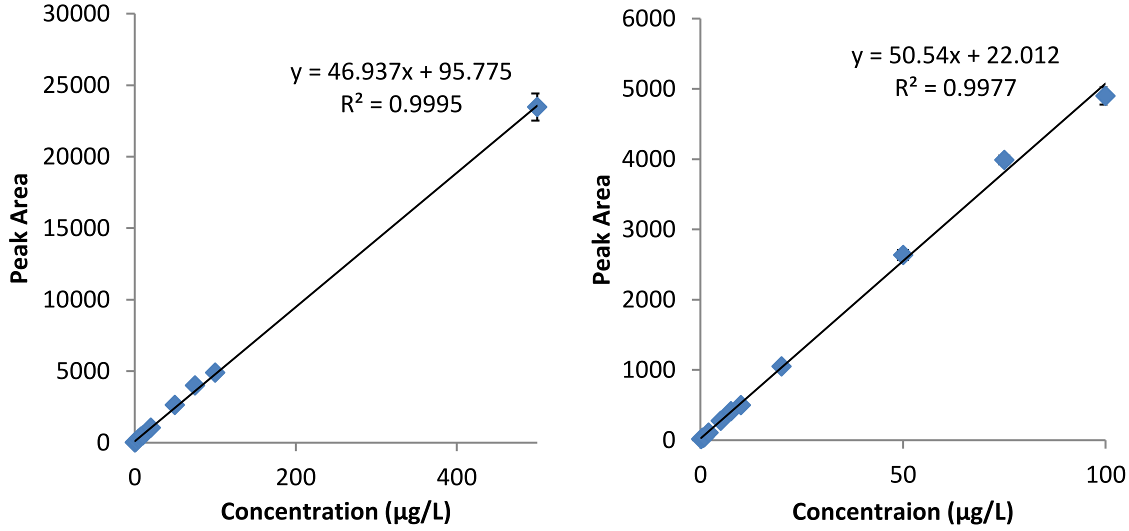
| | |
|---|---|
| **Column Temperature:** | 40 °C |
| **Mobile Phase Composition:** | A: 0.1% formic acid<br>B: 0.1% formic acid in 95% acetonitrile |
| **Flow Rate:** | 0.35 mL/min |
| **Injection Volume** | 10 µL |
| **Gradient** | t = 0 mins: 5 % B<br>t = 3.5 mins: 35 % B<br>t = 11 mins: 100 % B<br>t = 15 mins: 100 % B<br>t = 17 mins: 5 % B (held for 5 minutes to allow column to equilibrate |



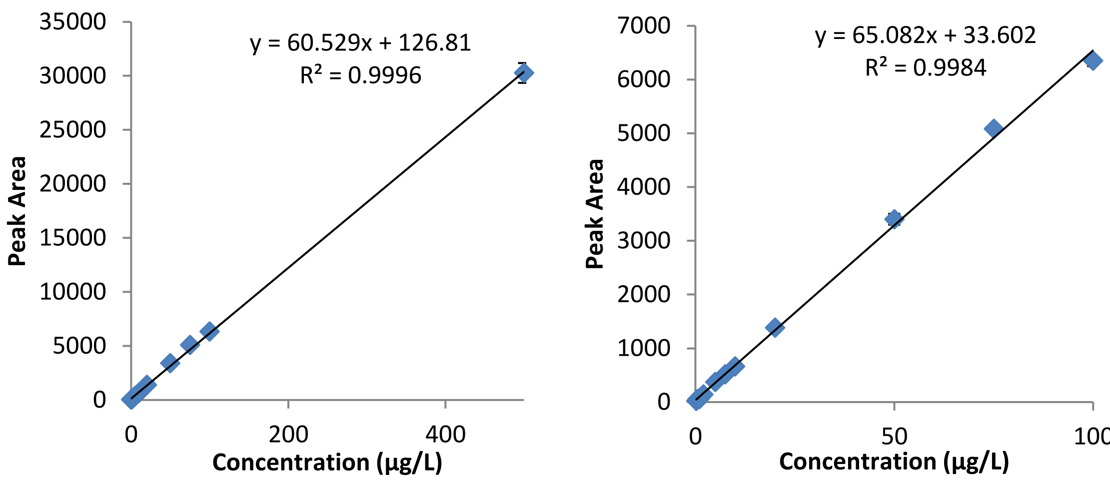

Appendix Figure 5: Separation of 100 ng/mL mixture of methamphetamine, amphetamine and pseudoephedrine on a C18 Zorbax eclipse XDB18 column with a flow rate of 0.35 mL/min



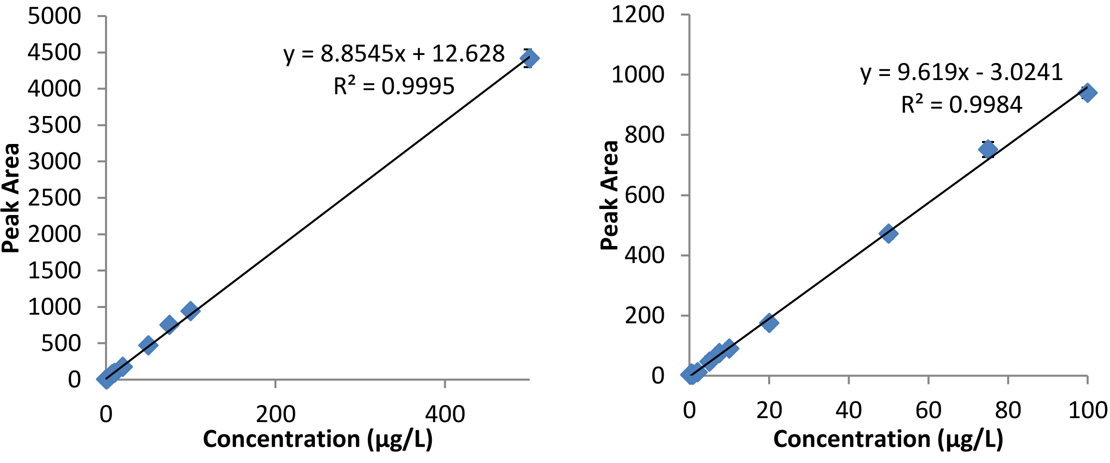

**Appendix Figure 6:** External calibration curve for methamphetamine 150.1 > 119.1 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows the range of 0.2 µg/L – 100 µg/L



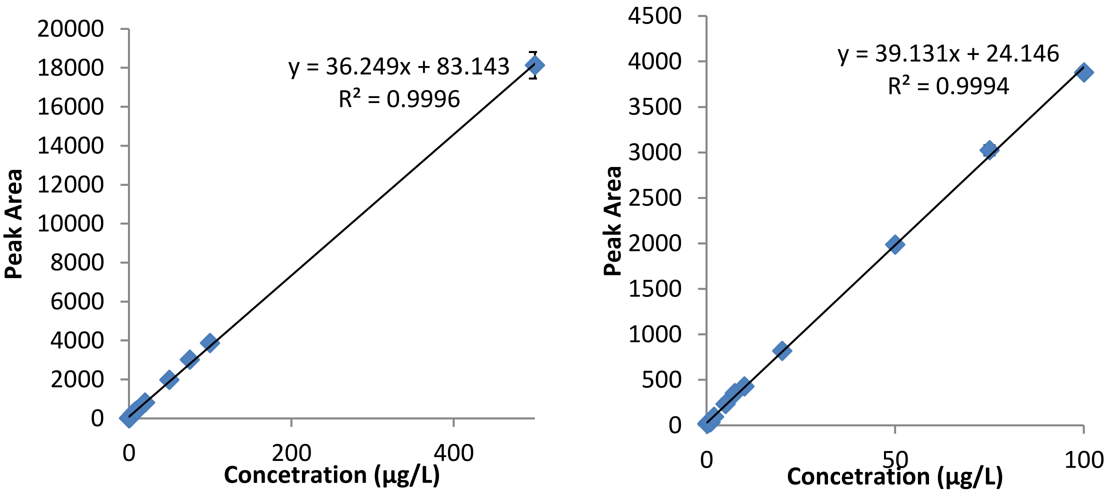

**Appendix Figure 7:** External calibration curve for amphetamine 136.1 > 119.1 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows the range of 0.2 µg/L – 100 µg/L



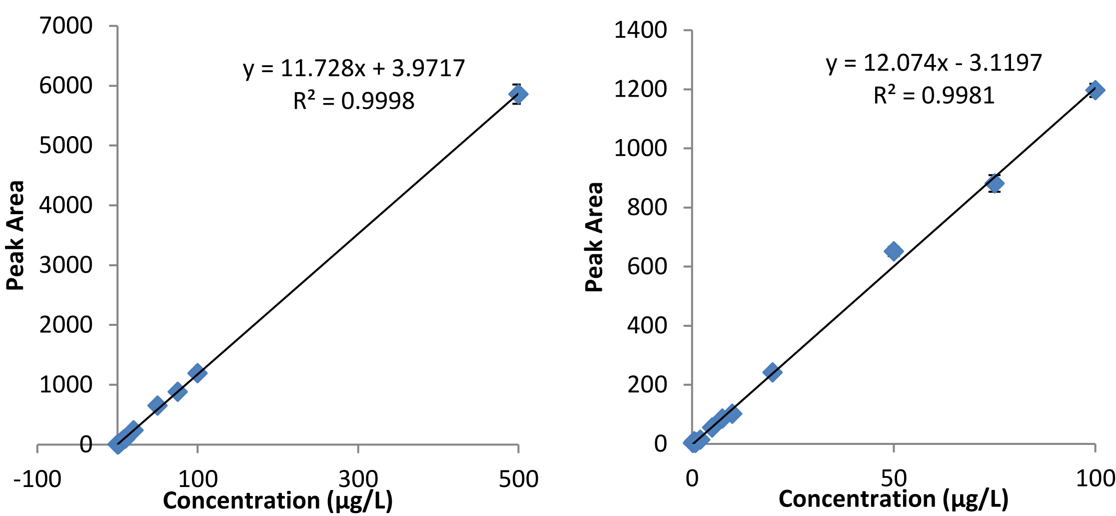

**Appendix Figure 8:** External calibration curve for amphetamine 136.1 > 91.1 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows the range of 0.2 µg/L – 100 µg/L



