Drugs of Abuse in Oral Fluid and Endogenous Post-Mortem Blood Concentrations of Gamma-Hydroxybutyrate

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By

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Abstract

Oral fluid is a versatile matrix that is proving more popular within forensic toxicology. Its use is multifaceted, and is the preferred matrix in therapeutic drug monitoring and roadside testing of drivers suspected to be under the influence of drugs. Benefits including the difficulty of adulteration, the ease and non-invasiveness of sample collection, the range of analytes that can be detected and decent correlations between concentrations in blood or plasma and oral fluid are some of the reasons for its attractiveness to practitioners and forensic toxicologists. Most often, oral fluid is collected using collection devices which can often include a stabilising buffer. When new collection devices are introduced to the market it is important that their applicability to drug testing is investigated to show they are fit for purpose.

One of the newest collection devices on the market is the NeoSAL™ collection device from Neogen. This collector was gravimetrically assessed for oral fluid volume collection and drug recovery. Collection volume adequacy of the NeoSAL™ device was compared to two commonly used, commercially available, collection devices: namely the Immunalysis Quantisal™ and the OraSure Intercept® *i*2™ collection devices. Results showed that the NeoSAL™ device is capable of collecting more than the volume stated by the manufacturer, similar to the Intercept® *i*2™ which also over-collected, whereas the Quanitsal™ device collected the stated volume. Drug recoveries from the NeoSAL™ collection pad for all drugs investigated in this thesis exceeded 57% (lower recoveries were observed for temazepam and diazepam).

Although amphetamine and methamphetamines are not often abused or encountered in forensic samples in Scotland, they are a global problem and effects of abuse can negatively impact a person's ability to drive by increasing recklessness and risk-taking. Neat and oral fluid collected using the NeoSAL™ device were used to develop and partially validate a method for the quantification of amphetamine, methamphetamine, MDMA, MDA and MDEA using GC-MS. MDEA was also assessed, but did not give acceptable results for accuracy and precision. A short-term autosampler stability study for the four acceptable

analytes showed that they were stable on the autosampler for up to 48 hours (~ 19 °C).

Opioid and benzodiazepine drugs are two of the most commonly abused drug groups in Scotland. They are often taken synchronously, and the latter is the most commonly prescribed and encountered drug group in Scotland. With the continuation of opioid epidemics and large numbers of people in opioid-treatment programmes, it is beneficial to have a sensitive and selective method that can be used for the simultaneous analysis of these two drug groups. Research has shown that both drug groups are common in drivers, although symptoms of use include loss of coordination, sedation, and drowsiness. An SPE procedure using LC-MS/MS detection was optimised for the extraction for the concurrent analysis of 5 benzodiazepines and 5 opioid drugs. The method was validated according to the guidelines for method validation in forensic toxicology (SWGTOX 2013). The validated method was successfully applied to paired oral fluid and blood samples collected from 16 benzodiazepine users. The NeoSAL™ collection device showed that good recoveries (>57% for all analytes but diazepam and temazepam), and good detection rates for the 10 analytes studied was possible. Oral fluid and blood results showed a good correlation between the analytes detected and in most cases where there was no overlap, it was possible to explain these discrepancies by metabolism, detection windows, low sample volume, and sensitivities of the respective analytical methods used.

Gamma-hydroxybutyrate (GHB) is a short-chain fatty acid that is not only endogenous to the mammalian body, but can also be prescribed medicinally and be used as a drug of abuse. A stability study (over 56 days) of GHB in neat oral fluid was carried out as none have been published in the literature. GHB stability is an important factor to assess due to its short detection window in the more traditional matrices blood and urine. A simple protein precipitation extraction procedure was used, and the analytical GC-MS method was adapted from the in-house method for analysis of GHB/beta-hydroxybutyrate (BHB) in blood. The method was partially validated and the stability of GHB was assessed at two concentrations at three temperatures (fridge ~ 4 °C, freezer ~ -21 °C, and room temperature ~ 20 °C). GHB appeared to be stable at all three temperatures for up to 56 days.

Endogenous post-mortem blood concentrations of GHB have been widely studied, however debate is still existent regarding cut-off concentrations that should be applied. Problematic interpretation arises from the post-mortem production, and inter- and intra- individual variation of GHB in the human body. 1811 cases between 2010 and 2016, which did not implicate GHB in the cause of death or where GHB was not suspected to have been used, were extracted from the in-house Forensic Medicine and Science (FMS) database. The majority of cases (51%) were deaths related to alcohol abuse. 76% of cases showed GHB concentrations <30 mg/L, and 94% of all cases had concentrations of less than 50 mg/L. Results also suggest that the use of a preservative may prevent *in vitro* formation of post-mortem GHB. 112 cases showed GHB concentrations in excess of 50 mg/L with advanced decomposition, therefore suggesting that decomposition changes may increase GHB concentrations. This was the largest dataset that ever studied endogenous post-mortem GHB concentrations, and results highlight the difficulty when applying cut-off concentrations to distinguish post-mortem or exogenous and endogenous concentrations.

Table of Contents

List of Tables

List of Figures

List of Equations

List of Accompanying Material

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Author's Declaration

"I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or at any other institution"

Signature _____

Printed Name _____

Definitions/Abbreviations

1,4-BD	1,4-Butanediol
%CV	Coefficient of Variation percentage
%ME	Matric Effect
%PE	Process Efficiency
%R	Percent Recovery
°C	Degree Celsius
µg	Micrograms
µm	Micrometre
6MAM/6-MAM	6-monoacetylmorphine
AA	Ammonium acetate
ACN	Acetonitrile
AMP	Amphetamine
ATS	Amphetamine-type stimulants
BCX	Benzenesulfonic Acid
BHB	Beta-hydroxybutyrate
BSTFA + 1% TMCS	N,O-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane
BZD	Benzodiazepine
CE	Collision energy
CI	Chemical ionisation
CNS	Central Nervous System
COD	Codeine
CYP2D6	Cytochrome P450 2D6
D-	Deuterated compound
DCM	Dichloromethane
DFC	Drug Facilitated Crime
DFSA	Drug Facilitated Sexual Assault
dH_2O	Deionised water
DHC	Dihydrocodeine
DIAZ	Diazepam
DMD	Demethyldiazepam, nodiazepam or nordazepam
DMRM	Dynamic Multiple Reaction Monitoring
DRE	Drug Recognition Expert
DRUID	Driving Under the Influence of Drugs, Alcohol and Medicines in Europe
DUID	Driving under the influence of drugs
EI	Electron Ionisation
ELISA	Enzyme-linked Immunosorbent assay
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
ESI	Electron Spray Ionisation
ETIZ	Etizolam
EtOAc	Ethyl acetate
EWDTS	European Workplace Drug Testing Society
FID	Flame Ionisation Dectector (or detection)
FMS	Forensic Medicine and Science
Frag	Fragmentor voltage

g	gram(s)
GBO	Greiner Bio One
GC-	Gas Chromatography
-MS	-Mass Spectrometry
-MS/MS	-Tandem Mass Spectrometry
GHB	Gamma-hydroxybutyrate
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
h	Hour(s)
i.d.	Internal diameter
IPA	Isopropanol
IS	Internal standard
L	Litre
LC	Liquid Chromatography
LCR	Large reservoir capacity
LLE	Liquid-liquid extraction
LLOQ	Lower Limit of Quantitation
LOD	Limit of Detection
LOQ	Limit of Quantitation
m	Metre
M	Molar
m/z	Mass to charge ratio
MALDI-QqQ-MS/MS	Matrix Assisted Laser Desorption/Ionization-Triple Quadrupole-Tandem Mass Spectrometry
MAMP	Methamphetamine
Man	Manufacturer
MDA	3,4-Methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-*N*-ethyl-amphetamine
MDMA	3,4-Methylenedioxy-methamphetamine
MeOH	Methanol
METH	Methadone
MF	Matrix Factor
mg	Milligrams
min	Minute(s)
mL	Milligrams
MMP	Methadone maintenance programme
MOR	Morphine
MP(A)	Mobile Phase (A)
MP(B)	Mobile Phase (B)
MRM	Multiple reaction monitoring
MSD	Mass Selective Detector
MTBE	*tert*-methyl butyl ether
N/A	Not available
N2	Nitrogen gas
NaF	Sodium fluoride
NaOH	Sodium hydroxide
ng	Nanogram
ng/mL	Nanogram per millilitre
NH_4OH	Ammonium hydroxide

NPS	Novel psychoactive substances
OXA	Oxazepam
PAR	Peak Area Ratio
PFPA	Pentafluoropropionic anhydride
pKa	Logarithmic acid dissociation constant
PM	Post-mortem
PMI	Post-Mortem Interval
POCT	Point of Care Testing
PP	Polypropylene
PPT	Protein precipitation
psi	Pounds per square inch
QC	Quality Control
R^2	Linear correlation coefficient
RSD	Relative standard deviation
ROSITA	ROadSIde Testing Assessment
rpm	Revolutions per minute
S/N	Signal to Noise Ratio
SAMSHA	Substance Abuse and Mental Health Services Administration
SCX	Strong cation exchanger
StDev/SD	Standard deviation
seq.	Sequential
SIM	Selected ion monitoring
SLE	Supported Liquid Extraction
SPE	Solid Phase Extraction
SUDAM	Sudden Unexpected Death in Alcohol Misuse
SVAI	Sample Volume Adequacy Indicator
SWGTOX	Scientific Working Group for Forensic Toxicology
TDM	Therapeutic Drug Monitoring
TEMA/TEMAZ	Temazepam
TIC	Total ion chromatogram
UCT	United Chemical Technologies
UK	United Kingdom
ULOQ	Upper Limit of Quantitation
UNODC	United Nations Office on Drugs and Crime
USA	United States of America
v/v	Volume to volume
w/v	Weight by volume
WDT	Workplace drug testing

1 Oral Fluid

1.1 Introduction

Blood and urine have been the most commonly analysed biological matrices in forensic toxicology for a long time. However, there are issues regarding the sampling of these traditional matrices – blood collection is very invasive and can cause the subject great distress, as well as requiring trained personnel (i.e. a phlebotomist). Urine collection, although not invasive, must be observed by the collector as adulteration of urine is easily achievable whether it be by diluting the sample, or by exchanging the sample for a different person's sample. It is therefore not surprising that in recent years much research has been focused on the use of alternative matrices to blood or urine, as well as cementing the usefulness of these alternatives within the field. These alternative matrices include hair, bones, oral fluid, vitreous humour, breath or sweat. All matrices have advantages and disadvantages, however sometimes it is the availability of specimens, the detection windows, or the invasiveness of the sample collection that must be acknowledged. Blood remains the most commonly analysed matrix in forensic toxicology, and for certain analytes it is possible to establish oral fluid/blood ratios. These can however be highly variable due to a number of issues, including the physiochemical nature of the analyte as well as the deposition of analytes in each matrix.

Oral fluid may not necessarily be considered an alternative matrix anymore as it is generally accepted in the field of forensic toxicology. The usefulness of oral fluid as a sample can depend on the nature of a case. It is not used post-mortem, but it can be used in workplace drug testing (WDT), therapeutic drug monitoring (TDM) and to detect drugs in drivers.

1.2 Composition

The term "saliva" is most widely used instead of "oral fluid". These terms are often used interchangeably although this is not always technically correct. "Saliva" refers to the secretions of the salivary glands specifically, which consists of about 99% water, 0.7% protein and 0.26% mucins (1), whereas "oral fluid" refers to all the different components that make up this matrix. These

components include secretions from salivary glands, cells shed from the oral cavity, remnants of previously eaten food, and other particulates.

Three main glands produce saliva in the oral cavity (Figure 1-1), and each gland produces a specific secretion. The parotid glands (*glandula parotis*) are located on the inside of the cheeks and produce serous fluid, which make up 25% (1) of oral fluid. *Glandula submandibularis*, or the submandibular glands, located on the floor of the mouth, produce sero-mucous fluid, which makes up 60-67% of oral fluid (but this percentage can decrease when salivation is stimulated, as parotid gland excretion increases to around 50% (1)).

The smallest and last of the main salivary glands, the sublingular gland (*glandula sublingualis*), is found under the tongue and produces mucous saliva, which constitutes only 3–4% of oral fluid components. Other minor glands are also present in the oral cavity and will spontaneously secrete salivary fluids as well (2).

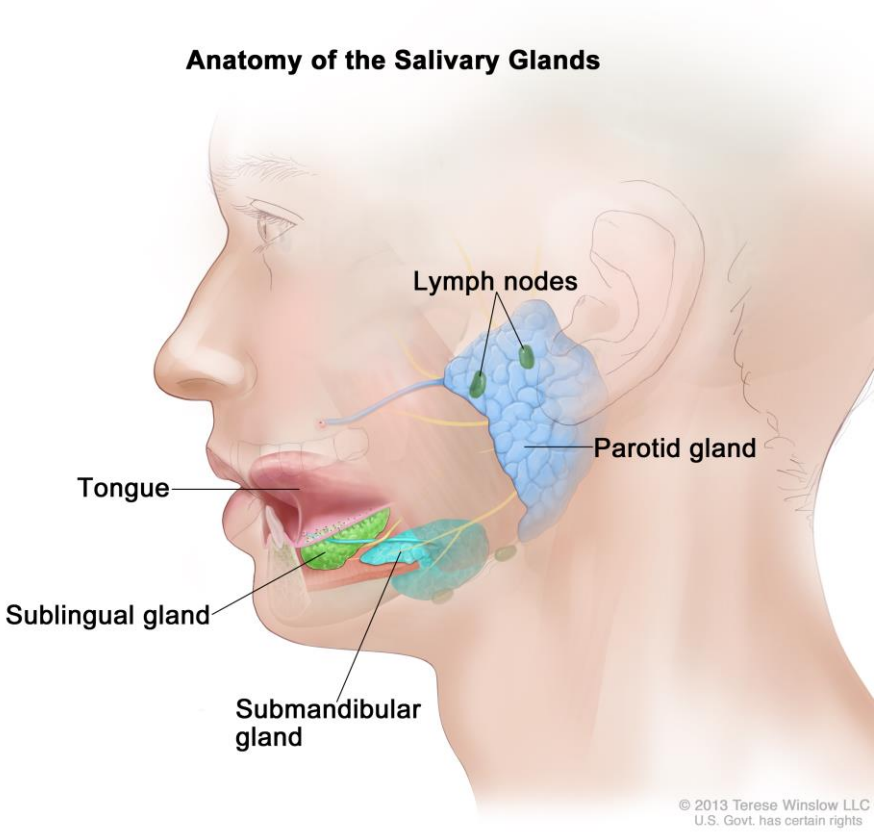

Figure 1-1 Location of three main salivary glands. For the National Cancer Institute © 2013 Terese Winslow LLC, U.S. Govt. has certain rights

“Oral fluid” (or “whole saliva”) is a mixture of the salivary secretions and may contain other components including mucosal transudate and crevicular fluid (1, 3), and cells shed from the oral cavity. There are a number of salivary proteins, electrolytes, and enzymes which exist in oral fluid at different concentrations. Each component has a different purpose, such as and including the prevention of bacterial growth, the breakdown of starch, or the prevention of tooth decay due to enamel preservation (2).

It is also known that production, as well as the composition, of oral fluid follows the circadian and circannual rhythm, meaning that production is dependent on the time of day and seasons (temperature) (2, 4).

The stimulation of oral fluid production can have a great effect on not only the alkalinity of the fluid, but also its viscosity, surface tension, buffer capacity, and obviously, flow rate. This was studied extensively by Gittings *et al,* and results showed that following stimulation by chewing Parafilm® wax, oral fluid had a lower viscosity due to the increased serous fluid secreted (higher aqueous content with less mucin). It also exhibited higher surface tension, increased pH due to increased electrolyte presence, increased bicarbonate (secreted by the parotid gland) concentrations, and increased the buffer capacity of oral fluid. The flow rate of serous fluids increased when salivation was increased by mechanoreceptor stimulation, as the parasympathetic nervous system activates the parotid gland. However, the authors reported a high degree of inter-individual variation (5).

1.3 Drugs in Oral Fluid

Unless oral fluid is exposed to drugs directly, analytes will most commonly be deposited in it through passive diffusion into epithelial layers in the oral cavity. All three glandular secretions may contain drug molecules. Although passive diffusion from blood or plasma into oral fluid is the predominant mechanism of drug deposition, active diffusion against the concentration gradient and ultrafiltration through membrane pores may occur (1).

Drug deposition in oral fluid is greatly dependent on lipid solubility, as well as the ionisation state of the analytes. Both are important factors to take into account when attempting to predict drug presence in this matrix.

Lipid solubility of analytes will affect their ability to cross the membranes, as ionised molecules are not usually able to cross this barrier. Membranes are lipophilic and so molecules must be unionised to diffuse across without help. Due to the pH of oral fluid (usually between 6.2 and 7.4 (6)) basic drugs, for example, can accumulate in oral fluid as they favour the slightly acidic environment; 6-monoacetylmorphine (6-MAM) has been found to stabilise in oral fluid (7). Neutral and acidic drugs in turn will favour plasma (8) (9).

The pKa values of analytes must be considered, as these indicate protonation states which will influence the movement of drugs into oral fluid from surrounding blood vessels. When molecules are ionised, they are more soluble in water which in turn will prevent back diffusion. The pKa will therefore also determine whether a drug will accumulate in oral fluid or whether it prefers the environment in other matrices.

Parent analytes and metabolites are detectable in oral fluid, although limited metabolism occurs close to the oral cavity (10). However, due to the increased volume of capillaries around the oral cavity, metabolites that are transported in the bloodstream may diffuse into oral fluid. It is therefore a matrix which can be used to determine either abstinence or recent use of substances. Moreover, the concentration of drug in oral fluid is dependent on the ratio of non-protein-bound drug in plasma (9, 11).

Similarly, the route of administration has an effect on the concentrations of drugs determined in oral fluid. Smoked drugs may accumulate in oral fluid resulting in an incorrect representation of the actual concentration of the substance due to the immediate exposure of the matrix to the drug. When drugs or medications are taken orally, or contamination of the oral cavity occurs through other means, drug concentrations can also be falsely elevated. However, due to the continual production of oral fluid, direct contaminants will be removed quickly. Drugs that are introduced into the body via injection will be

deposited in oral fluid through the above described diffusion from plasma through epithelial cells into the oral fluids (12).

1.4 Advantages and Limitations of Oral Fluid as a Matrix

In general, biological samples can typically be provided almost instantaneously. However, the collection of blood can cause distress to the subject, and urine sampling may be delayed as paruresis ("shy bladder syndrome") affects 3% of the world's population (approximately 220 million people) (13) - or simply cannot be provided due to dehydration. Oral fluid sampling can, however, be very simple.

Compared to some of the conventional matrices used in forensic toxicology, one of the main advantages of oral fluid sampling is that there is a lower risk of adulteration of the sample as the matrix offers not only gender neutral collection, but observed collection is easy and sampling can be fast. Not only is the likelihood of an adulterated sample greatly decreased, but the risk of spreading infections, which is possible when sampling infected blood or urine, is greatly reduced due to special sampling devices which reduce the exposure of the collector to the sample (11, 14).

However, although oral fluid has advantages over other alternative matrices, it does have certain limitations. The most problematic of these is the sample volume that can be collected. The sample volume collected is lower than that collected for both blood and urine, which are both collected readily. Certain systemic diseases, including diabetes or HIV/AIDS (15) can cause xerostomia (reduced oral fluid production/flow; "dry mouth syndrome"). This can also be symptomatic of depression and drug use (2), for example following the use of stimulants, cannabinoids, certain antidepressants and other medications. This means that sample collection may take longer than usual or a sample cannot be provided at all. Salivary production may be dependent on the time of day/collection as it follows a circadian rhythm (15), which in turn can also affect the collection of a sample of sufficient volume, or may increase or reduce the time required to obtain adequate samples.

A study conducted by Kauert in 2000 showed that in 21% of the 137 cases included in the study, it was not possible for subjects to provide oral fluid

samples (16). Nevertheless, it is possible to stimulate oral fluid production by either using a salivating agent in collection devices (often citric acid, sour sweets, or chewing gum (17, 18)) or by emulating chewing, or chewing Parafilm® wax (5), which can increase the production of oral fluid without a food stimulus being present (19). However this may end up reducing detectable drug concentrations (1). Similar to the effect that stimulation has on the production of oral fluid, the composition changes that occur following stimulation will affect the drug concentrations in oral fluid. As the pH of the matrix can be changed, as well as the water content, the pKa values and pharmacokinetic properties of the analytes of interest must be taken into consideration in order to determine how likely it is for the analytes to become trapped and therefore detectable in oral fluid. Studies have however found that there is limited drug decomposition due to the use of mouthwash and other foodstuffs (18).

A further factor which must be taken into consideration is the short detection window of most analytes in oral fluid, due to the disposition of drugs in the matrix. It is therefore often suggested that further matrices are analysed alongside oral fluid to determine metabolites and drug presence, like urine (20) or blood, and oral fluid alone is not a substitute for other matrices (18).

The cleanliness of the sample must also be recognised. Oral fluid can be readily contaminated with food and drink. For this reason, it is suggested that the collection of oral fluid samples should not occur for a minimum of 15 minutes following consumption of food or drink. Another source of contamination is the particulates that can be collected consisting of mucosal matter, protein or skin cells. It is therefore recommended that the oral fluid (21), especially expectorated samples, should be frozen before use to ensure homogeneity of the sample prior to analysis (freeze-thaw stability must however be taken into consideration as this can be an issue).

One aspect that can be considered a benefit of oral fluid sampling over the use of blood concerns sample volume. Although the collection of oral fluid may be problematic from “dry mouth” and only 1 – 2 mL can be collected readily, blood collection may be similarly difficult should the subject be an intravenous drug user, and in these cases sufficient blood cannot be collected for all the analyses.

Studies of randomly selected drivers have also shown that subjects are more likely to volunteer oral fluid samples compared to blood samples (22).

To overcome certain limitations that oral fluid presents to forensic toxicological analyses, it is possible to use synthetic oral fluid for validation and analysis procedures (most commonly as a blank, but it can also be used to make up calibration and quality control samples). Synthetic oral fluid may consist of distilled water with added components to reach the same consistency as real oral fluid.

Benefits of using synthetic oral fluid include analysts not having to handle biological materials, which may be hazardous and unsanitary, and also during the method development stages when synthetic oral fluid without preservative is used. In turn, however, the latter benefit may be a disadvantage when methods for oral fluid collected with, or diluted in, preservative solutions are developed. A further drawback may be the interferences that will become present when real oral fluid is used, as this matrix is less clean than its synthetic counterpart.

1.5 Specific Uses of Oral Fluid Testing

1.5.1 Therapeutic Drug Monitoring

Oral fluid has been used as a forensic toxicology matrix since the 1970s, with papers discussing the use of saliva over plasma in TDM published as early as 1978 (23). Review papers have been published outlining the benefits that oral fluid testing may have for forensic toxicology and sciences in general (12, 24, 25).

In instances of TDM, oral fluid may be considered a matrix of choice due to the non-invasiveness of the sampling. The ability to detect recent use, which is an important aspect of TDM cases, means that oral fluid is an advantageous matrix to use in these circumstances. A benefit over hair testing for TDM is that oral fluid concentrations show recent drug use, rather than drug intake from weeks previously (26).

For the determination of a patient’s adherence to or compliance with prescribed drug courses, the collected sample volume may be less important. This is because a qualitative analysis suffices to determine the presence or absence of

the drug and therefore volume is not a limiting factor. Drug concentrations can, however, be determined if the dose-response curve is linear (27).

Oral fluid has often been used to monitor the use of anticonvulsant drugs and has proved a reliable matrix for this (28-30). Literature suggest that when monitoring caffeine consumption (31), or monitoring of caffeine concentrations when used to treat apnoea in infants, oral fluid testing may be of benefit due to the short therapeutic window of caffeine, and again, the ability to determine recent drug ingestion in this particular matrix (32). Oral fluid should be considered an alternative to plasma or blood testing when it comes to therapeutic drug monitoring in children due to the non-invasiveness of the sampling but also because of the relationship between plasma and oral fluid drug concentrations for certain drugs (33-35).

Correlations between blood and oral fluid drug concentrations have been shown for drugs like ethanol (36) and codeine (37), and research has shown lacking correlations for lorazepam (and most benzodiazepines), THC (38), and methamphetamines (39). The authors, however, point out that due to the highly variable nature of the matrix and variable drug deposition times, drug findings must not be used to estimate drug concentrations in blood from oral fluid, or vice versa. A further study showed that for basic drugs, including amphetamines and opiate/opioid drugs, concentrations were higher in oral fluid than in blood, which corresponds to expected findings based on the physiological and physiochemical nature of these drugs (18, 40). Although blood and oral fluid concentrations can be difficult to compare (41), oral fluid and urine concentrations have been correlated with more success, especially for amphetamines, methadone, opiates, and benzodiazepines (26, 40).

Oral fluid has shown promise for the qualitative monitoring of opioid medication compliance in the United States, as results from paired oral fluid and urinalysis produced comparable results, with 191 positives for urine and 176 in oral fluid (with 1.4 and 1.3 drugs detected in urine and oral fluid, respectively) (42). The authors believe that these results were caused by the differences in drug detection windows, which are different for all analytes.

Although benefits exist, it is important to consider the pharmacokinetics of the monitored drugs in order to determine whether oral fluid is the most suitable matrix for the analytes of interest.

In diagnostic medicine, oral fluid has also proven to be a valuable matrix. Although concentrations of symptomatic molecules of interest may be low, with the development and increasing sensitivity of analytical methods, the list of successful applications grows steadily. Research has shown that the presence of particular proteins, or components can be clear indications for certain conditions: obstructive sleep apnoea, which can lead to brain damage, can be shown through oral fluid testing through increased presence of protein S100B, although the extent of damage cannot be determined (43). Monitoring of albumin during chemotherapy to monitor stomatitis, increased parotid lysozymes indicative of Sjögren's disease or changes in salivary compositions symptomatic of cystic fibrosis (44) are also possible. Studies have attempted to, and are showing promise for developing diagnostic tests utilising oral fluid to screen for breast cancer biomarkers (45), or viral infections including HIV and other infectious diseases (46-48).

1.5.2 Workplace Drug Testing

Oral fluid testing is especially important and valuable in instances of WDT. Again, it is both the non-invasiveness of the sampling and the determination of recent drug use that makes oral fluid advantageous to this type of testing – especially to evaluate work-related accidents (49). Similar to blood and urine, oral fluid can be used to detect alcohol consumption. Literature also suggests that oral fluid testing may be useful for the determination of exposure to low-molecular-weight molecules, including solvents or pesticides (50, 51). In cases of workplace testing, oral fluid may be the only matrix analysed, as it is primarily the presence of the parent drug analyte that is of importance initially.

In a study of an Australian workplace, Casolin *et al*. showed that urine samples may be more efficient at proving drug use than oral fluid, with an overall drug detection rate of 3.7% for urine and only 0.5% for oral fluid (52). It is therefore beneficial to have paired samples for testing, to ensure that results are accurate, although urine can remain positive for a longer time than oral fluid

and therefore is not immediately indicative of current impairment or recent use. Although advantages of salivary testing are explicitly expressed, certain drawbacks exist and ought to be taken into account.

1.5.3 Proficiency Testing

With the increased popularity of oral fluid as a testing matrix, laboratories have been implementing oral fluid proficiency testing (for example LGC Standards), although proficiency testing standards have been difficult to develop for commercial use because of the different analytical techniques used by laboratories and the highly variable nature of the matrix. The more oral fluid is established as a valuable and indispensable sample for certain aspects of forensic toxicology, the more laboratories will include it in their scope, thereby increasing proficiency assessment in oral fluid. Few papers have been published regarding the accuracy of this testing. One paper found good specificity, however a lack of sensitivity for certain drugs, including amphetamines and barbiturates, was reported (53).

1.5.4 Driving Under the Influence of Drugs

The usefulness of oral fluid testing for the determination of driving under the influence of drugs (DUID) depends heavily on the relevant country's laws and recommendations, drug prevalence, and foresight to matrices alternative to blood or urine.

On-site screening instrumentation is continually being developed and improved. In certain countries in Europe, oral fluid drug screening devices are already commonly used due to the ease of sampling and the rapidity of screening tests. An issue with point-of-care roadside testing is the potential for false negative results, due to the lack of sensitivity, but also false positives, from the lack of specificity that the devices used can exhibit (54) and due to cross reactivity of drugs (12). Reports from police officers confirm that oral fluid is the matrix of choice for DUID cases due to its availability, non-invasiveness as well as the sound correlation between oral fluid and blood concentrations (55).

The most successful roadside drug testing studies included the integrated DRUID (Driving Under the Influence of Drugs, Alcohol and Medicines) project which

brought together European countries to gain insights into the impairment caused by psychoactive substances, and their effects on road safety on European roads. The project spanned 5 years, running from October 2006 to October 2011, with 15 countries taking part. The study found that use of medicinal (opioid/opiate) drugs was more common in northern (Scandinavian) European countries, while alcohol, benzodiazepines and illicit drugs (or a combination) were more prolific in southern Europe. The DRUID study was preceded by the ROSITA (ROadSIde Testing Assessment) and ROSITA-2 (2003 – 2005) projects. Rather than focusing on the drugs of abuse consumed by drivers and drawing conclusions about missed impairment, the ROSITA project aimed to standardise a methodology to assess impairment at the roadside. Fewer countries took part (8 countries), and a total of 2850 subjects were tested. One of the main conclusions drawn from the ROSITA project specifically was the requirement for sensitive and standardised collection methodologies, especially for the use of oral fluid at the roadside. Both DRUID and ROSITA projects collected oral fluid at the roadside to evaluate drug concentrations in oral fluid.

Several studies have been conducted that use oral fluid to determine intoxication of drivers and the drugs causing the intoxication. Often these studies will compare oral fluid to blood drug concentrations, and thereby provide invaluable understanding and rapid testing for the forensic community. Frequently it is a random selection of drivers rather than drivers who have shown signs of DUID that form the study cohort. A study conducted in the United States of America (USA) in 2007 collected almost 6000 oral fluid samples from random night-time drivers, and analysis of samples showed that 14.4% of drivers were positive for an illegal substance (56). The main drug detected was marijuana (Δ^9-tetrahydrocannabinol (THC) in oral fluid, and its metabolite tetrahydrocannabinol carboxylic acid (THC-COOH) in blood). This is not a rare finding, and many studies focus specifically on cannabinoids (57-61) due to the new legality of cannabis in some states. However, it is also dependent on when the study was carried out, as well as in which country. An Italian study determined more cocaine and for metabolites positive cases than cannabis cases in their samples (62). The consensus is that oral fluid is a beneficial matrix for roadside testing where blood and urine cannot be collected, or urine is not used for on-site testing. Further benefits include that through detection windows the

use of oral fluid will lead to increased numbers of apprehensions of drivers with illegal levels of drugs in their systems (22). This is due to the determination of very recent drug use and the cut-off concentrations in oral fluid being lower, as well as no delay while waiting for the phlebotomist for sample collection, resulting in more convictions. Confirmation tests are being developed and used to confirm and quantify analyte concentrations in samples taken on-site. A laboratory-based confirmatory test is essential (63) for final reports or convictions of drug driving cases. Oral fluid has proved to be a valuable tool when testing for DUID as it removes the need for trained drug recognition experts (DREs) to make an assessment at the roadside (64, 65).

1.6 Cut-off Recommendations

A cut-off concentration is used to determine whether a drug response shows the presence of the analyte above a minimum concentration. These concentrations are specific to each different drug group but need to be achievable and relevant. Although cut-off concentrations are particularly important for screening techniques or on-site testing, guidelines should include recommendations for both screening and confirmation methods. Cut-off recommendations for oral fluid are important and they can differ per application. Special importance is placed on determining recent drug use or intoxication based on cut-offs, as certain drugs may be present endogenously (such as gamma-hydroxybutyrate) or may show late deposition in oral fluid (like codeine). Studies have shown great variation in oral fluid to blood concentration ratios, which is why separate cut-off concentrations should be utilised for each separate matrix, unless regression modelling is used to equate the thresholds between oral fluid and the secondary matrix (66). Cut-offs can be based on analytical methods but they can also be based on pharmacological effects of drugs and the application of the recommended cut-offs.

The European Workplace Drug Testing Society (EWDTS) publish guidelines for oral fluid testing for workplace monitoring frequently. These outline not only cut-off recommendations for quantitative analyses, but also for qualitative analyses. For roadside testing, cut-off concentrations set forth by the DRUID project are often followed as these are specific to examine intoxication or to establish potential impairment. Table 1-1 summarises recommended cut-offs set

by DRUID, EWDTS and the Substance Abuse and Mental Health Services Administration (SAMHSA).

Table 1-1 Cut-off recommendations proposed by DRUID, EWDTS and SAMHSA.

Analyte	DRUID (ng/mL) (67)	EWDTS (ng/mL) (68)	SAMHSA (ng/mL) (69)
6-MAM	5	2	2
Alprazolam	1	3	N/A
Amphetamine	25	15	15
Benzoylecogonine	10	8	8
Clonazepam	1	3	N/A
Cocaine	10	8	8
Codeine	20	15	15
Diazepam	5	3	N/A
Flunitrazepam	1	3	N/A
Lorazepam	1	3	N/A
MDA	25	15	15
MDEA	25	15	15
MDMA	25	15	15
Methadone	20	20	N/A
Methamphetamine	25	15	15
Morphine	20	15	15
Nordiazepam	1	3	N/A
Oxazepam	5	3	N/A
THC	1	2	2

Where DRUID - Driving under the Influence of Drugs, Alcohol and Medicines ; EWDTS – European Workplace Drug Testing Society; SAMHSA – Substance Abuse and Mental Health Services Administration; N/A – not available

Cut-off concentrations are determined based on statistical modelling as well as pharmacokinetic studies, as it is important that the selected cut-off concentration reflects the drug concentrations that can be found in the matrix. As already indicated, the concentration of drugs in oral fluid can depend on several factors, all of which must be taken into account when setting cut-off recommendations. This means that it is essential to set cut-offs that are not too conservative but also not too high, therefore preventing false positives and negatives.

The time between sampling and apprehension must also be taken into account when deliberating cut-off concentrations. As discussed, oral fluid is a valuable asset in assessing short-term drug use, but when analytical results between oral

fluid and blood drug concentrations are assessed, time between sampling may reduce diagnostic accuracies (61). Establishing cut-offs is a difficult task because of these variables in oral fluid sampling, sample volume and collection device used, and the analyte of interest, as certain drugs exhibit higher oral fluid concentrations than others, and some exhibit higher blood concentrations than oral fluid concentrations.

1.7 Collection of Oral Fluid/Overview of Collection Devices

1.7.1 Expectorate

For the collection of neat oral fluid without the use and help of a collection device, oral fluid can be expectorated into any container required. Expectoration into a polypropylene, plastic, glass or any other non-reactive container or tube is preferred.

Collecting the required volume of oral fluid can be a problem when collecting neat oral fluid, as the sample is supposed to flow naturally. Expectorated oral fluid is more challenging to deal with in the laboratory owing to the fact that the forced production of whole saliva increases the mucosal component making the sample more viscous and dirty.

For this particular reason, oral fluid collection devices have been developed, which aim to make collection of oral fluid easier, cleaner and faster.

1.7.2 Collection Devices

Oral fluid collection devices can be beneficial for the collection of oral fluid as they may make collection and storage easier, whether through the addition of salivating agents to the collection system, by having a preservative as part of the collection device, or by reducing the exposure of the analyst to the matrix. Ideally, the collection device would be able to collect the required, and stated, volume of oral fluid, have a consistent volume of buffer in the tube, and contain a tube that ensures analyte stability with a pad made from a material that prevents the adsorption of analytes. The drug recovery is of utmost importance for the device.

1.7.2.1 Pad-Based Devices

Pad-based collection devices are predominantly used for the collection of oral fluid. The collection itself is usually simple and generally follows the rules outlined below in Figure 1-2:

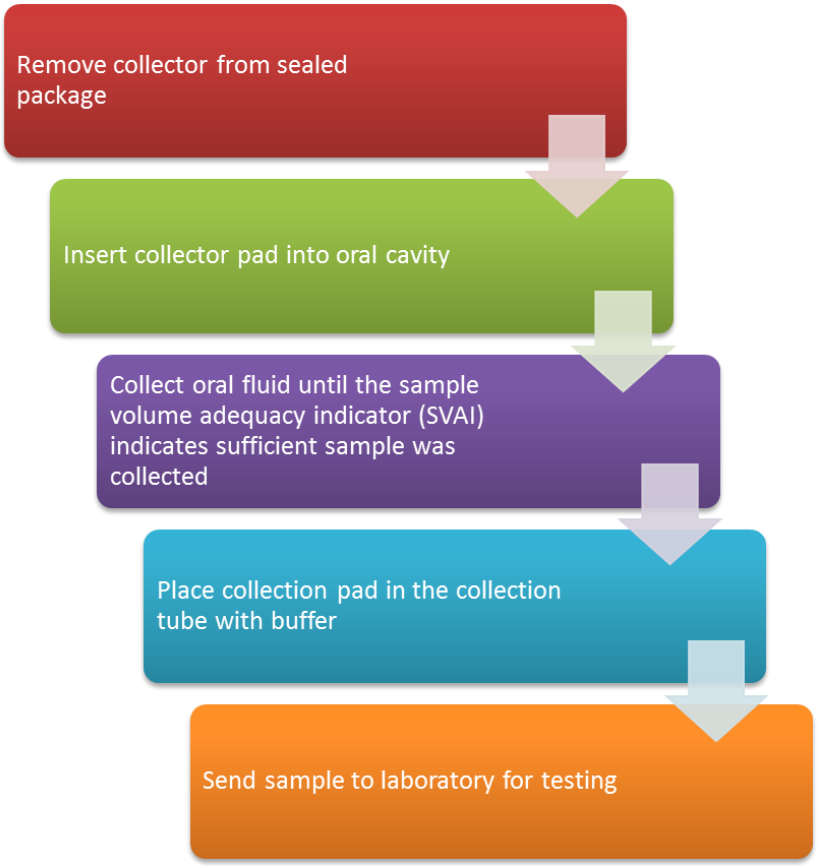

Figure 1-2 Generic steps for oral fluid collection using pad-based devices

The majority of collection devices utilise a cellulose or cotton collection pad, and it is common for the devices to contain a stabilising buffer to prevent microbial growth or drug degradation in the sample. A very common collection device is the Immunalysis™ Quantisal® device.

The Quantisal® (Figure 1-3) device is used by placing the collection pad between the cheek and gum. There is a sample volume adequacy indicator (SVAI) within the device which will turn blue when 1 mL of oral fluid has been collected. The pad is then removed from the mouth and placed into 3 mL of buffer contained in the collection tube. For sample preparation, the Quantisal® device requires the collection pad to be separated from the plastic stem to which it is attached. Laboratories are known to use a serum filter to compress the pad and remove as much sample volume from the pad as possible, or use a centrifugation step to

detach the pad from the stick. The buffer is a saline solution buffered with 100 mM phosphate buffer (pH 6), also containing the preservative ProClin™ 300 and food colour dyes giving it the typical blue colour. It also contains further, proprietary, stabilisers.

Figure 1-3 Immunalysis™ Quantisal®. Image reproduced with permission from Immunalysis™ Corporation

A new collection device on the market (introduced in 2016) is the pad-based Neogen® NeoSAL™ device (Figure 1-4). The NeoSAL™ collection kit is very similar to the widely used, and previously mentioned, Quantisal® collector. The two collectors collect different volumes of oral fluid, but the ratio of buffer to oral fluid collected for both devices is the same (see Table 1-2). The buffer that is used is a blue liquid at a pH 6. The exact components are proprietary but they are mirrored on the buffer used in the Quantisal™ device.

The NeoSAL™ device is used by placing the collection pad between the cheek and the gum (collection instructions are summarised in Appendix II). It is recommended that the subject does not eat or drink at least 15 minutes prior to collection.

Figure 1-4 Neogen® NeoSAL™ oral fluid collection device (in standard packaging)

Neogen® suggests a typical collection time of 1 – 2 minutes, but the SVAI will form a distinct blue line when the correct volume, 0.7 mL of oral fluid, has been collected. The blue cap on the collector is removed and snapped into the bottom of the tube. The collection swab is placed into the blue buffer (2.1 mL) and screwed shut. The tube should then be inverted four or five times. The collection tubes should be stored at 18 – 25 °C and extended exposure to sunlight should be avoided. Post-collection transportation can occur at room temperature, but devices should be stored at 5°C.

It is the laboratory procedure which differentiates the NeoSAL™ device from the Quantisal® device; the NeoSAL™ pad remains intact and attached to the white handle when it is discarded. Neogen® specifically states that the use of filters, plungers or centrifugation is not required for their device. The collection pad and handle can be discarded without any further removal of the excess fluid in the pad, as the buffer is meant to ensure “excellent drug recovery”.

The OraSure Technologies Inc., Intercept® *i*2™ device (shown in Figure 1-5) also requires an in-depth sample preparation step. The buffer tube is shaped differently to those of the Quantisal™ or NeoSAL™ devices as it has a small tip at the bottom. The device contains 0.8 mL buffer and is said to collect 1 mL of oral fluid. The sample collection process remains similar to the other pad-based collection devices. The collection stick with the attached pad is placed into the oral cavity. The device is equipped with a SVAI and when that turns blue (approximately 2 to 3 minutes), the collection stick is removed from the mouth

and slid into the collection tube containing the clear buffer. In order to prepare the sample for analysis, the tube is inverted so that the pad moves away from the tip of the tube. The tip is then broken off using a centrifuge tube and the collection tube is placed inside the centrifuge tube, with the broken end facing down. The centrifuge tubes required have a set of specific dimensions, or should be similar to those specified by OraSure Technologies. This is then centrifuged for 5 minutes which forces the oral fluid buffer mixture into the centrifuge tube, leaving behind the collection pad, and by containing this in the collection tube it prevents any reabsorption. The diluted oral fluid can then be stored at -20 °C for long term storage.

Figure 1-5 OraSure Technologies Intercept® i2™ collector. Image reproduced with permission from OraSure Technologies Inc.

The benefit of using either a centrifugation step or a filter is that minimal sample is lost, compared to when the collection pad is removed and discarded. However, it does make the sample preparation step more labour intensive and time consuming.

The Intercept® *i*2™ collector is also the only collector included in this study to contain a combination of sodium chloride (<1% w/v), sodium citrate dehydrate (<1% w/v), and citric acid monohydrate (<0.1% w/v) to stimulate salivation. This can be beneficial especially in cases of dry mouth syndrome. However, it can be relatively uncomfortable for the subject as the taste of salivation-inducing agents may not be palatable. Furthermore, care must be taken when evaluating the results, as citric acid or salt content may cause interferences during extraction and analysis, as well as potentially affecting the concentration of drug in plasma/blood. The 0.8 mL of preservative buffer solution contained in the vial is made up of <1% w/v of sodium chloride, <1% sodium azide and 99.94 – 99.98% w/v water.

Oasis Diagnostics® Corporation manufactures a pad-based, buffered oral fluid collection device collecting 1.0 to 1.1 mL of saliva into 2 mL of buffer. The Accu•SAL™ collection device (Figure 1-6) has an SVAI to indicate when sufficient sample has been collected, however should the donor not be able to produce a sufficient sample volume, the increments on the side of the collection tube make it possible to calculate the absolute quantity of sample provided. This is beneficial as it provides the possibility of an accurate dilution and back calculation for exact quantification.

Figure 1-6 Accu•SAL™, reproduced with permission from Oasis Diagnostics® Corporation

The buffer that is used is again proprietary, and the product information sheet provided suggests the donor pools saliva in the mouth for 1 – 2 minutes prior to sampling. The fluids are then collected. When the pad is placed into the buffer, the transport tube should be inverted 20 to 40 times. This is to ensure a good mixing of oral fluids and collection buffer.

The presence of a preservative buffer can be beneficial, and is often preferred for forensic use (to inhibit bacterial growth which may potentially change drug concentrations in the matrix). However, the presence of buffers can be a hindrance. This is especially true in analyses carried out on liquid chromatographic (LC) systems, as the buffer can readily interfere with the analysis. A further issue that must be taken into account when dealing with buffered oral fluid samples, is the dilution of oral fluid and thereby analyte concentrations. Dilution may result in a reduction of sensitivity; particularly should the buffer interfere with the analysis itself. For the extraction of analytes from oral fluid, a non-ionised state is usually preferred (unless the extraction

uses an ion-exchange mechanism) as this will then enable the extraction of drugs out of the matrix, therefore it is easier to extract drugs that are non-ionised in the pH environment of oral fluid.

Another pad-based collection device is the Sarstedt AG & Co Salivette® oral fluid collection device (

Figure 1-7) which can be supplied with citric acid to stimulate salivation, but can come without.

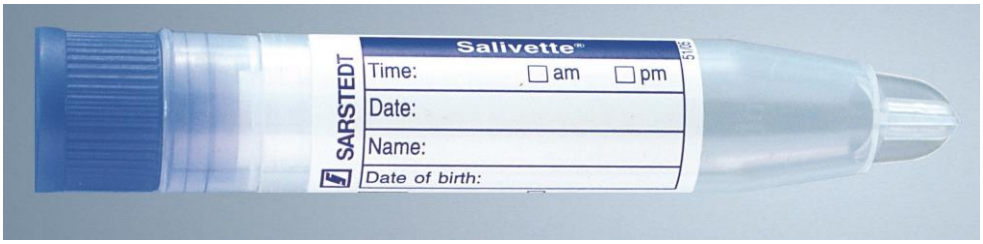

Figure 1-7 Sarsted Salivette® oral fluid collection device. Image reproduced with permission from Sarsted AG & Co.

Salivette® devices do not contain a stabilising buffer in the tube, but require the sponge to be chewed lightly and rolled around in the oral cavity for the collection. After 2 minutes in the mouth, the cotton sponge, which can also be made of a biocompatible synthetic fibre, is expelled back into the collection tube, and the cap is returned to the tube. It is important that the swab is not touched with the fingers either when transferring it into or out of the oral cavity. The tube is centrifuged to remove the saliva from the swab. Any extra debris or mucus strands are collected in the special tip of the collection tube. The upper layer can then be used for analysis. The advantage of this particular collection method is the sample volume – up to 1.5 mL is collected (70).

A further collection device that does not contain a buffer is the Oasis Diagnostics® Corporation Versi•Sal® collector (shown in Figure 1-8). It is similar in handling and appearance to the Quantisal® and other pad-devices but does not come with a stabilisation buffer.

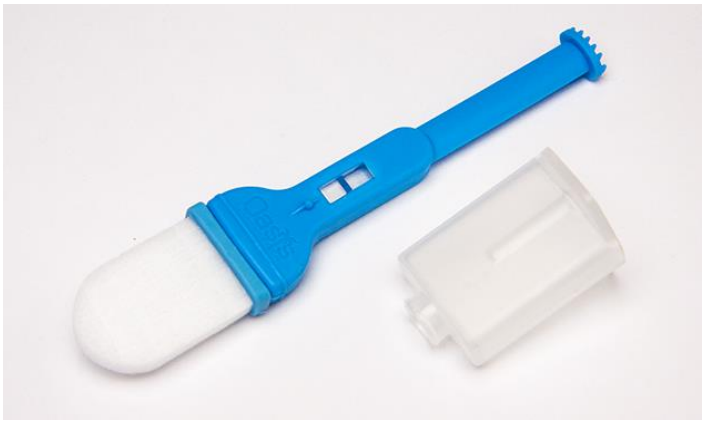

Figure 1-8 Oasis Diagnostics® Corporation Versi•SAL® oral fluid collector, reproduced with permission from Oasis Diagnostics® Corporation.

To collect oral fluid, the device is placed underneath the tongue until the blue SVAI line disappears indicating that adequate sample has been collected. The collector comes with a compression tube into which the pad is inserted. This compresses the pad and expels the collected sample into a standard Eppendorf tube. The sample can then be readily used for point-of-care tests (POCT) or can be sent to testing laboratories for analysis. However, Oasis Diagnostics® Corporation offers the device in several configurations, which can include a travel cap which ensures that sample integrity is maintained throughout the shipping process, or the "Bifurcating Compression Tube" which compresses the pad simultaneously into two sample tubes.

1.7.2.2 Non-pad-based collection devices

There are few collection devices that do not rely on a pad for collection.

The Greiner Bio One (GBO) collection kit (Figure 1-9) relies on 4 ml of the yellow saliva extraction solution which is used to rinse the oral cavity thoroughly for 2 minutes. The saliva/extraction solution mixture is then expectorated into the saliva collection beaker. The benefits of this method of collection, although not common, include that adsorption of drug molecules is not an issue as there is no collection pad. Dry-mouth syndrome is not an issue with this collection either. However, holding the mouthwash for two minutes can be distressful as this can be regarded as a long time and could be swallowed, which is not recommended. The extraction solution contains the dye tartrazine, which not only serves as an internal standard but at an acidic pH of 4.2, it ensures the stability of basic and neutral drugs.

Figure 1-9 Greiner Bio-One oral fluid collection kit, reproduced with permission from Greiner Bio-One

When the oral fluid is quickly transferred into the transfer tubes, the samples are ready for analysis. The sample is readily analysed photometrically for saliva content in U/mL (Units/millilitre) as the internal standard dye tartrazine enables a donor-specific quantification of oral fluid volume present. A quantification kit is available which includes 5 calibrators and 2 controls. The dye is determined photometrically between 450 – 520 nm, in calibrators, controls and saliva samples. A calibration curve based on the absorbance difference and the "saliva quantity as U/mL" is used to determine the volume of oral fluid/saliva in the sample - the underlying principle being the Beer-Lambert Law. With the collection procedure, it is then possible to quantify the analytes in the initial volume of oral fluid collected.

One main issue that is encountered when it comes to the forensic analysis of oral fluid samples is the availability of a secondary sample. Contrary to many other collection devices, which do not provide a second sample, the Greiner-Bio One kit provides sufficient sample volume for sample splitting.

One of the other collectors which contains no buffer or collection swab but addresses this issue is the SciTeck® Diagnostics, Inc. Saliva Spit Collector, shown in Figure 1-10.

Figure 1-10 SciTeck Saliva Spit Collector

The collector is placed in the mouth, between the lips and oral fluid is expectorated. The device can be turned so that an equal volume of oral fluid is collected in both sample tubes. The mouth piece can then be removed carefully so as to prevent sample spillage and purple caps are placed onto both tubes to secure the contents. Tamper-proof seals are provided for forensic use.

Table 1-2 summarises the specifications of some collection devices.

Table 1-2 Summary of Collection device specifications

Manufacturer and Collection Device	Volume of Oral Fluid Collected (mL)	Volume of Buffer (mL)	Type of Buffer/ Preservative used	Type of Collector
Neogen® NeoSAL™	0.7	2.1	Phosphate buffer, pH 6	Cotton pad
Greiner Bio-One	N/A*	6	Citrate buffer (to facilitate salivation) pH 4.2	Mouth wash
Oasis Diagnostics® Accu•SAL™	1 – 1.1	2	N/A	Cotton pad
Oasis Diagnostics® Versi•SAL®	1.2 – 1.4	N/A	N/A	Cotton pad
Immunalysis™ Quantisal®	1.0 (±10%)	3	Non-azide preservative, 100 mM phosphate buffer with ProClin™ 300	Cotton pad
OraSure Technologies Intercept® *i*2™	1.0	0.8 (14)	Buffer with Flag Blue dye (41), <1% w/v NaCl,<1% w/v sodium azide and water	Cotton pad impregnated with sodium chloride (3.5%)
Sarsted Salivette®	1.1 (±0.3)	N/A	N/A	Swab collector

*** no tests have been carried out determining the average volume of oral fluid collected using this device.**

1.7.2.3 On-Site Screening Systems

The main, on-site, oral fluid screening system is the Dräger DrugTest® System. It is a mobile screen kit which includes a volume adequacy indicator and sample collector. The analyser is a fully automated system which provides accurate screening on-site. It has 7 pre-installed analytical cut-offs, which are 50 ng/mL for amphetamine, 35 ng/mL for methamphetamine, 75 ng/mL for MDMA, and 5 ng/mL for THC. Studies have shown that it is a useful tool for on-site oral fluid screening (11, 71). Other on-site devices may not be automated and are referred to as Point of Care Test (POCT) kits. An example of an on-site POCT that is widely reported in the literature is the Securetec Detections-Systeme AG DrugWipe® -5+ (71, 72). Cut-off values for the POCT are lower than those of the Dräger DrugTest®: 50 ng/mL for amphetamine, 25 ng/mL for methamphetamine, 25 ng/mL for MDMA, 30 ng/mL for cocaine (BZE), 10 ng/mL for opiates (codeine) but 30 ng/mL for THC (73). Not only can these tests determine drug residues or levels for the main drug groups (cannabis, opiates, cocaine, amphetamines and methamphetamines (MDMA, ecstasy)) in oral fluid after suspected consumption, but can also be used to detect drug residues on all surfaces. POCT are often only qualitative, although some newer products claim to be semi- or fully quantitative.

1.8 Extraction Techniques

Clean-up procedures that are used to extract drug analytes from oral fluid are similar to those used for extraction from blood and urine. These may include solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PPT).

Oral fluid is considered a “dirty” matrix in the field of forensic toxicology owing to the numerous components contained in a sample. For this reason, it is very important to select an efficient sample clean-up technique prior to analysis. This can be especially true for samples collected using a buffer-based collection device, and extractions are dependent on analytical sensitivities and instrumentation used. Further details on the extraction techniques used for the analysis of drugs included in this thesis are found in subsequent Sections 2.1, 3.1, and 6.1.

1.9 Detection Techniques

Detection techniques vary greatly with the analyte of interest. Initial analyses can be immunoassays however these are not quantitative and therefore require further analysis to confirm and quantify the presence of the analytes.

Both gas and liquid chromatographic methods are very common for forensic toxicology. Gas chromatography – mass spectrometry (GC-MS) is the gold standard of analytical methods which most laboratories have available to them. Methods developed on GC are very reliable and robust which is why it is still the go-to method of analysis. Nevertheless, liquid chromatography – tandem mass spectrometry (LC-MS/MS) instrumentation and methods are becoming increasingly popular. It's main advantage over GC-MS methods are the increased sensitivity and selectivity due to the tandem mass spectrometer.

More detail regarding detection techniques for specific drugs tested for this thesis are given in subsequent Sections 2.1, 3.1, and 6.1.

2 Comparison of Neogen® NeoSAL™ and OraSure Intercept® *i*2™ Oral Fluid Collection Devices for Detection of Amphetamine and Methamphetamines

2.1 Introduction

The use and abuse of amphetamine and methamphetamines has been widely reported internationally. Globally, reports show higher seizures of methamphetamine (in East and South-East Asia and Northern America (74)) compared to those of amphetamine or 3,4-methylenedioxymethamphetamine (MDMA), although in Europe reports show the inverse (75). The 2013/14 Drug Seizures Report for England and Wales reported a 15% decrease in quantity of amphetamine seized from previous years – even though it was the second highest Class B drug seized. In 2015/2016 England and Wales Border Forces and Police jointly seized an average of 402 kg of amphetamines in 4418 drug seizures (76) which still noted a decrease from 2014/15 (77). In Scotland, 504.9 kg of amphetamines were seized by the Scottish Police forces in 2013/14, which notes an increase of 48% from 2012/13 (78). No methamphetamine was seized in Scotland in 2015/16, but 71.8 kg of amphetamine (Class B) were seized, having decreased from 118.4 kg in 2014/15 (79).

In 2016, 160 deaths (out of a total of 3744 deaths, 4.27%) were related to "any amphetamine[1]" drug in England and Wales (80), whereas amphetamines or ecstasy-type drugs were implicated in or contributed to 53 deaths in Scotland (out of a total of 867 deaths, 6.11%) (81).

The main attraction of the illegal use of these stimulants is enhanced cognitive and physical performance, which is what both amphetamine and methamphetamine were prescribed for originally (82). Historically, MDMA has been linked to the rave music scene and found its popularity through that. It is the ease of producing these drugs that constitutes one of their main attractions – clandestine laboratories are common and "recipes" for production are readily available online, and therefore it is very difficult to control the production of

[1] Reports did not specify which amphetamines were included in this.

these drugs (83). Addiction to these substances can occur readily, and tolerance is built up over time, meaning that an increased dosage is required to obtain the same physiological effects that had previously been caused by lower concentrations.

Amphetamine and methamphetamine are both Class B substances under the Misuse of Drugs Act 1971, however should amphetamine be prepared in a way that the route of administration is by injection, it falls within a different schedule of the Act, and therefore classed as a Class A drug. Methamphetamine (in its crystal form), MDEA and MDA are also Class A substances. The penalty associated with possession of a Class A substance can include a prison sentence of up to 7 years, an unlimited monetary fine or both. The penalties for supply or production are the same but the defendant could be punished with a lifetime prison sentence. For Class B substances, possession may result in a prison sentence of up to 5 years, and again an unlimited fine or both, whereas the supply or production can incur the latter or a prison sentence of up to 14 years.

2.1.1 Amphetamine and Methamphetamine

Amphetamine (Figure 2-1), known medically as benzedrine or dexedrine, and illicitly as “speed” or “billy” (among others), is a sympathomimetic phenethylamine derivative that affects the central nervous system (CNS). It was first synthesised in 1887 (82).

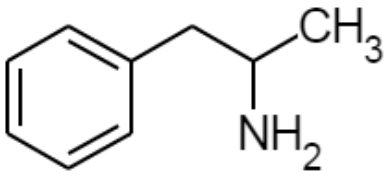

Figure 2-1 Chemical structure of amphetamine

Amphetamine exists as enantiomers; the *d*-isomer ((+)-(*S*)-amphetamine) is 3-4 times more potent than the *l*-isomer (*(R)*-amphetamine) (84, 85). Chiral analysis is usually required to differentiate between the isomers, although it is possible to separate the two isomers without chiral derivatisation (86). Due to its physiochemical effects on the dopamine receptors in the brain, *d*-amphetamine is prescribed in the treatment of attention deficit/hyperactivity disorder (ADHD) (87, 88). When amphetamine is prescribed medicinally, especially in the US, it is

commonly encountered under the tradename Adderall. Adderall is a blend of the two amphetamine isomers, usually consisting of 25% each of amphetamine aspartate monohydrate, *l*-amphetamine sulphate, *d*-amphetamine saccharate, and *d*-amphetamine sulphate (89).

Methamphetamine (Figure 2-2) is a highly addictive and highly potent stimulant drug which is commonly known as “meth”, “crystal meth”, or “ice” when it comes in its crystalline form. Methamphetamine, just as amphetamine, is an enantiomer and exists in two isomeric forms: the *l*- (*R*-(-)-methamphetamine) and the *d*- (*S*-(+)-methamphetamine) isomer. The methamphetamine *l*-enantiomer is used as a nasal decongestant, especially in the USA, as it has weaker CNS stimulant properties (85, 90). This is an important consideration during the analysis, as it is only the *l*-enantiomer which is approved for medical uses and finding the *d*-isomer in a sample proves use of an illicit substance (84).

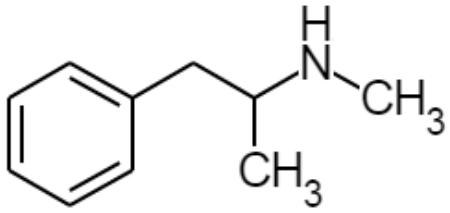

Figure 2-2 Chemical structure of methamphetamine

The primary metabolite of methamphetamine is amphetamine when the former is demethylated using CYP2D6 (91, 92), as shown in Figure 2-3.

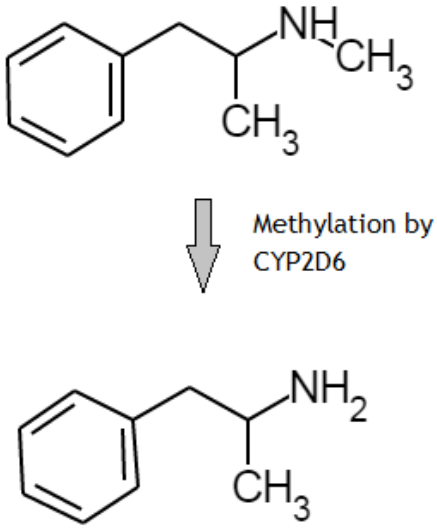

Figure 2-3 Metabolism of methamphetamine to amphetamine

The use of amphetamine and methamphetamine first started when both were marketed to promote alertness during World War II (89). Following 1945, the enantiomers were prescribed as an anti-depressant. The amphetamines are strongly related to endogenous neuromodulators including phenethylamines (93). Both amphetamine and methamphetamine have modern medical uses globally and can be prescribed, for weight loss or narcolepsy (94).

Amphetamine and methamphetamine are dopamine (as well as norepinephrine, and serotonin) reuptake inhibitors. When dopamine (or others) is released into the synaptic cleft, excess dopamine is reuptaken by the reuptake channels. Amphetamine, and methamphetamine, will completely block these reuptake channels and cause an excitatory neurotransmitter build-up (94). A dose-dependent response is observed, which explains why amphetamine can be prescribed medicinally when the dose is strictly controlled. The one difference that exists between amphetamine and methamphetamine is that methamphetamine is toxic to binding sites of dopamine and dopamine transporters in mammals (95). This toxicity and the desensitisation to the presence of the stimulus will lead to tolerance. Dopamine is also involved in the reward pathway. This means that methamphetamine is highly addictive due to the constant stimulation of the dopaminergic pathways. Pharmacokinetic properties of the two substances are described in Table 2-1.

Both amphetamine and methamphetamine undergo renal excretion and within the first 24 hours, 30% and 43% (respectively) are excreted unchanged in urine (85). Research has shown that the half-life of the amphetamine *l*-isomer can be up to 39% longer than its counter-isomer, and that acidification of urine can reduce elimination times (85).

Table 2-1 Pharmacokinetic characteristics of amphetamine and *d*-methamphetamine (85)

	Amphetamine	*d*-Methamphetamine
Volume of Distribution (V_D)	3.2 – 5.6 L/kg	3.0 – 7.0 L/kg
Half-Life	7 – 94 h	6 – 15 h
Plasma Protein Binding (Fb)	0.16	0.10 – 0.20
Blood/Plasma Ratio (b/p)	0.6 – 1.0	0.6 – 0.7
pKa	9.9	9.9

Most commonly, amphetamine is taken orally where methamphetamine is most commonly injected or inhaled through a pipe. Research shows, however, that methamphetamine is readily absorbed even when smoked or taken intranasally (96). Physiological effects can be increased with higher dosages but are also affected by the route of administration. Generally, amphetamine is a stimulant which causes increased alertness, excitement and heart rate. Methamphetamine is similar in that it increases feelings of arousal, awareness, and wakefulness, decreases the inhibitions and can increase risk taking. Both amphetamine and methamphetamine also decrease a user's appetite. When in acute overdose, symptoms for both substances are similar in that persons can exhibit agitation, headaches, hallucinations, hyperthermia and increased heart rates (85).

To achieve peripheral effects (increased heart rate) associated with amphetamine use, reports stated a threshold level of 20 µg/L in blood was needed (97). Medical doses of amphetamine can range from 3 to 50 mg (85). After intra-venous (IV) injection of 160 mg of *dl*-amphetamine, a plasma concentration of 0.59 mg/L was seen in a chronic amphetamine user (98). Methamphetamine single doses of 0.125 mg/kg or 12.5 mg showed average peak blood concentrations of 20 µg/L (85).

2.1.2 MDMA (3,4-methylenedioxymethamphetamine), MDA (3,4-methylenedioxyamphetamine) and MDEA (3,4-methylenedioxy-*N*-ethyl-amphetamine)

MDMA, or "ecstasy", is scheduled as a Class A drug in the UK under the Misuse of Drugs Act 1971. Its structure is shown in Figure 2-4, as are structures for MDA and MDEA. European MDMA is mainly manufactured in Belgium and the Netherlands, although it is now also imported from Canada (99). Chemicals required for the deamination reaction which is most common in Europe are purchased from Germany, Poland or Romania (99). MDMA content has been reported to have continually increased since 2009 (99). It first found its popularity in the rave and club scenes, where is became popular due to the increased sensation and experience of colours and sounds, as well as the "buzz" of amplified alertness it gave users.

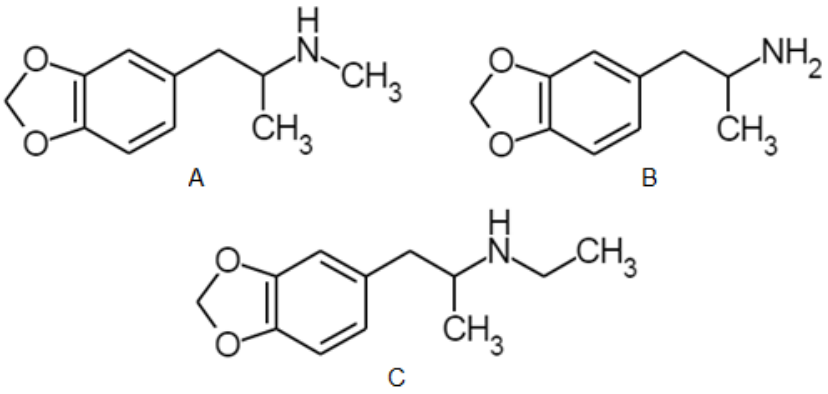

Figure 2-4 Chemical structures of MDMA (A), MDA (B) and MDEA (C)

Most commonly, MDMA is produced in tablet form, and the appearance of MDMA tablets differs widely, in shape, colour, branding and marking. MDMA is usually taken orally at doses of 100 - 150 mg (however, it is common to find what users expect to be ecstasy tablets, to contain both MDA and MDEA as well). Following a single dose of 100 – 125 mg, peak plasma concentrations averaged around 180 µg/L (85). MDMA is eliminated after 24 hours as MDMA (26%) and MDA (1%) to which it is metabolised, and both are pharmacologically active. Short-term adverse effects include anorexia, headaches, anxiety and insomnia, whereas long-lasting neurological changes, including the alteration of serotonergic and dopaminergic pathways, have been recorded (100, 101). Overdose or deaths by MDMA ingestion may be caused by hyperthermia following increased CNS stimulation, renal failure, and increased sweating due to the hyperthermia causing hyponatremia (increased consumption of fluids leading to cell death, and cerebral oedema). For MDMA, the volume of distribution (V_D) is 3 – 7 L/kg, the half-life between 4 and 12 hours, plasma protein binding has been found to be 0.65 and the blood/plasma ratio between 1.2 – 1.3. For MDEA and MDA, the blood/plasma ratio was found to be 1.0 and 1.2 – 1.3, respectively. Volumes of distribution, half-lives and plasma protein binding are unknown (82).

MDA is a drug of abuse in its own right, albeit also a minor metabolite of MDMA. The difference between the two molecules is the methyl group on the nitrogen. Colloquially it is known as “Eve”. It can be taken in various forms. It exhibits hallucinogenic properties when taken in large doses (hallucinations reported after ingestion of 98 mg/70 kg body weight (102)), and overdose symptoms include agitation, hypothermia, convulsions, and tachycardia. Although its metabolism has not yet been studied, urine concentrations in fatal cases show

large quantities of the parent drug which is indicative of excretion of a largely unchanged drug (i.e. MDA is not extensively metabolised).

No information is available in regards to therapeutic doses of MDA, however it is suggested that blood concentrations would not exceed 0.4 mg/L due to its similarity to amphetamine (85).

MDEA (3,4-methylenedioxy-*N*-ethyl-amphetamine, Figure 2-4) is an analogue of MDMA, but MDMA is not metabolised to MDEA or vice versa. Due to the similarity of their effects on the human body, both MDEA and MDMA are also scheduled as Class A drugs. MDEA is also taken orally in tablet form, however these tablets can contain a combination of MDA or MDMA at higher concentrations (85). An oral dose of 140 mg has been reported to produce peak plasma concentrations averaging at 0.26 mg/L (85).

MDEA metabolises via oxidative cleavage of its aromatic ring and is largely excreted in urine after 32 hours as the parent drug (19%) or as MDA (28%) (85). Small doses (30 – 100 mg) mildly stimulate the CNS; adverse reactions after ingestion may include hallucinations, agitation, and seizures. Studies have also suggested that MDEA causes less neuronal degeneration than MDMA, however, as higher concentrations of MDEA are required to produce the same effects as MDMA, MDEA cannot be considered a safer drug than MDMA (101). In overdose or toxic situations, after ingestion of usually between 100 – 200 mg of MDEA, symptoms will be similar to those shown in situations of acute intoxication with amphetamine, methamphetamine or MDMA (i.e. paranoia, increased heart rate, hyperthermia and hallucinations).

Similar to amphetamine and methamphetamine, all three analytes are monoamine reuptake inhibitors. This explains the similar physiological effects these substances have on the human body, as well as the presentation of symptoms and experiences of users when taking the substances.

2.1.3 Amphetamine and Methamphetamines in Ante-Mortem Forensic Toxicology

A number of studies have been performed investigating amphetamine and methamphetamines in oral fluid using various collection devices. Many of these

methods also employ analysis by GC-MS for confirmatory tests; however initial screening tests are often immunoassay techniques.

A review paper evaluated enzyme-linked immunosorbent assays (ELISA) specifically for amphetamine and methamphetamine as well as multidrug tests for oral fluid fortified with amphetamine-type stimulants (ATS). Cross-reactivity was assessed for ATS, and the authors concluded that further development of the assays is required. Cross-reactivity is prominent which may lead to false-positives. Issues with immunoassays for the amphetamines and false-positive results have been widely reported (55, 103). Nowadays, cross-reactivity is largely found with "designer drugs" that are structurally similar to the amphetamines (104, 105), including new psychoactive substances (NPS) (106) and synthetic cathinones (107).

It is important to investigate drug recoveries from collection devices as analytes behave very differently, and it must be assured that the drug concentrations determined in the sample correlate to the concentration present in neat oral fluid, and that no analytes are lost through the use of a collection device. One study, reported drug recoveries of amphetamine and methamphetamine exceeding 95% when using the Quantisal® device (108), when compared to other devices used in another study (including the Salivette, Intercept®, ORALscreen and the Hooded Collector devices) which showed recoveries of less than 59% (109). A study conducted by Langel *et al*. showed a mean amphetamine drug recovery of 89.7% using the Quantisal® device. Mean amphetamine recoveries ranged from 51.8 (Salivette device) – 103.1 (Intercept® device) %. A discussion as to why the recoveries varied so much from device to device was not given (14). Drug recoveries using various devices for MDMA have been studied. In the study conducted by Langel *et al* (14), mean recoveries ranging between 52.0 – 82.3% were reported for 10 collection devices. For both MDMA and amphetamine, recoveries found in this study were highest when oral fluid was collected using a plastic tube or using the Intercept® device (102.0% (RSD 3.0) and 101.1% (RSD 4.9), respectively). The lowest recovery for both analytes was found following oral fluid collection using the Salivette device (14).

One study investigated the recoveries of amphetamine and methamphetamine from the Quantisal® collection device (108). A serum separator was used to aid

the removal of oral fluid from the collection pad. This is common but not specifically instructed by the manufacturer. The drug extraction was performed using mixed-mode solid phase extraction (SPE) cartridges. Recoveries were reported to exceed 85%. The authors stated that the results were highly reproducible with all coefficients of variation (%CV) less than 10%. Although the linear ranges spanned a concentration range of 5 – 200 ng/mL for amphetamine and 2.5 – 100 ng/mL for methamphetamine, no limits of detection or quantitation were mentioned.

A further study compared amphetamine, methamphetamine, and MDMA concentrations in blood and oral fluid collected using the Intercept® sampling device. The method of extraction was not identified. The median oral fluid/blood ratios (OF/B) observed for amphetamine (n = 15), methamphetamine (n = 11) and MDMA (n = 4) were 7.2, 4.5 and 5.1, respectively. The authors stated that due to inter-individual variations of drug concentrations in both oral fluid and blood, OF/B ratios cannot be used to accurately estimate drug concentrations in blood based on concentrations found in oral fluid (41). Other studies (38, 110) have found higher ratios, which supports the notion that oral fluid concentrations should not be used to estimate blood concentrations. Gjerde (41) used the OraSure Intercept® sampling device, and other papers used other devices (such as the Saliva·Sampler™ device (38) or the Salivette® device (20)) so comparisons of recoveries and OF/B ratios must be done carefully.

Similar to these ratios, a study of methamphetamine and amphetamine in oral fluid, plasma and blood showed plasma concentrations were exceeded by concentrations in oral fluid, but that urine concentrations of these drugs were higher than oral fluid concentrations. The limit of quantification of the GC-MS method used was 2.5 ng/mL, but again the extraction technique was not specified (20). In this study oral fluid was stimulated using sour sweets, collected using an acidified Salivette® collection swab or using a non-acidified Salivette® swab. The use of salivating agents is known, but may change the composition of the oral fluid and thereby the concentrations of analytes present (as discussed in Section 1.2).

A similar study focused on amphetamine and methamphetamine concentrations in oral fluid following controlled dosing with 10 mg and 20 mg methamphetamine

tablets. Results showed that both amphetamine and methamphetamine were detectable in oral fluid for at least 24 hours. Similar to the previous study, oral fluid was collected by stimulation through citric acid as well as a neutral Salivette® swab. Instrumental analysis was carried out on GC-MS following an SPE extraction (39). The detection of amphetamine and methamphetamine in oral fluid has been studied and results showed that both analytes can be detected in oral fluid for up to 8 days. This is a longer window of detection than that for these analytes in both blood and urine at the recommended cut-off concentrations (111). This finding is important as this confirms that the use of oral fluid for the detection and monitoring of amphetamine and methamphetamine is worthwhile. The drawback of the study is that neither MDMA nor metabolites of methamphetamine (MDA or MDEA) were included. Furthermore, the authors comment that even though the detection window is large, individual differences in drug elimination rates must be considered.

A further study utilising the Salivette® swab, showed excellent recoveries for both amphetamine and methamphetamine (>75% for all concentrations and both analytes). The study also compared oral fluid concentrations to those detected in hair samples and urine samples submitted for testing by the police from drug users. Results showed significantly lower drug concentrations in hair than in oral fluid and urine and only one sample out of 12 gave a negative oral fluid result (112). The authors concluded that oral fluid is beneficial for confirmation of short-term drug use.

Although many studies have utilised GC-MS as the instrumentation of choice, a number of studies have developed methods on LC-MS/MS. One study developed a rapid method for the detection of amphetamine and methamphetamines (MDMA, MDA and MDEA) (113), for the analysis of expectorated oral fluid. The method gave good linearity, and limits of detection ranged from 0.15 – 0.5 ng/mL, and limits of quantitation from 0.5 – 1.0 ng/mL for analytes. The method was very sensitive, but that is to be expected on a tandem mass spectrometry method. The authors stated that due to the sometimes limited sample volume, it is advisable to use LC-MS/MS instrumentation. The authors did not report drug recoveries, which would have been interesting considering a simple PPT was used for the extraction.

As previously mentioned, all these analytes stimulate the CNS – this means that the use of these drugs will have an effect on motor skills too. This is particularly important as oral fluid is a sample taken ante-mortem in situations of WDT (49) or DUID. Amphetamine and methamphetamine are both substances that have been found in DUID cases, where plasma was tested (114), but also have also been found in oral fluid (115).

A number of studies have collected oral fluid samples at the roadside and analysed these for the presence of amphetamine and methamphetamines. The previously mentioned ROSITA projects included amphetamine and methamphetamines. Research has found that erratic driving, stopping and starting of the vehicle, no indication before turns, weaving, pulling into oncoming traffic, drifting off the road, speeding, and failing to stop at stop signs are the most common causes for drivers to be tested at the roadside (116). Observed behaviour and physiological changes, including pupil dilation, short attention spans, needle tracks, repetitiveness, rapid speech and agitation, were noted in drivers apprehended who then tested positive for either amphetamine or methamphetamines (116).

A study carried out in Australia evaluated the presence of methamphetamine, MDMA and THC in randomly selected drivers. 13176 roadside drug tests were performed. In this study, oral fluid samples for confirmatory, quantitative analyses were collected using the Cozart collection device. Drug recoveries from the device were tested in duplicate and recoveries for methamphetamine averaged 96%. A recovery study for MDMA was not conducted although recoveries are different for each analyte and analytes respond differently to collection devices. Presumptive positives in oral fluid were sent to the laboratory for confirmatory testing by GC-MS. Results showed a median methamphetamine concentration (269 positive cases) of 1136 ng/mL (ranging from 8 to 49000 ng/mL), and a median concentration of 2724 ng/mL for MDMA (118 positives, concentration ranging from 66 to 17000 ng/mL). Amphetamine was detected in 74% of samples that were positive for methamphetamine, with a median concentration of 90 ng/mL (range: 5 – 4800 ng/mL). MDA was detected in 80% of the samples where MDMA was positive, with concentrations ranging from 5 – 1700 ng/mL, and a median concentration of 181 ng/mL. None of the positive cases for MDA were negative for MDMA, but the ratio between MDA and MDMA

was established and authors stated that in only three cases did the ratio indicate MDA use rather than MDMA consumption (117). The maximum oral fluid concentrations found would indicate very recent use of the drugs, which is a concern considering the dataset were randomly selected drivers. Depending on the drug, on the time of use and the deposition rate of drug in oral fluid these concentrations could be proof of driving under the influence of drugs although maximum plasma concentrations may not have yet been reached.

In Finland, as part of the ROSITA2 project, oral fluid was taken in 153 cases of suspected DUID. A limitation of the study includes the low volume of oral fluid collected (224 µL) as amphetamine causes hypo-salivation, which meant that 39 cases were dismissed due to lack of sample. A further concern is that oral fluid samples were stored for up to two months before analysis was carried out. Even so, 100 samples tested positive in a range of 27.8 – 131000 ng/mL and a median concentration of 7440 ng/mL (118). Linear ranges or limits of detection and quantitation were not given, nor did it state the driving behaviour that caused the police to stop drivers other than that it was for “suspected drugged driving”.

Similarly, a study focused on drivers apprehended for driving under the influence of substances other than alcohol. 100 drivers were included and oral fluid was collected using the StatSure Saliva Sampler™. Concentrations found for amphetamine or methamphetamine were not reported, however the authors stated that MDA, MDMA and MDEA were not detected (40).

Table 2-2 Examples of studies testing amphetamines in oral fluid

Reference	Analyte	Neat/Collector	Recovery (%)	Extraction Technique	Analytical Method	Linear Range (ng/mL)	LOD/LOQ (ng/mL)
Drummer, 2007 (117)	MA, MDMA	Cozart	70 (MA)	N/A	GC-MS	5 – 2000	2.0/5 .0
Quintela, 2006 (108)	AM, MA	Quantisal™	85	SPE	LC-MS/MS	5 – 200 (AM) 2.5 – 100 (MA)	N/A
Andås, 2016 (111)	AM, MA	Intercept®	N/A	LLE	LC-MS/MS	6.8 – 68 (AM) 7.5 – 150 (MA)	AM: 1.4/4.1 MA: 1.5/7.5
Wood, 2003 (113)	AM, MA, MDMA, MDEA, MDA	Neat	N/A	PPT	LC-MS/MS	0.5 – 500	AM: 0.5/0.5, MA: 0.2/0.5, MDMA: 0.2/0.5, MDEA: 0.15/0.5, MDA: 0.5/1.0
Kim, 2008 (112)	MA, AM	Salivette	> 75.1	SPE	GC/MS	1 – 1000	MA: 0.1/1.0 AM: 1.0/3.0
Newmeyer, 2015 (119)	AM, MA	Quantisal™ Oral-Eze®	N/A	SPE	LC-MS/MS	1 - 500	0.5/1.0
Comiran, 2017 (120)	AM	Neat	>121.4	N/A dilution and vortex-mixing	LC-MS/MS	1 - 128	LOQ : 1.0
Bahmanabadi, 2016 (121)	MA	Neat	96	LLE	GC/MS	15 – 200	5.0/15.0
Poetzsch, 2016 (122)	MDMA	Quantisal™	95.3 ± 12.3	LLE	MALDI-QqQ-MS/MS	5 - 2000	1.0/5.0
Rositano, 2016 (60)	MA, MDMA	Alere DDS 805 AP	80	SLE	LC-MS Q-Trap	5 - 1250	5.0

SPE – solid-phase extraction; LLE – liquid-liquid extraction; PPT – protein precipitation; GC-MS – gas chromatography mass spectrometry; LC-MS/MS – liquid chromatography – tandem mass spectrometry, N/A – not available, MALDI-QqQ-MS/MS – matrix assisted laser desorption/ionization-triple quadrupole-tandem mass spectrometry.

2.2 Aims and Objectives

The evaluation of a novel collection device, the Neogen® NeoSAL™ oral fluid collection device, supplied by AgriYork 400 Ltd was the main aim of this project. The premier focus was the analysis of oral fluid collected using the device, for amphetamine drugs.

Objectives of the study included:

- Gravimetrical assessment of the Neogen® NeoSAL™ oral fluid collection device compared to the OraSure Technologies Intercept® *i*2™ and the Immunalysis™ Quantisal® oral fluid collection devices. Two main aspects were assessed for this:
    - Oral fluid collection adequacy of the NeoSAL™, Intercept® *i*2™ and Quantisal® devices;
    - Drug recoveries of amphetamine and methamphetamines from oral fluid collected using the NeoSAL™ and Intercept® *i*2™ devices.
- Establishing and partially validating a selective and sensitive analytical GC-MS method for amphetamine and methamphetamines in expectorated (neat) oral fluid and oral fluid collected using the NeoSAL™ device. The method aimed to meet the guidelines and cut-off recommendations put forward by the EWDTS for the quantitation of amphetamine drugs in oral fluid.

