Detecting and quantifying cannabinoids in oral fluid

by

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Certificate of authorship and originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

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DATE: 20/10/2015

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Publications and conference proceedings

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Other publications from related research activities conducted during candidature

J. Lewis, **A. Molnar**, S. Fu, D.J. Allsop, J. Copeland, 2015, 'Rapid elimination of carboxy-THC in a cohort of chronic cannabis users', International Journal of Legal Medicine, http://dx.doi.org/10.1007/s00414-015-1241-z.

D.J. Allsop, J. Copeland, M.M. Norberg, S. Fu, **A. Molnar**, J. Lewis, A.J. Budney, 2012, 'Quantifying the clinical significance of cannabis withdrawal', PLoS One, vol. 7, no. 9, p. e44864.

S. Fu, **A. Molnar**, P. Bowron, J. Lewis, H. Wang, 'Reduction of temazepam to diazepam and lorazepam to delorazepam during enzymatic hydrolysis', 2011, Analytical and Bioanalytical Chemistry, vol. 400, no. 1, pp. 153-164.

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- 48th Annual Meeting of the International Association of Forensic Toxicologists (TIAFT), Bonn, Germany.
- The 20th International Symposium on the Forensic Sciences (ANZFSS), Sydney, Australia.

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Abbreviations

11-OH-THC	11-hydroxy-delta-9-tetrahydrocannabinol
AS4760	Australian Standard 4760:2006
AS/NZS4308	Australian and New Zealand Standard 4308:2008
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
CBD	Cannabidiol
CBN	Cannabinol
CI	Chemical ionization
CID	Collision induced dissociation
CMC	Critical micelle concentration
CNS	Central nervous system
DDS	Drug Detection System
DRUID	Driving under the influence of drugs
DW	DrugWipe®
EIC	Extracted ion chromatogram
ESI	Electrospray ionisation
EtOAc	Ethyl acetate
FDA	United States Food and Drug Administration
FN	False negative
FP	False positive
GC	Gas chromatography
HPLC	High-performance liquid chromatography
LC	Liquid-chromatography
LLE	Liquid-liquid extraction
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
m/*z*	Mass-to-charge ratio
MDMA	3,4-methylenedioxy-*N*-methylamphetamine
MRE	Mean relative error
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
Na_2HPO_4	Di-sodium hydrogen orthophosphate
NaH_2PO_4	Sodium di-hydrogen orthophosphate
NaN_3	Sodium azide

NATA	National Association of Testing Authorities, Australia
NCI	Negative chemical ionization
NDSHS	National Drug Strategy Household Survey
NSW	New South Wales
PCDL	Personal Compound Database and Library
PCI	Positive chemical ionisation
PMME	Polymer monolith microextraction
POCT	Point of care test
PP	Polypropylene
QC	Quality control
QID	Four times a day
QQQ	Triple quadrupole
Q–TOF	Quadrupole time-of-flight
TOF	Time-of-flight
ROSITA	Roadside Testing Assessment
RSD	Relative standard deviation
SAMHSA	Substance Abuse and Mental Health Services Administration
SIM	Selected ion monitoring
SIR	Selected ion recording
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
THC	Delta-9-tetrahydrocannabinol
THCA-A	Delta-9-tetrahydrocannabinolic acid A
THC-COOH	11-nor-delta-9-tetrahydrocannabinol-9-carboxilic acid
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TN	True negative
TP	True positive
ULOD	Upper limit of detection
ULOQ	Upper limit of quantification
UNSW	University of New South Wales
WADA	World Anti-Doping Agency

Abstract

The main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), is the major target analyte for the detection of cannabis in oral fluid. While oral fluid has been used widely for drug testing purposes for a number of years, it has not been as thoroughly investigated as urine and blood testing procedures. This thesis aims to fill some of the gaps in knowledge regarding the detection of cannabinoids, particularly THC, in the oral fluid matrix.

THC is highly lipophilic and it is known that losses can occur when it comes in contact with plastic. Factors governing the interaction of THC with polypropylene in the oral fluid matrix were investigated using liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography–mass spectrometry (GC–MS) techniques. Preliminary results of the stability of THC in oral fluid stored in polypropylene containers indicated comparable THC losses under refrigerated and freezing conditions over a period of two weeks.