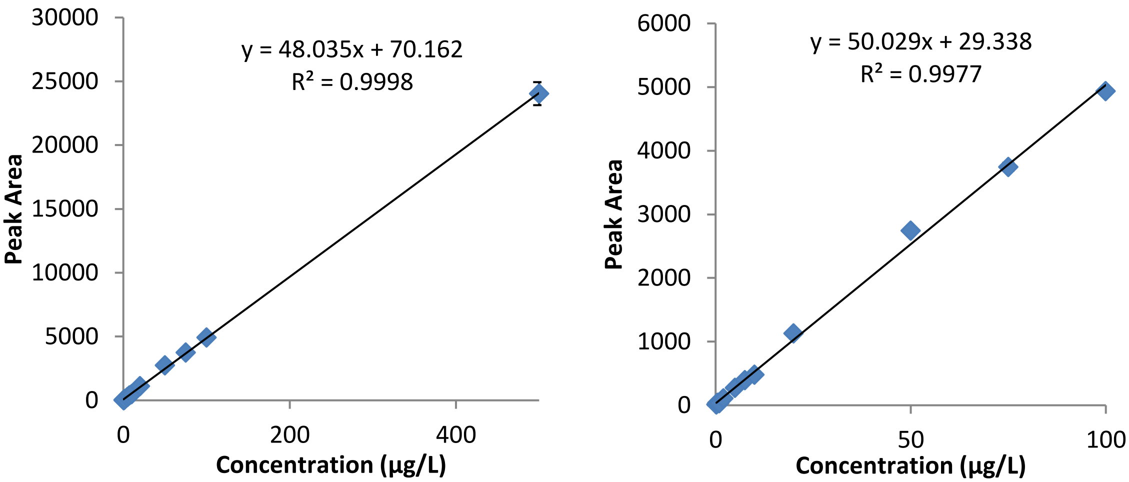

**Appendix Figure 9:** External calibration curve for ephedrine 166 > 117 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows the range of 0.2 µg/L – 100 µg/L



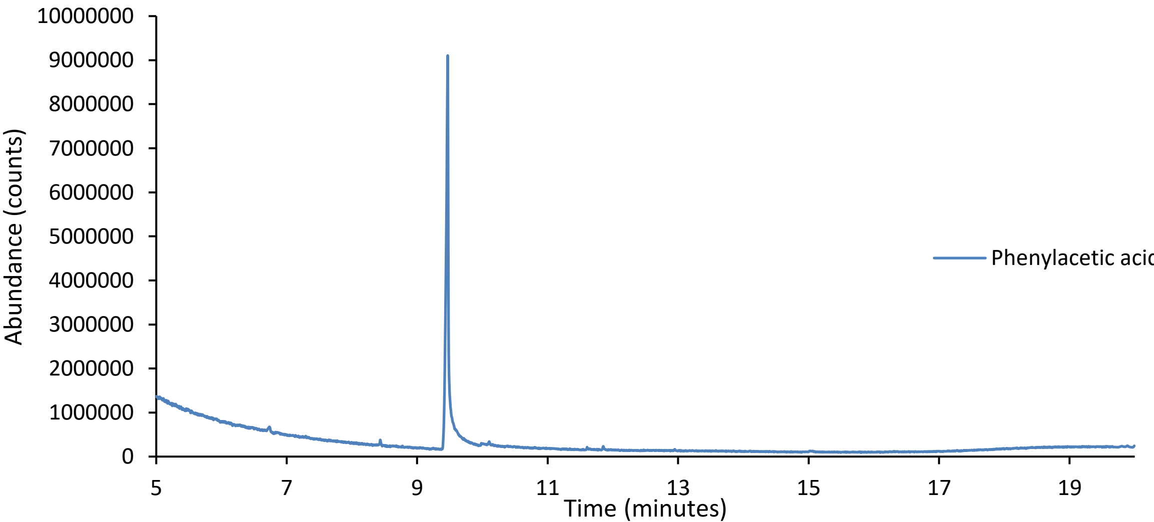

**Appendix Figure 10:** External calibration curve for ephedrine 166.1 > 148.1 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows the range of 0.2 µg/L – 100 µg/L



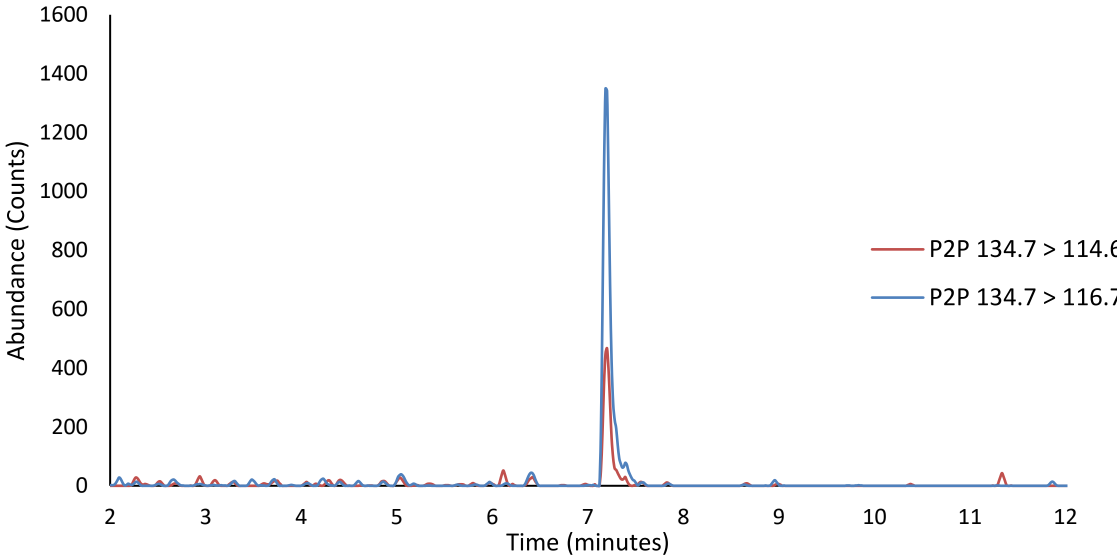

**Appendix Figure 11:** External calibration curve for pseudoephedrine 166 > 117 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows the range of 0.2 µg/L – 100 µg/L.




**Appendix Figure 12:** External calibration curve for pseudoephedrine 166.1 > 148.1 mass transition, left shows the range 0.2 µg/L – 500 µg/L whilst the range on the right shows the range of 0.2 µg/L – 100 µg/L




Appendix Figure 13: Chromatogram of phenylacetic acid standard showing retention time of 9.47 minutes




Appendix Figure 14: LCMS chromatogram of 1 µg/mL spike wastewater extract showing the selected mass transitions for 1-phenylpropan-2-one and retention of 7.20 minutes.

Appendix Derivation 1: Full derivation of pseudo first order equation for the derivatisation of P2P to P2P oxime using hydroxylamine hydrochloride

The reaction for the derivatisation is a bimolecular reaction as follows:

$$P2P + Hydroxylamine\ hydrochloride \rightarrow P2Poxime$$

$$when\ t = 0, [P2P]_t = [P2P]_0 - x\ and\ [Hydroxyl]_t = [Hydroxyl]_0 - x$$

Where $x$ is the concentration of each species reacted

$$rate = k[P2P]_t[Hydrox]_t$$

The change in P2P with time is given by:

$$-\frac{d[P2P]_t}{dt} = k[P2P]_t[Hydrox]_t$$

Substituting in $[P2P]_t = [P2P]_0 - x$ and $[Hydroxyl]_t = [Hydroxyl]_0 - x$ results in:

$$-\frac{d([P2P]_0 - x)}{dt} = k([P2P]_0 - x)([Hydroxyl]_0 - x)$$

$$-\frac{d[P2P]_0}{dt} + \frac{dx}{dt} = k([P2P]_0 - x)([Hydroxyl]_0 - x)$$

As$[P2P]_0$ is a constant $\frac{d[P2P]_0}{dt} = 0$

$$\frac{dx}{dt} = k([P2P]_0 - x)([Hydroxyl]_0 - x)$$

Rearranging results in:

$$\frac{dx}{([P2P]_0 - x)([Hydroxyl]_0 - x)} = k\,dt$$

Splitting the fraction on the LHS to simplify the integration results in:

$$\frac{1}{([P2P]_0 - x)([Hydroxyl]_0 - x)} = \frac{y}{[P2P]_0 - x} + \frac{z}{[Hydroxyl]_0 - x}$$

$$\frac{1}{([P2P]_0 - x)([Hydroxyl]_0 - x)} = \frac{y([Hydroxyl]_0 - x)}{([P2P]_0 - x)([Hydroxyl]_0 - x)} + \frac{z([P2P]_0 - x)}{([P2P]_0 - x)([Hydroxyl]_0 - x)}$$

As the sum of the numerators must equal 1

$$1 = y([Hydroxyl]_0 - x) + z([P2P]_0 - x)$$

$$1 = y[Hydroxyl]_0 - yx + z[P2P]_0 - zx$$

$$1 = y[Hydroxyl]_0 + z[P2P]_0 - x(y + z)$$

$$y + z = 0$$

$$y = -z$$

Substituting in y=-z and solving for z results in:

$$1 = -z[Hydroxyl]_0 + z[P2P]_0$$

$$1 = z([P2P]_0 - [Hydroxyl]_0)$$

$$z = \frac{1}{[P2P]_0 - [Hydroxyl]_0}$$

Substituting in the value for z and y (=-z) results in:

$$\frac{1}{([P2P]_0 - x)([Hydroxyl]_0 - x)} = -\frac{1}{[P2P]_0 - [Hydroxyl]_0}\left(\frac{1}{[Hydroxyl]_0 - [P2P]_0}\right) + \frac{1}{[Hydroxyl]_0 - x}\left(\frac{1}{[P2P]_0 - [Hydroxyl]_0}\right)$$

Which simplifies to:

$$\frac{1}{([P2P]_0 - x)([Hydroxyl]_0 - x)} = \frac{1}{[P2P]_0 - [Hydroxyl]_0}\left(\frac{1}{[Hydroxyl]_0 - x} - \frac{1}{[P2P]_0 - x}\right)$$