2.3 Ethical Approval

Ethical approval for the collection of blank matrices for method development and validation was granted through the University of Glasgow MVLS[2] Ethics Committee.

[2] College of Medical, Veterinary and Life Sciences

2.4 Materials and Methods

2.4.1 Chemicals and Reagents

Amphetamine, methamphetamine, MDMA, MDA and MDEA were purchased from Sigma Aldrich (Gillingham, UK). All certified drug standards were dissolved in methanol (MeOH) at a concentration of 1 mg/mL.

The deuterated standards used as internal standards, amphetamine-D_{11}, methamphetamine-D_{14}, MDA-D_5, MDMA-D_5 and MDEA-D_6, were purchased from Sigma Aldrich (Gillingham, UK). All certified deuterated drug standards were dissolved in MeOH at a concentration of 100 µg/mL.

MeOH, ethyl acetate (EtOAc), dichloromethane (DCM), isopropanol (IPA), tartaric acid (> 99%), glacial acetic acid (> 99%), sodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4.H_2O$), and concentrated ammonium hydroxide (NH_4OH, S.G. 0.91 25%) were purchased from VWR (Lutterworth, UK). All solvents were HPLC grade. Purified, deionised water was obtained from an in-house Millipore® Direct Q® 3UV-R system.

Pentafluoropropionic anhydride (PFPA) and anhydrous disodium hydrogen orthophosphate (Na_2HPO_4) were purchased from Sigma Aldrich (Gillingham, UK). Both were AnalaR grade.

Blank oral fluid was donated by volunteers from the FMS laboratory in line with procedures set out in ethical approval.

2.4.2 Preparation of Solutions

All solutions were prepared using Gilson PIPETMAN Classic™ accurate pipettes which were checked in house.

2.4.2.1 Stock Solutions

A stock solution was prepared by pipetting 1 mL of each amphetamine, methamphetamine, MDA, MDMA and MDEA certified drug reference standards at 1 mg/mL into a 100 mL volumetric flask. This was made up to the meniscus line with MeOH, resulting in a concentration of 10 µg/mL (0.01 mg/mL).

The same procedure was followed for the internal standard stock solution, but by pipetting the five internal standard certified drug reference standards at 100 µg/mL into a 10 mL volumetric flask. This was made up to the mark with MeOH. The resulting concentration was 10 µg/mL.

The solutions were transferred to labelled amber bottles and stored at -20°C for up to 6 months. The caps were covered with Parafilm® to prevent any leaks or loss due to evaporation.

Stock solutions for QC solutions were prepared as described above, but using different lot numbers of the reference standards.

2.4.2.2 Working Solutions

Using the amphetamines standard stock solution, two working solutions were prepared in MeOH.

To prepare a 1 µg/mL working solution 1 mL of amphetamine stock solution at 10 µg/mL was transferred into a 10 mL volumetric flask, and filled to the mark with MeOH.

In order to prepare a 0.1 µg/mL working solution, 100 µL of amphetamine stock solution at 10 µg/mL was transferred into a 10 mL volumetric flask and made up to the mark with MeOH.

Both solutions were stored at -20°C in the freezer for a maximum of 6 months, the caps secured with Parafilm®.

The internal standard working solution at 1 µg/mL was prepared by pipetting 1 mL of amphetamines internal standard stock solution at 10 µg/mL into a 10 mL volumetric flask and made up to the mark with MeOH. The solution was stored at -20°C in the freezer for a maximum of 6 months.

Working solutions for QC and spikes were prepared as described above but using the stock solutions prepared from the different lot numbers of reference standard.

2.4.2.3 0.1 M Phosphate Buffer (pH 6)

0.1M phosphate buffer was prepared by weighing 1.7 g of Na_2HPO_4 into a 1 L beaker. To this, 12.14 g of $NaH_2PO_4 \cdot H_2O$ was added. The mixture was dissolved in 800 mL of deionised water. The pH was adjusted to 6 (± 0.1) with 0.1 M monobasic NaH_2PO_4 or 0.1 M dibasic Na_2HPO_4 to lower or raise the pH, respectively, as required. This was then transferred to a 1 L volumetric flask, then made up to the mark with deionised water and mixed thoroughly. The buffer was stored at room temperature (20°C ± 2) for a maximum of 6 months.

2.4.2.4 1 M Acetic Acid

1 M acetic acid was prepared by pipetting 28.6 mL of glacial acetic acid (> 99%) into a 500 mL volumetric flask filled about half way with dH_2O. After mixing, it was made up to volume with dH_2O. The solution was stored at room temperature (20°C ± 2) for a maximum of 6 months.

2.4.2.5 1 mg/mL Tartaric Acid in EtOAc

100 mg of tartaric acid was dissolved in 100 mL of EtOAc. The solution was stored at room temperature (20°C ±2) for a maximum of 6 months.

2.4.2.6 Dicholoromethane:Isopropanol:Ammonium Hydroxide (DCM:IPA:NH_4OH; 78:20:2 v/v)

This solution was prepared daily, as required, and stored at room temperature. 78 mL of DCM and 20 mL of IPA were transferred to a reagent bottle and mixed. 2 mL of concentrated ammonia was added and sonicated for at least 5 minutes afterwards.

2.4.2.7 PFPA:EtOAc (2:1)

2 mL of PFPA was added to 1 mL of EtOAc in the fume hood. This solution was stored at room temperature and prepared fresh as required.

2.4.3 Oral Fluid Collection Devices

NeoSAL™, Intercept® *i*2™ and Quantisal® devices were supplied by AgriYork 400 Ltd (York, UK).

2.4.4 Assessment of Volume Adequacy Indicators – Gravimetric Analysis

Collection volumes of three oral fluid collection devices, namely, the Quantisal®, NeoSAL™, and Intercept® devices were assessed initially to investigate whether the volume of oral fluid actually collected was representative of the volume supposed to be collected. Few studies have called the actual volume into question (6, 14) and this is a vital step that very few researchers carry out prior to their analysis, as the volumes stated by the manufacturers are relied upon (123).

The collection volumes were assessed by weighing the collector including the pad prior to collection, as well as after the collection. The length of time to collect the sample was recorded. Assuming that oral fluid and collection buffer, share the same density as water (0.9982 g/mL at 20°C (124)), the difference in weight between post- and pre-collection of oral fluid gives the volume of oral fluid collected. The temperature in the laboratory was monitored on days where gravimetric assessments were carried out. The collection instructions for the NeoSAL™ device are shown in Appendix II.

2.4.5 Instrumentation

For the analysis, an Agilent Technologies 7890A Gas Chromatography (GC) System coupled with a 7683B Series injector attached to a 5975C inert XL Mass Selective Detector (MSD) with triple axis detector was used. Analysis was carried out using an Agilent HP-DB5 5% phenyl methyl siloxane column (30 m x 250 µm x 0.25 µm) with a maximum temperature of 350 °C. Data analysis and processing were done using the preinstalled ChemStation (Enhanced ChemStation, MSD ChemStation E.02.02.1431) software.

The GC-MS method used was an in-house method routinely used for the analysis of amphetamines. The GC-MS analysis oven temperature programme was held at 80 °C for 1 minute, then ramped to 300 °C at 10 °C/minute and held for 2 minutes. The run time was 25 minutes. The injection volume was 1 µL. Splitless injection was used, with a purge flow to split vent of 50 mL/minute at 2 minutes. The gain factor was set at 2 to ensure that the low concentrations

could be detected and quantified accordingly. Table 2-3 summarises the ions used to monitor the drugs included in the study.

Table 2-3 Ions (*m/z*) used for quantitation

Analyte	Quantification Ion *(m/z)*	Qualifier Ion 1 *(m/z)*	Qualifier Ion 2 *(m/z)*
AMP	190	118	91
MAMP	204	160	118
MDA	325	190	162
MDMA	339	204	162
MDEA	353	218	162
Deuterated Internal Standards			
AMP-D_{11}	194	128	98
MAMP-D_{14}	211	163	128
MDA-D_5	330	167	194
MDMA-D_5	344	208	165
MDEA-D_6	359	224	165

2.4.6 Oral Fluid SPE Extraction Procedure

The extraction method that was followed was the in-house extraction method for amphetamines from oral fluid collected using the Quanitsal® device. Blank oral fluid was always frozen before analysis, as this helps prevent possible breakdown of unstable analytes, bacterial growth in the sample (125), and aids the homogenisation when samples are subsequently mixed prior to use by allowing the precipitation of mucins in the sample.

Samples were prepared by adding 3 mL of 0.1 M pH 6 phosphate buffer to labelled test tubes. To this, 50 µL of 1.0 µg/mL internal standard solution was added, as well as relevant volumes of relevant solutions to produce QC and calibration samples (outlined in Section 2.4.7.2). The total volume of oral fluid/buffer mixture in the oral fluid collection devices was added to the test tubes, for all collection devices. This was done manually by squeezing the pad against the wall of the collection container in order to remove as much sample as possible from the saturated collection pad. These test tubes were then vortex mixed for 5 seconds before being placed in the Sigma 4-16 centrifuge, where the samples were centrifuged for 10 minutes at 2500 rpm.

UCT CleanScreen® DAU C18 mixed-mode SPE cartridges (ZSDAU020) were conditioned with 3 mL of MeOH, followed by 3 mL of dH_2O and 1 mL of pH 6, 0.1M phosphate buffer. The supernatant from the centrifuged samples was transferred onto the conditioned cartridges. When the supernatant had passed through the cartridges completely, 3 mL of dH_2O was added. This was followed with 1 mL 1M acetic acid. Finally, 3 mL of MeOH was added to the cartridges before drying under full vacuum for 5 minutes. The elution was carried out with 3 mL DCM:IPA:NH_4OH (78:20:2 v/v/v). 100 µL of tartaric acid in EtOAc (1 mg/mL) was added to stabilise the eluents, and then the solvent was evaporated to dryness under a constant, gentle stream of nitrogen gas with no heat. Samples were derivatised using 50 µL PFPA:EtOAc at room temperature for 5 minutes. The derivatisation agent was evaporated gently under a constant stream of nitrogen with no heat. Finally, samples were reconstituted in 50 µL of EtOAc, vortex mixed and then transferred into labelled GC autosampler vials for analysis by GC-MS.

10 µL of the amphetamines stock solution, as well as 10 µL of the amphetamines internal standard stock solution were used as system suitability checks before the analysis was run.

2.4.6.1 Preliminary Work

The linearity of unextracted standards at concentrations ranging from 1 to 200 ng/mL was first assessed to check the performance of the instrument. A blank was always run alongside the calibrators. Unextracted calibrators for the initial assessments of linearity were prepared using the volumes of working solutions given in Table 2-4.

The calibration range for initial work was 1 – 200 ng/50µL EtOAc. However this changed to 2 – 200 ng/50µL EtOAc. A quick assessment of the limits of detection, as outlined later in Section 2.4.7.3, of the instrument was also carried out at this point, to ensure that the sensitivity of the instrument would be able to detect concentrations below the EWDTS recommended 15 ng/mL (or 10.5 ng/0.7 mL of oral fluid) cut-off for amphetamines in oral fluid.

Table 2-4 Preparation of unextracted calibration range

Calibrator	Concentration (ng/50 µL EtOAc)	Concentration (ng/mL)	Volume (µL) of 0.1 µg/mL working solution	Volume (µL) of 1.0 µg/mL working solution
Cal 1	1.0	1.43	10	-
Cal 2	2.0	2.86	20	-
Cal 3	5.0	7.15	50	-
Cal 4	10.0	14.29	-	10
Cal 5	25.0	35.71	-	25
Cal 6	50.0	71.42	-	50
Cal 7	100.0	142.68	-	100
Cal 8	200.0	285.71	-	200

Where EtOAc – ethyl acetate

When acceptable R^2 values were obtained using unextracted standards, extractions were performed to ensure that an extracted calibration would also give good linearity. QC samples at known concentrations, outlined in Table 2-5, were injected alongside calibration standards to investigate whether the nominal and calculated concentrations of the QCs gave acceptable agreement. Agreement was deemed acceptable if the calculated QC concentrations were within ±20% of the nominal QC concentration.

2.4.7 Method Validation

All oral fluid collection devices were evaluated according to the European Guidelines for Workplace Drug Testing in Oral Fluid, in which the cut-off for amphetamine, methamphetamine, MDA, MDMA and other members of the amphetamine group was set at 30 ng/mL in previous guidelines. While the project was being carried out the guidelines changed, and the recommended cut-offs were reduced to 15 ng/mL.

2.4.7.1 Drug Recovery from Collection Devices

The recovery was tested for the Neogen® NeoSAL™ collector and OraSure's Intercept™ *i*2™ collector as a comparison. The intention was to include Immunalysis™' Quantisal® device as a third collector, however Alere (the suppliers of Immunalysis™ products in the UK) turned down any participation in this project and so no collectors were available for investigations other than for collection volume adequacy.

The recovery was investigated by spiking one set of quadruplicates per concentration and adding 50 µL internal standard working solution (at a concentration of 10 µg/mL) before the extraction. A second set of quadruplicates per concentration had internal standard added after extraction. Peak area ratios (PAR) of both sets of quadruplicates per concentration were compared to PAR of a set of unextracted quadruplicates per concentration.

Since oral fluid devices collect different volumes of oral fluid two methods of supplying the devices with oral fluid were selected. Oral fluid was collected by expectoration into plastic containers, frozen and defrosted before use. 5 mL of neat oral fluid was spiked at the two recovery concentrations tested, 30 ng/mL of oral fluid and 100 ng/mL of oral fluid, respectively. Then:

- Oral fluid collector pads were dipped ("Dipped") into the spiked oral fluid until the collection adequacy indicators turned blue indicating the correct volume of oral fluid for the device had been collected;

Or

- 0.7 mL and 1.0 mL of spiked oral fluid was then pipetted ("Pipetted") onto the collection pad of the NeoSAL and Intercept® *i*2™ devices, respectively.

Recoveries were tested by spiking neat oral fluid with 30 ng/mL and 100 ng/mL of amphetamine solution. For the NeoSAL™ device, that would give a drug concentration of 21 ng/0.7 mL and 70 ng/0.7 mL of oral fluid collected by the device, respectively. For the *i*2™ device, the concentrations would be 30 ng/mL and 100 ng/mL of oral fluid collected by the devices, respectively.

For these two recovery methodologies, two recoveries were calculated: the total (overall) recovery, as well as the recovery from the collection pad itself. Overall recovery refers to the extraction for the process, whereas recovery from the pad refers to the amount of drug that was recovered from the collection pad. This is important because studies (14, 126) have found that certain drugs absorb onto the collection pad and are therefore lost.

Recovery was calculated by using the mean PAR of the extracted standard to IS added after the SPE extraction (post-extraction spike, “After”) divided by the mean PAR of unextracted standard to IS. The drug recovery from the pad was calculated by dividing the mean PAR of extracted standard to IS added before the SPE extraction (pre-extraction spike; “Before”) by the mean PAR of unextracted standard to IS.

A further part of the investigation, which was repeated for both sets of collectors, as well as both types of matrix collection, involved calculating weight differences of oral fluid (i.e. oral fluid volume collected). This ensured that the amount of oral fluid collected was known, based on a density of 1 g/cm^3. For each of the collectors, the volume of buffer contained within the kits was also measured. Temperature was monitored accordingly.

2.4.7.2 Calibration Range and Controls

The calibrators were made up as described in Table 2-5. A calibration model to span the following concentration range was investigated: 2 ng/0.7 mL of oral fluid – 200 ng/0.7 mL of oral fluid.

Table 2-5 Preparation of calibration range in oral fluid

Calibrator	Concentration (ng/0.7 mL oral fluid)	Concentration (ng/mL)	Volume (µL) of 1.0 µg/mL working solution
Calibrators			
Cal 1	2.0	2.86	2.0
Cal 2	5.0	7.15	5.0
Cal 3	10.0	14.29	10.0
Cal 4	25.0	35.71	25.0
Cal 5	50.0	71.42	50.0
Cal 6	100.0	142.68	100.0
Cal 7	200.0	285.71	200.0
Quality Controls			
QC1	10.5	15.0	10.5
QC2	21.0	30.0	21.0
QC3	70.0	100.0	70.0

Linearity was assessed both using internal standard (50 µL of internal standard at a concentration of 1.0 µg/mL added) and without using internal standard for the same concentration ranges. The linearity was assessed in duplicate over 5 consecutive runs for both calibration samples.

2.4.7.3 Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

Both LOD and LLOQ are estimated by assessing the background noise of a sample. The LOD is determined by the sample that gives a signal-to-noise ratio (S/N) ≥3, and the S/N must be reproducible for all ions, the quantifier ion as well as both qualifier ions. LOD is assessed both in the presence and absence of matrix, giving the assay LOD (LOD in the presence of matrix) and the instrumentation LOD.

The lowest concentration that achieves acceptable detection, identification, accuracy and precision is considered the LLOQ. The S/N has to be ≥10, for all ions, and the calculated %CV must be less than or equal to 20%.

Three sources of blank oral fluid matrix were used, each spiked at decreasing concentrations and analysed in triplicate for at least 3 separate runs. The concentrations investigated were 0.5, 0.75, 1.0, 1.5, 2.0 and 5.0 ng/0.7 mL of oral fluid.

2.4.7.4 Interference from Common Drugs

Interferences from the most commonly encountered drugs of abuse and prescribed medications were investigated. These are summarised in Appendix III. In order to do this, mixtures of drug solutions at concentrations of 1 µg/mL were injected and run on the method described in Section 2.4.5. Results were examined visually, to ensure no peaks were found at the retention times of the analytes of interest.

2.4.7.5 Interference from Buffer and OF

As the NeoSAL™ collection device contains a buffer solution, it is important to assess whether it exhibits any interference with the method. To test this, blank NeoSAL™ buffer, and NeoSAL™ buffer spiked with 100 µL of standard mix at 1.0

µg/mL, giving a final concentration of 100 ng/mL, were put through the extraction method and compared with spiked and blank extracted neat oral fluid, as well as spiked and blank NeoSAL™ buffer/oral fluid mixture.

2.4.7.6 Accuracy and Precision

Accuracy and precision were evaluated using 3 pools of matrix sample spiked at 15, 30 and 100 ng/0.7 mL of oral fluid. Internal standard was added to the sample (50 µL of 1.0 µg/mL solution was added, resulting in a concentration of 50 ng/mL). Analysis was carried out in triplicate over 5 consecutive days, each with a freshly prepared calibration curve daily.

Accuracy was expressed as a percentage of the nominal concentration. The mean value should not differ more than 15% from the nominal value. The equation used to calculate accuracy is shown as Equation I below.

Equation I Calculation of Accuracy

$$\text{Accuracy (\%) at Concentration} = \left(\frac{X_x - N_x}{N_x} \right) \times 100$$

X – Grand mean of calculated concentration; N – nominal concentration

Precision (Equation II) is reported as the % coefficient of variation (%CV) and should be <20 % at each concentration.

Equation II Precision Calculation

$$\text{Precision (\%CV)} = \left(\frac{\text{Standard deviation of response(s)}}{\text{Mean response}} \right) \times 100$$

The mean concentration was calculated for each QC concentration (H (High) at 100 ng/0.7 mL oral fluid, M (Medium) at 30 ng/0.7 mL of oral fluid, L (Low) at 15 ng/0.7 mL oral fluid). In order to assess intra-day accuracy and precision, 4 replicates per concentration in one batch were used, whereas the inter-day accuracy and precision were assessed using calculated values over the 5 days of analysis.

2.4.7.7 Carryover

Carryover was assessed by injecting the highest extracted concentration (Calibration Standard 7, 200 ng/mL) twice followed by three extracted blank oral fluid samples. The chromatograms were evaluated visually to assess whether any carryover of drugs had occurred.

2.4.7.8 Autosampler Stability

A full method validation would require sample stability assessed over a period of time. However, only processed sample/autosampler stability was conducted over a period of 4 days (5 time points; Time 0, 24 h, 48 h, 72 h, 96 h), at approximately 19°C (± 0.5 °C). A full stability study was previously conducted for the method validation for the analytical method (amphetamines in blood) upon which this method is based. Finally, as there was a limited number of collection devices/ amount of collection device buffer available, the autosampler study was prioritised.

Blank oral fluid was spiked with drug standards to give two concentrations; 15 and 100 ng/mL of oral fluid. This was achieved by spiking the total volume of buffer/oral fluid mixture obtained from the NeoSAL™ device with 10.5 µL and 70 µL of the 1.0 µg/mL working solution, respectively. For each concentration, a set of samples (n = 4) were prepared containing internal standard. As each was run in quadruplicate, but the final reconstitution volume is 50 µL, samples had to be prepared in duplicate and combined to ensure that enough sample was available for the whole testing period. This meant a total of 4 samples and 4 collection devices were required and prepared at each concentration. The normal extraction procedure was followed for each concentration, and fresh spiked calibration standards were prepared and run alongside the samples each day.

The initial calculated concentrations, based on the linear calibration and regression equation obtained, were used as the average time 0 concentration. Subsequent calculated concentrations, based on the daily regression equation were then compared to the initial concentration. Later concentrations were divided by the initial average concentrations and then multiplied by 100 to

obtain the percentage of analyte remaining. If the concentration remained within ± 10% of the original concentration, the analyte was deemed stable.

2.5 Results and Discussion

2.5.1 Assessment of Volume Adequacy Indicators – Gravimetric Assessment

Preliminary data collected for the volume adequacy investigation are summarised in tables shown in Appendix VI (Table AIV-1, AIV-2, and AVI-3). Results are summarised in Table 2-6.

Table 2-6 Summary of gravimetric assessment data for the NeoSAL™, Intercept® *i*2™, and Quantisal® devices

Device	Mean weight before collection (g) (SD, %CV)	Mean weight after collection (g) (SD, %CV)	Mean weight difference (G) (SD, %CV)	Mean collection time (min:sec) (SD, %CV)	Mean total weight (incl. swab, g) (SD, %CV)
NeoSAL™	7.93 (0.03, 0%)	8.76 (0.13, 2%)	0.84 (0.13, 15%)	00:52.4 (00:04.8, 9%)	2.94 (0.13, 4%)
Intercept® *i*2™	9.92 (0.02, 0%)	11.16 (0.09, 1%)	1.24 (0.08, 6%)	01:37.1 (00:19.2, 20%)	2.04 (0.08, 4%)
Quantisal®	10.06 (0.06, 1%)	11.15 (0.20, 2%)	1.10 (0.19, 18%)	02:56.8 (01:33.3, 53%)	4.10 (0.19, 5%)

The NeoSAL™ collector collected an average of 0.84 mL of oral fluid (range 0.61 – 1.10 mL (%CV of 15%), assuming that oral fluid has the same density as water) as shown in Table 2-6. This is different to the volume that the manufacturer states is collected by the device, as outlined in Section 1.7.2.1. Based on this, there was a 20% difference between the collection volume stated by the manufacturer and the mean volume that was collected here.

The volumes obtained for the Intercept® device are also different to the volume collected stated by the manufacturer, outlined in Section 1.7.2.1.

The mean volume collected was 1.24 mL (range 1.11 – 1.41 mL, %CV 6%). Based on the results in Table 1-2, a 24% difference between the collection volume stated by the manufacturer and the mean volume collected here was found.

However, although the Intercept® device showed the greatest difference between stated collection volume and the mean collection volume found, it gave the most consistent results with the lowest coefficient of variation (6%). Therefore, the Intercept® device appears to be more reproducible and reliable at collecting oral fluid, but the mean collection volumes indicated that it is the least accurate at collecting the stated volume given by the manufacturer.

Table AVI-3 (in the Appendix) shows the volumes collected by the Quantisal® collection devices (mean 1.1 mL, range 0.88 - 1.28 mL). These were reported to only 2 decimal places as the gravimetric assessment of this collection device was carried out using a different scale to the one used previously for the NeoSAL™ and Intercept® devices. A 10% difference between the collection volume stated by the manufacturer and the mean volume collected in this study is shown, which is within the acceptable %range given by the manufacturer. The %CV was 18% which was similar to the %CV of the NeoSAL™ device but higher than that of the Intercept™ device.

The differences in oral fluid volumes collected in this study, compared to those stated by the manufacturers highlight the need of a gravimetric test before any oral fluid samples are analysed.

In the case of the NeoSAL™ device, 0.8 mL was collected rather than the stated 0.7 mL. Although this is not a large difference, calculating drug concentrations based on the stated volume not the collected volume can make a significant difference, which can lead to an overestimation of drug concentrations in the sample. In the case where cut-off concentrations are applied to results, this could mean the difference between an individual's result being below or above the cut-off. The information provided on collection times for the three devices shows the large variability between individuals to provide an adequate volume of oral fluid for the analysis.

2.5.2 Preliminary Work

Preliminary results of unextracted calibration standards, gave accurate and linear calibration models with a minimum R^2 value of 0.99. Examples of unextracted calibration graphs for each analyte are shown in Figure 2-5, and an

example of a TIC for Calibrator 7 (200 ng/mL of oral fluid) is shown in Figure 2-6. When the response for low calibration standard of 1 ng/0.7 mL gave inconsistent and irreproducible results a lowest calibration standard concentration of 2 ng/mL was selected. The calibration range used for further work was therefore 2 – 200 ng/mL. This was also used as the basis for LOD and LLOQ investigations. This initial work showed that the instrument and the analytical method were sufficiently sensitive to detect concentrations below the recommended cut-off of 15 ng/mL.

Consistent linear models ensured that QC concentrations could be calculated accurately.

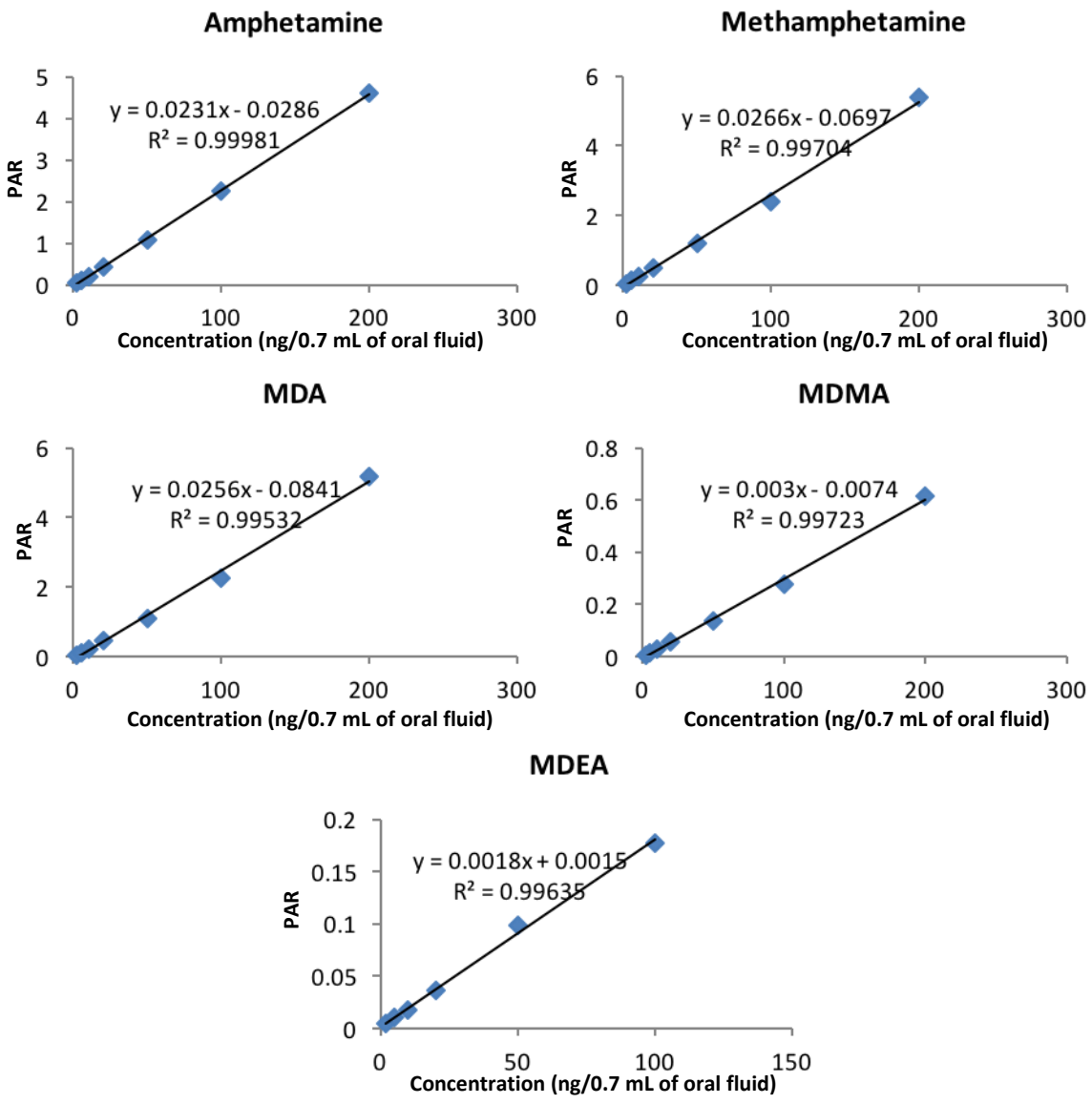

Figure 2-5 Examples of unextracted calibration graphs for each analyte

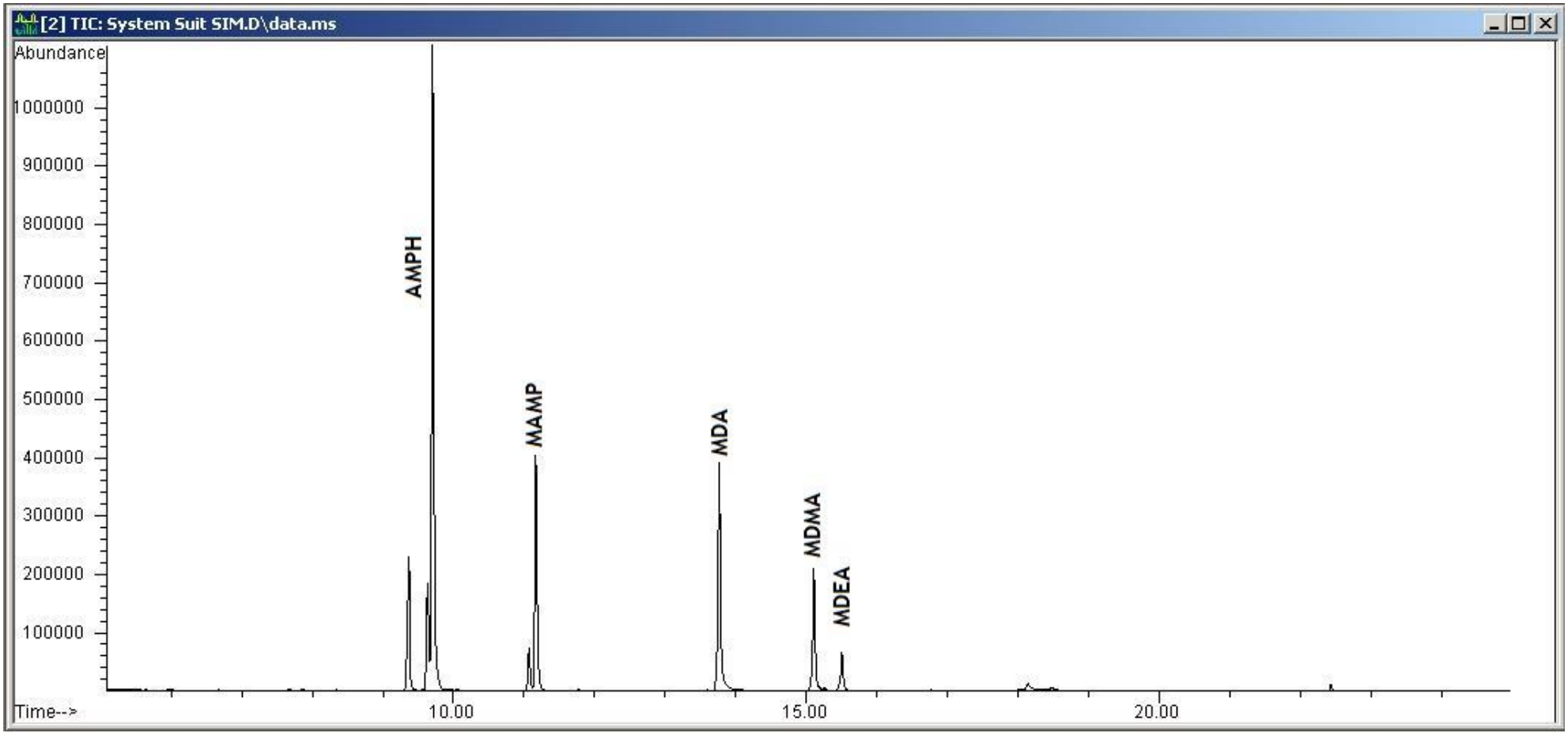

Figure 2-6 Example of TIC for Calibrator 7 (200 ng/0.7 mL of oral fluid)

2.5.3 Drug Recovery from Collection Devices

The recoveries obtained from the NeoSAL™ and Intercept® devices, which were dipped into spiked oral fluid and those where spiked oral fluid was pipetted directly on to the collection pad are shown in Table 2-7 (Intercept®) and Table 2-8 (NeoSAL™).

Table 2-7 Recovery from Intercept® *i2*™ Device (*n* = 4)

Analyte	Conc. (ng/0.7 mL oral fluid)	Recovery (%) (%CV)			
		Dipped		Pipetted	
		Overall	Pad	Overall	Pad
AMP	30	81 (5.1)	80 (2.2)	72 (4.4)	64 (1.7)
	100	71 (7.2)	75 (1.8)	63 (7.8)	63 (4.2)
MAMP	30	72 (3.1)	72 (3.1)	63 (1.6)	59 (4.6)
	100	69 (2.9)	73 (3.8)	60 (2.2)	61 (9.1)
MDA	30	72 (4.4)	81 (4.4)	57 (8.7)	53 (10.5)
	100	63 (4.8)	67 (7.1)	51 (12.1)	54 (4.7)
MDMA	30	30 (13.0)	33 (13.2)	88 (1.5)	85 (6.0)
	100	80 (3.3)	79 (9.4)	67 (1.6)	70 (6.9)
MDEA	30	44 (7.4)	48 (8.6)	64 (3.4)	50 (7.5)
	100	89 (2.1)	39 (4.8)	69 (1.7)	69 (4.5)

Recoveries for both devices where spiked oral fluid was pipetted onto the collection pad were generally lower than those where the collection pad was dipped into the spiked oral fluid. This can be explained by the limited sample volume (0.7 mL or 1 mL for the NeoSAL™ and Intercept® devices, respectively) that is pipetted onto the pad, compared to the potential excess volume of sample taken up by the pad, as was shown is likely to happen in Section 2.5.1. Recoveries for the Intercept® device are lower for amphetamine than the reported recovery using the Intercept® device of 103.1% (2.7 RSD) (14). This may be caused by being unable to follow manufacturer instructions with regards to sample preparation steps with regards to not being able to centrifuge the collection tubes to recover the full volume of buffer/oral fluid mixture.

Table 2-8 Recovery of amphetamine and methamphetamines from the NeoSAL™ device (*n* = 4)

Analyte	Conc. (ng/0.7 mL oral fluid)	Recovery (%) (%CV)			
		Dipped		Pipetted	
		Overall	Pad	Overall	Pad
AMP	30	78 (1.7)	77 (2.1)	64 (7.0)	63 (3.2)
	100	80 (7.1)	82 (3.8)	74 (7.7)	71 (5.2)
MAMP	30	79 (7.2)	78 (9.6)	72 (6.2)	69 (1.8)
	100	81 (8.4)	84 (4.4)	79 (4.2)	78 (9.4)
MDA	30	72 (4.6)	76 (12.1)	81 (7.6)	74 (4.)
	100	78 (4.2)	75 (4.1)	74 (2.1)	72 (4)
MDMA	30	72 (3.3)	74 (9.0)	69 (3.8)	64 (7)
	100	79 (7.1)	80 (1.9)	73 (3.6)	71 (6)
MDEA	30	69 (8.5)	70 (6.5)	66 (6.4)	62 (4)
	100	71 (1.1)	77 (7.4)	64 (12.1)	68 (2)

Recoveries obtained from the NeoSAL™ device, as shown in Table 2-8 were higher than those from the Intercept® device, shown in Table 2-7. One of the explanations for this may be that the sample preparation guidelines recommended by OraSure® were not followed as the required centrifuge buckets were not available. Therefore, some oral fluid and analytes may have been lost pre-extraction in the sample preparation step. Furthermore, no investigations into whether the saliva-stimulating products on the pad cause any interference have been carried out.

2.5.4 Calibration Model and Linearity

A linear calibration was found for all drugs, with a minimum R^2 value of 0.99, for both calibration assessments - with and without internal standard. An 8 point calibration curve (2.0 – 200.0 ng per 0.7 mL of oral fluid) was determined to be acceptable with an R^2 value of a minimum of 0.99. All calibration ranges were linear and no weighting was required. To assess the actual linearity of the calibration ranges, residual plots were created. These are shown in Figure 2-7. Although slight trends could be identified, the residuals are random enough to assume a linear regression is best suited. Results of the calibration model including internal standards are shown in Table 2-9 and linearity results for calibrations excluding internal standards are summarised in Table 2-10.

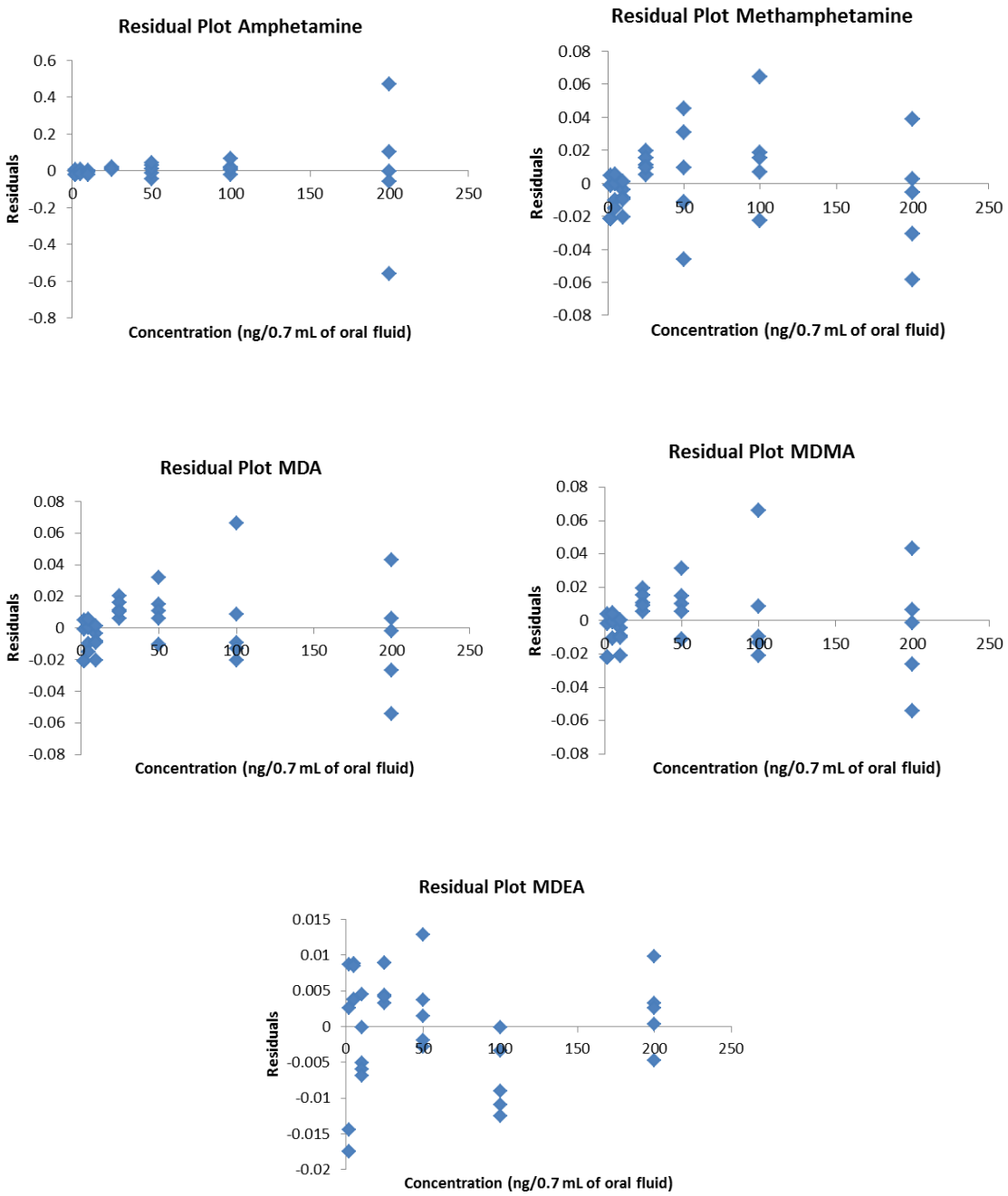

Figure 2-7 Residual plots for amphetamine, methamphetamine, MDA, MDMA, and MDEA

Table 2-9 Calibration model and linearity for amphetamine and methamphetamines with internal standard

Drug	Calibration Range (ng/0.7 mL of oral fluid)	Mean R^2 (n = 5)	%CV
AMP	2 - 200	0.9992	0.38
MAMP	2 - 200	0.9994	0.05
MDA	2 - 200	0.9995	0.07
MDMA	2 - 200	0.9995	0.05
MDEA	2 - 200	0.9990	0.10

Figure 2-8 shows example calibration graphs for all analytes.

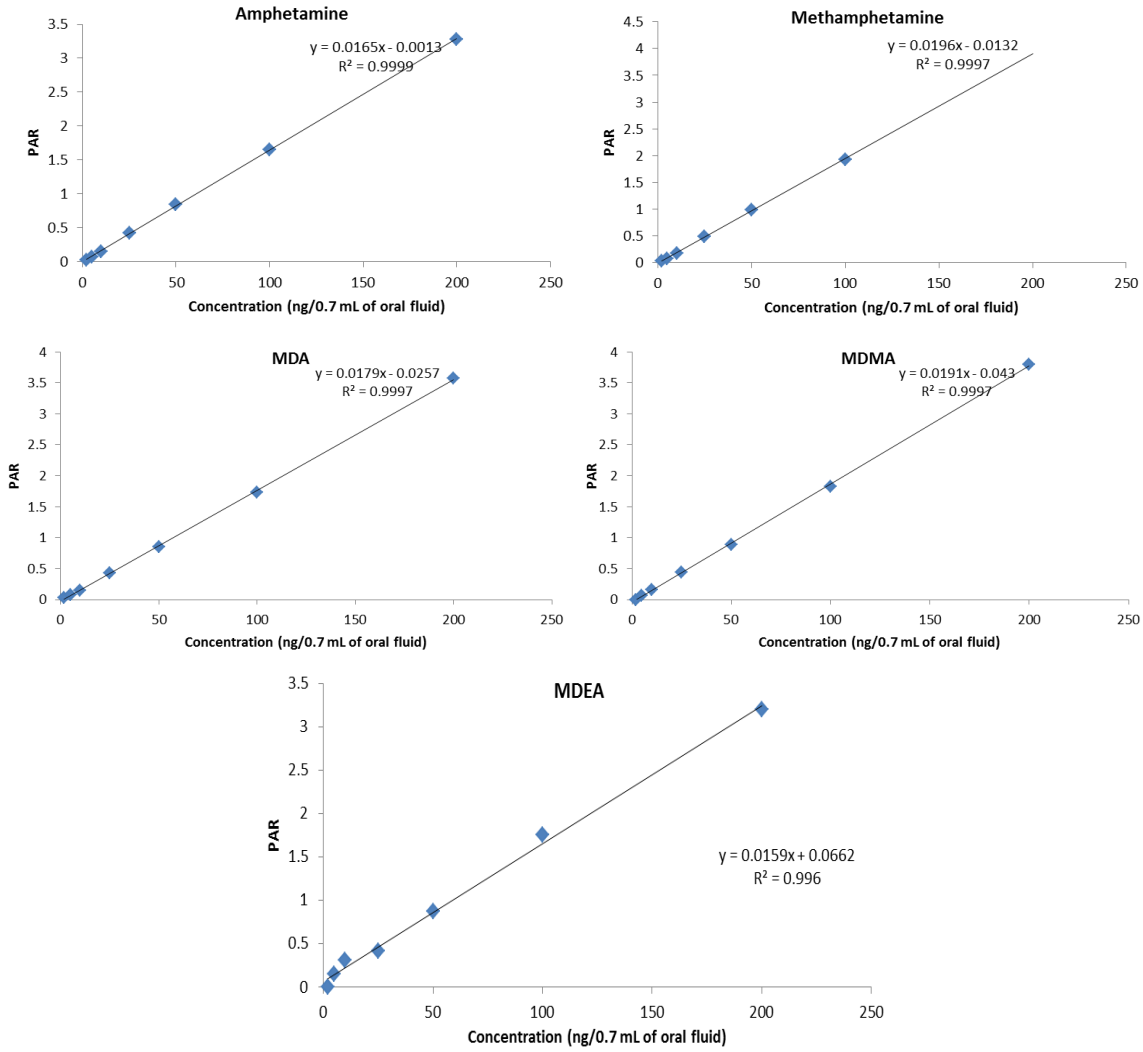

Figure 2-8 Example calibration graphs for amphetamine, methamphetamine, MDA, MDMA, and MDEA with internal standard

Table 2-10 Calibration model and linearity for amphetamine and methamphetamines without internal standard

Drug	Calibration Range (ng/0.7 mL of oral fluid)	Mean R^2 (n = 5)	%CV
AMP	2 - 200	0.9990	0.09
MAMP	2 – 200	0.9990	0.11
MDA	2 – 200	0.9989	0.04
MDMA	2 - 200	0.9983	0.16
MDEA	2 - 200	0.9981	0.16

Figure 2-9 shows example calibration graphs for all analytes.

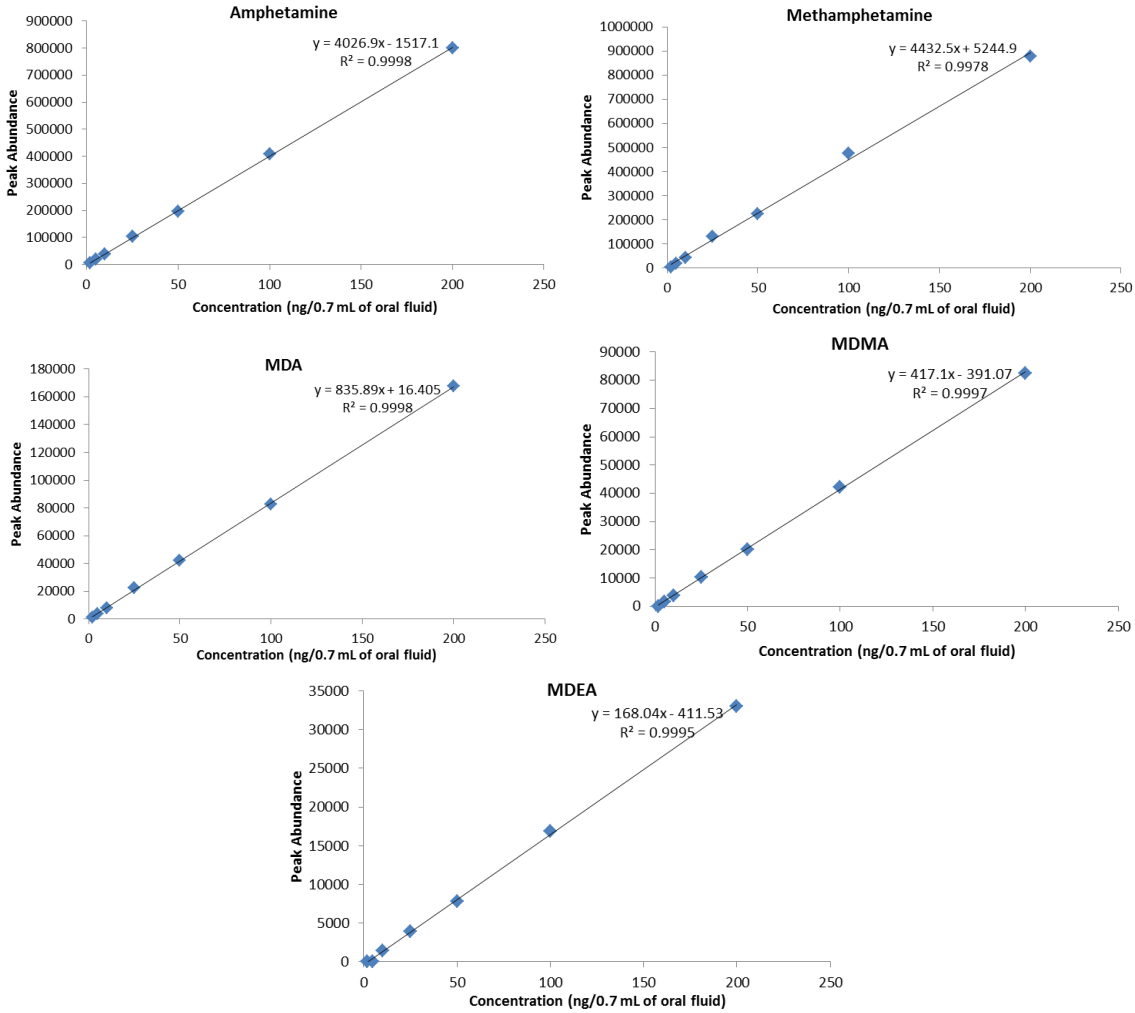

Figure 2-9 Example calibration graphs for amphetamine, methamphetamine, MDA, MDMA, and MDEA without internal standard

2.5.5 Limits of Detection and Quantitation

Both instrument and assay LOD were assessed, and the results are presented in Table 2-11. The LLOQ was based on the lowest calibration standard, as lower concentrations were not reproducible over the three consecutive runs required by SWGTOX for LOQ determination.

Table 2-11 Instrument and Assay LOD, and LLOQ for amphetamine and methamphetamines

Drug	Instrument LOD (ng/mL)	Assay LOD (ng/0.7 mL of oral fluid)	LLOQ (ng/0.7 mL of oral fluid)
AMP	0.5	0.5	2.0
MAMP	0.5	0.5	2.0
MDA	0.5	0.5	2.0
MDMA	0.5	0.5	2.0
MDEA	0.5	0.75	2.0

2.5.6 Interference from Common Drugs

Visual examination of the spectra produced by commonly encountered drugs of abuse in neat oral fluid, unextracted and spiked into NeoSAL™ buffer did not show any potential interference. Peaks and common drugs of abuse did not elute at the retention times set for the analytes. Therefore, no interferences were found which would cause a false positive interpretation of the spectra.

2.5.7 Interference from Buffer and Oral Fluid

Visual examination of the spectra produced by blank buffer injected after extraction did not show any potential interference. No peaks were detected for the ions monitored for the analytes, nor did any peaks elute at the retention times set for the analytes. Relative retention times (i.e. the relative retention time of the analyte compared to the retention time of the internal standard) were monitored closely and shifts in retention times were observed for both. Therefore, no interferences were found that would cause false-positive interpretation of the spectra.

2.5.8 Accuracy and Precision

Accuracy and precision results for all five amphetamine and methamphetamine drugs included in the study are shown in the tables below. Intra-day results are summarised in Table 2-12.

Table 2-12 Intra-day accuracy and precision results for amphetamine and methamphetamines

Analyte	QC (ng/mL)	Calc. Conc. (ng/mL; *n* = 5); (StDev))	Accuracy (%) (*n* = 4)	Precision (CV%) (*n* = 4)
AMP	Low (15)	13.6 (1.1)	83 - 103	1.6 – 4.1
	Medium (30)	28.4 (2.5)	84 – 108	2.3 – 3.7
	High (100)	92.2 (5.2)	86 - 100	1.3 – 3.0
MAMP	Low (15)	12.8 (1.3)	74 – 94	2.8 – 3.9
	Medium (30)	26.9 (1.2)	83 - 94	2.5 – 4.0
	High (100)	91.3 (8.5)	80 - 99	1.3 – 4.0
MDA	Low (15)	12.4 (0.4)	80 – 87	2.0 – 4.3
	Medium (30)	27.2 (1.9)	80 - 96	2.6 – 4.0
	High (100)	90.8 (6.2)	83 - 96	1.4 – 4.5
MDMA	Low (15)	12.2 (1.4)	80 - 90	2.5 – 3.8
	Medium (30)	27.5 (1.6)	83 - 95	2.6 – 4.3
	High (100)	87.6 (7.0)	78 – 95	1.0 – 4.5
MDEA	Low (15)	8.5 (6.6)	43 - 90	-29.3 – 3.5
	Medium (30)	18.8 (6.8)	52 – 82	2.2 – 5.0
	High (100)	75.0 (5.6)	68 - 77	3.2 – 4.4

Intra-day accuracy ranged from 43 – 108%. The highest intra-day precision observed was 5% for MDEA, and all other inter-day precision values were less than 4.5%. The lowest inter-day accuracy was recorded for MDEA at 43%. MDEA gave the most inconsistent results and showed most variation of calculated concentrations – an accuracy issue was observed with MDEA, all calculated concentrations were lower than the known concentration. All low QC concentrations were found to be less than ±26% of the nominal concentration. Both accuracy and precision results were highest on the last day of the validation, due to a drop in sensitivity of the analytical method, which affected especially MDEA which had already shown the greatest variability. An intra-day precision of -29.3 was calculated for the low concentration QC on the final day. A drop in accuracy and precision was observed for all analytes but was worst for MDEA.

The same can be said for the inter-day accuracy and precision results, as shown in Table 2-13.

Table 2-13 Inter-day accuracy and precision results for amphetamine and methamphetamines

Drug	QC (ng/mL)	Calc. Conc. (ng/mL; *n* = 5); (StDev))	Mean Accuracy (%) (*n* = 5)	Mean Precision (%CV) (*n* = 5)
AMP	Low (15)	13.6 (1.1)	91	7.9
	Medium (30)	28.4 (2.5)	95	9.0
	High (100)	92.2 (5.2)	92	5.6
MAMP	Low (15)	12.8 (1.3)	85	10.0
	Medium (30)	26.9 (1.2)	90	4.6
	High (100)	91.3 (8.5)	91	9.3
MDA	Low (15)	12.4 (0.4)	83	3.4
	Medium (30)	27.2 (1.9	91	6.9
	High (100)	90.8 (6.2)	91	6.9
MDMA	Low (15)	12.2 (1.4)	81	11.3
	Medium (30)	27.5 (1.6)	92	5.7
	High (100)	87.6 (7.0)	88	7.9
MDEA	Low (15)	8.5 (6.6)	57	77.2
	Medium (30)	18.8 (6.8)	63	36.2
	High (100)	75.0 (5.6)	75	7.4

It is clear again from Table 2-13, that MDEA gives the most variable results. Accuracy was low for the low concentration QC with 57%, especially when compared to the other analytes which, on average, showed a collective mean accuracy of 89%. %CV was ≤11.3% for all analytes and QC concentrations, with the exception of MDEA at the low and medium concentrations.

2.5.9 Carryover

No carryover was observed in the blank oral fluid samples run after two consecutive injections of the highest standard (200 ng/mL) for all the drugs included.

2.5.10 Autosampler Stability

Samples were quantifiable for all of the drugs investigated up to 48 hours on the autosampler. For both the low and high concentrations, nominal concentrations varied from the actual calculated concentrations. Results for MDEA, as it had previously shown to be inaccurate, were excluded from the stability study.

Table 2-14 shows calculated concentrations and the final recoveries for the low concentration (15 ng/mL) QC samples tested over 96 hours. Table 2-15 shows concentrations calculated over the testing period for the high concentration QC (100 ng/mL).

Table 2-14 Autosampler stability over a testing period of 96 hours with internal standard at low concentration (15 ng/mL, *n* = 4)

Drug	Calculated Concentration (ng/0.7 mL oral fluid)					Recovery (%)
	T_o (0 Hrs)	T_1 (24 Hrs)	T_2 (48 Hrs)	T_3 (72 Hrs)	T_4 (96 Hrs)	
AMP	14.9 (100%)	11.2 (75%)	12.4 (84%)	N/A	N/A	84
MAMP	11.3 (100%)	11.1 (98%)	11.1 (98%)	N/A	N/A	98
MDA	11.4 (100%)	12.1 (107%)	13.4 (117%)	N/A	N/A	117
MDMA	11.8 (100%)	11.5 (105%)	12.4 (105%)	N/A	N/A	105

Table 2-15 Autosampler stability over a 96 hour testing period with Internal standard at high concentration (100 ng/mL, *n* = 4)

Drug	Calculated Concentrations (ng/0.7 mL oral fluid)					Recovery (%)
	T_o (0 Hrs)	T_1 (24 Hrs)	T_2 (48 Hrs)	T_3 (72 Hrs)	T_4 (96 Hrs)	
AMP	87.2 (100%)	87.8 (101%)	84.1 (97%)	N/A	N/A	97
MAMP	90.9 (100%)	83.7 (98%)	82.3 (98%)	N/A	N/A	91
MDA	90.7 (100%)	87.1 (96%)	84.8 (94%)	N/A	N/A	94
MDMA	91.1 (100%)	87.3 (96%)	81.9 (90%)	N/A	N/A	90

MDMA showed substantially higher drug concentrations for both high and low QC. This finding has been previously observed (127). From the tables, it is also clear that the use of the peak area ratio (PAR) makes up for the loss of analyte, as the recoveries were more acceptable when internal standard was used compared to when it was not. Although internal standards were used for this study, the actual stability of the deuterated compounds themselves was not studied.

For T_3 (72 hrs) only one out of the four quadruplicates showed a response. Although a response was shown for one of the samples at T = 72 hours, it was excluded from the above data as it was not reproducible. The calculated

concentrations for the one quadruplicate were not particularly low, but as only one response was given, it was excluded as none of the analytes showed a response at 96 hours. Other studies did not find such a reduction in concentration - one study showed no significant degradation of methamphetamine for 70 hours on the autosampler (128).

In a real lab setting, the reconstitution volume must also be taken into account – as this was only 50 µL, it may have evaporated before a reanalysis could take place. This will not have been an issue in this case as it was ensured that sufficient sample was going to be available for the testing period.

2.6 Conclusions

A method has been partially validated according to the SWGTOX guidelines for the use of the NeoSAL™ device for the analysis of oral fluid for amphetamine and methamphetamines.

The drug recovery for these drugs using the NeoSAL™ device was investigated and acceptable drug recoveries from the device were obtained, ranging from 63 – 79% for a spiked drug concentration of 30 ng/mL, and 64 – 84% for a spiked drug concentration of 100 ng/mL. It was observed that recoveries were higher when the collection pad was dipped into spiked oral fluid, compared to the recoveries obtained when the spiked oral fluid was pipetted directly onto the pad. This is likely to be a result of a larger quantity of drug being present on dipped collection pads.

The method was validated for the detection of amphetamine, methamphetamine, MDMA and MDA in oral fluid, even though poor accuracy and precision observed at low concentrations. The LLOQ of the method was determined to be 2 ng/mL which is still 7.5 times lower than the cut-off concentrations recommended by the EWDTS for these drugs. This LLOQ is also lower than those reported by other authors who used GC-MS as analytical method for quantitation of these analytes (112, 121). MDEA was found to be inaccurate and precision results failed, which is why this analyte was excluded from the stability results. Stability results showed that analytes tested appeared relatively stable on the autosampler for up to 48 hours at the two concentrations

investigated. No analytical interferences were found for any of the drugs of abuse, endogenous matrix components, or stabilising buffer were found.

Volume adequacies were also investigated and it was found that the volume collected was greater than the collection volume stated by the manufacturer. Overall, it was found that analytes in processed samples remained stable for at least 48 hours.

2.7 Future Work

Although short-term processed sample stability of amphetamine and methamphetamines was investigated, further investigation into benchtop, fridge and freezer stability (at 21 °C, -20 °C and 4 °C, respectively) should be carried out for this oral fluid device. This is especially important for non-processed samples, so where the oral fluid was collected but the sample not yet prepared for analysis. A longer timeframe for this would be required. A freeze-thaw cycle study would help to mimic the actual transport procedure from sample collection to laboratory analysis. The manufacturers recommend the storage of the collection devices at either in the fridge or at room temperature, but not in the freezer.

Further investigation into the effect of the oral fluid pH is warranted, as although a number of sources were pooled for stability and recovery studies and the pooled sample pH was tested, a study using oral fluid at a range of different pH would be important and useful.

Although the extraction method described above is validated, a further investigation into the applicability of other extraction techniques for this analysis should be carried out. Different types of extraction procedures, as well as different SPE cartridges should be trialled. It would be beneficial to find a faster and more efficient extraction technique as SPE is a laborious methodology.

Finally, the analysis of authentic oral fluid samples from individuals consuming amphetamine or methamphetamines would be beneficial. This would test and validate the method even further. However, amphetamine and

methamphetamines are not commonly abused in Scotland and users are not likely to be seen by drug addiction services, therefore it was not possible to obtain samples for this.

For future work, it would also be recommended and important to weigh collection devices before and after sample collection. This would be helpful when establishing whether the collectors have over- or under-collected.

2.8 Acknowledgements

Oral fluid collection devices were supplied by AgriYork 400 Ltd. Further devices and materials were obtained from Greiner-Bio One directly as well as from OraSure Technologies for the Intercept® *i*2™ device.

3 Method Development and Extraction Optimisation for Benzodiazepines and Opioids from Oral Fluid Using LC-MS/MS

3.1 Introduction

3.1.1 Benzodiazepines

Benzodiazepines are one of the most commonly used group of drugs in Scotland, and were the most commonly seized Class C substances in England and Wales until March 2016 with 1964 seizures[3] (77). For Class C drugs, the penalty for possession can be up to 2 years in prison with an unlimited fine or both, while supply and production of these substances can result in up to 14 years in prison with an unlimited fine or both. Benzodiazepines are commonly prescribed, highly abused, and are usually taken orally, with adult prescription doses of up to 15 - 30 mg of diazepam daily to treat anxiety, 30 – 60 mg of oxazepam daily for anxiety, and 10 – 40 mg of temazepam when treating insomnia short-term (85, 129). Diazepam (prescribed as Valium®) metabolises to oxazepam (medicinally prescribed as Serax®), temazepam (Euhypnos®) and desmethyldiazepam (nordiazepam, nordazepam, DMD) in the body. Structures and metabolic pathways are shown in Figure 3-1.

[3] Drug seizure statistics for the end of 2016 have not been published at the time of writing.

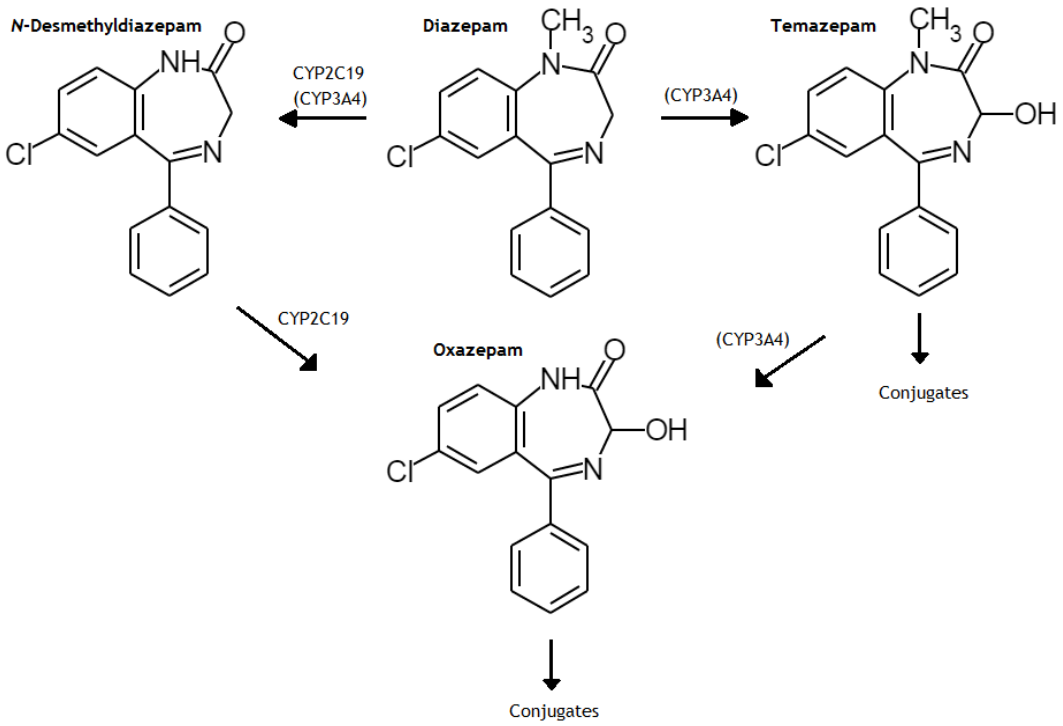

Figure 3-1 Metabolism of diazepam to nordiazepam, temazepam, and oxazepam

Side effects and overdose symptoms of benzodiazepines include drowsiness, lack of coordination, CNS depression (including difficulty breathing), and blurred vision (85).

Therapeutically, these drugs will treat symptoms of anxiety or alcohol withdrawal as benzodiazepines are CNS depressants. For diazepam, nordiazepam, oxazepam, temazepam, and etizolam, the analytes included in the study, adverse effects, or overdose symptoms include extreme sedation and confusion. It is possible for physical dependence to develop.

The chemical structure of etizolam is shown below in Figure 3-2. Etizolam is a derivative of thienotriazolodiazepine, and has been used in Asia and Europe since 1983 (85), but was first reported to the EMCDDA in 2011 (130). Etizolam is a medication that has been prescribed in Japan since 1983 for anxiety and as a strong muscle relaxant. An adult etizolam dose can range from 0.5 to 3 mg per day (in countries where it is prescribed medicinally, not in the UK) (85).

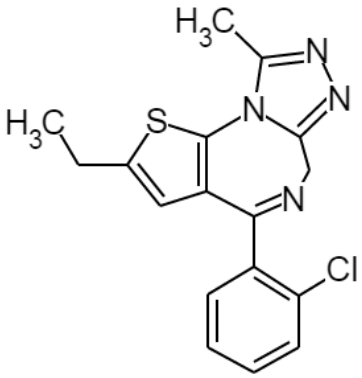

Figure 3-2 Chemical structure of etizolam

Pharmacokinetic properties for diazepam, its metabolites, and etizolam are shown in Table 3-1.

Table 3-1 Pharmacokinetic characteristics and pKa of benzodiazepines included in this study (85)

Parameter	Diazepam	Nordiazpeam	Oxazepam	Temazepam	Etizolam
Volume of Distribution (V_D/ L/kg)	0.7 – 2.6	0.5 – 2.5	0.7 – 1.6	0.8 – 1.0	0.7 – 1.1
Half-Life (h)	21-37	31 – 97	4 – 16	3 – 13	7 – 15
Plasma Protein Binding (Fb)	0.96	0.97	0.87 – 0.94	0.97	0.93
Blood/Plasma Ratio (b/p)	0.6	0.6	0.9 – 1.1	0.5	?
pKa	3.4	3.5 (base) 12.0 (acid)	1.7 (base) 11.6 (acid)	1.3 (base)	2.6 (base)

3.1.1.1 Benzodiazepines in Ante-Mortem Samples

As benzodiazepines are commonly prescribed medicinally these drugs have been extensively studied in oral fluid due to monitoring programmes in clinical settings. In the UK, the Department of Transport has selected blood concentrations, used in England and Wales, which are indicative of impaired driving ability for these analytes. These concentrations are based on studies and concentrations reported by toxicologists. Cut-off concentrations of 0.55, 0.3, and 1 mg/L have been set for diazepam, oxazepam, and temazepam respectively (131). Average blood concentrations in drivers of 1.1 mg/L (*n* = 49) have been found for oxazepam, but concentrations of up to 8 mg/L have been reported (85). Temazepam concentrations ranged between 0.10 – 12 mg/L in 1827 impaired drivers (average 0.66 mg/L) and other reported drug driving cases reported similar results (85). Blood concentrations of drivers arrested for DUID

showed nordiazepam concentrations of up to 6.4 mg/L, but averaged between 0.1 and 1.0 mg/L for several other studies (85). In Scotland, an older study of Strathclyde drivers found blood concentrations of benzodiazepines averaging from 1.9 mg/L for temazepam to 0.7 mg/L for desmethyldiazepam, and 0.8 mg/L for diazepam (132). Between 2012 and 2015, benzodiazepines were found to be positive in 14 out of 118 blood samples of driver and motorcyclist fatalities (133), making benzodiazepines the third most common drug group found in this dataset (following cannabinoids and opiates).

Oral fluid concentrations of benzodiazepines in drivers are infrequently reported. However, over a 24-hour testing period in Glasgow in 2005, benzodiazepines tested positive in 37 (2.7%) of 1396 oral fluid samples. The most common analyte found was temazepam, which was found in 14 cases with a mean concentration detected of 37 ng/mL (median, 14 ng/mL, and range 4 – 189 ng/mL, with an LOD and LOQ of 0.3 and 0.5 ng/mL, respectively). Diazepam, desmethyldiazepam and oxazepam were found in 9, 9, and 5 cases, respectively. Mean concentrations for these three analytes were reported at 15 ng/mL (median 15 ng/mL, range 5 – 28 ng/mL) for diazepam, 46 ng/mL (median 16 ng/mL, range 4 – 221 ng/mL) for desmethyldiazepam, and 14 ng/mL (median 10 ng/mL, range 4 – 33 ng/mL) for oxazepam (134). In Australia, 8% of 853 oral fluid samples tested positive for benzodiazepines, with diazepam the most common analyte (3.5%) (135). This shows that there is evidence of these drugs being taken by drivers who will be in charge of a motor-vehicle and possibly under the influence of drugs.

Studies have found that oral fluid concentrations of benzodiazepines should not be used to estimate benzodiazepine blood concentrations, as the oral fluid to blood concentration ratios are low and show a lot of variation (136). This is an important consideration should oral fluid samples be taken at the roadside in cases of DUID, as the collection of blood is therefore still necessary for an accurate assessment of impairment and accurate drug quantitation, unless oral fluid cut-offs are used. That being said, authors have commented that the detected concentration of diazepam in oral fluid is reflective of the unbound drug in plasma, therefore making oral fluid a good diagnostic tool for TDM (137, 138). However, other authors have commented that urine is the preferred matrix to be tested for cases of TDM as benzodiazepines show low

concentrations in oral fluid due to their chemical nature (139), especially oxazepam (26). Low concentrations observed, for especially diazepam, in oral fluid are due to the weakly acidic (reflected in the high protein binding to albumin Table 3-1) chemical nature of benzodiazepines.

Table 3-2summarises various published methods of analysis of benzodiazepines in oral fluid. Some papers were excluded from the table as not all information regarding LOD/LOQ, analytical technique, extraction technique or recoveries was given.

Table 3-2 Published methods for the detection of benzodiazepines from oral fluid

Reference	Analyte*	Neat/Collector	Recovery	Extraction Technique	Analytical Method	Linear Range (ng/mL)	LOD/LOQ (ng/mL)
Cone, 2007 (140)	DIAZ, DMD, OXA, TEMAZ	Intercept®	N/A	SPE (HCX)	GC-MS-CI	2 – 2000	LOQ: 0.5 for all
Kintz, 2005 (141)	DIAZ, DMD, OXA, TEMAZ	Intercept®	>80%	LLE	LC-MS/MS	0.1 - 20	LOQ 0.1 for all
Badawi, 2009 (142)	DIAZ, DMD, OXA	Neat/StatSure Saliva-Sampler	DIAZ 102%, DMD 103%, OXA 94.8%	SPE (Bond Elut)	LC-MS/MS	0.5 – 100 µg/kg	LOQ: 0.5 µg/kg for all
Wylie, 2005 (143)	DIAZ, DMD, OXA, TEMAZ	Omni-Sal™	DIAZ 65%, DMD 57%, OXA 77%, TEMAZ 109%	SPE (Bond Elut)	LC-MS/MS	5 – 200	LOD: DIAZ 0.3, DMD 0.4, OXA 0.8, TEMAZ 0.5 LOQ: DIAZ 0.9, DMD 1.2, OXA 2.5, TEMAZ 1.6
Quintela, 2005 (144)	DIAZ	Salivette®	72.2%	LLE	LC-MS/MS	0.2 - 25	LOD 0.1
Ngwa, 2007 (145)	DIAZ, DMD, OXA, TEMAZ	Intercept®	DIAZ 94.5%, DMD 90.5%, OXA 99%, TEMAZ 90.5%	SPE (Bond Elut)	LC-MS/MS	0.1 - 20	LOD: 0.05 for all LOQ: 0.1 for all
Moore, 2007 (146)	DIAZ, DMD, OXA, TEMAZ	Quantisal™	DIAZ 82.8%, DMD 83.3%, OXA 64.7%, TEMAZ 84.2%	SPE	LC-MS/MS	0.4 - 50	LOQ: DIAZ 1.0, DMD 0.5, OXA 5.0, TEMAZ 0.5

*** overlapping analytes between this study and the published study. LOD – limit of detection; LOQ – limit of quantitation; N/A – not available**

In a study into the drug recoveries from various oral fluid collectors, good recoveries (>80%) were found for diazepam using the majority of collectors with the exception of two. The Salivette® and the OraTube™ collectors showed poor recoveries of 15.9% and 39.8%, respectively. The reason for the discrepancy between reported Salivette® benzodiazepine recoveries is unclear, but it is possible that the extraction used by Langel (14), was not the most efficient; however the extraction was not specified. The highest recovery (97.1%) however, was observed when oral fluid was expectorated into a plastic tube. Out of the collectors tested, the highest recovery found was 95.7% using the Salicule device (14)[4]. A recovery of >80% for diazepam, desmethyldiazepam, oxazepam and temazepam was found using the Intercept® device (141).

With the emergence and influx of new benzodiazepine drugs, many of which are not medicinally prescribed, it is important to have a method available in which new drugs are included. However, benzodiazepines are also taken with other illicit drugs. Paired oral fluid and urine samples showed a 98.3% correlation in the detection of these analytes, authors highlighted the importance of oral fluid testing for benzodiazepines as they are often taken concurrently with opioid/opiate analytes, such as methadone. In these cases it is especially important to monitor drug consumption as the simultaneous use of benzodiazepines and methadone can increase the likelihood of drug- and highly impaired driving deaths (147).

3.1.2 Opioids

The 4 opioids targeted and included in the study were morphine, codeine, 6-MAM, dihydrocodeine, and methadone. Morphine and methadone are Class A drugs under the Misuse of Drugs Act 1971. Out of all 29,949 Class A drug seizures in England and Wales in 2015/16, morphine was seized 136 times (constituting 0.5% of all seizures, but a reduction from 173 seizures in the previous year) and methadone was seized 547 times (1.8%, down from 686 seizures the year previously) (77). Similarly to the reduction in seizures of morphine and methadone in England and Wales, seizures of methadone decreased in Scotland

[4] The paper describing the recoveries was not included in Table 3-2 as no information pertaining to the analysis was given and it focused on drug recoveries from various collectors rather than the extraction and analysis of benzodiazepines and opioids.

from 2014/15 to 2015/16 as well. In 2015/16, 11 seizures of methadone were made by Police Scotland, where 34 seizures had been made in the year previously. Morphine seizures, on the other hand, increased from 3 to 18 between 2014/15 and 2015/16 (79). For Class A drugs, penalties for possession, supply or production, remain an unlimited fine and/or a prison sentence of up to 7 years for possession, or a lifetime prison sentence for supply and production.

Codeine and dihydrocodeine are both Class B drugs under the Misuse of Drugs Act 1971, and as such, the penalties for possession can incur a prison sentence of up to 5 years, whereas production or supply could incur a prison sentence of up to 14 years. On top of this, an unlimited fine may also be part of the charge. For codeine and dihydrocodeine, as they are mainly prescribed medication, seizure information is not available.

Table 3-3 Pharmacokinetic characteristics and pKa of opioid drugs included in the study (85)

Parameter	Morphine	Codeine	Dihydrocodeine	Methadone
Volume of Distribution (V_D)	2 – 5 L/kg	2.5 – 3.5 L/kg	1.0 – 1.3 L/kg	4 - 7 L/kg
Half-Life	1.3 – 6.7 h	1.2 – 3.9 h	3.4 – 4.5 h	15 – 55 h
Plasma Protein Binding (Fb)	0.35	0.07 – 0.25	0.20	0.87
Blood/Plasma Ratio (b/p)	1.0	0.9	1.0	0.7 – 0.8
pKa	8.21	8.21	14.15 (acid) 9.33 (base)	8.94

All four opioid included in this study are prescription drugs. A normal adult dose of codeine would range from 30 mg to a maximum daily dose of 240 mg, depending on tolerance, and requirement. Codeine is also present in proprietary blends of non-narcotic analgesics and antihistamines or other pain killers (like paracetamol, cocodamol). Dihydrocodeine has a recommended daily limit of 192 mg, whereas maximum dihydrocodeine tartarate doses are 50 mg daily but can be increased to 60 – 120 mg for chronic or severe pain (129). The presence of dihydrocodeine in forensic matrices may however result from hydrocodone metabolism. Methadone is most commonly used in heroin substitution (maintenance) programmes although it was originally synthesised as a morphine substitute. In cases of prior heroin addiction, or developed tolerance, daily doses can be up to 180 mg of methadone, although much lower doses have

caused death in non-tolerant adults. Normal-release doses of morphine in tablet-form range from 15 to 30 mg (85, 129). Side effects and overdose symptoms following opioid ingestion include pin-point pupils, mood swings, confusion, constipation, and breathing problems (85).

Physical dependence on any of these drugs is highly likely. Following random mandatory drug tests of six adult prisons in Scotland between April and September 1997, 226 cases were positive for opioids (148).

A difficulty faced by toxicologists when interpreting detected concentrations of opioid drugs, especially morphine and codeine arises from their metabolic relationships, shown below in Figure 3-3. Diamorphine the active constituent of heroin breaks down in the body very rapidly to give 6-MAM, which rapidly converts to morphine. When heroin is used, codeine is often present at a lower concentration from the presence of acetylcodeine as in impurity in “street” heroin. Codeine breaks down to morphine in the body. Careful interpretation is therefore required when these substances are found in toxicology samples.

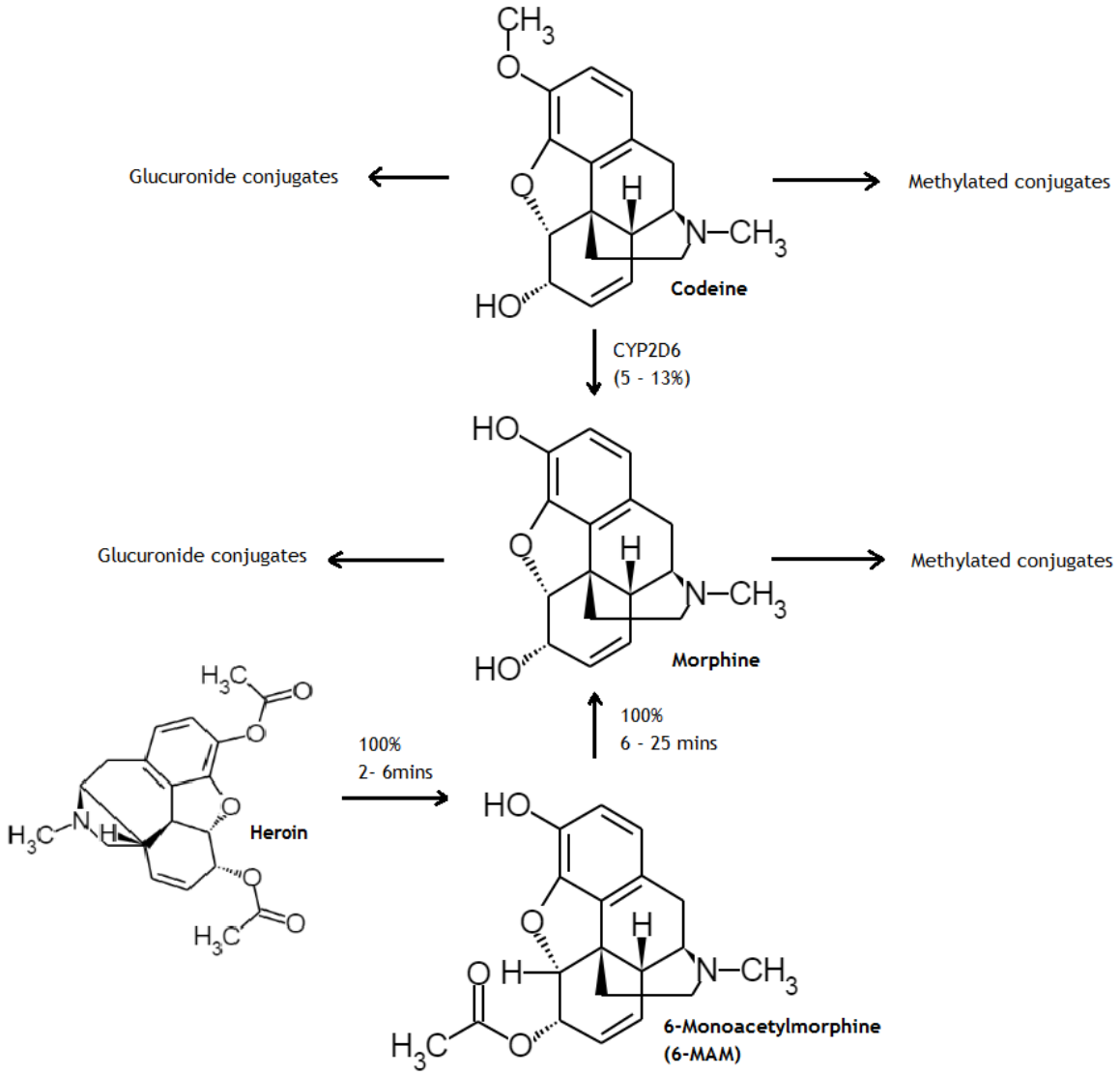

Figure 3-3 Metabolic relations between opioid/opiate analytes

The chemical structures of dihydrocodeine and methadone are shown below in Figure 3-4.