Delving further into the circumstances surrounding the absorptive tendencies of THC, no significant difference was found in terms of THC loss to plastic when the concentration ranged from 25–1000 ng/mL in the same volume of oral fluid. Varying the oral fluid volume (0.5–1.5 mL) while keeping THC at a constant concentration showed an upward trend with more loss associated with lower volumes. This indicated that THC adsorption is increased with greater plastic surface area to oral fluid volume. The use of Triton® X-100 significantly decreased the adherence of THC to the plastic tubes and increased the THC transfer (>96%) at all volumes tested. Addition of a surfactant to an accurately measured volume of oral fluid is a potential way to reduce the adsorption effect, while avoiding inconsistencies with oral fluid volumes generally found when using commercial collection devices. Degradation of THC during storage was also studied over a 4-week period and it was found that azide did not seem to play a significant role in preserving THC in oral fluid.

Sativex®, an oromucosal spray containing THC and cannabidiol (CBD), is indicated for the treatment of spasticity in multiple sclerosis in the United Kingdom (UK) and a number of other countries. The introduction of Sativex® to the Australian market may have implications for patients who drive since the THC may be detected by roadside drug testing procedures. Studies were carried out to determine whether or not patients taking Sativex® will test positive to THC using the DrugWipe® II Twin and Cozart® Drug Detection System (DDS) screening tests. Detectable levels of THC, CBD and cannabinol (CBN) in their oral fluid were also confirmed by LC–MS/MS. It was found that Sativex® users may test positive for THC by roadside drug testing within 2–3 h of use. Confirmatory analysis can identify Sativex® treatment through use of THC/CBD ratios, however, these ratios would unlikely be sufficient to differentiate non-medicinal cannabis use from Sativex® use if both are taken concurrently.

Analytical methods are continually evolving as more sensitive, more reliable and more user-friendly instrumentation and procedures are developed. The potential of using novel nanospray LC–chip–MS to detect and quantify cannabinoids in oral fluid was evaluated. The system was found to be unsuitable for routine analysis procedures; however it may have potential in other fields if used with a highly sensitive tandem MS.

The results presented in this thesis provide new insight into some of the difficulties faced with the detection and quantification of cannabinoids in oral fluid. The importance of determining the most appropriate collection and storage procedures for oral fluid specimens is highlighted, as is the interpretation of positive screening and confirmatory results when medicinal cannabis products are inevitably introduced.

Chapter 1:

Introduction

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1.1 Cannabis

1.1.1 Facts and figures

The *Cannabis sativa* plant (Cannabinacea family) contains psychoactive compounds and is mainly cultivated for recreational drug use (Figure 1-1). Marijuana is the most common term given to a mixture of cannabis leaves and flowering tops while hashish is typically the name given to the resin. Both marijuana and hashish are smoked as a rolled cigarette or through a water pipe or bong [1]. Smoking cannabis results in a nearly instantaneous effect on the nervous system. Hashish is also cooked with food and eaten, however this route takes longer to give an effect since absorption through the gastrointestinal tract is quite slow [2].

Figure 1-1: Cannabis plant and inset (top to bottom) a joint being rolled, cookies, and lighting a bong.

Cannabis is the most widely used illicit drug in Australia and around the world [3]. The World Drug Report published by the United Nations Office on Drugs and Crime

estimated that there were 125–227 million cannabis users around the world in 2012 [4]. According to the 2010 National Drug Strategy Household Survey, one in three people (35.4%) in Australia aged 14 and over, used cannabis at least once in their life and 10.3% had used in the previous twelve months [5]. This high prevalence of illegal cannabis use in the community has led to much interest and research into the detection of cannabis in bodily fluids.

1.1.2 Cannabinoids and metabolites

Cannabis contains over 100 cannabinoids that are derivatives of 2-(2-isopropyl-5-methylphenyl)-5-pentylresorcinol [6, 7]. These include CBN, CBD, Δ^8-tetrahydrocannabinol (Δ^8-THC), Δ^9-tetrahydrocannabinolic acid A (THCA-A) and the major psychoactive constituent, THC (Figure 1-2 and Figure 1-3). THC is present in cannabis in amounts usually 20 times that of Δ^8-THC. The potency of marijuana samples were found to have risen from 3.4% THC in 1993 to 11.9% THC in 2011 in the United States [4, 6].