Integrating the equation from t=0 (x=0) to t results in:

$$\int_0^x \frac{1}{([P2P]_0 - x)([Hydroxyl]_0 - x)}\,dx = \int_0^t k\,dt$$

Substituting in $\frac{1}{([P2P]_0 - x)([Hydroxyl]_0 - x)} = \frac{1}{[P2P]_0 - [Hydroxyl]_0}\left(\frac{1}{[Hydroxyl]_0 - x} - \frac{1}{[P2P]_0 - x}\right)$ yields:

$$\frac{1}{[P2P]_0 - [Hydroxyl]_0}\left(\int_0^x \frac{1}{[Hydroxyl]_0 - x}\,dx - \int_0^x \frac{1}{[P2P]_0 - x}\,dx\right) = \int_0^t k\,dt$$

$$\frac{1}{[P2P]_0 - [Hydroxyl]_0}[(-\ln([Hydroxyl]_0 - x) - (-\ln([P2P]_0 - x))]_{x=0}^{x} = kt$$

$$\frac{1}{[P2P]_0 - [Hydroxyl]_0}\left[\ln\left(\frac{[P2P]_0 - x}{[Hydroxyl]_0 - x}\right)\right]_{x=0}^{x} = kt$$

$$\frac{1}{[P2P]_0 - [Hydroxyl]_0}\left(\ln\left(\frac{[P2P]_0 - x}{[Hydroxyl]_0 - x}\right) - \ln\left(\frac{[P2P]_0}{[Hydroxyl]_0}\right)\right) = kt$$

$$\frac{1}{[P2P]_0 - [Hydroxyl]_0}\ln\left(\frac{[Hydroxyl]_0([P2P]_0 - x)}{[P2P]_0([Hydroxyl]_0 - x)}\right) = kt$$

Substituting $[P2P]_0 - x = [P2P]_t$

$$\frac{1}{[P2P]_0 - [Hydroxyl]_0}\ln\left(\frac{[Hydroxyl]_0[P2P]_t}{[P2P]_0[Hydroxyl]_t}\right) = kt$$

As $[Hydroxyl] \gg [P2P]$ $[Hydroxyl]_0 \approx [Hydroxyl]_t$

$$-\frac{1}{[Hydroxyl]_0}\ln\left(\frac{[P2P]_t}{[P2P]_0}\right) = kt$$

$$\ln\left(\frac{[P2P]_t}{[P2P]_0}\right) = -kt[Hydroxyl]_0$$

$$\ln[P2P]_t = -kt[Hydroxyl]_0 + \ln[P2P]_0$$

$$\ln[P2P]_t = -k't + \ln[P2P]_0$$

If the natural logarithm of P2P concentration is plotted against time, the result should be a linear relationship with slope of -k' (k'= k[Hydroxyl]$_0$) and intercept of ln[P2P]$_0$.

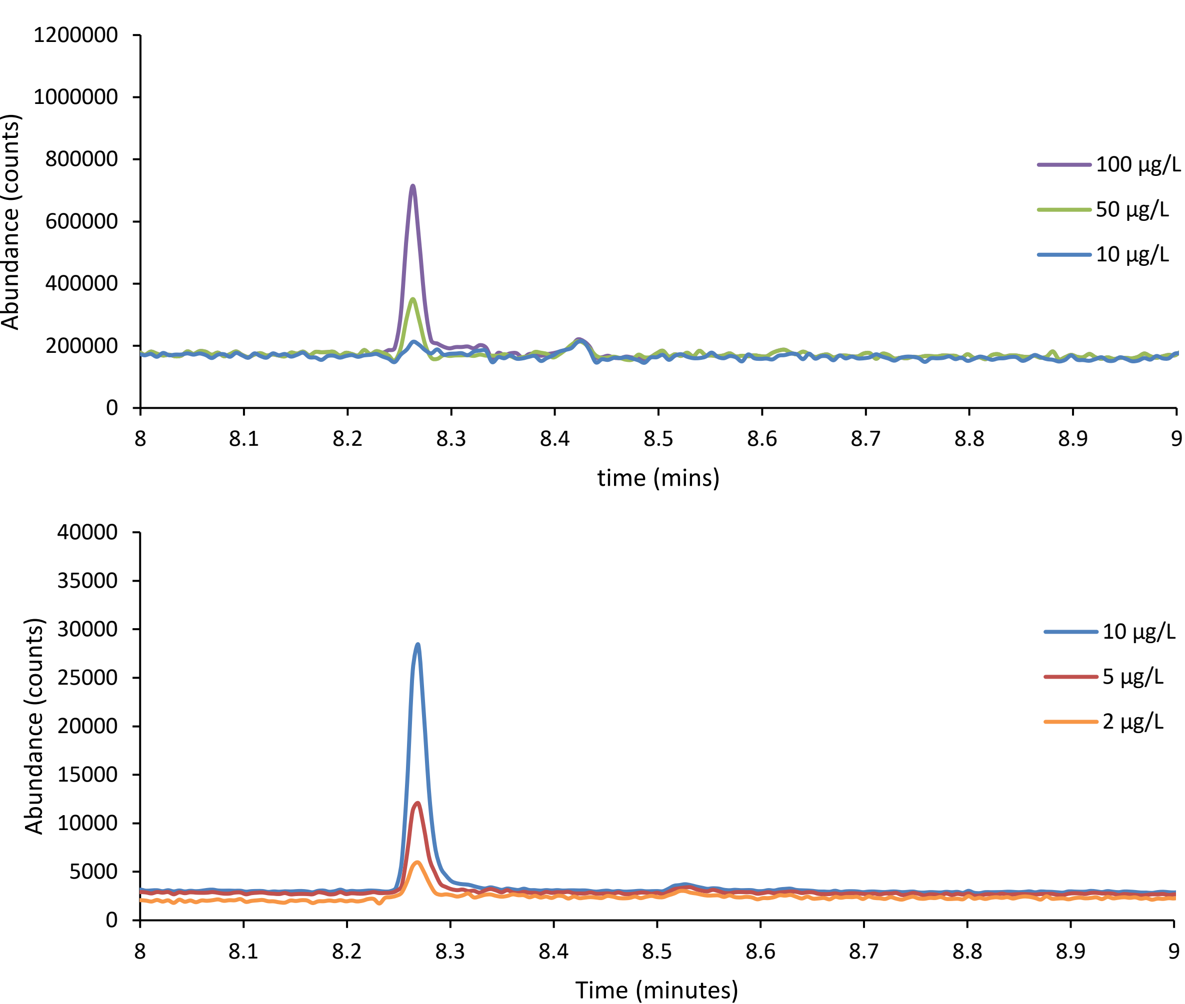
As peak area is proportional to concentration for P2P. i.e. $[P2P] = cA_{P2P}$ where c is a constant and is the response factor for P2P and A is the Peak area. Substituting this in yeilds

$$\ln cA_{P2P_t} = -k't + \ln cA_{P2P_0}$$

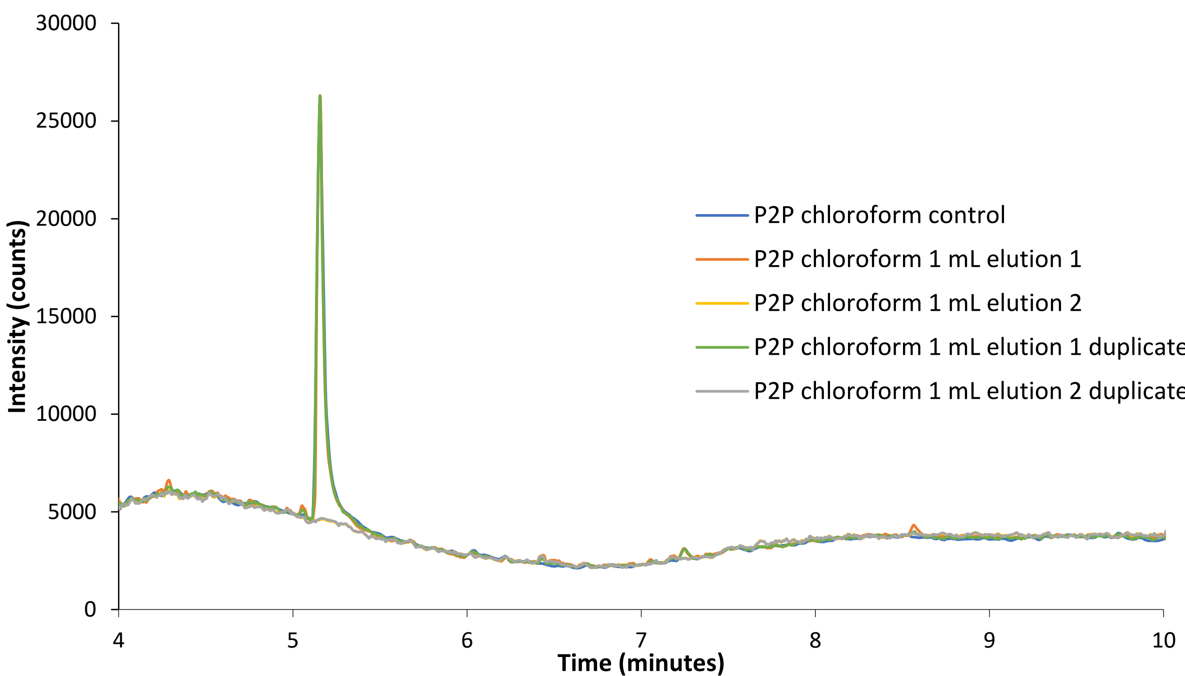
$$\ln A_{P2P_t} + \ln c = -k't + \ln A_{P2P_0} + \ln c$$

$$\ln A_{P2P_t} = -k't + \ln A_{P2P_0}$$

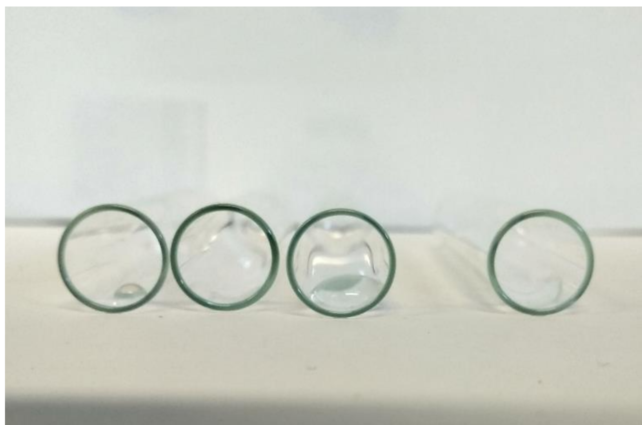
When plotting ln peak area vs time, the intercept becomes the natural logarithm of the peak area for P2P at t = 0 the slope remains constant and as a result the graph is shifted either up or down.