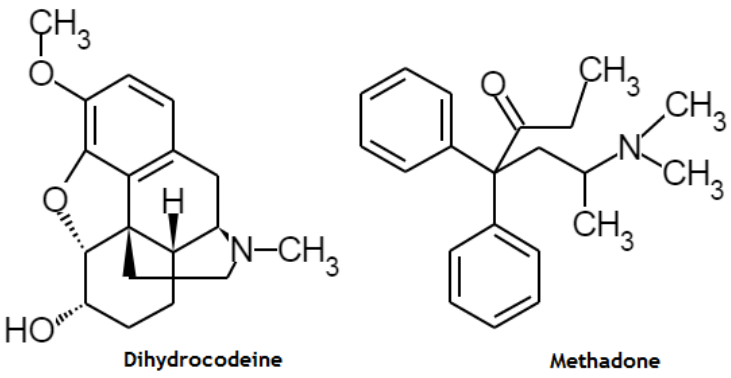

Figure 3-4 Chemical structures of dihydrocodeine and methadone

3.1.2.1 Opioids in Ante-Mortem Cases

Due to their popularity, studies have researched opioid concentrations in DUID cases. Codeine blood concentrations between 2.6 – 7.0 mg/L have been reported for impaired drivers, but lower concentrations have been described (85). Morphine blood concentrations of 0.045 mg/L (range 0.017 – 0.104 mg/L) were found in injured vehicle operators. The cut-off set by the UK Government (England and Wales) for drug impairment for morphine is 0.08 mg/L in blood. A minimum concentration of 0.04 mg/L in blood in healthy volunteers has been shown to impair motor skills. Drivers detained for reckless driving showed blood dihydrocodeine concentrations of 0.04 – 1.0 mg/L (average 0.4 mg/L) in blood. A methadone concentration of 0.5 mg/L (131) in blood is enough to be convicted of DUID. Impaired drivers showed methadone concentrations of 0.26 mg/L and 0.31 mg/L in 114 drivers and 14 drivers, respectively. The Scottish DUID study between 1995 and 1998 by Seymour *et al.*, testing a total of 752 samples, showed that opioids/opiates were used by drivers. Especially methadone, which was found in a total of 35 cases over the testing period, was taken in conjunction with benzodiazepine drugs. The concentrations detected for methadone were within the therapeutic limits of the drug, however it was not known whether apprehended drivers were on a methadone maintenance programme (MMP) and in turn whether the methadone was taken illegally. Morphine was detected in 12 cases (34%) in which methadone also tested positive. Over the testing period, morphine positives became more frequent (from 15 cases in the first year to 38 in the final year of testing). Just as was observed with methadone, benzodiazepine positives were often seen in morphine-positives cases (n = 33) (132). Although this study provides an insight into the use of drugs in drivers, it is out of date and the numbers of cases are not reflective of the actual DUID cases on Scottish roads in 2017. In 118 fatalities on Scottish roads between 2012 and 2015, opiates were found to be the second most common drug group, with 14% of all blood specimens submitted being positive for opiate drugs (133). In this study, codeine was the most commonly found opiate (found in 7.6% of cases).

Opioid quantitation in oral fluid is a little trickier than that of benzodiazepines, due to their low lipid solubility, especially morphine, which has a higher affinity for plasma (Table 3-3). A benefit of oral fluid analysis is that it can be used to

determine recent heroin use as both the parent molecule (diamorphine) as well as its major metabolite 6-monoacetylmorphine (6-MAM) can be present. Due to the very rapid metabolism of diamorphine, it is very uncommon to find the parent molecule in blood or urine; the differentiation between the presence of 6-MAM from heroin use, or as an eventual metabolite of either codeine or morphine, is possible using oral fluid as matrix and is important to forensic toxicology (12, 149).

Reported concentrations of opioid/opiates in oral fluid following DUID arrests are rare, but one study showed 31 codeine oral fluid positives (mean 139 ng/mL, median 50 ng/mL, range 4 – 1504 ng/mL), 17 dihydrocodeine oral fluid positives (mean 371 ng/mL, median 190 ng/mL, range 8 – 1315 ng/mL), 7 morphine positives (mean 1119 ng/mL, median 61 ng/mL, range 9 – 7442 ng/mL) and 6 methadone positives (mean concentration 1578, median 667 ng/mL, and range 8 – 6949 ng/mL) in a total of 1396 submitted oral fluid samples (134). The oral fluid samples were voluntarily donated by drivers stopped at random, so are not biased due to exhibited signs of intoxication. Although this study was conducted over 10 years ago, it does highlight that these analytes were found on the roads in drivers that would have not been stopped if not for the purpose of this study. A slightly more recent study conducted in Australia, showed 14% of drivers stopped at random who provided oral fluid samples for testing were positive for opioid drugs (4.8% for 6-MAM, 3.3% for methadone)(135).

Apart from cases of DUID, reports have suggested that the use of oral fluid in cases of TDM, especially in opioid monitoring, is beneficial as there is a good overlap in detection rates between oral fluid and urine samples (42). Some authors recommend the use of oral fluid over urine (26).

Care must be taken in interpreting codeine and morphine findings, as codeine can break down to morphine. Concentrations between the two analytes were successfully correlated in one study, and morphine was found in 18.4% of samples that tested positive for codeine (150).

A number of studies have published oral fluid methods for these analytes, some of which are summarised below to give an overview of the different extraction and analytical procedures employed by laboratories. Due to the low disposition

of opioids in oral fluid, sensitive techniques like LC-MS/MS are often used for the analyses. Not all papers were included as it was important to included papers with as much analytical information as possible (which is why the study by Langel *et al* (14) regarding drug recoveries from oral fluid collection devices was omitted from Table 3-2 and Table 3-4.

Table 3-4 Published methods of detection of opiate/opioid drugs in oral fluid

Reference	Analyte	Neat/Collector	Recovery	Extraction Technique	Analytical Method	Linear Range (ng/mL)	LOD/LOQ (ng/mL)
Dams, 2007 (151)	6MAM, MOR	Salivette®	75.4% MOR, 83.7% COD, 89.4% 6MAM*	PPT	LC-MS/MS	1 - 500	LOD: 0.25 – 5.0 LOQ: 1.0 – 10.0
Cone, 2007 (140)	6MAM, COD, DHC, MOR	Intercept®	N/A	SPE (HCX)	GC-MS-EI	2 - 2000	LOQ: 2.0 for all
Kim, 2002 (37)	COD, MOR	Neat (citric acid stimulatated) or Salivette®	N/A	SPE (UCT)	GC-MS	1.25 – 1000	LOD 2.5 for all, LOQ 2.5 for all
West, 2017 (150)	COD, MOR	Quantisal™	N/A	LLE	LC-MS/MS	1.0 – 320	LOQ: 1.0 for both
Liu, 2015 (152)	METH, MOR, COD, 6MAM	Neat	N/A	LLE/Direct injection	LC-MS/MS (MOR, COD, 6MAM), GC-MS (METH)	1 - 100	LOD: METH/MOR/6MAM 0.5, COD 1.0 LOQ: METH 0.25, MOR/6MAM 0.5, COD 1.0
Badawi, 2009 (142)	MOR, 6MAM, COD, METH	Neat/StatSure Saliva-Sampler	MOR 119%, 6MAM 108%, COD 102% METH 91.3%	SPE (Bond Elut)	LC-MS/MS	0.5 – 100 µg/kg	LOQ: 0.5 µg/kg for all
Wylie, 2005 (143)	MOR, 6MAM, COD, DHC, METH	Omni-Sal™	MOR 52%, 6MAM 93%, COD 98%, DHC 92%, METH 96%	SPE (Bond Elut)	LC-MS/MS	5 – 200	LOD: MOR 0.5, 6MAM 0.3, COD/DHC 0.5, METH 0.7 LOQ: MOR 1.7, 6MAM 1.0, COD 1.8, DHC 1.7, METH 2.2

*** Average recovery for three tested concentrations. LOD – limit of detection; LOQ – limit of quantitation; SPE – solid phase extraction; LLE – liquid-liquid extraction; PPT – protein precipitation; GC-MS-EI – gas chromatography mass spectrometry with election ionisation; LC-MS/MS – liquid chromatography – tandem mass spectrometry**

If oral fluid is collected using a collection device, drug recoveries from the device must be taken into account. Drug recoveries from the Quantisal® collection device averaged at 82.7% for morphine and 99.7% for codeine (14). The same study found that the Salivette® oral fluid collector gave the lowest recoveries for these two analytes with 35.2% and 39.0% for morphine and codeine, respectively. The other collection devices gave recoveries for both analytes of greater than 76.3%. Some studies have used the Intercept® oral fluid collection device – Langel *et al*. reported drug recoveries of 92.4 mg/L and 116.0 mg/L for morphine and codeine, respectively (14). A further study found mean concentrations of 178.9 ng/mL, 210.5 ng/mL, 1306.2 ng/mL for morphine, codeine, and dihydrocodeine, respectively (140) after oral fluid was collected with the Intercept® device, although no recovery study was carried out.

Several detection techniques are used for the quantitation of opioids, but LC-MS/MS is now one of the most common techniques due to its improved sensitivity compared to GC-MS. SPE is most commonly used in cases where methods are quantitative rather than for screening, as it provides a more rigorous sample clean up.

3.1.3 Aims and Objectives

The aim of this project was to develop an analytical liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the simultaneous analysis and quantitation of opioid and benzodiazepine drugs in both expectorated oral fluid and matrix collected using the Neogen® NeoSAL™ oral fluid collection device. This was done to investigate the suitability of the NeoSAL™ device for the detection of opioids and benzodiazepines.

An extraction procedure had to be developed or chosen and optimised for the simultaneous extraction of diazepam, desmethyldiazepam, etizolam, oxazepam, temazepam, morphine, 6-MAM, codeine, dihydrocodeine and methadone. Tested parameters in the determination process of the most suitable extraction were the overall efficiency of the extraction process, matrix effect evaluation (the chosen extraction had to provide the cleanest extract so as to leave matrix components that could interfere with the detection and quantification), as well as overall drug recovery.

Initial extraction methods tested included published and in-house extractions for the analytes of interest in blood, but also those specifically developed for the analytes in oral fluid.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Drug standards for morphine, 6-MAM, codeine, dihydrocodeine, methadone, diazepam, oxazepam, temazepam, demethyldiazepam and etizolam were obtained from Sigma Aldrich (Gillingham, UK). All certified drug standards were dissolved in MeOH at a concentration of 1 mg/mL.

Deuterated internal standards, morphine-D_5, 6-MAM-D_3, codeine-D_3, dihydrocodeine-D_6, methadone-D_3, diazepam-D_5, oxazepam-D_5, temazepam-D_5, and desmethyldiazepam-D_5, were purchased from Sigma Aldrich (Gillingham, UK). All certified deuterated drug standards were dissolved in MeOH at a concentration of 100 µg/mL. Diazepam-D_5 was used as the internal standard for the quantification of etizolam as this is common procedure at FMS and deuterated etizolam is very costly. Ammonium acetate (HLPC grade), acetic acid, formic acid, ammonium formate, sodium acetate trihydrate and ammonium carbonate were also purchased from Sigma Aldrich (Gillingham, UK).

Concentrated ammonium hydroxide solution (28%), methyl-*tert*-butyl-ether (MTBE), cyclohexane (HPLC grade), MeOH (HPLC grade), EtOAc, acetonitrile (ACN), isopropanol, dichloromethane (DCM), and concentrated hydrochloric acid were all purchased from VWR International Ltd (Lutterworth, UK).

3.2.2 Preparation of Drug and Internal Standard Solutions

3.2.2.1 Preparation of Stock Solutions

Stock solutions were prepared individually in MeOH for all drugs. They were prepared from drug reference standards.

Two solutions were prepared – one for assessment of matrix effects and to make calibrators, and a separate stock solution for the preparation of quality controls.

All stock solutions were prepared at a concentration of 10 µg/mL. For drug standards that came at a concentration of 1 mg/mL, 50 µL was pipetted into a 5 mL volumetric flask and made up to the mark with MeOH, giving a final concentration of 10 µg/mL. The solutions were stored, labelled, in the freezer for up to 6 months.

Deuterated internal standard solutions at initial concentrations of 100 µg/mL were prepared to 10 µg/mL by pipetting 500 µL into a 5 mL volumetric flask and making it up the mark with MeOH. Separate internal standard stock solutions were prepared for each drug. The solutions were stored, labelled, in the freezer for 6 months.

3.2.2.2 Preparation of Internal Standard Solution

For the analysis, an IS working solution with a final concentration of 100 ng/mL was prepared by adding 100 µL of each IS stock solution (10 µg/mL) to a 10 mL volumetric flask and making it up to the mark with MeOH. This solution was also stored in the freezer for up to 6 months.

3.2.2.3 Preparation of Matrix Effect/Unextracted Solution for Extraction Development

Throughout the stages of development and optimisation of the extraction procedure, an unextracted drug solution was prepared, which was also used to test matrix effects. To reconstitute in 250 µL, an unextracted solution was prepared by pipetting 28 µL of each drug and IS stock solution at 10 µg/mL, into a 10 mL volumetric flask, and made up to the mark with deionised water with 0.1% formic acid and 2 mM ammonium acetate (Mobile Phase (A) (MP (A))). This was prepared as and when required.

For the assessment of a reconstitution volume of 500 µL, the unextracted/matrix effects solution was prepared by pipetting 14 µL of each drug and internal standard stock solution (at 10 µg/mL) into a 10 mL volumetric flask and making it up to the mark with MP (A).

Finally, for the assessment of a reconstitution volume of 1000 µL, 7 µL of each drug and internal standard stock solution (10 µg/mL) was pipetted into a 10 mL volumetric flask and made up to the mark with MP (A).

3.2.3 Preparation of Solutions and Reagents

- **2 M Ammonium acetate**

2 M Ammonium acetate is prepared by dissolving 15.4 g of ammonium acetate powder in 100 mL of deionised water. It was stored at room temperature for up to 3 months.

- **2 mM, 3 mM, 4 mM, 5 mM and 10 mM ammonium acetate**

For the method development, different molarities of ammonium acetate were prepared. The volumes of 2M ammonium acetate solution shown in Table 3-5 were added to 500 mL of deionised water.

Table 3-5 Volumes used to prepare various molarities for method validation

Final Molarity	Volume of 2 M Ammonium Acetate (mL)	Final Volume (mL)
2 mM	0.5	500
3 mM	0.75	500
4 mM	1.0	500
5 mM	1.25	500
10 mM	2.5	500

When the most suitable molarity was chosen, the volume was finally doubled, so 1 mL of 2 M ammonium acetate was pipetted into 1 L of deionised water.

- **2 mM ammonium acetate in MeOH**

1 mL of 2 M ammonium acetate was added to a 1 L volumetric flask and made up to the mark with MeOH.

- **10 mM ammonium formate**

0.3154 g of ammonium formate powder were weighed and dissolved in 500 mL of deionised water.

- **Acetate buffer pH 4.5**

2.93 mg of sodium acetate trihydrate were dissolved in 400 mL of deionised water in a 500 mL volumetric flask. To that, 1.62 mL of glacial acetic acid was added and diluted to 500 mL with deionised water.

- **1 M and 0.1 M acetic acid**

To prepare 1 M acetic acid, 28.6 mL of glacial acetic acid were pipetted into a 500 mL volumetric flask and made up to the mark with deionised water. The solution was stored at room temperature (20 °C) for up to 6 months.

To prepare 0.1 M acetic acid, 10 mL of 1 M acetic acid were added to a 100 mL volumetric flask and made up to the mark with deionised water. This solution was stored at room temperature for up to a month.

- **0.1 M phosphate buffer pH 6**

0.1M phosphate buffer was prepared by weighing 1.7 g of Na_2HPO_4 into a 1 L beaker. To that, 12.14 g of $NaH_2PO_4 \cdot H_2O$ was added. The mixture was dissolved in 800 mL of dH_2O. The pH was adjusted to 6 (± 0.1) with 0.1 M monobasic sodium or 0.1 M dibasic sodium to lower or raise the pH, respectively. This was then transferred to a 1 L volumetric flask, then made up to the mark with deionised water and mixed thoroughly. The buffer was then stored at room temperature for a maximum of 6 months.

- **0.1 M hydrochloric acid**

5 mL of 1M hydrochloric acid was pipetted into a 50 mL volumetric flask and made up to the mark with deionised water.

- **1 M sodium hydroxide**

22 g of sodium hydroxide were added to 500 mL of deionised water.

- **0.1 M sodium hydroxide**

5 µL of 1 M sodium hydroxide were added to a 50 mL volumetric flask and made up to the mark with deionised water.

- **0.01 M sodium hydroxide**

500 µL of 0.1 M sodium hydroxide solution was pipetted into a 5 mL volumetric flask and made up to the mark with deionised water.

- **Reconstitution solution**

100 mL of reconstitution solution was prepared by transferring 2.5 mL of MeOH to a 100 mL volumetric flask. This was made up to the mark with deionised water. To this, 100 µL of 2 M ammonium acetate and 100 µL of formic acid were added.

- **EtOAc with 2% ammonium hydroxide**

98 mL of EtOAc were measured out in a measuring cylinder. To this, 2 mL of concentrated ammonium hydroxide was added. The solution was prepared freshly as required.

- **Dichloromethane:isopropanol:ammonium hydroxide (DCM:IPA:NH_3; 78:20:2 w/v)**

This elution solution was prepared by adding 2 mL of ammonium hydroxide to 20 mL of isopropanol. This was added to 78 mL of dichloromethane. The solution was sonicated for at least 5 minutes and was prepared fresh daily, as required.

- **MeOH with 2% ammonium hydroxide**

To 98 mL of MeOH, 2 mL concentrated ammonium was added.

- **Acetone:dichloromethane (1:1 v/v)**

Proportionate volumes of acetone and DCM were added to a measuring cylinder, always dependent on the volume required for the extraction. Usually 100 mL was prepared, by combining 50 mL of acetone and 50 mL of DCM. The solution was mixed and sonicated, and clearly labelled.

- **1 M Ammonium carbonate ($(NH_4)_2CO_3$)**

50 mL of 1 M ammonium carbonate ($(NH_4)_2CO_3$) was prepared by dissolving 4.8 g of ammonium carbonate salt powder was dissolved in 48.76 mL of deionised water. 4.8 g of salt was transferred into a 50 mL volumetric flask and made up to the mark with deionised water.

- **0.2 M Ammonium carbonate ($(NH_4)_2CO_3$)**

Using the 1 M ammonium carbonate solution prepared, a 0.2 M ammonium carbonate solution was prepared to be used for extraction purposes. 10 mL of 1 M ammonium carbonate solution was transferred into a 50 mL volumetric flask and made up to the mark with deionised water. The solution was shaken and sonicated, and was stored at room temperature for up to 1 year.

- **EtOAc:heptane (4:1 v/v)**

To prepare EtOAc:heptane (4:1), four parts of EtOAc were added to 1 part heptane. The solution was prepared at the required volume as required.

- **Formic acid (2M, 1.25M, 1M and 0.5M)**

Formic acid was prepared at various concentrations. 2 M formic acid was prepared by pipetting 762 µL of formic acid into a 10 mL volumetric flask filled with approximately 2.5 mL of deionised water. This was mixed and finally made up the 10 mL mark with deionised water.

1.25M formic acid was prepared by diluting 4.96 mL of formic acid in approximately 25 mL of deionised water in a 100 mL volumetric flask before being made up the 100 mL mark with further deionised water.

From the 1.25 M formic acid solution, 1 M and 0.5 M formic acid was prepared by dilution. 1M formic acid was prepared by diluting 40 mL of 1.25 M formic acid to 50 mL with deionised water. 10 mL of this solution was diluted to 20 mL to give a final molarity of 0.5 M formic acid.

- **Mobile Phase (A)**

Mobile phase (A) was prepared by adding 1000 µL of formic acid (99 – 100%) and 1000 µL of 2M ammonium acetate to a 1 L measuring cylinder and making up to the mark with deionised water. The solution was sonicated for 15 minutes before being used on the LC-MS/MS.

- **Mobile Phase (B)**

Mobile phase (B) was prepared by adding 1000 µL of formic acid (99 – 100%) and 1000 µL of 2 M ammonium acetate to a 1 L measuring cylinder and made up to the mark with MeOH. The solution was sonicated for 15 minutes before being used on the LC-MS/MS.

3.2.4 Oral Fluid Collection

Oral fluid was collected using Neogen® NeoSAL™ oral fluid collection devices (hereafter referred to as “buffered oral fluid samples” or “buffered oral fluid”), or expectorated directly into a polypropylene (PP) collection tube (hereafter referred to as “neat oral fluid” or “expectorated oral fluid”). Expectorated oral fluid samples were frozen and defrosted before use. This was done as previously outlined to ensure the homogeneity of the samples, prevent bacterial growth, and allow for the precipitation of mucins present in the specimen (125).

3.2.5 SPE Cartridges

SPE cartridges used are outlined in Table 3-6. United Chemical Technologies (UCT) cartridges were obtained from Chromatography Direct Ltd (Runcorn, UK)

or supplied by UCT (PA, USA). Agilent Bond Elut Certify LRC cartridges were supplied by Crawford Scientific (Strathaven, UK). Biotage and Waters extraction cartridges were manufacturer samples sent for evaluation, free of charge.

The table below summarises cartridge chemistry and manufacturers of the columns tested (Table 3-6). All cartridges were mixed-mode as this can be beneficial to a large range of compounds.

Table 3-6 SPE cartridges tested

Man.	Name	Chemical Phase	Average pore size (Å)	Pore Volume (cm^3/g)	Surface Area (m^2/g)	Sorbent Amount (mg)	Tube Size (mL)
UCT	Clean Screen® DAU	C8 + BCX (Benzene-sulfonic Acid)	60	0.77	500	200	10
	Clean Screen Xcel® I	C8 + SCX	60	0.77	500	130	6
	XtrackT® DAU	C8 + Ion Exchange (Benzene-sulfonic Acid)	60	0.77	500	200	6
Agilent	Bond Elut Certify® LCR	C8 + SCX	71	N/A	500	130	10
Waters	Oasis® MCX	Mixed mode, reverse-phase strong cation-exchange	30 µm particle size	N/A	N/A	30	1
	Oasis® PRiME HLB	Hydrophilic-lipophilic balanced copolymer	N/A	N/A	N/A	30	1
Biotage	Isolute® HCX-5	Mixed-mode C4 and strong cation exchange (SO_3^-)	54 (particle diameter 55 µm)	N/A	521	130	10

Man. – Manufacturer; BCX - Benzenesulfonic Acid, SCX - Strong Cation Exchanger; LCR - Large Reservoir Capacity; N/A – not available

3.2.6 Instrumentation

An Agilent 1260 Infinity LC-MS/MS triple quadrupole mass spectrometer system with an electron ionisation (ESI) source was used. The Agilent 1260 Infinity series degasser was connected to the Agilent 1260 Infinity series binary pump which was coupled with an Agilent 1260 Infinity series Autosampler SL and an Agilent 1260 Infinity series Thermostated Column Compartment (TCC). A Phenomenex

Gemini® column 5 µm C18 110 Å (150 x 2.1 mm) with Phenomenex Gemini® C18 guard column (40 x 2.0 mm) was used.

Both positive and negative ionisation modes were possible using the turbo ion-spray interface however for this study only positive ionisation was used. Nitrogen gas was used as the collision gas. Data analysis and processing as well as system control was done using the preinstalled Agilent MassHunter Workstation software (B.01.05) – Mass Hunter Software Workstation Software Quantitative Analysis Version B.05.02 Build 5.2.365.0 for QQQ and Mass Hunter Workstation Software Qualitative Analysis Version B.05.00 Build 5.0.519.13 Service Pack 1. Table 3-7 (LC) and Table 3-8 (MS) summarises the initial parameters used for the method development.

Table 3-7 Summary of LC parameters used for first development experiments

LC Parameters	
Column	Phenomenex Gemini® 5 µm C18 110 Å (150 x 2 mm) with Phenomenex Gemini® C18 guard column (40 x 2.0 mm)
Mobile Phase	2 mM ammonium acetate in water/2 mM ammonium acetate in MeOH (50:50)
Column Temperature	40 °C
Flow Rate	0.3 mL/min

Table 3-8 Summary of MS parameters used for method development

MS Parameters	
Operating Mode	Positive ionisation mode
Nebulizer Pressure	15 psi
Gas Flow	11 L/min
Gas Temperature	300 °C
Capillary Voltage	4000 V

All samples were shaken using the IKA Vibrax® VXR basic optoelectronically controlled shaker.

3.2.6.1 Operation Mode: MRM vs. DMRM

Using multiple reaction monitoring (MRM or DMRM – conventional multiple reaction monitoring) for LC-MS/MS is the way of monitoring the fragmentation of analyte precursor ions for targeted product ion identification and subsequent quantitation.

However, once the transitions to be monitored are chosen, peak shapes and points across a peak must be measured. In some instances, the desired sensitivity will not be achievable using MRM. When this is the case, dynamic multiple reaction monitoring (DMRM) can be used. For this, selected retention windows are specifically selected in which a specific ion transition is monitored. The time windows are based on the previously identified retention times of the analytes in question. By monitoring specific retention windows for the analytes, the number of MRM transitions is decreased thereby increasing the sensitivity and accuracy of the detection as both cycle and dwell times can be optimised for the best possible run (153).

MRM transitions were originally used throughout the development of the liquid chromatographic and mass spectrometric methods. One these were optimised, and the retention times of each analyte were known, it was possible to switch the method to DMRM by assigning time windows to each ion transition.

3.2.7 Optimisation of Mass Spectrometric Parameters

The initial step to begin method development was the post-column infusion of drugs at a concentration of 0.5 µg/mL. Previously, 1 µg/mL were infused, however the abundances of the majority of the peaks were too high so the infusion solutions were diluted further to give a final concentration of approximately 0.5 µg/mL.

3.2.7.1 Fragmentor Voltage; Fragmentation and Collision Energy

The fragmentor voltage, parent and product ions, and subsequently the collision energies for each individual analyte were determined using the Agilent MassHunter Workstation Acquisition – Data Acquisition for Triple Quad B.04.01 (B4114 SP6). Post-column infusion of an infusion solution for each analyte and internal standard was carried out using ESI, using positive ionisation mode.

3.2.7.2 Nebuliser Gas Pressure

Four different nebulizer gas pressures were investigated: 10, 15, 20 and 30 psi. Most commonly, the nebulizer pressure is set at 30 psi; however it is a parameter that can be altered to achieve better abundances.

3.2.7.3 Gas Flow

The gas flow is often set to 11 L/min, however it can be set at 6 L/min. These two flows were investigated.

3.2.7.4 Gas Temperature

Gas temperatures were investigated by changing the temperature in the system control centre of the Agilent MassHunter Workstation Acquisition – Data Acquisition for Triple Quad B.04.01 (B4114 SP6). Often the temperature of the gas is set at 350 °C; however, temperatures can range from 250 - 350 °C. Therefore it was decided to investigate the effect of temperatures of 250, 300, 325, and 350 °C on the abundances of each analyte.

3.2.7.5 Injection Volume

The injection volume can be altered in order to increase the quantity of the analytes going into the mass spectrometer. This can aid with the method's sensitivity. For this, injection volumes of 10, 20 and 30 µL were assessed.

3.2.7.6 Column Temperature

Column temperature, as well as stationary phase make up can affect the resolution as well as the separation of analytes. Column temperatures of 25, 40, and 50 °C were investigated.

3.2.8 Optimisation of Liquid Chromatography Parameters

3.2.8.1 Aqueous Phase Additives

Chromatography has been reported to improve with the addition of volatile buffers, which help the separation of molecules, as well as the peak shape of the chromatograms. They can also stabilise the pH of the mobile phase which in turn help develop reproducible chromatography. MeOH was used as the organic phase for all of the investigations.

For this investigation, the addition of 1000 µL of each 10 mM ammonium acetate and 10 mM ammonium formate, to both mobile phases (aqueous and organic) were investigated. Following initial tests using an isocratic mobile phase of 50%

MP (A) to 50% MP (B) was used as this gave the best retention and chromatography for all analytes under investigation.

3.2.8.2 Molarity of Ammonium Acetate

Reports published in the literature use a range of molarities, and therefore it was important to determine which molarity worked best for this particular investigation. The molarity of the aqueous phase additive was investigated following the determination of which additive would give the best overall chromatography. The molarities investigated were: 2, 3, 4, 5, and 10 mM of ammonium acetate. 1000 µL were added to each 1 L mobile phase.

3.2.8.3 Formic Acid addition

Organic acids, which are volatile, are often added to mobile phases for the use in HPLC systems as they aid the chromatographic resolution. The use of formic acid is common in LC-MS/MS methods. Formic acid also acts as a stabilising buffer and will evaporate readily in the LC interphase, which a phosphate buffer would not. Due to its nature it also donates protons during positive ionisation mode, as well as not suppressing ionisation in the mass spectrometer (154).

In order to determine which concentration of formic acid gave the best results, three concentrations were investigated. These were: 0.001%, 0.01% and 0.1%, by adding different volumes of formic acid: 10 µL in 1 L to give 0.001%, 100 µL in 1 L to give 0.01%, and 1000 µL in 1 L to give 0.1%.

3.2.8.4 Isocratic Elution vs. Gradient Elution

Initially, an isocratic elution at 50:50 aqueous to organic phase was used. This gave enough peak height, and using the ion transitions it was possible to use this phase ratio to investigate the other parameters of the method. By changing the aqueous and organic phase percentage ratios, the best possible chromatographic separation was achieved. The different gradient systems tested are summarised below in Table 3-9.

Table 3-9 Gradient profiles tested

Time (min)	System 1		Time (min)	System 2		System 3	
	MP(A)%	MP(B)%		MP(A)%	MP(B)%	MP(A)%	MP(B)%
0 - 8	95	8.00	**8.00**	95	5	97.5	2.5
8.00	95	9.00	**9.00**	40	60	40	60
9.00	40	15.00	**15.00**	40	60	40	60
25.00	40	15.01	**15.01**	20	80	20	80
25.01	95	20.00	**20.00**	20	80	20	80
30.00	95	20.01	**20.01**	95	5	97.5	2.5
			25.00	95	5	97.5	2.5

3.2.9 Optimisation of Extraction Procedure

In order to provide the cleanest and most suitable extract to introduce to the analytical method developed, different extraction procedures were assessed.

Due to the complex nature of the matrix, an efficient sample clean-up and extraction is required in order to achieve the best possible drug recovery. SPE, liquid-liquid extraction (LLE) and supported liquid extraction (SLE+) were used. At the same time as investigating the extraction efficiencies as different extraction methods, the Matrix Effect (ME) was also calculated. For evaluation, a non-matrix extracted sample was also prepared.

It is important to investigate ion suppression/enhancements early on in the extraction optimisation in order to ensure sufficient sample clean-up to prevent a unreliable of analytical results (154).

All extractions were assessed for drug recovery, matrix effects, and process efficiency at a concentration of 25 ng/mL for all SPE, SLE+, and LLE extractions. The below equations (Equation III, Equation IV, and Equation V) were used to assess process efficiency, matrix effects, and drug recovery, respectively, as per Matuszewski (155).

Equation III Process Efficiency (%) calculation

$$\text{Process Efficiency (\%)} = \left(\frac{\text{PRE}}{\text{UES}}\right) \times 100$$

Equation IV Matrix Effect (%) calculation

$$\text{Matrix Effect (\%)} = \left(\frac{\text{POST}}{\text{UES}}\right) \times 100$$

Equation V Recovery (%) calculation

$$\text{Recovery (\%)} = \left(\frac{\text{PRE}}{\text{POST}}\right) \times 100$$

Where PRE = peak area of analyte added before extraction, POST = peak area of analyte added after extraction, and UES = peak area of analyte without extraction.

Three types of samples were prepared in duplicate. Samples had working solutions and internal standard solutions added before ("PRE") or after ("POST") extraction. An unextracted standard ("UES") was also analysed in duplicate. In the case of post-extraction addition of analytes to the sample, to keep the extraction comparable, MeOH was added at a correlating volume (i.e. instead of adding 70 µL of IS solution, 70 µL of MeOH was added to the sample instead and the internal standard was only added post-extraction). This giving a final concentration of 25 ng/mL at which these parameters were investigated initially. Peak areas of "UES", "PRE" and "POST" were used for calculations.

At early stages of the investigation, buffered oral fluid samples (samples collected using the NeoSAL™) were extracted as well. However, the results were not reproducible and the extractions were not giving acceptable results while only using up resources as there was only a limited supply of collection devices. It was therefore decided not to go forward with further extractions of buffered samples until an extraction technique was developed for expectorated oral fluid. Once this has been achieved, the extraction technique was applied to buffered oral fluid samples.

3.2.10 Liquid-Liquid Extraction (LLE) Optimisation

All LLE samples were prepared in duplicate, i.e. PRE samples prepared in duplicate, POST samples prepared in duplicate, and a duplicate set of UES. Extractions were tested at final concentrations of 25 ng/mL of analytes and 10 ng/mL of internal standards.

3.2.10.1 LLE Method 1

The preliminary LLE procedure followed used 100 µL of blank oral fluid matrix spiked with 25 µL of a benzodiazepine drug mixture (1 µg/mL), and 25 µL spiked with the opiate/opioid drug mixture (1 µg/mL). 100 µL of IS (1 µg/mL) was also added to the two sets of extracts – once before the extraction (pre-) and once post-extraction in order to assess process efficiency, matrix effects as well as extraction recoveries (as outlined by Matuszewski (155), Section 3.2.9).

To the spiked oral fluid 200 µL of 0.1 M phosphate buffer (pH 6) or sodium hydroxide at 0.01, 0.001, 0.0001 or 0.00001 M or ammonium hydroxide (1, 5 or 10%) was added. 1 mL of methyl-*tert*-butyl-ether (MTBE) was used as the solvent. After vortexting for 5 minutes, the mixture was micro-centrifuged for 5 minutes at 5000 rpm. Finally, the organic top layer was carefully removed, transferred to an LC vial and the solvent was evaporated under a gentle stream of nitrogen, at less than 40 °C. Reconstitution was performed in 250 µL of 2.5% MeOH in deionised water.

3.2.10.2 LLE Method 2

An extraction technique used prior to a drug screen for 32 drugs, including some of the drugs included in this study, from oral fluid collected using the Intercept® collection device (156) was also tested. This method reported to give good results for many of the analytes included in this study. This was especially interesting as the method was specifically focused on drug extraction from preserved oral fluid. The method was adapted to accommodate an oral fluid volume of 700 µL (as this is the volume of oral fluid that the NeoSAL™ collection device supposedly collects) compared to the 500 µL used in the literature. Expectorated oral fluid was spiked with 70 µL of a drug working solution at 100 ng/mL, and 70 µL of IS mix at the same concentration. 250 µL of 0.2 M ammonium carbonate (pH 9) was added to the oral fluid as a salt. To that, 1.3 mL of solvent mixture (EtOAc:heptane, 4:1 v/v) was added. Samples were then shaken using the previous IKA Vibrax® VXR basic optoelectronically controlled shaker for 5 and 10 minutes at 1500 rpm, and then centrifuged at 3000 rpm for 5 minutes. The organic layer was transferred and evaporated under a gentle

stream of nitrogen gas, heated at 40 °C. 250 µL of reconstitution solution was used.

3.2.10.3 LLE Method 3: Initial Investigation

Following a literature search, the LLE procedure outlined by Liao *et al*. (157) was trialled. The method, although used for the extraction of morphine and codeine from plasma, was adapted to extract the drugs of interest by altering the additives to the extraction to change the pH of the solution in order to aid the ionisation of the drugs and thereby the extraction.

The original procedure that was followed used 700 µL of oral fluid spiked with 70 µL of IS solution at 100 ng/mL (final concentration 10 ng/mL) and 70 µL of drug working solution at 250 µg/mL (final concentration 25 ng/mL). When the extraction was a post-extraction spike, the corresponding volumes of IS and working solution were substituted by MeOH. To this, 50 µL of MeOH and purified water (50:50) were added, as well as 100 µL of 0.1 M sodium hydroxide. 3 mL of EtOAc were used as the extraction solvent. Samples were shaken for 15 minutes, and then centrifuged at 3000 rpm/10 minutes. The upper organic layer was transferred into clean vials, and evaporated under a gentle stream of nitrogen at less than 40 °C. An initial reconstitution volume of 250 µL was used.

Amendments were made to the published method and included adapting the volume of matrix used (500 µL in the original article, 700 µL for this study), and adapting the volume of working solution used to spike the matrix with (50 µL and 70 µL, respectively).

3.2.10.4 LLE Method 3: Molarity Experiments

The ionic strength of the sodium hydroxide solution added to the extraction was investigated by testing 1 M (pH 14), 0.1 M (pH 13), 0.01 M (pH 12), 0.001 M (pH 11), and 0.0001 M (pH 10) sodium hydroxide.

3.2.10.5 LLE Method 3: Reconstitution Volume and Shaking Time Experiments

Reconstitution volumes of 250, 500, and 1000 µL were investigated.

Starting with the shaking time outlined by Liao *et al.* (157) of 15 minutes, different shaking times were tested. These were 5, 10, 15, 20, 25, and 30 minutes.

3.2.10.6 LLE Method 3: Extraction Solvent Experiments

The original published method employed EtOAc as the extraction solvent. However, both MTBE and DCM were also tested as extraction solvents. 3 mL of a combination of EtOAc and heptane (in a ratio of 4:1) were also used to see whether the use of a combination of extraction solvents would prove beneficial to the extraction efficiencies. This particular combination was previously used successfully by Øiestad *et al.* (156).

3.2.11 Solid Phase Extraction Optimisation

For SPE, recovery (%), process efficiency (%), and matrix effect (%) were assessed as was previously done for the LLE procedures. Likewise, all PRE-, POST-, and UES- samples were prepared in duplicate. These were also spiked at a final concentration of 25 ng/mL (using 70 µL of a working solution at 250 ng/mL) and 10 ng/mL of IS (using 70 µL of an IS working solution of 100 ng/mL). Initial assessments were made for only neat oral fluid, and unless otherwise stated, methods were only applied to neat oral fluid.

3.2.11.1 SPE Using UCT Columns

Various SPE procedures were carried out to test their suitability for the extraction of these analytes in oral fluid. The simplest utilised the United Chemical Technologies (UCT) Inc. mixed-mode C18 Clean Screen® DAU cartridges. The procedure followed were the sample extraction procedures for basic analytes from blood, serum, urine, and tissue samples and were adapted for the extraction from oral fluid, as well as procedures developed by UCT.

The SPE cartridges themselves were selected for their ease of use. Clean Screen® DAU cartridges are the most commonly applied SPE cartridges as they are suitable to extract a large set of chemically different analytes. Furthermore, based on the increased viscosity of oral fluid samples (compared to more common matrices, like blood or urine), XtrackT® DAU cartridges were included

in the comparison. These contain the same sorbent chemistry with a slightly larger particle size, which aids the flow rate of sample through the sorbent thereby theoretically increasing extraction efficiency.

Both of these cartridges were tested using the in-house methodology for the extraction of both opioids/opiates and benzodiazepines from oral fluid, as well as the UCT method of extraction of benzodiazepines (and designer benzodiazepines) from oral fluid. The previous method was adapted in the sense that the analysis would have been carried out on GC-MS, but the final derivitisation step was foregone for a reconstitution step in mobile phase for injection into the LC-MS/MS instead.

Upon recommendation from UCT representatives, Clean Screen® Xcel I cartridges were also tested as these, although again similar in sorbent chemistry, do not need any preconditioning (the sample can be directly applied to the column) which makes for a speedier extraction.

The extraction procedures were also adapted to ensure that the most suitable eluting agent was used. The use of an alkaline elution solvent is recommended for basic drugs as this breaks any ionic bonds binding the drug molecules to the sorbent beds.

Table 3-10 summarises the extraction procedures followed for the optimisation steps. The procedure followed for the XtrackT® DAU cartridge was the same procedure as for the CleanScreen® DAU cartridges, as it is the same sorbent phase.

Table 3-10 UCT CleanScreen SPE extraction procedures tested

Extraction Procedure	UCT1	UCT2	UCT3	UCT4	UCT5	UCT6	UCT7	UCT8
	UCT BZD	**UCT BZD**	**FMS Opiates (inc Methadone)**	**Miller *et al*. (2008) (158)**	**FMS BDZ**	**UCT BDZ with adapted elution**	**UCT BDZ with seq. elution**	**N/A**
Analytes of Interest specified	BZD	BZD	Opiates/Opioids	BZD & Opiates	BZD	BZD	BZD	BZD/Opioids
Suggested SPE Cartridge	CSDAU106	Xcel® I	ZDAU020	ZDAU020	ZDAU020	CSDAU106	CSDAU106	N/A
SPE Cartridge Used	DAU020 XtrackT DAU	Xcel® I	ZDAU020	ZDAU020	ZDAU020	ZDAU020	ZDAU020	ZDAU020
Condition	3 mL MeOH 3 mL dH_2O 3 mL Buffer	**Not Required**	3 mL MeOH 3 mL dH_2O 1 mL Buffer	3 mL MeOH 3 mL dH_2O 1 mL Buffer	3 mL MeOH 3 mL dH_2O 2 mL Buffer	3 mL MeOH 3 mL dH_2O 3 mL Buffer	3 mL MeOH 3 mL dH_2O 3 mL Buffer	3 mL MeOH 3 mL dH_2O 1 mL Buffer
Load Sample								
Wash	3 mL acetate buffer pH 5 3 mL DCM	3 mL acetate buffer pH 5 3 mL DCM	2 mL dH_2O 2 mL 0.1 M HCl 3 mL MeOH	3 mL Buffer† 1 mL 1.0 M AA	2 mL dH_2O 2 mL Buffer:ACN (80:20 v/v) 2 mL cyclohexane **Dry 1 min** 3 mL dH_2O	3 mL acetate buffer pH 5 3 mL DCM	3 mL acetate buffer pH 4.5 3 mL DCM	3 mL dH_2O 3 mL 0.1 M AA 3 mL MeOH
Dry under high vacuum for 5 mins (*10 mins)								
Elution	3 mL EtOAc:NH_4OH (98:2 v/v)	3 mL EtOAc:NH_4OH (98:2 v/v)	3 mL DCM:IPA:NH_4OH (78:20:2 v/v)	2 mL MeOH:NH_4OH (98:2 v/v)	3 mL EtOAc:NH_4OH (98:2 v/v)	3 mL DCM:IPA:NH_4OH (78:20:2 v/v)	3 mL EtOAc:NH_4OH (98:2 v/v) **Dry 2 mins** 3 mL DCM:IPA:NH_3 (78:20:2 v/v)	3 mL EtOAc:ACN:NH_4OH (20:78:2 v/v)
Comments	*Manufacturer specifies designer BZD*	-	*Extraction from neat oral fluid. GC-MS method*	*Hair Method †0.1M pH 5 phosphate buffer*	*Extraction from oral fluid*	-	*Acetate buffer pH 5 was recommended as wash step*	-

Where dH_2O - deionised water; MeOH – methanol; DCM – dichloromethane; IPA – isopropanol; NH_4OH - ammonium hydroxide solution; EtOAc - ethyl acetate; ACN – acetonitrile; BZD – benzodiazepine drugs; FMS – Forensic Medicine and Science (in-house procedures); AA - acetic acid; seq. – sequential; Buffer – unless otherwise stated, Buffer refers to 0.1M pH 6 phosphate buffer. All UCT SPE cartridges are named "CleanScreen®".

As some of the washing or loading steps were not optimised for both sets of drug groups, both wash steps (and elution steps, if present) were analysed separately in the case of non-retention of the drugs to the sorbent material due to ionisation state. With the intent of finding the analytes in the wash steps, the extraction could then be optimised to efficiently extract both sets of analytes.

The acetate buffer wash step used in the UCT6 procedure was initially carried out with acetate buffer pH 5, however, a lower pH was found to be more efficient. Acetate buffer pH 4.5 was used as pH 4 is usually recommended to prevent the hydrolysis of 6-MAM (159).

3.2.11.2 SPE Using Bond Elut Certify Columns

Table 3-11 is a summary of the extraction procedures tested using the Agilent Bond Elut Certify cartridges. The sorbent bed is slightly different to the UCT CleanScreen® cartridges, and therefore the methodologies differ.

Following initial assessments of the extraction procedure outlined by Wylie *et al*. for concurrent extraction of benzodiazepines and opioids/opiates from oral fluid, recoveries for some analytes (morphine, oxazepam and temazepam especially) were not deemed acceptable for oral fluid collected using the NeoSAL™ collector. Recoveries reported in the original study were acceptable and the sole amendment that was made to the extraction procedure was the substitution of dichloromethane for chloroform in the elution solution. In an attempt to find where the analytes with poor recoveries were being lost, the extraction was carried out with the elimination of each of the wash steps (for example, the addition of 50 µL of MeOH to the extraction cartridges was eliminated but the rest of the procedure was followed as described), but also the elution step using acetone:DCM, as this was not supposed to collect analytes of interest.

Further optimisation attempts included the optimisation of elution solvent. Several solvents were used for elution optimisation: namely, acetone and dichloromethane separately but also combined (1:1), cyclohexane, and an elution with DCM:IPA:NH_4OH.

Table 3-11 Bond Elut Certify SPE extraction procedures tested

Extraction Procedure	BE1	BE2	BE3	BE4	BE5
	Wylie *et al*. (2005)(143)	Agilent for "Basic Drugs"	Agilent for Opiates	Agilent for BZD	Wylie *et al*. (2005)(143)
Analytes of Interest specified	BZD & Opiates	Basic drugs	Opiates in urine	BZD	BZD & Opiates
Suggested SPE Cartridge	Bond Elut Certify® LCR (130 mg/10mL)				
SPE Cartridge Used	Bond Elut Certify® LCR (130 mg/10mL)				
Condition	2 mL MeOH 2 mL Buffer	2 mL MeOH 2 mL dH_2O 1 mL Buffer	2 mL MeOH 2 mL Buffer*	2 mL MeOH 2 mL dH_2O 1 mL Buffer	2 mL MeOH 2 mL Buffer
Load Sample					
Wash	1 mL dH_2O 0.5 mL 0.01 M AA **Dry 10 min** 50 µL MeOH	2 mL dH_2O 2 mL 0.1 M HCl 3 mL MeOH	2 mL dH_2O 2 mL 0.1 M Acetate buffer (pH 4) 2 mL MeOH	2 mL dH_2O 2 mL 20% ACN in Buffer	1 mL dH_2O 0.5 mL 0.01 M AA **Dry 10 min** 50 µL MeOH
Dry under high vacuum for 2 mins (5 mins for Basics & Benzos)					
Elution	4 mL acetone:chloroform (1:1 v/v) 2x 1.5 mL EtOAc:NH_4OH (98:2 v/v)	2 mL MeOH + 2% NH_4OH	2 mL DCM:IPA:NH_3 (78:20:2 v/v)	2 mL hexane† 2 mL DCM:IPA:NH_3 (78:20:2 v/v)	4 mL acetone:DCM (1:1 v/v) DCM:IPA:NH_3 (78:20:2 v/v)
Comments	*Chloroform was substituted with DCM for the elution step*		** 0.1M phosphate buffer pH adjusted to 8 or 9 with 10 M KOH*	*Extraction from urine. Analysis on GC-MS* †not collected	*Adapted elution solution: DCM:IPA:NH_3 (78:20:2 v/v) instead of EtOAc:NH_4OH (98:2 v/v)*

Where dH_2O - deionised water; MeOH – methanol; DCM – dichloromethane; IPA – isopropanol; NH_4OH - ammonium hydroxide solution; EtOAc - ethyl acetate; ACN – acetonitrile; BZD – benzodiazepine drugs; AA - acetic acid; Buffer – unless otherwise stated; Buffer refers to 0.1 M pH 6 phosphate buffer; HCl – hydrochloric acid

Following initial success, this extraction procedure - using the DCM:IPA:NH_4OH in the elution steps – was then applied to oral fluid collected using the NeoSAL™ device. As part of sample preparation, 3.5 mL of 0.1 M pH 6 phosphate buffer was added to neat oral fluid samples, which caused the pH of the spiked oral fluid to remain at pH 6. As the NeoSAL™ buffer contained in the collection device is also pH 6, no extra phosphate buffer was added to samples, similarly to what happened earlier in the UCT extraction procedures. In order to potentially aid the extraction of analytes from the buffer (and in order to break the buffer equilibrium), formic acid at two molarities was used to acidify sample. 1.25, 1, 0.5 , 0.001, and 0.000 1M formic acid (1 and 3.5 mL) or 2% ammonium hydroxide

(1 mL; to basify samples) were added to collected samples and fractions were analysed separately.

3.2.11.3 SPE/SLE Using Biotage and Waters Columns

Finally, Table 3-12, summarises SPE and SLE+ cartridges tested manufactured by both Waters Scientific and Biotage.

Table 3-12 Biotage and Waters SPE extraction procedures tested

Extraction Procedure	Biotage Generic Extraction of Basic Drugs	Biotage SLE+ drugs of abuse panel after collection with Quantisal™ device	Waters Drugs of Abuse in preserved saliva	Waters Oasis® PRiME HLB
Analytes of Interest specified	Basic drugs	Drugs of Abuse panel	Drugs of Abuse	Basic drugs procedure from the Waters Method Development Tool
Suggested Cartridge	Isolute® HCX, HCX-3, HCX-5 (100 mg/1 mL)	Isolute® SLE+ 400 µL	Oasis® MCX (30 mg/1cc)	Oasis® PRiME HLB (30 mg/1cc)
Cartridge Used	Isolute® HCX-5 (130 mg/10 mL)	Isolute® SLE+ 400 µL	Oasis® MCX (30 mg/1cc)	Oasis® PRiME HLB (30 mg/1cc)
Condition	1 mL MeOH 1 mL 0.05 M ammonium acetate buffer pH 6	-	2 mL MeOH 2 mL dH_2O	2 mL MeOH 2 mL dH_2O
Load Sample (let absorb for SLE+)				
Wash	1 mL 0.05 M ammonium acetate buffer pH 6 1 mL 1 M AA **Dry 30 seconds** 1 mL MeOH	-	2 mL 0.1 M HCl 2 mL MeOH	2 mL dH_2O:MeOH (95:5 v/v)
No drying steps				
Elution	1 mL MeOH:NH_4OH (95:5 v/v)	1 mL DCM **Wait 5 mins** 1 mL DCM **Wait 5 mins and let pass through**	3 mL 98mL (ACN:IPA (40:60 v/v)):2 mL NH_4OH	3 mL 98mL (ACN:IPA (40:60 v/v)):2 mL NH_4OH
Comments				*Exhibiting a concentration factor of 1.4*

Where dH_2O - deionised water; MeOH – methanol; DCM – dichloromethane; IPA – isopropanol; NH_4OH - ammonium hydroxide solution; EtOAc - ethyl acetate; ACN – acetonitrile; HCl – hydrochloric acid; AA - acetic acid; Buffer – unless otherwise stated, Buffer refers to 0.1 M pH 6 phosphate buffer.

3.2.12 Sample Reconstitution

To reduce matrix effects, the sample reconstitution volume was investigated. The initial volume of reconstitution solution used was 250 µL. Further reconstitution volumes analytically assessed were 500 and 1000 µL in order to reduce the matrix effects.

3.3 Results and Discussion

3.3.1 Optimisation of Mass Spectrometric Parameters

3.3.1.1 Fragmentor Voltage; Fragmentation and Collision Energy

Table 3-13 summarises the optimum fragmentor voltages, collision energies as well as the ions monitored for each of the analytes.

Table 3-13 Ion transitions monitored for all analytes of interest and internal standards

Compound	Precursor Ion (m/z)	Quantifier Ion (m/z)	Qualifier Ion (m/z)	Frag[A] (V)	CE[B] (V)
6MAM	328.1	211.0	165.0	135	26
Codeine	300.1	215.1	165.0	140	50
DHC	302.1	201.0	199.0	150	32
Diazepam	285.1	193.0	154.0	160	34
DMD	271.0	164.9	139.9	130	32
Etizolam	343.1	314.0	259.0	140	25
Methadone	310.1	265.1	105.0	100	13
Morphine	286.1	201.0	165.0	140	25
Oxazepam	287.0	269.0	241.0	125	13
Temazepam	301.0	283.0	255.0	120	11
Internal Standards					
6MAM-D_3	331.1	211.0	N/A	165	26
Codeine-D_3	303.2	165.1	N/A	140	50
DHC-D_6	308.2	202.0	N/A	140	34
Diazepam D_5	291.1	154.0	N/A	150	28
DMD-D_5	276.0	140.0	N/A	140	34
Methadone-D_3	314.4	268.1	N/A	115	12
Morphine-D_3	289.1	201.0	N/A	140	25
Oxazepam-D_5	292.1	246.0	N/A	120	23
Temazepam-D_5	306.1	260.0	N/A	110	21

[A] Fragmentor Voltage. [B] Collision energy.

3.3.1.2 Nebuliser Gas Pressure

All analytes gave different responses to the four nebuliser gas pressures tested. This is shown in Figure 3-5. The most marked difference was noticed with methadone at 15 psi. Three other analytes also gave the best response at this pressure. Hence, a nebuliser pressure of 15 psi was chosen.

Gas pressure can affect analyte ionisation as it is possible that ionisation is not effective enough if the pressure selected is too low, whereas a pressure which is too high compared to the optimum pressure can disperse the charged particles too much. The optimisation is essential to ensure the maximum number of charged droplets is dispersed in the right trajectory to reach the mass spectrometer.

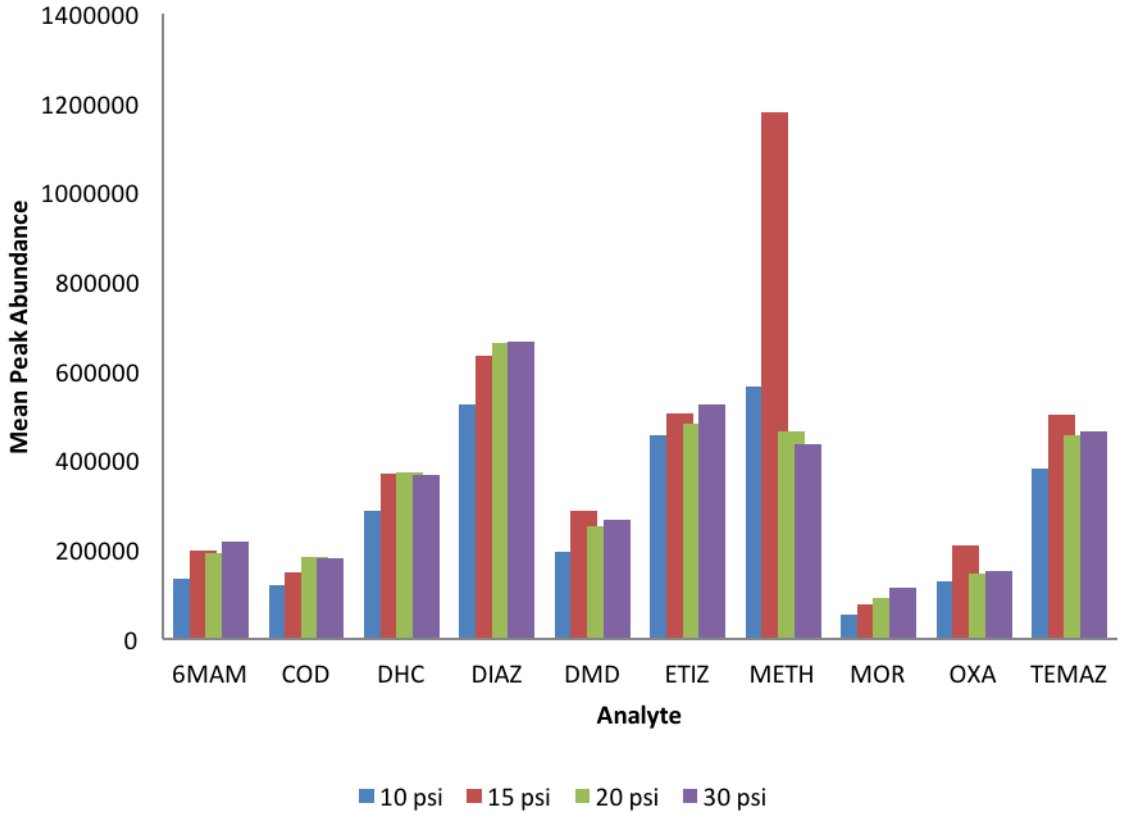

Figure 3-5 Optimisation of nebuliser pressure (psi) for all analytes.

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3.3.1.3 Gas Flow

For all analytes a gas flow of 11 L/min had a beneficial effect on the peak abundances, as show in Figure 3-6.

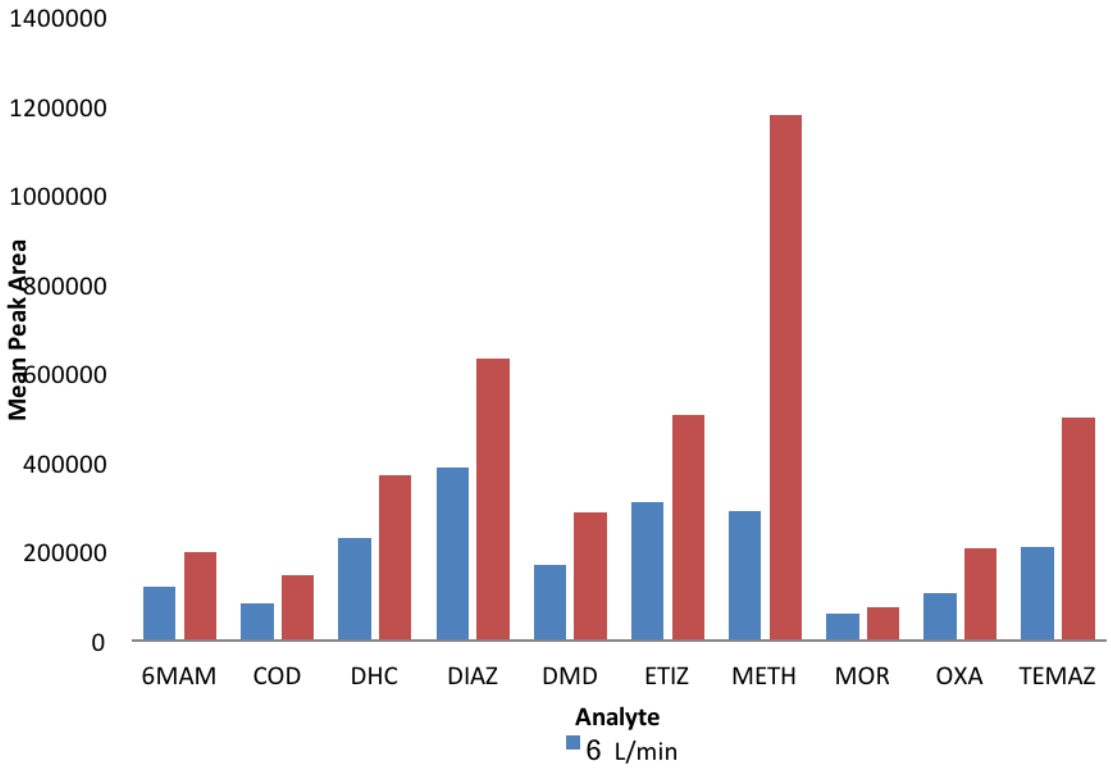


Figure 3-6 Mean peak abundances for analytes at 6 and 11 L/min gas flow rate.

3.3.1.4 Gas Temperature

Out of the four temperatures investigated, the highest mean peak area abundance for the majority of analytes was achieved at a temperature of 325 °C. Results of the investigation are summarised in Figure 3-7.

With the exception of methadone, etizolam, 6-MAM, and morphine and dihydrocodeine, a gas temperature of 325 °C gave the highest abundances for the analytes in question. However, for 6-MAM, morphine and etizolam there was little difference between the responses seen at 325 °C and the highest observed response at 350 °C.

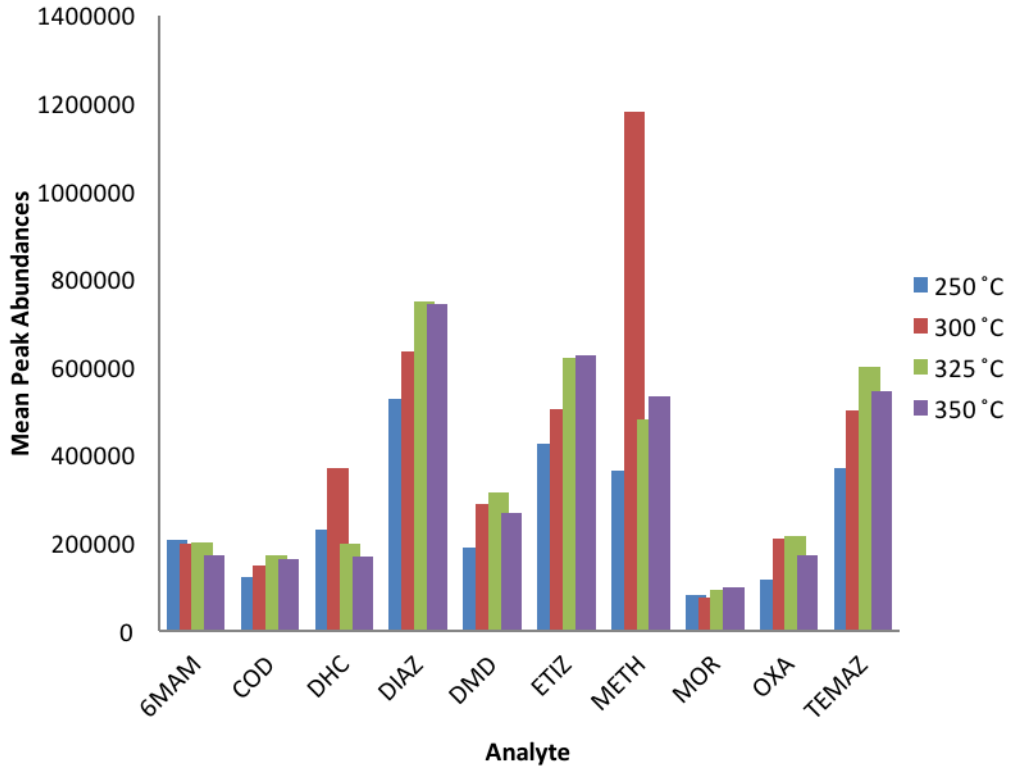


Figure 3-7 Mean peak abundances at different gas temperatures (°C) for analytes investigated

3.3.1.5 Injection Volume

The results for the three injection volumes tested are shown in Figure 3-8.

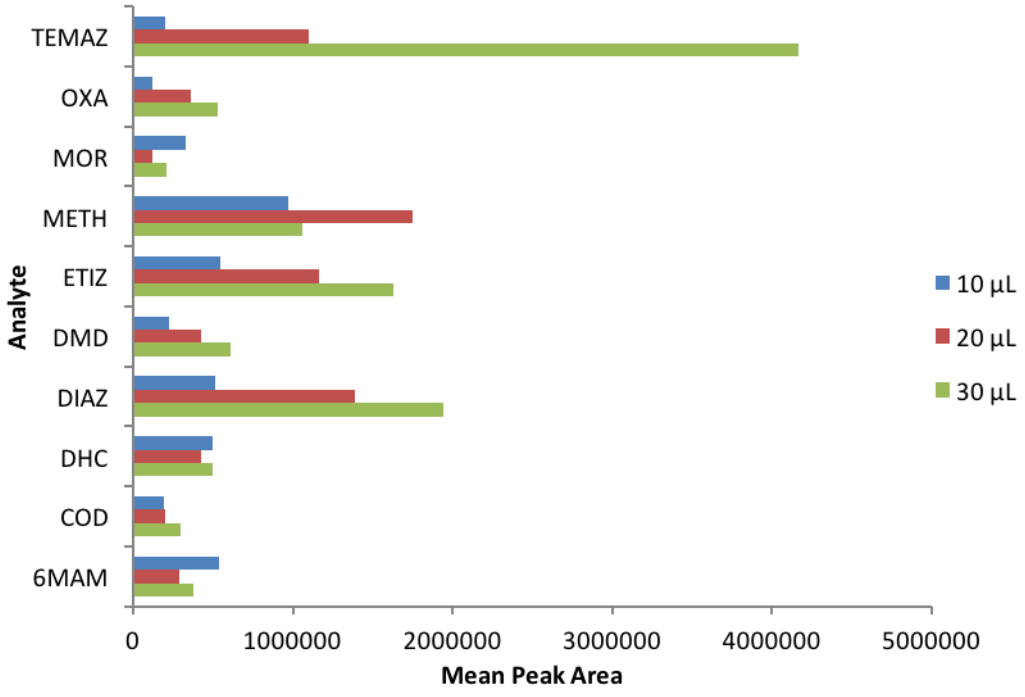


Figure 3-8 Effect of injection volume (10, 20, and 30 µL) on peak abundance

Initially, 10 µL were injected onto the column, but both 20 and 30 µL were tested. Although S/N ratios improved when increasing the injection volume, %ME increased as well, and so it was decided that due to the lower matrix effects and analytical response that did not improve dramatically, 20 µL would be used as the final injection volume.

3.3.1.6 Column Temperature

The effects of three column temperatures on the peak abundance of the analytes were investigated. Results are shown below (Figure 3-9).

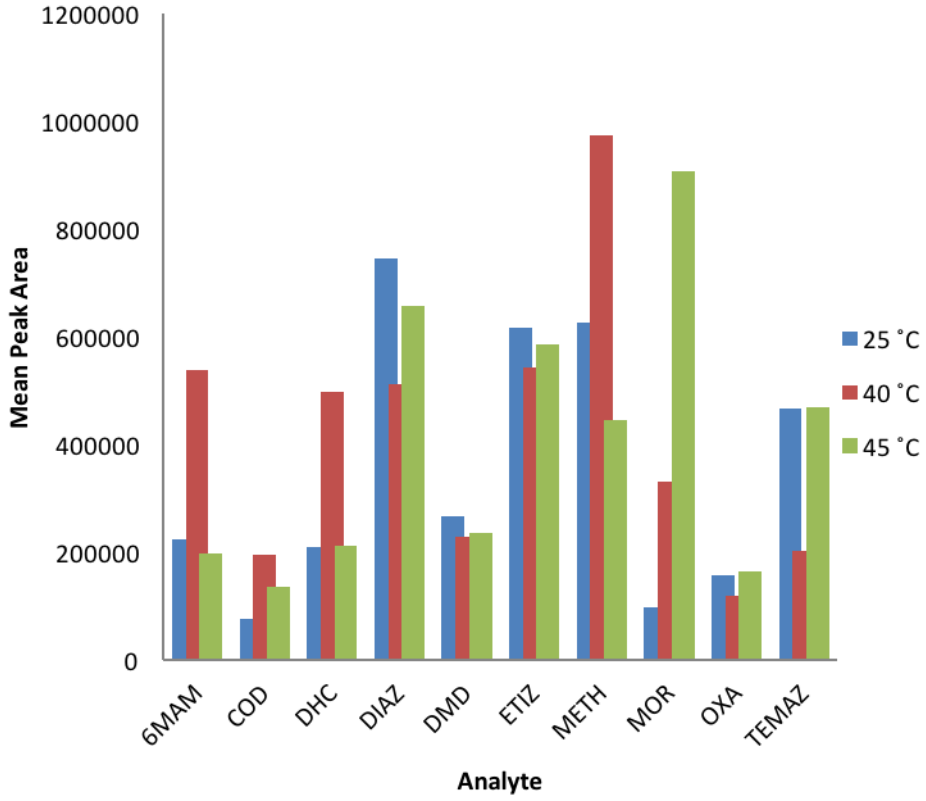


Figure 3-9 Effect of column temperature (25, 40 , and 45 °C) on analyte abundance

A column temperature of 25 °C gave the highest mean peak abundance for etizolam, desmethyldiazepam and diazepam. However, a temperature of 40°C was chosen for the analysis, as 6-MAM, codeine, dihydrocodeine, and methadone all had the highest mean peak abundances at this temperature. Only morphine and temazepam, for which a column temperature of 45 °C gave the highest peak abundances, showed a large difference in abundances. Even so, peak abundances at 40 °C were still sufficiently high to warrant the selection of this temperature.

3.3.1.7 Summary of Final Mass Spectrometric Parameters

The final mass spectrometric parameters chosen for the method are summarised in the table below (Table 3-14).

Table 3-14 Optimised mass spectrometric parameters

MS Parameter	
Nebuliser gas pressure	15 psi
Gas flow	11 mL/min
Gas temperature	325 °C
Injection Volume	20 µL
Column temperature	40 °C

3.3.2 Optimisation of Liquid Chromatographic Parameters

3.3.2.1 Aqueous Phase Additives

Figure 3-10 shows the mean peak areas that were achieved on an isocratic (50:50) run with the addition of 10 mM of either ammonium acetate and ammonium formate to the aqueous phases.

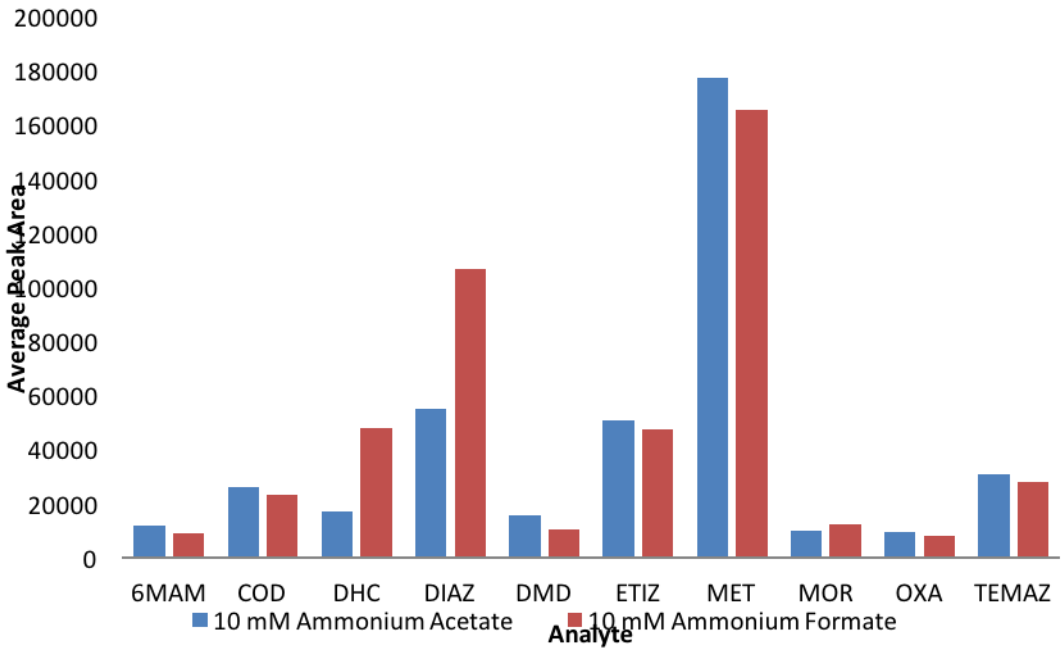


Figure 3-10 Comparison of additives to the aqueous phase

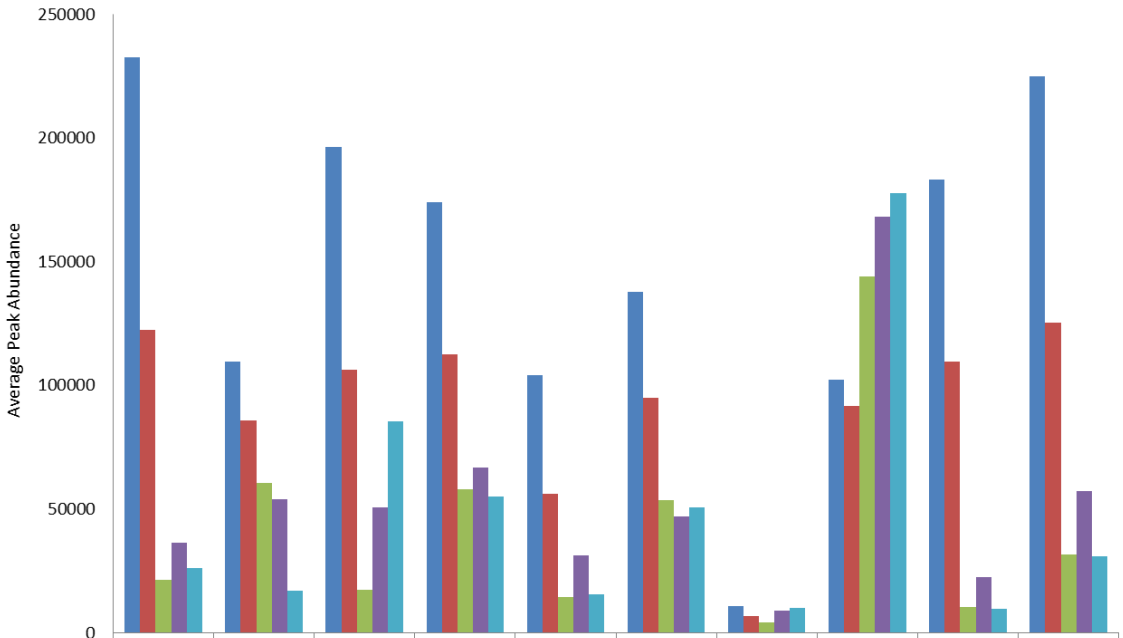
For all but morphine, DHC, and diazepam, ammonium acetate gave the highest mean peak areas. Peak shapes also improved for the 7 analytes analytes which gave better responses using ammonium acetate.

The pH of the additives is an important factor that must be considered as well. The pH measured for 10 mM ammonium acetate was 3.42, compared to a pH of 6.20 for 10 mM ammonium formate. When considering the pKa values of the analytes, it is understandable that a lower pH would help the ionisation of the molecules and helps to explain why relative peak shapes were better with the addition of ammonium acetate.

Therefore ammonium acetate was the additive of choice for future work.

3.3.2.2 Molarity of ammonium acetate

The mean peak abundances found for each analyte at the tested molarities of ammonium acetate are summarised below in Figure 3-11.

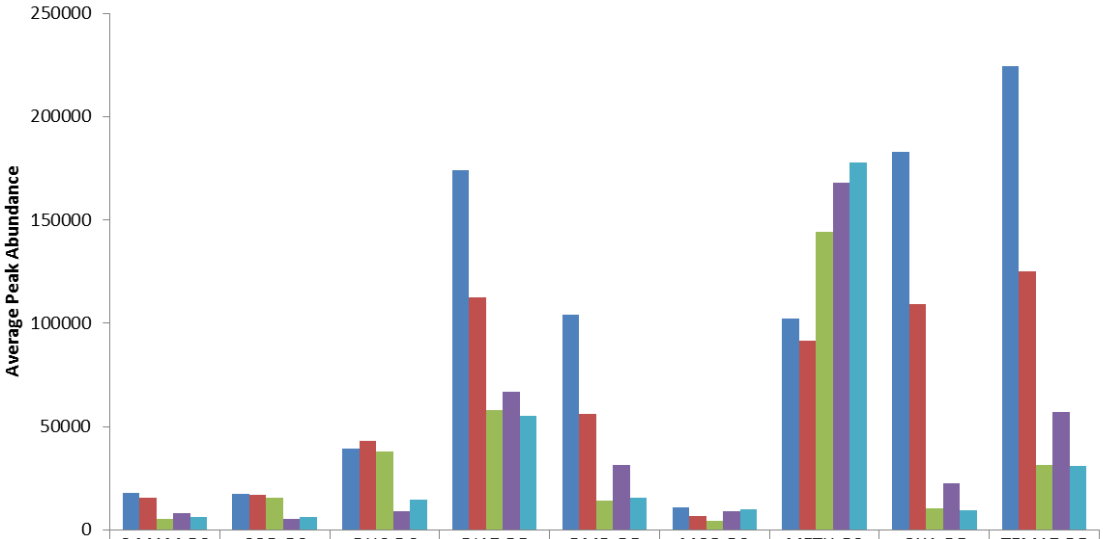


	6MAM	COD	DHC	DIAZ	DMD	ETIZ	MOR	METH	OXA	TEMAZ
2 mM	232317	109371	196111	174003	103977	137687	10648	102193	182976	224642
3 mM	122392	85691	106251	112373	56010	94716	6656	91544	109348	125149
4 mM	21162	60423	17408	57793	14296	53548	4133	144049	10411	31501
5 mM	36382	53999	50668	66720	31273	46880	8970	168164	22449	57152
10 mM	26032	16879	85434	55059	15594	50747	10123	177602	9658	30874

Figure 3-11 Mean peak abundances observed for analytes at increasing molarities of ammonium acetate

From Figure 3-11 it becomes apparent that 2 mM ammonium acetate gave the best responses for the majority of analytes. Therefore, it was decided that 2 mM ammonium acetate would be used. A similar pattern was observed for the internal standard mean peak abundances found, that are shown in Figure 3-12. 2

mM ammonium acetate ensured the highest peak abundance for the internal standards for all analytes apart from methadone which preferred an ionic strength of 10 mM, and dihydrocodeine which showed the highest mean peak abundance at 3 mM.



	6-MAM-D3	COD-D3	DHC-D6	DIAZ-D5	DMD-D5	MOR-D3	METH-D3	OXA-D5	TEMAZ-D5
2 mM	18050	17406	39338	174003	103977	10648	102193	182976	224642
3 mM	15613	16863	43159	112373	56010	6656	91544	109348	125149
4 mM	5437	15459	38045	57793	14296	4133	144049	10411	31501
5 mM	7865	5248	8814	66720	31273	8970	168164	22449	57152
10 mM	5995	6014	14511	55059	15594	10123	177602	9658	30874

Figure 3-12 Mean peak abundances observed for internal standard analytes at increasing molarities of ammonium acetate

3.3.2.3 Concentration of Formic Acid

The results of the addition of 0.1, 0.01 and 0.001% formic acid are summarised in Table 3-15.

Table 3-15 Mean peak abundances at different concentrations of formic acid (*n* = 4)

Analyte	Formic Acid Concentration (%)		
	0.1	0.01	0.001
6MAM	197736	6160	5926
COD	126011	5971	4868
DHC	370803	13975	13507
DIAZ	634018	6292	6925
DMD	287981	2212	2338
ETIZ	840067	111707	13209
MOR	76189	369	95
METH	1430982	350766	354967
OXA	153318	807	814
TEMAZ	232829	1531	1591

From the abundances shown, a concentration of 0.1% formic acid gave the highest responses for all analytes and was therefore chosen for future work.

3.3.2.4 Summary of Final Liquid Chromatographic Parameters

Table 3-16 summarises the final LC parameters that were chosen for the method.

Table 3-16 Optimised liquid chromatographic parameters

LC Parameter	
Aqueous Phase Additive	Ammonium acetate
Molarity of Ammonium Acetate	2 mM
Concentration of Formic Acid	0.1%

The gradient profile selected is shown in 3.2.8.4 (System 3). The final TIC for the analysis, as well as examples of peaks for the quantifier ions for all analytes and internal standards are shown below in Figure 3-13.