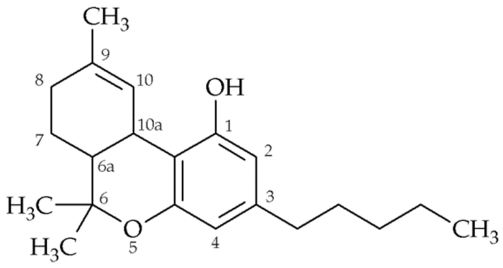

Figure 1-2: Chemical structure of THC (Δ^9-tetrahydrocannabinol) shown with dibenzofuran numbering.

THC affects the central nervous system (CNS) by slowing down the messages travelling between the brain and the body. This is a result of the binding of THC to cannabinoid CB_1 receptors and activating multiple pathways which induces the euphoric feeling associated with cannabis use [8]. THC is generally taken in small doses (a few milligrams) and effects last for 2 to 4 hours after use [8]. Cannabis has both hallucinogenic and depressant properties resulting in CNS effects such as a sense of relaxation, loss of inhibition, impaired coordination, reduced concentration, hallucinations, anxiety, reduced brain function and paranoia [1].

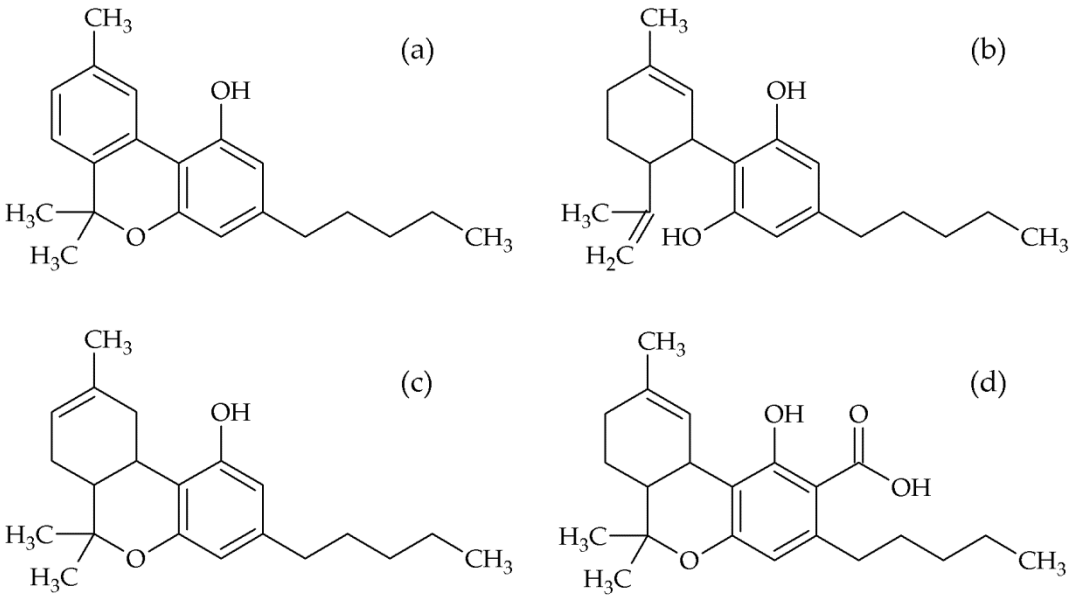

Figure 1-3: Other major constituents in cannabis, (a) CBN; (b) CBD; (c) Δ^8-THC; (d) THCA-A.

The other major cannabinoids, CBN and CBD are often present in large amounts; however, they have little psychoactive activity. THCA-A can also be found in cannabis in abundance and while this compound is inactive, it is converted into the active THC through smoking [2].

In the body, THC is primarily metabolised in the liver where the hepatic cytochrome P450 enzyme oxidises THC to the active metabolite 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC). This is then oxidised to the inactive 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and the conjugated THC-COOH-glucuronide (Figure 1-4) [2, 9]. A number of other minor metabolites are also formed, including the active 8β-hydroxy-Δ^9-THC and inactive 8α-hydroxy-Δ^9-THC and 8α,11-dihydroxy-Δ^9-THC [2]. THC-COOH and 11-OH-THC are most important for biological sample testing procedures.