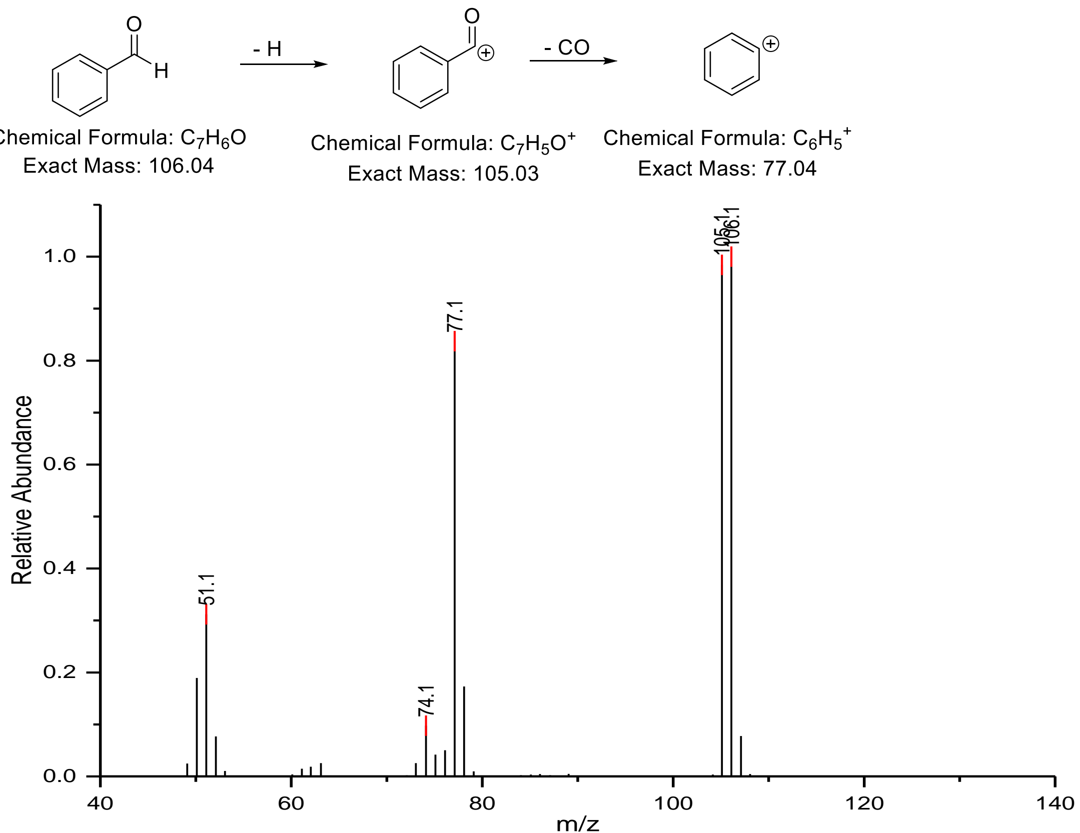

**Appendix Figure 15:** Chromatograms comparing the scan GCMS method (top) to the SIM method (bottom). It can clearly be seen that the SIM method was much more sensitive than that the scan with the non-detection of `1-phenylpropan-2-one in the scan method at 10 µg/L, a concentration well above the LLOD for the SIM method.



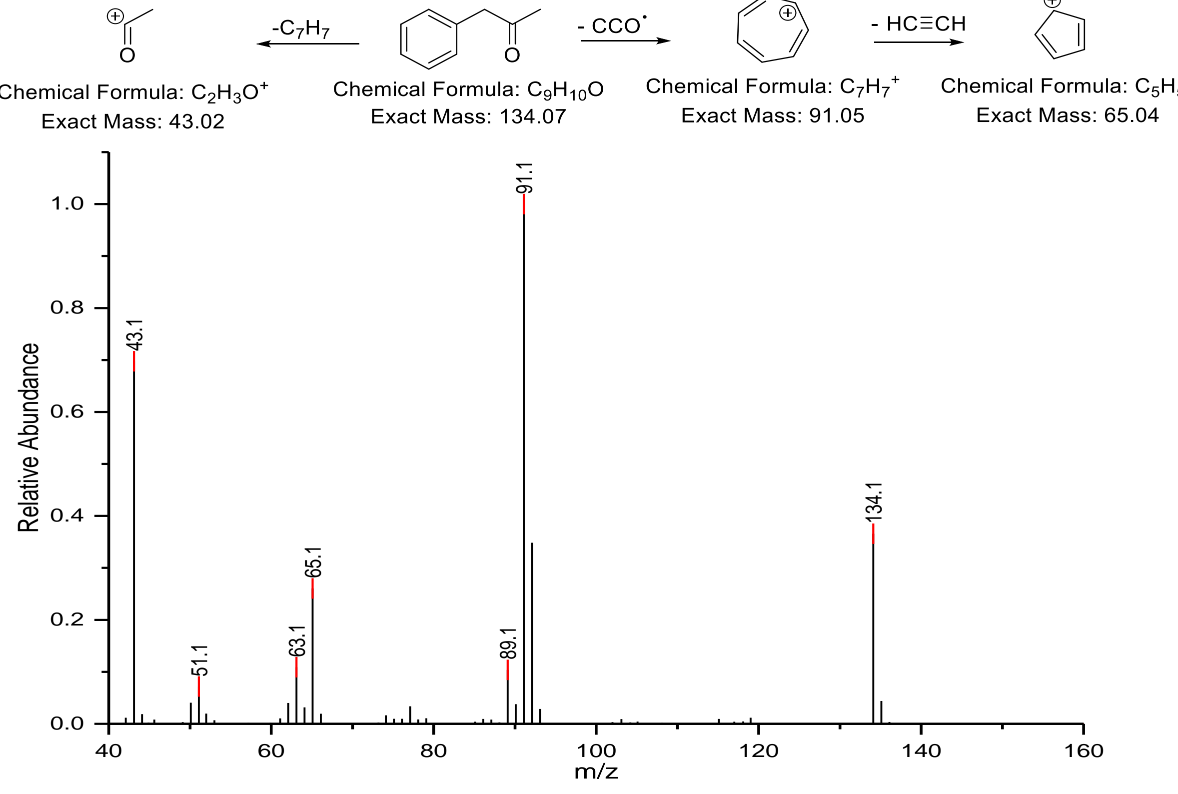

**Appendix Figure 16**: Chromatogram comparing the elution of P2P in the solid phase extraction process using 1mL chloroform compared to the control, spiked blank. GCMS data acquired using the Varian GCMS with the instrumental parameters outlined in section **6.3.2**.



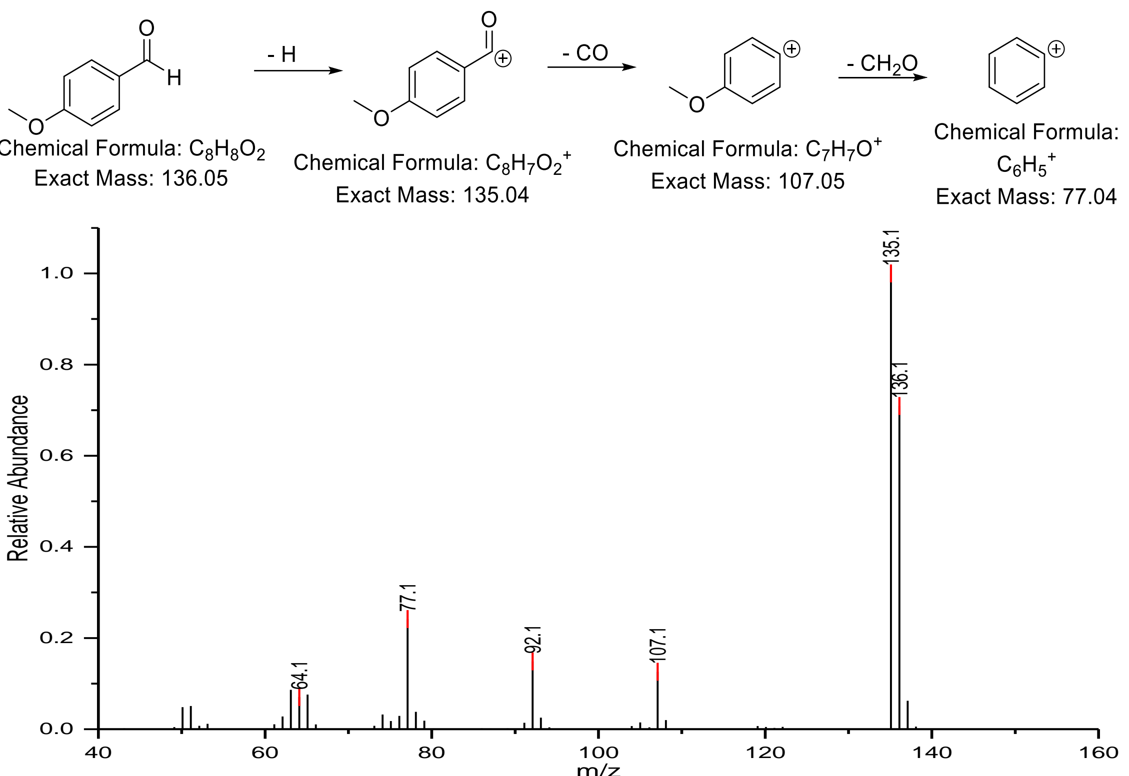
Appendix Figure 17: Side view of droplet with images of in order from left to right each tube is: 10 µL milli Q water on silanised glass tube, 10 µL milli Q water on unmodified glass tube, 100 µL milli Q water on silanised glass tube and 100 µL milli Q water on unmodified glass tube.