TIC

6MAM-D_3

6MAM

Codeine-D_3

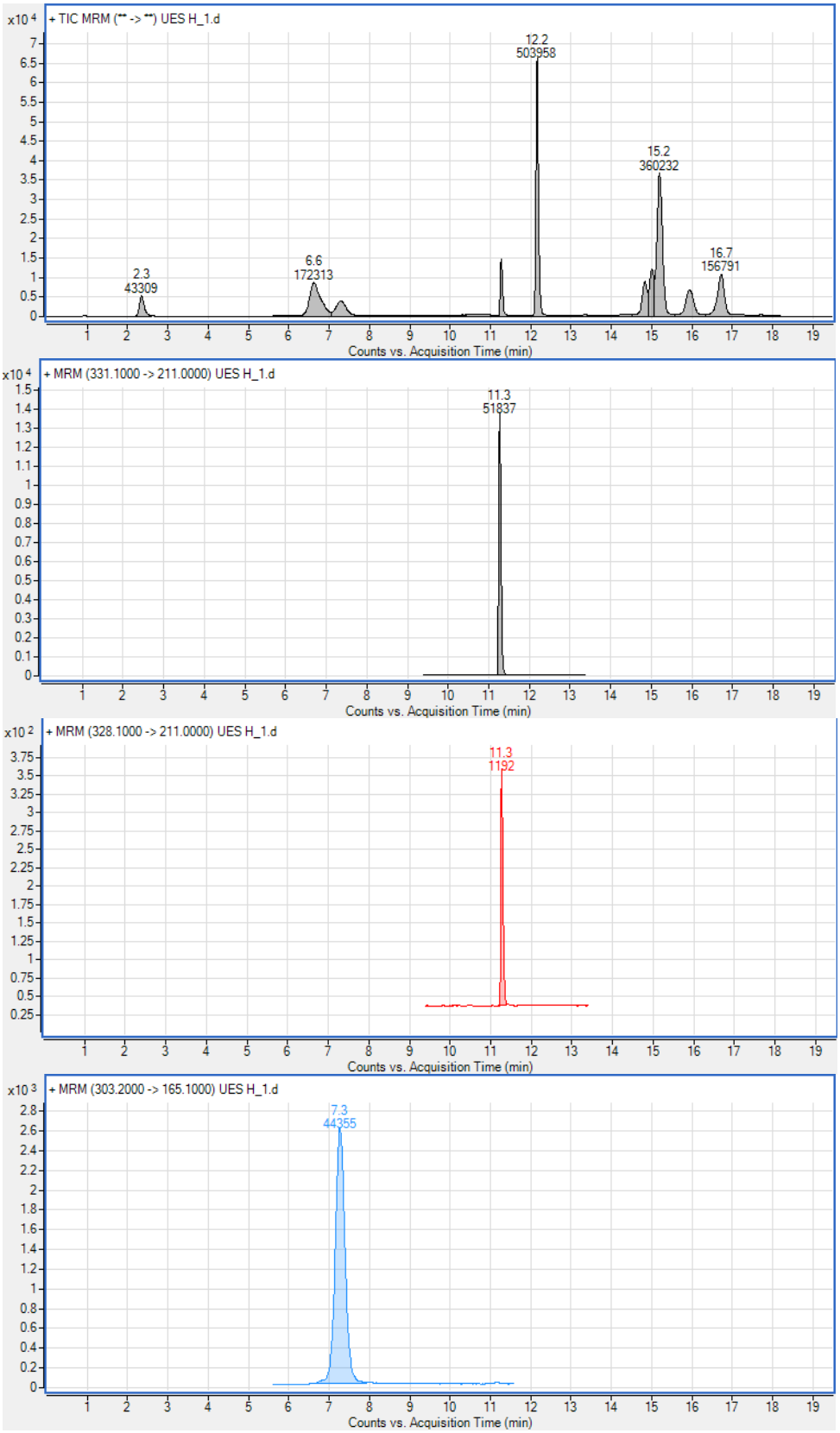
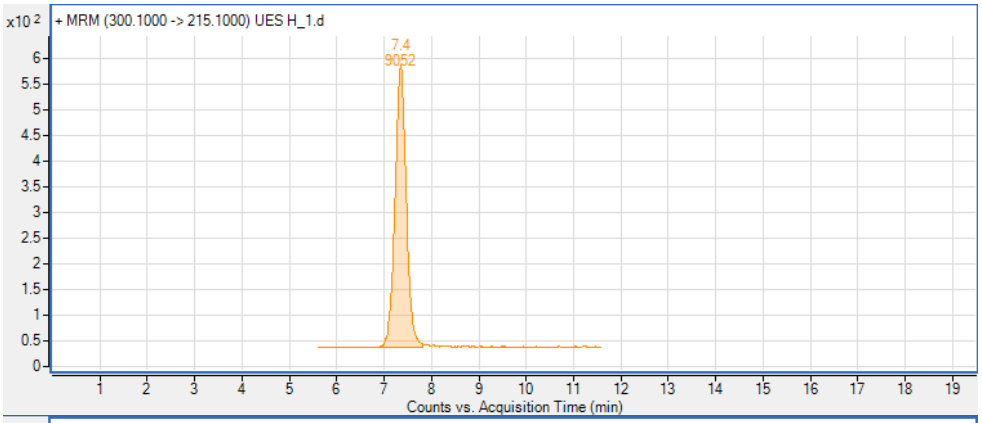
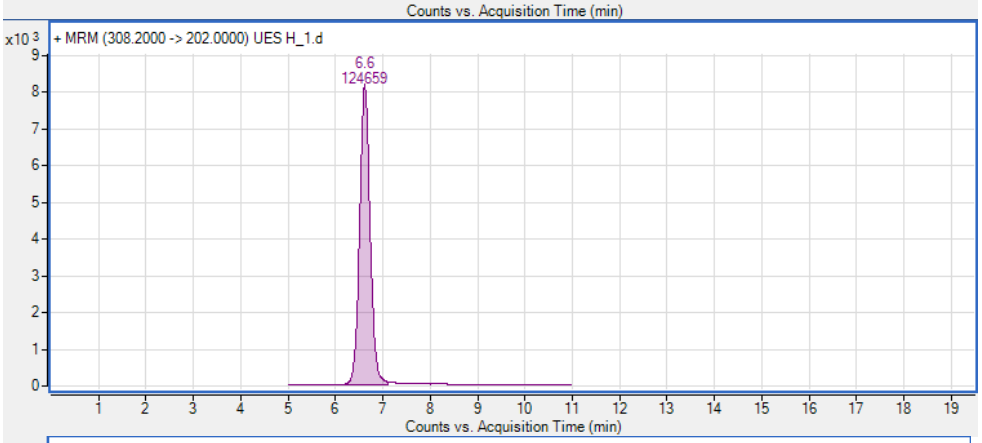


Figure 3-13 TIC and quantifier peaks for analytes and internal standards

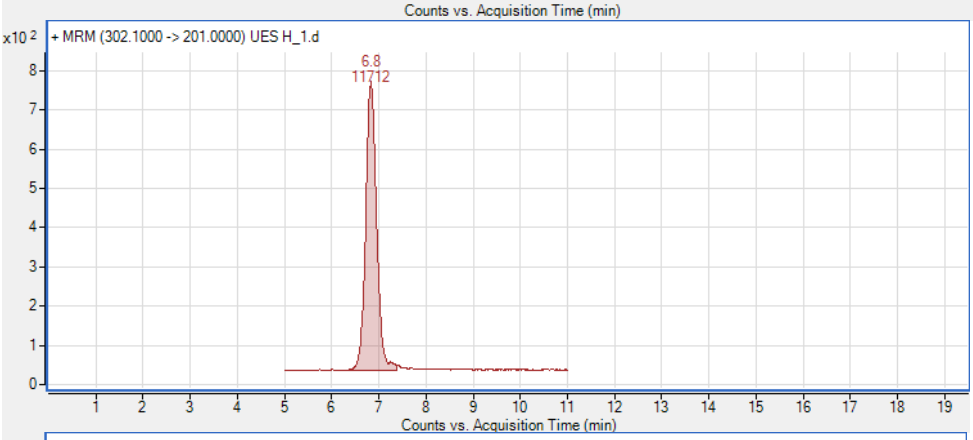
Codeine



DHC-D_6



DHC



Diazepam-D_5

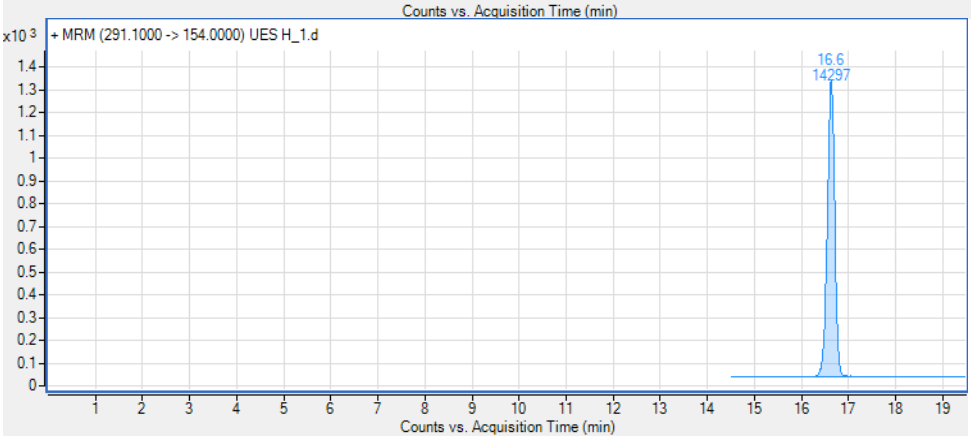
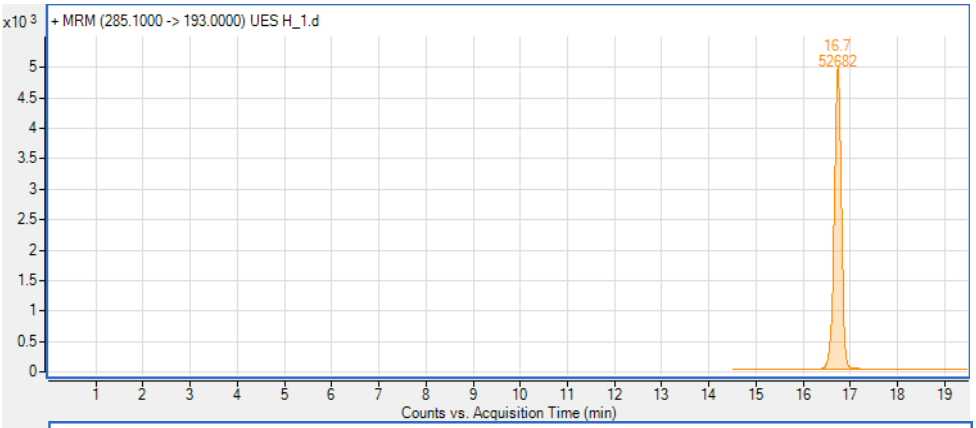
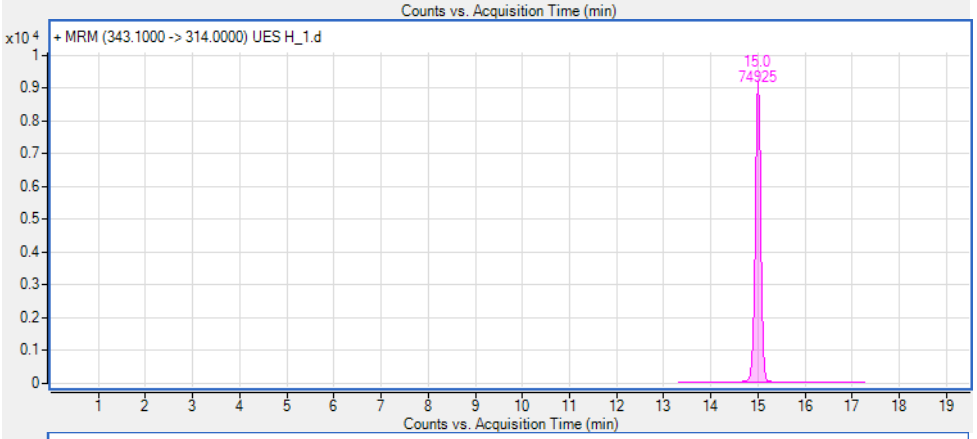


Figure 3-13 Cont'd TIC and quantifier peaks for analytes and internal standards

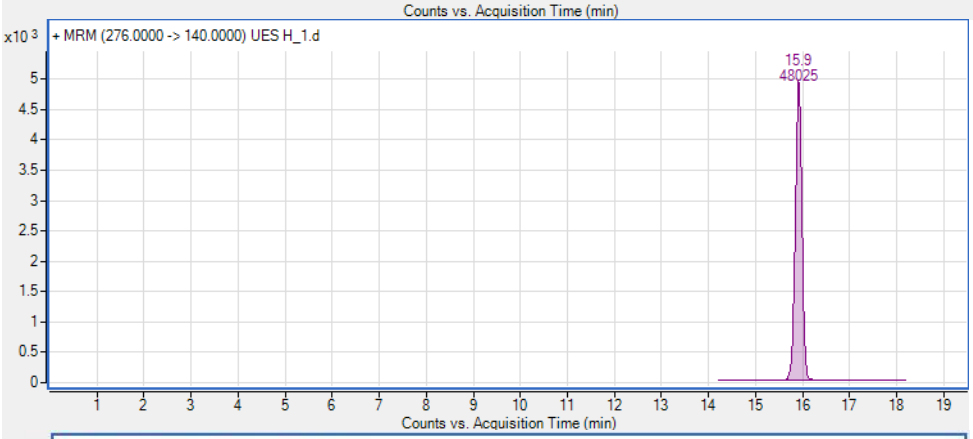
Diazepam



Etizolam



DMD-D_5



DMD

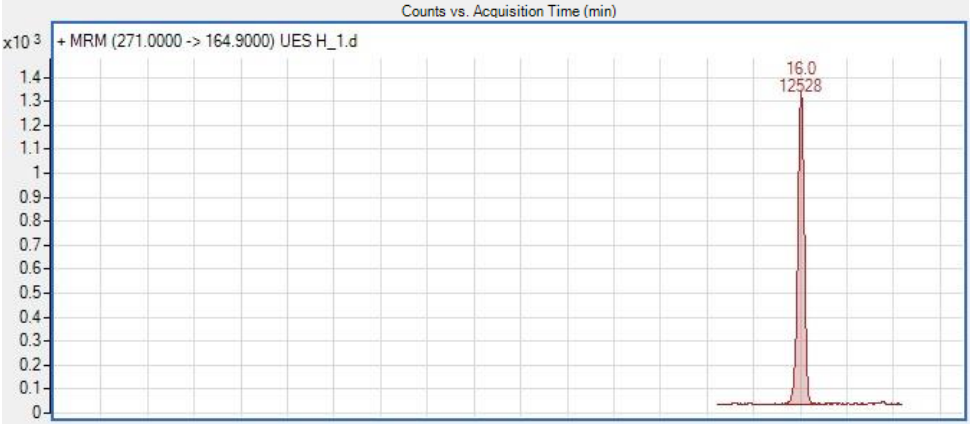
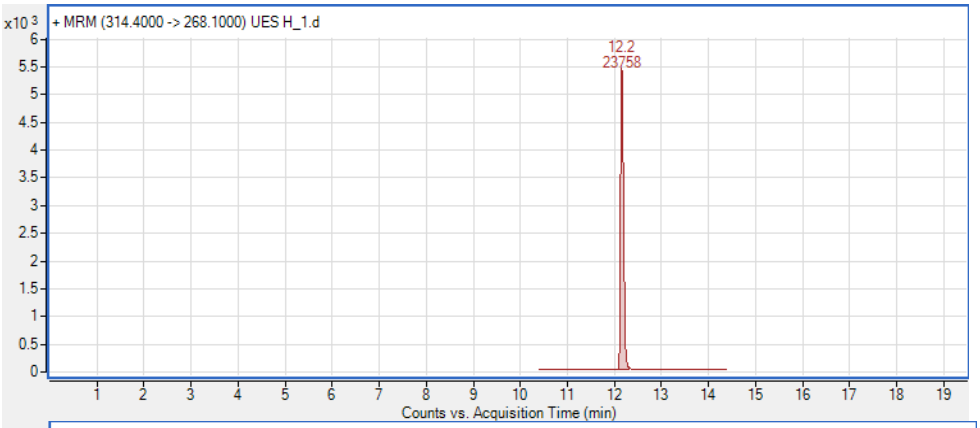
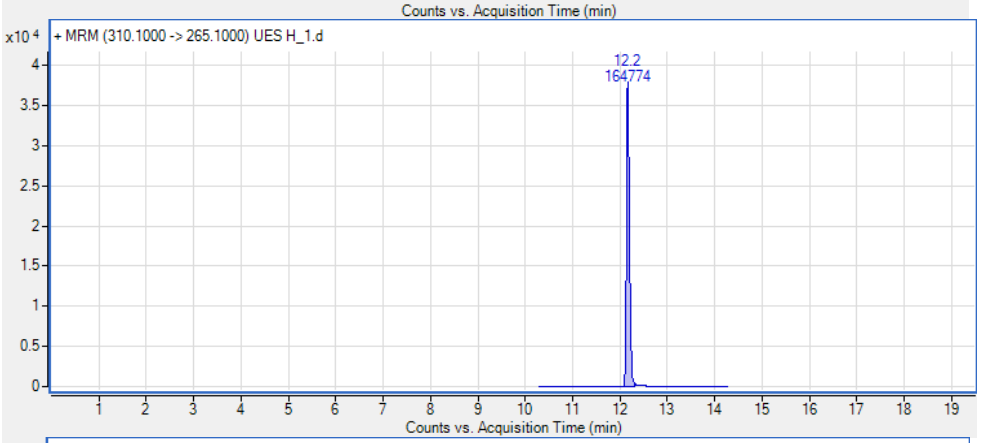


Figure 3-13 Cont'd TIC and quantifier peaks for analytes and internal standards

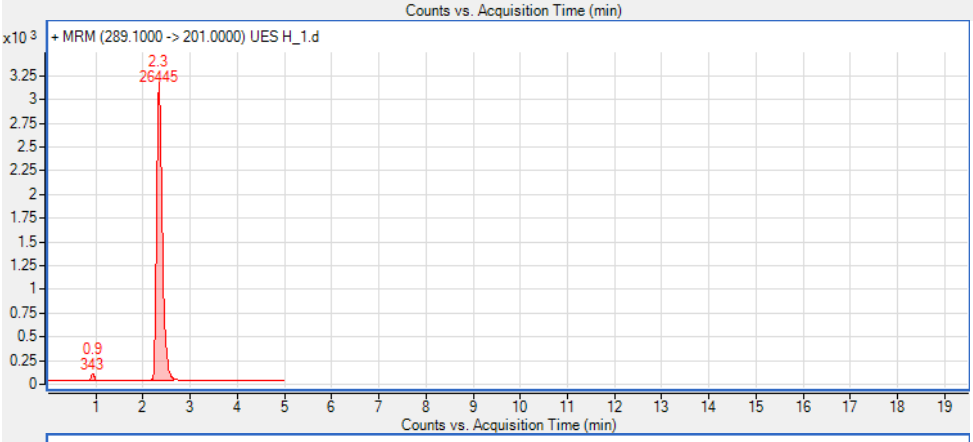
Methadone-D_3



Methadone



Morphine-D_3



Morphine

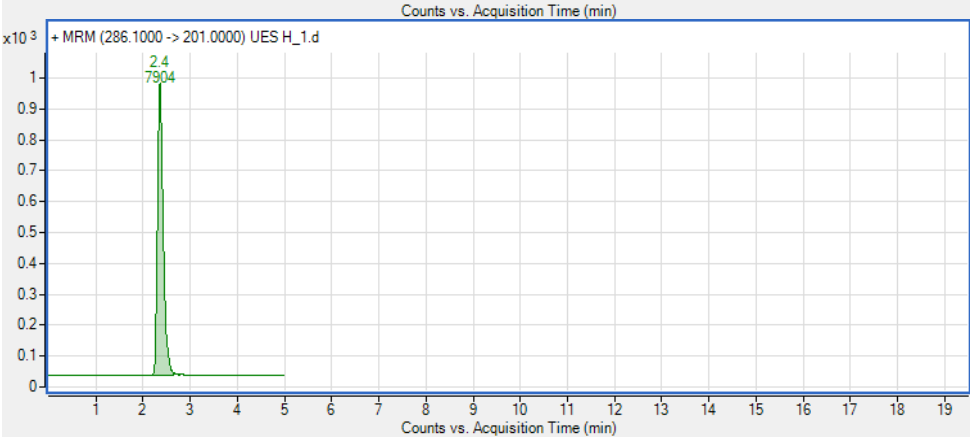
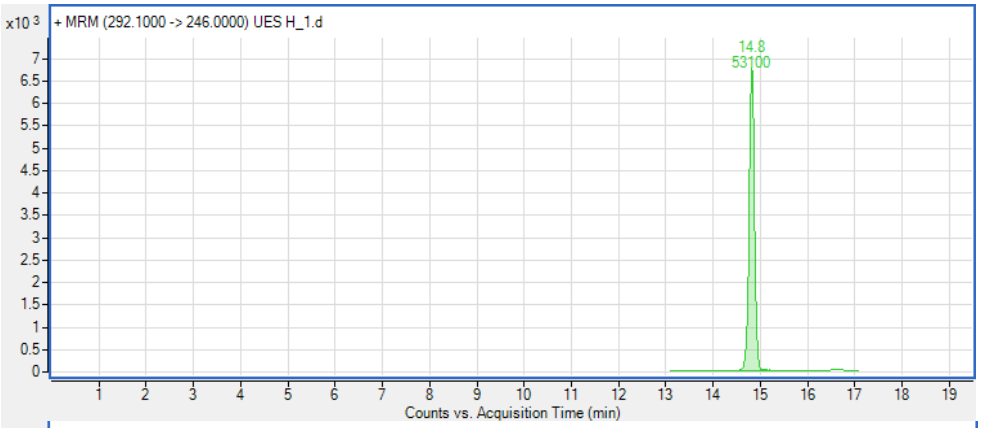
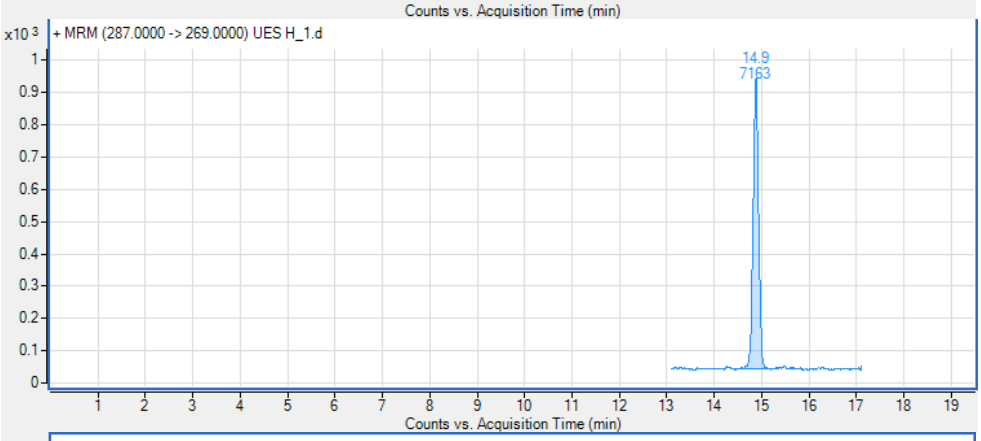


Figure 3-13 Cont'd TIC and quantifier peaks for analytes and internal standards

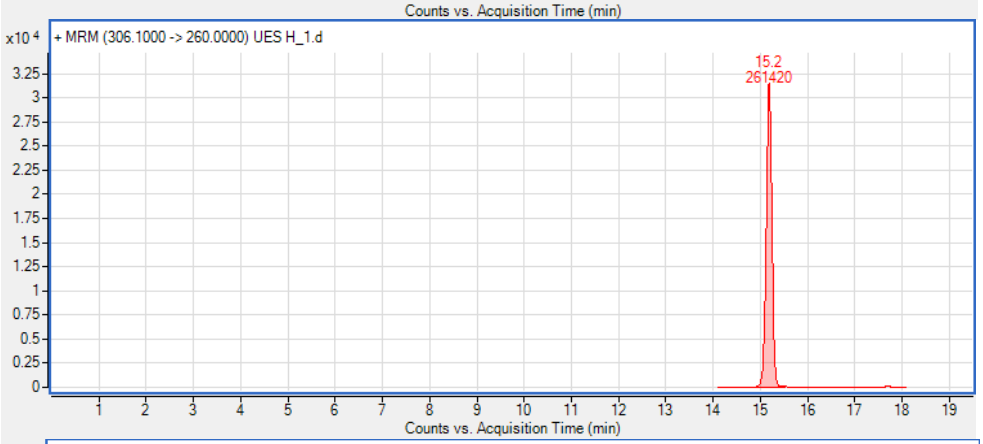
Oxazepam-D_5



Oxazepam



Temazepam-D_5



Temazepam

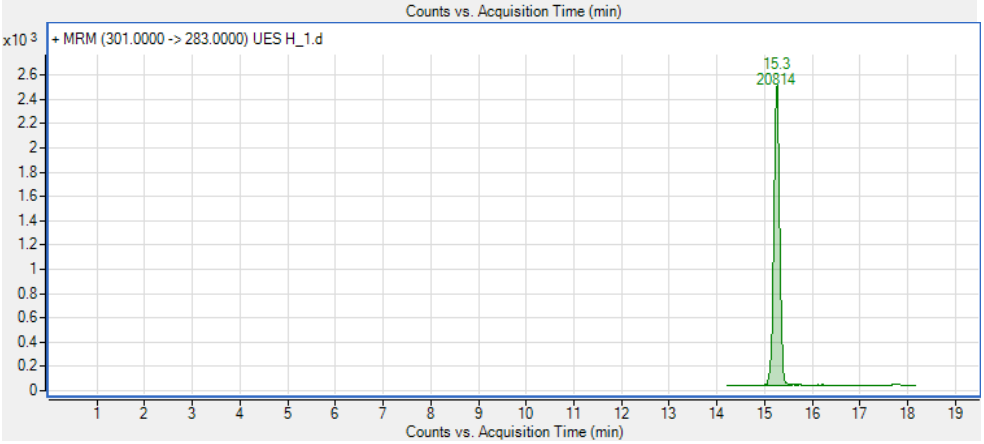


Figure 3-13 Cont'd TIC and quantifier peaks for analytes and internal standards

3.3.3 Optimisation of LLE Procedure

The selection and optimisation of the extraction procedure was the key aspect of method development, as results lacked reproducibility and this was a problem. The evaluation of the extractions was assessed using calculations of process efficiencies, matrix effects and drug recovery. Initially, extractions were carried out in duplicate but were repeated on subsequent days.

3.3.3.1 LLE Method 1

Initial LLE procedures for LLE Method 1, as described in Section 3.2.10, yielded the results shown in Table 3-17, Table 3-18, and Table 3-19. Results were deemed 'acceptable' when recoveries and process efficiencies ranged between 85 and 115%, and matrix effects were within ±25%.

Table 3-17 Process efficiency (%), matrix effect (%), and recovery (%) results for LLE with pH 6 0.1 M phosphate buffer, sodium hydroxide 0.1 M and 0.01 M

Analyte	0.1 M Phos. Buff (pH 6)			NaOH 0.1 M (pH 13)			NaOH 0.01 M (pH 13)		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	18.7	0.2	18.6	N/A	-82.9	N/A	0.4	-15.9	0.5
COD	5.6	-1.2	5.7	60.7	-3.2	62.6	60.5	-4.3	63.3
DHC	1.9	-1.5	2.0	58.1	-1.7	59.1	57.7	-1.9	58.8
DIAZ	101.7	9.8	92.7	106.7	8.2	98.6	108.3	8.9	99.5
DMD	96.7	3.5	93.4	95.1	3.7	91.7	106.6	7.4	99.2
ETIZ	86.0	11.0	77.5	93.6	9.2	85.7	91.3	11.0	82.2
METH	63.1	8.4	58.1	83.7	10.5	75.8	84.6	0.5	84.2
MOR	4.7	2.7	4.6	0.3	28.2	0.2	3.1	9.1	2.9
OXA	97.9	7.7	90.9	31.5	4.1	30.3	93.5	8.4	86.3
TEMA	97.4	5.5	92.3	102.2	3.5	98.8	105.9	8.6	97.5

Where NaOH – sodium hydroxide; Phos. Buff – phosphate buffer

LLE using phosphate buffer gave acceptable results for the benzodiazepines. Recoveries for the benzodiazepines ranged from 77.5 – 90.9% and matrix effects showed some enhancement (3.5 – 11.0%, which is acceptable matrix effects). However, results for the opiates were poor. Although both drug groups are basic, opioids have a higher polarity than benzodiazepines. This meant that the use of

phosphate buffer at pH 6 did not provide the ideal conditions for the opioid drugs to be non-ionised, and in turn extracted. Therefore, it was decided to basify the solutions in order to achieve optimum conditions for the highest yield of non-ionised analytes. Ionic strengths from 0.1 M sodium hydroxide to 0.00001 M sodium hydroxide were tested to see how it affected the extraction. This was done to investigate the pH range from pH 14 to pH 9 in order to ensure the drugs were non-ionised. For basic drugs, this would be the optimum state for analyte extraction.

Recoveries improved for codeine, dihydrocodeine, and methadone, but recoveries for 6-MAM and morphine when 0.1 M sodium hydroxide was added were negligible. No major improvement was seen when 0.001 M sodium hydroxide was used. The first improvement was found when a molarity of 0.00001 M of sodium hydroxide was used (pH 9). When this was used, although recoveries for dihydrocodeine were low and unacceptable for morphine (2.9%), all other analytes gave good results for process efficiencies, matrix effects and recoveries.

Table 3-18 Process efficiency (%), matrix effect (%), and recovery (%) results for LLE with sodium hydroxide 0.001 M, 0.0001 M and 0.00001 M

Analyte	NaOH 0.001 M (pH 11)			NaOH 0.0001 M (pH 10)			NaOH 0.00001 M (pH 9)		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	96.4	17.9	81.8	88.9	28.4	69.3	86.1	15.3	74.7
COD	5.6	-1.2	5.7	60.7	-3.2	62.6	60.5	-4.3	63.3
DHC	1.9	-1.5	2.0	58.1	-1.7	59.1	57.7	-1.9	58.8
DIAZ	101.7	9.8	92.7	106.7	8.2	98.6	108.3	8.9	99.5
DMD	96.7	3.5	93.4	95.1	3.7	91.7	106.6	7.4	99.2
ETIZ	86.0	11.0	77.5	93.6	9.2	85.7	91.3	11.0	82.2
METH	63.1	8.4	58.1	83.7	10.5	75.8	84.6	0.5	84.2
MOR	4.7	2.7	4.6	0.3	28.2	0.2	3.1	9.1	2.9
OXA	97.9	7.7	90.9	31.5	4.1	30.3	93.5	8.4	86.3
TEMA	97.4	5.5	92.3	102.2	3.5	98.8	105.9	8.6	97.5

Where NaOH – sodium hydroxide.

Table 3-19 Process efficiency (%), matrix effect (%), and recovery (%) results for LLE with ammonium hydroxide at 1, 5 and 10%

Analyte	NH_4OH 1% (pH 11)			NH_4OH 5% (pH 11.39)			NH_4OH 10% (pH 11.7)		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	90.0	12.4	80.1	37.4	18.2	31.7	10.4	9.7	9.5
COD	68.8	17.1	58.8	66.7	11.9	59.6	61.8	18.7	52.1
DHC	63.5	16.8	54.4	62.5	9.5	57.1	59.1	18.5	49.9
DIAZ	109.2	12.8	96.8	93.2	-2.8	95.9	103.4	13.9	90.8
DMD	110.7	14.3	96.9	102.6	7.2	95.6	100.6	14.8	87.7
ETIZ	91.9	16.8	78.7	79.1	0.3	78.8	86.4	15.4	74.8
METH	75.2	-0.2	75.4	90.4	-2.9	93.1	68.2	-5.0	71.8
MOR	49.6	9.0	45.5	13.6	14.1	11.9	8.1	10.1	7.4
OXA	113.8	16.7	97.5	101.9	8.4	94.0	98.8	14.3	86.5
TEMA	109.0	14.5	95.2	107.7	14.3	94.3	99.3	13.2	87.7

Where NH_4OH – ammonium hydroxide.

Using ammonium hydroxide in the extraction showed best results when 1% was used. The matrix effects ranged from –0.2 – 17.1% at this concentration. As the concentration of NH_4OH was increased, the recoveries of morphine and 6-MAM decreased. The benzodiazepines gave similar recoveries for the three concentrations.

Although some of these procedures provided acceptable results, when the extractions were repeated, results were not reproducible. For example, matrix effects for methadone in the extraction using phosphate buffer were 8.4% initially, but the following time, matrix effects of 54.1% were observed. Recoveries of morphine using 1% ammonium hydroxide were 45.5% initially, but when the extractions were repeated, the recovery dropped to 3.6%. This may have been caused by the sample matrix and the extractions not producing a sufficiently clean extract to give reproducible results. None of these methods were chosen.

3.3.3.2 LLE Method 2

Results for process efficiencies, matrix effects and drug recoveries using this extraction procedure (as well as increased shaking time for the procedure) are summarised below in Table 3-20.

Table 3-20 Process efficiency (%), matrix effect (%), and recovery (%) results for LLE procedure (156) with 5 and 10 minute shaking times

Analyte	5 minutes			10 minutes		
	PE%	ME%	R%	PE%	ME%	R%
6MAM	53.7	-40.7	90.6	9.1	3.5	8.8
COD	41.4	-38.5	67.3	5.9	0.6	5.8
DHC	26.9	-41.6	46.1	4.4	0.1	4.4
DIAZ	22.5	-83.9	139.9	3.8	-13.0	4.4
DMD	87.5	-53.6	188.5	14.9	-10.9	16.7
ETIZ	64.9	-24.4	85.9	8.0	-5.3	8.4
METH	64.6	-16.3	77.2	6.2	-2.8	6.4
MOR	7.0	-18.5	8.6	1.8	-7.8	1.9
OXA	37.8	-26.2	51.2	6.0	-3.0	6.2
TEMAZ	46.3	-29.4	65.5	6.4	-7.6	6.9

The results shown for 5 and 10 minute shaking times were run on the same day, immediately after one another. Although matrix effects were low when the shaking time was increased, the mean drug recoveries for all analytes decreased. This was repeated to see whether these results were a reliable representation of the extraction procedure (Table 3-21).

Table 3-21 Process efficiency (%), matrix effect (%), and recovery (%) results for repeated LLE procedure (156) with 5 and 10 minute shaking times

Analyte	5 minutes			10 minutes		
	PE%	ME%	R%	PE%	ME%	R%
6MAM	63.1	-2.4	64.7	63.6	-2.0	65.0
COD	49.7	-1.5	50.5	44.2	0.5	43.9
DHC	43.1	1.2	42.6	36.1	-0.4	36.2
DIAZ	28.8	-96.3	787.5	11.4	-71.0	39.2
DMD	60.9	-70.2	204.0	99.0	-46.6	185.5
ETIZ	81.7	1.7	80.3	54.2	-17.6	65.8
METH	63.8	-7.2	68.7	59.3	-4.4	62.0
MOR	20.8	-4.6	21.8	15.2	-7.8	16.4
OXA	51.1	-7.6	55.4	44.4	-20.3	55.7
TEMAZ	44.3	-16.5	53.1	47.7	-40.4	80.1

It is clear that the initial results did not match the results obtained for the repeated extraction. This method was not reproducible and there was clearly a large matrix effect with diazepam and desmethyldiazepam, as these were outwith ±25%. This calls into question the reliability and accuracy of the method reported and no further attempts were made to improve or develop this procedure.

3.3.3.3 LLE Method 3 Initial Investigation

A procedure outlined by Liao *et al* for the rapid extraction of morphine and codeine from plasma (157) was used, with amendments (as outlined in Section 3.2.10). Results are shown below in Figure 3-14.

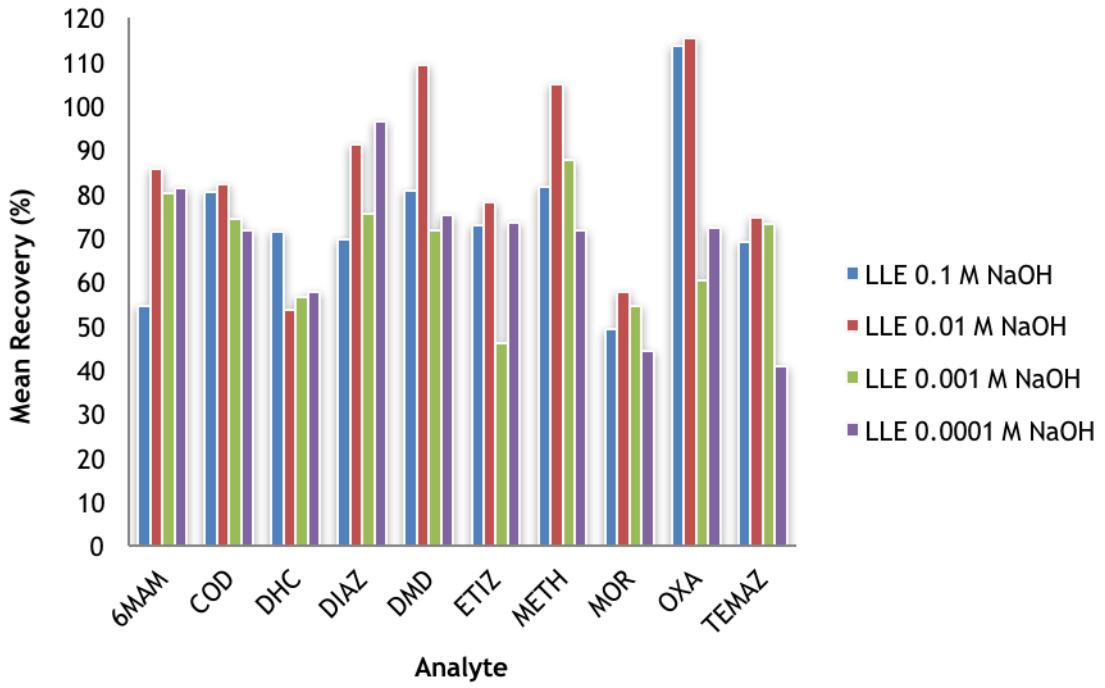


Figure 3-14 Summary of LLE recovery (%) results from extraction adapted from (157) using different concentrations of sodium hydroxide

Where LLE – liquid-liquid extraction; NaOH – sodium hydroxide

3.3.3.4 LLE Method 3 Molarity Experiments

From this experiment, it was found that 0.01 M sodium hydroxide gave the best recoveries. DHC and morphine gave the lowest recoveries at 53.7% and 57.8%, respectively. Oxazepam recoveries were a little high at 115.3% due to matrix effects. Corresponding matrix effects are shown below (Table 3-22).

Table 3-22 Matrix effects (%) for LLE with different molarities of sodium hydroxide

Analyte	0.1 M NaOH	0.01 M NaOH	0.001 M NaOH	0.0001 M NaOH
6MAM	-12.5	-4.2	-4.1	-6.0
COD	-7.7	-1.3	-2.9	-5.5
DHC	2.4	4.7	-11.9	7.0
DIAZ	-47.5	-53.0	-43.2	-54.8
DMD	-24.5	-28.5	-20.4	-25.5
ETIZ	-52.9	-40.8	-17.4	-28.4
METH	9.5	-2.5	-10.6	-11.3
MOR	-10.7	-9.7	-5.0	-7.0
OXA	-49.4	-37.7	-18.8	-20.1
TEMAZ	-11.6	-22.9	-44.4	-20.2

Where NaOH – sodium hydroxide.

Matrix effects were not acceptable for diazepam for all molarities tested, and this explains the low process efficiencies observed.

3.3.3.5 LLE Method 3 Reconstitution Volume and Shaking Time Experiments

Using a reconstitution volume of 1000 µL improved matrix effects for diazepam and desmethyldiazepam, although these were still high at -24.1% and 32.0%, respectively. Results for the LLE procedure using different concentrations of sodium hydroxide, although having previously given drug recoveries all above 54% when reconstitution was done in 250 µL, did not show recoveries exceeding 28% (for temazepam) when samples were reconstituted in 1000 µL and shaken for 5 minutes. Due to the lower recovery of temazepam, the shaking time was investigated at different reconstitution volumes.

Therefore, it was determined that the shaking time would be tested as well as different reconstitution volumes to optimise the method. The shaking time can increase drug recovery (160) and increasing the reconstitution volume has been proven to reduce matrix effects by dilution.

Results for various reconstitution volumes and different shaking times are shown in Table 3-23 (250 µL reconstitution volume), Table 3-24 (500 µL reconstitution volume), and Table 3-25 (1000 µL reconstitution volume).

Table 3-23 Process efficiency (%), matrix effect (%), and recovery (%) results for increasing shaking times and a reconstitution volume of 250 µL

Analyte	5 minutes			10 minutes			15 minutes			20 minutes			25 minutes			30 minutes		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	77.2	-19.9	96.4	72.6	-6.4	77.6	84.6	-3.9	88.0	92.6	-5.9	98.4	168.3	-12.7	192.8	164.0	1.2	162.1
COD	73.5	-18.0	89.6	69.9	3.3	67.6	69.1	-5.5	73.1	76.0	-6.0	80.8	133.9	-4.1	139.7	114.5	-2.4	117.3
DHC	67.0	-21.2	85.1	59.7	1.2	59.0	59.6	-5.0	62.8	65.0	-4.6	68.1	47.5	-2.9	48.9	40.1	-1.5	40.7
DIAZ	23.7	-73.9	90.9	21.1	-88.9	190.7	9.0	-88.5	78.6	22.6	-85.2	152.7	28.6	-73.2	106.6	16.7	-68.9	53.7
DMD	113.5	-43.7	201.8	51.4	-55.0	114.0	84.7	-46.7	158.8	32.1	-69.8	106.3	67.3	-51.3	138.1	24.3	-87.4	193.1
ETIZ	74.1	-58.7	179.6	81.8	-47.9	156.9	64.1	-31.2	93.2	73.5	-45.9	135.8	64.1	-39.3	105.7	55.7	-73.9	213.6
METH	77.9	-8.0	84.6	95.9	6.3	90.2	73.3	-11.4	82.7	82.0	-14.2	95.6	96.3	-10.9	108.1	68.1	-12.6	78.0
MOR	50.6	10.8	45.6	69.1	10.6	62.5	43.3	-9.0	47.6	45.0	-32.5	66.8	32.4	-7.4	35.0	28.9	-4.5	30.3
OXA	67.1	-51.1	137.2	48.2	-50.4	97.2	66.0	-15.0	77.7	65.7	-33.4	98.5	62.1	-47.2	117.4	59.8	-49.9	119.4
TEMA	62.2	-31.8	91.1	45.4	-55.4	101.8	40.5	-41.1	68.8	24.1	-52.1	50.4	61.9	-42.4	107.6	35.2	-86.3	257.0

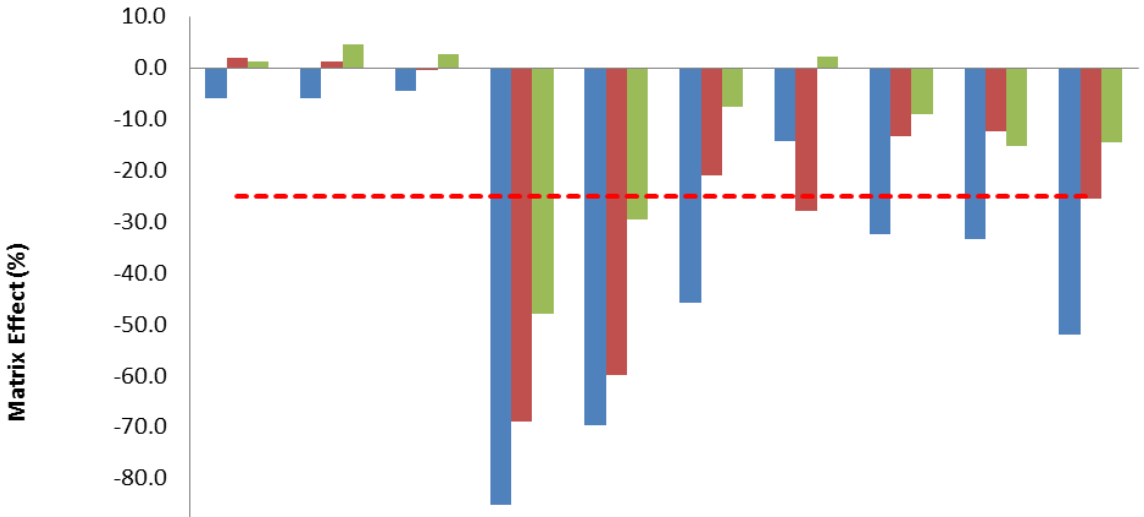
Table 3-24 Process efficiency (%), matrix effect (%), and recovery (%) results for increasing shaking times and a reconstitution volume of 500 µL

Analyte	5 minutes			10 minutes			15 minutes			20 minutes			25 minutes			30 minutes		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	110.9	-3.4	114.8	113.3	-25.3	151.7	102.8	-3.6	106.6	117.6	1.9	115.4	83.0	3.4	80.3	83.8	3.6	80.9
COD	70.5	1.2	69.7	73.5	-25.5	98.7	64.9	-6.5	69.4	70.1	1.1	69.3	74.1	0.3	73.8	83.0	-0.9	83.7
DHC	77.2	-0.8	77.8	84.7	-29.8	120.6	64.1	-7.6	69.3	73.1	-0.4	73.4	78.0	-1.1	78.9	86.1	2.0	84.4
DIAZ	9.0	-70.3	30.5	26.6	-66.2	78.5	27.5	-63.4	75.3	28.8	-69.1	93.3	32.4	-62.7	86.7	23.2	-73.3	86.8
DMD	62.6	-50.7	126.9	38.7	-82.7	223.5	72.6	-40.7	122.4	74.7	-59.8	185.8	33.2	-41.6	56.9	41.3	-59.8	102.8
ETIZ	71.4	-32.9	106.5	59.8	-23.1	77.7	58.7	-11.5	66.4	72.8	-21.0	92.2	58.9	-17.3	71.2	73.1	-15.2	86.1
METH	93.3	2.3	91.2	100.9	-1.7	102.6	91.6	-15.2	108.1	76.3	-27.9	105.7	88.6	-0.4	89.0	96.0	-7.1	103.3
MOR	44.3	5.0	42.2	47.3	-4.5	49.5	37.7	-16.1	45.0	50.7	-13.3	58.5	67.9	6.7	63.6	67.2	5.4	63.7
OXA	81.4	-29.5	115.3	91.7	-24.2	120.9	89.6	-7.5	96.9	133.3	-12.4	152.2	62.8	-15.1	74.0	75.7	-16.9	91.1
TEMA	59.5	-46.2	110.6	58.3	-72.1	209.0	83.5	-18.6	102.5	102.2	-25.6	137.4	49.6	-23.7	65.0	66.6	-41.7	114.3

Table 3-25 Process efficiency (%), matrix effect (%), and recovery (%) results for increasing shaking times and a reconstitution volume of 1000 µL

Analyte	5 minutes			10 minutes			15 minutes			20 minutes			25 minutes			30 minutes		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	84.8	7.7	78.7	88.6	10.7	80.1	73.2	3.5	70.7	82.1	1.2	81.1	100.1	19.4	83.8	99.8	8.7	91.8
COD	63.3	5.0	60.3	71.8	6.1	67.7	59.8	4.6	57.2	64.6	4.6	61.8	82.2	7.3	76.6	81.3	-4.2	84.9
DHC	67.9	6.9	63.5	79.4	6.9	74.3	57.7	3.5	55.7	68.0	2.6	66.3	65.0	10.5	58.8	65.9	-1.7	67.0
DIAZ	108.7	-24.1	143.2	91.6	-43.8	163.1	89.8	-51.0	183.4	56.9	-47.9	109.2	57.7	-48.6	112.3	36.2	-52.9	76.9
DMD	31.4	-32.0	46.1	130.5	-15.1	153.7	99.5	-31.8	145.9	88.8	-29.5	125.9	113.4	9.2	103.9	69.4	-34.9	106.6
ETIZ	72.8	-6.3	77.7	81.3	1.6	80.1	77.9	-1.9	79.4	66.5	-7.5	71.9	37.0	-22.3	47.7	48.1	-28.4	67.2
METH	84.7	5.3	80.4	86.1	10.3	78.0	75.3	-1.1	76.2	59.1	2.2	57.8	165.0	14.4	144.3	156.3	30.4	119.8
MOR	44.6	-9.8	49.4	66.8	7.6	62.1	40.5	-13.3	46.7	50.8	-9.0	55.9	76.0	N/A	N/A	51.4	14.5	44.9
OXA	79.7	-4.0	83.0	95.5	-1.5	97.0	76.7	-10.0	85.3	74.4	-15.3	87.9	78.5	26.7	61.9	63.6	-15.3	75.1
TEMA	25.6	-0.4	25.7	94.4	0.2	94.3	74.4	-10.8	83.4	75.9	-14.5	88.7	124.4	49.3	83.4	84.3	-22.4	108.6

Optimal results for the majority of analytes were achieved when the sample was shaken for 20 minutes and reconstituted in 1000 µL, as recoveries were too high exceeded 140% for desmethyldiazepam and 180% for diazepam, when a shaking time of 15 minutes was employed. Matrix effects were greatly reduced when a larger reconstitution volume was used. At a shaking time of 20 minutes, the matrix effects (%) shown in Figure 3-15 were observed for the three reconstitution volumes.



	6MAM	COD	DHC	DIAZ	DMD	ETIZ	METH	MOR	OXA	TEMAZ
250 µL	-5.9	-6.0	-4.6	-85.2	-69.8	-45.9	-14.2	-32.5	-33.4	-52.1
500 µL	1.9	1.1	-0.4	-69.1	-59.8	-21.0	-27.9	-13.3	-12.4	-25.6
1000 µL	1.2	4.6	2.6	-47.9	-29.5	-7.5	2.2	-9.0	-15.3	-14.5

Figure 3-15 Matrix effects (%) observed for three different reconstitution volumes (250, 500, and 1000 µL) when shaken for 20 minutes

Desmethyldiazepam showed matrix effects marginally outwith the recommended ±25% threshold at 29.5% when a reconstitution volume of 1000 µL was used. Additionally, diazepam matrix effects were still too high at this reconstitution volume. Increasing the shaking time to 25 and 30 minutes did not show improvements for any of the criteria for any of the analytes.

3.3.3.6 LLE Method 3: Extraction Solvent Experiment

In an attempt to improve the extraction even further, other solvents were used for previously selected parameters (shaking time of 20 minutes, reconstitution in 1000 µL of reconstitution solution). 3 mL of DCM and MTBE (which had been used in the first LLE procedure) were used with both 0.1M and 0.01M sodium hydroxide. Based on their electronegativities and polarities, it was assumed that

EtOAc would prove the most successful extraction solvent for the analytes in question. Results of this experiment are shown in Figure 3-16.

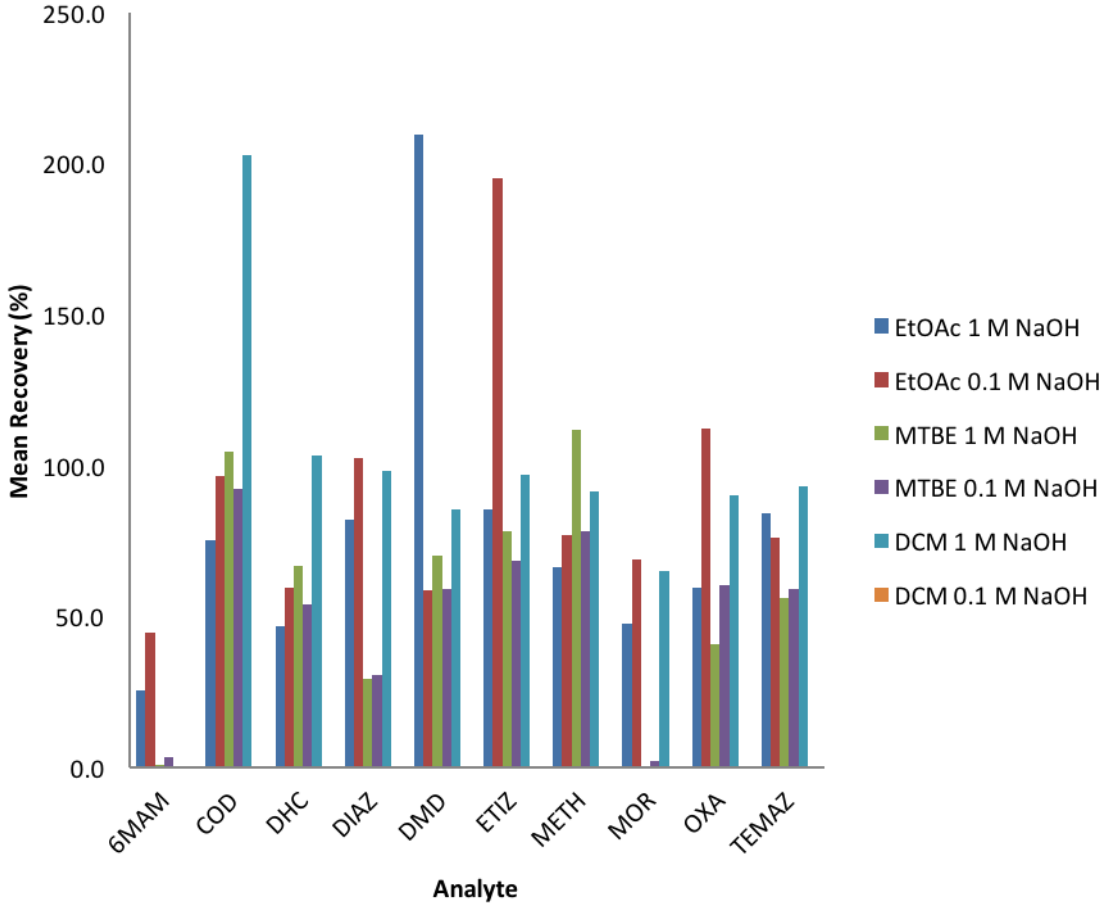


Figure 3-16 Mean recovery (%) for each solvent attempted with 1 M and 0.1 M sodium hydroxide solution, 20 minutes shaking time and a reconstitution volume of 1000 µL (*n* = 4) EtOAc – ethyl acetate; MTBE – methyl-*tert*-butyl-ether; DCM – dichloromethane

No drugs were recovered when DCM with 0.1 M sodium hydroxide was used. Although DCM and 1M sodium hydroxide were successful at extracting methadone, etizolam, desmethyldiazepam, diazepam, morphine, oxazepam and temazepam, 6-MAM was not recovered at all. 6-MAM and morphine recoveries were negligible with MTBE. The optimum solvent for the extraction of 6-MAM (although recoveries were low) was found to be EtOAc, which was also the only solvent to extract all analytes. However, the matrix effects with EtOAc were unacceptable for 6 out of the 10 drugs.

The associated matrix effects are shown in Figure 3-17.

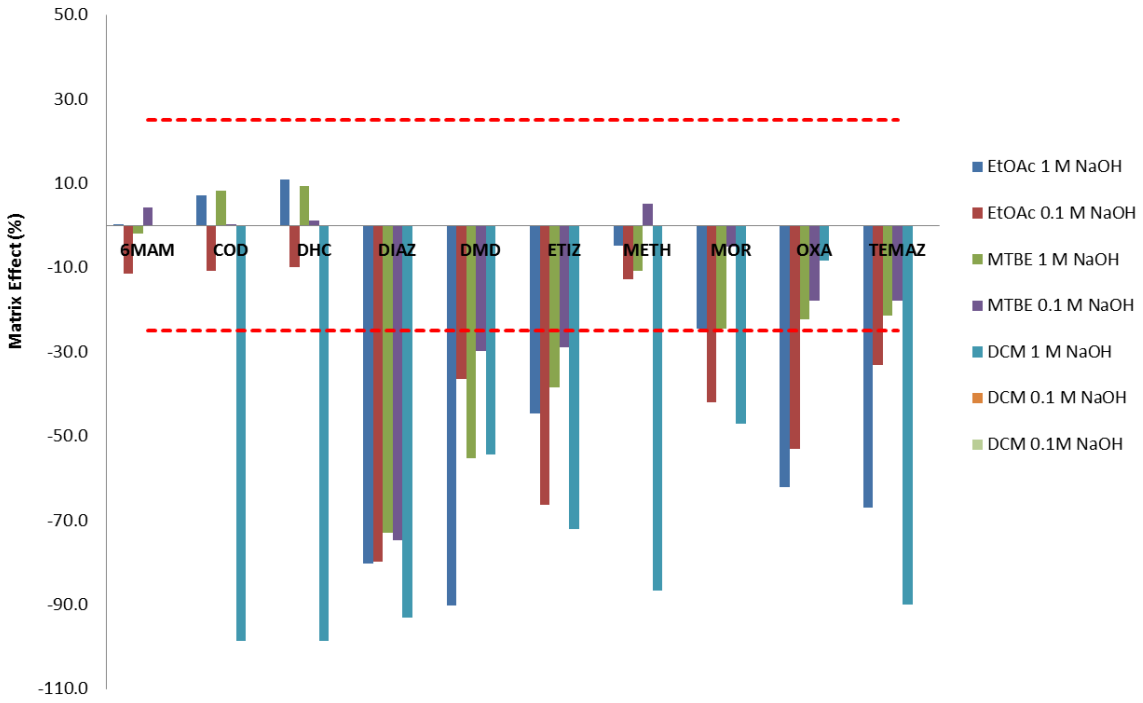


Figure 3-17 Matrix Effects (%) results for LLE using different solvents and 1M and 0.1M sodium hydroxide.
Where EtOAc – ethyl acetate; MTBE – methyl-*tert*-butyl-ether; DCM - dichloromethane

The results for the extraction using a combination of EtOAc and heptane (4:1) are shown in Table 3-26. The samples were shaken for 20 minutes as this had proven itself to be the optimum shaking time, and a reconstitution volume of 1000 µL was used.

Table 3-26 Process efficiency (%), matrix effect (%), and recovery (%) results for LLE extractions with EtOAc and EtOAc:heptane (4:1)

Analyte	EtOAc			EtOAc:heptane (4:1)		
	PE%	ME%	R%	PE%	ME%	R%
6MAM	82.1	1.2	81.1	89.6	2.7	87.3
COD	64.6	4.6	61.8	71.5	-10.5	79.9
DHC	64.6	4.6	61.8	58.9	-8.2	64.2
DIAZ	56.9	-47.9	109.2	92.5	-37.2	147.4
DMD	88.8	-29.5	125.9	74.9	-42.9	131.1
ETIZ	66.5	-7.5	71.9	19.0	-61.8	49.7
METH	59.1	2.2	57.8	159.8	0.0	159.7
MOR	50.8	-9.0	55.9	59.2	14.6	51.7
OXA	74.4	-15.3	87.9	38.1	-47.7	73.0
TEMAZ	75.9	-14.5	88.7	55.2	-46.1	102.2

Table 3-26 shows that although the combination of EtOAc and heptane, increased drug recoveries for most analytes (compared to using only EtOAc), the matrix effects were also increased. Matrix effects most drastically worsened for etizolam (from -7.5% for the extraction using only EtOAc to -61.8% when using the combination of EtOAc and heptane). On the other hand, matrix effects improved for diazepam (-47.9% for EtOAc and -37.2% for the solvent combination), although the ion suppression found was still outwith the recommended ±25%. Matrix effect was the limiting factor found in the EtOAc only extraction procedure; the synchronous use of both extraction solvents did nothing to improve these. For morphine and DHC, the synchronous use of both extraction solvents seemed to inverse the matrix effects from ion suppression to enhancement, and vice versa. For the remaining analytes, with the exception of methadone and diazepam, ion suppression worsened with the addition of heptane to the extraction.

3.3.4 Optimisation of SPE Procedure

3.3.4.1 SPE Using UCT Columns

Table 3-27 shows the results of extraction procedure for designer benzodiazepines (recommended by UCT, outlined in Table 3-10) from oral fluid with altered elution solutions. Here, UCT1 refers to the original extraction procedure outlined by UCT for the extraction of designer benzodiazepine drugs from oral fluid. Analytes were eluted with 3 mL of EtOAc with 2% ammonium hydroxide. UCT6 describes the same extraction procedure but elution of analytes was achieved using a standard elution solution of DCM:IPA:NH_4OH (78:20:2 v/v). UCT7 used both elution solutions in a sequential elution with a 2 minute drying step between elution steps. Eluates were collected into the same vial. Recoveries of opioid drugs improved when the chlorinated elution solution was used. Sequential elution did not improve the recovery from using DCM:IPA:NH_4OH only. Matrix effects remained unacceptable for DHC.

Table 3-27 Extraction procedure optimisation of changing elution solutions

Analyte	UCT1 Elution EtOAc:NH_4OH (98:2 v/v)			UCT6 Elution DCM:IPA:NH_4OH (78:20:2 v/v)			UCT7 Elution EtOAc:NH_4OH (98:2 v/v) then DCM:IPA:NH_4OH (78:20:2 v/v)		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	54.0	3.8	40.4	88.2	-7.0	94.8	80.1	-9.7	88.7
COD	41.1	1.6	40.4	98.6	-3.7	102.4	85.8	-5.6	90.8
DHC	179.8	692.3	22.7	374.5	109.0	179.2	785.7	-11.6	888.7
DIAZ	65.0	2.7	63.3	63.8	-5.1	67.2	60.8	-9.3	67.0
DMD	174.3	73.0	100.8	125.1	14.5	109.3	132.6	25.2	105.9
ETIZ	77.1	-1.1	77.9	96.1	-3.3	99.5	95.4	-2.0	97.3
METH	58.6	-1.0	59.2	68.4	-26.6	93.2	53.7	-26.6	73.2
MOR	7.0	-4.7	7.4	92.6	-3.2	95.6	79.5	-0.1	79.5
OXA	71.0	1.8	69.7	105.6	-1.2	106.9	101.2	-3.7	105.0
TEMAZ	52.0	4.2	49.9	61.3	-0.5	61.6	54.5	-5.7	57.8

Where DCM – dichloromethane; IPA – isopropanol; NH_4OH – ammonium hydroxide solution; EtOAc – ethyl acetate.

A procedure for the extraction of the analytes of interest from hair samples (158) was tested for its efficacy for the extraction from neat oral fluid ("UCT4"). Although initial drug recoveries were found to be good, matrix effects for the benzodiazepine drugs were too great for the method to be used. This was clear when extractions were repeated over several consecutive days and no reproducibility was found (Table 3-28). From the results presented, it is clear that there is an inherent problem with the extraction based on the very high recoveries found for several analytes at random times (75334.5% for diazepam during the fourth run). Furthermore, the matrix effects observed were also not reproducible over the five analyses. The authors of the original work reported drug recoveries of >75% for all analytes. The discrepancy may have been caused by instrumentation issues that persisted throughout the extraction procedure optimisation stages or it may have been a problem with the matrix, as 100% ion suppression was observed for some analytes. The extraction procedure was also applied to a matrix different from the published method, and this would of course also cause different results.

The procedure UCT4 (158) was applied to buffered oral fluid samples; however, the elution steps eluted the blue dye particles from the buffer that are usually retained on the sorbent bed. Therefore, reconstituted samples were slightly blue in colour. For a working laboratory this would not be acceptable as not only could the unknown components of the buffer create instrumentation issues by blocking or dirtying the system, it could also affect the analysis by interfering with the drug molecules.

Results were not consistent and followed no pattern when reproducibility was tested. Below are the results for two duplicate extractions, extracted (UCT3) and analysed on two consecutive days (Table 3-29).

Table 3-28 Reproducibility results for extraction procedure UCT4 (158)

Analyte	Analytical Results														
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	91.1	-4.7	95.5	0.0	-100.0	0.0	84.5	-0.8	85.2	94.3	2.6	91.9	29.5	-24.3	39.0
COD	86.0	-10.7	96.3	128.6	-99.3	17393.8	93.9	3.4	90.9	85.4	-0.4	85.8	94.7	-12.6	108.4
DHC	78.9	-5.9	83.8	138.7	-99.6	32169.7	76.2	2.8	74.1	71.7	-5.7	76.0	78.6	-12.5	89.8
DIAZ	62.7	-50.4	126.4	51.2	-3.1	52.9	40.4	-100.0	767380.4	21.7	-100.0	75334.5	76.6	-20.8	96.7
DMD	50.3	-63.9	139.4	34.3	-45.6	63.0	66.5	-38.2	107.5	39.2	-60.2	98.3	70.7	-29.0	99.4
ETIZ	69.0	-36.6	108.9	60.1	15.1	52.2	80.8	-13.1	93.0	72.2	-26.4	98.0	73.5	-25.5	98.6
METH	117.0	26.8	92.2	194.4	-99.7	60423.7	99.6	8.9	91.4	23.0	-85.9	163.1	48.6	-57.8	115.1
MOR	76.4	-23.8	100.2	167.5	-99.7	56400.0	67.2	-9.4	74.2	80.2	-0.1	80.3	66.6	-25.1	88.9
OXA	63.5	-45.3	116.0	54.5	3.6	52.6	81.5	-13.2	93.8	54.8	-44.9	99.5	1033.0	1779.5	55.0
TEMA	64.3	-41.8	110.5	54.6	-1.6	55.5	78.9	-19.9	98.5	54.9	-42.4	95.4	780.2	140.8	324.0

Table 3-29 Reproducibility of process efficiency (%), matrix effect (%), and recovery (%) results from neat oral fluid for extraction procedure UCT3

Analyte	Day 1, Duplicate 1			Day 1, Duplicate 2			Day 2, Duplicate 1			Day 2, Duplicate 2		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	65.2	-30.8	94.3	86.1	-18.6	105.7	68.5	-43.0	120.1	76.5	-22.1	98.1
COD	169.9	-97.1	5953.9	50.8	-52.0	105.8	95.8	0.3	95.4	178.6	85.4	96.3
DHC	172.1	127.8	75.5	216.5	18.5	182.7	1003.4	1840.5	51.7	81.7	-17.8	99.4
DIAZ	38.6	-62.0	101.4	88.4	-9.8	98.0	47.6	-54.1	103.7	84.7	-14.4	98.9
DMD	62.0	-30.6	89.2	76.9	-25.4	103.1	56.8	-41.4	97.0	75.3	-29.1	106.3
ETIZ	81.5	-8.6	89.2	98.4	-2.4	100.8	75.1	-28.0	104.3	91.1	-12.3	104.0
METH	105.9	19.4	88.7	81.6	-11.7	92.4	57.2	-50.2	114.8	70.8	-29.5	100.3
MOR	56.2	-40.3	94.2	89.8	-7.3	96.9	54.7	-49.2	107.7	94.2	-12.5	107.7
OXA	69.7	-20.4	87.5	98.6	-1.8	100.5	72.7	-31.3	105.9	94.0	-8.2	102.4
TEMAZ	38.0	-61.2	97.9	96.7	-1.5	98.2	44.4	-56.9	102.9	93.0	-9.8	103.1

At a later stage, the sequential extraction (UCT7) was repeated again but this time for both expectorated and NeoSAL™/buffered oral fluid. A lack of consistency and reproducibility was found for 6-MAM where other analytes showed decent (i.e. results being more consistently reproducible than previously observed) reproducibility over two runs on consecutive days, for expectorated oral fluid. Results for this are shown in Table 3-30. Matrix effects were found to be high for morphine for both runs but matrix effects for methadone improved from the first to the second run.

Table 3-30 Repeated reproducibility of process efficiency (%), matrix effect (%), and recovery (%) results from neat oral fluid for extraction with sequential elution (UCT7)

Analyte	Run 1			Run 2		
	PE%	ME%	R%	PE%	ME%	R%
6MAM	80.2	-14.7	94.0	39.0	-19.8	48.6
COD	84.9	-2.6	87.2	87.3	-1.4	88.5
DHC	84.8	-3.1	87.5	87.8	-2.6	90.1
DIAZ	42.1	-0.6	42.3	52.9	-5.3	55.9
DMD	75.4	-4.2	78.7	70.5	-7.9	76.6
ETIZ	84.3	-3.8	87.6	84.8	-7.0	91.1
METH	53.9	-36.1	84.4	82.1	-21.9	105.1
MOR	40.8	-42.0	70.2	63.9	-35.6	99.1
OXA	86.9	2.7	84.6	78.9	2.9	76.7
TEMAZ	38.2	1.5	37.6	46.0	0.9	45.6

Buffered oral fluid on the other hand showed inconsistent results with diazepam and temazepam, although all other analytes showed reproducibility. Recoveries for both diazepam and temazepam were much lower than other analytes with the exception of methadone, which had a clear problem with ion suppression. Results are summarised in Table 3-31.

Table 3-31 Reproducibility of process efficiency (%), matrix effect (%), and recovery (%) results from oral fluid collected with the NeoSAL™ device for extraction with sequential elution

Analyte	Run 1			Run 2		
	PE%	ME%	R%	PE%	ME%	R%
6MAM	96.2	-1.8	97.9	98.2	-7.6	106.2
COD	93.1	5.0	88.7	96.0	6.2	90.3
DHC	93.9	2.3	91.8	93.6	2.8	91.0
DIAZ	25.1	-4.5	26.3	37.8	-9.0	41.5
DMD	60.2	-13.1	69.3	56.7	-16.5	67.8
ETIZ	66.8	-5.9	71.0	75.2	-9.0	82.7
METH	7.7	-91.0	86.2	10.4	-89.0	94.7
MOR	72.7	-16.5	87.0	81.8	-7.3	88.2
OXA	76.2	-4.2	79.5	74.1	-4.0	77.1
TEMAZ	23.0	-8.8	25.2	36.3	-9.3	40.1

Separate extraction procedures used by FMS for opiates (Procedure UCT3 in Table 3-10) and benzodiazepines (Procedure UCT5 in Table 3-10) from blood and oral fluid were also tested on neat oral fluid. Following poor results, and results that showed better extraction efficiencies for benzodiazepines when an optimised opioid/opiate extraction method was followed, drug groups were analysed separately using both procedures.

The results are shown in Table 3-32. 6-MAM, codeine and dihydrocodeine gave good results when extracted following the benzodiazepine procedure. The matrix effects obtained for these three analytes were acceptable when extracted on this procedure, but were outside of the ±25% threshold when the opioid/opiate extraction procedure was followed. It must be noted that the FMS opiate extraction procedure is used with GC-MS analyses on which matrix effects are less of an issue. The only analyte that gave both acceptable recovery and matrix effect on the opioid method was morphine and the recovery using this extraction was greater than twice that of the benzodiazepine method. Acceptable matrix effects were also observed for etizolam, diazepam, oxazepam and temazepam. The latter two however were not recovered well using this procedure (24.2% and 8.4%, respectively). Neither extraction method gave good

results for methadone, nor for desmethyldiazepam. For the latter, recoveries of 162.5% were found using the benzodiazepine extraction and matrix effects of -34.6% were found following the opiate extraction. Both are unacceptable. For these reasons, neither extraction method was chosen for future work.

Table 3-32 Analytical results obtained when drug groups were separated and extracted using the FMS routine procedures

Analyte	Extraction Procedure UCT5 (FMS Benzodiazepine Method)			Extraction Procedure UCT3 (FMS Opiate Method)		
	PE%	ME%	R%	PE%	ME%	R%
6MAM	75.7	-7.7	82.1	49.6	-42.7	86.5
COD	72.6	-7.0	78.0	62.8	-33.4	94.3
DHC	63.3	-6.1	67.4	71.1	-33.6	106.9
DIAZ	92.1	-7.1	99.1	104.6	-14.1	121.8
DMD	165.5	1.8	162.5	113.7	-34.6	173.9
ETIZ	79.8	-14.5	93.4	88.0	-19.2	108.9
METH	43.0	-57.7	101.7	109.7	-32.2	161.7
MOR	38.5	-11.8	43.7	84.2	-7.9	91.5
OXA	79.4	3.2	76.9	20.2	-16.6	24.2
TEMAZ	88.2	10.3	79.9	6.8	-19.5	8.4

Where FMS – Forensic Medicine and Science.

These results could potentially be explained by the different chemistries of these analytes. The excellent recoveries for morphine can be explained by the elution solution used for the opiates method, as this employs DCM:IPA:NH_4OH (78:20:2 v/v), which previous results have shown to be favoured by morphine, and in turn explains the lack of recovery of both temazepam and oxazepam as these are not eluted using this particular elution solution.

3.3.4.2 SPE Using Bond Elut Certify Columns

The recoveries shown in Table 3-33, using the extraction procedure (Extraction Procedure BE1 in Table 3-11) outlined in (143), are from the analysis of the EtOAc with 2% ammonium hydroxide eluate (after drying and reconstitution in 1000 µL of reconstitution solution). Recoveries for the full procedure were low for the benzodiazepines (especially temazepam with 5.6%, oxazepam with 21.1%) but also for morphine (recovery 13.2%). Excellent recoveries were found

for methadone (98.1%), although a high matrix effect creates doubt about the high recovery for 6-MAM (99.0%).

When the acetone:DCM wash step was removed, however, drug recoveries for all the benzodiazepines improved dramatically. The recovery of morphine increased to 98.5%, however, corresponding matrix effect was -97.8% and therefore the process efficiency was only 2.2%. It is believed that the NH_4^+ ion from the ammonium hydroxide solution substitutes the drugs bound to the sorbent bed, thereby explaining the increased drug recoveries. The analytes are not retained on the sorbent bed, but are eluted instead. The removal of the other wash steps did not seem to have a major effect on the drug recovery of any of these three analytes. An attempt was made to alter the wash steps to influence the cleanliness of the sample (i.e. reduce the matrix effects that made the results for methadone, morphine and codeine unacceptable), improve drug recoveries to avoid collecting both elution fractions, and finally to improve reproducibility of extraction results.

Table 3-33 Process efficiency (%), matrix effect (%), and recovery (%) results obtained when wash steps were eliminated from the extraction procedure BE1 for oral fluid collected with the NeoSAL™ collection device

Analyte	Eliminated Step												Full Procedure (BE1)		
	Deionised Water			0.01M Acetic Acid			MeOH			Acetone:DCM (1:1)					
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	79.9	3.6	77.1	80.2	5.4	76.1	75.7	6.6	71.0	87.0	0.6	86.5	66.5	-32.8	99.0
COD	140.4	58.1	88.8	146.0	93.0	75.7	140.6	94.0	72.5	164.9	93.3	85.3	63.0	-12.3	71.8
DHC	58.4	-2.7	60.0	63.3	-2.7	65.0	60.6	-2.4	62.1	71.3	-4.2	74.4	55.4	-5.4	58.5
DIAZ	41.3	3.9	39.7	0.6	5.1	0.6	11.2	4.3	10.7	98.1	-6.8	105.2	19.2	-20.4	24.2
DMD	67.5	0.0	67.5	6.9	-1.9	7.0	27.5	-6.8	29.5	75.9	-21.4	96.7	44.2	-17.0	53.3
ETIZ	41.9	19.7	35.0	0.8	19.3	0.7	11.4	21.5	9.4	100.5	18.6	84.7	27.8	-19.6	34.5
METH	98.6	-4.1	102.9	103.4	1.1	102.3	77.5	-9.8	86.0	43.7	-43.4	77.3	57.0	-41.9	98.1
MOR	30.9	27.8	24.2	28.0	36.6	20.5	24.7	29.2	19.1	2.2	-97.8	98.5	10.5	-20.5	13.2
OXA	35.4	3.6	34.2	3.7	6.5	3.5	6.9	5.8	6.5	80.6	-1.7	81.9	18.1	-14.2	21.1
TEMAZ	13.9	1.4	13.7	2.0	2.7	2.0	2.9	-0.3	2.9	94.4	-5.3	99.8	4.6	-18.4	5.6

Optimisation of elution solvents was attempted following the presented results. The use of these solvents for the elution step (solvents were substituted for the acetone:DCM (1:1) elution step) and the analysis of both the "new elution" as well as the subsequent elution using EtOAc and 2% ammonium hydroxide showed no improvement of drug recovery on the whole. Drug recoveries improved for temazepam and oxazepam when cyclohexane was used prior to the elution with EtOAc with 2% ammonium hydroxide (55.5% and 57.1%, respectively), especially when compared to initial results when the acetone:DCM fraction was discarded.

An elution using 3 mL of acetone:DCM (1:1) followed by an elution with 2 mL of DCM:IPA:NH_4OH (i.e. BEC5) showed recoveries in the DCM:IPA:NH_4OH fraction exceed 50% for all analytes except temazepam and oxazepam. The analysis of the acetone:DCM fraction showed that both analytes were eluted here.

As none of the other solvents tested gave better results than the original solutions used, it was decided that the elution fractions would be combined rather than being collected and analysed separately. Analysis was carried out on the combined fraction sample and compared to the results obtained from separated fraction samples. Unexpectedly, the matrix effect for desmethyldiazepam improved when the eluents were combined, although was still high at 27.4%. As this method proved most fruitful, this was carried out for expectorated (Table 3-34) and oral fluid collected using the NeoSAL™ device (Table 3-35).

Analyte recoveries for all analytes in neat and buffered oral fluid were acceptable when the elution fractions were combined. Higher matrix effects were observed for temazepam and desmethyldiazepam. However it was determined that owing to the use of a deuterated internal standard for both analytes, these results would be tolerable.

Table 3-34 Analytical results for combined and separate elution steps observed for expectorated oral fluid, BE5

Analyte	Elution 1 (Acetone:DCM 4 mL)			Elution 2 (DCM:IPA:NH_4OH 2 mL)			Combined		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	N/A	-5.9	N/A	78.1	2.5	76.2	87.5	1.4	86.3
COD	N/A	-2.8	N/A	69.6	-0.1	69.6	74.6	1.5	73.5
DHC	N/A	-10.5	N/A	69.8	1.8	68.5	72.3	5.4	68.5
DIAZ	42.2	-1.0	42.6	62.8	10.8	56.6	106.2	8.3	98.0
DMD	46.6	79.4	26.0	131.7	92.2	68.5	130.5	27.4	102.4
ETIZ	30.6	-9.4	33.8	68.5	10.8	61.8	103.5	8.4	95.5
METH	0.3	-15.5	0.3	76.0	0.9	75.2	82.8	5.1	78.8
MOR	N/A	-21.0	N/A	61.0	-12.3	69.5	61.7	-10.9	69.3
OXA	61.6	-2.9	63.4	44.8	19.4	37.5	113.2	17.3	96.5
TEMAZ	107.7	24.9	86.2	25.7	38.9	18.5	134.2	39.1	96.4

Where N/A – not available due to non-present peaks.

Table 3-35 Analytical results for combined and separate elution steps observed for oral fluid collected using the NeoSAL™ device (BE5)

Analyte	Elution 1 (Acetone:DCM 4 mL)			Elution 2 (DCM:IPA:NH_4OH 2 mL)			Combined		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	1.5	-8.2	1.6	96.9	9.2	88.7	84.2	0.1	84.2
COD	N/A	-0.3	N/A	90.7	2.8	88.2	73.1	-0.7	73.6
DHC	0.1	1.0	0.1	92.7	6.2	87.3	72.1	2.0	70.7
DIAZ	44.4	2.8	43.2	45.9	1.7	45.1	87.9	-2.8	90.4
DMD	30.0	-3.6	31.1	79.3	8.8	72.9	127.2	41.7	89.7
ETIZ	25.0	6.8	23.4	60.9	-1.2	61.6	84.0	-3.1	86.7
METH	0.2	-28.2	0.3	7.3	-91.8	88.7	6.4	-91.6	76.1
MOR	2.3	-97.6	98.5	81.9	-5.3	86.5	69.4	-8.9	76.2
OXA	42.1	5.7	39.9	46.7	2.5	45.6	91.8	3.8	88.5
TEMAZ	95.2	14.6	83.1	4.9	2.4	4.8	111.4	24.2	89.7

Where N/A – not available due to non-present peaks.

Matrix effects for desmethyldiazepam increased from 27.4% to 41.7% when neat and buffered oral fluid was used, respectively. Matrix effects for methadone were very high for the buffered oral fluid, and therefore it was decided that

going forward, methadone would not be quantitated, as this would not be possible with such high matrix effects. For all other analytes both recoveries and matrix effects were found to be acceptable.

Before issues with reproducibility became a major concern with this method for neat oral fluid, it was applied to the oral fluid samples collected using the NeoSAL™ collection device. The buffer in the collection device is pH 6 and therefore no extra phosphate buffer was added to samples. Initial results of this did not show improved recoveries.

When buffered samples were acidified, the acetone:DCM fraction was not analysed due to the dirtiness (large white flakes, potentially salt from the buffer) of the reconstituted samples. Therefore, results below show the analytes present in the EtOAc 2% ammonium hydroxide fraction only. Recoveries observed for the benzodiazepines in the NeoSAL™ buffered oral fluid were why the buffer was then acidified to potentially aid the release and ionisation states of these analytes. Low recoveries found for morphine can be explained as previous results showed improved recoveries when using DCM:IPA:NH_4OH for the elution.

The use of a higher molarity of formic acid especially improved recoveries of desmethyldiazepam and diazepam. Recoveries increased from 53.3% to 76.5% for desmethyldiazepam following the addition of 1mL of 1.25M formic acid, and diazepam recoveries increased to 61.0% (from 24.2% originally). Matrix effects observed for methadone also improved following the addition of acid (-41.9% with no addition, to -2.4% when 1 mL of 2M formic acid was added). These results are shown in Table 3-36 and Table 3-37.