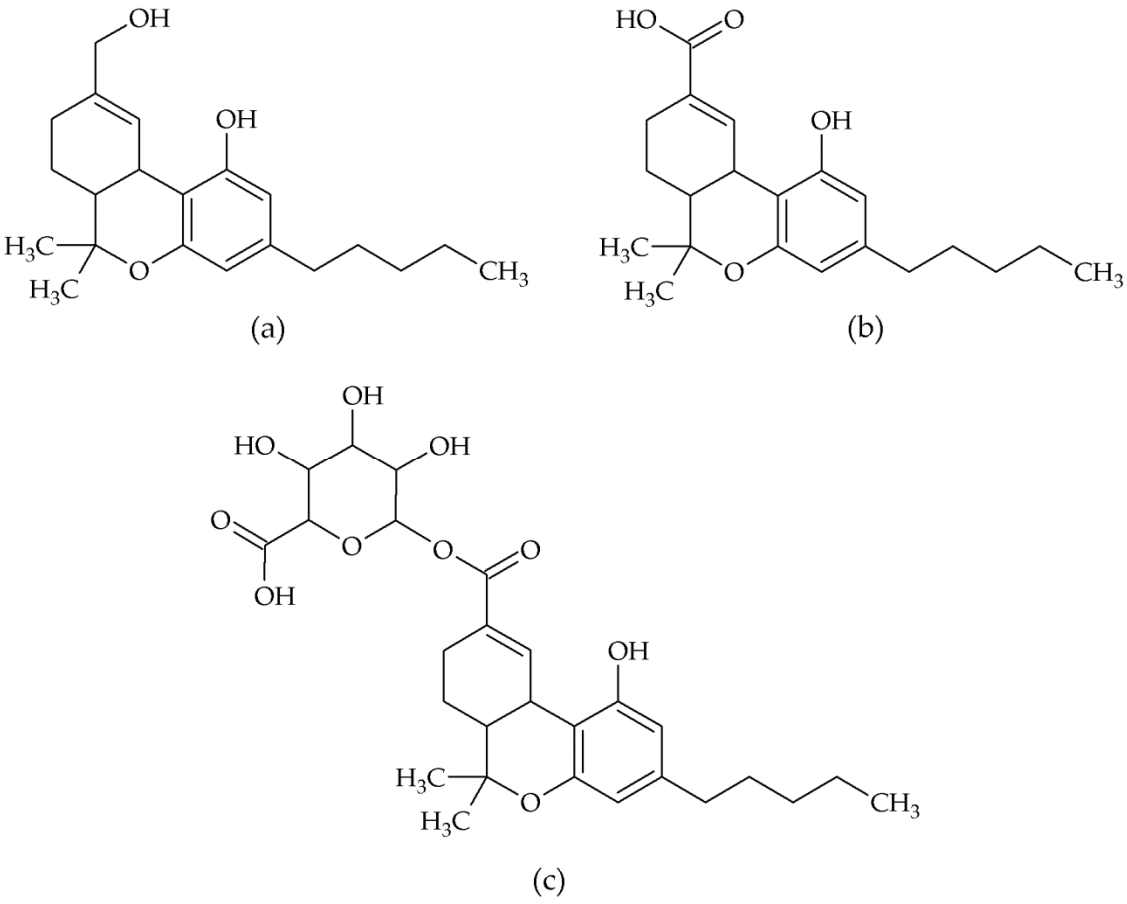

Figure 1-4: Structures of the major THC metabolites; (a) 11-OH-THC; (b) THC-COOH; and (c) THC-COOH-glucuronide.

1.1.3 Motivations for detection

In addition to testing in post-mortem cases, cannabis testing is already commonly performed in the workplace and on the roadside in jurisdictions around the globe. This section outlines these and some of the other reasons that organisations implement drug testing to monitor and deter cannabinoid use, e.g. for doping control and patient care.

It is important to control the instances of driving whilst under the influence of cannabis since the effects of the drug greatly decrease the user's motor functions and ability to react to hazardous situations on the road. There is much research data available that shows recent cannabis use can cause similar impairment to blood alcohol concentrations above the legal limit of 0.05% [10]. In New South Wales (NSW) it is illegal to drive while any illicit drug including THC, is present in an individuals'

oral fluid or blood (Road Transport (Safety and Traffic Management) Act 1999 No 20). Persons caught committing this offence can be fined and have their licence suspended for a period of time in NSW [11]. Thus, appropriate and accurate methods to determine if someone is driving with a prohibited drug in their system must be available to police this. These laws are not limited to NSW; as all Australian states and territories have now employed similar roadside drug testing programs. Roadside testing plays a role in deterring people from driving under the influence of drugs as has been proven with the implementation of mobile random breath testing (RBT) units for reducing drink-driving incidents.