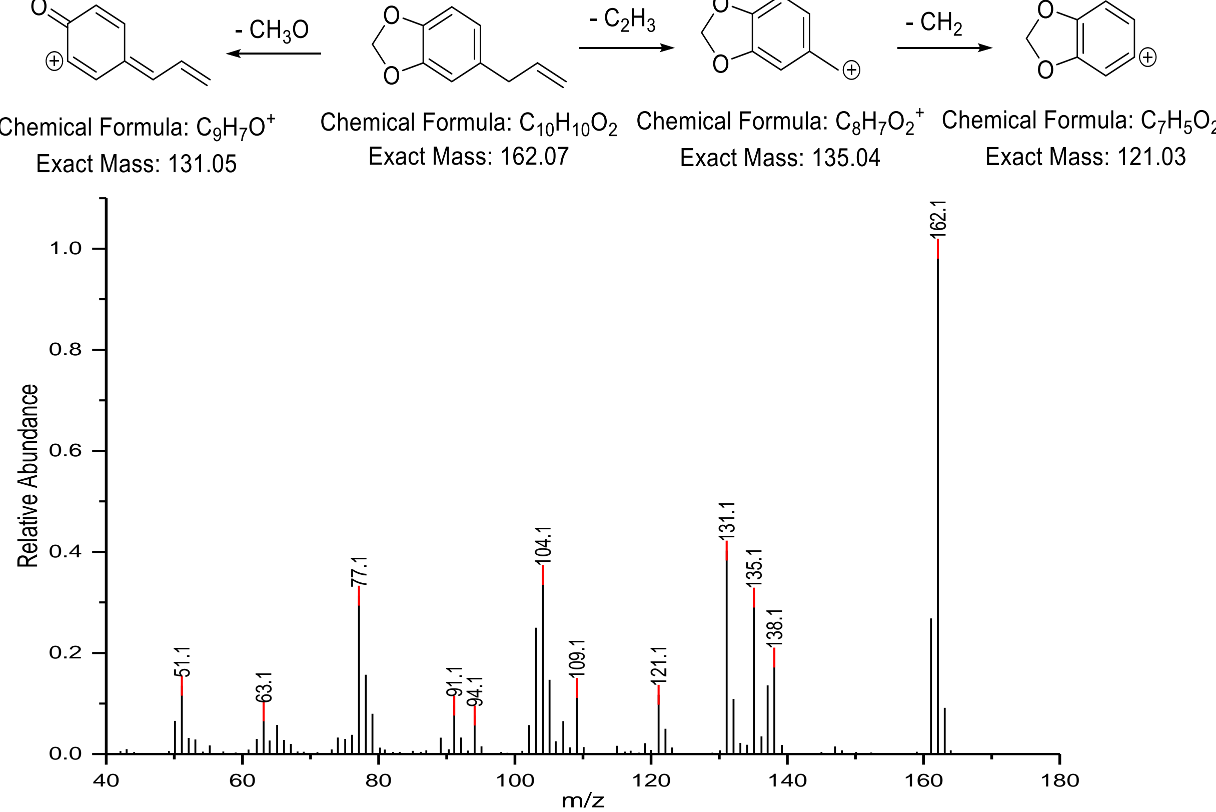

Appendix Figure 18: Mass fragmentation pattern of benzaldehyde showing the molecular ion at 106 m/z and formation of the fragments at 105 and 77 m/z.




Appendix Figure 19: Mass fragmentation pattern of 1-phenylpropan-2-one showing the molecular ion at 134 m/z and formation of the fragments at 91, 65 and 43 m/z.




Appendix Figure 20: Mass fragmentation pattern of 4-methoxybenzaldehyde showing the molecular ion at 136 m/z and formation of the fragments at 135, 107 and 77 m/z.




Appendix Figure 21: Mass fragmentation pattern of safrole showing the molecular ion at 162 m/z and formation of the fragments at m/z = 135,131 and 121.

- $CH_2O$ → - $CH_2O$ → - C≡O →

Chemical Formula: $C_8H_6O_3$
Exact Mass: 150.03

Chemical Formula: $C_7H_5O_2^+$
Exact Mass: 121.03

Chemical Formula: $C_6H_3O^+$
Exact Mass: 91.02

Chemical Formula: $C_5H_3^+$
Exact Mass: 63.02

- C≡O ↓ - C≡O → - $H_2$ →

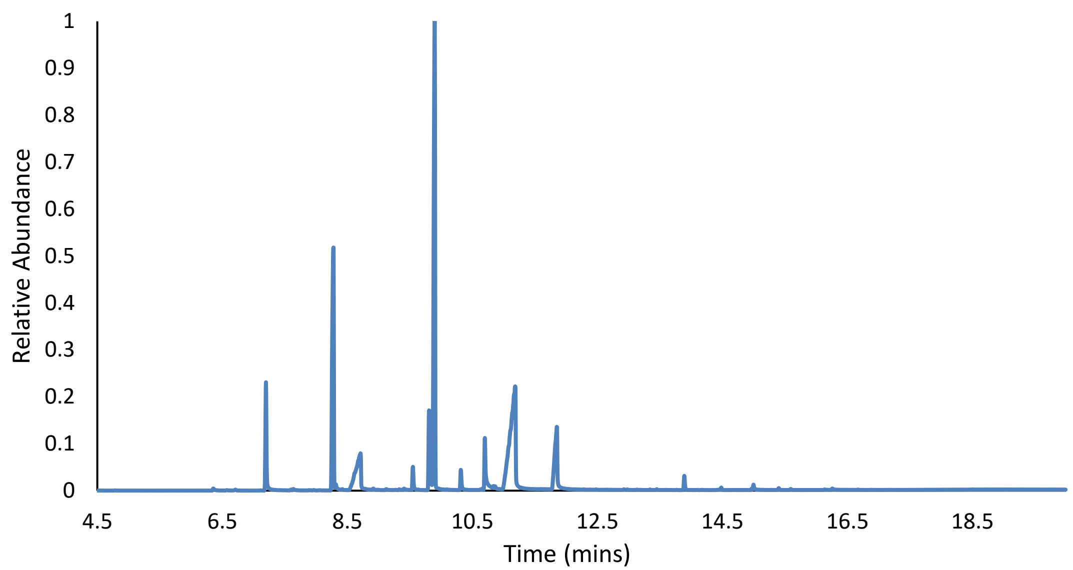
Chemical Formula: $C_6H_5O^+$
Exact Mass: 93.03

Chemical Formula: $C_5H_5^+$
Exact Mass: 65.04

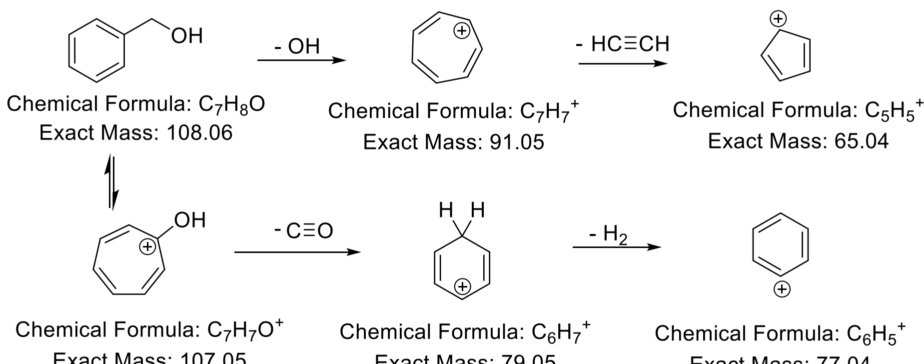
Chemical Formula: $C_5H_3^+$
Exact Mass: 63.02

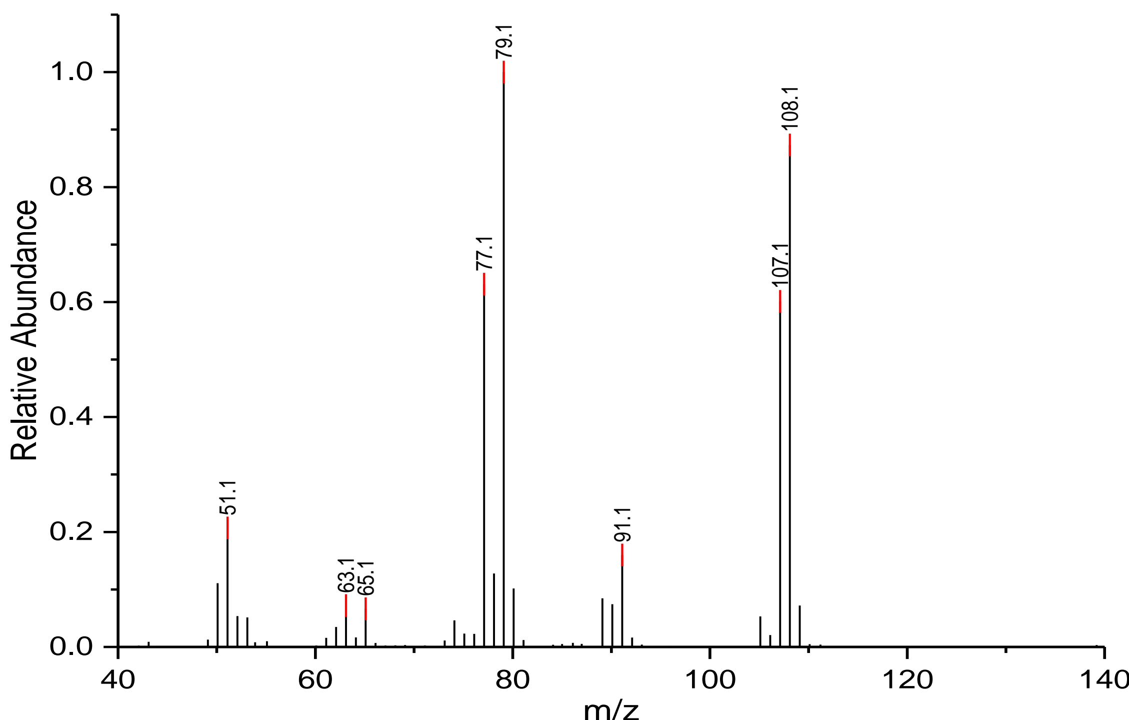
Relative Abundance
1.0
0.8
0.6
0.4
0.2
0.0
149.1
150.1
121.1
91.1
63.1
65.1
40 60 80 100 120 140 160
m/z

Appendix Figure 22: Mass fragmentation pattern of piperonal showing the molecular ion at 1µm/z and formation of the fragments at 121, 91, 65 and 63 m/z.