Table 3-36 Analytical results for the EtOAc with 2% ammonium hydroxide fraction for unacidified NeoSAL™ oral fluid, and acidified buffered oral fluid (extraction procedure BE1)
Oral fluid collected using the NeoSAL™ device was acidified with 2, 1, 1.25, 0.5, and 0.001M formic acid

Analyte	NeoSAL			1.25 M FA (1 mL)			1.25 M FA (3.5 mL)			2.0 M FA (1 mL)			1.0 M FA (1 mL)			0.5 M FA (1 mL)			0.001 M FA (1 mL)		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	66.5	-32.8	99.0	62.4	-28.0	86.7	60.4	6.5	56.7	59.4	-0.4	59.6	62.2	11.2	55.9	65.7	7.8	60.9	62.2	3.5	60.1
COD	63.0	-12.3	71.8	66.7	-13.1	76.8	72.6	-0.4	72.9	70.9	2.7	69.0	60.5	3.8	58.2	62.1	7.4	57.8	63.4	1.4	62.6
DHC	55.4	-5.4	58.5	57.5	-4.0	59.9	43.7	0.9	43.3	43.3	2.9	42.1	45.1	-1.1	45.6	49.5	0.3	49.4	42.4	-0.8	42.7
DIAZ	19.2	-20.4	24.2	47.8	-21.6	61.0	55.2	1.5	54.4	57.3	1.1	56.7	35.1	6.5	33.0	36.5	8.2	33.7	37.7	-5.9	40.1
DMD	44.2	-17.0	53.3	64.2	-16.1	76.5	64.0	-12.0	72.7	67.1	-5.7	71.1	63.1	-1.7	64.2	66.8	-4.5	69.9	49.2	-16.9	59.2
ETIZ	27.8	-19.6	34.5	51.6	-19.0	63.6	72.1	1.1	71.3	71.7	2.2	70.1	54.4	6.0	51.3	56.8	7.9	52.6	40.3	-2.3	41.3
METH	57.0	-41.9	98.1	62.5	-44.9	113.5	73.1	-1.1	73.9	73.8	-2.4	75.7	81.2	2.5	79.3	82.9	2.2	81.1	67.9	-10.1	75.5
MOR	10.5	-20.5	13.2	18.0	-19.9	22.4	9.4	3.3	9.1	8.2	3.1	7.9	12.0	15.9	10.4	14.7	23.5	11.9	13.5	6.3	12.7
OXA	18.1	-14.2	21.1	25.0	-14.3	29.2	43.4	-1.8	44.2	40.1	1.1	39.6	32.6	5.4	30.9	29.7	-0.3	29.7	34.1	2.4	33.3
TEMA	4.6	-18.4	5.6	11.6	-16.4	13.9	25.6	-4.4	26.8	28.0	-1.5	28.5	16.2	8.2	15.0	11.5	3.4	11.1	19.2	-3.2	19.9

Where FA – formic acid.

Table 3-37 Analytical results for the EtOAc with 2% ammonium hydroxide fraction for oral fluid collected using the NeoSAL™ device, acidified with 0.001, 0.0001 M formic acidand basified with 2% ammonium hydroxide (extraction procedure BE1)

Analyte	0.001 M FA (1 mL)			0.0001 M FA (1 mL)			2% NH_4OH (1 mL)		
	PE%	ME%	R%	PE%	ME%	R%	PE%	ME%	R%
6MAM	62.2	3.5	60.1	69.2	5.0	65.9	68.5	4.3	65.7
COD	63.4	1.4	62.6	69.1	1.6	68.1	65.3	0.2	65.2
DHC	42.4	-0.8	42.7	50.4	0.8	50.0	60.3	-1.6	61.2
DIAZ	37.7	-5.9	40.1	38.8	-6.9	41.6	10.3	-6.9	11.1
DMD	49.2	-16.9	59.2	65.6	-21.2	83.2	25.1	-10.3	28.0
ETIZ	40.3	-2.3	41.3	45.0	-2.3	46.1	18.0	-3.6	18.7
METH	67.9	-10.1	75.5	69.2	-21.0	87.6	69.7	-6.3	74.4
MOR	13.5	6.3	12.7	13.7	7.9	12.7	4.5	5.2	4.3
OXA	34.1	2.4	33.3	35.7	0.9	35.4	11.1	4.1	10.7
TEMAZ	19.2	-3.2	19.9	10.5	-5.0	11.0	4.7	-1.4	4.8

Where FA – formic acid; NaOH – sodium hydroxide.

A reproducibility study for expectorated oral fluid was carried out following increased variation observed in recovery and matrix effect results. It was initially only carried out for neat oral fluid to conserve collection devices. Analysis of quadruplicate samples was carried out over 5 consecutive days. Mean drug recovery (Figure 3-18) and mean matrix effects for all analytes over five runs are shown in Figure 3-19.

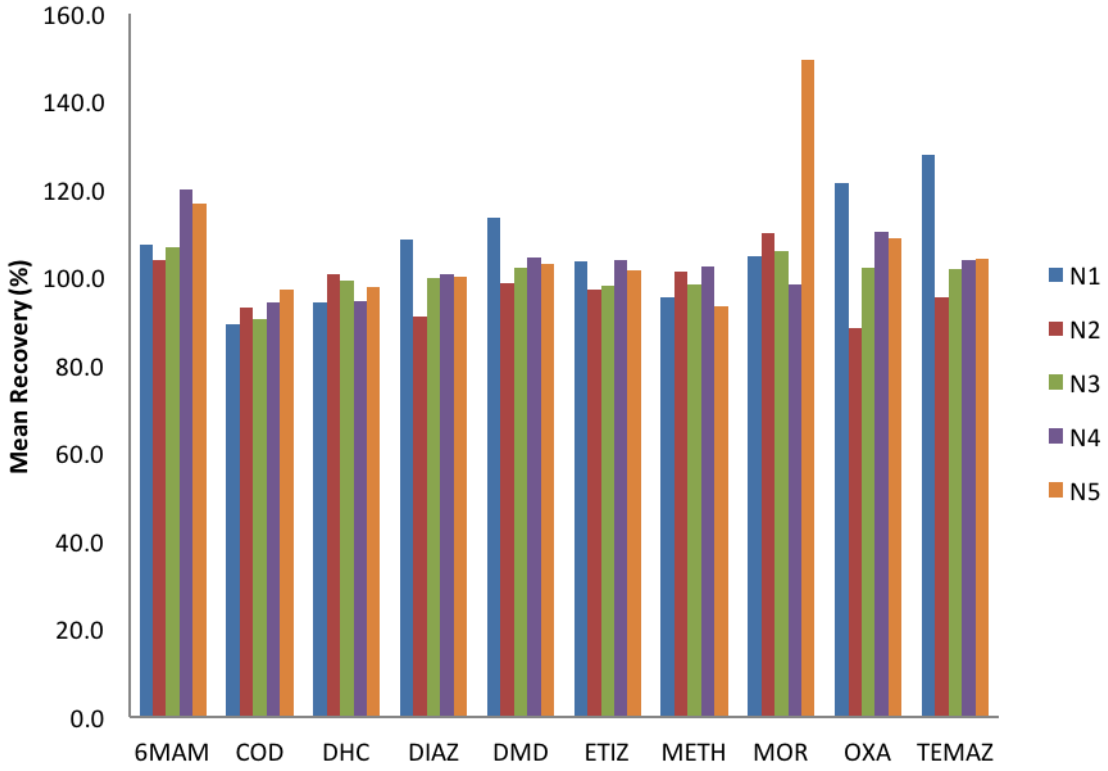


Figure 3-18 Mean recovery (%) from neat oral fluid over five consecutive runs N1-N5 represents the run number (*n* = 4)

Recoveries that ranged between 85 and 115% were deemed 'acceptable'. Although recoveries for all drugs were acceptable for all five runs (with the exception of morphine in run 5 with a recovery of 149.4%, and oxazepam and temazepam in run 1 with recoveries of 121.5% and 128.0%, respectively), variation was observed. Oxazepam showed greatest variation with recoveries ranging from 88.4% to 121.5%, a difference of 33.1%. Morphine recoveries showed a %CV of 18%, whereas oxazepam recoveries showed a %CV of 11%.

The greatest variation, and inevitably the reason why this extraction method was not chosen for the rest of the analysis, was seen for matrix effects. Matrix effects for morphine ranged from -97.3 to 36.2%. Variation is shown in Figure 3-19. However it is important to consider that for this initial test, only one source of oral fluid was used. This suggests that the observed variation could be due to the extraction technique rather than the oral fluid matrix itself.

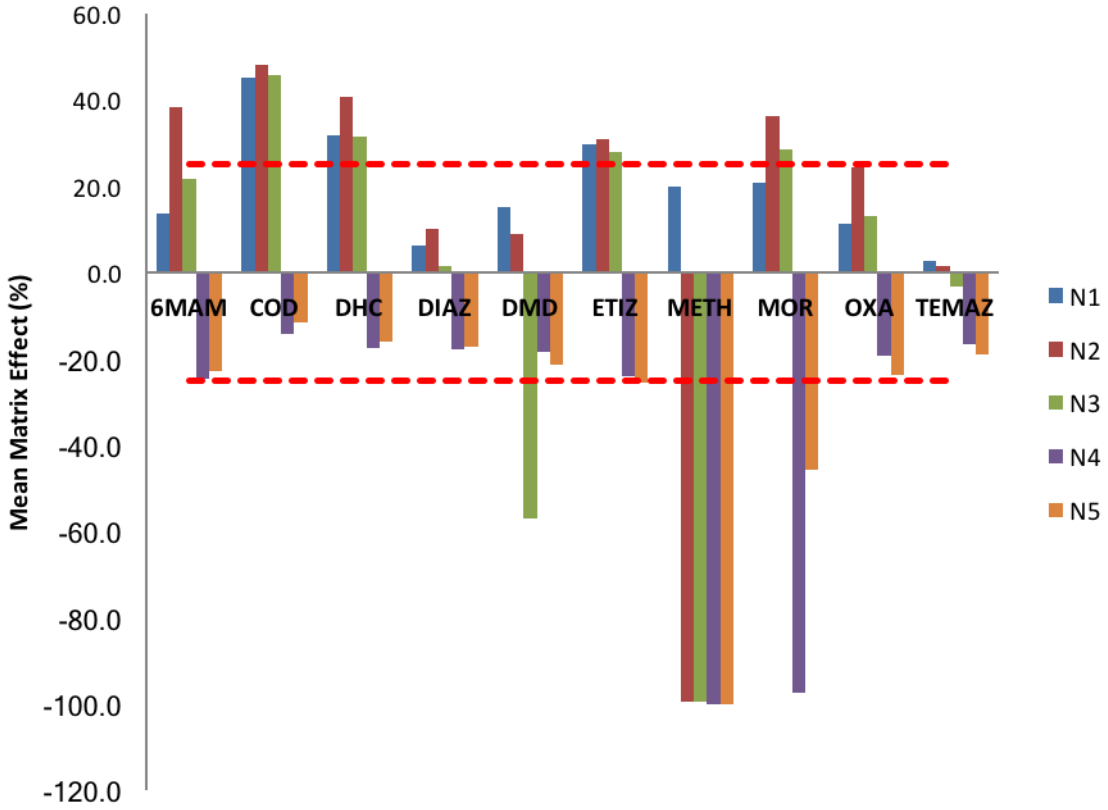


Figure 3-19 Mean matrix effect (%) from neat oral fluid over five consecutive runs N1-N5 represents the run number (*n* = 4)

The pattern that is followed for most analytes is that ion enhancement was observed over the first three runs and ions were suppressed for the latter two. Although variation is unavoidable, especially for a matrix like oral fluid, it is unclear why these results were obtained. The lack of reproducibility meant that the method could not be further validated.

3.3.4.3 SPE/SLE+ Using Biotage Columns

Two SPE products from Biotage were tested: Isolute® SLE+ 400 µL as well as the Isolute® HCX-5 SPE cartridges. Matrix Effect and recovery data for both extraction techniques are shown in Figure 3-20.

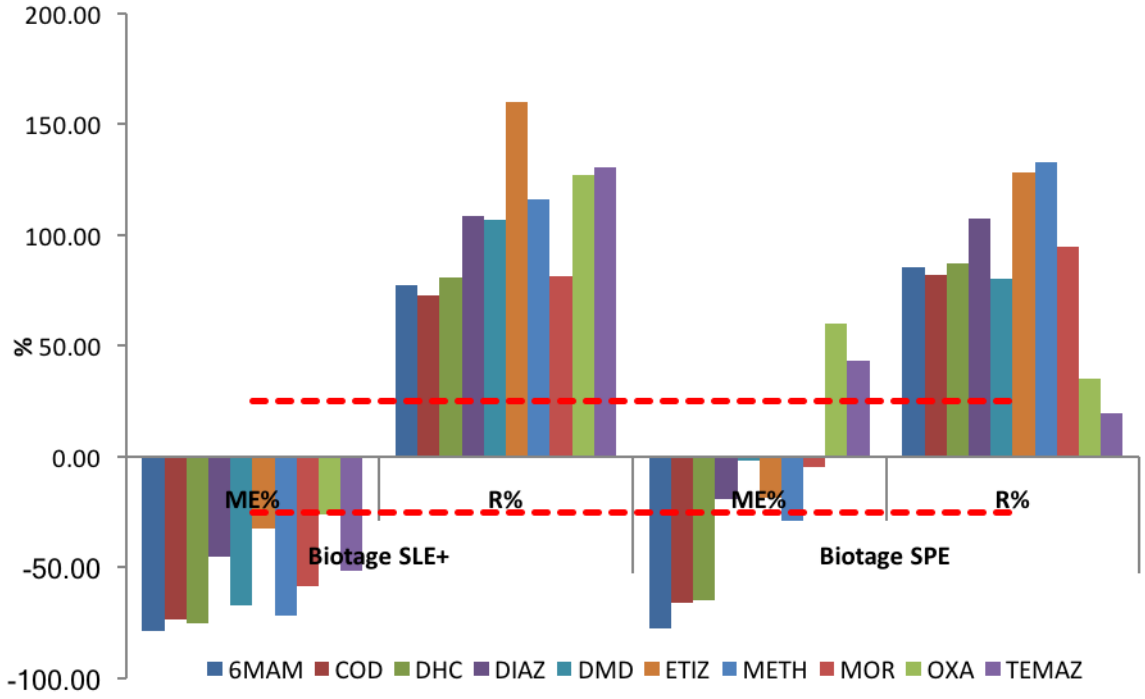


Figure 3-20 Recovery (%) and matrix effect (%) for analytes using Biotage SLE+ and Biotage HCX-5 SPE cartridges

Table 3-38 below summarises the values corresponding to Figure 3-20. From the table it can be seen that all matrix effect results obtained for SLE+ are unacceptable as they are outwith the acceptable range of ±25%. The majority of results exceeded 50% ion suppression (8 out of 10 analytes). A possible explanation for the observed matrix effects is that the SLE+ method is very simple. It includes very few wash steps, and therefore provides only limited sample clean-up. With a lack of wash steps there is an increased likelihood that the method is not able to remove all the matrix components that cause ion suppression or enhancement.

The recovery observed for etizolam was the highest (>150%) out of all the analytes. One possible explanation for the results obtained is that etizolam is the only analyte that does not have its own deuterated internal standard, but rather deuterated diazepam is used. Etizolam results improved fractionally when extracted using the HCX-5 columns, but recoveries still exceeded 125%.

Recoveries for the opiate/opioid drugs (with the exception of methadone) were acceptable, however benzodiazepine analyte recoveries exceed 100% and were therefore not confidently accepted.

Table 3-38 Summary of matrix effect (%) and recovery (%) results obtained for Biotage Extractions

Analyte	Isolute® SLE+ 400 µL			Isolute® HCX-5 SPE		
	%PE	ME%	R%	%PE	ME%	R%
6MAM	16.4	-78.8	77.3	18.9	-77.9	85.3
COD	19.1	-73.7	72.5	27.8	-66.2	82.2
DHC	20.1	-75.2	81.0	30.6	-64.8	86.9
DIAZ	59.5	-45.2	108.5	87.3	-19.0	107.7
DMD	35.0	-67.2	106.8	78.8	-1.9	80.3
ETIZ	107.8	-32.6	159.9	104.7	-18.4	128.3
METH	32.9	-71.6	115.8	94.1	-29.2	132.9
MOR	33.8	-58.5	81.3	90.3	-4.9	94.9
OXA	94.2	-25.9	127.1	56.0	60.0	35.0
TEMAZ	62.9	-51.8	130.6	27.7	43.5	19.3

Recoveries for the Isolute® HCX-5 SPE cartridges were good (i.e. were greater than 80%) for the opiate drugs. The recoveries for diazepam, etizolam, and DMD were lower than for the SLE+ extraction, but were more acceptable. Oxazepam and temazepam recoveries were very low (35.0% and 19.3%, respectively). Matrix effects were still unacceptable as they exceeded ±25% for the majority of analytes (with the exception of diazepam and desmethyldiazepam and morphine) and ranged from -77.9% for 6-MAM to 60.0% for oxazepam. The Isolute® HCX-5 SPE columns have a C4 sorbent bed, where the UCT Clean Screen® columns have C8 sorbent beds; therefore it is possible that the sorbent beds are playing a role in the extraction efficiencies.

3.3.4.4 SPE Using Waters Columns

Two SPE cartridges manufactured by Waters Scientific were tested, viz Oasis® MCX and HLB PRiME cartridges. The matrix effects are visually presented in Figure 3-21. Both cartridges were only evaluated using neat oral fluid.

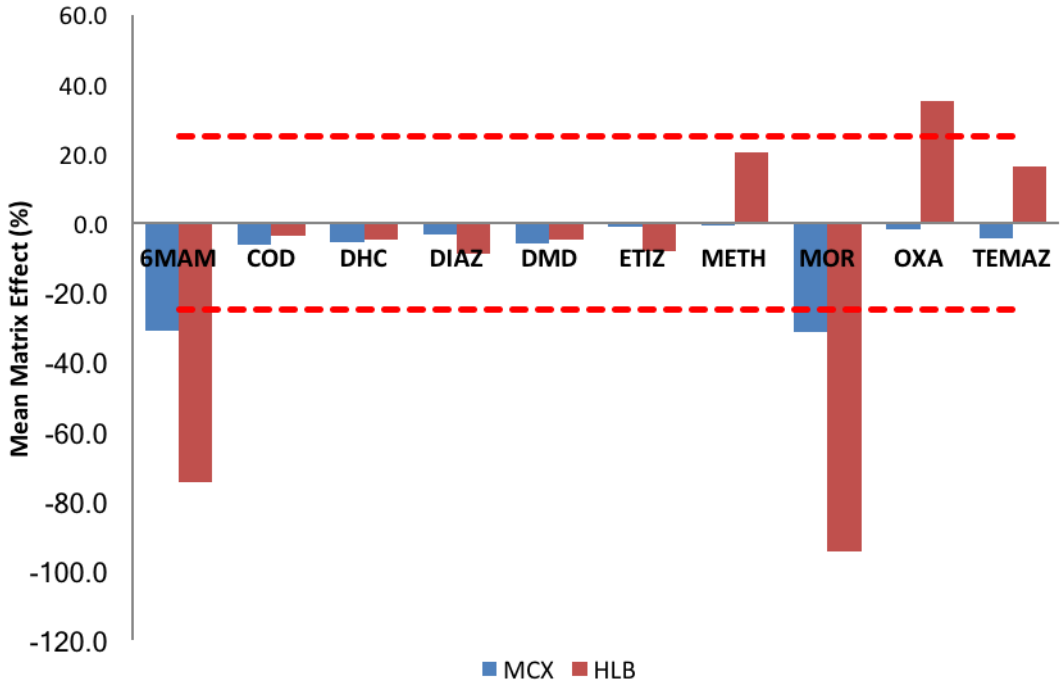


Figure 3-21 Mean matrix effect (%) results for Waters Oasis® MCX and Oasis® PRiME HLB SPE cartridges (*n* = 4)
Where the red dotted lines indicate the accepable limits of ±25% matrix effects

Matrix effects (%) ranged from -6.3% to -0.8% for all analytes except 6-MAM and morphine (30.9% and -31.2%, respectively) when extracted using the MCX cartridges. The HLB PRiME cartridges showed similar results in that matrix effects were unacceptable for both 6-MAM and morphine (-74.4% and -94.5%, respectively) but to a greater extent than the MCX cartridges. Oxazepam showed matrix effects exceeding the accepted ±25% range with matrix effects of 35.1%. All other matrix effects were acceptable, although in general slightly greater than those observed for the MCX cartridges.

Recoveries were similarly good for the majority of analytes, as shown in Figure 3-22.

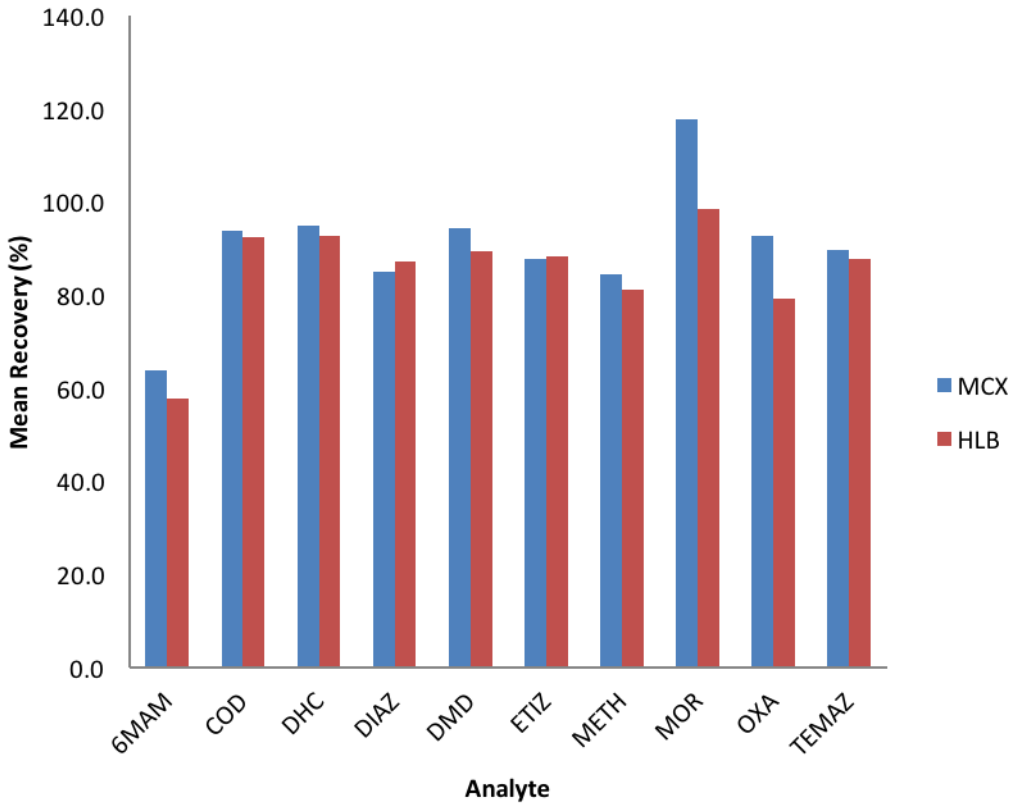


Figure 3-22 Mean recoveries (%) observed for Waters Oasis MCX and Oasis® PRiME HLB SPE columns

The lowest recoveries for both cartridgeswere observed for 6-MAM (63.8% with MCX and 57.7% using the PRiME HLB cartridges). Although these recoveries would be acceptable if reproducible, this analyte is important and higher recoveries would be desired. Low recovery values may have resulted from the ion suppression that is exhibited for this analyte in both extractions. Overall the MCX cartridge gave better results compared to the HLB column.

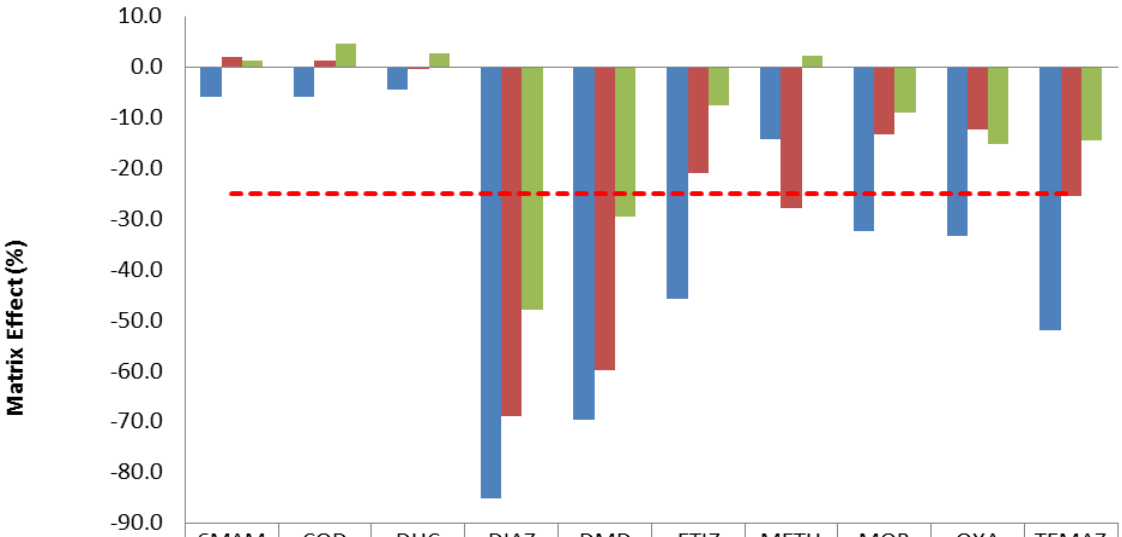
However, although the results for recovery were very good, the use of either of these columns in a routine laboratory would be costly, as these are over double the price of other SPE cartridges. It was therefore decided that a different procedure using different, more cost-effective SPE cartridges would be chosen. Furthermore, 6-MAM and morphine also showed about 30% ion suppression which is not ideal.

3.3.5 Sample Reconstitution

Initially samples were reconstituted in 250 µL of mobile phase when recovery and matrix effect studies were completed at 20 ng/mL. Following initial matrix

effect tests, as well as sensitivity assessments including linearity tests and LOD evaluations, the reconstitution volume was increased to 1000 µL of mobile phase. This reduced matrix effects, especially for diazepam, to an acceptable level, for the extraction procedure that was finally chosen for the analyses. Reconstitution in 500 µL showed less acceptable matrix effects and even with an increased injection volume did not give results that were better than results obtained at the 1000 µL reconstitution volume.

An example of the effect the reconstitution volume had on matrix effects is shown below in Figure 3-23.



	6MAM	COD	DHC	DIAZ	DMD	ETIZ	METH	MOR	OXA	TEMAZ
250 µL	-5.9	-6.0	-4.6	-85.2	-69.8	-45.9	-14.2	-32.5	-33.4	-52.1
500 µL	1.9	1.1	-0.4	-69.1	-59.8	-21.0	-27.9	-13.3	-12.4	-25.6
1000 µL	1.2	4.6	2.6	-47.9	-29.5	-7.5	2.2	-9.0	-15.3	-14.5

Figure 3-23 Effect of reconstitution volumes on observed matrix effect (%)

The results shown are for the LLE extraction mentioned previously employing 3 mL of EtOAc as extraction solvent, 50 µL of deionised water to MeOH (in a ratio of 1:1), and 100 µL of 0.1 M sodium hydroxide solution. Samples were shaken for 20 minutes. Although the observed matrix effects (%) for diazepam, and desmethyldiazepam, were still unacceptable, effects improved when the reconstitution volume was increased. The same was observed for all other analytes. For etizolam, morphine, oxazepam, and temazepam the matrix effects improved so much that they became acceptable when a reconstitution volume of 1000 µL was used.

Before using the increased reconstitution volume, limits of detection were assessed for the lowest calibrators. Although the LOD increased from its original concentration, it was still considered acceptable for the concentration ranges assessed. Thus it was decided that a reconstitution volume of 1000 µL was acceptable and beneficial to the extraction procedure.

3.3.6 Summary of Extraction Procedure Selected for Further Work

Although all tested extractions had both advantages and disadvantages over other extractions, the extraction procedure that was finally chosen was UCT7, as this gave the most reproducible and acceptable results for all analytes. Even though some recoveries were low, they were reproducible and sufficed to reach recommended cut-offs.

To expectorated oral fluid samples, 2 mL of 0.1M pH6 phosphate buffer was added. No extra buffer was added to NeoSAL™ oral fluid samples. Samples were vortex mixed for a minimum of 5 seconds and then centrifuged for 10 minutes at 3000 rpm.

SPE columns were conditioned sequentially with 3 mL of each MeOH, deionised water, and 0.1 M pH 6 phosphate buffer. Samples were loaded onto the UCT ZDAU020 extraction cartridgesand were allowed to pass through completely. 3 mL of acetate buffer (pH 4.5) was added, followed by 3 mL of dichloromethane. Cartridgeswere dried under full vacuum for 10 minutes. Before eluting with 3 mL of EtOAc with 2% ammonium hydroxide, tips were cleaned and excess solution removed. Cartridgeswere dried under full vacuum for a further two minutes after the first elution step. 3 mL of DCM:IPA:NH_4OH (78:20:2 v/v) were added and eluted into the same vials as the first elution.

Samples were then dried under a gentle stream of nitrogen at <40 °C, and were reconstituted in 1000 µL of 2.5% MeOH in deionised water with 0.1% formic acid and 2 mM ammonium acetate.

3.4 Conclusion

A sensitive LC-MS/MS method for the simultaneous detection and quantification of 9 benzodiazepines and opioid drugs (namely, diazepam, desmethyldiazepam, etizolam, oxazepam, temazepam, morphine, 6-MAM, codeine, and dihydrocodeine) was developed for expectorated oral fluid and oral fluid collected using the NeoSAL™ collection device.

Several LLE and SPE procedures were tested for the simultaneous extraction of benzodiazepine and opioid drugs. Matrix effects and drug recoveries were tested, and both proved a problem for certain analytes: methadone was particularly affected by matrix effects, whereas recoveries for temazepam and oxazepam were usually low. Morphine recoveries were very low until the elution solution was optimised.

LLE methods tested showed some promise, in the sense that certain alterations to methodologies showed improved recoveries and reduced matrix effects for certain analytes. However due to time limitations, it was not possible to optimise these to a suitable level for validation purposes.

A lot of optimisation was carried out using the BE1 procedure using the Bond Elut Certify® cartridges. Reproducibility issues arose, and these were most likely caused by the variation in the packing material of these SPE cartridges. The FMS laboratory has had previous issues with this, as they had been using these cartridges for the extraction of buprenorphine until a sudden, and unexplained, drop in recovery and poor reproducibility caused them to change their extraction procedure.

The most promising extraction techniques were SPE using UCT7 method, as well as the SPE extractions using Waters Oasis MCX and Oasis® PRiME HLB SPE columns. The UCT7 procedure was finally chosen for further validation, as the Oasis® cartridges were over double the price, which was not reasonable for further work.

The UCT7 method showed the most promising results with the fewest compromises, and after a initial method validation experiments it was deemed

the most acceptable method. The recovery of morphine improved considerably when elution was carried out using DCM:IPA:NH_4OH (78:20:2 v/v). While this method did have lower recoveries for diazepam, it was decided that this was an acceptable compromise. Matrix effects for methadone in both oral fluid compositions were too variable and it was therefore decided that the quantification of methadone would not be possible.

3.5 Future Work

One of the main issues with the extraction and procedure outlined above is the low recovery for diazepam. Due to its frequent use and prescription in the UK, an extraction yielding a higher drug recovery would be beneficial. However, due to time constraints, the low recoveries with matrix effects varying within ±10% (within the acceptable criteria of ±25%) were deemed acceptable and there was no concern with false negatives or positives as a result of this.

Furthermore, it could be beneficial to optimise the analytical method so that the run time is shortened. For busy laboratories, this would be ideal, as a 27 minute run time, although acceptable, is on the long side.

A further investigation should be made into the effectiveness of different chromatographic columns for this study. Although the Gemini C18 column gave good, reproducible results, there are other columns with other compositions and particle sizes that would be worth investigating. Changing column specifications may aid improved retention, chromatography and peak shape. Using a different column could also improve the retention time of analytes like morphine, for example. Retention times were acceptable as they did not show much shifting; however, in some instances a later retention time is preferred. Although it was attempted to achieve this by altering mobile phase compositions with no dramatic improvement, changing columns could provide a more significant change.

The inclusion of other analytes would be beneficial to widen the scope of the method and, in turn, its applicability.

4 Method Validation of Simultaneous Extraction and Analysis of Benzodiazepine and Opioid Drugs from Oral Fluid Using LC-MS/MS

4.1 Introduction

This chapter assesses the validity of the previously developed method for the simultaneous extraction and analysis of diazepam, desmethyldiazepam, etizolam, oxazepam, temazepam, morphine, 6-monoacetylmorphine (6-MAM), codeine, dihydrocodeine, and methadone.

The method validation was carried out according to the SWGTOX guidelines (161). Assessments of linearity, accuracy and precision, limits of detection and quantification, carry over, interferences, ion suppression and enhancement (Matrix Effect), dilution integrity, and stability were carried out.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

Chemicals and reagents previously described in Section 3.2.1 were used for the method validation.

4.2.2 Preparation of Solutions and Reagents

All stock, and working solutions, as well as all reagents used for SPE were prepared as previously described in Section 3.2.2. Oral fluid was collected as outlined in Section 3.2.4.

4.2.2.1 Preparation of Calibrators and Quality Controls

Calibration solutions were prepared from the stock solutions (10 µL/mL). Quality Controls (QC) were prepared from separately prepared stock solutions at the same concentration. The solutions were selected in a way that spiking 700 µL of oral fluid with 70 µL of working solution would give the final concentrations listed in Table 4-1. Two volumes of working solutions are described below, 10 mL and 20 mL. QC1 was prepared by adding 8.4 µL of each drug stock solution to a 20 mL volumetric flask and then making to the mark with MeOH. A volume of

10 mL was not prepared for Cal 1 or QC1 as the volumes to pipette were too low and it would not be possible to accurately pipette these. Therefore, 20 mL were prepared, as outlined below.

Table 4-1 Preparation of calibration and Quality Control (QC) solutions

Calibrator	Volume (µL) of stock solution (10 µg/mL) added to volumetric flask		Working Solution Concentration (ng/mL)	Final Concentration (ng/mL)
	10 mL	20 mL		
Calibrators				
Cal 1	-	5.6	10.0	1.0
Cal 2	7	-	25.0	2.5
Cal 3	14	-	50.0	5.0
Cal 4	28	-	100.0	10.0
Cal 5	70	-	250.0	25.0
Cal 6	140	-	500.0	50.0
Cal 7	280	-	1000.0	100.0
Quality Controls (QC)				
QC1	-	2.1	15.0	1.5
QC2	10.5	-	150.0	15.0
QC3	56	-	800.0	80.0
QC4	21	-	30.0	3.0

QC concentrations were selected following SWGTOX guidelines, with QC3 being 80% of the highest calibrator and QC1 being 3 times of lowest working range. The lowest calibrator was changed from a final concentration of 0.5 ng/mL to 1 ng/mL due to sensitivity issues and therefore QC4 was introduced at three times of the new low working range of 1 ng/mL. QC4 was prepared to assess matrix effects and recoveries, as well as stability. QC1 was used for bias and precision measurements, and is lower than QC4 as in initial calibration models the lowest calibration point used was 0.5 ng/mL. All concentration calculations were done in a way that took the collection volume of 0.7 mL of oral fluid into consideration such that the reported concentrations were in ng/mL.

4.2.3 Oral Fluid Collection

Oral fluid was collected in the same manner as described in 3.2.4. Expectorated oral fluid (or "neat oral fluid", latterly also referred to as "neat") was collected into a plastic tube, and frozen prior to analysis. Oral fluid collected using the NeoSAL™ device ("buffered oral fluid") was collected as required, and if required, stored in the fridge as recommended by the manufacturer.

4.2.4 SPE Cartridges

United Chemical Technologies (UCT) cartridges were obtained from Chromatography Direct Ltd (Runcorn, UK) or supplied by UCT (PA, USA). Clean Screen® ZDAU020 columns were used for the extraction.

4.2.5 Instrumentation

Instrumentation and software used were previously outlined in Section 3.2.6.

The LC-MS/MS parameters that were developed in the preceding chapter were used for the method validation and are summarised in Table 4-2.

Table 4-2 Summary of LC-MS/MS parameters

LC Parameters	
Column	Phenomenex Gemini® 5 µm C18 110 Å (150 x 2 mm) with Phenomenex Gemini® C18 guard column (40 x 2.0 mm)
Mobile Phase	Gradient profile shown in Figure 4-1
Column Temperature	40 °C
Flow Rate	0.3 mL/min
Mobile Phase (A)	Deionised water with 2 mM ammonium acetate and 0.1% formic acid
Mobile Phase (B)	Methanol with 2 mM ammonium acetate and 0.1% formic acid
MS Parameters	
Operating Mode	Positive ionisation mode
Nebulizer Pressure	15 psi
Gas Flow	11 L/min
Gas Temperature	300 °C
Capillary Voltage	4000 V

The gradient profile used for the analysis is shown below in Figure 4-1.

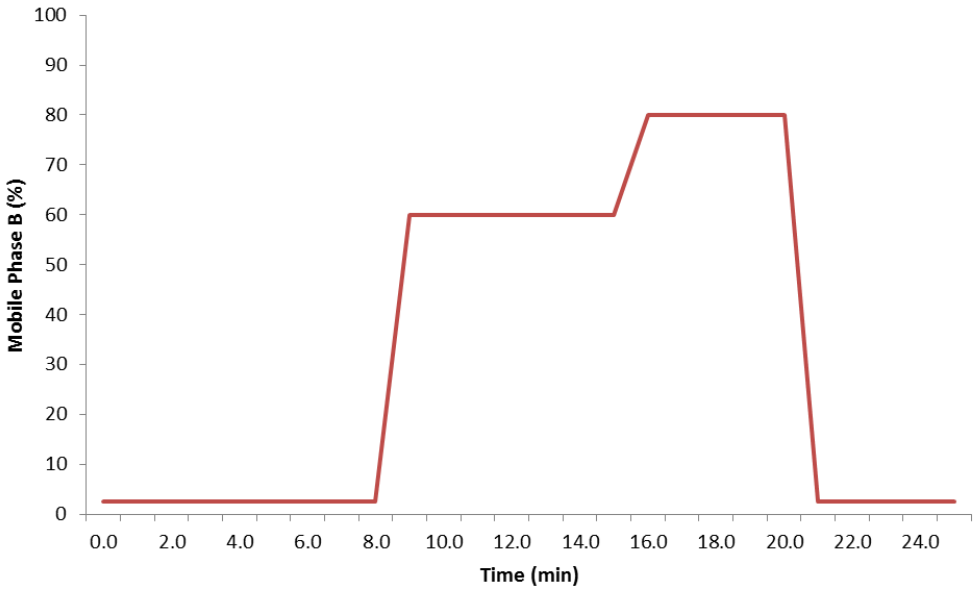


Figure 4-1 Mobile phase gradient system

4.2.6 SPE Procedure for Sample Extraction

The SPE procedure summarised in Section 3.3.6 was selected

To neat oral fluid samples, 2 mL of 0.1 M pH 6 phosphate buffer was added. No extra buffer was added to NeoSAL™ oral fluid samples, as these contain collection buffer. Work carried out in Section 3.2.11.1 did not show that the addition of buffer was beneficial for these samples. Samples were vortex mixed for a minimum of 5 seconds and then centrifuged for 10 minutes at 3000 rpm.

Clean Screen® ZDAU020 SPE cartridges were sequentially conditioned with 3 mL each of MeOH, deionised water, and 0.1M pH6 phosphate buffer. Samples were loaded onto the extraction cartridges and were allowed to pass through completely. 3 mL of acetate buffer (pH 4.5) was added, followed by 3 mL of dichloromethane. Cartridges were dried under full vacuum for 10 minutes. Before eluting with 3 mL of EtOAc with 2% ammonium hydroxide, tips were cleaned and excess solution removed. Cartridges were dried under full vacuum for a further two minutes after the first elution step. 3 mL of DCM:IPA:NH_4OH (78:20:2 v/v) were added and eluted into the same vials as the first elution.

Samples were then dried under a gentle stream of nitrogen at 36 °C. Reconstitution occurred in 1000 µL of 2.5% MeOH in deionised water with 0.1% formic acid and 2 mM ammonium acetate.

4.3 Method Validation

4.3.1 Calibration Model and Linearity

Linearity is assessed by injecting a series of calibrators, prepared freshly, over five consecutive runs. The linear range is deemed acceptable when the linear regression equation, correlation efficient (R^2) is a minimum of 0.99 when plotting peak area ratios (PAR) versus spiked concentration.

Linearity was initally assessed over a range spanning from final concentrations of 0.5 ng/mL to 100 ng/mL for both neat and buffered oral fluid. The lowest calibration point was changed to 1 ng/mL due to a lack of sensitivity. Residual plots were created to assess actual linearity.

4.3.2 Limits of Detection and Limits of Quantitation

Using a serial dilution, oral fluid was spiked at decreasing concentrations. Initially, only concentrations down to 0.5 ng/mL were tested, but due to the high S/N ratios shown by the benzodiazepine drugs, this was further decreased to 0.1 ng/mL. Concentrations of 0.8, 0.6, 0.5, 0.4, 0.2, and 0.1 ng/mL were assessed. LOD and LLOQ were assessed in duplicate over four consecutive runs.

4.3.3 Accuracy and Precision

Intra- and inter-day accuracy and precision were assessed for both neat and buffered oral fluid over a total of 5 runs at three concentrations; 1.5 ng/mL (QC1, “Low”), 15 ng/mL (QC2, “Medium”) and 80 ng/mL (QC3, “High”), respectively. QC samples were prepared as outlined in Section 4.2.2.1 in quadruplicate and were run alongside a freshly prepared calibration curve to be used to calculate the concentrations.

4.3.4 Drug Recovery from Collection Device

Drug recoveries from the NeoSAL™ collection device were assessed in a similar manner as to what had been previously done in Section 2.4.7.1. Oral fluid was spiked at two concentrations, namely 3 ng/mL (low concentration) and 80 ng/mL (high concentration). QC solutions were used to spike oral fluid. Collection pads

were either dipped into the spiked oral fluid, or 0.7 mL of spiked oral fluid was pipetted onto the collection pad.

The drug recovery from the collection device was assessed using Equation III, Equation IV, and Equation V in 3.2.9.

4.3.5 Recovery and Matrix Effect

Matrix effect/factor was evaluated as part of the selection of the most fitting extraction procedure, and was assessed as previously stated using the Matuszewski method (155), using the equations detailed in Equation III, Equation IV, and Equation V in Section 3.2.9, where neat refers to an unextracted standard (UES), "PRE" refers to samples spiked with internal standard before) the extraction, and "POST" refers to samples which were spiked with internal standard after extraction. Ten sources of oral fluid, both buffered and neat, were used to test matrix effects and recovery as suggested by SWGTOX guidelines, all samples were analysed in duplicate.

Matrix effects and recoveries were investigated using QC4 at concentrations of 3 ng/mL ("Low") and 80 ng/mL (QC3; "High").

Recoveries and matrix effects using the NeoSAL™ device were evaluated by spiking oral fluid samples at the relevant concentrations and then dipping the collection pad into the oral fluid. This method, although having previously shown to potentially give higher recoveries, seemed the most representative of the collection device being used in a real-world setting.

4.3.6 Selectivity and Specificity

Potential interferences from blank matrix (n = 10), as well as a number of analytes not included in the method were investigated to determine whether the developed method is specific and selective for the analytes included. An exhaustive list of analytes included in specificity assessments can be found in Appendix III.

4.3.7 Carryover

Carryover was assessed at a concentration of 200 ng/mL to ensure that even at high concentrations, should there also be oral contamination from drug use before the collection of oral fluid, no false positives would result. High concentrations were injected in duplicate and followed with three injections of reconstitution solution. Chromatograms were analysed visually by comparing the total ion chromatogram (TIC) for the injected high concentrations, and the subsequent mobile phase injections were examined for any drug peaks.

4.3.8 Dilution Integrity

Dilution integrity is an important aspect of forensic testing as the concentrations may exceed expected concentrations, especially in cases of overdose or in oral fluid cases due to contamination from orally administered drugs. Although this project does not focus on post-mortem cases, patients may be taking more than their recommended or prescribed dose of medication. In these cases, where concentrations may exceed the ULOQ (highest calibrator), serial dilution may have to be carried out in order to quantify samples. It is an important aspect to study. However, it is likely that sufficient sample for further dilution is not available; especially when only a limited sample volume is available or the whole sample has been used for analysis.

To assess the dilution integrity of samples, blank oral fluid was spiked at a concentration of 200 ng/0.7 mL of oral fluid, and then diluted with further blank oral fluid at 1:2, and 1:5. This was completed in quadruplicate per dilution, and injected alongside fresh calibration standards. Using the regression equation used to assess the linearity, concentrations of the dilutions were calculated. When calculated concentrations do not differ by more than 15% of the nominal concentration and %CV ≤15%, dilution integrity was determined to be maintained.

4.3.9 Stability

A stability study of these compounds is important as there is possible conversion of both 6-MAM and codeine breakdown to morphine over time. This in turn will

cause a falsely increased morphine concentration. Stability of only buffered oral fluid (collected using the NeoSAL™ device) was assessed.

The NeoSAL™ device contains a stabilising buffer; however, a stability study must still be carried out to investigate the stability of drugs when collected using this collection device. Stability was assessed at two concentrations: 80 ng/mL ("High") and 15 ng/mL ("Low"). These concentrations were selected based on SWGTOX recommendations, but also at concentrations high enough to ensure that the peaks could be quantified accurately. The following temperature parameters were investigated:

1. Autosampler stability/processed sample stability (approximately 18 °C) over 72 hours.

2. Benchtop stability at 20.9 °C (±0.2 °C)

3. Fridge stability at 4.6 °C (±1.0 °C)

4. Freezer stability at -22.3 °C (±0.2 °C)

Freezer stability was assessed for the buffered oral fluid samples, although storage at -21 °C is not recommended for the collection device. Autosampler, or processed sample stability is essential to this study as the run time of the analytical method means that the run time of a batch can exceed 24 hours. Therefore it is important to assess whether processed samples at the end of the batch, for example, are still viable and give reliable results.

Buffered oral fluid stability samples were prepared in triplicate for each concentration for each storage condition. Collection device pads were dipped into spiked oral fluid, as the recovery from the pad study showed higher recoveries from dipped pads versus pipetted pads. The saturated pads were then returned to the collection tube and screwed closed. Collectors were inverted five times to ensure mixing of oral fluid and pad, as recommended by the manufacturer. Labelled collection tubes were then placed into labelled test tube racks, and placed on a specific place on the benchtop, in the fridge or in the freezer, until ready for analysis.

Samples were removed from the fridge or freezer and were allowed to thaw or come to room temperature prior to analysis. Internal standard (70 µL of a 100 µg/mL working solution) was added to labelled test tubes prior to the extraction. A fresh calibration was prepared each day of the stability study. Due to the long run time of the analytical method, and in order to not delay analysis of stability samples, only two QC were run alongside samples and calibration samples. QC samples at 15 ng/mL "Low" and 80 ng/mL "High" were prepared in triplicate, as outlined in 4.2.2.1. QC samples were evaluated each day to ensure the accuracy of the analysis, and were only found acceptable if the calculated concentration did not deviate more than ±20% from the nominal concentration.

For the evaluation of stability, calculated concentrations at the relevant time points were compared to the concentrations calculated at Time 0 (T_0). Theoretical recoveries were calculated by dividing the observed concentration by the initial concentration (assumed to be 100% recovery). Concentrations were calculated based on the linear regressions of the curve and used peak area ratios between abundances of internal standard peaks and analyte peaks.

4.4 Results and Discussion

4.4.1 Calibration Model and Linearity

Linearity was assessed for all drugs over a range of 1 to 100 ng/mL. Table 4-3 summarises the R^2 values and the linear regression models for the analytes in question. No weighting was used, as this was not deemed necessary. Residual plots (not shown) created for each analyte showed a random distribution which indicates that the best fit for the calibration is a linear regression.

Table 4-3 Linearity for all analytes (*n* = 5)

Analyte	Internal Standard (IS)	Neat		Buffered	
		Mean R^2	%CV	Mean R^2	%CV
6MAM	6-MAM-D_3	0.997	0.13	0.996	0.30
COD	Codeine-D_3	0.997	0.13	0.996	0.25
DHC	DHC-D_6	0.999	0.06	0.998	0.20
DIAZ	Diazepam-D_5	0.998	0.13	0.997	0.33
DMD	DMD-D_5	0.996	0.26	0.995	0.40
ETIZ	Diazepam-D_5	0.997	0.23	0.993	0.21
METH	Methadone-D_3	0.997	0.17	0.996	0.22
MOR	Morphine-D_3	0.997	0.21	0.996	0.27
OXA	Oxazepam-D_5	0.996	0.21	0.996	0.18
TEMAZ	Temazepam-D_5	0.997	0.14	0.994	0.25

Good linearity was found for all analytes in neat and buffered oral fluid as shown in Figure 4-2, and %CVs were found to be acceptable for all analytes as well.

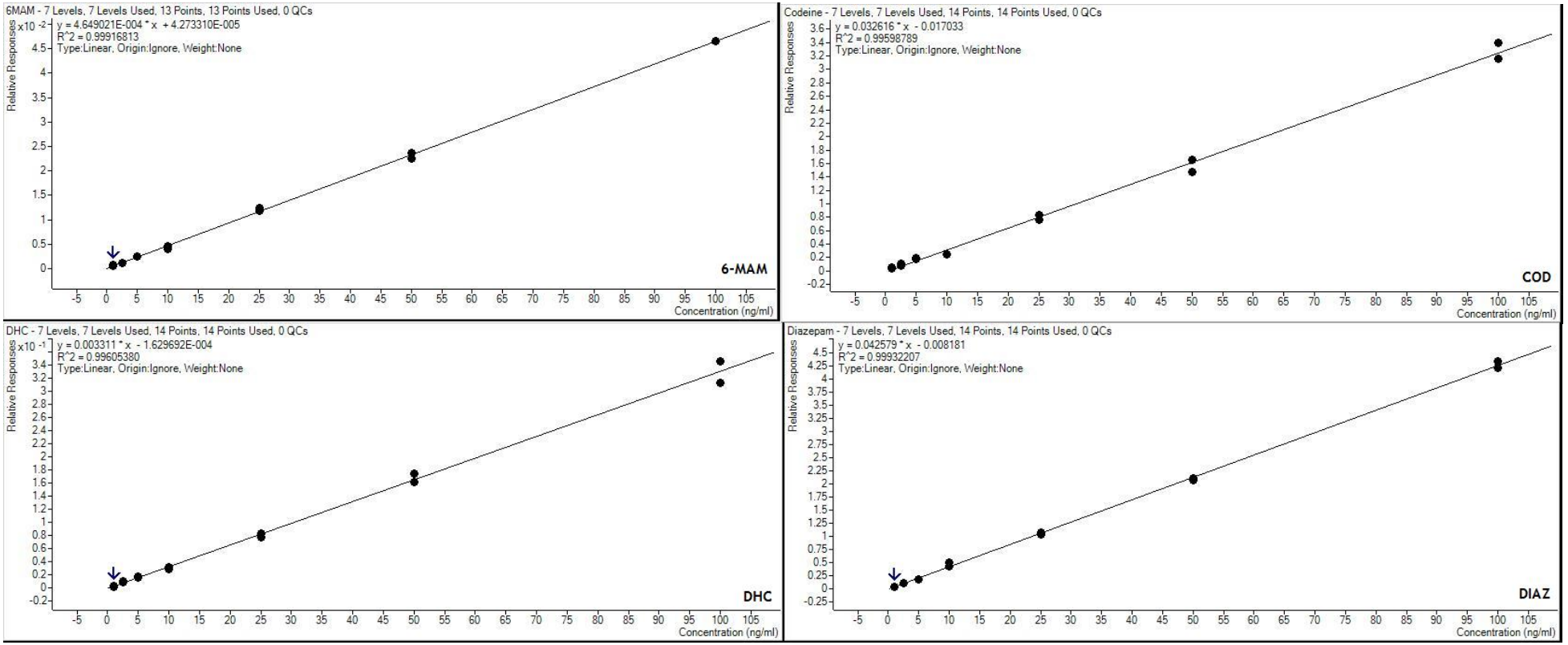


Figure 4-2 Examples of linearity graphs for all analytes

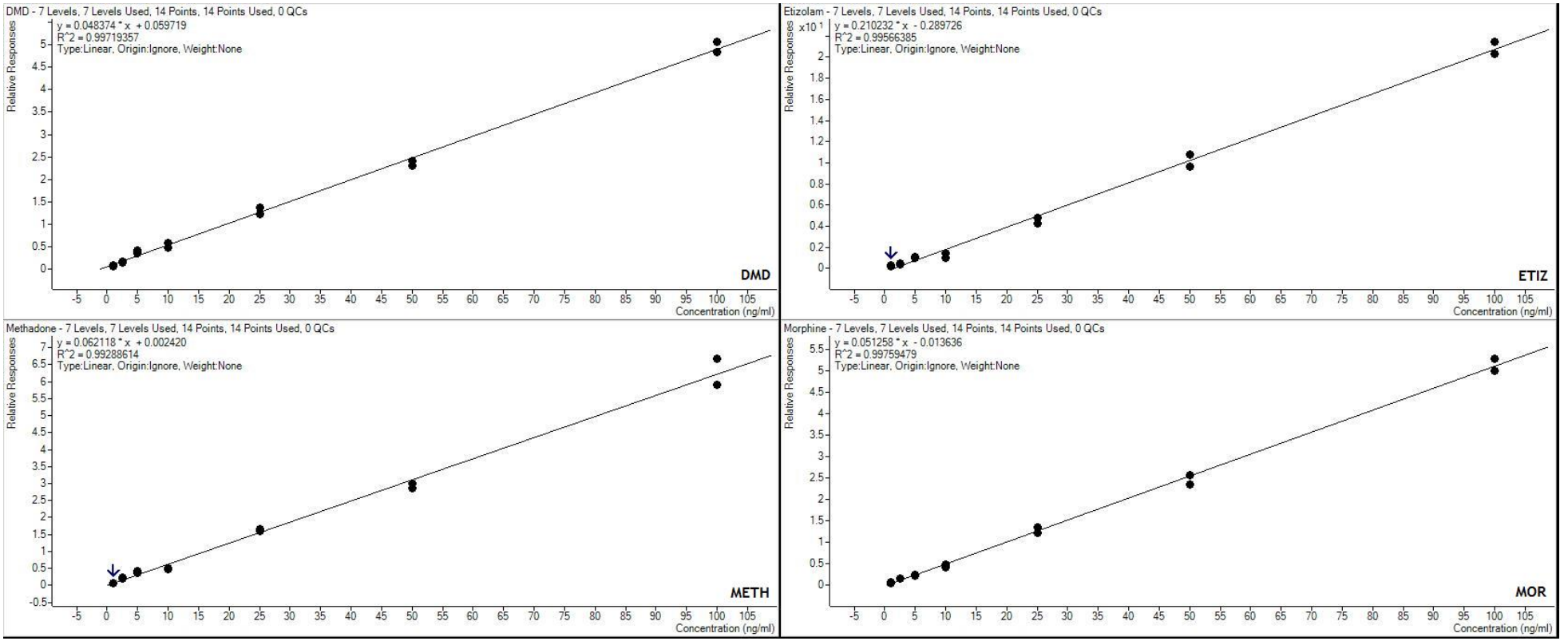


Figure 4-2 Cont'd Examples of linearity graphs for all analytes

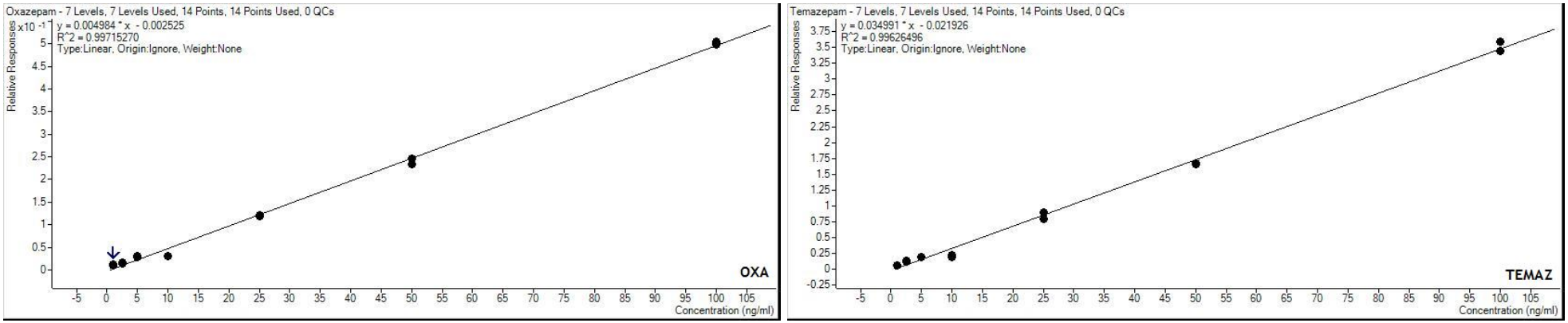


Figure 4-2 Cont'd Examples of linearity graphs for all analytes

4.4.2 Limits of Detection and Limits of Quantitation

Results for both limits of detection and quantitation are summarised in Table 4-4 below.

Table 4-4 Summary of Limits of Detection (LOD) and Quantitation (LLOQ)

Analyte	LOD	LLOQ
6MAM	0.6	0.8
COD	0.6	0.8
DHC	0.4	0.5
DIAZ	0.2	0.5
DMD	0.4	0.5
ETIZ	0.5	1.0
METH	N/A	N/A
MOR	0.8	1.0
OXA	0.8	1.0
TEMAZ	0.8	1.0

LOD – limit of detection; LLOQ – lower limit of quantitation.

Generally, the limits observed are similar to those published in the literature, as previously outlined in Table 3-2 (for benzodiazepines) and Table 3-4 (for opioids). Limits of detection and quantitation were lower for methadone than they were for any of the other analytes investigated. For the majority of the analytes the LOD and LOQ was 0.5 ng/mL, which is lower than the lowest calibrator. Due to the low recoveries observed for temazepam the limit of quantitation was set to 1.0 ng/mL as variation was shown in concentrations lower than that. Peaks for analytes where the LLOQ were set at 1.0 ng/mL were observable but were dependent on many variables, such as instrument cleanliness and other factors outside the analyst's control, and therefore to be able to quantify with the greatest confidence, these LLOQs were chosen.

It was not possible to accurately establish limits of detection and quantitation for methadone as it was later shown that the analytical results cannot be accurately assessed due to the overwhelming matrix effects observed for this analyte.

4.4.3 Accuracy and Precision

Table 4-5 and Table 4-6 show the results of intra- and inter-day accuracy and precision for neat oral fluid, respectively.

Table 4-5 Intra-day accuracy and precision results for neat oral fluid

Analyte	QC[5]	Calc. Conc. (ng/mL; *n* = 5); (StDev))	Accuracy (%) (*n* = 4)	Precision (%CV) (*n* = 4)
6MAM	Low	1.5 (0.2)	83 – 115	22.1 – 23.4
	Medium	15.3 (0.5)	99 – 108	5.0 – 13.5
	High	77.8 (3.1)	94 – 103	2.2 – 5.4
COD	Low	1.6 (0.1)	95 – 118	1.0 – 25.4
	Medium	15.4 (0.5)	99 – 106	2.8 – 5.8
	High	43.3 (2.8)	101 – 109	0.3 – 2.4
DHC	Low	1.7 (0.1)	104 – 122	2.1 – 5.9
	Medium	15.7 (0.5)	100 – 108	3.1 – 8.2
	High	80.4 (4.3)	98 – 109	1.0 – 5.8
DIAZ	Low	1.8 (0.1)	109 – 120	2.0 – 23.5
	Medium	16.2 (0.6)	103 – 111	2.3 – 6.8
	High	82.2 (1.4)	101 – 105	0.9 – 7.8
DMD	Low	1.5 (0.3)	87 – 133	4.0 – 17.6
	Medium	14.4 (0.6)	89 – 100	0.4 – 11.1
	High	79.5 (2.0)	96 – 103	0.3 – 1.2
ETIZ	Low	1.5 (0.1)	87 – 111	1.2 – 18.4
	Medium	15.7 (0.5)	101 – 109	2.3 – 22.0
	High	80.8 (3.6)	95 – 105	1.5 – 10.9
MOR	Low	1.6 (0.1)	94 – 111	2.1 – 21.9
	Medium	15.7 (0.7)	98 – 111	3.3 – 5.1
	High	81.4 (2.1)	98 – 104	0.7 – 4.2
OXA	Low	1.7 (0.1)	107 – 123	2.3 – 24.4
	Medium	15.4 (0.7)	98 – 109	0.7 – 9.2
	High	80.7 (3.3)	96 – 106	1.0 – 2.6
TEMAZ	Low	1.6 (0.2)	95 – 115	1.3 – 8.9
	Medium	14.2 (0.7)	89 – 102	1.8 – 11.0
	High	80.6 (4.6)	92 – 109	0.7 – 9.6

[5] QC concentrations are: 1.5 ng/mL for Low QC, 15 ng/mL for Medium QC, and 80 ng/mL for High QC

All calculated concentrations were within ±15% of the nominal concentrations, with the exception of the low QC for 6-MAM which had an accuracy of 83% on one of the days. The precision for this analyte at the low concentration was also higher, and therefore less good, than for most other analytes.

Table 4-6 Inter-day accuracy and precision results for neat oral fluid

Analyte	QC	Calc. Conc. (ng/mL; *n* = 5); (StDev))	Mean Accuracy (%) (*n* = 5)	Mean Precision (%CV) (*n* = 5)
6MAM	Low	1.5 (0.2)	102.1	14.6
	Medium	15.3 (0.5)	102.3	3.2
	High	77.8 (3.1)	97.3	3.9
COD	Low	1.6 (0.1)	108.3	8.5
	Medium	15.4 (0.5)	102.5	3.4
	High	43.3 (2.8)	104.1	3.3
DHC	Low	1.7 (0.1)	116.6	6.9
	Medium	15.7 (0.5)	104.8	2.9
	High	80.4 (4.3)	100.6	5.3
DIAZ	Low	1.8 (0.1)	117.5	4.9
	Medium	16.2 (0.6)	108.2	3.8
	High	82.2 (1.4)	102.8	1.7
DMD	Low	1.5 (0.3)	98.9	19.2
	Medium	14.4 (0.6)	95.7	4.2
	High	79.5 (2.0)	99.4	2.5
ETIZ	Low	1.5 (0.1)	97.1	9.4
	Medium	15.7 (0.5)	104.3	3.1
	High	80.8 (3.6)	101	4.5
MOR	Low	1.6 (0.1)	105	7.0
	Medium	15.7 (0.7)	104.8	4.7
	High	81.4 (2.1)	101.8	2.5
OXA	Low	1.7 (0.1)	116.8	7.4
	Medium	15.4 (0.7)	102.4	4.4
	High	80.7 (3.3)	100.8	4.1
TEMAZ	Low	1.6 (0.2)	105.9	9.6
	Medium	14.2 (0.7)	95	5.1
	High	80.6 (4.6)	100.8	6.1

As would be expected, the precision at the low QC concentrations was highest (least good) for most analytes. The widest range of intra-day accuracy was observed for the low concentration desmethyldiazepam. Although the intra-day accuracy results gave a greater range, precision results for neat low QC desmethyldiazepam were less than 20%, which was not observed for buffered oral fluids samples (see Table 4-7). Inter-day results were all less than ±20% for all analytes, for both accuracy and precision. Low concentration desmethyldiazepam precision was 98.9%, but the highest accuracy was observed for diazepam at 117.5%.

Intra- and inter-day accuracy and precision results for buffered oral fluid (oral fluid collected using the NeoSAL™ device) are shown in Table 4-7 (Intra-day) and Table 4-8 (Inter-day). Calculated concentrations of all three QCs for both neat and buffered oral fluid were found to be within ±15%, with the exception desmethyldiazepam, of the nominal concentrations.

Table 4-7 Intra-day accuracy and precision results for buffered oral fluid

Analyte	QC	Calc. Conc. (ng/mL; *n* = 5); (StDev))	Accuracy (%) (*n* = 4)	Precision (%CV) (*n* = 4)
6MAM	Low	1.6 (0.1)	96 - 116	6.5 – 16.8
	Medium	15.7(1.1)	97 – 115	3.0 – 7.6
	High	79.4 (1.0)	98 – 101	2.2 – 5.2
COD	Low	1.5 (0.2)	88 – 109	5.4 – 11.6
	Medium	15.8 (0.2)	104 – 107	4.3 – 8.6
	High	80.1 (1.8)	98 – 102	0.6 – 4.1
DHC	Low	1.6 (0.1)	96 – 111	0.9 – 9.4
	Medium	16.2 (0.4)	104 – 111	2.8 – 8.5
	High	78.2 (1.7)	95 – 100	0.3 – 4.4
DIAZ	Low	1.6 (0.1)	99 – 115	1.9 – 8.8
	Medium	16.5 (0.3)	108 – 112	1.9 – 15.5
	High	81.7 (1.1)	100 – 104	0.5 – 4.1
DMD	Low	1.4 (0.3)	76 – 109	7.1 – 23.1
	Medium	14.9 (0.7)	95 – 104	1.6 – 11.9
	High	79.2 (2.7)	96 – 102	0.7 – 5.6
ETIZ	Low	1.6 (0.2)	80 – 116	10.4 – 18.4
	Medium	15.6 (0.9)	100 – 113	7.6 – 17.2
	High	77.3 (2.7)	92 – 100	0.7 – 3.9
MOR	Low	1.7 (0.3)	85 – 130	3.9 – 11.5
	Medium	16.2 (0.5)	105 – 112	3.1 – 7.2
	High	81.4 (1.7)	100 – 104	2.8 – 5.2
OXA	Low	1.7 (0.1)	105 – 122	1.9 – 14.5
	Medium	15.3 (0.2)	100 – 104	4.0 – 14.4
	High	78.9 (3.3)	95 – 105	1.4 – 5.2
TEMAZ	Low	1.6 (0.4)	78 – 137	2.0 – 14.7
	Medium	14.0 (0.6)	89 – 98	3.1 – 5.4
	High	78.4 (2.2)	96 – 102	2.7 – 4.7

The greatest variability of intra-day accuracy was observed for temazepam for the Low QC (1.5 ng/mL), ranging from 78 – 137%. This would have been caused by the low drug recoveries yielded by the extraction procedure giving rise to greater variability at the low concentration. Precision results were less than 20% for all analytes except etizolam at low concentrations. The drug recovery for desmethyldiazepam in oral fluid collected using the NeoSAL™ device was lower

than that found from neat oral fluid. This could explain the greater variability in the accuracy and precision results as it is possible that the analyte remains stuck on the collection pad or that the preservative buffer interferes with the extraction. In the case of methadone, it is likely that the variation in both accuracy and precision data occurred due to the high matrix effects found for this analyte.

Table 4-8 Inter-day accuracy and precision results for buffered oral fluid

Analyte	QC	Calc. Conc. (ng/mL; *n* = 5); (StDev))	Mean Accuracy (%) (*n* = 5)	Mean Precision (%CV) (*n* = 5)
6MAM	Low	1.6 (0.1)	109	7.8
	Medium	15.7(1.1)	105	7.2
	High	79.4 (1.0)	99	1.3
COD	Low	1.5 (0.2)	102	9.9
	Medium	15.8 (0.2)	106	1.5
	High	80.1 (1.8)	100	2.2
DHC	Low	1.6 (0.1)	104	6.5
	Medium	16.2 (0.4)	108	2.7
	High	78.2 (1.7)	98	2.2
DIAZ	Low	1.6 (0.1)	105	7.1
	Medium	16.5 (0.3)	110	1.9
	High	81.7 (1.1)	102	1.3
DMD	Low	1.4 (0.3)	91	18.6
	Medium	14.9 (0.7)	99	4.4
	High	79.2 (2.7)	99	3.5
ETIZ	Low	1.6 (0.2)	104	15.7
	Medium	15.6 (0.9)	104	5.6
	High	77.3 (2.7)	97	3.4
MOR	Low	1.7 (0.3)	114	17.6
	Medium	16.2 (0.5)	108	2.8
	High	81.4 (1.7)	102	2.1
OXA	Low	1.7 (0.1)	112	6.7
	Medium	15.3 (0.2)	102	1.4
	High	78.9 (3.3)	99	4.2
TEMAZ	Low	1.6 (0.4)	106	22.8
	Medium	14.0 (0.6)	94	4.2
	High	78.4 (2.2)	98	2.8

Inter-day results for the NeoSAL™ device showed that the method is both accurate and precise even at the low concentrations. 112% accuracy was the highest found for low QC of oxazepam. With the exception of temazepam for QC1 (22.8%), all precision results were found to be less than 20%. Again, it is likely that the lack of precision is caused by the low drug recoveries yielded by the method. However, as stated before, although the recoveries for temazepam are low, the chromatography and peak S/N ratios are still acceptable to detect concentrations lower than the recommended cut-off.

4.4.4 Drug Recovery from Collection Device

Drug recoveries from the NeoSAL™ collection device were studied for all analytes at high and low concentrations (80 and 3 ng/mL, respectively). As previously seen in earlier work (Section 2.5.3), dipped recoveries (recoveries observed when the collection pad was dipped into spiked oral fluid) were greater than when spiked oral fluid was directly pipetted onto the collection pad. Figure 4-3 summarises the recoveries found.

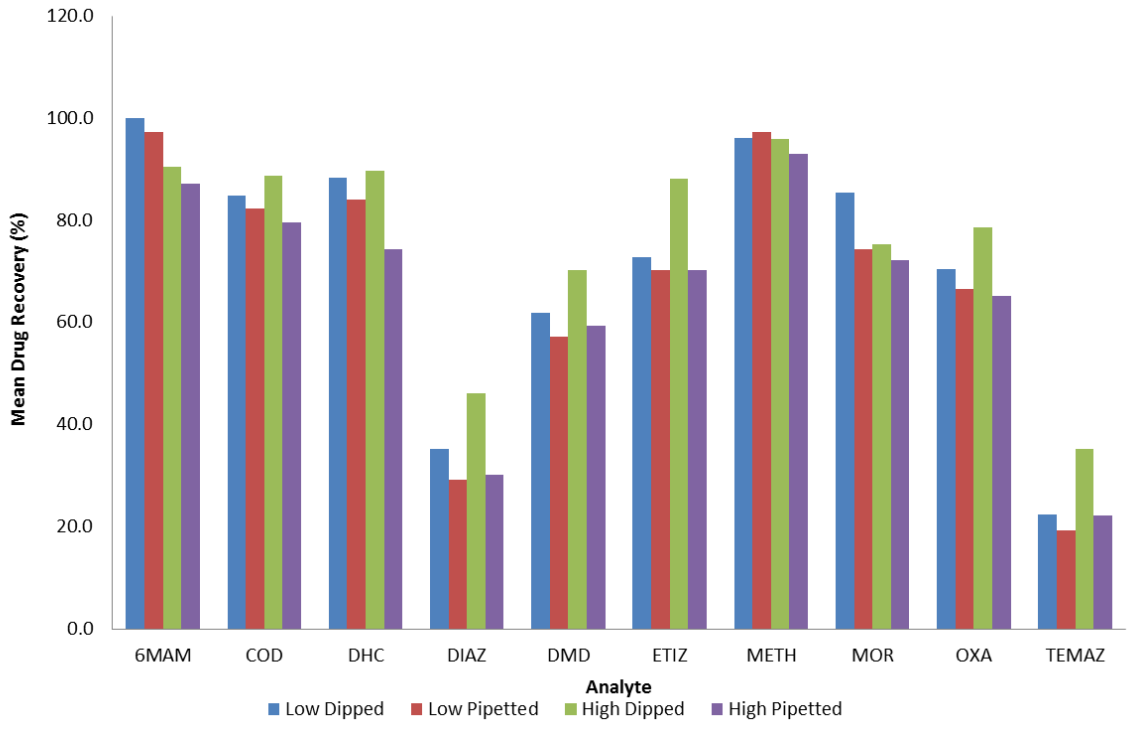


Figure 4-3 Summary of drug recoveries from the NeoSAL™ collection device (*n* = 10 for each variable)

Following the transfer of oral fluid onto the collection pad (whether by pipetting or dipping), the collection pad was placed into the buffer tube, the cap secured and the collector inverted several times, as suggested by the manufacturer. Collectors were then left for approximately 5 minutes. For sample preparation, although not specified by the manufacturer, the pads were pressed against the inside of the collection tube to squeeze out as much of the oral fluid/buffer saturating the pad as possible. Although this was done to increase and maximise the volume of oral fluid/buffer mixture recovered from the pad, this will have introduced variability to the sample as it could not be ensured that each pad was squeezed in the same manner. The irregularities in squeezing, from human involvement could have had an effect on analytical performance and measurement – i.e. reproducibility. Preliminary investigation data (not shown) demonstrated that the volume of oral fluid/buffer available for analysis was lower when the pad was not squeezed.

An explanation for the generally lower recoveries is that the analytes could be stuck on the pad, especially in the case of temazepam and diazepam.

On the whole, dipped recoveries were higher than recoveries where the spiked oral fluid was pipetted onto the collection pad. A possible explanation for this is the slight over-collection of the device (i.e. more than the stated 0.7 mL of oral fluid is collected) resulting in an increased amount of analyte on the pad. The %CV observed for all analytes for all testing variables, although not included in Figure 4-3, did not exceed ±20%

4.4.5 Recovery and Matrix Effects

Oral fluid is a highly variable matrix; it is therefore not surprising to see that the calculated matrix effects showed considerable variability. All samples were extracted in duplicate and %CV was calculated for the raw data. Within sample variation did not exceed 10%, although the variation across all 10 sources exceeded ±25%. It must be noted that recoveries showed discrepancies every time they were investigated.

As seen from Table 4-9, matrix effects, recoveries and associated %CV showed more variation for sources spiked at the low concentration. At the low

concentration, the only drug that exhibited ion enhancement was codeine for all 10 sources of neat oral fluid. For 6-MAM, the observed matrix effects varied from ion suppression of -23.4% to ion enhancement of 20.2%. The extreme range of matrix effects found for the low concentration codeine, and even morphine and oxazepam show that care must be taken when it comes to low concentrations of drug in oral fluid, because both ion enhancement and suppression are possible, depending on the source of oral fluid.

Table 4-9 Recovery (%) and matrix effect (%) results for neat oral fluid (*n* = 10)

Analyte	High Concentration (80 ng/mL)			Low Concentration (3 ng/mL)		
	Recovery (% (%CV))	Mean ME (% (%CV)	ME Range (%)	Recovery (% (%CV))	Mean ME (% (%CV)	ME Range (%)
6MAM	89.1 (11)	-19.2 (8)	-24.5 – -13.3	112.0 (23)	1.9 (22)	-23.4 – 20.2
COD	85.7 (6)	-9.6 (6)	-18.7 – -2.4	85.7 (10)	16.0 (7)	4.6 – 26.2
DHC	85.7 (7)	-11.0 (6)	-17.2 – -5.3	85.5 (7)	-6.3 (6)	-13.5 – 3.0
DIAZ	48.3 (12)	-9.8 (6)	-16.7 – -2.9	48.9 (14)	-5.7 (5)	-13.4 – 0.9
DMD	79.6 (5)	-14.0 (8)	-23.0 – -9.2	75.8 (13)	-11.7 (8)	-23.3 – -1.1
ETIZ	86.6 (4)	-14.7 (6)	-22.0 – -9.2	81.0 (11)	-7.7 (9)	-16.0 – 12.4
METH	80.8 (23)	-20.5 (17)	-42.0 – -10.9	81.7 (14)	-15.3 (16)	-24.5 – 5.8
MOR	63.9 (13)	-24.7 (24)	- 33.0 – -17.7	67.5 (16)	-16.4 (14)	-26.8 – 10.3
OXA	80.0 (5)	-8.4 (7)	-13.7 – -3.3	78.3 (10)	-1.3 (7)	-15.0 – 6.8
TEMAZ	33.7 (23)	-9.0 (16)	-18.2 – -4.0	36.0 (17)	-6.1 (6)	-13.4 – 1.7

Where ME – matrix effect.

Recoveries exceeded 60% for all analytes in both low and high concentrations with the exception of diazepam and temazepam, which were considerably lower at 48.3% and 33.7% approximately, respectively. During the extraction procedure selection, the method used did show low recoveries for diazepam, however due

to the LLOQ and LOD of the method, low concentrations of the drug can still be accurately and confidently quantified. Furthermore, even with low recoveries the recommended cut-off concentrations were met.

Table 4-10 summaries matrix effects and drug recoveries from buffered oral fluid for high and low concentrations.

Table 4-10 Matrix effects and recoveries for buffered oral fluid

Analyte	High Concentration (80 ng/mL)			Low Concentration (3 ng/mL)		
	Recovery (% (%CV))	Mean ME (% (%CV))	ME Range (%)	Recovery (% (%CV))	Mean ME (% (%CV)	ME Range (%)
6MAM	89.5 (18)	-4.6 (18)	-19.8 – 7.8	106.3 (11)	-13.1 (18)	-25.1 –11.4
COD	89.8 (4)	3.3 (20)	-2.6 – 11.0	87.8 (12)	0.7 (6)	-6.4 – 9.7
DHC	90.2 (5)	-1.3 (16)	-7.9 – 4.6	89.2 (6)	7.9 (7)	2.0 – 21.9
DIAZ	45.9 (20)	-14.9 (26)	-32.9 – -0.6	32.5 (9)	-15.9 (10)	-25.1 – 2.3
DMD	70.0 (12)	-17.9 (15)	-30.2 – -4.2	61.7 (27)	-24.2 (13)	-31.3 – -0.8
ETIZ	87.9 (12)	-7.7 (19)	-11.6 – -3.8	75.3 (18)	-13.2 (7)	-22.9 – 0.5
METH	96.2 (28)	-64.5 (81)	-91.0 – -21.9	98.0 (29)	-87.5 (30)	-90.9 – -79.2
MOR	76.9 (27)	-12.4 (20)	-29.1 – 3.5	90.9 (21)	-16.9 (10)	-29.3 – 0.5
OXA	80.0 (6)	-6.1 (27)	-13.0 – 3.0	71.2 (22)	-23.2 (8)	-29.2 – -13.4
TEMAZ	37.7 (19)	-5.2 (26)	-10.9 – 1.5	21.6 (27)	-25.4 (8)	-34.6 – - 18.8

Where ME – matrix effect.

It is evident from the table that the buffer in the oral fluid collection device causes slightly less ion suppression than is shown in neat oral fluid for the majority of drugs, with the exception of diazepam (matrix effects of -9. 8% versus -14.9% for neat versus buffered oral fluid, respectively). Care must be taken with methadone detected in buffered oral fluid samples collected with the

NeoSAL™ collection device, as calculated matrix effects ranged from -91.0% to -21.9%, averaging -64.5% and -87.5%, for high and low concentrations, respectively. Neither of these results were acceptable. Consequently, methadone in buffered oral fluid samples cannot be quantified accurately or with any confidence.

Results from neat and buffered oral fluid are consistent, in that low recoveries were found for temazepam and diazepam regardless of buffer presence. Recoveries from the buffered samples were lower than those from neat oral fluid for diazepam at the low QC, desmethyldiazepam at both QC concentrations, and temazepam, which showed lower recoveries for the low concentration QC than in neat oral fluid. Methadone showed higher recoveries from buffered oral fluid than from neat. However the increased recoveries may be due to the highly variable matrix effects for this analyte. Based on the results in Table 4-9 and Table 4-10, it follows that benzodiazepines favour neat oral fluid to buffered oral fluid, as the recoveries from the latter are lower. The inverse seems to be true for the opioids where recoveries from buffered oral fluid exceeded those from neat oral fluid.

A possible explanation for the higher drug recoveries observed from neat oral fluid than the buffered oral fluid for benzodiazepines is the presence of the collection pad. This has previously been shown to reduce recoveries for THC (14), and similarly, it was observed for desmethyldiazepam. Recoveries averaged 77. 7% for both concentrations tested for neat oral fluid, whereas recoveries averaged 65.8% for both concentrations in buffered oral fluid. This is a decrease in drug recovery by 13%. As recoveries from neat oral fluid are generally higher and acceptable, it follows that the analyte must be retained on the collection pad and not lost during the extraction (for example).

A further factor that could explain decreased recoveries from the collection pad is the preservative buffer. Components of the buffer are not known, and so it is possible that sodium ions were present that could interfere with the analytes in the oral fluid samples.

From Table 4-9 and Table 4-10, it can be seen that morphine is the only analyte that shows higher recoveries from the buffered samples compared to the neat

samples. An average recovery of 65.7% was seen for neat oral fluid versus 83.91% for buffered oral fluid. However, increased variability was found in the buffered samples for matrix effects. Caution must therefore be observed when interpreting the drug concentrations as better drug recoveries from buffered oral fluid samples were observed.

Matrix effects were more than double for methadone when oral fluid was collected using the NeoSAL™ device. This leaves the assumption that either the preservative buffer (with unknown components) or the materials of the collection pad are affecting the results. Methadone is the only analyte where such a dramatic increase was observed, however other analytes did show an increase in matrix effects for buffered oral fluid samples as well, in particular the benzodiazepines (with the exception of etizolam). The low concentration buffered samples all showed a noticeable worsening of ion suppression compared to both the neat counterparts but also the high concentration buffered samples.

However, the use of mean matrix effects is problematic as this may skew the results in a way that they look better than they might actually be. For example, the observed range of matrix effects for 6-MAM in neat oral fluid at low concentration was -23.4% – 20.2% but the mean matrix effect was 1.9%. Therefore, the mean matrix effect may not be completely reflective of the actual effects that the matrix has on the peak abundances, and perchance on the quantitation. Equally, certain matrix source may show matrix effects outwith the accepted ±25%, but if an average is taken, the matrix effects become acceptable – as was the case for high concentration desmethyldiazepam, which showed matrix effects ranging from -31.3% (unacceptable) to -0.8% for buffered oral fluid at low QC (acceptable), with a mean value of -24.2% (just about acceptable).