Safety in the workplace is a priority and many companies have implemented drug testing programs in order to deter drug use and maintain a safe environment for employees while complying with the relevant occupational health and safety legislation. Drug use is of particular concern in industries such as aviation, trucking, mining and construction, where concentration and hand-eye coordination skills for operating heavy machinery are vital. Some companies also have pre-employment drug screening programs in place [10].

Doping agents are commonly steroidal-type drugs that effectively enhance physical performance; however the World Anti-Doping Agency (WADA) has encountered a high prevalence of samples from athletes containing cannabinoids. It appeared that some of the effects of cannabis such as reduced pain and anxiety have been beneficial to the performance of some athletes. In 2004 WADA prohibited the use of cannabinoids in-competition [12].

Cannabis is known to be an effective pain reliever and as a result, a number of medicinal cannabis products have been developed in recent years. Cannabis itself is also used illegally for treatment of chronic pain, nausea and vomiting in, for example, cancer patients. Pharmaceutical companies have developed medicines containing cannabinoids that can be offered by prescription in countries where they have been approved. Marinol® (dronabinol) contains synthetic THC and is used in the United States to treat loss of appetite associated with weight loss in patients with acquired

immune deficiency syndrome (AIDS) [13]. Nabiximols is a standardised cannabis extract containing primarily THC and CBD [14]. Sativex® is an example of a nabiximols and has been approved in a number of countries for treatment of spasticity in multiple sclerosis patients [15]. Close monitoring of patients using medicinal cannabis is required to avoid potential dependence; and patients using illegal cannabis may be at risk of adverse interactions with any prescribed drugs they may also be taking. Monitoring use by analysing bodily fluids can be useful in these situations. It has also recently been accepted that cannabis dependence does occur and now programs are in place to help addicts through withdrawal [16, 17]. Drug testing can therefore be utilised in withdrawal programs to help monitor abstinence.

1.2 Cannabis detection in biological matrices

Most commonly, cannabis testing is performed using urine, whole blood or plasma samples, and now oral fluid is becoming a popular choice. Some matrices relevant to drug testing are briefly outlined in the following sections.

1.2.1 Urine

Routine urine testing involves the detection of cannabis metabolites, THC-COOH and THC-COOH-glucuronide. The other major metabolite, 11-OH-THC, is also of interest in research studies [18]. Due to its relatively large window of detection, urine testing is useful for determining if a person has used drugs within the last few days. Additionally, urine testing can detect the metabolites of THC regardless of the route of consumption. Urine has been thoroughly researched as a matrix for drug testing and testing procedures are well-established. However, urine testing has some significant disadvantages, such as its ease of adulteration either through dilution or substitution, particularly due to privacy issues with collection. The large and varied window of detection observed can also pose problems when interpreting results, as the metabolites of THC may be found in the urine up to 12 days after a single oral

dose [2] and even longer if the subject is a regular user. Although drug testing of any kind cannot itself confirm that an individual is impaired, urine testing is widely accepted as a means of identifying the risk of impairment. However, due to the poor correlation between concentration in urine and level of impairment, there is much debate as to whether urine testing should be used for workplace and roadside programs, since it does not give an indication of recent use which many believe should be the focus of such testing.

1.2.2 Plasma

Plasma contains more lipophilic and proteinaceous compounds that make it a more complex matrix compared to urine [19]. Sensitivity of methods must be higher for plasma as THC and its metabolites are present in smaller concentrations. THC has a plasma half-life of about 2 hours; after which it is converted into its metabolites. Therefore the detection of THC rather than the carboxy metabolite in plasma, is a good way to detect recent use [20]. It is more difficult to extract THC from whole blood, but this is sometimes done when plasma samples are not readily available. Plasma and whole blood are most useful for post-accident and post mortem analyses, as these samples are typically collected in hospitals and morgues where specialised personnel and facilities are already available. Due to the requirements for the collection of blood and the invasiveness of its sampling, this matrix is not ideal for on-site testing. There is also some evidence that fat-stored THC can be redistributed into the blood of chronic users following extensive exercise, and this may have implications for the interpretation of any on-site test results [21].