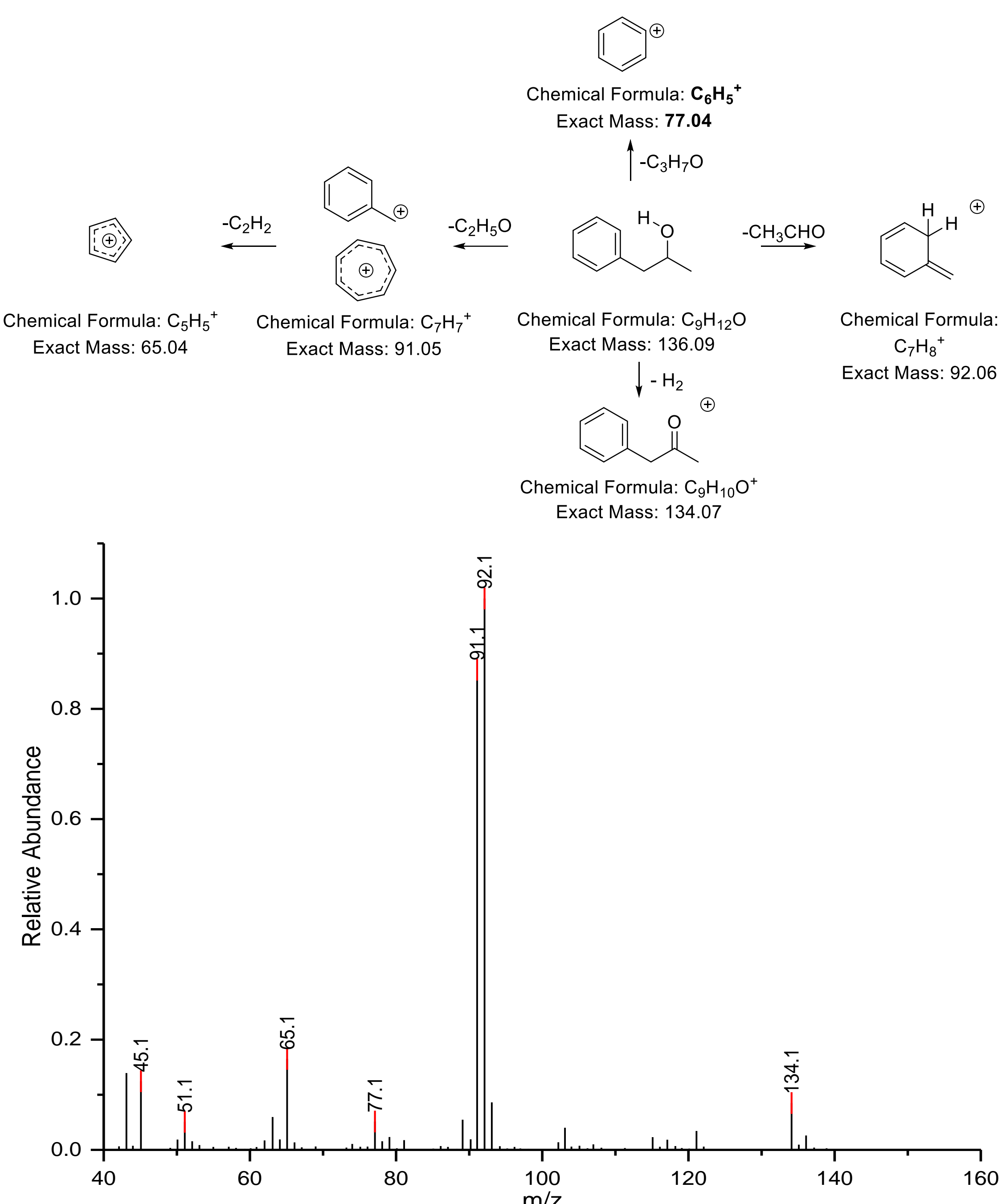



**Appendix Figure 23: SCAN GCMS chromatogram showing the presence of large peaks with retention times of 7.20, 8.32, 8.71, 9.81, 10.7, 11.19, 11.85 and 13.89 minutes.**



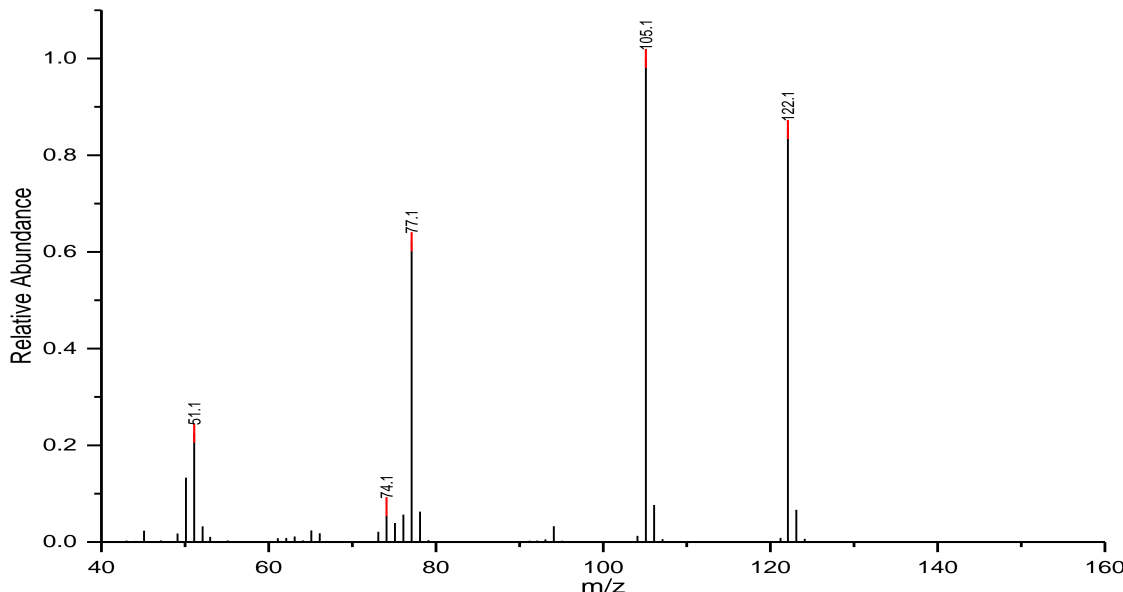



**Appendix Figure 24: Fragmentation pattern of the peak eluting at 7.20 minutes in the spiked wastewater sample believed to be due to the formation of benzyl alcohol. The expected fragmentation of benzyl alcohol (m/z =108) shows the formation of mass fragments with m/z of 107 ,91, 79, 77 and 65.**

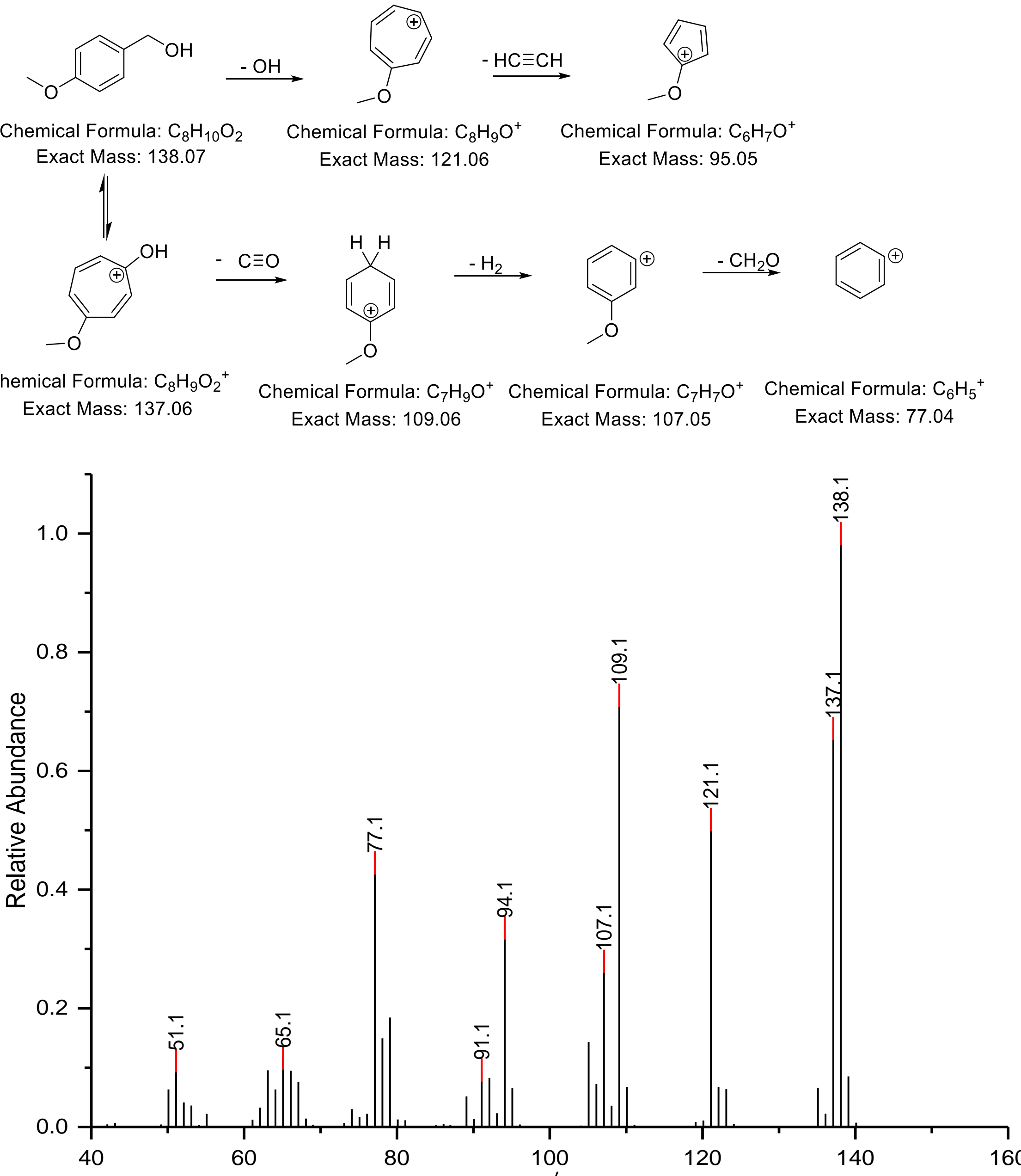



Appendix Figure 25: Fragmentation pattern of the peak eluting at 8.32 minutes in the spiked wastewater sample believed to be due to the formation of 1-phenylpropan-2-ol. The expected fragmentation of 1-phenylpropan-2-ol (m/z =136) shows the formation of mass fragments with m/z of 136, 134, 92, 91 and 65.

The molecular ion for 1-phenylpropan-2-ol occurs at 136 m/z as can be seen in the mass spectrum. The presence of the peak at m/z 134.1 is likely to be a result of not only fragmentation of 1-phenylpropan-2-ol but also due to 1-phenylpropan-2-ol eluting on the tail of the 1-phenylpropan-2-one peak.