Mean process efficiencies for both neat and buffered oral fluid, at high and low concentrations (80 ng/mL and 3 ng/mL, respectively), of the method used are shown in Table 4-11.

Table 4-11 Process efficiencies (%) and %CV for neat and buffered oral fluid at high (80 ng/mL) and low (3 ng/mL) concentrations

Analyte	Process Efficiency (%) (%CV)			
	Neat High	Neat Low	Buffered High	Buffered Low
6MAM	71.7 (7)	112.2 (20)	86.0 (22)	91.4 (8)
COD	77.3 (2)	99.3 (11)	92.7 (4)	88.2 (11)
DHC	76.1 (4)	79.8 (4)	89.0 (4)	96.2 (8)
DIAZ	43.5 (12)	46.2 (15)	38.7 (17)	27.3(8)
DMD	68.4 (6)	67.2 (17)	57.4 (18)	48.0 (22)
ETIZ	73.9 (6)	74.2 (6)	81.1 (13)	65.4 (20)
METH	62.9 (13)	69.0 (15)	35.3 (84)	12.3 (41)
MOR	48.0 (13)	55.8 (13)	67.6 (32)	74.7 (16)
OXA	73.2 (4)	77.3 (13)	75.1 (8)	54.7 (14)
TEMAZ	30.6 (23)	33.8 (16)	35.9 (22)	16.1 (19)

4.4.6 Selectivity and Specificity

Potential endogenous interferences from oral fluid were investigated. Total ion chromatograms were assessed visually to see whether components gave a response at the MRM transitions monitored for the set of drugs included in the method. Figure 4-4 shows the total ion chromatogram (TIC) for an extracted Standard 7 at a concentration of 100 ng/mL for neat oral fluid ((A); grey), for buffered oral fluid ((B); red) and the TIC of an extracted blank matrix sample without IS ((C); green) and with internal standard ((D); blue), respectively. A peak at 1 minute can be seen in blank matrix samples as well as the neat exacted sample. It is also present in the buffered oral fluid sample, however at a much lower abundance.

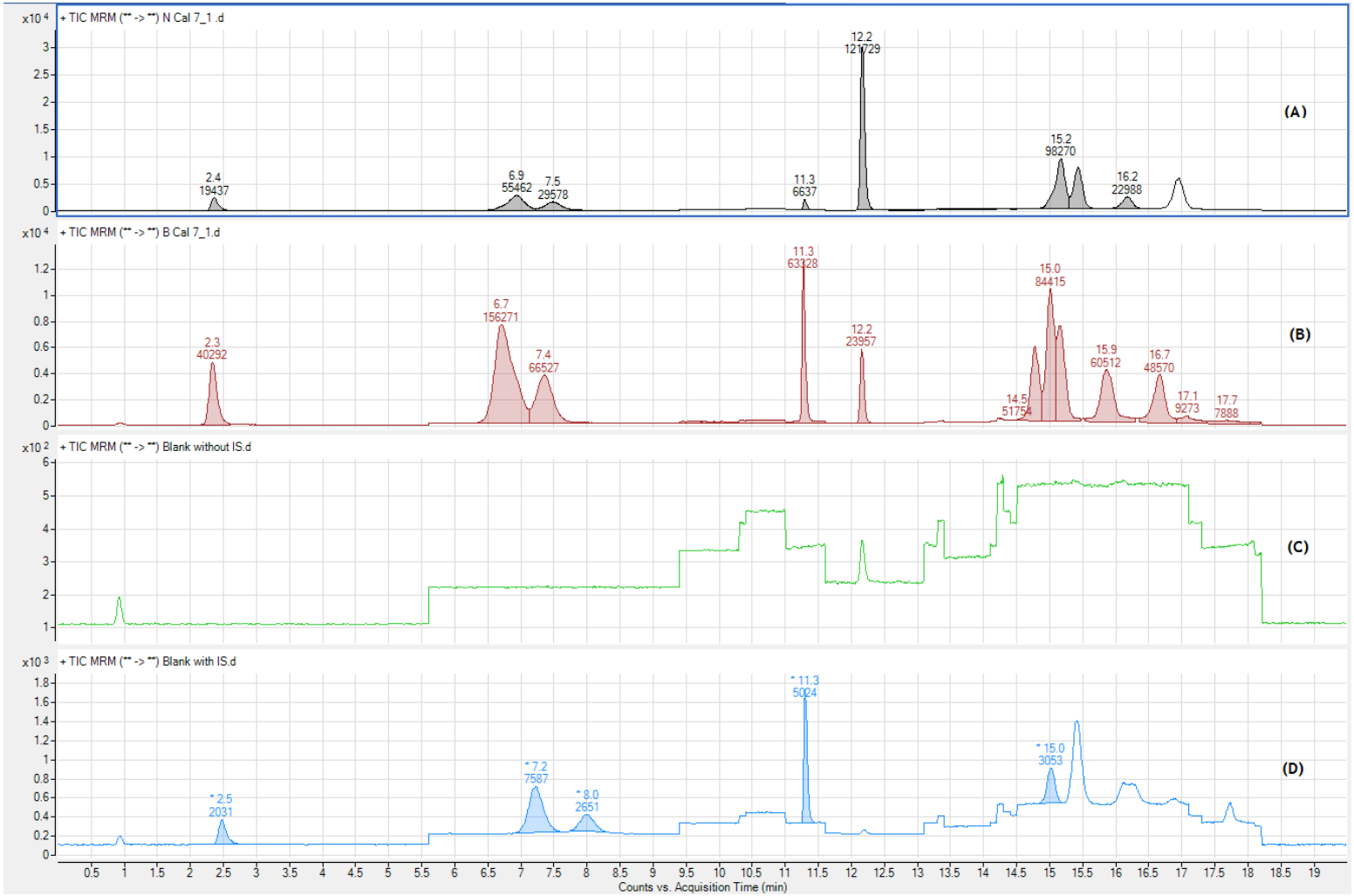


Figure 4-4 Total Ion Chromatogram (TIC) for Standard 7 (100 ng/mL) for neat and buffered oral fluid versus TIC for extracted blank matrix with and without internal standard (IS).

Where (A) is Std 7 in neat oral fluid, (B) Std 7 in buffered oral fluid, (C) is blank oral fluid, and (D) is blank oral fluid with internal standard)

A peak that exists in both chromatograms is found at 0.9 minutes (approximately), but is not integrated. This means that it must be an endogenous compound that is not removed during the extraction procedure or the dead volume, which is common for LC analysis. As the first analyte elutes at 2.5 minutes, respectively, no interference was exhibited by the peak at 0.9 minutes.

The selectivity of other common drugs of abuse was tested and results are shown in Figure 4-5. Possible interferences were found for fentanyl, midazolam, and the synthetic cannabinoids solution. The retention times for these do not exactly match however, this may be caused by analytical and instrumental variation. Therefore, MRM transitions monitored for each analyte were examined and it was found that peaks were not present for all of the potential interference peaks. Finally, ratios between quantifier and qualifier ions were compared and these were different. Therefore it was determined that none of the tested analytes gave analytical interferences.

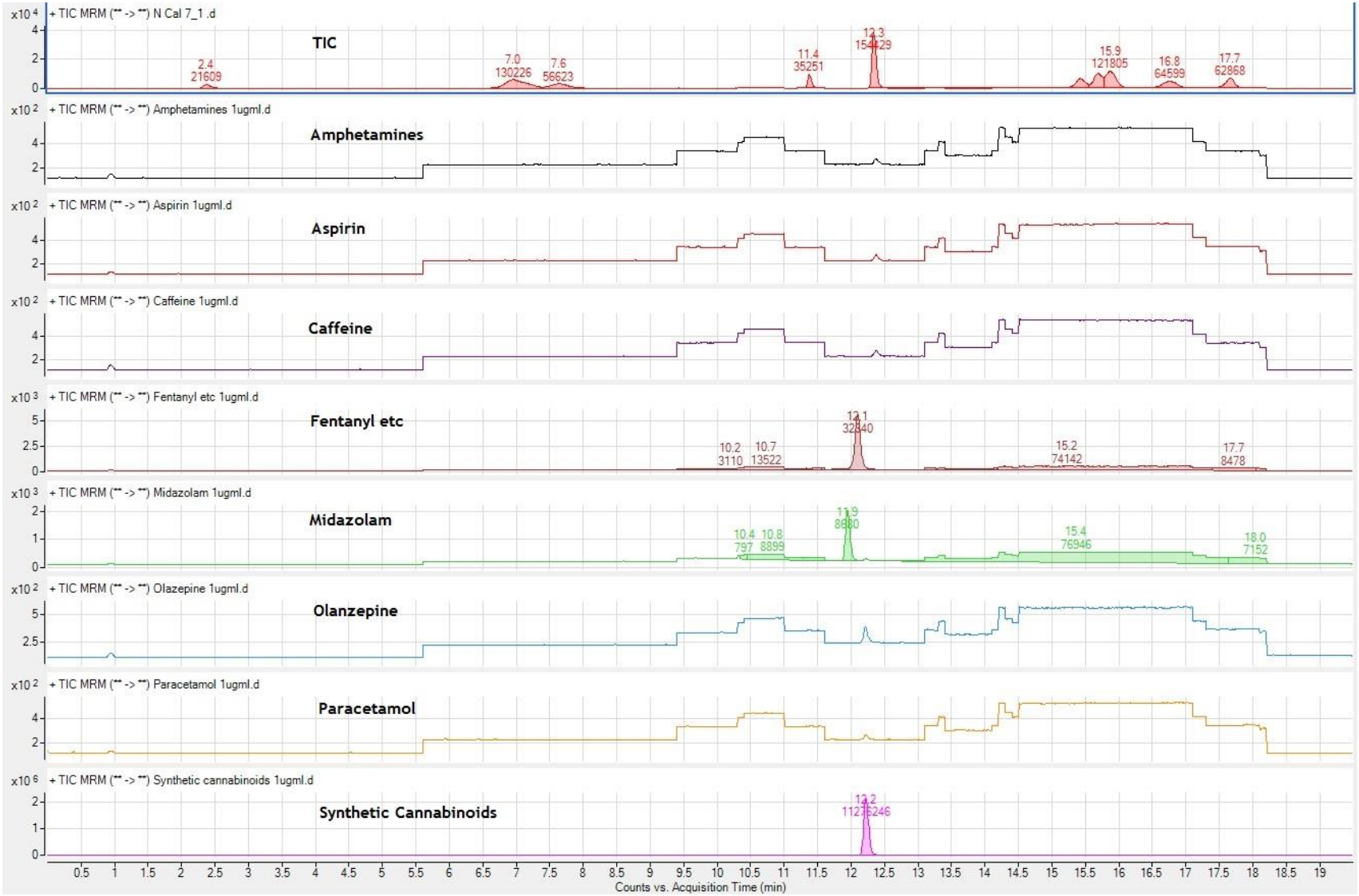


Figure 4-5 TIC of highest standard and interference samples

4.4.7 Carryover

Carryover was assessed at 200 ng/mL. A visual examination of the three flushes following the carryover sample showed no peaks that could be considered carryover.

4.4.8 Dilution Integrity

Dilution integrity was assessed for two dilution factors (1:2 and 1:5). Results are shown in below in Table 4-12.

Table 4-12 Dilution integrity results for dilution factors 1:2 and 1:5

Analyte	1:2 Dilution		1:5 Dilution	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
6MAM	92.2	0.9	87.5	0.9
COD	87.4	1.2	103.2	2.5
DHC	89.6	4.1	76.8	11.6
DIAZ	89.7	2.0	88.9	1.0
DMD	85.6	3.6	86.4	1.7
ETIZ	91.1	0.7	74.5	0.8
METH	115.2	0.8	87.2	1.1
MOR	100.2	0.5	84.9	1.4
OXA	87.2	11.4	72.6	0.4
TEMAZ	89.5	1.2	69.0	0.2

Although temazepam accuracy decreased when a greater dilution factor was investigated, it can be assumed that dilution of expectorated samples is possible and accurate analyte quantification may still be possible when samples are diluted 1:2. When samples were further diluted (1:5) however, etizolam, dihydrocodeine, oxazepam, and temazepam accuracies were outwith acceptable accuracy criteria.

4.4.9 Stability

Based on the matrix effects previously presented in this chapter, the quantitation of methadone is not reliable, and methadone results were therefore excluded from stability results.

Autosampler stability (approximately 18 °C) for all analytes was assessed over 72 hours. This time frame was selected to reflect a potential loss of instrumentation in a routine laboratory. Results for high and low concentrations are summarised in Table 4-13 and Table 4-14, respectively. Most analytes proved stable over the autosampler testing period for both concentrations. All T_0 concentrations were found to be ±15% of the nominal concentrations for both high and low concentrations. Contrary to a previous publication reporting the stability of 6-MAM in oral fluid collected using the Intercept® device (162), 6-MAM can be considered stable on the autosampler for up T_0 72 hours in this study.

Table 4-13 Autosampler stability high concentration (80 ng/mL, *n* = 3) results

Analyte	Calculated Concentrations (ng/mL)				Final Recovery (%)
	T_0 (0 h) (Recovery %)	T_1 (24 h) (Recovery %)	T_2 (48 h) (Recovery %)	T_3 (72 h) (Recovery %)	
6MAM	80.1 (100%)	84.4 (105%)	83.1 (104%)	68.0 (85%)	85
COD	71.5 (100%)	73.9 (103%)	77.3 (108%)	79.7% (111%)	111
DHC	75.5 (100%)	73.9 (98%)	77.4 (102%)	75.0 (99%)	99
DIAZ	84.1 (100%)	76.8 (91%)	78.4 (93%)	75.3 (90%)	90
DMD	73.6 (100%)	73.9 (100%)	73.7 (100%)	76.5 (104%)	104
ETIZ	84.6 (100%)	55.8 (66%)	61.6 (73%)	104.8 (124%)	124
MOR	74.9 (100%)	81.5 (109%)	79.0 (105%)	67.5 (90%)	90
OXA	69.3 (100%)	72.7 (105%)	70.6 (102%)	70.3 (101%)	101
TEMAZ	71.4 (100%)	72.9 (102%)	73.9 (103%)	70.3 (98%)	98

Etizolam showed an increase at 72 hours on the autosampler, however it can be assumed that this is due to analytical error as etizolam is the only analyte without its own deuterated internal standard. An increase was also observed for codeine, which showed a theoretical average recovery of 111%, but still less

than etizolam with 124%. The stability of etizolam in oral fluid is not well studied and therefore this increase should be considered an outlier. The reduction in recovery of 6-MAM may be due to its conversion into morphine, however, morphine concentrations also reduced by 10%.

In the low concentration stability samples, etizolam was reduced to 25% of its initial T_0 concentration by 72 hours, which was different to what was observed for its high concentration counterpart.

Table 4-14 Autosampler stability low concentration (15 ng/mL, *n* = 3) results

Analyte	Calculated Concentrations (ng/mL)				Final Recovery (%)
	T_0 (0 h) (Recovery %)	T_1 (24 h) (Recovery %)	T_2 (48 h) (Recovery %)	T_3 (72 h) (Recovery %)	
6MAM	12.6 (100%)	10.7 (85%)	10.9 (87%)	10.1 (80%)	80
COD	12.3 (100%)	12.5 (101%)	11.8 (98%)	11.2 (91%)	91
DHC	15.5 (100%)	12.5 (81%)	12.0 (78%)	10.2 (66%)	66
DIAZ	13.6 (100%)	14.5 (107%)	11.9 (88%)	11.9 (88%)	88
DMD	12.9 (100%)	14.4 (112%)	10.4 (84%)	10.4 (84%)	84
ETIZ	14.2 (100%)	10.7 (76%)	8.2 (58%)	3.5 (25%)	25
MOR	14.0 (100%)	15.8 (112%)	15.8 (113%)	7.2 (51%)	51
OXA	14.9 (100%)	11.0 (74%)	10.4 (69%)	11.3 (76%)	76
TEMAZ	12.6 (100%)	11.4 (91%)	11.3 (90%)	10.3 (82%)	82

Morphine was found to be stable for up to 48 hours at low concentrations on the autosampler, but reduced to 51% after 72 hours. Etizolam degraded but again it is unclear whether this is an outlier, or an accurate representation of its behaviour in solution in this environment. It is unlikely to be an outlier as the previous reinjections for method development and validation work showed a reduction in concentration, so a lower recovery could be reflective of a lack of stability.

Room temperature stability was assessed over a period of 96 hours and then on days 8 and 10. Results for high and low concentrations are shown in Table 4-15 and Table 4-16, respectively.

No results were obtained for diazepam, desmethyldiazepam, etizolam and morphine on day 8 as the calibration failed for these analytes on that day. The analyte with the highest theoretical recovery was etizolam with a recovery of 140%. Although this is reflective of the results obtained for the autosampler stability assessment, looking at the trend of the drug recovery at time points preceding the day 10 assessment, the result is questionable. QC samples for etizolam passed, and the calibration showed an R^2 value of 0.993. However, variation (%CV) exceeded 15% on day 10 and therefore the results should not be considered accurate. Other analytes showed slight increases from T_0, however all calculated concentrations remained within 15% of the nominal concentration and could therefore be put down to analytical variation.

Table 4-15 Room temperature high concentration (80 ng/mL) results (*n* = 3)

Analyte	Calculated Concentrations (ng/mL)							R (%)
	T_0 (0 h)	T_1 (24 h)	T_2 (48 h)	T_3 (72 h)	T_4 (96 h)	T_5 (D 8)	T_6 (D 10)	
6MAM	80.1 (100%)	81.3 (101%)	81.7 (102%)	79.2 (99%)	81.7 (102%)	74.5 (93%)	82.6 (103%)	103
COD	71.5 (100%)	71.6 (100%)	76.0 (106%)	71.2 (100%)	75.3 (105%)	71.6 (100%)	82.6 (115%)	115
DHC	75.5 (100%)	71.6 (95%)	76.4 (101%)	76.5 (101%)	78.8 (104%)	74.0 (98%)	75.7 (127%)	100
DIAZ	84.1 (100%)	74.8 (89%)	76.6 (91%)	76.0 (90%)	82.8 (99%)	N/A	83.5 (99%)	99
DMD	73.6 (100%)	72.1 (98%)	75.5 (103%)	71.3 (97%)	76.6 (104%)	N/A	85.9 (117%)	117
ETIZ	84.6 (100%)	69.0 (82%)	85.9 (101%)	66.6 (79%)	74.6 (98%)	N/A	112.2 (140%)	140
MOR	74.9 (100%)	75.0 (100%)	77.9 (104%)	74.1 (99%)	75.4 (101%)	N/A	78.1 (104%)	104
OXA	69.3 (100%)	68.1 (98%)	68.8 (99%)	65.4 (94%)	69.9 (101%)	67.8 (98%)	68.0 (98%)	98
TEMAZ	71.4 (100%)	69.2 (97%)	72.4 (101%)	70.0 (98%)	72.9 (102%)	73.1 (102%)	78.7 (110%)	110

R – Final theoretical recovery (%)

Table 4-16 summarises the stability of low concentration spikes at room temperatures. The lowest recovery was observed for DHC (75%) on day 10. The remaining analytes would be deemed relatively stable at room temperature (20.9 °C (±0.2)) over the testing period, and samples left sitting at this temperature would still show decent drug recoveries.

Table 4-16 Room temperature low concentration (15 ng/mL) results (*n* = 3)

Analyte	Calculated Concentrations (ng/mL)							R (%)
	T_0 (0 h)	T_1 (24 h)	T_2 (48 h)	T_3 (72 h)	T_4 (96 h)	T_5 (D 8)	T_6 (D 10)	
6MAM	12.6 (84%)	16.9 (134%)	11.6 (92%)	12.3 (97%)	10.4 (83%)	11.4 (90%)	11.7 (93%)	93
COD	12.3 (100%)	12.5 (101%)	11.6 (94%)	12.3 (100%)	12.0 (98%)	11.8 (96%)	11.8 (96%)	96
DHC	15.5 (100%)	12.5 (81%)	11.9 (77%)	11.3 (73%)	11.8 (76%)	11.4 (74%)	11.6 (75%)	75
DIAZ	13.6 (100%)	14.5 (107%)	11.7 (86%)	11.7 (87%)	12.8 (95%)	N/A	10.4 (77%)	77
DMD	12.9 (100%)	14.4 (112%)	10.8 (84%)	9.2 (72%)	6.7 (52%)	N/A	11.1 (86%)	86
ETIZ	14.2 (100%)	10.6 (75%)	9.4 (66%)	13.7 (97%)	12.3 (87%)	N/A	10.8 (76%)	76
MOR	14.0 (100%)	14.6 (104%)	14.1 (100%)	15.5 (111%)	11.5 (82%)	N/A	11.4 (81%)	81
OXA	14.9 (100%)	11.0 (74%)	10.4 (69%)	11.3 (76%)	9.0 (60%)	10.6 (71%)	11.6 (78%)	78
TEMAZ	12.6 (100%)	11.3 (90%)	11.0 (88%)	11.0 (88%)	12.0 (96%)	11.1 (88%)	12.1 (97%)	97

R – Final theoretical recovery (%) Fridge stability results are shown below in Table 4-17 (high concentration, 80 ng/mL) and Table 4-18 (low concentration, 15 ng/mL).

All analytes, except etizolam and methadone, showed recoveries of ≥100%. The highest recovery was observed for oxazepam at 113%. However, all calculated concentrations, even those exceeding 100% recovery, were within 20% of the nominal, T_0, concentration an analytes are considered stable when stored in the fridge. This would be in accordance with the storage recommendations given by the manufacturer.

At low concentrations (Table 4-18), oxazepam showed the greatest reduction in analyte recovery. The final calculated recovery was outwith the 15% of the nominal concentration of 15 ng/mL. All other analytes, other than dihydrocodeine and oxazepam, showed recoveries exceeding 80% and could therefore be considered stable at these conditions.

Table 4-17 Fridge high concentration (80 ng/mL) results (*n* = 3)

Analyte	Calculated Concentrations (ng/mL)							R (%)
	T_{o0} (0 h)	T_1 (24 h)	T_2 (48 h)	T_3 (72 h)	T_4 (96 h)	T_5 (D 8)	T_6 (D 10)	
6MAM	80.1 (100%)	77.2 (96%)	78.3 (98%)	77.6 (97%)	81.7 (102%)	72.2 (90%)	80.2 (100%)	100
COD	71.5 (100%)	68.0 (95%)	73.9 (103%)	70.3 (98%)	71.4 (100%)	67.5 (94%)	80.2 (112%)	112
DHC	75.5 (100%)	68.0 (90%)	72.6 (96%)	73.6 (97%)	73.0 (97%)	69.8 (92%)	85.7 (124%)	100
DIAZ	84.1 (100%)	70.5 (84%)	73.5 (87%)	80.6 (96%)	93.0 (111%)	N/A	88.9 (106%)	106
DMD	73.6 (100%)	69.0 (94%)	68.9 (94%)	66.6 (90%)	74.0 (101%)	N/A	80.5 (109%)	109
ETIZ	84.6 (100%)	69.1 (82%)	72.7 (86%)	63.7 (75%)	67.7 (80%)	N/A	81.4 (96%)	96
MOR	74.9 (100%)	71.7 (96%)	74.8 (100%)	72.5 (97%)	72.2 (96%)	N/A	78.5 (105%)	105
OXA	69.3 (100%)	65.8 (95%)	67.3 (97%)	94.5 (93%)	66.4 (96%)	70.1(101%)	78.3 (113%)	113
TEMAZ	71.4 (100%)	65.9 (92%)	69.6 (97%)	68.5 (96%)	73.6 (103%)	69.7 (98%)	77.9 (109%)	109

R – Final theoretical recovery (%)

Table 4-18 Fridge low concentration (15 ng/mL) results (*n* = 3)

Drug	Calculated Concentrations (ng/mL)							R (%)
	T_{o0} (0 h)	T_1 (24 h)	T_2 (48 h)	T_3 (72 h)	T_4 (96 h)	T_5 (D 8)	T_6 (D 10)	
6MAM	12.6 (100%)	16.8 (133%)	12.6 (100%)	11.7 (93%)	10.2 (81%)	11.2 (89%)	11.9 (95%)	95
COD	12.3 (100%)	12.5 (101%)	12.2 (99%)	12.4 (101%)	11.9 (97%)	11.9 (97%)	11.9 (97%)	97
DHC	15.5 (100%)	12.5 (81%)	11.8 (77%)	11.2 (73%)	11.7 (76%)	11.5 (74%)	11.6 (76%)	76
DIAZ	13.6 (100%)	15.5 (114%)	10.7 (79%)	12.5 (92%)	13.3 (98%)	N/A	12.5 (92%)	92
DMD	12.9 (100%)	14.4 (112%)	10.8 (84%)	9.3 (72%)	6.8 (53%)	N/A	11.0 (85%)	85
ETIZ	14.2 (100%)	10.9 (77%)	9.3 (66%)	11.0 (78%)	11.5 (82%)	N/A	11.5 (82%)	82
MOR	14.0 (100%)	15.3 (109%)	14.4 (103%)	15.7 (112%)	11.4 (82%)	N/A	11.7 (84%)	84
OXA	14.9 (100%)	11.0 (74%)	10.4 (69%)	11.3 (76%)	10.7 (72%)	10.6 (71%)	10.6 (71%)	71
TEMAZ	12.6 (100%)	11.4 (91%)	11.1 (88%)	10.9 (87%)	12.0 (95%)	11.0 (88%)	12.2 (97%)	97

R – Final theoretical recovery (%)

Freezer stability results are summarised in Table 4-19 and Table 4-20 for high and low concentrations, respectively. The manufacturer does not recommend the storage of the NeoSAL™ device in this temperature environment, and it is possible that the variations observed for certain analytes are caused by interferences from the frozen buffer or the cellulose pad.

Analyte recoveries all exceeded 92% (observed for dihydrocodeine). The cause of the increase for the majority of analytes on day 10 is not known. QC and calibration graphs for all analytes were acceptable.

Table 4-19 Freezer high concentration (80 ng/mL) results (*n* = 3)

Analyte	Calculated Concentrations (ng/mL)							R (%)
	T_o (0 h)	T_1 (24 h)	T_2 (48 h)	T_3 (72 h)	T_4 (96 h)	T_5 (D 8)	T_6 (D 10)	
6MAM	80.1 (100%)	81.8 (102%)	78.0 (97%)	77.3 (97%)	78.1 (98%)	73.1 (91%)	84.0 (105%)	105
COD	71.5 (100%)	71.9 (100%)	72.7 (102%)	71.4 (100%)	70.4 (98%)	68.1 (95%)	84 (117%)	117
DHC	75.5 (100%)	71.9 (95%)	71.4 (95%)	74.5 (99%)	72.6 (96%)	69.4 (92%)	75.7 (92%)	92
DIAZ	84.1 (100%)	75.0 (89%)	72.4 (86%)	72.2 (86%)	64.4 (77%)	N/A	82.1 (98%)	98
DMD	73.6 (100%)	72.5 (98%)	70.2 (95%)	68.1 (93%)	78.0 (106%)	N/A	87.6 (119%)	119
ETIZ	84.6 (106%)	59.5 (70%)	70.0 (83%)	106.5 (126%)	69.2 (82%)	N/A	96.0 (113%)	113
MOR	74.9 (100%)	75.1 (100%)	73.6 (98%)	72.8 (97%)	72.4 (97%)	N/A	82.9 (111%)	111
OXA	69.3 (100%)	68.8 (99%)	67.4 (97%)	68.1 (98%)	67.2 (95%)	66.1 (95%)	68.0 (98%)	98
TEMAZ	71.4 (100%)	70.0 (98%)	68.9 (96%)	71.2 (100%)	69.8 (98%)	69.2 (97%)	75.8 (106%)	106

R – Theoretical Recovery (%)

Low concentration recoveries are summarised in Table 4-20. Greater variation was observed compared to the variation observed at the higher concentration. Lowest recoveries were seen for dihydrocodeine (76%), diazepam (73%), and oxazepam (78%). Only etizolam showed recoveries greater than 100%. All other analytes that showed recoveries between 87% and 98% can be considered stable.

Table 4-20 Freezer low concentration (15 ng/mL) results (*n* = 3)

Analyte	Calculated Concentrations (ng/mL)							R (%)
	T_o (0 h)	T_1 (24 h)	T_2 (48 h)	T_3 (72 h)	T_4 (96 h)	T_5 (D 8)	T_6 (D 10)	
6MAM	12.6 (100%)	15.2 (121%)	14.5 (115%)	11.5 (91%)	10.4 (82%)	11.0 (87%)	12.2 (96%)	96
COD	12.3 (100%)	12.6 (102%)	11.7 (95%)	12.4 (100%)	11.9 (96%)	11.9 (96%)	12.2 (98%)	98
DHC	15.5 (100%)	12.6 (81%)	11.8 (77%)	11.3 (73%)	11.6 (75%)	11.6 (75%)	11.7 (76%)	76
DIAZ	13.6 (100%)	16.6 (123%)	11.3 (84%)	12.1 (89%)	12.6 (93%)	N/A	9.9 (73%)	73
DMD	12.9 (100%)	14.4 (112%)	10.8 (84%)	10.8 (84%)	6.8 (52%)	N/A	11.2 (87%)	87
ETIZ	84.6 (100%)	59.5 (70%)	70.0 (83%)	106.5 (126%)	69.2 (82%)	N/A	96.0 (113%)	113
METH	13.4 (100%)	13.6 (102%)	11.1 (83%)	11.7 (87%)	10.9 (82%)	11.8 (88%)	11.9 (89%)	89
MOR	14.0 (100%)	15.7 (112%)	14.5 (104%)	16.1 (115%)	11.4 (81%)	N/A	11.8 (84%)	84
OXA	14.9 (100%)	11.0 (74%)	10.4 (69%)	11.3 (76%)	10.6 (71%)	10.6 (71%)	11.6 (78%)	78
TEMAZ	12.6 (100%)	11.3 (90%)	7.8 (62%)	11.1 (88%)	11.9 (95%)	11.2 (89%)	12.3 (98%)	98

R – Theoretical Recovery (%)

In vitro conversion of 6-MAM to morphine does not appear to be an issue as 6-MAM was found to be stable (recoveries for low concentrations ranged from 93% at benchtop temperatures, to 96% at -20°C, and high concentration recoveries ranging from 100% at approximately 4 °C to 105% for storage at -20 °C). A further factor that must be taken into account when assessing the theoretical recoveries of the analytes are the slightly low recoveries found for this collection device. This was found to be an issue especially for temazepam, diazepam, and desmethyldiazepam. This means that it is possible that more analyte was present but not recovered from the collection device.

4.5 Conclusion

The previously developed method was successfully validated for linearity, accuracy and precision, stability, dilution integrity, carryover, matrix effects and recovery for diazepam, desmethyldiazepam, oxazepam, temazepam, etizoloam, morphine, 6-MAM, codeine, and dihydrocodeine in both neat and buffered (collected using the NeoSAL™ collection device) oral fluid. The method

proved sensitive, as well as selective, both endogenous and drugs of abuse were not found to interfere with the quantitation of the analytes of interest in this study.

Neat oral fluid samples were more precise between runs; however, intra-day results showed wide ranges of precisions for both neat and buffered oral fluid. Desmethyldiazepam showed the widest range intra-day accuracies for neat oral fluid, while this was found to be true for temazepam in buffered oral fluid. Drug recoveries were particularly low for diazepam and temazepam from the collection device (46% for a concentration of 80 ng/mL, and 33% for 3 ng/mL for diazepam from buffered oral fluid, and 38% and 22% for temazepam for the same concentrations, respectively). However, recoveries from neat oral fluid were higher for both analytes (49% for both concentrations for diazepam, and 34% and 36% for high and low concentrations, respectively, for temazepam). For diazepam, especially these discrepancies indicate that the analyte may be retained on the collection pad of the collection device. That being said, recoveries of both analytes were found to be lower in the development stages as well, but it can be seen that the use of the collection device does not improve low recoveries. Some potential interferences from other analytes, especially other benzodiazepines, were ruled out due to the mismatched ion ratios, as well as the lack of both quantitation and qualifier ion peaks for the interferences investigated. Collection of neat oral fluid samples for this method and extraction procedure is preferred as neat oral fluid results were comparatively slightly better than those obtained for buffered oral fluid.

Limits of detection and quantitation found for the analytes in this method were reasonably similar to those published in the literature. In some instances the described method was more sensitive, and in some it was less sensitive, however the comparison of LC-MS/MS methods is more challenging than that of GC-MS methods, for example. Furthermore, there are no published methods regarding these analytes and the collection device used for this study.

Methadone was excluded from the validation process following the investigation of matrix effects for both sets of oral fluid, as these proved unacceptable (outwith ±25%) for this analyte.

Although the developed method has some faults (including low recoveries for certain analytes as well as previously mentioned unacceptable matrix effects for methadone), the method was validated successfully. Matrix effects for methadone in both oral fluid compositions were too variable and it was therefore decided that the quantification of this analyte was not possible. Low recoveries for temazepam and oxazepam were known and therefore it was possible to explain certain variability present in validation results. Nonetheless, the method was sensitive enough to detect concentrations lower than the cut-off concentrations recommended by the EWDTS.

4.6 Future Work

In order to further understand the effects of matrix components on the extraction procedure, and to see the variation that exists, it would be beneficial to include more than 10 sources of blank matrix in the assessment of matrix effects. Unfortunately, this was not possible as the donation of oral fluid to the project was voluntary and people are less comfortable donating oral fluid than urine, for example.

Applying this method to authentic oral fluid samples from real drug users would be beneficial in further testing the robustness and workability of the developed method. Carryover needs to be tested at a higher concentration.

Although the stability of these analytes has been extensively studied, with the exception of etizolam as this is a newer drug, stability of the analytes in neat oral fluid should be studied. It would then be possible to comfortably compare stability results from the NeoSAL™ device to neat oral fluid stability data that was collected on the same instrumentation. Further investigations should be made into whether diazepam and temazepam are retained on the collection pad of the NeoSAL™ device, as recoveries from the collection pad were especially low for these two analytes.

Finally, a dilution integrity study diluting the oral fluid with deionised water should be attempted, as it is probably not recommended to dilute authentic oral fluid samples with oral fluid collected from another source.

5 Application of Developed and Validated Analytical Method to Authentic Oral Fluid Samples

5.1 Introduction

The final assessment of the validity and practicality of an analytical method is the analysis of case, or authentic, samples. In order to achieve this for the previously presented developed and validated method for the simultaneous analysis of benzodiazepine and opiate/opioid drugs, authentic case samples were collected and analysed.

5.2 Ethical Approval

Ethical approval was granted by the North West – Liverpool Central Research Ethics Committee (REC reference: 17/NW/0329) (Appendix VI). Ethical approval was granted for the collection of 15 paired blood and oral fluid samples. Oral fluid was collected by expectoration and using the NeoSAL™ collection device. These samples were collected at the Muirhouse Medical Group in Edinburgh at the discretion of Professor Roy Robertson. The sample number was selected as this was an attainable number for the GP so as to not interfere in his treatment of the participants. However, 16 samples were received and analysed.

The criteria for inclusion in the study required participants to be:

- Over 18 years of age,
- Prescribed or using benzodiazepine drugs,
- Mentally and physically fit,
- Consenting to provide both blood and oral fluid samples,
- Understanding of written and verbal English.

5.3 Materials and Methods

5.3.1 Authentic Samples

Paired blood and oral fluid samples, expectorated and collected using the NeoSAL™ collection device, were collected from patients prescribed benzodiazepines. Study participants were selected at the discretion of Prof. Roy Robertson of the Muirhouse Medical Practice. 16 participants were recruited over a period spanning approximately 3 months (June – August 2017), and samples were taken immediately after consent was given. Prof. Robertson, who collected the oral fluid samples, was shown how to collect the samples; however no official training was provided prior to the beginning of sampling.

A total of 16 paired samples were collected which included 8 samples provided from males (average age 49 years, median age 48.5 years, range 37 – 62 years) and 8 female participants (average age 46 years, median age 46.5 years, ranging from 30 years to 65 years). The frequency of participants in different age groups is shown in Figure 5-1.

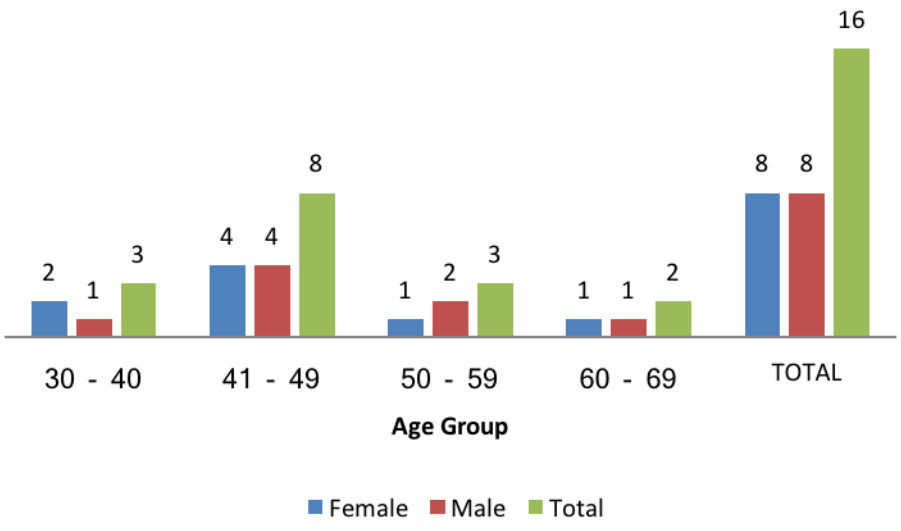


Figure 5-1 Frequency and Age Ranges (*n* = 16)

Following consent, participants were asked a few questions and a questionnaire was filled out with the GP (an example of the questionnaire can be found in Appendix V). Questions included the type of prescription medications participants used, what kind (if at all) and how often they used non-prescribed benzodiazepines, and what other drugs they consumed. It was made clear to the

participants that their health care would not be influenced or altered based on the answers they gave or the results from the analysis.

Out of the 16 participants, 4 participants (25%) admitted to using unprescribed benzodiazepine drugs (diazepam, MSJ or MSQ9, the latter two being forms of diazepam that are not “Valium”). Out of these four, one participant stated they used the unprescribed benzodiazepine when available, one participant admitted to 2-3 times weekly, and one admitted to 60 mg daily. 69% of participants (*n* = 11) stated they did not use unprescribed benzodiazepines. One participant did not answer the question. Results also showed that 5 (31%) participants admitted to recreational use of cannabis, one admitting to using heroin, and one admitted to using methadone although not being prescribed this.

Table 5-1 summarises the prescription medication, and daily dosage where available, and drugs that participants admitted to taking. MSJ Diazepam is a brand name for diazepam tablets, which can be purchased online, manufactured by Sri Lankan based J.L. Morison Son & Jones (Ceylon) PLC (163).

Table 5-1 Prescribed medication and other drug use of participants of the study

Case Number	Prescribed Medication (and dose, if known)	Other
1	Suboxone, lamotrigine, duloxetine, naproxen	Diazepam
2	Chlorpromazine diazepam, mirtazapine, methadone, pregabalin	N/A
3	Methadone, oxybutynin, sildenafil	Diazepam (MSQ/9)
4	Clonazepam, buprenorphine	Methadone
5	Methadone (75 mg daily), nitrazepam (17.5 mg daily)	N/A
6	Diazepam (5 mg), zopiclone, zirtle (?) (10 mg daily)	N/A
7	Buprenorphine/naloxone 16 mg daily, diazepam 25 mg daily	Cannabis
8	DHC, methadone, diazepam	N/A
9	Diazepam (as Valium, 30 mg daily)	Cannabis
10	DHC, diazepam, trazodone, gabapentin, tolterodine, cyclizine	N/A
11	Methadone, diazepam, mirtazapine, phenoxymethylpenicillin	Cannabis
12	Methadone, mirtazapine, diazepam	MSJ & heroin
13	Methadone, diazepam	Cannabis
14	Methadone (80 mg daily), diazepam (30 mg daily), gabapentin (300 mg daily), mirtazapine (45 mg daily)	Cannabis
15	Dihydrocodeine, amitriptyline, fluoxetine	Not prescribed diazepam
16	Cocodamol, dihydrocodeine, mirtazapine, diazepam	N/A

Other – drugs and substances participants admitted to taking, whether non-prescribed benzodiazepines or other drugs taken regularly (exceeding 1x a week)

5.3.1.1 Oral Fluid Samples

Prior to analysis, oral fluid samples were examined visually and observations were recorded throughout the sample preparation stages. This included noting down the actual volumes obtained from each sample. For buffered, NeoSAL™

samples, the volume refers to the oral fluid/buffer mixture. Findings are summarised in Table 5-2.

Table 5-2 Observations and volumes of authentic oral fluid samples

#	Collection Date	Expectorated/Neat[6]		NeoSAL™ Collected	
		Observations	Volume (µL)	Observations	Volume (µL)
1	01/06/17	Sample of mainly phlegm	700	Swab soaked and almost disintegrating	950
2	N/A	Very little sample	80	No buffer present	400
3	02/06/17	Very little sample	100	Particulates floating at bottom of tube	1200
4	06/06/17	Mucousy- phlegm, sticky and opaque	150	Black stains on pad	1150
5	13/06/17	Very small sample, phlegm on the side of collection container	50	Green in colour	950
6	16/06/17	Cloudy	600	N/A	1450
7	19/06/17	Bright yellow, almost hard, and particularly unpleasant	600	Pad tinted yellow	1450
8	21/06/17	Cloudy and sticky	300	Stiff pad	1350
9	22/06/17	N/A	160	Pad very floppy and saturated	1450
10	N/A	Yellowish sample	450	Orange speckled pad	1625
11	28/07/17	Yellow, grainy in appearance, brown	700	N/A	1800
12	04/08/17	Very little sample, dirty, particulates in sample	100	Stiff pad, slightly discoloured	1250
13	04/08/17	Phlegm, very little sample available	80	Saturated pad	1850
14	17/08/17	Impossible to pipette, only phlegm and food debris.	N/A	Slimy coating on pad	1250
15	22/08/17	Too little sample volume present to be able to use	N/A	N/A	950
16	22/08/017	Sample container labelled as “17”. No sample present.	N/A	N/A	1450
		Mean Volume (%CV)	313 (83)		1283 (28)

Neat samples from participants 14 – 16 were not analysed as they were not fit-for-purpose. The lowest volume obtained from the collectors was 400 µL, which

[6] Neat samples 14 – 16 were not appropriate for analysis and were therefore excluded from calculations of mean volume present. Only NeoSAL™ collected samples 14 – 16 were analysed.

was for the sample where the collection tube had no buffer present. It is unclear whether the device did not contain buffer when it was used for the collection, or whether the buffer had leaked or evaporated. None of the buffered oral fluid samples were present at the assumed volume of 2.1 mL. For later samples, the collection tube was weighed in order to determine how much oral fluid the mixture potentially contained. Doing this relied on previous data collated during the gravimetric assessment of collection volume adequacy (as described in Section 2.4.4). From this, it is known how much, on average, a collection device prior to oral fluid collection would and should weigh (i.e. an mean weight of 7.93g with %CV 0.3% for an unused collector, but a mean weight of 8.76g with a %CV of 2.0% for a used collector, were observed previously). By taking the difference between this value and the weight of the collection devices following collection, the difference should in theory be indicative of the volume of oral fluid collected. These results are summarised in Table 5-3.

Table 5-3 Collector weights on receipt for samples 11 - 16

Sample Number	Weight of collector (g)	Weight of unused NeoSAL™ collector (g)	Volume of Oral Fluid Collected (mL)
11	8.69	7.93	0.77
12	8.32		0.40
13	8.78		0.85
14	8.25		0.33
15	8.09		0.17
16	8.91		0.99
Mean	8.51		0.58
%CV	4%		57%

Theoretically, if the device collected 0.7 mL of oral fluid, as specified by the manufacturer, the weight following collection should be 8.63 g (based on a mean 'empty' collection device weight of 7.93 g). However, the average weight found for the used collectors was 8.5084. Based on the assumption that the weight of 1 mL of oral fluid is equivalent to 1 g, this means that the average volume of oral fluid collected was 0.58 mL (range 0.17 – 0.99 mL), which is lower than the theoretical amount by 0.12 mL. The variation of oral fluid collection between devices was large at 57%. It is however a concern that in one sample received,

only 0.17 mL of oral fluid appeared to have been collected. It was previously shown that the NeoSAL™ collector does have a tendency to collect 20% more oral fluid than stated by the manufacturer. This is not reflected by the results displayed above. It is a possibility that the lower volumes of oral fluid collected were a result of the xerostomia caused by the use of benzodiazepine and opioid/opiate drug use. A further reason could be low or varying buffer volumes in the collection tubes. For example, Sample 2, the NeoSAL™ device sample tube did not contain any buffer. This can be an issue for stability of the oral fluid sample but also calls into the question the collection device itself. A previously published article has noted the differences in buffer volume present (164).

5.3.2 Preparation of Calibration Standards, Internal Standards, and Quality Controls

A full calibration was prepared in neat and buffered oral fluid and run alongside authentic samples. The linear range was 1 ng/mL to 100 ng/mL. Calibration samples were prepared using solutions described in Section 4.2.2.1 and the volumes described in Table 4-1 in Section 4.2.2.1 were used to spike calibration standards at the correct concentrations. As before, 70 µL of internal standard working solution (at a concentration of 10 µg/mL) was added to all calibrators, QC and authentic samples, giving a final concentration of 100 ng/mL.

Three QC samples were freshly prepared in triplicate at concentrations of 1.5 ng/mL (QC1, Low), 15 ng/mL (QC2, Medium), and 80 ng/mL (QC3, High) for each analysis. Two blank samples were prepared, one without the addition of internal standard and one spiked with 70 µL of the previously mentioned internal standard solution.

For the opiate blood extraction, all drug standards (calibrators, internal standards, and QC) were prepared by a member of the Forensic Medicine and Science (FMS) laboratory. The calibration range spanned 25 – 500 ng/mL for morphine, dihydrocodeine, and codeine, and 5 – 200 ng/mL for 6-MAM.

5.3.3 Extraction Procedures

The oral fluid collection followed the same procedure as previously outlined in Section 4.2.3. Oral fluid samples were extracted using the extraction procedure previously validated (Section 3.3.6).

Blood samples were analysed for opiate drugs (6-MAM, codeine, morphine and dihydrocodeine) using the routine and validated GC-MS analysis used by the FMS laboratory, for which the extraction procedure is outlined below. All SPE extractions utilised UCT ZDAU020 Clean Screen® extraction columns. The procedure used by FMS at the University of Glasgow was followed to extract opiates from blood. This included 5 mL of 0.1 M pH 6 phosphate buffer/deionised water (1:2) added to all samples prior to vortex mixing for a minimum of 5 seconds and a 10 minute centrifugation step at 3000 rpm. SPE cartridges were conditioned by sequentially adding 3 mL MeOH, 3 mL deionised water, 1 mL 0.1 M pH 6 phosphate buffer. Samples were then allowed to pass through the extraction cartridges. Following this, cartridges were washed with 3 mL deionised water, 2 mL 0.1 M pH 4.5 sodium acetate buffer. Finally, 3 mL of MeOH were added before the cartridges were dried under full vacuum for 10 minutes. Excess liquid was removed from the needle tips. Elution occurred using 2.5 mL DCM:IPA:NH_4OH (78:20:2 v/v/v). All samples were evaporated to dryness under a gentle stream of nitrogen at a temperature of less than 40 °C. When samples had dried, they were allowed to cool before the derivatisation step. For this, 50 µL of BSTFA with 1% TMCS was added to each sample vial. Vials were capped, vortex mixed for a minimum of 5 seconds, and transferred to a heating block at 90 °C (± 4.5 °C) for 15 ± 2 minutes. Vials were allowed to cool before samples were transferred into labelled autosampler vials.

Benzodiazepines were extracted from blood using SPE, using a routine in-house, validated, extraction procedure. A calibration range (ranging from 50 to 2000 ng/mL for diazepam and metabolites, and ranging from 5 to 200 ng/mL for etizolam) was prepared and used for quantification. 3.5 mL of pH 6 Buffer Mix (pH 6 0.1 M phosphate buffer: deionised water, 1:2 v/v) was added to all standards, spikes and samples, before vortex mixing and centrifugation at 2500 rpm for 10 minutes. UCT Clean Screen® ZDAU020 SPE cartridges were conditioned with 3 mL of MeOH, 3 mL deionised water and 2 mL pH 6 phosphate

buffer. Samples were added to the cartridgesand allowed to pass through, before cartridgeswere washed with 2 mL deionised water, 2 mL phosphate buffer/acetonitrile (80/20) and dried under full vacuum for 1 minute. Following this, 2 mL of cyclohexane were added, and cartridgeswere again dried for 1 minute under full vacuum. 3 mL of deionised water were added before a further drying step under full vacuum for 5 minutes. 3 mL EtOAc with 2% ammonium hydroxide was used for elution. Samples were placed on the evaporating block and dried under a stream of nitrogen at 40 °C. Samples were reconstituted in 1.5 mL 50% MeOH:50% deionised water before being transferred to the autosampler.

5.3.4 Instrumentation

The instrumentation used for the analysis of oral fluid samples was the same instrumentation used for both method development and method validation, as outlined in 3.2.6.

Blood opiates were analysed on an Agilent Technologies 7890A GC system with a 7683B Series Injector coupled with a 5975C inert XL MSD with triple axis detector was used for the analysis. An Agilent DB-1 (30 m x 250 µm x 0.25 µm) column, with guard column, was installed and used for the analysis. The LOQ for the method was 25 ng/mL for morphine, codeine, and dihydrocodeine, and 5 ng/mL for 6-MAM.

Blood benzodiazepines were analysed on an Agilent Technologies 1260 Infinity HPLC system coupled with an AB-Sciex 3200 Q-Trap MS/MS system. Data was processed and quantitated using the pre-installed Analyst software (Version 1.6.3). The LOQ for the method was 50 ng/mL for diazepam, desmethyldiazepam, temazepam, and oxazepam, and 5 ng/mL for etizolam.

5.3.5 Calculation of Analyte Concentrations in Oral Fluid Samples

As described in Table 5-2, only very few of the authentic samples gave the volumes that were expected in the method development process (i.e. 0.7 mL expectorated oral fluid, 2.8 mL buffer/oral fluid mixture yielded from NeoSAL™ device). For neat oral fluid, many of the samples collected did not have a

volume of 700 µL so the amount available was used and resultant concentrations were multiplied up to reflect this.

For buffered oral fluid samples it was assumed that the correct volumes of oral fluid were collected. Therefore, buffered oral fluid samples were not multiplied up and the concentrations were determined by the quantitation software.

5.4 Results and Discussion

Results of both blood and oral fluid analyses are summarised in Table 5-4 below. As it had not been possible to accurately quantify methadone during the development and validation stages, only the presence of methadone is acknowledged in Table 5-4. Methadone was also not included in the analyses carried out on the blood samples (as it is not included in the FMS method for opiates). Neat oral fluid samples submitted from patients 14, 15, and 16 were not suitable for analysis and therefore no results are shown for these.

For concentrations that were found to be less than the lowest calibrator, concentrations were reported as less than the lowest calibrator. The LLOQ was adjusted to reflect the volume available, by visually assessing S/N ratios of the lowest calibrators.

From the table it can be seen that oxazepam and temazepam were more commonly detected in buffered oral fluid samples compared to neat oral fluid. For temazepam this is especially surprising as the drug recoveries found for this analyte during the method validation were lower for the collection device than those found for neat oral fluid. However, drug stability must be taken into account. Samples were not always frozen upon collection, but rather stored in the fridge. Although this may have been sufficient for NeoSAL™ oral fluid, this will not have been considered the best storage conditions for blood or neat oral fluid. In one instance samples were also stored overnight in a warehouse without air conditioning or temperature monitoring and were transferred in a non-temperature controlled delivery van.

Table 5-4 Summary of analytical results for oral fluid and blood samples for all 16 cases

Case #	Sample	Sample Volume Analysed	Concentration (ng/mL)									
			6MAM	MOR	COD	DHC	DIAZ	DMD	OXA	TEMAZ	ETIZ	METH
1	Blood	1000 µL	NEG	NEG	NEG	NEG	169.0	246	NEG	NEG	46	N/A
	Neat	700 µL	NEG	<1.0	18.8	NEG	7.9	137.1	NEG	NEG	6.3	PRESENT
	Buffered	950 µL	NEG	<5.0	29.0	NEG	7.0	24.0	NEG	NEG	9.2	PRESENT
2	Blood	1000 µL	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	8.0	N/A
	Neat	80 µL	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NOT PRESENT
	Buffered	400 µL	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	<1.0	NOT PRESENT
3	Blood	1000 µL	NEG	NEG	NEG	NEG	<50.0	100.0	NEG	NEG	NEG	N/A
	Neat	100 µL	53.3	1.37	NEG	NEG	NEG	NEG	NEG	NEG	NEG	PRESENT
	Buffered	120 µL	>100.0	NEG	36.7	NEG	2.3	11.6	NEG	NEG	NEG	PRESENT
4	Blood	1000 µL	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	N/A
	Neat	150 µL	368.4	36.0	NEG	NEG	NEG	NEG	NEG	NEG	NEG	PRESENT
	Buffered	1150 µL	30.6	<5.0	NEG	NEG	NEG	NEG	NEG	NEG	NEG	PRESENT
5	Blood	1000 µL	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	N/A
	Neat	50 µL	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	PRESENT
	Buffered	950 µL	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	PRESENT
6	Blood	1000 µL	NEG	NEG	NEG	NEG	120.0	132.0	NEG	NEG	NEG	N/A
	Neat	600 µL	NEG	NEG	NEG	NEG	111.0	93.91	NEG	NEG	NEG	PRESENT
	Buffered	1450 µL	NEG	NEG	NEG	NEG	84.5	31.9	NEG	NEG	NEG	PRESENT

Table 5-4 Summary of analytical results for oral fluid and blood samples for all 16 cases

Case #	Sample	Sample Volume Analysed	Concentration (ng/mL)									
			6MAM	MOR	COD	DHC	DIAZ	DMD	OXA	TEMAZ	ETIZ	METH
7	Blood	1000 µL	NEG	NEG	NEG	45.0	486.0	285	<50.0	<50.0	NEG	N/A
	Neat	600 µL	37.3	2.6	NEG	NEG	>100.0	157.6	13.2	78.2	NEG	PRESENT
	Buffered	1450 µL	10.6	NEG	NEG	NEG	>100.0	38.7	7.9	46.7	NEG	PRESENT
8	Blood	1000 µL	NEG	NEG	<25.0	>500.0	161.0	342.0	<50.0	<50.0	NEG	N/A
	Neat	300 µL	NEG	NEG	23.7	>100.0	31.3	69.3	NEG	NEG	NEG	PRESENT
	Buffered	1350 µL	NEG	NEG	<5.0	>100.0	12.6	32.3	<1.0	<2.5	NEG	PRESENT
9	Blood	1000 µL	NEG	<25.0	197.0	NEG	482.0	636.0	<50.0	<50.0	NEG	N/A
	Neat	160 µL	NEG	NEG	62.0	>100.0	53.7	14.0	<2.5	NEG	NEG	NOT PRESENT
	Buffered	1450 µL	87.4	99.9	>100.0	NEG	42.8	90.8	8.7	<5.0	NEG	NOT PRESENT
10	Blood	1000 µL	NEG	NEG	<25.0	>500.0	270.0	327.0	<50.0	<50.0	NEG	N/A
	Neat	450 µL	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Buffered	1625 µL	NEG	NEG	6.9	>100.0	13.0	25.8	<5.0	<2.5	NEG	PRESENT
11	Blood	1000 µL	NEG	NEG	NEG	NEG	174.0	304.0	<50.0	NEG	NEG	N/A
	Neat	700 µL	NEG	NEG	NEG	NEG	80.2	129.3	12.2	<5.0	NEG	PRESENT
	Buffered	1800 µL	NEG	NEG	NEG	NEG	63.7	46.5	5.4	<5.0	NEG	PRESENT
12	Blood	1000 µL	NEG	NEG	NEG	NEG	90.0	530.0	60.0	<50.0	NEG	N/A
	Neat	100 µL	NEG	NEG	NEG	NEG	98.6	584.3	92.1	<5.0	NEG	PRESENT
	Buffered	1250 µL	NEG	NEG	NEG	NEG	5.6	59.2	10.3	<5.0	NEG	PRESENT

Table 5-4 Summary of analytical results for oral fluid and blood samples for all 16 cases

Case #	Sample	Sample Volume Analysed	Concentration (ng/mL)									
			6MAM	MOR	COD	DHC	DIAZ	DMD	OXA	TEMAZ	ETIZ	METH
13	Blood	1000 µL	NEG	NEG	NEG	NEG	508.0	894.0	NEG	NEG	NEG	N/A
	Neat	80 µL	NEG	NEG	NEG	NEG	214.1	563.9	<5.0	<5.0	NEG	PRESENT
	Buffered	1850 µL	NEG	NEG	NEG	NEG	32.5	>100.0	9.8	<5.0	NEG	PRESENT
14	Blood	1000 µL	NEG	NEG	NEG	NEG	1344.0	1169.0	<50.0	<50.0	NEG	N/A
	Neat	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Buffered	1250	>100.0	>100.0	89.7	NEG	>100.0	56.9	<5.0	<5.0	NEG	PRESENT
15	Blood	1000 µL	NEG	65.0	<25.0	>500.0	61.0	100.0	NEG	NEG	8.0	N/A
	Neat	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Buffered	950 µL	>100.0	>100.0	>100.0	>100.0	NEG	<5.0	NEG	NEG	<1.0	NOT PRESENT
16	Blood	1000 µL	NEG	NEG	51.0	>500.0	428.0	395.0	NEG	NEG	NEG	N/A
	Neat	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Buffered	1450 µL	NEG	6.8	>100.0	>100.0	>100.0	47.7	<5.0	<5.0	NEG	NOT PRESENT

Where N/A – not available.

Three cases showed etizolam to be present (Case 1, 2, and 15). It was possible to detect etizolam in both buffered and neat oral fluid samples in **Case 1**, at a concentration of 6.3 ng/mL in neat oral fluid, and 9.2 ng/mL in buffered oral fluid. Diazepam, which the participant had admitted to taking, was found in both oral fluid samples (7.9 ng/mL and 7.0 ng/mL for neat and buffered oral fluid, respectively) and in blood at a concentration of 169.0 ng/mL. Codeine was found to be positive in both neat and buffered oral fluid (18.8 ng/mL and 29.0 ng/mL, respectively), but was not found to be positive in blood. The results found are indicative of codeine and diazepam, and etizolam, use, although codeine was not prescribed, but could have been obtained over-the-counter.

The remaining 15 cases showed good correlation between prescribed medication and detected positive results in oral fluid.

As the participants were selected based on their prescription and use of benzodiazepines, it is not surprising that the majority of cases showed the presence of benzodiazepines in oral fluid (and blood). Only 12.5% of cases (2 out of 16) showed no presence of a single benzodiazepine in blood or oral fluid – however in two of these cases the participants were prescribed benzodiazepine drugs that were not included in the scope of the validated method (**Case 4** – clonazepam and **Case 5** – nitrazepam). In **Case 4**, it must be assumed that methadone was taken "illegally" as it was not prescribed. This was reflected by oral fluid samples as the presence was found in both samples. For **Case 4**, 6-MAM and morphine were also present in both oral fluids at different concentrations. Concentrations were higher in the neat oral fluid than buffered sample, however it should be noted that a low volume of neat oral fluid was available therefore this may encompass analytical error due to multiplying up. The ratios that were found to be present between 6-MAM and morphine were similar in both neat and oral fluid. Morphine had to be reported as <2.5 ng/mL in buffered oral fluid as calibrators 1 and 2 (1 and 2.5 ng/mL, respectively) were outwith acceptable limits. In **Case 5** only methadone was prescribed apart from nitrazepam, and methadone was detected in both oral fluid samples.

Case 2 was known to be prescribed methadone and diazepam, yet neither prescribed drug was detected in oral fluid or blood samples. Blood analysis showed only etizolam present at 8.0 ng/mL, and this analyte was also found in

buffered oral fluid but at <1.0 ng/mL (i.e. not quantified). It was not found in the neat oral fluid sample but this is most probably due to low sample volume.

In **Case 13** the participant was prescribed methadone and diazepam. Methadone, although not quantified, was detected in both oral fluid samples. Diazepam and desmethyldiazepam were found in all blood and oral fluid samples (508.0 ng/mL and 894.0 ng/mL in blood, 214.1 and 563.9 ng/mL in neat oral fluid, and 32.5 and >100.0 ng/mL in buffered oral fluid for diazepam and desmethyldiazepam, respectively). Oxazepam and temazepam were found to be negative in the blood sample, however both were found at <5 ng/mL in neat oral fluid and oxazepam was present in buffered oral fluid at 9.8 ng/mL where temazepam was found at <5.0 ng/mL. It is possible that the lack of detection in blood was due to the LLOQ of the benzodiazepine blood method.

In 14 cases at least one benzodiazepine was found. In one case (**Case 15**), diazepam was present detected at 61.0 ng/mL in blood but was not detected in oral fluid. Its metabolite desmethyldiazepam was detected at a concentration of less than 5 ng/mL in buffered oral fluid, but 100.0 ng/mL were found in the blood. Temazepam and oxazepam were both not detected. The participant was being prescribed dihydrocodeine, amitriptyline, and fluoxetine only. Case 15 is also a further case in which etizolam was found to be positive at a concentration of 8 ng/mL in blood but although it was present in the oral fluid sample, it was not possible to quantify as the concentration was less than the lowest calibrator.

Case 3 was similar in that only methadone out of the analytes of interest was prescribed. The participant however admitted to taking diazepam/MSQ/9. Diazepam and desmethyldiazepam were detected in the blood and buffered oral fluid sample, but neither were detected in the neat sample. However, the lack of either analyte in the neat oral fluid may again be down to the limited sample volume available for the analysis (100 µL). 6-MAM was present in both neat and buffered oral fluid (53.3 ng/mL in neat and >100.0 ng/mL in buffered oral fluid). Codeine was found in the buffered oral fluid sample, but the low sample volume of neat oral fluid may explain the negative codeine result. Codeine was also negative in the blood, so it is possible that codeine was found at concentrations lower than the LLOQ of the analytical method. Morphine was only detected in neat oral fluid but not in buffered, but was negative in the blood sample. 6-MAM

was not present in the blood sample which would suggest that 6-MAM has already been metabolised to morphine. Cone (9) suggests that the presence of 6-MAM in oral fluid without the presence of morphine is indicative of recent snorting or smoked use of heroin, but the blood results do not corroborate this. It is possible that the results did not match due to the lower LOQ of the GC-MS method of analysis of opiates in blood.

In only one case (**Case 12**) did the participant admit to taking a benzodiazepine that was not prescribed to them by a medical professional. Prescription information shows that both methadone and diazepam were prescribed to the participant, but participant 12 admitted to taking MSJ and heroin.(163). Methadone, diazepam, nordiazepam, oxazepam, and temazepam were all present in both oral fluid samples. Blood analysis for benzodiazepines also showed the presence of diazepam and metabolites at a concentration of 90.0 ng/mL of diazepam, 530.0 ng/mL of desmethyldiazepam, 60.0 ng/mL of oxazepam, and <50.0 ng/mL of temazepam. The concentrations of diazepam, desmethyldiazepam, oxazepam, and temazepam correlated well with the neat oral fluid although concentrations found in neat oral fluid were slightly higher (for example desmethyldiazepam 530.0 ng/mL in blood but 584.3 ng/mL in neat oral fluid).

In the remaining cases it was possible to detect prescribed drugs in all samples tested. In **Case 16**, cocodamol (codeine and paracetamol), DHC, and diazepam were prescribed to the participant. DHC and codeine were both detected in blood and buffered oral fluid. Concentrations of diazepam, DHC, and codeine in buffered oral fluid exceeded 100 ng/mL. DMD, oxazepam, and temazepam (most likely as breakdown products from diazepam) were also detected. In the blood sample, diazepam and desmethyldiazepam were found at concentrations of 428.0 ng/mL and 395.0 ng/mL, respectively, which ties in with the results found in the oral fluid sample. Morphine was detected at a concentration of 6.8 ng/mL in the buffered oral fluid sample, but was not detected in the blood sample. The presence of morphine in this case is likely to be from codeine metabolism.

In **Case 9**, only diazepam was prescribed to the participant. The individual also admitted to cannabis consumption. However, blood and oral fluid analysis showed the presence of opiates as well as confirming diazepam use. Codeine and

DHC were found in neat oral fluid, at 62.0 ng/mL and >100 ng/mL, respectively. Buffered oral fluid showed high concentrations of codeine (>100 ng/mL), morphine (99.9 ng/mL), and 6-MAM (87.4 ng/mL). Buffered oral fluid was found to be negative for DHC as no qualifier ions were present. The discrepancies of opiate results found in neat and buffered oral fluid samples may be explained due to the low sample volume of neat oral fluid (160 µL). Results of neat oral fluid and blood would suggest codeine use due to the lack of 6-MAM, whereas results of buffered oral fluid could be indicative of heroin and codeine use. Codeine and morphine were detected in blood at concentrations of 197.0 ng/mL and <25.0 ng/mL, respectively. These concentrations are indicative of codeine use. 6-MAM was negative in the blood sample, but 6-MAM is relatively unstable and may consequently have biotransformed into morphine. Diazepam was found to be present in both oral fluid samples, as well as in the blood sample. Desmethyldiazepam was also detected in all three samples. Temazepam was present in buffered oral fluid and blood. The lack of temazepam in neat oral fluid could have been caused by degradation to non-detectable amounts or the low sample volume (160 µL instead of 700 µL), resulting in concentrations that could not be detected.

Similar to this, only diazepam was prescribed in **Case 6**. The analyte, as well as its metabolite desmethyldiazepam, was found in oral fluid and blood. Neither oxazepam nor temazepam were detected. Methadone was present in buffered and neat oral fluid but not prescribed, and the participant did not admit to taking this.

Diazepam was the only benzodiazepine prescribed in **Case 7** and diazepam was found in both neat and buffered oral fluid samples as well as in the blood sample. DMD was present in all three samples as well. Temazepam and oxazepam were found in blood and oral fluid samples. Oral fluid concentrations for temazepam were higher than in other cases (78.2 ng/mL, where in other neat samples it was either found to be negative, but 46.7 ng/mL in buffered oral fluid where it was <5.0 ng/mL in 50% of cases), and <50.0 ng/mL were found in the blood sample. The oral fluid results could be indicative of recent temazepam use, although this is not supported by the blood results. 6-MAM was also present in both oral fluid samples (37.3 ng/mL in neat and 10.6 ng/mL in buffered oral fluid). DHC was present in blood (45.0 ng/mL) but not in either oral fluid sample

– morphine was found at a low concentration in neat oral fluid (2.6 ng/mL), but not in buffered oral fluid or blood samples.

In Cases 11, 13, and 14 methadone and diazepam were both prescribed. **In Case 11**, both were detected in neat and buffered oral fluid, as well as in blood. Diazepam, DMD, oxazepam and temazepam were also found in both oral fluid samples at similar concentrations (except for DMD – 46.5 ng/mL in buffered oral fluid vs. 129.3 ng/mL in neat oral fluid), where only diazepam, DMD, and oxazepam were detected in the blood. Methadone was detected in buffered and neat oral fluid in **Case 13**. Here, diazepam, DMD, oxazepam, and temazepam were found in both oral fluid samples, but only diazepam and desmethyldiazepam were found in the blood. The presence of DMD, oxazepam and temazepam in the oral fluid is most likely due to the breakdown of diazepam. Desmethyldiazepam concentrations were higher than diazepam concentrations in blood and oral fluid samples. 6-MAM, morphine, and codeine were found in the buffered oral fluid sample in **Case 14**, but were all negative in blood, which could indicate that the analytes had not yet reached the bloodstream, if it had been very recent (snorted or smoked) use. Diazepam and all three of its metabolites were found in both blood and oral fluid samples although concentrations of all of these analytes were higher in the blood.

In **Case 8** methadone, diazepam and dihydrocodeine were prescribed. Found in all matrices, codeine was present at low concentrations in blood (<25.0 ng/mL), and both oral fluid samples (23.7 ng/mL in neat oral fluid, and <5 ng/mL in buffered oral fluid). Dihydrocodeine was found at a concentration of over 500.0 ng/mL in the blood sample and >100 ng/mL in both oral fluid samples, which is in keeping with the prescription information provided. Diazepam was present in oral fluid, as well as DMD. Methadone was detected (and prescribed), and further breakdown potential breakdown products of diazepam, oxazepam and temazepam (at concentrations less than 2.5 ng/mL) were found in neat oral fluid and in buffered oral fluid, respectively.

Dihydrocodeine was detected and prescribed in **Cases 10** and **15**. Unfortunately no result for neat oral fluid is available for **Case 10** due to a bad injection, and no sample was available for retesting or reinjection. However, buffered oral fluid showed the presence of DHC, codeine (both also present in the blood, at

concentrations of >500.0 ng/mL and <25.0 ng/mL, respectively), diazepam (13.0 ng/mL) and breakdown products DMD (25.8 ng/mL), oxazepam, and temazepam (the latter two at concentrations <2.5 ng/mL). Methadone was detected in the oral fluid. Correspondingly, dihydrocodeine was detected in blood and buffered oral fluid samples (>500.0 ng/mL in blood and >100.0 ng/mL in buffered oral fluid), as were codeine anddiazepam and all three of its metabolites included in the study. In **Case 15,** 6-MAM detected in buffered oral fluid (>100.0 ng/mL) was not present in blood. This could be indicative of 6-MAM having already been metabolised to morphine in the blood, or that the concentrations of 6-MAM present in the bloodstream were too low to be detected on the GC-MS. Morphine, codeine, and dihydrocodeine (the latter prescribed to the participant) were also found to be positive in both blood and oral fluid. The blood sample was positive for diazepam and desmethyldiazepam (DMD was also detected in the oral fluid). **Case 15** was also the final case that tested positive for etizolam in both the blood (8.0 ng/mL) and in the buffered oral fluid (<1.0 ng/mL).

Detection ratios between blood and oral fluid ratios were relatively consistent. Issues were found with diazepam and desmethyldiazepam, for a number of cases diazepam was found in higher concentrations than desmethyldiazepam in the neat oral fluid, but the opposite was seen in buffered oral fluid (i.e. desmethyldiazepam concentrations were higher than diazepam concentrations) which was not unsurprising based on recovery results observed previously.

5.5 Conclusion and Future Work

A short study was performed to test the applicability of the NeoSAL™ oral fluid collection device. The results showed good potential of the device to detect opiates and benzodiazepines, as the majority of cases that were positive in blood were positive in buffered oral fluid (a lack of overlap was found for 6-MAM, however it has a short detection window in blood and is more likely to be found in oral fluid, so this discrepancy was not found to be surprising). This clearly illustrates the problems attributed to real life use of the collection device as well as the analytes themselves. A more extensive study would be beneficial using paired samples, maybe including only cases where adequate sample volume is collected. This would make it possible to rule out errors caused by multiplying up concentrations or no detection due to low volume.

Some of the anomalies between paired oral fluid samples may be explained by the low sample volumes obtained through both collection methods, and possibly the stability issues due to the lack of control of storage conditions, especially during transportation between GP practice and laboratory, and more obviously the poor quality of the samples received.

The correlation between prescribed drugs and the detection in oral fluid proves that the developed method is fit–for-purpose as it can accurately detect the presence of analytes as well as their metabolites. However, further testing to include a larger cohort of samples would be beneficial.

6 Stability of GHB in Oral Fluid

6.1 Introduction

Stability of gamma-hydroxybutyrate (GHB) in biological matrices has been investigated by several authors (165-171). The two main matrices that have been studied are the most commonly used matrices in forensic toxicology: blood and urine. In the case of blood stability, both ante- and post-mortem stability studies have been conducted.

The studies have shown that GHB is stable in post-mortem blood in a number of circumstances (168), whether it be between sexes (171) or between fridge and freezer storage (165, 170). In cases where stability is indeed an issue, the problem is solved when a preservative is added to the sample (165, 169), although this could elevate GHB blood concentrations, depending on the preservative used (172). This is likely to happen as there is endogenous biotransformation of GHB's precursor GBL in basic conditions and GHB conversion to GBL in acidic conditions.

Although GHB has lost much of its fame, it is still encountered in laboratories throughout the UK and Europe. It remains especially popular among subcultures that take part in "chemsex" (173, 174). Studies conducted in Norway and Sweden tested drivers who were believed to be under the influence of drugs and GHB was found in a number of cases. The majority of studies published tested blood GHB concentrations (175), but serum has also been tested (176, 177). Studies similar to this have not been frequently conducted in the United Kingdom and studies that focused on analysing road-side drivers' blood for drug use have not included GHB (like the DRUID programme, or ROSITA projects) (178, 179). One roadside testing study that did include GHB found a mean GHB/GBL concentration of 126 mg/L (median 120 mg/L, range 80 – 190 mg/L, *n* = 5) (179). Pre-empting any studies that could be conducted analysing for GHB in oral fluid, is the question of stability. The stability of GHB in oral fluid has not yet been investigated and can be seen as the basis for any further work on GHB in oral fluid.

Three papers (180-182) exist in the literature about the detection of GHB in oral fluid/saliva. The most recent paper was published by De Paoli (181) in 2011 concerning endogenous concentrations of the drug in oral fluid (*n* = 120, range 0.15 – 3.33 mg/L, mean 1.29 mg/L, median 1.13 mg/L). The paper suggests that samples were stable, as derivatives were found to be stable for up to 24 hours at 5 °C, and oral fluid samples stored at –22.5 °C were found to be stable for 30 days. However, no stability study of GHB in oral fluid has ever been published. The importance of a stability study for GHB is to be able to differentiate between exogenous and endogenous concentrations and to show that exogenous concentrations are stable over a testing period. This prevents exogenous concentrations being confused with endogenous concentrations although GHB was ingested.

Oral fluid will not replace blood or urine as the matrix of choice when it comes to the analysis of drugs in a forensic setting (18); yet, it is a matrix that can be used as an alternative to the previously mentioned matrices. This may especially be true due to the comparable concentrations observed between oral fluid and blood over time. Oral fluid can be used at the roadside to test for DUID cases. However, with a lack of methods of testing for GHB in oral fluid, as well as the absence of a dataset of (endogenous) concentrations that can be expected for GHB in oral fluid, this is a difficult task.

A number of studies involving drivers have found GHB concentrations exceeding endogenous concentrations (178). Dresen studied serum samples of DUID drivers (176): 5 out of 247 (2.02 %) of subjects were positive for GHB as well as other drugs (amphetamines, THC and cocaine). The serum GHB concentrations in their study ranged from 28.1 – 166.0 mg/L. Dresen suggested that GHB should be taken into account when drug screening roadside samples, especially when there is no proof of consumption of any of the common drugs of abuse. Jones *et al*. (183), who conducted a substantial amount of research on GHB and DUID drivers, found a mean concentration of 89 mg/L in blood (median 82 mg/L, highest 340 mg/L, *n* = 548), of which 215 cases had only GHB in their systems (mean blood concentrations 91 mg/L, median 83 mg/L, highest 270 mg/L). GHB was determined in blood samples taken from DUID cases in Sweden between 1998 and 2007. The same study also found that 29% of drivers, on average, were arrested 3 times per driver with GHB in their systems. The authors also

suggested that due to the short half-life of GHB, it is possible that the GHB concentrations at the time of driving were substantially higher than those reported.

A study conducted by Barker *et al*. was aimed at gauging the reasons behind GHB use as well as users' feelings towards driving under the influence of GHB (184). Even though Barker's focus group participants stated that the majority would not recommend driving following GHB ingestion, "many participants" would still drive, for a number of reasons. The first reason mentioned by the focus group is the short-lived effect GHB has, as well as the lack of a test to prove GHB ingestion or "drunkenness". Participants also suggested that the dosage determined the ability to drive and that, as a user of GHB, it is possible to recognise loss of motor-function and therefore determine whether it is safe to drive or not (184). These results support the findings in the papers presenting concentrations (176, 183).

Most commonly, literature reports concentrations in serum or whole blood. However, blood testing is very invasive, and therefore it would be of use to investigate the stability of GHB in oral fluid to assess its practicality and effectiveness as a matrix to test for GHB in drivers and in other forensic cases.

6.2 Aims and Objectives

The aims and objectives of this research were to adapt and validate an in-house gas chromatography - mass spectrometry (GC-MS) method for the quantitative determination of GHB in oral fluid.

This method was then applied to assess the stability of GHB in oral fluid over 56 days. 6 days were initially chosen to mimic the expected turn-around time of a routine working laboratory (or more specifically FMS) but were then extended to 56 days to investigate longer term storage, as this was the number of samples prepared for analysis with the volume of oral fluid available.

6.3 Materials and Methods

6.3.1 Chemicals and Reagents

Gamma-hydroxybutyrate (GHB) and the internal standard (IS), GHB-D_6 (both at a concentration of 1 mg/mL), and N,O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA + 1% TMCS) were purchased from Sigma Aldrich (Gillingham, UK).

Acetonitrile (ACN) and MeOH (MeOH were purchased from VWR (Lutterworth, UK). Deionised water (dH_2O) was obtained from an in-house Millipore® system.

All solutions and chemicals were prepared and pipetted using Gilson PIPETMAN Classic™ accurate pipettes, calibrated in house.

Oral fluid was donated from one female, drug-free volunteer. However, it is important to remember that GHB is an endogenous compound to the mammalian system and therefore donated oral fluid is not GHB-free. In cases where oral fluid was required from different sources, oral fluid was voluntarily and anonymously donated from staff and students at FMS. Oral fluid was pipetted using an accurate, calibrated Gilson MICROMAN® positive displacement pipette.

6.3.2 Preparation of Solutions

6.3.2.1 Stock Working Solutions

Using the certified drug reference standard of GHB in MeOH, 2 stock working solutions were prepared: a working solution of 100 µg/mL, and one of 1 µg/mL.

To prepare the 100 µg/mL solution, 500 µL of the certified drug reference standard at 1 mg/mL was pipetted into a 5 mL volumetric flask and made up to 5 mL with MeOH.

In order to prepare a working solution of 1 µg/mL, 50 µL of the 100 µg/mL solution were pipetted into a 5 mL volumetric flask and filled to the 5 mL mark with MeOH.

The solutions were stored at -20°C in the freezer for a maximum of 6 months.

6.3.2.2 Calibrators, Quality Controls and Internal Standards

Calibrators were prepared by pipetting the correct volume into a 5 mL volumetric flask and making it up to the mark with MeOH. The calibration range and quality control samples were prepared according to the volumes outlined Table 6-1.

For QC solutions, a new stock solution was prepared from a new drug reference standard. It was ensured that the lot numbers of drug reference standards used for calibration stock solutions and QC stock solutions were different.

Table 6-1 Preparation of calibrators and QCs

Solution	Concentration (mg/L)	Volume of stock solution [100 µg/mL] used (µL)	Final Volume (mL)
Calibrators			
Calibrator 1	1.0	50	5
Calibrator 2	2.5	125	5
Calibrator 3	5.0	250	5
Calibrator 4	10.0	500	5
Calibrator 5	25.0	1250	5
Calibrator 6	40.0	2000	5
Calibrator 7	45.0	2250	5
Calibrator 8	50.0	2500	5
Quality Controls			
QC 1	4.2	210	5
QC 2	42.0	2100	5

To prepare an internal standard working solution at a concentration of 10 µg/mL solution, 50 µL of the certified drug reference standard for GHB-D_6 was pipetted into a 5 mL volumetric flask and made up to 5 mL with MeOH. 100 µL of this was added to every sample.

6.3.3 Instrumentation

The method that was used was the routine FMS method for analysis of BHB/GHB in blood and urine (185), which was subsequently adapted and validated for the detection and quantification of GHB in oral fluid.

An Agilent Technologies 7890A GC system with a 7683B Series Injector coupled with a 5975C inert XL MSD with triple axis detector was used for the analysis. An Agilent DB-5 5% phenyl methyl siloxane (30 m x 250 µm x 0.25 µm) column was installed and used for the analysis. The oven programme started at an initial temperature of 60 °C, held for two minutes and then ramped to 180 °C at 20 °C/minute. The first ramp was followed by a second ramp at 50 °C/minute until 250 °C was reached. This was held for one minute. The total run time was 10 minutes. Temperatures of the ion source, transfer line, and injector were 200, 250, and 250, respectively. The method was run in full scan, a splitless injection was used and the carrier gas flow was set to 1.2 mL/min.

Data analysis was carried out using the Agilent ChemStation® software (Enhanced ChemStation, MSD ChemStation E.02.02.1431).

The ions monitored for GHB throughout the SIM run were *m/z* 233, 204 and 117 and *m/z* 239 and 241 for the deuterated internal standard, where the underlined ions indicate quantifier ions. To ensure system suitability, a system suitability sample of 10 µL of stock solution and 10 µL of internal standard stock solution was injected prior to each analysis. Peak areas and retention times were monitored to ensure an adequate analysis.

6.3.4 GC-MS Method Optimisation

A short investigation was made into the effect of different split ratios of the analytical method on the results. The method developed by Hassan (185) was set as a split-injection method due to the sensitivity of the instrumentation used for the analysis. Therefore, initially, the split ratio of the method was set at 80:1. Different split ratios ranging from 20:1 to 90:1 were investigated when preliminary injection of GHB at a concentration of 50 mg/L gave a chromatogram that showed detector saturation. The gain factor was also investigated. This was originally set to 3 and assessment was made of the most suitable setting for this analysis.

Temperature programmes of the method were not optimised, as both unextracted and extracted standards showed acceptable chromatograms, and

the final run time of the above described method was 10.4 minutes, which was determined to be acceptable and would not require any further shortening.

Primarily, the analytical method was carried out in SCAN mode. This was later amended to SIM mode to give greater sensitivity (monitoring the *m/z* ratios mentioned in Section 6.3.3).

6.3.5 Extraction Procedure and Derivatisation Optimisation

In FMS, GHB is extracted from blood using a simple protein precipitation step using 500 µL of acetonitrile. The use of both 500 µL of acetonitrile and MeOH were both assessed, as both of these solvents were used in the literature (181, 186).

500 µL of acetonitrile was added to 250 µL of oral fluid spiked with 250 µL of standard working solution (Calibrator 8, 50 mg/L) and 100 µL of internal standard. Samples were vortex mixed before being centrifuged using a Heraeus Instruments Biofuge Pico microcentrifuge at 1300 rpm for 15 minutes. Following centrifugation, the supernatant was transferred to a 3.5 mL vial using a glass Pasteur pipette. Samples were evaporated under nitrogen gas, with the heating block set at 40 °C. Once the samples had been evaporated, 75 µL of BSTFA + 1 % TMCS were added for derivatisation and placed in a heating block set to 90 °C for 10 minutes. This was then transferred into correctly labelled GC autosampler vials and then placed on the autosampler for analysis.

For this particular project only expectorated oral fluid was used rather than oral fluid collected using a collection device, as endogenous concentrations of GHB are low, and therefore difficult enough to detect using GC-MS instrumentation without the added complication of an extraction buffer often contained in oral fluid collection devices. This was done five times using as many sources of blank oral fluid as possible (10 sources).

Drug recoveries were assessed for both sets of extractions to determine which was the most effective at extracting GHB from oral fluid. This was achieved by comparing peak area ratios (PAR) of extracted samples (spiked either pre-

extraction or post-extraction) and PAR of unextracted samples at the same spiked concentration.

Finally, the reconstitution volume, or the volume of derivatising agent used, was also investigated. 25, 50, and 75 µL were assessed to determine whether a lower volume would have an advantageous effect on the chromatography.

6.3.6 Method Validation

The partial method validation was completed according to SWGTOX guidelines (161). The only part of the validation that did not follow the guidelines was the evaluation of Accuracy and Precision. The guidelines state that analysis must occur on five consecutive days, however in this case there were only 4 days of analysis, and not on consecutive days due to instrument downtime.

6.3.6.1 Linearity

The calibration model was chosen to cover a wide range of concentrations. An 8-point calibration range covering concentrations from 1 mg/L to 50 mg/L was selected: using concentrations shown in Table 6-1. Residual plots were used to assess actual linearity.

Blank oral fluid was spiked using 250 µL of the 10 GHB working solutions (preparation outlined in Table 6-1) at different concentrations. Blank oral fluid spiked with 100 µL of internal standard (10 µg/mL) was also analysed but was not included on the calibration curves. The linearity was assessed by running five separate calibration curves over five consecutive days. The curves were produced by plotting peak area ratios (PAR) of GHB against the spiked analyte concentration.

6.3.6.2 Limits of Detection (LOD) and Quantitation (LLOQ)

Both the limit of detection and the limit of quantitation were assessed by injecting a series of diluted extracted standards ranging from 0.01 – 2.00 mg/L. The concentrations tested were 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0 mg/L. These concentrations overlapped with concentrations included in the calibration range, but this was done to ensure that the low calibrators would give a strong enough

signal to be included. The lowest concentrations were prepared by diluting the lowest calibrator to the correct concentration.

6.3.6.3 Accuracy and Precision

Accuracy and precision were calculated over a total 5 day period, using the equations detailed in Section 2.4.7.6.

These variables were investigated at two different concentrations: 4.2 and 42 mg/L (QC1 and QC2, respectively). QC samples were prepared as outlined in Table 6-1, over four different runs. Each concentration was assessed using six replicates.

The percentage coefficient of variance (%CV) was calculated and for the method to be deemed acceptable %CV must be ≤20%.

6.3.6.4 Selectivity and Specificity

The selectivity and specificity of a method must be assessed in order to ensure that the method is valid and worthwhile. To assess matrix interferences, 10 sources of blank matrix were spiked at a concentration of 50 mg/mL, and injected following the normal procedure, without the addition of an internal standard.

Interferences from common drugs of abuse and common prescription medication were also assessed. Analytes included in this are summarised in Appendix III.

6.3.6.5 Carryover

Carryover was assessed by injecting the highest standard of the calibration curve (50 mg/L) twice followed by three flushes of EtOAc. Chromatograms were visually assessed for any evidence of carryover.

6.3.6.6 Stability

A short time frame of 6 days was selected for the stability study initially. This was done in order to replicate a real-life scenario that would be found in a working laboratory. However, the stability study was extended to 56 days (8

weeks/2 months), with the first six days analysed initially and then one sample at the beginning of the week for up to 8 weeks.

Stability of GHB in oral fluid was assessed at two concentrations – 42 mg/L ("High") and 4.2 mg/L ("Low"). Stability samples were prepared in quadruplicate. Three temperature conditions were assessed: Samples were stored at room temperature (approximately 23 ± 1.5 °C), in the fridge (approximately 4 ± 1.5 °C) and in the freezer (approximately -22 ± 1.5 °C). Temperatures were monitored to ensure that in the case of outlier points of the stability study, it would be possible to determine whether a change in temperature caused the variation. Freeze-thaw analysis and autosampler stability were not assessed for this investigation.

For each run, two QC concentrations were run in triplicate, prepared freshly for each stability day as shown previously. A fresh calibration was also prepared for each of the assessment days. Two blank samples were run as well, one with internal standard present and one without. 100 µL of internal standard (10 µg/mL) were added to all calibrators, QC, one of the blanks, as well as to the stability study samples assessed on that particular day.

6.3.6.7 Statistical Analysis

In order to assess the significance of variation observed in stability study samples, IBM® SPSS® Statistics Data Editor (Version 21, Release 21.0.0.0) software was used to run a simple analysis of variance (ANOVA). Variation was deemed statistically insignificant when $p \leq 0.005$.

6.4 Results and Discussion

6.4.1 GC-MS Method Optimisation

The final, and optimum, conditions that were used included an oven programme which was held at 60 °C for 2 minutes, then ramped at 20 °C/minute to 180 °C and then finally ramped at 50 °C/minute to 250 °C. The final temperature was held for 1 minute, post-run. The injection volume was 1 µL. A split ratio of 60:1 was used, with a split flow of 60 mL/min. Ultimately, the gain factor was set to

4 as lower gain factors caused problems with the instrumentation and the method run.

The total run time for the method was 10.4 minutes, with GHB eluting at approximately 7.65 minutes and GHB-D_6 at 7.62 minutes.

The method was initially used in full SCAN mode to detect all ions and was later changed to a SIM method, monitoring the ions mentioned in Section 6.3.3. Improvements in peak shape, peak abundances, and the general noise of the TIC were observed. A TIC of extracted Calibrator 6 (40 mg/L) run in scan mode is shown in Figure 6-1, and the associated mass spectrum is shown in Figure 6-2.

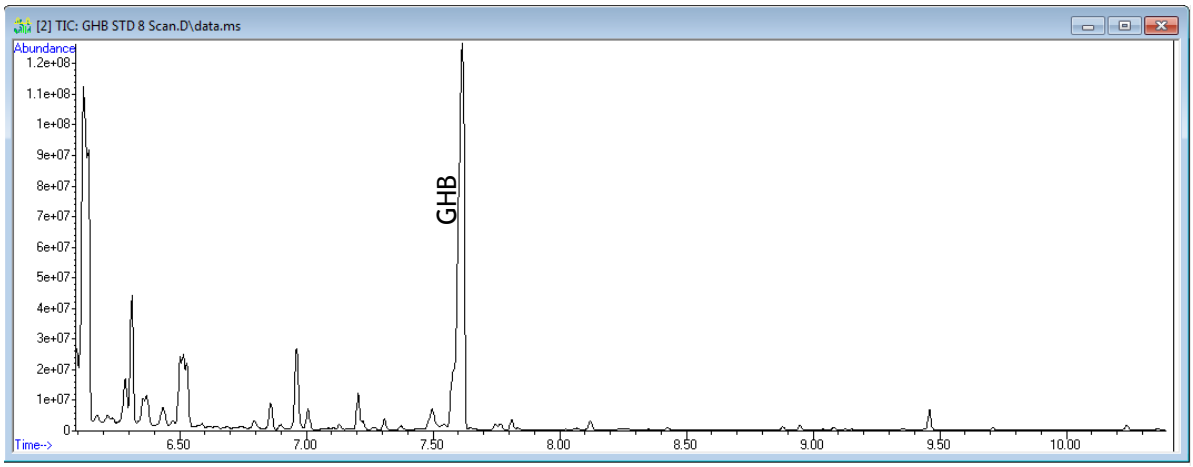


Figure 6-1 GHB Calibrator 6 (40 mg/L) TIC in scan mode

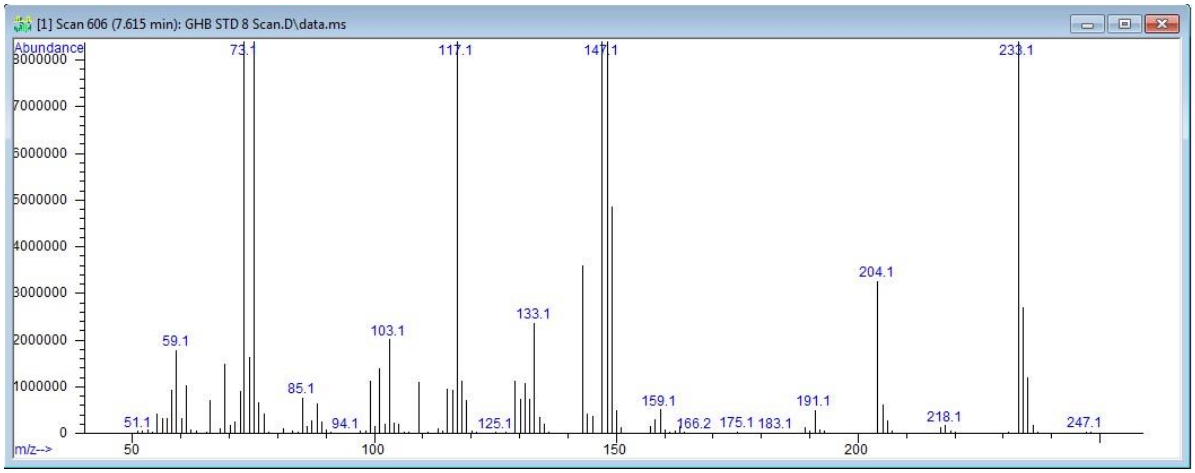


Figure 6-2 Mass spectrum of GHB peak in TIC of GHB Calibrator 6 (40 mg/L)

A noisy chromatogram is seen between 0 and 6 minutes. This was dramatically improved when SIM mode was used, as can be seen from the TIC (Figure 6-3).

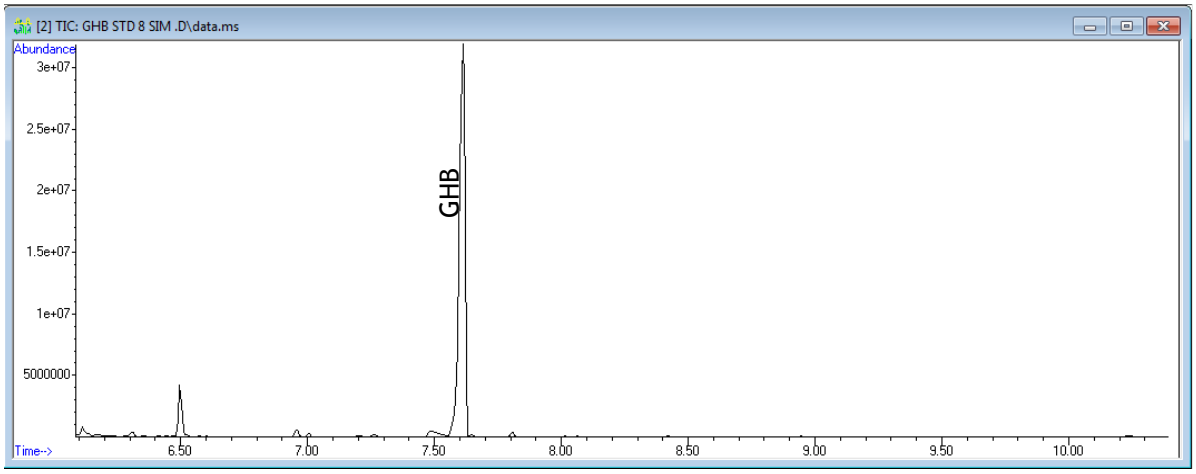


Figure 6-3 GHB Calibrator 6 (40 mg/L) in SIM mode

Although some noise and peaks are present, the peak at 6.50 minutes improved in shape and even the peak for the *m*/z 233 ion observed at about 7.60 minutes improved. The mass spectrum observed for the GHB peak in SIM mode is shown in Figure 6-4.

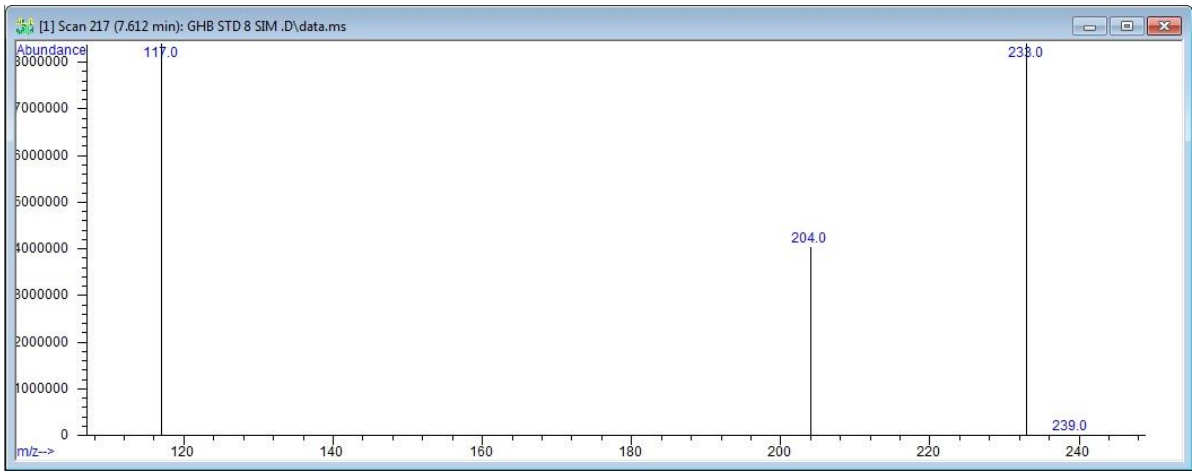


Figure 6-4 Mass spectrum observed for GHB peak in Calibrator 6 (40 mg/L) when run in SIM mode

Different split ratios were investigated to determine which split ratio would be the most suitable for the analysis. During this time, Calibrator 6(40.0 mg/L) and Standard 7 (45.0 mg/L) were also added to the calibration range, due to the results obtained as the previous high standard was saturating the detector. Finally, a split ratio of 60:10 was chosen.

6.4.2 Extraction Procedure and Derivatisation Optimisation

Drug recoveries from protein precipitation using MeOH and acetonitrile were assessed. A mean drug recovery of 91% (%CV 3%) was found for the extraction

using acetonitrile. This recovery was determined to be acceptable, and so this method of extraction was used, especially as protein precipitation with MeOH only gave a mean drug recovery of 65% (%CV 5%).

6.4.3 Method Validation

6.4.3.1 Linearity

Calibration curves were established by calculating the peak area ratio (PAR) of the standard response to the IS response and plotting this against concentration. An example calibration curve is shown in Figure 6-5. Residual plots (not shown), showed a slight inverse funnel indicating more variation at lower concentrations, closer to the LOD and LOQ of the method. However, it was determined that the dispersion of the points was random enough to use a linear fit for the calibration graph.

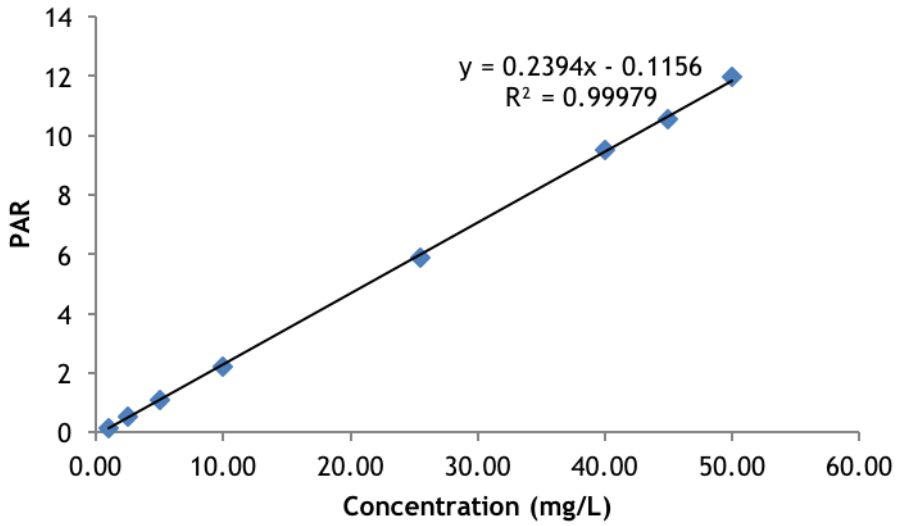


Figure 6-5 Example of a calibration curve for GHB in oral fluid (1 - 50 mg/L)

Calibration curves showed an R^2 value of greater than 0.999 (and a minimum of 0.99, $n = 5$).

6.4.3.2 Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

Due to limited analytical sensitivity the LOD (n = 18) for this method was determined to be 0.05 mg/L. The average signal-to-noise ratio determined for this concentration was 4.9. In the early stages, the LOD was found to be 0.01 mg/L, however this was not reproducible so a higher concentration had to be

selected. The reproducibility issues will have most likely been caused by the dirtiness of the sample following the extraction.

The LLOQ was determined to be 1.0 mg/L, and this, on average, gave an S/N of 12.8 for qualifier and quantifier ions. Initially, lower concentrations gave higher S/N ratios, but it was found that this was not reproducible and therefore a higher concentration of 1.0 mg/L was set as LLOQ.

The %CV calculated for the quadruplicate runs was less than 20% for each concentration investigated, both for LOD and LLOQ when reproducible concentrations were selected.

6.4.3.3 Accuracy and Precision

The mean concentration is calculated for each QC (QC1 at 4.2 mg/L and QC2 at 42 mg/L, *n* = 12). Intra-day results are summarised in Table 6-2, inter-day results in Table 6-3.

Table 6-2 Intra-day accuracy and precision results (*n* per concentration = 6)

QC (mg/L)	Calculated Concentration (mg/L)	Accuracy (%) (*n* = 6)	Precision (%CV) (*n* = 6)
QC1 (4.2)	4.9	88 - 91	1.7 – 4.2
QC2 (42.0)	51.7	76 - 114	2.5 – 15.7

Inter-day accuracy was assessed over four days.

Table 6-3 Inter-day accuracy and precision results (*n* per concentration = 6)

QC (mg/L)	Calculated Concentration (mg/L)	Accuracy (%) (*n* = 4)	Precision (%CV) (*n* = 4)
QC1 (4.2)	4.9	116.7	24.8
QC2 (42.0)	51.7	123	19.6

Variability was an issue with the low QC at 4.2 mg/L, which can be seen by the high range of intra-day precision results, as well as the high inter-day %CV/precision (%). Inter-day accuracies for both QC samples were unacceptable. Accuracy and precision was not assessed at the LLOQ.

The highest QC calculated higher than the presumed concentration, however, it was just within 20% of the nominal concentration and was therefore accepted.

6.4.3.4 Selectivity and Specificity

The analysis was carried out in both full SCAN and SIM mode and no inferences were found from assessed common drugs of abuse and common prescription medications.

6.4.3.5 Carryover

Carryover (*n* = 10) was not found to be an issue with this method. The highest calibrator was injected three times followed by five EtOAc washes and none of the washes showed any peaks which would indicate the presence of either GHB or GHB-D_6.

6.4.3.6 Stability

The calculated concentration over a 56-day testing period of the stability study, did not show a lot of variation at 4.2 and 42.0 mg/L. Stability was determined by concentrations remaining within 20% of initial theoretical recovery.

In order to accurately assess the variation observed in the stability samples, calibrators and QC samples were assessed for their acceptability. For each of the runs, standard deviation and %CV were calculated for QC controls. A concentration for each of the calibrators was also calculated. In order to be deemed acceptable, the calculated concentrations for both QC and calibrators had to be within ±15% of the nominal concentration, and %CV had to be within ±15%.

Analyses were run on the blank samples included in each run. Although the LLOQ was 1.0 mg/L for all time points, GHB peaks that were detected in the blanks. These were not quantified as they were all lower than 1.0 mg/L. S/N was monitored for each of these, and the ratios were within ±20% of each other. They were further expected to be present due to the endogenous nature of the analyte in question.

The stability of the high concentration spike is shown in Figure 6-6. The concentrations were stable with slight increases at Days 4 and 5, which are likely to be due to analytical variation, before settling back down. The mean

concentration over the testing period was calculated to be 44.1 mg/L (%CV 2.9%), 44.0 mg/L (%CV 3.1%), and 43.3 mg/L (%CV 3.6%) for fridge, freezer, and storage at room temperature, respectively.

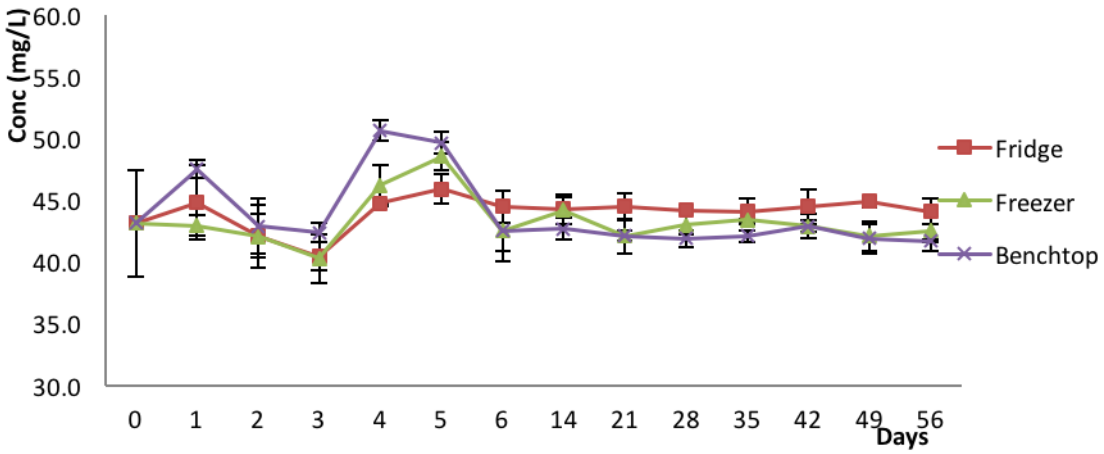


Figure 6-6 Stability of oral fluid spiked at 42.0 mg/L ("High")

All calculated concentrations were within ±20% of the nominal concentration, with the exception of Day 4 benchtop which calculated at a concentration of 50.7 mg/L. For Day 4, QC and calibrators were acceptable, and were within ±15% of the accepted concentrations, with an R^2 value of 0.996. At a high concentration of 42 mg/L, final theoretical recoveries of GHB from oral fluid over 56 days were 97% for room temperature storage conditions, 102% for samples stored in the fridge, and 98% for freezer stability sample.

When one assesses the stability at 4.2 mg/L, as shown in Figure 6-7, it seems like that there is a slight variation in concentration at Days 4 and 5, however, when observing the differences in concentration, the variation is not statistically relevant.

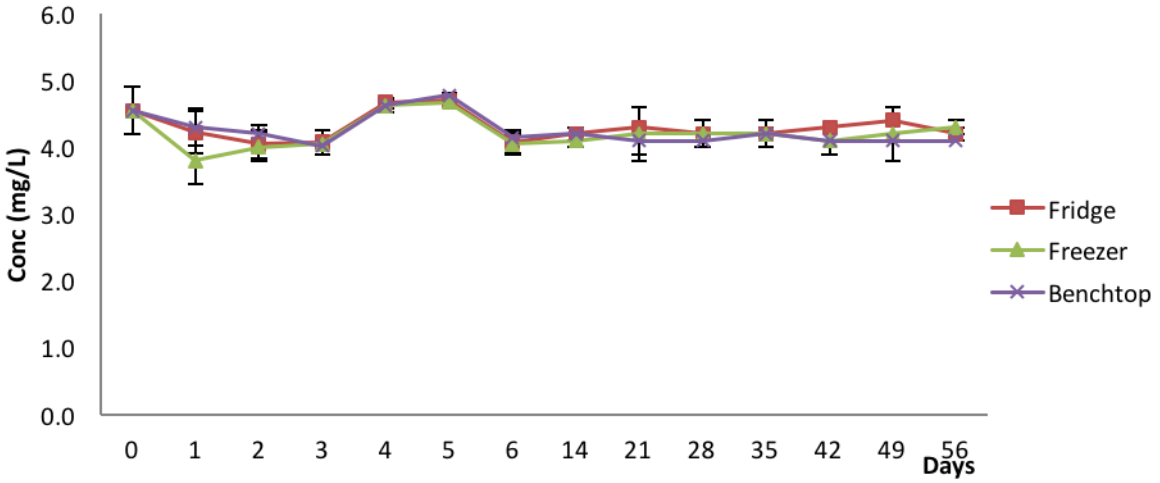


Figure 6-7 Stability of oral fluid spiked at 4.2 mg/L ("Low")

Calculated concentrations for each time point was within ±15% of the nominal concentration, whereas high concentrations were only within ±20%. The highest %CV was found for Day 0, where a %CV of 7.8 was observed on Day 0. Over the course of the testing period, %CV decreased and did not exceed 4.3% and 4.8% for room temperature and fridge assessments. Freezer stability showed maximum %CV of 9.5%. Again, the slight variation around Days 4 and 5 are most likely down to instrumental and analytical variation, as this was observed for both concentrations for all three storage conditions tested. Final theoretical recoveries of GHB were 90% for benchtop samples, 92% for fridge samples and 95% for freezer samples. Not much difference is observable for these three recoveries, and GHB seems to be stable at all three temperatures.

Even with slight variation, as also indicated by the line of standard deviation, although a bit of variation occurs, the concentrations in the final analysis are very similar, across both stability concentrations. ANOVA statistical analysis carried out on both concentrations worked and variation was found to be statistically insignificant as *p* was greater than 0.05 for all temperature conditions.

6.5 Conclusion and Future Work

In conclusion it can be seen that the method was partially validated according to the SWGTOX guidelines, however the method is not sensitive enough to reproducibly quantify endogenous GHB concentrations. Especially inter-day accuracy and precision were not acceptable according to SWGTOX. The cause of

this may have been the lengthy extraction procedure, which could have introduced variability to the results.

GHB appeared to be stable at both concentrations (4.2 mg/L and 42.0 mg/L) at all three testing temperatures for up to 56 days. Although GHB appears stable over a period of 56 days, it would be advisable if a longer-term stability study were conducted, as well as testing a higher spiked concentration. Furthermore, the stability study should include assessment of freeze-thaw stability of GHB in oral fluid as well as an autosampler stability to investigate the stability of processed samples.

Due to the nature of oral fluid, it would also be advisable to switch the method from a GC-MS to a GC-MS/MS method or to a LC-MS/MS for increased sensitivity. Although a simple protein precipitation using acetonitrile was used, the sample preparation step was too time-consuming due to the aqueous nature of the matrix. Due to the high aqueous component of oral fluid, samples were very difficult to evaporate and blow down using a gentle stream of nitrogen as advised in the extraction and sample preparation procedure. Therefore it would be advisable to explore other extraction procedures that would be less time consuming, such as LLE procedures.

It would also have been interesting to evaluate the effect of pH on the analysis, as GHB is converted to its prodrug gamma-butyrolactone (GBL) in acidic conditions. This should still at least be attempted, dependent on the sensitivity of the available instrumentation.

A further limitation of the study was that the pH of oral fluid was not taken into account and not measured for every time point included. This could be carried out in conjunction with the investigation into whether the use of a collection device, probably with a buffer solution would affect the stability and the drug recovery.

Finally, it would be worth applying this method to oral fluid case samples, where the known use of exogenous GHB is documented. This would test whether the actual employability of the method.

7 Endogenous Concentrations of Gamma-Hydroxybutyrate (GHB) in Post-mortem Blood from Deaths Unrelated to GHB Use 2010 – 2016

7.1 Introduction

Various investigations into endogenous concentrations of gamma-hydroxybutyrate (GHB) in post-mortem blood have been made (166, 169, 187-195). Where much debate has been sparked due to its use in drug-facilitated crimes (DFC) including drug-facilitated sexual assault (DFSA), further controversy arises in forensic toxicology when attempting to select a cut-off concentration that can be used to actively and accurately distinguish between endogenous and exogenous or even GHB produced post-mortem. GHB is an endogenous molecule, which when taken exogenously can increase sexual promiscuity and if spiked into a drink, is very hard to detect as its only distinguishing feature is a slightly salty taste. Additionally, detection windows of GHB in ante-mortem blood and urine samples are very narrow and it becomes impossible to distinguish exogenous from endogenous GHB after a few hours - thereby increasing its attractiveness to be used in DFSA or DFC scenarios. At post-mortem, however, it has been found that concetrations of endogenous GHB can increase, and the exact reason for this phenomenon has not yet been established.

Possible mechanisms have been explored to explain the production of GHB post-mortem. One mechanism, as suggested by Moriya and Hashimoto, is by glycolysis of bacteria. This causes the conversion of glucose to succinate, which can be reduced to succinic semialdehyde, which can be further reduced to GHB (visually represented in Figure 7-1). Post-mortem concentrations in this paper ranged from 1.33 – 44.3 mg/L, with an average of 9.80 mg/L. A median concentration was not reported (194).

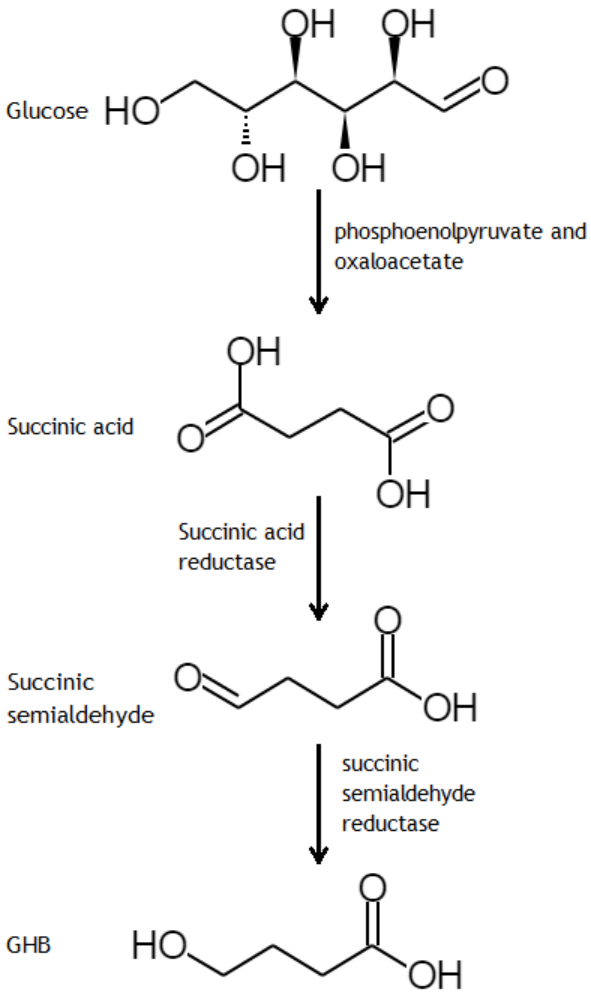


Figure 7-1 Possible post-mortem production of GHB by bacterial glycolysis (194)

On the other hand, Elliott *et al.* postulated the influence of putrefactive micro-organisms on GHB production. No relationship between putrefactive compounds (*Clostridium perfringens*, *Clostridium sordellii*, *Escherichia coli*, *Proteus vulgaris*, *Enterococcus faecalis*, *Aeromonoas hydrophila*, and *Pseudomonas aeruginosa*) and GHB concentrations was established. Elliott however suggested that a correlation may become clear on a longer timescale (196).

As levels of GHB increase post-mortem, it is advisable to establish a cut-off level to distinguish between endogenous and exogenous GHB. The cut-off of 50 mg/L commonly used was suggested by Kintz *et al.* in 2004 (189), however this level should be reviewed, as higher levels have been reported, which do not necessarily imply illicit use. It has also been suggested that it would be advantageous to use a preservative, sodium fluoride rather than sodium citrate, for post-mortem blood samples to inhibit in vitro formation of post-mortem GHB (169), however inconclusive results call for further investigation.

A further factor that must be considered when investigating post-mortem concentrations of GHB is the post-mortem interval (PMI) and the potential role that advancing decomposition plays (196). Post-mortem interval describes the time elapsed since a person has died and the discovery of the body. Several factors are used to determine the PMI, which can provide an estimation of the time of death should this not be known. Putrefactive, decomposition changes, and insect infestation can be used to estimate PMI (197-199).

Concentrations of endogenous post-mortem GHB reported in the literature are summarised in Table 7-1 below.

Table 7-1 Post-mortem endogenous concentrations of GHB as reported in the literature

Reference	Matrix	Mean (range) GHB (mg/L)	*n*
Elliott *et al.*, 2004 (196)	Unpreserved blood	7 (0 – 16)	6
	Preserved blood	13 (4 – 24)	3
Moriya and Hashimoto, 2005 (200)	Femoral blood	5 (2 - 8)	23
Kintz, 2004 (189)	Femoral blood	30 (17 – 44)	5
	Cardiac blood	17 (0.4 – 409)	71
Elliott, 2004 (193)	Unpreserved blood	12 (2 – 29)	38
	Preserved blood	13 (4.0 – 25)	17
Fieler *et al.*, 1998 (195)	Blood	25 (0 – 168)	20
Bosman *et al.*, 2003 (201)	PM Blood	6 – 40	14
	PM Blood	10 - 29	3
Busardò *et al.*, 2014 (202)	Femoral blood	6 (0.54 – 24.12)	30
Fjeld *et al.*, 2012 (203)	Femoral blood (preserved)	53.7 (10.5 – 201.5)*	18
Castro *et al.*, 2016 (187)	Femoral blood	8.43 (1.82 – 15.80)	32
Andresen-Streichert *et al.*, 2015 (204)	Femoral blood	11.9 (<0.6 – 28.7)	61
	Heart blood	15.2 (<0.6 – 65.3)	56
Lelong *et al.*, 2014 (191)	Peripheral blood	25.1 (<1.0 – 246.0)	31
	Cardiac blood	17.8 (<1.0 – 176.6)	20
Busardo *et al.*, 2017 (205)	Peripheral blood	3.64 (0.7 – 32.1)*◊	22

*** Initial analysis; PM – post-mortem; ◊ – mean given is the geometric mean**

7.1.1 Aims and Objectives

The aim of this retrospective data study was to investigate endogenous concentrations of GHB in post-mortem blood. This was achieved by collating all cases that were analysed for beta-hydroxybutyrate (BHB). BHB analysis is usually requested in cases of suspected alcohol/diabetic ketoacidosis. The BHB analysis simultaneously tests for GHB. Cases where GHB use was specifically implicated were excluded as well as cases where GHB analysis was requested. By doing this, it was possible to evaluate the concentrations of GHB found in cases where alcoholic and/or diabetic ketoacidosis were suspected.

The other main objective was to attempt to investigate if there was a correlation between decomposition changes and advancement with GHB concentrations.

7.2 Ethical Approval

Ethical approval for this project was granted by the West of Scotland Research Ethics Service (WoSRES), REC reference 16/WS/0236 (Appendix VII). A signed letter from the Head of the Scottish Fatalities Investigation Unit, David Green, giving permission to access and review data from FMS for scientific research and publication purposes can be found in Appendix VIII.

7.3 Methodology

7.3.1 Case Selection

The department of Forensic Medicine and Science (FMS) is located within the University of Glasgow and includes both Forensic Pathology and Toxicology. The toxicology department dealt with approximately 1600 – 1700 cases per year during the time of the study, but the case load has since as increased to around 3300 cases per year. Throughout the duration of the study, the majority of cases originated from the west coast of Scotland including the city of Glasgow and the surrounding urban areas, which are recognised as the most densely populated zones in Scotland.

Initially, all analytical data from BHB/GHB cases from 2007 to 2012 was retrieved from the in-house database. This was later extended to include cases from 2013 to 2016. At FMS, GHB and BHB are analysed simultaneously due to their molecular similarity, as shown in Figure 7-2.

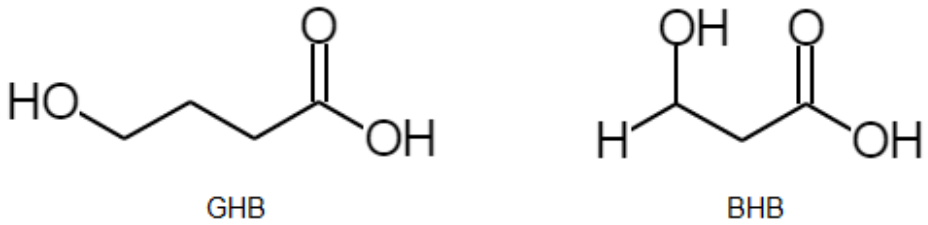


Figure 7-2 Chemical structures of GHB and BHB

The method of analysis of BHB/GHB changed in 2010 from a gas chromatography – flame ionisation detection (GC-FID) method to the later described gas chromatography – mass spectrometry (GC-MS) method. To ensure a homogenous dataset and to exclude variation in results caused by different techniques of analysis, cases predating 2010 were excluded. Other case information, including demographic data, pathological findings as well as the cause of death is stored on the in-house database.

For this investigation, all information pertaining to the toxicology results and pathology findings was extracted. From pathology reports, information pertaining to sex, date of birth, age at death, category and cause of death, but also the date last seen alive and dates of post-mortem examination were extracted. Findings relating to post-mortem changes were extracted for cases with GHB concentrations exceeding 50 mg/L, and any post-mortem changes that would be considered indicative of advanced decomposition were extracted from the reports. Results of the toxicological analysis were extracted, and only GHB concentrations were noted. Cases where full pathological findings were not available due to the pathologist being based outwith FMS, were omitted. Moreover, specifically for this investigation, cases where GHB was implicated in the police reports, mentioned in the cause of death, or the analysis for GHB was specifically requested were also excluded.

Using the dates and times given for when the deceased was last seen alive and the date of the post-mortem examination, both of which were extracted from the database, two intervals were calculated – the post-mortem interval (PMI) (the last time the deceased was seen alive and the post-mortem examination), as well as the 'delay' between the date upon which the deceased was declared dead and the post-mortem examination. The time or date that the deceased was last seen alive, as well as time and date of the body discovery was taken from the post-mortem or police reports. If the time or date that the deceased was declared dead (time of death) was not clear, the time of body discovery was used as time of death. These times were used to calculate the PMI or interval. The delay was calculated using the time of death (or declared time of death) taken from post-mortem/police reports to the date of the post-mortem examination, the date of which was extracted from the database. These two time intervals were calculated as GHB can be produced post-mortem, and therefore post-mortem delays and intervals were considered an important factor to investigate. It was postulated that it would be possible to correlate GHB concentrations and increased delays and intervals.

7.3.2 Toxicological Analysis

The method that was used in the toxicological analysis is the method published by Hassan and Cooper (206). It was originally developed and validated for the analysis of BHB only, but was later revalidated in accordance with best practice recommendations (207) for GHB analysis.

7.3.3 Criteria for Batch Acceptability

In order to establish the acceptability and validity of the data, it was evaluated according to certain criteria. Namely:

- A minimum of 4 points on the calibration curve, which is accepted in routine working laboratories, but Quality Control (QC) samples must also be acceptable.
- GHB must not be detected in quantifiable concentrations (as GHB is endogenous, samples will always contain traces) in the blank standards.

- Concentration of QC samples must be as close to the nominal concentration as possible (±15%), thereby ensuring accurate quantification.
- Ion ratios must be within ±20% relative to the control or standard ion ratio. This ensures that the correct analyte peaks are identified, and thereby accurate quantitation of the analyte is possible.
- Chromatographic peaks should resemble Gaussian distribution, to ensure that random variation can be identified and to identify potential column overloading or extended interaction between analytes and the analytical stationary phases.
- A minimum correlation coefficient of ≥0.99 must be obtained, as this shows linear correlation providing accuracy as 99% of results can be explained by the correlation.
- Retention times should be similar for both sample and ions and must not deviate more than 1 – 2% from retention times of controls or calibrators. This ensures that the peaks observed are the peaks for the analytes of interest. This must be true for analytes as well as their deuterated internal standards.
    - Deuterated-GHB should elute slightly earlier than undeuterated-GHB.
- QC points must lie within ±3 standard deviations from the mean. If the calculated concentrations deviate from the nominal concentrations outside of this range, the analysis is inaccurate and the quantitation of analytes is not reliably reportable.

These criteria were applied to all cases in order to assess the validity and acceptability of the data, and any case that did not meet the specified criteria was excluded. The validity of the data was assessed to ensure that the reported concentrations were accurate and reliable as this would affect the legitimacy of the results presented.

7.4 Results and Discussion

7.4.1 Demographics

Between July 2010 and December 2016, a total of 1811 cases were submitted for BHB analysis and were selected for this study based on the inclusion criteria mentioned in Section 7.3.3. 1267 of the cases were males (69.9%) and 544 females (30%).

The mean age for each year is shown in Table 7-2. 2016 showed the oldest mean age at death per year of 56.0 years. The mean for the whole study was lower at 53.2 years.

Table 7-2 Mean ages at death between 2010 - 2016 and associated standard deviations

Year	Mean age at death (years) (Standard deviation)
2010	49.1 (14)
2011	49.8 (14)
2012	53.1 (14)
2013	53.3 (14)
2014	55.5 (13)
2015	55.4 (14)
2016	56.0 (13)
Total	53.2 (14)

Mean age at death was 54 years (SD 13.75) for females and 53 years (SD 13.41) for males, also sharing a median age of 55 years. The mean age per year per sex are shown below in Figure 7-3.

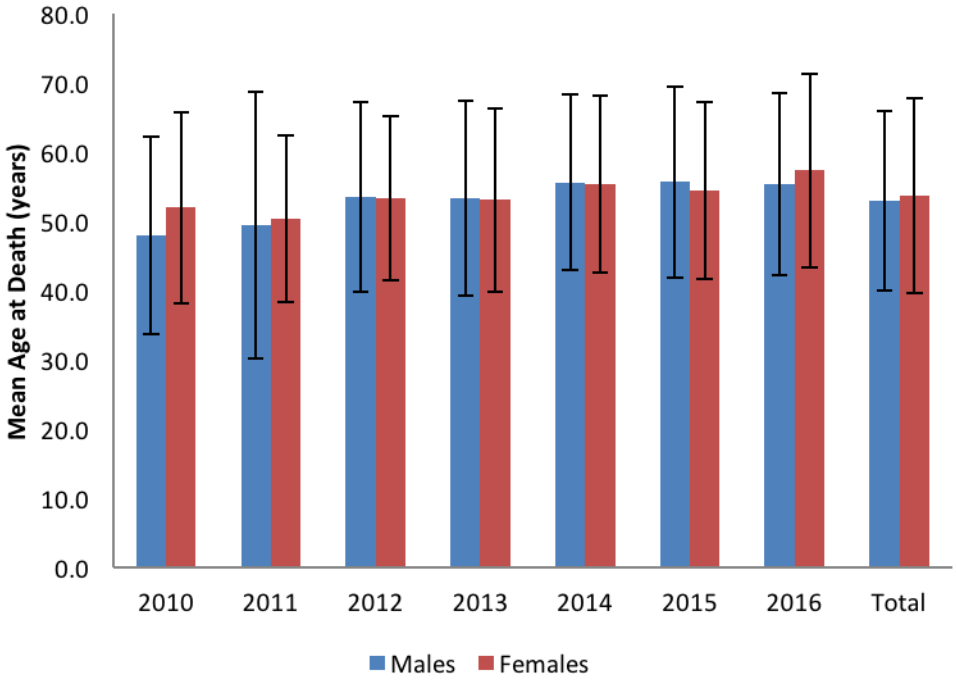


Figure 7-3 Mean age at death per year

This shows that the mean age at death was higher for females than for males. Only in 2015 was there a noticeable difference in mean age between males and females with 55.7 years for males and 54.5 years for females. Females died at older ages than males in 2016 where the mean ages were 57.4 and 55.4 years, respectively.

The spread of the categories of deaths for this particular data set is shown in Figure 7-4.

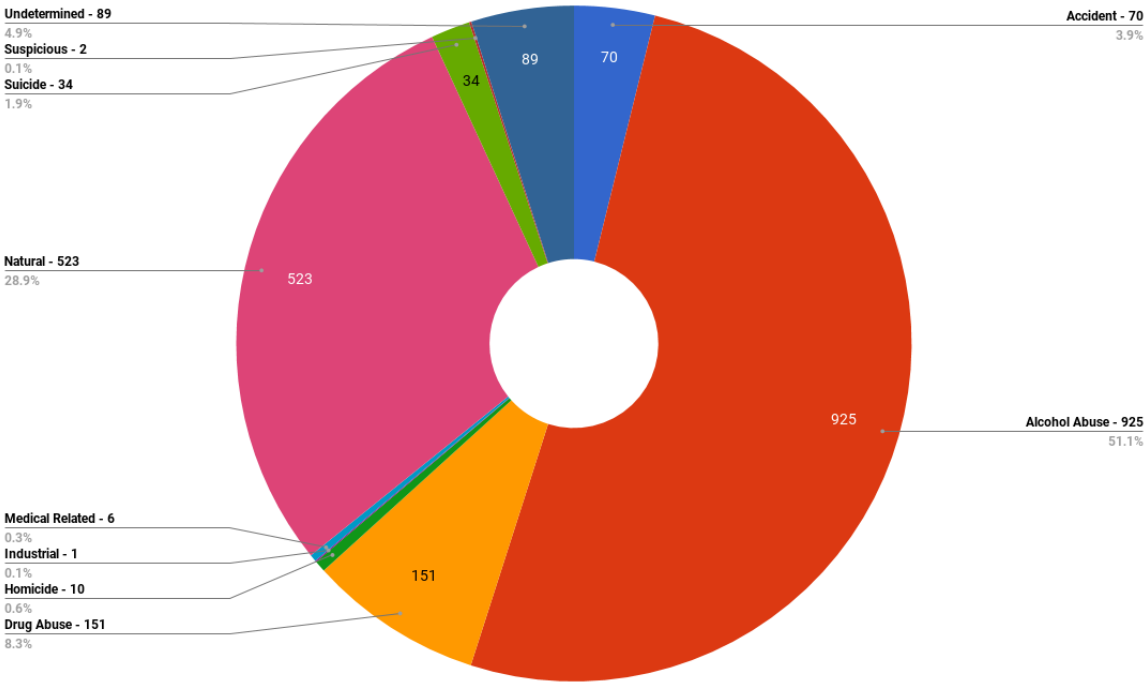


Figure 7-4 Spread of categories of deaths (*n* = 1811)

The majority of cases (51%) fell under the alcohol abuse category as manner of death, with the remaining being classified as natural (29%), drug abuse (8%), undetermined cases (5%), accidental (4%), suicide (2%), homicide (1%), medical related (0.3%), suspicious (0.11%), and industrial (0.06%). Cases where this information was unobtainable were omitted. The spread is explained by the fact that BHB analysis is carried out on cases where alcoholic ketoacidosis is suspected, or acetone is detected in the alcohol analysis. Alcohol ketoacidosis is a disorder that occurs in persons who have a history of increased alcohol consumption and reduced nutritional intake. This metabolic disturbance can also be associated with sudden death in persons with severe alcoholism (Sudden Unexpected Death in Alcohol Misuse – SUDAM) (208). With reduced nutrition and increased alcohol consumption, alcoholic ketoacidosis affects glucose metabolism which results in the formation and accumulation of ketone bodies. This in turn reduces body pH and this is termed “acidosis”. To determine alcoholic ketoacidosis, BHB analysis is carried out as BHB is a biomarker for alcohol consumption and is more concentrated in the human body compared to other ketone bodies (such as acetone, which does not contribute to ketoacidosis).

Figure 7-5 shows the frequencies of the manners of death separated by sex. This shows that a greater number of deceased cases of males were submitted for BHB/GHB analysis and more male cases were associated with alcohol abuse.

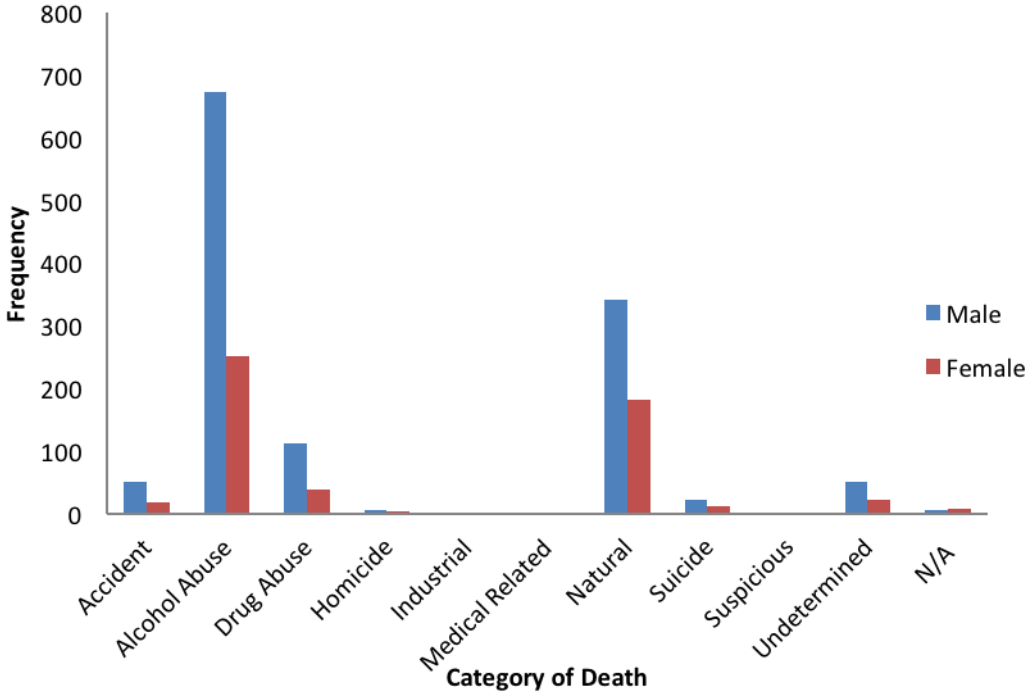


Figure 7-5 Frequencies of canner of death divided by sex

7.4.2 GHB Concentrations

Out of 1811 cases, a total of 412 cases did not report a GHB concentration to be included in the study. In 52 (2.8%) cases GHB was not detected, and in 360 cases (87%) GHB concentrations were below the lowest calibrator (10 mg/L). For ease, all cases where no GHB concentration was provided were classed as <10 mg/L for the inclusion in concentration ranges.

The highest reported GHB concentration was 421 mg/L, with a median concentration of 24 mg/L. The spread is shown in Figure 7-6. All GHB concentrations were rounded so as to not include decimal places.

76% (*n* = 1383) of cases showed a GHB concentration of less than 30 mg/L, and 94% (*n* = 1697) showed concentrations between 0 and 50 mg/L. Two cut-offs have been suggested to differentiate between exogenous and endogenous GHB, 50 and 30 mg/L (196, 209). Based on the results found in this case series where

GHB was not implicated in any of the cases, the 50 mg/L cut-off should be considered.

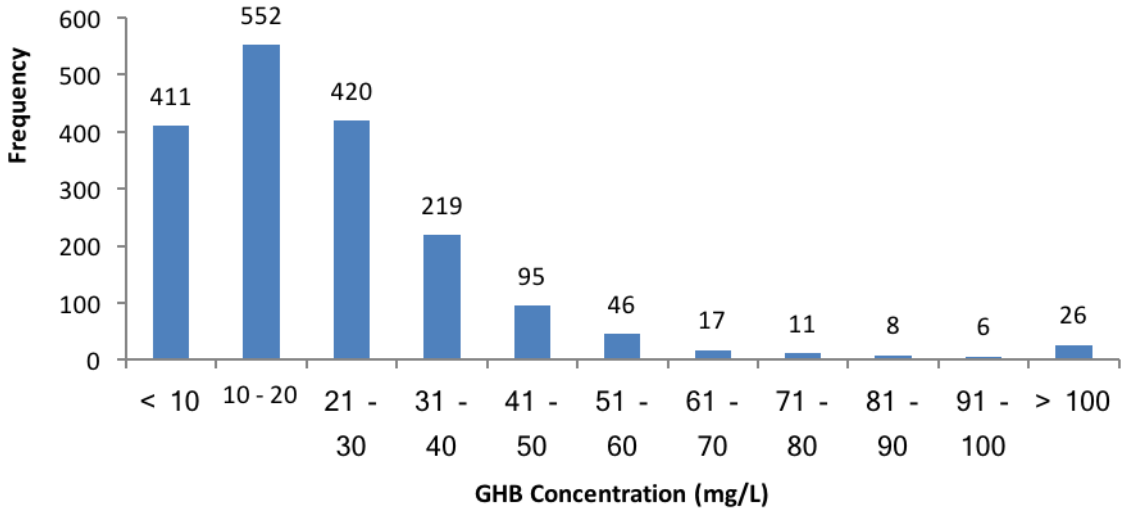


Figure 7-6 GHB Concentration Ranges (mg/L)

However, 6% (*n* = 114) cases showed concentrations in excess of 50 mg/L.

Femoral blood was collected in all of the cases included in this study. The site of collection was not included in a number of studies referenced. It has been suggested that the collection and analysis of post-mortem urine or vitreous humour may help and backup post-mortem blood findings; however, elevated concentrations of urinary GHB concentrations have been reported (189, 192). In addition to this, post-mortem urine samples may not always be available or may be deemed not suitable for analysis due to putrefactive changes or low sample volume. The possible contamination of post-mortem urine must also be considered. It is not uncommon for urine to be contaminated with blood, especially in cases of moderate-to-advanced decomposition, thereby falsely elevating urinary GHB concentrations.

Median concentrations for each year, as well as the ranges are shown in Table 7-3. As the data are not normally distributed, median GHB concentrations are shown rather than mean GHB concentrations.

Table 7-3 Median and ranges of GHB concentration (mg/L) between 2010 - 2016

Year	Median GHB Concentration (mg/L)	Range of GHB Concentrations (mg/L)
2010	27	<10 - 101
2011	31	<10 - 128
2012	25	<10 - 401
2013	25	<10 - 228
2014	23	<10 - 194
2015	22	<10 - 147
2016	23	<10 - 424

All median concentrations, similarly to the mean concentrations observed for each year, do not show much variation. The highest median concentration (31 mg/L) observed was seen in 2011, and the lowest median concentration of 22 mg/L was observed in 2015.

Median concentrations for males and females are shown in Figure 7-7. The median concentration found for females over the testing period was 24 mg/L (range <10 – 424 mg/L), while for males the GHB concentration averaged at 28 mg/L (range <10 – 228 mg/L).

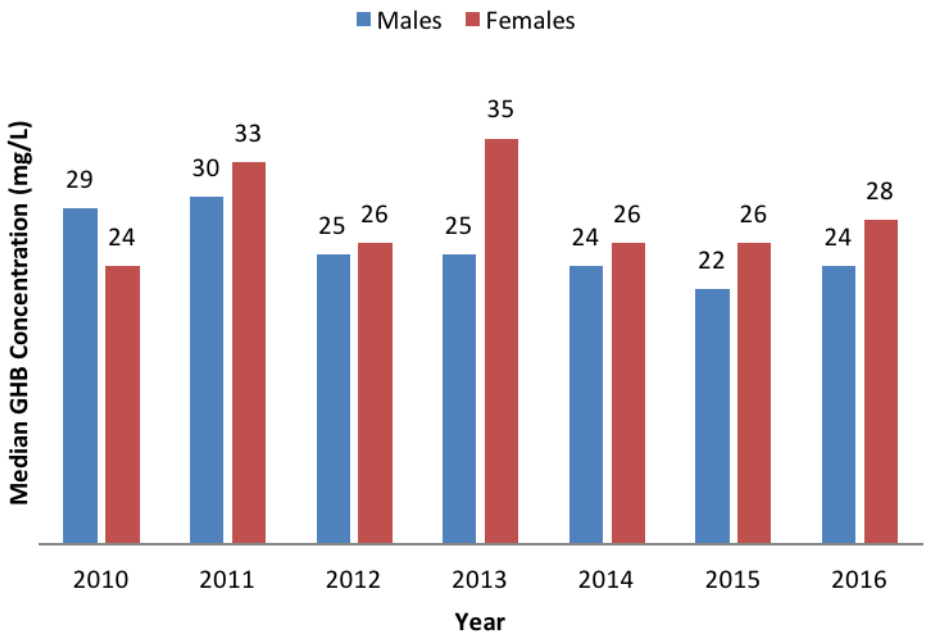


Figure 7-7 Median GHB concentrations observed over the testing period for both sexes

Median concentrations observed for females were higher in all years but in 2010.

7.4.3 Effects of Decomposition and Post-Mortem Interval (PMI)

For a large number of cases, decomposition changes were described in the post-mortem reports. However, understandably, the details varied greatly from report to report and there is no established scientific way of categorising and reporting the extent of decomposition. This has previously been described as a limiting factor in determining the role played by decomposition in increasing post-mortem GHB concentrations (210). Very few authors are able to provide PMI-related information when reporting GHB concentrations. Only one study was able to provide this data (200), and due to the limiting factor of non-standardised reporting of decomposition, very little information is available. Two studies have been able to successfully divide cases into categories of PMI and find a mean GHB concentration for each group (187, 202). Castro (187) found that their samples were able to be divided into four PMI categories where a maximum GHB concentration was found at a PMI of 72 hours. Interestingly, no statistically significant difference was found between GHB concentrations measured between 24 and 48 hours, although the authors conclude that PMI does influence post-mortem GHB concentrations. Similar findings were reported by Busardò *et al.* (202), where cases were divided into three PMI groups and GHB concentrations increased with increasing PMI.

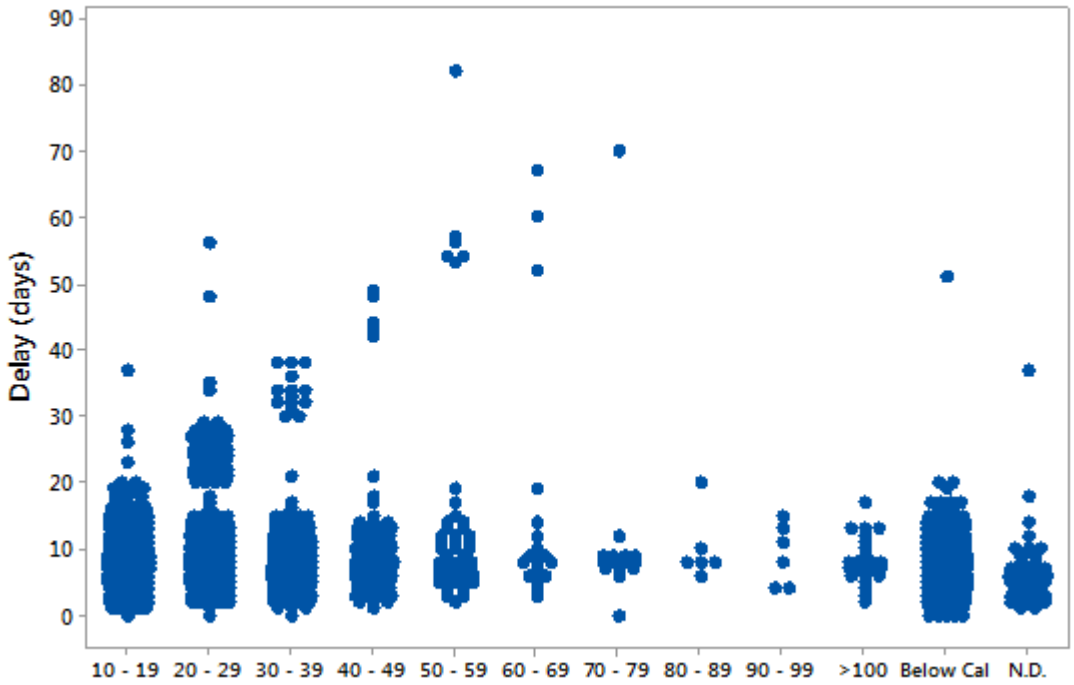


Figure 7-8 Spread of post-mortem delay (days) versus post-mortem GHB concentration (mg/L)

Figure 7-8 shows the non-linear relationship between post-mortem delay (days) and post-mortem GHB concentration (mg/L). For scaling, four cases where the delay exceeded 100 days were excluded. 65% of cases had a delay between 0 and 9 days. For 11 cases it was not possible to establish or estimate a delay, which were also excluded from Figure 7-8. The frequency of post-mortem delay (time passed since the deceased was declared dead and the post-mortem examination) is shown in Figure 7-9.

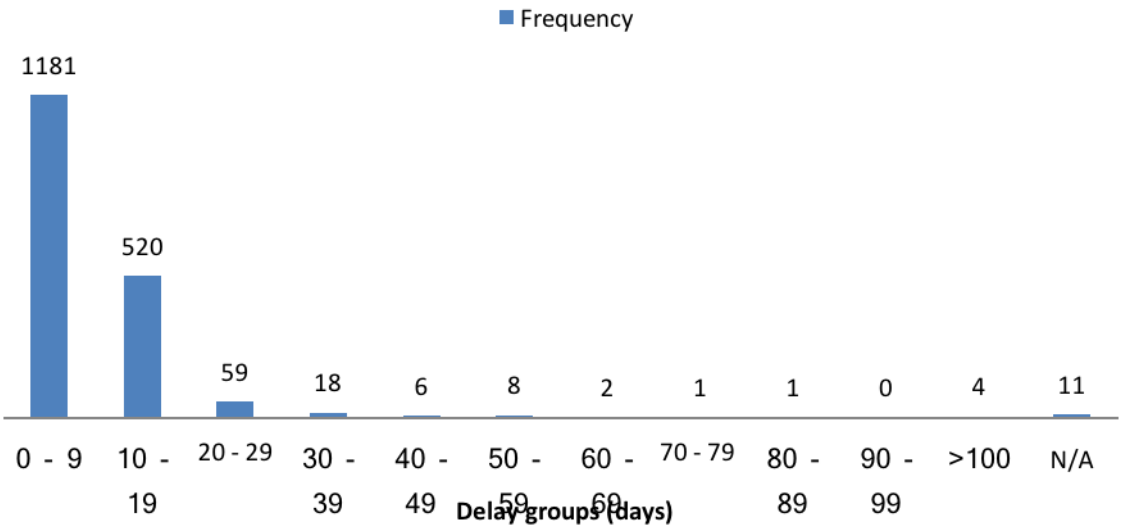


Figure 7-9 Frequency of post-mortem delay (days)

Although all 26 cases where GHB concentrations exceeded 100 mg/L noted some form of decomposition change in the post-mortem report, in 22 cases (85%) moderate-to-advanced decomposition changes were recorded. This supports the notion of post-mortem production of GHB previously mentioned in the literature. Due to the complex nature of the molecule, however, some cases with GHB concentrations of <50 mg/L, even some cases showing GHB concentrations lower than the LLOQ of 10 mg/L, had advanced stages of decomposition. Cases with GHB concentrations ≥50 mg/L are outlined in Table 7-4. Unfortunately, it was not possible to determine any relationship between the case types and the increased concentrations of GHB, especially as the dataset is biased towards deaths associated with alcohol abuse.

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
1	U	F	66	54	1a. Alcoholic ketoacidosis 1b. Chronic alcohol abuse	Alcohol Abuse	5	Rigor mortis passed, patchy skin slippage, lividity on body
2	U	M	63	101	1a: Ischaemic heart disease 1b: Coronary artery atheroma	Natural	8	Rigor mortis passed, lividity on body
3	U	M	39	51	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol Abuse	14	Rigor mortis passed, lividity on body, areas of pallor, blistering
4	U	M	37	57	1a: Acute pancreatitis	Alcohol Abuse	6	Rigor mortis present, lividity present
5	U	F	32	75	1a: Unascertained	Undetermined	8	Lividity on back, rigor mortis worn off
6	U	F	67	99	1a: Ischaemic heart disease 1b: Coronary artery atheroma 2a: Chronic alcohol abuse associated with fatty liver disease	Alcohol Abuse	8	Skin slippage on chest, arms and neck, fungal growth on nose. Rigor mortis passed, hypostasis present. Green discolouration of abdomen
7	U	M	60	59	1a: Ischaemic and valvular heart disease 2a: Acute alcohol intoxication	Alcohol Abuse	4	Lividity on back, rigor mortis worn off
8	U	M	42	76	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol Abuse	<1	Advanced state of decomposition, black discolouration, swelling of face, protrusion of tongue, green and purple discolouration of torso, skin slippage over trunk. Marbling present
9	U	M	55	128	1a: Ischaemic heart disease 1b: Coronary artery atheroma 2a: Diabetes mellitus	Natural	8	Fungal growth in both eyes, marbling, skin slippage as well as drying of fingers
10	U	F	40	51	1a: Sudden death associated with chronic alcohol abuse	Alcohol Abuse	7	Hypostasis on back, rigor mortis worn off
11	U	F	38	96	1a: Myocardial fibrosis	Natural	4	Lividity on back, rigor mortis worn off
12	U	M	55	62	1a: Bronchopneumonia 2a: Diabetes mellitus, ischaemic heart disease and chronic alcohol abuse	Natural	12	Lividity on back, rigor mortis worn off

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
13	U	F	40	58	1a: Alcohol related fatty change of the liver	Alcohol Abuse	10	Lividity on back, rigor mortis worn off
14	U	M	65	58	1a: Diabetic ketoacidosis 1b: Bronchopneumonia and diabetes mellitus 2a: Ischaemic heart disease	Natural	7	Green discolouration of abdomen, rigor mortis passed, lividity present
15	U	M	53	60	1a: Sudden death associated with chronic alcohol abuse	Alcohol Abuse	6	Drying of fingertips and toes. Green discolouration, rigor mortis passed. Hypostasis on back.
16	U	F	49	60	1a: Fatty degeneration and cirrhosis of the liver 1b: Chronic alcohol abuse	Alcohol Abuse	7	Lividity on back, rigor mortis worn off
17	U	M	71	144	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol Abuse	8	Moderate decomposition, generalised green discolouration, parchmenting of face, drying of hands and feet and patchy blistering
18	U	M	32	158	1a: Diazepam, dihydrocodeine and codeine intoxication	Drug Abuse	8	Hypostasis over face, front of trunk and back of body. Generalised green discolouration of abdomen, marbling over arms, abdomen and upper arms
19	U	F	49	51	1a: Infective exacerbation of chronic obstructive pulmonary disease 2a: Chronic drug abuse associated with hepatitis C infection	Alcohol Abuse	7	Generalised brown discolouration, hypostasis over back of body and patchy over thighs
20	U	M	48	73	1a: Unascertained	Undetermined	8	Advanced state of decomposition, green discolouration, skin slippage
21	U	F	58	52	1a: Ischaemic heart disease 1b: Coronary artery atheroma 2a: Fatty degeneration of the liver associated with chronic alcohol abuse	Alcohol Abuse	6	Hypostasis over front of body, rigor mortis passed
22	U	M	72	56	1a: Ischaemic heart disease	Natural	6	Green discolouration

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
					1b: Coronary artery atheroma 2a: Oesophageal cancer			
23	U	M	56	72	1a: Acute alcohol intoxication	Alcohol Abuse	8	Rigor mortis passed, hypostasis present over front
24	U	M	52	67	1a. Fatty degeneration of the liver, 1b. Chronic alcohol abuse	Alcohol Abuse	10	Rigor mortis and lividity on back of body
25	P	F	51	123	1a: Methadone intoxication	Drug Abuse	9	Advanced state of decomposition, generalised green discolouration, marbling with fungal growth, skin slippage and early mummification of fingers
26	U	F	34	51	1a: Acute and chronic myocardial damage 1b: Suspected amphetamine toxicity	Drug Abuse	5	Hypostasis over back of body, rigor mortis passed
27	P	M	45	193	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol Abuse	14	Moderate decomposition. Generalised green and dark brown discolouration of skin. Heavy maggot infestation
28	U	M	48	82	1a: Cardiac enlargement	Natural	20	Green discolouration, skin slippage
29	U	F	63	57	1a: Acute alcohol intoxication	Alcohol Abuse	82	Rigor mortis passed, hypostasis present over back
30	U	M	58	56	1a: Hypertensive and ischaemic heart disease 2a: Diabetes mellitus	Natural	57	Hypostasis over body, rigor mortis wearing off
31	P	M	54	60	1a: Acute alcohol intoxication 2a: Fatty degeneration of the liver due to chronic alcohol abuse	Alcohol Abuse	5	Patchy green discolouration, lividity on back of body
32	U	M	53	53	1a: Sudden death associated with chronic alcohol abuse	Alcohol Abuse	53	Degree of decomposition with some black and green discolouration, skin slippage
33	P	M	70	54	1a: Ischaemic and valvular heart	Alcohol Abuse	54	Early decomposition changes

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
					disease 2a: Chronic obstructive pulmonary disease			
34	P	M	82	54	1a: Bronchopneumonia	Natural	7	Green discolouration, lividity present on back
35	P	F	49	56	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol Abuse	56	Rigor mortis wearing off
36	P	M	52	70	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol Abuse	9	Early marbling, rigor mortis passed, lividity over back
37	P	M	62	58	1a: Co-codamol intoxication	Alcohol abuse	15	Pressure parchmenting, purple discolouration of face, fungal patches on back, drying of fingers and toes
38	P	M	59	69	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol abuse	8	Rigor mortis still present, lividity on back of body. Green discolouration of abdomen.
39	P	F	70	55	1a: Congestive cardiac failure 2a: Electrolyte imbalance	Natural	13	N/A
40	P	M	61	88	1a: Chronic obstructive pulmonary disease	Alcohol abuse	8	Advanced decomposition changes. Sunken and autolytic eyes, nose and lips desiccated. Generalised dark green skin discolouration, extensive skin slippage and marbling. Scaly skin changes on both feet
41	P	M	31	70	1a: Heroin intoxication	Drug abuse/ Natural	8	Rigor mortis passing, lividity on back of body
42	P	M	50	81	1a: Ischaemic heart disease 1b: Coronary artery atheroma	Natural	8	Advanced decomposition, black/brown skin discolouration of face and torso, skin slippage on chest, neck and legs
43	P	M	73	90	1a: Ischaemic heart disease 1b: Coronary artery atheroma	Natural	4	Advanced decomposition. Skin covered in grey granular material due to submersion in water. Grey and brown skin discolouration, skin slippage and disintegration of earlobes.
44	P	M	35	62	1a: Suspected cardiac arrhythmia	Alcohol abuse	52	Moderately advanced decompositionSunken

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
					due to long QT syndrome			eyeballs, generalised green/black discolouration, parchmentation present
45	P	M	39	87	1a: Hanging	Suicide	8	Rigor mortis worn off, hypostasis on front of body. Skin slippage on arms and legs. Green discolouration of skin on lower abdomen
46	P	M	46	58	1a: Heroin intoxication 2a: Cirrhosis of the liver	Alcohol abuse	6	Lividity of face on body, skin slippage on legs. Rigor mortis worn off
47	P	F	49	53	1a: Alcoholic ketoacidosis 1b: Chronic alcohol abuse associated with fatty degeneration and cirrhosis of the liver	Alcohol abuse	17	Moderate decomposition. Rigor mortis had passed, pungent smell. Green skin discolouration of face, skin and hair slippage
48	P	M	44	219	1a: Unascertained	Alcohol abuse	4	Advanced decomposition. Loss of soft tissue, extensive black/brown discolouration of remaining skin, skin slippage. Infestation of maggots
49	P	M	43	115	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol abuse	8	Advanced decomposition, drying of skin, skin slippage and marbling. Insect activity caused loss of eyes. Black skin discolouration, mummification of extremities, fungal growth. Presence of insect pupae
50	P	M	61	59	1a: Complications of alcoholic liver disease	Alcohol abuse	5	Skin slippage of trunk, arms and legs, green discolouration of face and trunk. Venous staining. Hypostasis present, rigor mortis had passed in limbs
51	P	F	36	53	1a: Alcohol and tramadol intoxication	Accident	2	N/A
52	P	F	49	53	1a: Fatty degeneration of the liver 1b: Chronic alcohol abuse	Alcohol abuse	8	Advanced decomposition, extensive black discolouration, areas of skin slippage. Rigor mortis had passed, Hypostasis present.
53	P	M	39	100	1a: Sudden death associated with chronic alcohol abuse	Alcohol abuse	3	Rigor mortis had passed, decomposition noted, brown/purple and green

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
								discolouration of skin. Skin slippage on abdomen and legs
54	P	M	57	130	1a: Fatty liver disease	Alcohol abuse	6	Advanced decomposition, black discolouration of face with tissue disintegration and liquefaction. Brown and black skin changes on body, skin slippage present
55	P	F	71	67	1a: Paracetamol and codeine toxicity 2a: Cardiac enlargement and fatty liver disease	Natural	9	N/A
56	P	M	57	99	1a: Alcoholic ketoacidosis 1b: Chronic alcoholism 2a: Ischaemic heart disease	Alcohol abuse	11	N/A
57	P	M	54	228	1a: Alcoholic ketoacidosis	Alcohol abuse	5	Fairly advanced decomposition, putrefaction and maggot holes present consistent with moderate maggot infestation. Mummification of face, hands, and feet
58	P	M	72	60	1a: Fatty degeneration of the liver 1b: Alcohol abuse	Alcohol abuse	10	Rigor mortis wearing off
59	P	F	60	59	1a: Alcoholic ketoacidosis 1b: Chronic alcoholism	Alcohol abuse	9	N/A
60	P	F	49	54	1a: Lobar pneumonia 2a: Chronic alcohol abuse	Alcohol abuse	5	Sunken eyes, generally advanced decomposition, mixture of putrefaction, greenish discolouration and mummification
61	P	M	48	79	1a: Unascertained	Undetermined	7	Rigor mortis had passed, moderate decomposition, black, brown and purple discolouration of body, patchy fungal growth, venous marbling and skin slippage present
62	P	M	61	194	1a: Bronchopneumonia 1b: Chronic obstructive pulmonary	Alcohol abuse	17	Moderate decomposition. Purple skin discolouration, areas of skin slippage. Early

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
					disease 2a: Chronic alcohol abuse			marbling, insect casts present.
63	P	M	56	54	1a: Chronic obstructive pulmonary disease	Natural	19	Rigor mortis passed, green discolouration of abdomen
64	P	M	42	59	1a: Sudden unexpected death in epilepsy	Alcohol abuse	8	Decomposition related dark discolouration, skin slippage over legs, parchmented skin loss
65	P	M	72	92	1a: Ischaemic heart disease 1b: Coronary artery atheroma 2a: Chronic alcoholism	Alcohol abuse	15	Rigor mortis had passed early mummification of fingers and toes. Green discolouration
66	P	M	48	77	1a: Bronchopneumonia 2a: Chronic alcoholism	Alcohol abuse	6	Moderately advanced decomposition, leathery brown discolouration of face and hands, green/brown discolouration of body and skin slippage. Early mummification changes of fingers and toes
67	P	M	36	100	1a: Heroin intoxication	Alcohol abuse	12	Rigor mortis passed, lividity present. Skin slippage on arms, early marbling on right side of body
68	P	M	41	78	1a: Unascertainable	Undetermined	6	Moderately advanced decomposition, generalised green/black discolouration, and widespread skin slippage. Maggot infestation
69	P	M	72	127	1a: Unascertainable	Undetermined	7	Moderately advanced decomposition changes. Green/black skin discolouration, skin slippage, maggots present. Decomposition swelling of face, some patchy parchmentation
70	P	M	54	150	1a: Unascertained	Undetermined	2	Moderate decomposition, generalised green/black colouration of body, skin slippage, and focal insect infestation. Mummification of side of face
71	P	M	52	62	1a: Heroin intoxication 2: Chronic obstructive pulmonary	Drug abuse	4	Rigor mortis passed, patchy green discolouration over legs, venous staining,

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
					disease			drying and partial mummification of fingers, skin slippage over legs
72	P	M	55	90	1a: Alcoholic liver disease	Alcohol abuse	13	Early decomposition changes, grey reddish skin discolouration, skin slippage over torso and face
73	P	F	50	61	1a: Suspected alcohol related death	Alcohol abuse	19	Rigor mortis had worn off, skin blistering and decomposition changes including green discolouration of abdomen and trunk
74	P	M	65	79	1a: Unascertained	Undetermined	9	Moderate decomposition changes, widespread grey and purple, green/black skin discolouration. Extensive skin slippage, venous marbling, heavy insect infestation. Ears decomposed
75	P	M	47	65	1a: Unascertained	Alcohol abuse	14	Moderate decomposition changes, green discolouration and areas of extensive skin slippage over body
76	P	F	76	131	1a: Cardiac enlargement 1b: Aortic valve disease 2: Chronic alcohol abuse	Alcohol abuse	13	Moderate decomposition changes, bloated face with protruding tongue. Green skin discolouration, extensive marbling, and blistering
77	P	M	84	118	1a: Unascertained	Natural	10	Bad state of decay, insect predation, advanced decomposition, extensive skin slippage, yellow parchmentation and drying of extremities
78	P	M	50	79	1a: Cardiac enlargement 2a: Chronic alcohol abuse	Alcohol abuse	12	Early stages of decomposition, rigor mortis had passed, generalised lividity, green discolouration, patchy skin slippage, blister formation and mould growth over face, arms and torso, mummification changes of hands, forehead and nose
79	P	M	48	88	1a: Complications of liver cirrhosis	Natural	10	Moderate decomposition changes, black skin discolouration, parmented skin loss on upper

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
								torso, skin slippage, early mummification changes
80	P	M	65	59	1a: Coronary artery atheroma and ketoacidosis	Alcohol abuse	7	Marked mummification and extensive fungal growth
81	P	M	64	70	1a: Alcoholic ketoacidosis	Alcohol abuse	9	Variable decomposition changes, brownish/black skin discolouration, skin slippage over the head, generalised green discolouration of body and focal marbling
82	P	M	57	70	1a: Ischaemic heart disease 1b: Coronary artery atheroma 2a: Alcoholic liver disease	Alcohol abuse	9	Rigor mortis passed and lividity present
83	P	M	43	51	1a: Methadone intoxication	Drug abuse	9	Early decomposition, generalised black discolouration of the face, arms and shoulders. Rigor mortis had worn off, patchy marbling
84	P	M	28	54	1a: Alcoholic ketoacidosis	Alcohol abuse	10	Advanced decomposition, right hand starting to fuse with carpet. Mummification of face, patchy fungus formation, early maggot infestation
85	P	M	56	147	1a: Unascertained	Undetermined	7	Extensive decomposition, skin blackening, venous staining, skin slippage, patchy mould growth over body
86	P	M	65	67	1a: Complications of chronic alcoholism 2a: Chronic obstructive pulmonary disease	Alcohol abuse	9	Rigor mortis passed, lividity present. Green discolouration of next and abdomen
87	P	M	56	52	1a: Subdural haematoma 1b: Presumed fall	Accident	19	Rigor mortis wearing off
88	P	M	57	54	1a: Sudden death associated with chronic alcohol abuse	Alcohol abuse	6	Rigor mortis established, extensive green discolouration, mummification of fingers and toes

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
89	P	M	36	127	1a: Morphine intoxication	Drug abuse	13	Extensive decomposition, grey/black skin discolouration over entire body. Hands and fingers mummified, wet decomposition changes, flattening of face, patchy white mould growth
90	P	F	63	140	1a: Ischaemic heart disease	Natural	7	Extensive decomposition, green discolouration and venous marbling, skin blistering over left side of body, mummification of fingers
91	P	M	41	75	1a: Unascertained	Undetermined	9	Extensive decomposition, dark grey/green skin discolouration, maggot and pupae infestation, skin slippage and skin loss on legs, trunk and arms
92	P	F	33	126	1a: Unascertained	Undetermined	13	Early decomposition changes, greenish discolouration, patchy marbling
93	P	F	41	54	1a: Fatty change of the liver 1b: Chronic alcohol abuse	Alcohol abuse	7	Fairly advanced decomposition, sunken eyes and mummification changes, skin slippage in non-mummified aspects of body
94	P	F	50	68	1a: Suspected alcohol related death	Alcohol abuse	8	Fairly advanced mummification, patchy fungal growth
95	P	M	76	52	1a: Ischaemic heart disease 1b: Coronary artery atheroma	Natural	5	Early decomposition changes, slight drying and yellow discolouration of skin. Green discolouration of abdominal wall. Rigor mortis passed
96	P	F	68	60	1a: Alcoholic ketoacidosis 2a: Hypothermia	Alcohol abuse	6	Advanced decomposition, eyes absent, mummification, fungus formation and maggot infestation
97	P	M	65	53	1a: Alcoholic ketoacidosis 1b: Chronic alcohol abuse	Alcohol abuse	12	Rigor mortis passed and lividity present. Green discolouration of trunk and arms, venous marbling, parchmented skin loss.
98	P	M	50	56	1a: Alcoholic ketoacidosis	Alcohol abuse	11	Rigor mortis worn off

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
99	P	M	85	122	1a: Ketoacidosis	Natural	11	Decomposition and mummification, mouth sealed shut from mummification. Mould growth on face
100	P	M	42	69	1a: Complications of chronic alcohol misuse and gastrointestinal haemorrhage	Alcohol abuse	8	Marked decomposition changed, grey/green discolouration of body, skin slippage and skin loss over head, trunk, arms, and legs
101	P	F	63	54	1a: Ischaemic heart disease 1b: Coronary artery atheroma	Natural	12	Rigor mortis passed, pressure pallor and green discolouration of abdomen
102	P	M	44	59	1a: Ischaemic heart disease 1b: Coronary artery atheroma 2a: Type 1 diabetes mellitus	Natural	11	Rigor mortis passed. Reddening of face, green/black discolouration of trunk, venous marbling. Partial skin slippage
103	P	M	63	120	1a: Diabetic ketoacidosis (insulin-dependent diabetes mellitus) and coronary artery atheroma	Natural	7	Parchmentation of skin, patchy mould growth all over body
104	P	M	57	54	1a: Fatty degeneration of the liver with ketoacidosis	Alcohol abuse	17	Dark grey skin discolouration, mould growth, skin slippage and skin loss in areas
105	P	M	50	78	1a: Fatty degeneration of the liver and possible codeine intoxication	Alcohol abuse	7	Moderately advanced decomposition, greenish/black discolouration, skin slippage and some mummification
106	P	M	34	217	1a: Sudden death associated with chronic alcohol abuse	Alcohol abuse	7	Generalised early decomposition, skin slippage, blister formation, early mummification of fingertips. Maggots in eye sockets
107	P	M	64	83	1a: Ischaemic heart disease 1b: Coronary artery atheroma	Alcohol abuse	6	Early decomposition changes, green discolouration of face, marling of abdomen, extensive skin slippage on legs. Presence of beetles
108	P	F	54	424	1a: Unascertained	Alcohol abuse	6	Fairly advanced decomposition with green/black discolouration. Mummification of both hands and feet
109	P	M	76	61	1a: Right lower lobe pneumonia 1b: Chronic obstructive pulmonary	Alcohol abuse	5	Lividity present

Table 7-4 Demographic information and decomposition changes in cases with GHB concentrations >50 mg/L

Case	Preserved (P)/ Unpreserved (U)	Sex	Age	[GHB] mg/L	Cause of Death	Manner of Death	PMI (days)	Decomposition Changes
					disease 2a: Fatty infiltration of the liver			
110	P	F	58	57	1a: Alcoholic ketoacidosis with fatty degeneration of the liver 1b: Chronic alcohol misuse	Alcohol abuse	3	Faint rigor mortis present in limbs
111	P	M	43	154	1a: Unascertainable	Undetermined	6	Moderately advanced decomposition, green/brown/black discolouration of skin, skin slippage and venous marbling, early fungal growth
112	P	M	48	63	1a: Diabetic ketoacidosis	Natural	3	Fairly advanced decomposition, black generalised colouration of skin, skin slippage. Mummification of trunks, arms, and legs

It was not possible to formulate any hypotheses based on the delay between the time the individual was last seen alive to the post-mortem examination and the PMI. The information provided in both the police and pathology reports was not reliable enough to make any scientific judgements. The fact that the exact time of death was not known in some cases does not aid the drawing of conclusions as these would be inaccurate as they were based on estimations which may not be reflective of the actual events.