**Appendix Figure 26: Fragmentation pattern of the peak eluting at 8.71 minutes in the spiked wastewater sample believed to be due to the formation of Benzoic acid. The expected fragmentation of Benzoic acid (m/z =122) shows the formation of mass fragments with m/z of 105, 77 and 51.**




**Appendix Figure 27: Fragmentation pattern of the peak eluting at 9.81 minutes in the spiked wastewater sample believed to be due to the formation of 4-methoxybenzene methanol. The expected fragmentation of 4-methoxybenzene methanol (m/z =138) shows the formation of mass fragments with m/z of 137, 121, 109,107 and 77.**

Chemical Formula: $C_8H_8O_3$
Exact Mass: 152.05

- C≡O

Chemical Formula: $C_8H_7O_3^+$
Exact Mass: 151.04

Chemical Formula: $C_7H_7O_2^+$
Exact Mass: 123.04

- $H_2$

Chemical Formula: $C_7H_5O_2^+$
Exact Mass: 121.03

- C≡O

Chemical Formula: $C_6H_5O^+$
Exact Mass: 93.03

- C≡O

Chemical Formula: $C_5H_5^+$
Exact Mass: 65.04

Relative Abundance
1.0
0.8
0.6
0.4
0.2
0.0
51.1
63.1
65.1
77.1
93.1
105.1
122.1
135.1
152.1
40
60
80
100
120
140
160
m/z

**Appendix Figure 28: Fragmentation pattern of the peak eluting at 10.70 minutes in the spiked wastewater sample believed to be due to the formation of 3,4-(methylenedioxy)benzyl alcohol. The expected fragmentation of 3,4-(methylenedioxy)benzyl alcohol (m/z =152) shows the formation of mass fragments with m/z of 151, 123, 121, 93 and 65.**

- OH
- CO
- $CH_2O$
- $CH_4$
- C≡O

Chemical Formula: $C_8H_8O_3$
Exact Mass: 152.05

Chemical Formula: $C_8H_7O_2^+$
Exact Mass: 135.04

Chemical Formula: $C_7H_7O^+$
Exact Mass: 107.05

Chemical Formula: $C_6H_5^+$
Exact Mass: 77.04

Chemical Formula: $C_6H_3O^+$
Exact Mass: 91.02

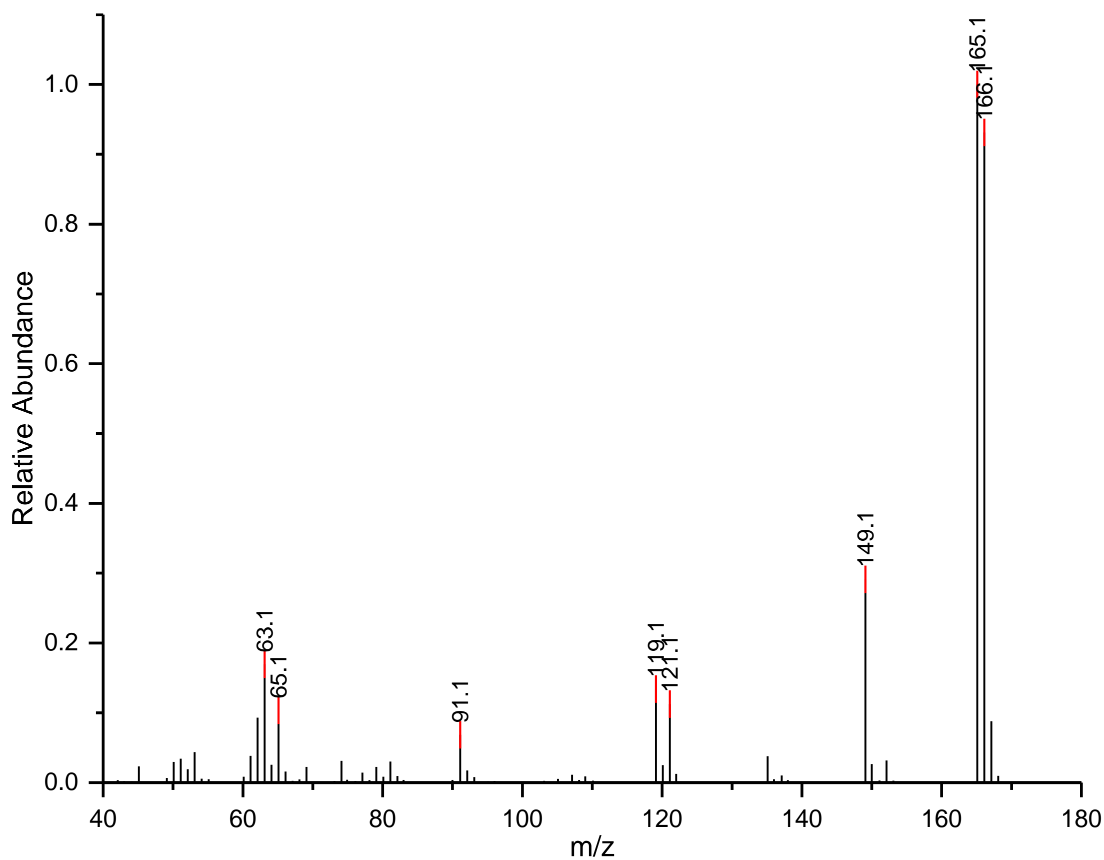
Chemical Formula: $C_5H_3^+$
Exact Mass: 63.02

Relative Abundance

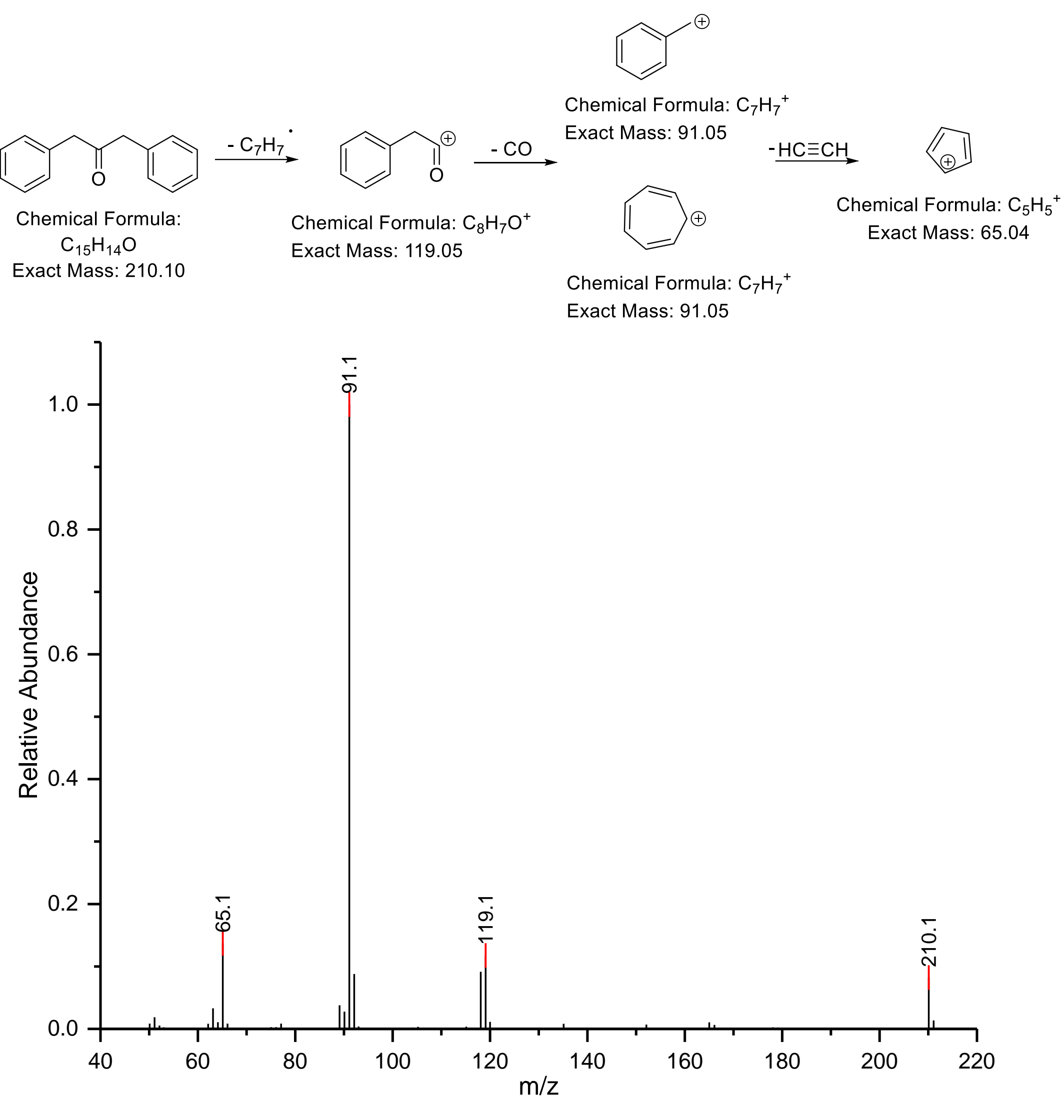
m/z

63.1
77.1
81.1
92.1
107.1
135.1
152.1

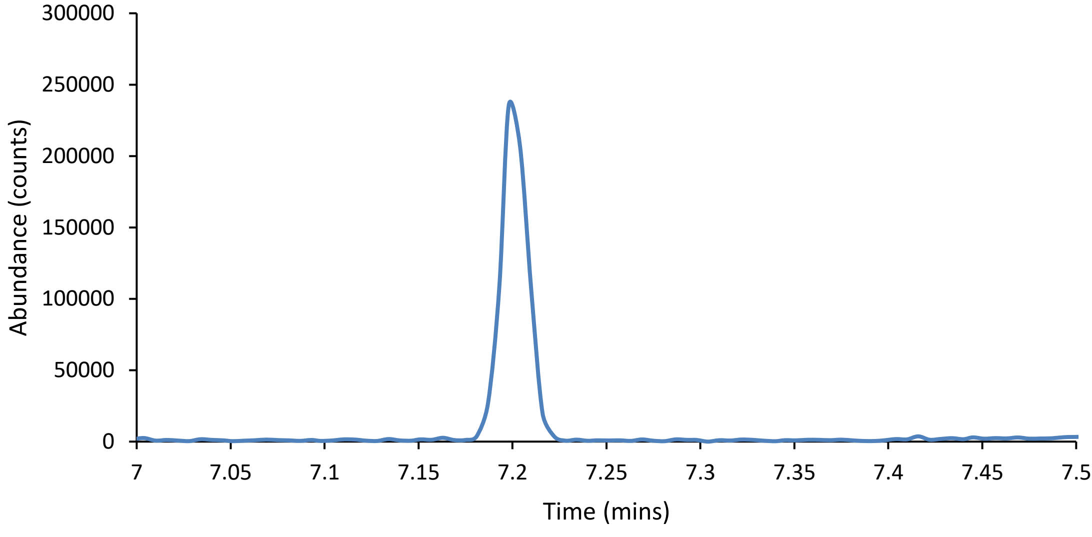
**Appendix Figure 29: Fragmentation pattern of the peak eluting at 11.19 minutes in the spiked wastewater sample believed to be due to the formation of 4-methoxybenzoic acid. The expected fragmentation of 4-methoxybenzoic acid (m/z =152) shows the formation of mass fragments with m/z of 135, 107, 91, 77 and 63.**