For 98% (*n* = 1771) of the 1811 cases, the delay between discovery of the body and the last time they were seen alive was equal to or exceeded 2 days. In order to establish any sort of time frame then, it must be assumed that the time the deceased was last seen alive would be the date of the death. This however is based on assumptions and may not be reflective of the actual PMI. This in turn will then prevent the accurate judgement and drawing of conclusions.

In some instances (*n* = 11, 0.6%) no information was available to even presume the date of declaration of death.

7.4.4 Effect of Preservative in Collection Vial

Of 1811 cases included in the study, blood samples collected in 1549 cases (86%) contained a preservative (sodium fluoride) and showed GHB concentrations ranging from "Below Calibration/Not Detected" – 424 mg/L (median 23 mg/L). The remaining 262 cases (14%) were cases where blood was unpreserved and exhibited concentrations of GHB ranging from 10 – 158 mg/L (median 28 mg/L))[7]. The frequencies of preserved and unpreserved blood and the total number of cases with the corresponding average GHB concentrations are shown in Figure 7-10.

Unfortunately, it was not possible to investigate and compare any paired blood samples, as the analysis was only completed on one sample per case.

[7] All concentrations detected lower than the LLOQ of 10 mg/L were noted as "Below Calibration" or "Not Detected" and were not included in the results described

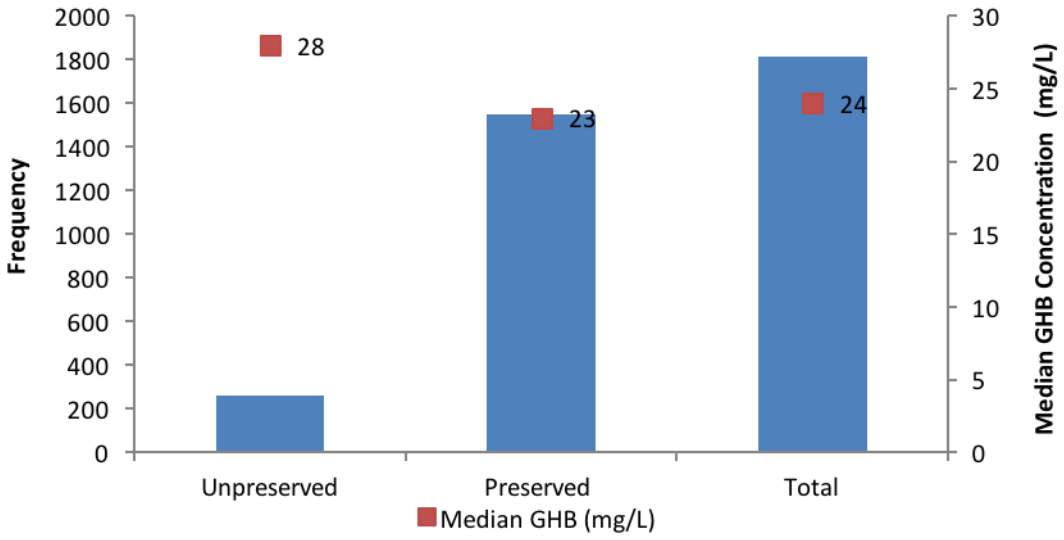


Figure 7-10 Frequency of unpreserved, preserved and the total number of cases with the associated median GHB concentrations (mg/L)

The frequencies (%) of the different concentrations measured in each preserved and unpreserved data set are show in Figure 7-11.

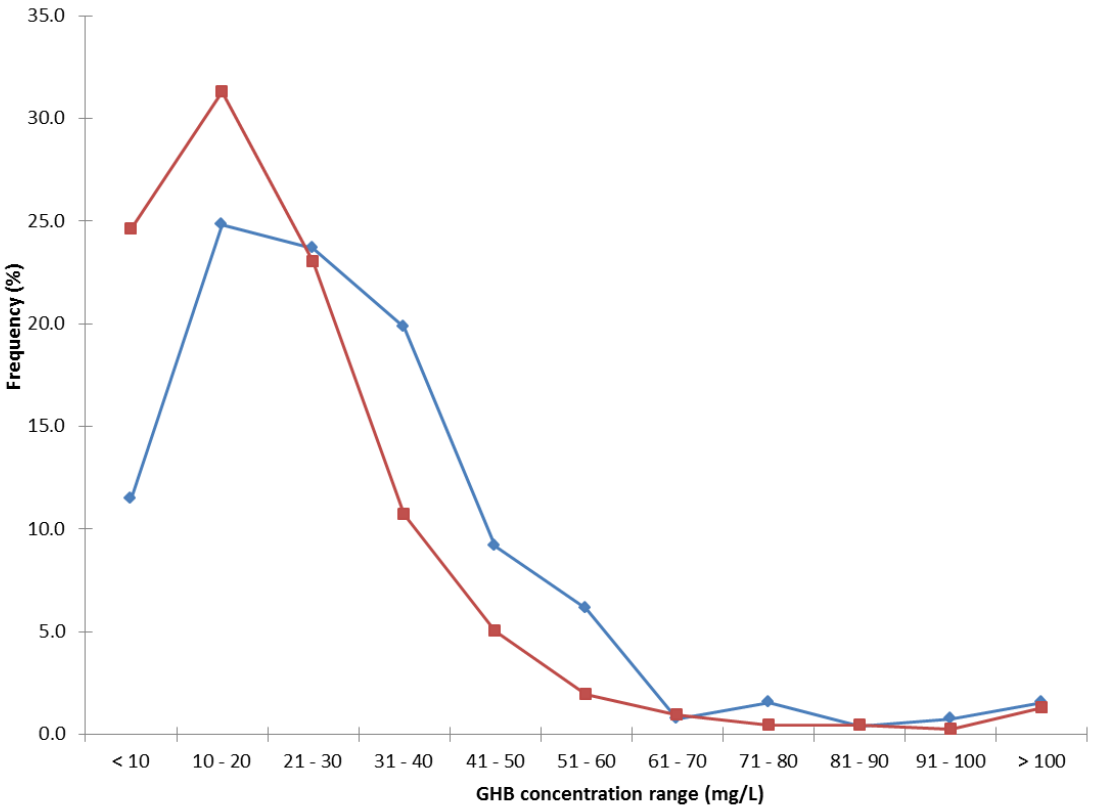


Figure 7-11 GHB concentration ranges for preserved and unpreserved post-mortem blood samples

There was a higher percentage of cases with GHB concentrations <30 mg/L for preserved samples (79%) than unpreserved samples (60%). However, when the

<50 mg/L cut-off is used, the difference in results becomes less pronounced (95% cases preserved and 89% cases unpreserved). Although the results between preserved and unpreserved findings are not drastically different, it suggests the importance of using preservatives in blood samples in order to prevent *in vitro* formation of GHB at post-mortem (171, 211), although studies have shown that GHB tends to be stable in blood over an extended period of time, especially when blood samples are frozen immediately after collection (194, 203). However, the results presented here show that high concentrations of GHB can be measured in both unpreserved and preserved blood samples, even though a higher proportion was seen in unpreserved samples. When applying the recommended cut-off concentration of 50 mg/L, the difference becomes less pronounced, almost negating the advice of using a preservative. Nevertheless, these results do not make it possible to determine whether the addition of preservative prevents further *in vitro* formation of post-mortem GHB when compared to blood collected without the addition of a preservative.

7.5 Conclusions

Elevated GHB concentrations were reported in post-mortem femoral blood samples where GHB use was not implicated in the death. The proposed cut-off level of 50 mg/L used to differentiate between exogenous use and endogenous production of GHB effectively identified endogenous levels or endogenous production in the vast majority of the cases (94%, *n* = 1699). However, a further 112 cases (6.2%) had concentrations in excess of 50 mg/L with no apparent explanation, with a maximum concentration of 424 mg/L observed.

Of these 112, moderate-to-advanced decomposition changes were reported in 60 cases. The lack of standardisation of how decomposition changes were reported prevents any further investigation into the potential role and effect of decomposition on GHB concentrations. It was also not possible to assess the effect of PMI on the GHB concentrations due to the unreliability of the available information regarding the times when the subjects were last seen alive.

A higher proportion of preserved blood samples showed a higher percentage of cases with GHB concentrations less than 30 mg/L, than unpreserved blood samples. This may support the premise of improved stability with the use of a

preservative to decrease formation of GHB *in vitro*. However, this may just be due to the lower GHB concentrations in those cases. A comparison between paired preserved and unpreserved blood samples collected post-mortem would provide an opportunity to investigate the role of a preservative in preventing or deceasing *in vitro* formation of GHB in post-mortem blood.

Establishing cut-off concentrations that can be used to differentiate between endogenous and exogenous GHB is important, especially when decomposition may play a role too. It is important to test other matrices as blood should not be used as a stand-alone matrix when it comes to the interpretation of post-mortem concentrations. Where available, urine should be tested in positive GHB cases, however there are limitations concerning the use of post-mortem urine, including the potential of contamination of the sample when post-mortem changes occur. Additionally, each case must be assessed individually taking into account all circumstances and information available to ensure correct interpretations are provided.

7.6 Future Work

For a less-biased dataset it would be beneficial to include cases where GHB was implicated in the cause of death or the analysis for GHB was specifically requested by the pathologist. Including cases pre-2010 would also be interesting, although the concentrations determined may not be comparable due to the differences in analytical method used for quantitation.

Retesting of samples, especially for cases where GHB was found in concentrations greater than 100 mg/L, would be of use to establish GHB stability in blood or more generally what the change in concentration would be. This would be interesting when comparing preserved and unpreserved blood samples.

In order to more specifically evaluate the effect of the manner and cause of death (i.e. hanging, drowning, drug abuse, *etc.*) on GHB concentration, it would be interesting to obtain further details from the database, like weight and height to establish BMI for example, to be included in the dataset. Finally it would be highly interesting to compare ante-mortem and post-mortem samples when available.

Conclusion

Oral fluid is a versatile matrix that has many applications and uses in both clinical and forensic toxicology. Its benefits over more traditional matrices include the range of analytes that can be detected, the ease and non-invasiveness of the collection and that sample adulteration is more difficult. However, it is known that blood concentrations cannot be accurately estimated from oral fluid concentrations and *vice versa*. Moreover, collecting an adequate sample volume may be difficult. Issues also exist when a buffered collection device is used, as this may cause interferences and issues with the sensitive instrumentation used for these analyses.

The main focus of the presented work was to evaluate the usability of the NeoSAL™ oral fluid collection device for various analytes of forensic interest.

The NeoSAL™ device was gravimetrically evaluated against other commercially available collection devices to assess any advantages and disadvantages it may have. Neogen®, the manufacturers of the NeoSAL™ device, state that the NeoSAL™ device collects 0.7 mL of oral fluid within 1 to 2 minutes. Assessment of the collection volume however showed that on average the device over-collects by 20%. Comparatively, the Intercept® *i*2™ over-collected as well whereas the Quantisal™ collection device collected within the 1 mL ±10% acknowledged by the manufacturer. Drug recoveries for the NeoSAL™ device were assessed for all analytes included in this work. Two processes were used to assess drug recovery: in the first instance, the collection pad was dipped into oral fluid, spiked at the relevant concentrations, until the sample volume adequacy indicator indicated sufficient sample had been collected. In the second instance, spiked oral fluid was pipetted onto the collection pad. The drug recovery was assessed for all analytes included in the study (amphetamine, methamphetamine, MDMA, MDA, MDEA, 6-MAM, morphine, codeine, dihydrocodeine, methadone, diazepam, desmethyldiazepam, oxazepam, temazepam and etizolam). The lowest recoveries observed were for diazepam and temazepam when the oral fluid was pipetted onto the collection pad. The lowest recovery observed for the amphetamine and methamphetamines, was for MDEA, whereas all other analytes gave recoveries >75%.

Amphetamine and methamphetamines are not commonly abused in Scotland, but they are abused globally, and effects of abuse can negatively impact a person's ability to drive. A method of extraction and analysis using GC-MS for amphetamine and methamphetamines was optimised for the NeoSAL™ device. The method was modified from a method that had previously been used for the extraction from oral fluid collected using the Quantisal® device. As the ratios of collected oral fluid to collection device buffer of both devices are the same, only minimal optimisation was required. The method was validated according to SWGTOX, however MDEA was neither accurate nor precise and therefore had to be excluded from further validation assessments, such as the autosampler stability study that was performed. Autosampler stability was assessed at two concentrations. Analytes were deemed stable for up to 48 hours on the autosampler. Further stability experiments were not carried out mainly due to a limited supply of collection devices. The method was sufficiently sensitive to detect amphetamine and methamphetamine concentrations lower than the cut-off concentrations recommended by the European Workplace Drug Testing Society (EWDTS).

The concurrent use of opioid and benzodiazepine drugs is well documented, and globally both drug groups are commonly abused. Therefore, an SPE method using LC-MS/MS analysis was developed for the simultaneous extraction of analytes from both drug groups. Several extraction procedures were tested, including protein precipitation, SPE using various SPE cartridges from different manufacturers, as well as LLE procedures and SLE columns. Although Waters Oasis HLB PRiME extraction cartridges gave the best overall results for matrix effects, process efficiency and drug recovery, they were not chosen for further work due to their cost. Excessive cost of consumables is not acceptable for laboratories with a high case load, and therefore a compromise between cost efficiency and drug recovery was made. Finally after optimisation, an SPE procedure using UCT Clean Screen® C18, mixed-mode ZDAU020 cartridges and a sequential elution using 3 mL of EtOAc with 2% ammonium hydroxide and 3 mL DCM:IPA:NH_4OH (78:20:2 v/v) was chosen. Recoveries for the benzodiazepines were lower than those for the opioid drugs, but even with these lower recoveries it was possible to detect concentrations lower than those recommended by the EWDTS as cut-off concentrations. Although matrix effects showed a lot of

variation for all analytes tested, for both neat and buffered oral fluid, the matrix effects observed for methadone in oral fluid collected using the NeoSAL™ device exceeded the acceptable limits and therefore methadone was excluded from further testing in both neat and buffered oral fluid testing. A stability study showed that all analytes were relatively stable within the 7-day testing period. To show that the method was robust and could be used in real-world settings, it was applied to neat and buffered oral fluid collected from 16 benzodiazepine drug users. A good overlap between concentrations detected in both oral fluid samples, as well as for the analytes detected in blood, was found. This makes it possible to conclude that the method is sensitive and accurate enough to test real, authentic, samples. The most likely reason for lacking overlap of detection of analytes in all three samples, is the deficit of sample volume collected in all instances, but may also be caused due to differences in the LOQ of the blood and oral fluid methods and detection windows of analytes. Although the volume of NeoSAL™ samples available for analysis was an issue, the detection of benzodiazepines and opioids in authentic samples was possible, and drug recoveries for both these and the amphetamines were acceptable. Therefore it must be concluded that the NeoSAL™ device, on the whole, is fit–for-purpose.

Finally the stability of GHB in neat oral fluid was assessed. The method used for the analysis was adapted from the method used to extract GHB from blood. It was found that GHB is stable for up to 56 days, not only when samples are stored in the fridge or freezer, but also when they are stored on the benchtop. Variation was assessed using a simple ANOVA test, and it was found that variation was not significant. It is also important to remember that GHB is an endogenous molecule and much inter- and intra-individual variation exists. This is especially true for GHB blood concentrations, and it is also known that GHB can be produced *in vitro* and concentrations can increase post-mortem.

To assess both possible *in* vitro formation and post-mortem increases of GHB, a retrospective database study on reported GHB concentration in post-mortem blood in cases unrelated to GHB use was performed. Cases between 2010 and 2016 that had a BHB/GHB analysis were extracted from the in-house database. Cases where GHB was specifically requested, tested or implicated in the cause of death were excluded from the dataset so as to assess purely endogenous post-mortem GHB. 1811 cases were included in the study, which is the largest dataset

to be evaluated for post-mortem GHB to date. Results showed that the proposed cut-off of 50 mg/L for the differentiation between endogenous and exogenous GHB must be used cautiously, as even in cases where no advanced decomposition changes were noted, concentration in excess of 100 mg/L were reported. Experts recommend the use a preservative in blood sample storage vials to prevent *in vitro* formation of post-mortem GHB; however, results suggest that even with a preservative present concentrations observed in samples may be difficult to interpret.

List of References

1. Spiehler V, Cooper G. Drugs-of-Abuse Testing in Saliva or Oral Fluid. In: Jenkins AJ, Caplan YH, editors. Drug Testing in Alternate Biological Specimens. Totowa, NJ: Humana Press; 2008. p. 83-99.

2. Ekström J, Khosravani N, Castagnola M, Messana I. Saliva and the Control of Its Secretion. In: Ekberg O, editor. Dysphagia: Diagnosis and Treatment. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. p. 19-47.

3. Maurer HH. Advances in analytical toxicology: the current role of liquid chromatography-mass spectrometry in drug quantification in blood and oral fluid. Anal Bioanal Chem. 2005;381(1):110-118.

4. Dawes C. Circadian rhythms in human salivary flow rate and composition. J Physiol. 1972;220(3):529-545.

5. Gittings S, Turnbull N, Henry B, Roberts CJ, Gershkovich P. Characterisation of human saliva as a platform for oral dissolution medium development. Eur J Pharm Biopharm. 2015;91:16-24.

6. Crouch DJ. Oral fluid collection: The neglected variable in oral fluid testing. Forensic Sci Int. 2005;150(2–3):165-173.

7. Presley L, Lehrer M, Seiter W, Hahn D, Rowland B, Smith M, *et al*. High prevalence of 6-acetylmorphine in morphine-positive oral fluid specimens. Forensic Sci Int. 2003;133(1-2):22-25.

8. Blood. Britannica Academic: Encyclopædia Britannica; 2016.

9. Cone EJ, Huestis MA. Interpretation of oral fluid tests for drugs of abuse. Ann N Y Acad Sci. 2007;1098:51-103.

10. Yamahara H, Lee VHL. Drug metabolism in the oral cavity. Adv Drug Deliver Rev. 1993(12):25-40.

11. Toennes SW, Steinmeyer S, Maurer HJ, Moeller MR, Kauert GF. Screening for drugs of abuse in oral fluid—correlation of analysis results with serum in forensic cases. J Anal Toxicol. 2005;29(1):22-27.

12. Bosker WM, Huestis MA. Oral fluid testing for drugs of abuse. Clin Chem. 2009;55(11):1910-31.

13. Soifer S. International Paruresis Association, Inc. 2017.

14. Langel K, Engblom C, Pehrsson A, Gunnar T, Ariniemi K, Lillsunde P. Drug testing in oral fluid—evaluation of sample collection devices. J Anal Toxicol. 2008;32(6):393-401.

15. Aps JK, Martens LC. Review: The physiology of saliva and transfer of drugs into saliva. Forensic Sci Int. 2005;150(2-3):119-131.

16. Kauert GF. Drogennachweis in Speichel vs. Serum. Blutalkohol. 2000(37):76-83.

17. Moritsuka M, Kitasako Y, Burrow MF, Ikeda M, Tagami J, Nomura S. Quantitative assessment for stimulated saliva flow rate and buffering capacity in relation to different ages. J Dent. 2006;34(9):716-720.

18. Drummer OH. Drug testing in oral fluid. Clin Biochem Rev. 2006;27(3):147-159.

19. Dodds MW, Johnson DA. Influence of mastication on saliva, plaque pH and masseter muscle activity in man. Arch Oral Biol. 1993;38(7):623-626.

20. Huestis MA, Cone EJ. Methamphetamine disposition in oral fluid, plasma, and urine. Ann N Y Acad Sci. 2007;1098:104-121.

21. Jickells S, Negrusz A. editors. Clarke's Analytical Forensic Toxicology. London: Pharmaceutical Press; 2008.

22. Van der Linden T, Legrand SA, Silverans P, Verstraete AG. DUID: oral fluid and blood confirmation compared in Belgium. J Anal Toxicol. 2012;36(6):418-421.

23. Danhof M, Breimer DD. Therapeutic drug monitoring in saliva. Clin Pharmacokinet. 1978;3(1):39-57.

24. Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta. 2007;383(1-2):30-40.

25. Wille SM, Baumgartner MR, Fazio VD, Samyn N, Kraemer T. Trends in drug testing in oral fluid and hair as alternative matrices. Bioanalysis. 2014;6(17):2193-2209.

26. Vindenes V, Yttredal B, Oiestad EL, Waal H, Bernard JP, Mørland JG, *et al*. Oral fluid is a viable alternative for monitoring drug abuse: detection of drugs in oral fluid by liquid chromatography-tandem mass spectrometry and comparison to the results from urine samples from patients treated with methadone or buprenorphine. J Anal Toxicol. 2011;35(1):32-39.

27. Mucklow JC. The use of saliva in therapeutic drug monitoring. Ther Drug Monit. 1982;4(3):229-248.

28. Rylance GW, Butcher GM, Moreland T. Saliva carbamazepine levels in children. Br Med J. 1977;2:1481.

29. Liu H, Delgado MR. Therapeutic drug concentration monitoring using saliva samples. Focus on anticonvulsants. Clin Pharmacokinet. 1999;36(6):453-470.

30. Patsalos PN, Berry DJ. Therapeutic Drug Monitoring of Antiepileptic Drugs by Use of Saliva. Ther Drug Monit. 2013;35(1):4-29.

31. Horning MG, Brown L, Nowlin J, Lertratanangkoon K, Kellaway P, Zion TE. Use of saliva in therapeutic drug monitoring. Clin Chem. 1977;23(2 PT. 1):157-164.

32. de Wildt SN, Kerkvliet KT, Wezenberg MG, Ottink S, Hop WC, Vulto AG, *et al*. Use of saliva in therapeutic drug monitoring of caffeine in preterm infants. Ther Drug Monit. 2001;23(3):250-254.

33. Aman MG, Vinks AA, Remmerie B, Mannaert E, Ramadan Y, Masty J, *et al*. Plasma pharmacokinetic characteristics of risperidone and their relationship to saliva concentrations in children with psychiatric or neurodevelopmental disorders. Clin Ther. 2007;29(7):1476-1486.

34. Flarakos J, Luo W, Aman M, Svinarov D, Gerber N, Vouros P. Quantification of risperidone and 9-hydroxyrisperidone in plasma and saliva from adult and pediatric patients by liquid chromatography-mass spectrometry. J Chromatogr A. 2004;1026(1-2):175-183.

35. Lena SM, Hutchins P, Wood CB, Turner P. Salivary theophylline estimation in the management of asthma in children. Postgrad Med J. 1980;56(652):85-87.

36. Jones AW. Distribution of ethanol between saliva and blood in man. Clin Exp Pharmacol Physiol. 1979;6(1):53-59.

37. Kim I, Barnes AJ, Oyler JM, Schepers R, Joseph RE, Cone EJ, *et al*. Plasma and oral fluid pharmacokinetics and pharmacodynamics after oral codeine administration. Clin Chem. 2002;48(9):1486-1496.

38. Langel K, Gjerde H, Favretto D, Lillsunde P, Øiestad EL, Ferrara SD, *et al*. Comparison of drug concentrations between whole blood and oral fluid. Drug Test Anal. 2014;6(5):461-471.

39. Schepers RJ, Oyler JM, Joseph RE, Cone EJ, Moolchan ET, Huestis MA. Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. Clin Chem. 2003;49(1):121-132.

40. Vindenes V, Lund HME, Andresen W, Gjerde H, Ikdahl SE, Christophersen AS, *et al*. Detection of drugs of abuse in simultaneously collected oral fluid,

urine and blood from Norwegian drug drivers. Forensic Sci Int. 2012;219(1):165-171.

41. Gjerde H, Mordal J, Christophersen AS, Bramness JG, Mørland J. Comparison of drug concentrations in blood and oral fluid collected with the Intercept® sampling device. J Anal Toxicol. 2010;34(4):204-209.

42. Conermann T, Gosalia AR, Kabazie AJ, Moore C, Miller K, Fetsch M, *et al*. Utility of oral fluid in compliance monitoring of opioid medications. Pain Physician. 2014;17(1):63-70.

43. Traxdorf M, Wendler O, Tziridis K, Bauer J, Scherl C. S100B in serum and saliva: a valid invasive or non-invasive biomarker in obstructive sleep apnea? Eur Rev Med Pharmacol Sci. 2016;20(22):4766-4774.

44. Mandel ID. The diagnostic uses of saliva. J Oral Pathol Med. 1990;19(3):119-125.

45. Takayama T, Tsutsui H, Shimizu I, Toyama T, Yoshimoto N, Endo Y, *et al*. Diagnostic approach to breast cancer patients based on target metabolomics in saliva by liquid chromatography with tandem mass spectrometry. Clin Chim Acta. 2016;452:18-26.

46. Corstjens PL, Abrams WR, Malamud D. Saliva and viral infections. Periodontol 2000. 2016;70(1):93-110.

47. Malamud D, Abrams WR, Bau H, Wang J, Chen Z, Corstjens P, *et al*. Oral-based techniques for the diagnosis of infectious diseases. J Calif Dent Assoc. 2006;34(4):297-301.

48. Corstjens PL, Abrams WR, Malamud D. Detecting viruses by using salivary diagnostics. J Am Dent Assoc. 2012;143(10 Suppl):12S-8S.

49. Caplan YH, Goldberger BA. Alternative specimens for workplace drug testing. J Anal Toxicol. 2001;25(5):396-399.

50. Michalke B, Rossbach B, Göen T, Schäferhenrich A, Scherer G. Saliva as a matrix for human biomonitoring in occupational and environmental medicine. Int Arch Occup Environ Health. 2015;88(1):1-44.

51. Weber TJ, Smith JN, Carver ZA, Timchalk C. Non-invasive saliva human biomonitoring: development of an in vitro platform. J Expos Sci Environ Epidemiol. 2017;27(1):72-77.

52. Casolin A. Comparison of urine and oral fluid for workplace drug testing. J Anal Toxicol. 2016;40(7):479-485.

53. Clarke J, Wilson JF. Proficiency testing (external quality assessment) of drug detection in oral fluid. Forensic Sci Int. 2005;150(2-3):161-164.

54. Musshoff F, Hokamp EG, Bott U, Madea B. Performance evaluation of on-site oral fluid drug screening devices in normal police procedure in Germany. Forensic Sci Int. 2014;238:120-124.

55. Verstraete AG. Oral fluid testing for driving under the influence of drugs: history, recent progress and remaining challenges. Forensic Sci Int. 2005;150(2-3):143-150.

56. Kelley-Baker T, Moore C, Lacey JH, Yao J. Comparing drug detection in oral fluid and blood: data from a national sample of nighttime drivers. Traffic Inj Prev. 2014;15(2):111-118.

57. Anzillotti L, Castrignanò E, Strano Rossi S, Chiarotti M. Cannabinoids determination in oral fluid by SPME-GC/MS and UHPLC-MS/MS and its application on suspected drivers. Sci Justice. 2014;54(6):421-426.

58. Wille SM, Di Fazio V, Toennes SW, van Wel JH, Ramaekers JG, Samyn N. Evaluation of Δ(9) -tetrahydrocannabinol detection using DrugWipe5S® screening and oral fluid quantification after Quantisal™ collection for roadside drug detection via a controlled study with chronic cannabis users. Drug Test Anal. 2015;7(3):178-186.

59. Wille SM, Di Fazio V, Ramírez-Fernandez MeM, Kummer N, Samyn N. Driving under the influence of cannabis: pitfalls, validation, and quality control of a UPLC-MS/MS method for the quantification of tetrahydrocannabinol in oral fluid collected with StatSure, Quantisal, or Certus collector. Ther Drug Monit. 2013;35(1):101-111.

60. Rositano J, Harpas P, Kostakis C, Scott T. Supported liquid extraction (SLE) for the analysis of methylamphetamine, methylenedioxymethylamphetamine and delta-9-tetrahydrocannabinol in oral fluid and blood of drivers. Forensic Sci Int. 2016;265(Supplement C):125-130.

61. Gjerde H, Langel K, Favretto D, Verstraete AG. Detection of illicit drugs in oral fluid from drivers as biomarker for drugs in blood. Forensic Sci Int. 2015;256:42-45.

62. Strano-Rossi S, Anzillotti L, Castrignanò E, Felli M, Serpelloni G, Mollica R, *et al*. UHPLC-ESI-MS/MS method for direct analysis of drugs of abuse in oral fluid for DUID assessment. Anal Bioanal Chem. 2011;401(2):609-624.

63. Strano-Rossi S, Castrignanò E, Anzillotti L, Serpelloni G, Mollica R, Tagliaro F, *et al*. Evaluation of four oral fluid devices (DDS®, Drugtest 5000®, Drugwipe 5+® and RapidSTAT®) for on-site monitoring drugged driving in comparison with UHPLC-MS/MS analysis. Forensic Sci Int. 2012;221(1-3):70-76.

64. Veitenheimer AM, Wagner JR. Evaluation of oral fluid as a specimen for DUID. J Anal Toxicol. 2017;41(6):517-522.

65. Edwards LD, Smith KL, Savage T. Drugged driving in Wisconsin: oral fluid versus blood. J Anal Toxicol. 2017;41(6):523-529.

66. Gjerde H, Langel K, Favretto D, Verstraete AG. Estimation of equivalent cutoff thresholds in blood and oral fluid for drug prevalence studies. J Anal Toxicol. 2014;38(2):92-98.

67. European Workplace Drug Testing Society (EWDTS). Analytical evaluation of oral fluid screening devices and preceding selection procedures. 2010. [Online: http://www.bast.de/Druid/EN/deliverales-list/downloads/Deliverable_3_2_2.pdf?__blob=publicationFile&v=1]

68. European Workplace Drug Testing Society (EWDTS). European Guidelines for Workplace in Oral Fluid. 2015. p. 1-40. [Online: http://www.ewdts.org/data/uploads/documents/ewdts-oral-fluid-2015-05-29-v02.pdf]

69. Substance Abuse and Mental Health Services Administration (SAMHSA) H. Mandatory Guidelines for Federal Workplace Drug Testing Programs. 2015. p. 28053-28101.

70. Flanagan RJ, Taylor AA, Watson ID, Whelpton R. Fundamentals of Analytical Toxicology. 1st ed: Chichester, UK: Wiley; 2008. 544 p.

71. Wille SM, Samyn N, Ramírez-Fernández MeM, De Boeck G. Evaluation of on-site oral fluid screening using Drugwipe-5(+), RapidSTAT and Drug Test 5000 for the detection of drugs of abuse in drivers. Forensic Sci Int. 2010;198(1-3):2-6.

72. Van der Linden T, Wille SM, Ramírez-Fernandez M, Verstraete AG, Samyn N. Roadside drug testing: comparison of two legal approaches in Belgium. Forensic Sci Int. 2015;249:148-155.

73. Pehrsson A, Blencowe T, Vimpari K, Langel K, Engblom C, Lillsunde P. An evaluation of on-site oral fluid drug screening devices DrugWipe 5+ and Rapid STAT using oral fluid for confirmation analysis. J Anal Toxicol. 2011;35(4):211-218.

74. United Nations Office on Drugs and Crime (UNODC). World Drug Report 2017. United Nations Office on Drugs and Crime, 2017.

75. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), Europol. EU Drug Markets Report: In-Depth Analysis. Luxembourg: European Monitoring Centre for Drugs and Drug Addiction-Europol Joint publications, 2016.

76. UK Government. United Kingdom Drug Situation: Focal Point Annual Report 2016. United Kingdom Focal Point at Public Health England, London. 2017.

77. Home Office. Seizures of drugs in England and Wales, year ending 31 March 2016. Home Office, London, UK. 2016.

78. Scottish Government. Drug Seizures by Police Scotland, 2013-14. Edinburgh, UK. 2015.

79. UK Government. Drug Seizures and Offender Characteristics, 2014-15 and 2015-16. Scottish Government, Edinburgh, UK. 2017.

80. UK Government. Deaths related to drug poisoning in England and Wales: 2016 registrations. Office for National Statistics, London, UK. 2017.

81. Scottish Government. Drug-related deaths in Scotland in 2016. National Records of Scotland, Edinburgh, UK. 2017.

82. Baselt R. Disposition of Toxic Drugs and Chemicals in Man. 11 ed. Foster City, California: Biomedical Publications; 2017.

83. United Nations Department of Public Information. UN General Assembly Special Sessions on the World Drug Problem Amphetamine-Type Stimulants. United Nations Department of Public Information; New York, USA. 1998.

84. Cody JT, Schwarzhoff R. Interpretation of methamphetamine and amphetamine enantiomer data. J Anal Toxicol. 1993;17(6):321-326.

85. Baselt RC. Disposition of Toxic Drugs and Chemicals in Man. 11 ed. Foster City, California: Biomedical Publications; 2017.

86. Paul BD, Jemionek J, Lesser D, Jacobs A, Searles DA. Enantiomeric separation and quantitation of (+/-)-amphetamine, (+/-)-methamphetamine, (+/-)-MDA, (+/-)-MDMA, and (+/-)-MDEA in urine specimens by GC-EI-MS after derivatization with (R)-(-)- or (S)-(+)-alpha-methoxy-alpha-(trifluoromethy)phenylacetyl chloride (MTPA). J Anal Toxicol. 2004;28(6):449-455.

87. Glaser PE, Thomas TC, Joyce BM, Castellanos FX, Gerhardt GA. Differential effects of amphetamine isomers on dopamine release in the rat

striatum and nucleus accumbens core. Psychopharmacology 2005;178(2-3):250-258.

88. Heal DJ, Smith SL, Findling RL. ADHD: current and future therapeutics. Curr Top Behav Neurosci. 2012;9:361-290.

89. Heal DJ, Smith SL, Gosden J, Nutt DJ. Amphetamine, past and present — a pharmacological and clinical perspective. J Psychopharmacol. 2013;27(6):479-496.

90. Mendelson JE, McGlothlin D, Harris DS, Foster E, Everhart T, Jacob P, *et al*. The clinical pharmacology of intranasal l-methamphetamine. BMC Clin Pharmacol. 2008;8(1):4.

91. de la Torre R, Yubero-Lahoz S, Pardo-Lozano R, Farré M. MDMA, methamphetamine, and CYP2D6 pharmacogenetics: what is clinically relevant? Front Genet. 2012;3:235.

92. Karch SB, Drummer OH. Synthetic Stimulants. Karch's Pathology of Drug Abuse, 5th ed: Boca Raton; CRC Press; 2015. p. 273-364.

93. Pesce G, Adler-Graschinsky E. Comparison of the effects of beta-phenylethylamine and d-amphetamine on rat isolated atria. J Pharmacol Exp Ther. 1983;227(1):205-214.

94. Goodman, Gills. The Pharmacological Basis of Therapeutics. Rall TW, Nies AS, Taylor P, editors. USA: Pergamon Press, Inc.; 1990.

95. Itzhak Y, Ali SF. Behavioral consequences of methamphetamine-induced neurotoxicity in mice: relevance to the psychopathology of methamphetamine addiction. Ann N Y Acad Sci. 2002;965:127-135.

96. Harris DS, Boxenbaum H, Everhart ET, Sequeira G, Mendelson JE, Jones RT. The bioavailability of intranasal and smoked methamphetamine. Clin Pharmacol Ther. 2003;74(5):475-486.

97. Morselli PL, Placidi GF, Maggini C, Gomeni R, Guazelli M, De Lisio G, *et al*. An integrated approach for the evaluation of psychotropic drug in man. Psychopharmacologia. 1976;46(2):211-217.

98. Anggård E, Gunne LM, Niklasson F. Gas chromatographic determination of amphetamine in blood, tissue, and urine. Scand J Clin Lab Invest. 1970;26(2):137-143.

99. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Recent changes in Europe's MDMA/ecstasy market. Luxembourg: 2016.

100. Stone DM, Merchant KM, Hanson GR, Gibb JW. Immediate and long-term effects of 3,4-methylenedioxymethamphetamine on serotonin pathways in brain of rat. Neuropharmacology. 1987;26(12):1677-1683.

101. Colado MI, O'Shea E, Green AR. Acute and long-term effects of MDMA on cerebral dopamine biochemistry and function. Psychopharmacology 2004;173(3-4):249-263.

102. Baggott MJ, Siegrist JD, Galloway GP, Robertson LC, Coyle JR, Mendelson JE. Investigating the mechanisms of hallucinogen-induced visions using 3,4-Methylenedioxyamphetamine (MDA): a randomized controlled trial in humans. PLoS ONE. 2010;5(12):e14074.

103. Concheiro M, de Castro A, Quintela O, Cruz A, López-Rivadulla M. Confirmation by LC-MS of drugs in oral fluid obtained from roadside testing. Forensic Sci Int. 2007;170(2-3):156-162.

104. Regester LE, Chmiel JD, Holler JM, Vorce SP, Levine B, Bosy TZ. Determination of designer drug cross-reactivity on five commercial immunoassay screening kits. J Anal Toxicol. 2015;39(2):144-151.

105. Apollonio LG, Whittall IR, Pianca DJ, Kyd JM, Maher WA. Matrix effect and cross-reactivity of select amphetamine-type substances, designer analogues, and putrefactive amines using the Bio-Quant direct ELISA presumptive assays for amphetamine and methamphetamine. J Anal Toxicol. 2007;31(4):208-213.

106. Petrie M, Lynch KL, Ekins S, Chang JS, Goetz RJ, Wu AHB, *et al*. Cross-reactivity studies and predictive modeling of "Bath Salts" and other amphetamine-type stimulants with amphetamine screening immunoassays. Clin Toxicol. 2013;51(2):83-91.

107. de Castro A, Lendoiro E, Fernández-Vega H, Steinmeyer S, López-Rivadulla M, Cruz A. Liquid chromatography tandem mass spectrometry determination of selected synthetic cathinones and two piperazines in oral fluid. Cross reactivity study with an on-site immunoassay device. J Chromatogr A. 2014;1374(Supplement C):93-101.

108. Quintela O, Crouch DJ, Andrenyak DM. Recovery of drugs of abuse from the Immunalysis Quantisal™ oral fluid collection device. J Anal Toxicol. 2006;30(8):614-616.

109. Crouch DJ, Day J, Baudys J. Evaluation of Saliva/Oral Fluid as Alternate Drug Testing Specimen - Final Report (NISTR 7139). Gaitherburg, MA: National Institute of Standards and Testing; 2004. p. 1-70.

110. Wille SM, Raes E, Lillsunde P, Gunnar T, Laloup M, Samyn N, *et al*. Relationship between oral fluid and blood concentrations of drugs of abuse in drivers suspected of driving under the influence of drugs. Ther Drug Monit. 2009;31(4):511-519.

111. Andås HT, Enger A, Øiestad Å, Vindenes V, Christophersen AS, Huestis MA, *et al*. Extended detection of amphetamine and methamphetamine in oral fluid. Ther Drug Monit. 2016;38(1):114-119.

112. Kim E, Lee J, Choi H, Han E, Park Y, Choi H, *et al*. Comparison of methamphetamine concentrations in oral fluid, urine and hair of twelve drug abusers using solid-phase extraction and GC-MS. Ann Toxicol Anal. 2008;3(20):145-53.

113. Wood M, De Boeck G, Samyn N, Morris M, Cooper DP, Maes RA, *et al*. Development of a rapid and sensitive method for the quantitation of amphetamines in human plasma and oral fluid by LC-MS-MS. J Anal Toxicol. 2003;27(2):78-87.

114. Musshoff F, Madea B. Driving under the influence of amphetamine-like drugs. J Forensic Sci. 2012;57(2):413-419.

115. Barnes AJ, Scheidweiler KB, Kolbrich-Spargo EA, Gorelick DA, Goodwin RS, Huestis MA. MDMA and metabolite disposition in expectorated oral fluid after controlled oral MDMA administration. Ther Drug Monit. 2011;33(5):602-608.

116. Logan BK, Fligner CL, Haddix T. Cause and manner of death in fatalities involving methamphetamine. J Forensic Sci. 1998;43(1):28-34.

117. Drummer OH, Gerostamoulos D, Chu M, Swann P, Boorman M, Cairns I. Drugs in oral fluid in randomly selected drivers. Forensic Sci Int. 2007;170(2-3):105-110.

118. Engblom C, Gunnar T, Rantanen A, Lillsunde P. Driving under the influence of drugs-amphetamine concentrations in oral fluid and whole blood samples. J Anal Toxicol. 2007;31(5):276-280.

119. Newmeyer MN, Concheiro M, da Costa JL, Flegel R, Gorelick DA, Huestis MA. Oral fluid with three modes of collection and plasma methamphetamine and amphetamine enantiomer concentrations after controlled intranasal l-methamphetamine administration. Drug Test Anal. 2015;7(10):877-83.

120. Comiran E, Barreto F, Meneghini LZ, Carlos G, Fröehlich PE, Limberger RP. Method validation and determination of lisdexamfetamine and amphetamine in

oral fluid, plasma and urine by LC–MS/MS. Biomed Chromatogr. 2017;31(3):e3812.

121. Bahmanabadi L, Akhgari M, Jokar F, Sadeghi HB. Quantitative determination of methamphetamine in oral fluid by liquid–liquid extraction and gas chromatography/mass spectrometry. Hum Exp Toxicol. 2016;36(2):195-202.

122. Poetzsch M, Steuer AE, Hysek CM, Liechti ME, Kraemer T. Development of a high-speed MALDI-triple quadrupole mass spectrometric method for the determination of 3,4-methylenedioxymethamphetamine (MDMA) in oral fluid. Drug Test Anal. 2016;8(2):235-240.

123. Amaratunga P, Clothier M, Lorenz Lemberg B, Lemberg D. Determination of dextromethorphan in oral fluid by LC–MS-MS. J Anal Toxicol. 2016;40(5):360-366.

124. National Centre for Biotechnology Information. PubChem Compound Database CID=962 [6 May 2016]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/962.

125. Whembolua GL, Granger DA, Singer S, Kivlighan KT, Marguin JA. Bacteria in the oral mucosa and its effects on the measurement of cortisol, dehydroepiandrosterone, and testosterone in saliva. Horm Behav. 2006;49(4):478-483.

126. Kauert GF, Iwersen-Bergmann S, Toennes SW. Assay of Delta9-tetrahydrocannabinol (THC) in oral fluid-evaluation of the OraSure oral specimen collection device. J Anal Tox. 2006;30(4):274-277.

127. Swortwood MJ, Boland DM, DeCaprio AP. Determination of 32 cathinone derivatives and other designer drugs in serum by comprehensive LC-QQQ-MS/MS analysis. Anal Bioanal Chem. 2013;405(4):1383-97.

128. Slawson MH, Taccogno JL, Foltz RL, Moody DE. Quantitative analysis of selegiline and three metabolites (N-desmethylselegiline, methamphetamine, and amphetamine) in human plasma by high-performance liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry. J Anal Toxicol. 2002;26(7):430-437.

129. Joint Formulary Committee. British National Formulary. BMJ Group and Pharmaceutical Press, London, UK; 2017 [cited 2017 29 Nov].

130. Manchester KR, Lomas EC, Waters L, Dempsey FC, Maskell PD. The emergence of new psychoactive substance (NPS) benzodiazepines: A review. Drug Test Anal. (in print)

131. UK Government. Table of Drugs and Limits. In: Department of Transport. London.

132. Seymour A, Oliver JS. Role of drugs and alcohol in impaired drivers and fatally injured drivers in the Strathclyde police region of Scotland, 1995–1998. Forensic Sci Int. 1999;103(2):89-100.

133. Hamnett HJ, Ilett M, Izzati F, Smith SS, Watson KH. Toxicological findings in driver and motorcyclist fatalities in Scotland 2012–2015. Forensic Sci Int. 2017;274(Supplement C):22-26.

134. Wylie FM, Torrance H, Seymour A, Buttress S, Oliver JS. Drugs in oral fluid Part II. Investigation of drugs in drivers. Forensic Sci Int. 2005;150(2-3):199-204.

135. Chu M, Gerostamoulos D, Beyer J, Rodda L, Boorman M, Drummer OH. The incidence of drugs of impairment in oral fluid from random roadside testing. Forensic Sci Int. 2012;215(1):28-31.

136. Gjerde H, Langel K, Favretto D, Verstraete AG. Detection of 4 benzodiazepines in oral fluid as biomarker for presence in blood. Ther Drug Monit. 2014;36(2):252-256.

137. Choo RE, Huestis MA. Oral fluid as a diagnostic tool. Clin Chem Lab Med. 2004;42(11):1273-1287.

138. de Gier JJ, t Hart BJ, Wilderink PF, Nelemans FA. Comparison of plasma and saliva levels of diazepam. Brit J Clin Pharmaco. 1980;10(2):151-155.

139. Laloup M, Ramirez Fernandez MdM, Wood M, Maes V, De Boeck G, Vanbeckevoort Y, *et al*. Detection of diazepam in urine, hair and preserved oral fluid samples with LC-MS-MS after single and repeated administration of Myolastan® and Valium®. Anal Bioanal Chem. 2007;388(7):1545-1556.

140. Cone EJ, Clarke J, Tsanaclis L. Prevalence and disposition of drugs of abuse and opioid treatment drugs in oral fluid. J Anal Toxicol. 2007;31(8):424-433.

141. Kintz P, Villain M, Concheiro M, Cirimele V. Screening and confirmatory method for benzodiazepines and hypnotics in oral fluid by LC-MS/MS. Forensic Sci Int. 2005;150(2):213-220.

142. Badawi N, Simonsen KW, Steentoft A, Bernhoft IM, Linnet K. Simultaneous screening and quantification of 29 drugs of abuse in oral fluid by solid-phase extraction and ultraperformance LC-MS/MS. Clin Chem. 2009;55(11):2004-2018.

143. Wylie FM, Torrance H, Anderson RA, Oliver JS. Drugs in oral fluid Part I. Validation of an analytical procedure for licit and illicit drugs in oral fluid. Forensic Sci Int. 2005;150(2-3):191-198.

144. Quintela O, Cruz A, Castro Ad, Concheiro M, López-Rivadulla M. Liquid chromatography–electrospray ionisation mass spectrometry for the determination of nine selected benzodiazepines in human plasma and oral fluid. J Chromatogr B. 2005;825(1):63-71.

145. Ngwa G, Fritch D, Blum K, Newland G. Simultaneous analysis of 14 benzodiazepines in oral fluid by solid-phase extraction and LC-MS-MS. J Anal Toxicol. 2007;31(7):369-376.

146. Moore C, Coulter C, Crompton K, Zumwalt M. Determination of benzodiazepines in oral fluid using LC-MS-MS. J Anal Toxicol. 2007;31(9):596-600.

147. Miller KL, Puet BL, Roberts A, Hild C, Carter J, Black DL. Urine drug testing results and paired oral fluid comparison from patients enrolled in long-term medication-assisted treatment in Tennessee. J Subst Abuse Treat. 2017;76:36-42.

148. Gore SM, Bird AG, Strang JS. Random mandatory drugs testing of prisoners: a biassed means of gathering information. J Epidemiol Biostatistics. 1999;4(1):3-9.

149. Jenkins AJ, Oyler JM, Cone EJ. Comparison of heroin and cocaine concentrations in saliva with concentrations in blood and plasma. J Anal Toxicol. 1995;19(6):359-374.

150. West RE, Guevara MG, Mikel C, Gamez R. Detection of hydrocodone and morphine as metabolites in oral fluid by LC-MS/MS in patients prescribed codeine. Ther Drug Monit. 2017;39(1):88-90.

151. Dams R, Choo RE, Lambert WE, Jones H, Huestis MA. Oral fluid as an alternative matrix to monitor opiate and cocaine use in substance-abuse treatment patients. Drug Alcohol Depend. 2007;87(2-3):258-267.

152. Liu H-C, Lee H-T, Hsu Y-C, Huang M-H, Liu RH, Chen T-J, *et al*. Direct injection LC–MS-MS analysis of opiates, methamphetamine, buprenorphine, methadone and their metabolites in oral fluid from substitution therapy patients. J Anal Toxicol. 2015;39(6):472-480.

153. Stone P, Kuhlmann F, Schlabach T, Miller K. New dynamic MRM mode improves data quality and triple quad quantification in complex analyses. Santa Clara, CA, USA: Agilent Technologies; 2009. p. 1-7.

154. Mallet CR, Lu Z, Mazzeo JR. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. Rapid Commun Mass Spectrom. 2004;18(1):49-58.

155. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem. 2003;75(13):3019-3030.

156. Øiestad EL, Johansen U, Christophersen AS. Drug screening of preserved oral fluid by liquid chromatography-tandem mass spectrometry. Clin Chem. 2007;53(2):300-309.

157. Liao Q, Deng Y, Xie Z, Pan B, Zhang L. Rapid simultaneous determination of codeine and morphine in plasma using LC-ESI-MS/MS: application to a clinical pharmacokinetic study. J Sep Sci. 2009;32(2):202-211.

158. Miller EI, Wylie FM, Oliver JS. Simultaneous detection and quantification of amphetamines, diazepam and its metabolites, cocaine and its metabolites, and opiates in hair by LC-ESI-MS-MS using a single extraction method. J Anal Toxicol. 2008;32(7):457-469.

159. Karch SB. Workplace drug testing. Karch SB, editor. Boca Raton: CRC Press; 2008.

160. Hii TM, Lee HK. Liquid–Liquid Extraction in Environmental Analysis. Handbook of Sample Preparation: John Wiley & Sons, Inc.; Hoboken, New Jersey; 2010. p. 39-51.

161. Scientific Working Group for Forensic Toxicology (SWGTOX) Standard practices for method validation in forensic toxicology. J Anal Toxicol. 2013;37(7):452-474.

162. Wood M, Laloup M, Ramirez Fernandez MeM, Jenkins KM, Young MS, Ramaekers JG, *et al*. Quantitative analysis of multiple illicit drugs in preserved oral fluid by solid-phase extraction and liquid chromatography-tandem mass spectrometry. Forensic Sci Int. 2005;150(2-3):227-238.

163. MSJ (Diazepam) Information & Advice on substance.org.uk - Reducing the harm from substance misuse [updated September 201112/09/2017]. Available from: http://www.substance.org.uk/topic/37-msj-guide-diazepam.aspx.

164. Kintz P, Jamey C, Ameline A, Richeval C, Raul J-S. Characterization of metizolam, a designer benzodiazepine, in alternative biological specimens. Toxicol Anal Clin. 2017;29(1):57-63.

165. Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. Forensic Sci Int. 2006;161(2-3):158-62.

166. Castro AL, Dias M, Reis F, Teixeira HM. Gamma-hydroxybutyric acid endogenous production and post-mortem behaviour – The importance of different biological matrices, cut-off reference values, sample collection and storage conditions. J Forensic Leg Med. 2014;27:17-24.

167. Fjeld B, Burns ML, Karinen R, Larssen B, Smith-Kielland A, Vindenes V. Long-term stability of GHB in post-mortem samples and samples from living persons, stored at -20 degrees C, using fluoride preservatives. Forensic Sci Int. 2012;222(1-3):47-51.

168. Jones AW, Gladh S, Windberg CN, Johansen SS. Stability of γ-hydroxybutyrate in blood samples from impaired drivers after storage at 4°C and comparison of GC-FID-GBL and LC-MS-MS methods of analysis. J Anal Toxicol. 2015;4(39):294-299.

169. Stephens RG, Coleman DE, Baselt RC. In vitro stability of endogenous GHB in postmortem blood. J Forensic Sci. 1999;44(1):231.

170. Marinetti LJ, Isenschmid DS, Hepler BR, Kanluen S. Analysis of GHB and 4-methyl-GHB in postmortem matrices after long-term storage. J Anal Toxicol. 2005;29(1):41-47.

171. Zörntlein SW, Kopp A, Becker J, Kaufmann TJ, Röhrich J, Urban R. In vitro production of GHB in blood and serum samples under various storage conditions. Forensic Sci Int. 2012;214(1-3):113-117.

172. LeBeau MA, Montgomery MA, Jufer RA, Miller ML. Elevated GHB in citrate-buffered blood. J Anal Toxicol. 2000;24(5):383-384.

173. Ahmed A-K, Weatherburn P, Reid D, Hickson F, Torres-Rueda S, Steinberg P, *et al*. Social norms related to combining drugs and sex ("chemsex") among gay men in South London. Int J Drug Policy. 2016;38:29-35.

174. Hockenhull J, Murphy KG, Paterson S. An observed rise in γ-hydroxybutyrate-associated deaths in London: Evidence to suggest a possible link with concomitant rise in chemsex. Forensic Sci Int. 2017;270:93-97.

175. Al-Samarraie MS, Karinen R, Morland J, Opdal MS. Blood GHB concentrations and results of medical examinations in 25 car drivers in Norway. Eur J Clin Pharmacol. 2010;66(10):987-998.

176. Dresen S, Kempf J, Weinmann W. Prevalence of gamma-hydroxybutyrate (GHB) in serum samples of amphetamine, metamphetamine and ecstasy impaired drivers. Forensic Sci Int. 2007;173(2-3):112-116.

177. Lott S, Musshoff F, Madea B. Estimation of gamma-hydroxybutyrate (GHB) co-consumption in serum samples of drivers positive for amphetamine or ecstasy. Forensic Sci Int. 2012;221(1-3):98-101.

178. Moeller MR, Kraemer T. Drugs of abuse monitoring in blood for control of driving under the influence of drugs. Ther Drug Monit. 2002;24(2):210-21.

179. Burch HJ, Clarke EJ, Hubbard AM, Scott-Ham M. Concentrations of drugs determined in blood samples collected from suspected drugged drivers in England and Wales. J Forensic Leg Med. 2013;20(4):278-289.

180. De Paoli G, Bell S. A rapid GC-MS determination of gamma-hydroxybutyrate in saliva. J Anal Toxicol. 2008;32(4):298-302.

181. De Paoli G, Walker KM, Pounder DJ. Endogenous γ-hydroxybutyric acid concentrations in saliva determined by gas chromatography-mass spectrometry. J Anal Toxicol. 2011;35(3):148-152.

182. Grootveld M, Algeo D, Silwood CJ, Blackburn JC, Clark AD. Determination of the illicit drug gamma-hydroxybutyrate (GHB) in human saliva and beverages by ^{1}H NMR analysis. Biofactors. 2006;27(1-4):121-136.

183. Jones AW, Holmgren A, Kugelberg FC. Driving under the influence of gamma-hydroxybutyrate (GHB). Forensic Sci Med Pat. 2008;4(4):205-211.

184. Barker JC, Karsoho H. Hazardous use of gamma hydroxybutyrate: driving under the influence. Subst Use Misuse. 2008;43(11):1507-1520.

185. Hassan HMA. An Evaluation of the Stability and Prevalence of Alcohol and Related Biomarkers in Biological Matrices with Applications to the Interpretation of Medico-Legal Cases. PhD Thesis; Glasgow: University of Glasgow; 2011.

186. Kintz P, Goullé J-P, Cirimele V, Ludes B. Window of detection of γ-hydroxybutyrate in blood and saliva. Clin Chem. 2001;47(11):2033-2034.

187. Castro AL, Tarelho S, Dias M, Reis F, Teixeira HM. Comparison of endogenous GHB concentrations in blood and hair in death cases with emphasis on the post mortem interval. Int J Legal Med. 2016;130(4):959-965.

188. Erdmann F, Zandt D, Auch J, Schütz H, Weiler G, Verhoff MA. [Investigations concerning the threshold value between endogenous and exogenous GHB (liquid ecstasy)]. Arch Kriminol. 2006;217(5-6):129-136.

189. Kintz P, Villain M, Cirimele V, Ludes B. GHB in postmortem toxicology discrimination between endogenous production from exposure using multiple specimens. Forensic Sci Int. 2004;143(2-3):177-181.

190. Saudan C, Augsburger M, Kintz P, Saugy M, Mangin P. Detection of exogenous GHB in blood by gas chromatography-combustion-isotope ratio mass spectrometry: Implications in postmortem toxicology. J Anal Toxicol. 2005;29(8):777-781.

191. Lelong J, Brunet B, Kintz P, Mura P. Determination of GHB concentrations in postmortem samples: A case series and comparison between matrices. Toxicol Anal Clin. 2014;26(2):S22.

192. Elliott S. The presence of gamma-hydroxybutyric acid (GHB) in postmortem biological fluids (Letter to the editor). J Anal Toxicol. 2001;25(2):152.

193. Elliott SP. Further evidence for the presence of GHB in postmortem biological fluid: Implications for the interpretation of findings. J Anal Toxicol. 2004;28(1):20-26.

194. Moriya F, Hashimoto Y. Endogenous gamma-hydroxybutyric acid levels in postmortem blood. Leg Med. 2004;6(1):47-51.

195. Fieler EL, Coleman DE, Baselt RC. gamma-hydroxybutyrate concentrations in pre- and postmortem blood and urine. Clin Chem. 1998;44(3):692.

196. Elliott S, Lowe P, Symonds A. The possible influence of micro-organisms and putrefaction in the production of GHB in post-mortem biological fluid. Forensic Sci Int. 2004;139(2-3):183-190.

197. Arnaldos MI, Garcia MD, Romera E, Presa JJ, Luna A. Estimation of postmortem interval in real cases based on experimentally obtained entomological evidence. Forensic Sci Int. 2005;149(1):57-65.

198. Catts EP. Problems in estimating the postmortem interval in death investigations. Journal of Agricultural Entomology. 1992;9(4):245-255.

199. Goff ML. Estimation of postmortem interval using arthropod development and successional patterns. Forensic Sci Rev. 1993;5:81-94.

200. Moriya F, Hashimoto Y. Site-dependent production of gamma-hydroxybutyric acid in the early postmortem period. Forensic Sci Int. 2005;148(2-3):139-142.

201. Bosman IJ, Lusthof KJ. Forensic cases involving the use of GHB in The Netherlands. Forensic Sci Int. 2003;133(1):17-21.

202. Busardò FP, Bertol E, Vaiano F, Baglio G, Montana A, Barbera N, *et al*. Post mortem concentrations of endogenous gamma hydroxybutyric acid (GHB) and in vitro formation in stored blood and urine samples. Forensic Sci Int. 2014;243:144-148.

203. Fjeld B, Burns ML, Karinen R, Larssen B, Smith-Kielland A, Vindenes V. Long-term stability of GHB in post-mortem samples and samples from living persons, stored at −20°C, using fluoride preservatives. Forensic Sci Int. 2012;222(1):47-51.

204. Andresen-Streichert H, Jensen P, Kietzerow J, Schrot M, Wilke N, Vettorazzi E, *et al*. Endogenous gamma-hydroxybutyric acid (GHB) concentrations in post-mortem specimens and further recommendation for interpretative cut-offs. Int J Legal Med. 2015;129(1):57-68.

205. Busardò FP, Mannocchi G, Giorgetti R, Pellegrini M, Baglio G, Zaami S, *et al*. Stability of endogenous GHB in vitreous humor vs peripheral blood in dead bodies. Forensic Science International. 2017;274(Supplement C):64-69.

206. Hassan HMA, Cooper GAA. Determination of ß-Hydroxybutyrate in blood and urine using gas chromatography—mass spectrometry. J Anal Toxicol. 2009;33(8):502-507.

207. Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Sci Int. 2007;165(2–3):216-224.

208. Templeton AH, Carter KLT, Sheron N, Gallagher PJ, Verrill C. Sudden unexpected death in alcohol misuse—an unrecognized public health issue? Int J Environ Red Publ Health. 2009;6(12):3070-3081.

209. Elliott S, Smith C, Cassidy D. The post-mortem relationship between beta-hydroxybutyrate (BHB), acetone and ethanol in ketoacidosis. Forensic Sci Int. 2010;198(1-3):53-57.

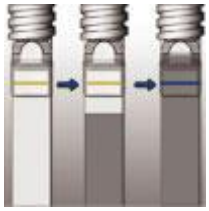
210. Percival S, Janaway R, Wilson A. Decomposition of Human Remains. Microbiology and Aging: Flintshire, UK: Humana Press; 2009. p. 313-334.

211. Shima N, Miki A, Kamata T, Katagi M, Tsuchihashi H. Endogenous level and in vitro production of GHB in blood from healthy humans, and the interpretation

of GHB levels detected in antemortem blood samples. J Health Sci. 2005;51(2):147-154.

Appendices

Appendix II: NeoSAL™ Oral Fluid Collection Instructions

1		Peel open package and remove collector. Do not eat or drink at least 15 minutes prior to collection.
2		Pool saliva in mouth. Insert collector pad in mouth between cheek and gums.
3		When a distinct blue line forms, remove collector from mouth. Typical collection time is 1-2 minutes.
4		Remove blue cap and snap into bottom of the tube. Insert saturated collector pad into tube.
5		Tighten white cap. Gently invert tube 4 or 5 times. Send sample to testing laboratory.

Appendix IV: Volume adequacy data for the NeoSAL™, Intercept® *i*2™, and Quantisal® devices

Table AIV-1 Volume adequacy data for NeoSAL device (*n* = 25)

#	Weight before collection (g)	Weight after collection (g)	Weight Difference (g)	Collection Time (min:sec)	Total Weight (incl. swab, g)
1	7.9437	9.0428	1.0991	01:20.0	3.1991
2	7.9406	8.6720	0.7314	00:50.8	2.8314
3	7.9308	8.6005	0.6697	00:56.2	2.7697
4	7.9395	8.8937	0.9542	00:59.8	3.0542
5	7.9653	8.9125	0.9472	01:01.0	3.0472
6	7.9069	8.7940	0.8871	00:37.5	2.9871
7	7.9590	8.8592	0.9002	00:48.7	3.0002
8	7.9066	8.9412	1.0346	01:00.4	3.1346
9	7.9383	8.8152	0.8769	00:41.5	2.9769
10	7.9203	8.6253	0.7050	00:42.9	2.8050
11	7.8964	8.7848	0.8884	00:51.5	2.9884
12	7.9005	8.8775	0.9770	00:45.0	3.0770
13	7.9768	8.9691	0.9923	00:54.5	3.0923
14	7.9346	8.7572	0.8226	00:56.5	2.9226
15	7.9244	8.8488	0.9244	00:56.8	3.0244
16	7.9231	8.6061	0.6830	01:00.2	2.7830
17	7.9048	8.6522	0.7474	00:46.9	2.8474
18	7.9705	8.9103	0.9398	00:55.3	3.0398
19	7.9294	8.7258	0.7964	00:51.3	2.8964
20	7.9539	8.8918	0.9379	00:57.3	3.0379
21	7.9189	8.6255	0.7066	00:54.5	2.8066
22	7.9042	8.5877	0.6835	00:45.2	2.7835
23	7.9057	8.7363	0.8306	00:46.3	2.9306
24	7.9505	8.5649	0.6144	00:50.6	2.7144
25	7.8909	8.8835	0.9926	00:54.4	3.0926
Mean	7.9256	8.7614	0.8358	00:52.4	2.9358
StDev	0.0270	0.1320	0.1261	00:04.8	0.1261
%CV	0%	2%	15%	9%	4%

Where StDev – Standard deviation; %CV – coefficient of variation

Table AIV-2Collection adequacy for OraSure Intercept® *i*2™ device (*n* = 25)

#	Weight before collection (g)	Weight after collection (g)	Weight Difference (g)	Collection Time (min:sec)	Total Weight (incl. swab, g)
1	9.8991	11.1952	1.2961	01:02.8	2.0961
2	9.9282	11.0644	1.1362	01:26.3	1.9362
3	9.9258	11.0400	1.1142	01:14.1	1.9142
4	9.9281	11.1715	1.2434	01:16.4	2.0434
5	9.9400	11.2846	1.3446	01:54.1	2.1446
6	9.9309	11.1080	1.1771	01:36.8	1.9771
7	9.9510	11.2548	1.3038	01:21.4	2.1038
8	9.8956	11.1877	1.2921	01:45.1	2.0921
9	9.9422	11.1691	1.2269	02:04.5	2.0269
10	9.8951	11.0251	1.1300	01:20.5	1.9300
11	9.9153	11.1278	1.2125	01:46.8	2.0125
12	9.9028	11.3130	1.4102	01:28.7	2.2102
13	9.9524	11.2862	1.3338	01:49.6	2.1338
14	9.8910	11.1020	1.2110	01:35.7	2.0110
15	9.9373	11.2349	1.2976	01:14.7	2.0976
16	9.8901	11.1099	1.2198	02:01.1	2.0198
17	9.9605	11.1643	1.2038	02:06.1	2.0038
18	9.9163	11.1744	1.2581	02:06.3	2.0581
19	9.9027	11.0601	1.1574	01:40.9	1.9574
20	9.9552	11.2126	1.2574	01:31.1	2.0574
Mean	9.9230	11.1643	1.2413	01:37.1	2.0413
StDev	0.0232	0.0845	0.0780	00:19.2	0.0780
%CV	0%	1%	6%	20%	4%

Where StDev – Standard deviation; %CV – coefficient of variation

Table AIV-3 Collection adequacy data for Quantisal® (*n* = 25)

#	Weight before collection (g)	Weight after collection (g)	Weight Difference (g)	Collection Time (min:sec)	Total Weight (incl. swab, g)
1	9.98	11.14	1.16	01:16.4	4.16
2	10.06	11.34	1.29	01:55.2	4.29
3	10.06	11.12	1.06	01:12.3	4.06
4	10.09	11.25	1.16	01:41.1	4.16
5	10.03	11.23	1.20	01:56.1	4.2
6	10.01	11.28	1.27	01:44.3	4.27
7	10.12	11.20	1.08	01:58.5	4.08
8	10.05	11.21	1.16	01:36.3	4.16
9	10.08	11.22	1.14	01:54.6	4.14
10	10.11	11.21	1.10	02:21.1	4.1
11	9.91	10.89	0.98	01:27.9	3.98
12	10.11	11.24	1.13	02:56.9	4.13
13	10.04	11.03	0.98	02:30.1	3.98
14	10.04	11.90	1.85	02:36.0	4.85
15	10.19	11.10	0.91	06:21.3	3.91
16	10.09	10.99	0.90	05:50.0	3.9
17	10.10	10.99	0.89	03:07.2	3.89
18	10.08	11.08	1.00	03:37.0	4
19	10.07	11.22	1.15	04:00.9	4.15
20	10.10	11.18	1.08	03:00.0	4.08
21	10.00	10.88	0.88	03:06.0	3.88
22	10.09	11.02	0.93	04:46.0	3.93
23	10.09	11.10	1.01	03:41.0	4.01
24	9.93	10.98	1.05	06:45.3	4.05
25	10.02	11.05	1.03	02:19.0	4.03
Mean	10.06	11.15	1.10	02:56.8	4.10
StDev	0.06	0.20	0.19	01:33.3	0.19
%CV	1%	2%	18%	53%	5%

Where StDev – Standard deviation; %CV – coefficient of variation

Appendix V: Drugs included in Selectivity/Specificity/Interference Studies

Drugs used to assess Selectivity and Specificity		
Caffeine	Oxazepam	Diltiazem
Benzylpiperazine (BZP)	Chlordiazepoxide	Cyclizine
PMMA	7-amino flunitrazepam	Chlorpheniramine
Mephedrone	Ketamine	Mirtazapine
Methamphetamine	Diphenhydramine	3-methoximethcathinone
Amphetamine	Lignocaine	Bupedrone
MDEA	Tramadol	3,4-dimethylmethcathinone
PMA	Methadone	Methylone
MDA	Procyclidine	Ethylone
MDMA	Amitriptyline	4-ethylmethcathinone
Aspirin	Promethazine	Methedrone
Paracetamol	Sertraline	Pentylone
Diazepam	Citalopram	4-methylethcathinone
Nordiazepam	Chlorpromazine	Butylone
Temazepam	Zolpidem	Pentedrone
Olanzapine	Midazolam	Fentanyl

PMMA=p-methoxy-N-methylamphetamine; MDEA=3,4-methylenedioxy-N-ethylamphetamine; PMA=p-methoxyamphetamine; MDA=3,4-methylenedioxyamphetamine; MDMA=3,4-methylenedioxy-methamphetamine

Drugs included in the optimised/developed methods were not included in the selectivity studies. All were at a concentration of 1 µg/mL.

Appendix X: Publications, Presentations, and Awards in Support of this Thesis

Publications

- Ann-Sophie Korb, Gail A.A. Cooper. "Endogenous Concentrations of GHB in Postmortem Blood from Deaths Unrelated to GHB Use". Journal of Analytical Toxicology – Special Issue, 2014; 38 (8): 582 – 588

Poster Presentations

- Ann-Sophie Korb, Gail Cooper. "Endogenous Concentrations of Gamma-Hydroxybutyrate (GHB) in Post-Mortem Blood from Deaths Unrelated to GHB Use". Presented at and in proceedings of the Annual Meeting of the Society of Forensic Toxicologists, *Grand Rapids, MI, USA* (October 2014)

- Ann-Sophie Korb, Fiona M. Wylie, Karen S. Scott, and Gail A. A. Cooper. "Short-term Stability of Gamma-hydroxybutyrate (GHB) in Oral Fluid". Presented at and in proceedings of the Annual Meeting of the Society of Forensic Toxicologists, *Atlanta, GA, USA* (October 2015)

Oral Presentations

- Ann-Sophie Korb. "What is GHB and GBL?" Presented at the GHB Workshop at the Annual Meeting of the Society of Forensic Toxicologists, *Atlanta, GA, USA* (October 2015)

- Ann-Sophie Korb, Karen S. Scott, Fiona M. Wylie. "Investigation into Applications of the NeoSAL™ Oral Fluid Collection Device for the Determination of Amphetamine and Metabolites". Presented at and in proceedings of the Annual Meeting of the Society of Forensic Toxicologists, *Dallas, TX, USA* (October 2016)

Award

- Leo Dal Cortivo / Young Forensic Toxicologist Award for the best Poster Presentation at Society of Forensic Toxicologists (SOFT) 2015, Annual Business Meeting, *Atlanta, GA, USA* (October 2015) for Ann-Sophie Korb, Fiona M. Wylie, Karen S. Scott, and Gail A. A. Cooper. "Short-term Stability of Gamma-hydroxybutyrate (GHB) in Oral Fluid".

classified as accidental (N=11), alcohol-related (N=237), drug-related (N=23), homicide (N=1), natural (N=91), suicide (N=9), medical related (N=1) and undetermined (N=14). Six cases had GHB concentrations in excess of 100 mg/L with advanced decomposition changes noted in five of these cases. Moderate to advanced decomposition was also noted in 50% (N=15) of the cases with GHB concentrations in excess of 50 mg/L but less than 100 mg/L. Approximately one third of the blood samples tested contained a preservative and although a higher proportion of these samples had GHB concentrations < 10 mg/L or not detected (~30% preserved v 11% unpreserved), there were still cases with GHB concentrations > 51 mg/L (~6% preserved v 11% unpreserved).

Conclusion: The findings in this study support other published investigations highlighting the difficulties and dangers of only using a cut-off to establish endogenous levels compared with exogenous use of GHB in post-mortem blood, especially when decomposition has reached advanced stages. The use of a preservative may be advantageous but more research must be conducted.

References

1. Kintz P, Villain M, Cirimele V, Ludes B. GHB in postmortem toxicology discrimination between endogenous production from exposure using multiple specimens. Forensic Science International. 2004;143(2-3):177-81.

2. Villain M, Cirimele V, Ludes B, Kintz P. Ultra-rapid procedure to test for gamma-hydroxybutyric acid in blood and urine by gas chromatography-mass spectrometry. Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences. 2003;792(1):83-7.

3. Hassan HMA, Cooper GAA. Determination of ß-Hydroxybutyrate in Blood and Urine Using Gas Chromatography—Mass Spectrometry. Journal of Analytical Toxicology. 2009;33(8):502-7

Short-term Stability of Gamma-hydroxybutyrate (GHB) in Oral Fluid.

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Background/Introduction: Gamma-hydroxybutyrate (GHB) is a short-chain fatty acid endogenous to mammalian tissues. As GHB has been predominantly studied in blood and urine, there is limited information regarding endogenous concentrations in oral fluid or the stability of GHB in this matrix. De Paoli *et al* found endogenous concentrations of GHB in oral fluid ranging from 0.15 – 3.33 mg/L (median 1.13 mg/L) (1). Oral fluid is no longer considered an alternative matrix, and is frequently collected when monitoring an individual's compliance with drug treatment programmes, in workplace drug testing programmes as well as in roadside testing. Collection of oral fluid is non-invasive, and can be carried out with ease without specialist collection facilities. Studies conducted in Germany and Sweden, showed drivers with elevated concentrations of GHB following arrest on suspicion of driving under the influence of drugs (DUID). Research also shows that following GHB ingestion, subjects would still drive. Evaluation of the short-term stability of GHB is important to better understand and interpret GHB concentrations measured in oral fluid.

Objective: The objectives of the study were to develop and validate a gas chromatography-mass spectrometry (GC/MS) method for the quantitative determination of GHB in oral fluid, and to evaluate the short-term stability of GHB in oral fluid. The short time frame was selected based on the expected turn-around time of routine working laboratories from time of collection, to the reporting of results.

Methods: Analysis for GHB was carried out using deuterated GHB as internal standard (IS; 100 µL at 10 mg/L), over a calibration range of 0.01 – 50 mg/L. The analytical method was adapted and validated for oral fluid from an in-house method optimised for the analysis of GHB in blood and urine.

Drug-free oral fluid was collected from a female volunteer and was frozen prior to the analysis. Oral fluid samples were spiked with GHB at 3 concentrations, 0.42, 4.2 and 42 mg/L. The IS was added prior to extraction. Acetonitrile was used to facilitate protein precipitation, following which the samples were centrifuged at 3000 rpm for 15 minutes. The acetonitrile was removed and evaporated to dryness at 40°C under nitrogen. Derivatisation with 75 µL of BSTFA + 1% TMCS was carried out prior to analysis using an Agilent 5975C/7890A GC/MS system with a DB5 column. The method was validated in accordance with SWGTOX guidelines. The stability of GHB in oral fluid was assessed at three different temperatures; room temperature (24°C), refrigeration (4°C) and frozen (-22°C). Samples were analysed in duplicate and injected in duplicate.

Results: The method was successfully validated according to recommendations set in the SWGTOX guidelines, including linearity, specificity, sensitivity, carryover, bias, precision and accuracy.

Investigation into Applications of the NeoSAL™ Oral Fluid Collection Device for the Determination of Amphetamine and Metabolites.

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Background/Introduction: Oral fluid (OF) is no longer considered an alternative matrix, and is often collected when monitoring compliance with drug treatment programmes, in workplace and roadside drug testing. New guidelines for OF testing are constantly emerging making it important to critically assess new collection devices.The newest collection device is the Neogen® NeoSAL™ device. It is a pad-based device that contains 2.1 mL of buffer, and is stated to collect 0.7 mL of OF. No data on drug recoveries and applicability of the device exists and this is what this study aimed to change.

Objective: The objective of the study was to evaluate the applicability and advantages of the new Neogen® device for the analysis of amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxy-N-ethyl-amphetamine (MDEA) in OF samples. A partial validation was performed according to SWGTOX guidelines, collection volume adequacy and drug recoveries assessed, in accordance with cut-offs recommended by the European Workplace Drug Testing Society (EWDTS). All QCs and samples were made up using 0.7 mL of OF.

Methods: The analysis for these analytes was carried out using the respective deuterated compounds as internal standard (IS; 35 ng/0.7 mL of OF), over a calibration range of 2–200 ng/0.7 mL of OF. An in-house gas chromatography-mass spectrometry (GC/MS) method of analysis for amphetamines in OF was used. A sample volume of approximately 2.8 mL (assuming 2.1 mL of buffer and 0.7 mL of OF) as per the manufacturer's specification was used. An SPE procedure was followed for sample preparation.

The drug recovery was assessed at two concentrations; 30 and 100 ng/0.7 mL of OF. Two methods were followed when assessing drug recoveries from the

NeoSAL™ device: the collection pad was dipped into spiked OF, and 0.7 mL of spiked OF was pipetted onto the pad. Each evaluation was assessed using pre- and post-extraction IS addition. Processed-sample stability was assessed at two QC concentrations (15 and 100 ng/0.7 mL of OF). Samples were extracted, and left on the autosampler over the 5-day testing period. Bias and precision were evaluated at QC concentrations of 15, 30 and 100 ng/0.7 mL of OF.

Results: Calibration graphs were linear, showing r2 values of 0.999 for all curves. The limit of detection (LOD) was determined to be 0.5 ng/mL (where S/N ≥3 for all ions); Although bias and precision were not studied at 0.75 ng/mL, S/N ≥10 for all ions (limit of quantitation (LOQ)). Carryover was not seen for concentrations up to 1000 ng/mL. None of the 40 drugs investigated showed interferences. Inter-day analytical accuracy (bias) and precision (n = 5) were 81–98%, and 3% for all three QC concentrations.The analysis of processed-samples showed analytes were stable for at least 72 hours on the autosampler (19 ±0.5 °C), showing drug concentrations 67–105% and 84–97% of Day 0 concentrations at the two QCs over the testing period. Drug recoveries ranged from 63–81% for 30 ng/0.7 mL, and 64–81% for 100 ng/0.7 mL, which were higher than Intercept® i2™ recoveries (range 44–80%) found in previous work. Gravimetric work (n = 25) carried out shows that the NeoSAL™ device collects an average of 0.84 mL (ranging from 0.61–1.1 mL, median 0.89 mL, %CV 15%) of OF compared to the stated 0.7 mL.

Conclusion/Discussion: Novel data was collected on the newest commercially available OF collection device. Although it is stated that the collection volume of the NeoSAL™ device is 0.7 mL, gravimetric work shows that OF can be collected in excess of the stated collection volume and drug recoveries for the amphetamine drugs from the device assessed were good.