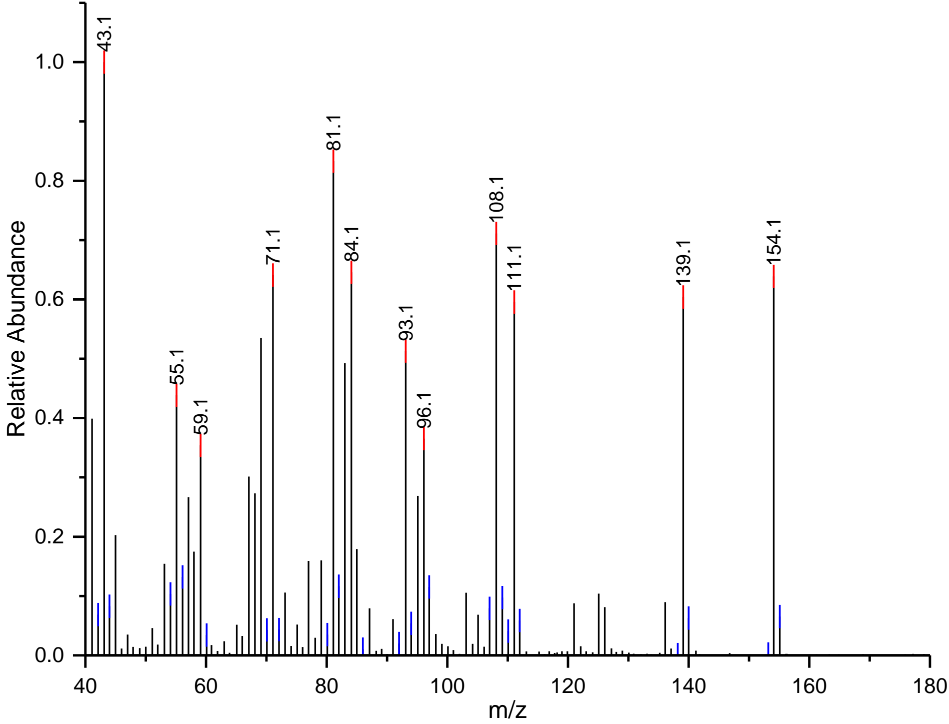



**Appendix Figure 30:** **Fragmentation pattern of the peak eluting at 11.85 minutes in the spiked wastewater sample believed to be due to the formation of 3,4-(methylenedioxy)benzoic acid. The expected fragmentation of 3,4-(methylenedioxy)benzoic acid (m/z =155) shows the formation of mass fragments with m/z of 165, 149, 121, 119, 91 and 63.**

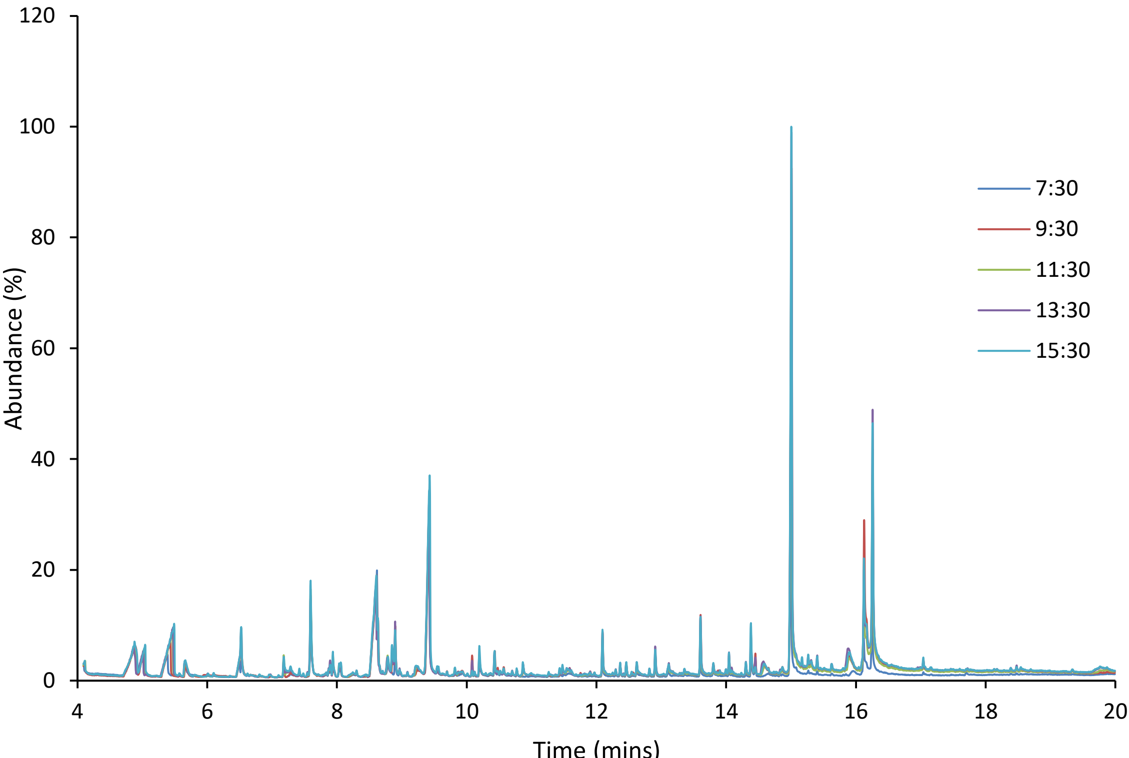



**Appendix Figure 31: Fragmentation pattern of the peak eluting at 13.89 minutes in the spiked wastewater sample believed to be due to the formation of 1,3-diphenylpropan-2-one. The expected fragmentation of 1,3-diphenylpropan-2-one (m/z =210) shows the formation of mass fragments with m/z of 119, 91and 65.**




**Appendix Figure 32: (Top) 108 m/z extracted ion chromatogram showing its presence in a 05/05/2018 Christies beach wastewater sample at 7.20 mins. (Bottom) corresponding mass fragmentation pattern observed for the peak at 7.20 minutes believed to be due to the presence of eucalyptol in the sample. Note the extremely low intensity of the mass fragments with m/z at 107 and 79.**




**Appendix Figure 33:** **Overlay of chromatograms obtained from the GCMS analysis of extracted Christies Beach grab wastewater samples taken every 2 hours from 7:30 am until 3:30 pm on 22/01/2018.**

**Appendix Example Calculation 1:** **Calculation of drug target residue per day 1000 people served by a wastewater treatment facility.**

$$A_{meth\ 161.1 \rightarrow 91.1} \quad = \ 8171.2\ counts.min$$

The line of best fit from the external calibration is given by:

$$A_{meth\ 161.1 \rightarrow 91.1} \quad = 40.218 \times C_{meth\ extract} - 3.42$$

Where $A_{meth\ 161.1 \rightarrow 91.1}$ is the peak area for the methamphetamine peak with mass transition 161.1 → 91.1 and $C_{meth}$ is the concentration of methamphetamine in the extract in µg/L. Substituting in $A_{meth\ 161.1 \rightarrow 91.1}$ and solving for $C_{meth}$ results in 113.2 µg/L. Using the recovery of methamphetamine throughout the extraction procedure, 98 %, and the preconcentration factor (CF) of 500 the concentration of methamphetamine in the 50 mL sample can be calculated.

$$C_{meth\ 50mL} = \frac{C_{meth\ extract} \ \times CF}{recovery\ (\%)} \times 100\%$$

Solving the above equation results in a concentration of 231.0 ng/L. This value is then used to calculate the mass load of methamphetamine in the wastewater per day. This is done by multiplying the $C_{meth\ 50mL}$ by the flowrate of the wastewater treatment facility.

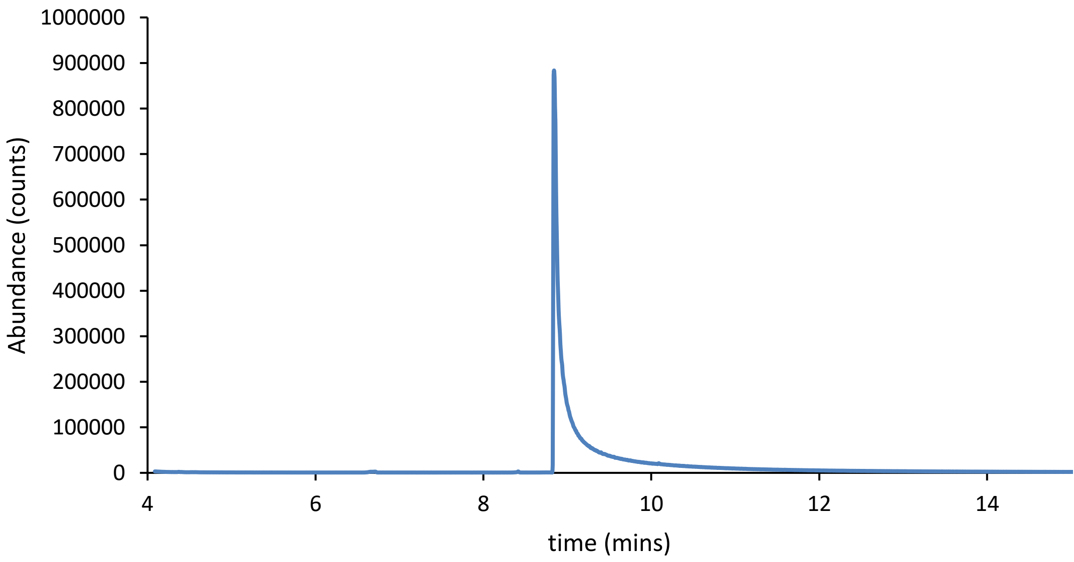
$$m_{meth} = C_{meth\ extract} \times FR$$

$$m_{meth} = 15710.62\ mg/day$$

Knowing that approximately 39% of methamphetamine is excreted unchanged and the population served by the wastewater treatment facility is approximately 230,000 the amount consumed per 1000 people can be calculated to be 175.15 mg/day/1000 people.

**Appendix Table 3: Mass loads of methamphetamine, amphetamine and pseudoephedrine in a Coombabah extracted wastewater sample. Mass loads were calculated from LCMS peak area using an external calibration.**

| Compound | Mass load in WW (mg/day/1000 people) |
|---|---|
| Methamphetamine | 175 |
| Amphetamine | 9.0 |
| Pseudoephedrine | 995 |




**Appendix Figure 34: SIM Chromatogram obtained from the analysis of methamphetamine (4 µg/mL). showing the broad tailing peak**