1.2.3 Hair

There has been much research into testing hair for drugs, showing its capability of detecting THC among many other substances. Hair is primarily analysed to detect past use. This matrix is most useful for approximating the time and duration of past events as the growth rate of the hair and distance of the hair section from the scalp can provide this type of information. However, an issue described with hair analysis

is the difficulty in distinguishing between the drug and metabolites that are within the hair and external contamination [22]. Hair does not have any real application for roadside testing unless simple exposure to cannabis smoke is of interest and the external THC collected through washing steps is utilised.

1.2.4 Sweat

Drugs of abuse are also found in human sweat. The exact mechanism by which the drugs are excreted into sweat is unknown, but one hypothesis is that the parent drugs diffuse passively into sweat glands from the blood [23]. A common detection method involves sweat patches that are placed on the body for a number of hours or even days to collect excreted sweat which can then be analysed for the presence of drugs [23]. This may be useful for patient care and monitoring, though it is unsuitable to sample in roadside testing, due in part to the time frame required for the process. Quick screening tests have also been developed, for example the DrugWipe® "F" is an immunoassay screening test that can detect a number of illicit drugs including THC after being wiped across the forehead a few times [24]. While this indicates that sweat is probably the least invasive matrix to collect, it is prone to issues including passive exposure since the parent compound is being detected. Furthermore, although presence of drugs in sweat can indicate recent use, there is still some delay for the drug to be excreted [25]. When compared to oral fluid where the physical deposition of cannabinoids occurs immediately after smoking, sweat does not appear to be the most appropriate matrix when impairment or recent use is the issue.

1.2.5 Oral fluid

Healthy adults produce approximately 500–1500 mL of oral fluid daily, though the rate varies throughout the day according to the circadian rhythm and a multitude of other factors [26]. Fluids are secreted into the oral cavity primarily by the parotid, submaxillary and sublingual glands [26, 27]. A number of minor glands also contribute to the secretions. Oral fluid composition varies between individuals and at different times for the same individual, but consists mostly of water (>97%) with

proteins (enzymes, immunoglobulins) such as lysozyme, lactoferrin, alpha-amylase, lipase and proteinase; and electrolytes such as sodium, potassium, chloride and bicarbonate [10, 26]. These components are important for the protection and lubrication of oral tissues, cleaning and beginning the digestion process [28]. Bacteria, epithelial cells, blood cells and food debris are also present in oral fluid [28].

Oral fluid testing is advantageous for determining recent use since the target analyte is the parent THC compound, which is deposited in the oral cavity directly from smoking. After these deposits have been depleted, the THC content in the mouth may even correlate to plasma levels in the blood. Huestis and Cone [29] found that after the initial deposits from smoking had been depleted, the concentration of THC in oral fluid was well correlated to plasma concentrations until 4 hours after use. Kauert *et al.* [30] found that the similar elimination rates of THC from oral fluid and plasma are merely coincidental. Nevertheless, detecting THC in oral fluid is indicative of recent use since most of the THC detected will be from the primary deposit following smoking, which is typically lost within a few hours. Additionally, a study by Menkes *et al.* revealed there is a good correlation between oral fluid THC concentrations and observed symptoms of intoxication [31]. Toennes *et al.* [32] and Laloup *et al.* [33] also obtained data indicating that the detection of THC in oral fluid is a good predictor for THC also being found in plasma and therefore the subject being under the influence of cannabis.

Detecting THC in oral fluid following the ingestion of cannabis is more challenging. Niedbala *et al.* [34] found mean peak concentrations of 23.3 and 25.3 ng/mL of THC in oral fluid collected from the left and right sides of the mouth, respectively, after subjects smoked a single cigarette containing 20–25 mg of THC. Comparatively, the mean peak concentrations detected following the ingestion of brownies containing the same amount of THC was only 3.4 and 4.8 ng/mL in oral fluid collected from the left and right sides of the mouth, respectively. Additionally, Milman *et al.* [35] noted that orally administered capsules of Marinol® (synthetic THC) are unlikely to result in detectable concentrations of THC appearing in the oral fluid. It seems that, unlike

urine testing which targets the metabolites of THC, oral fluid testing is likely to fail if cannabis has been orally ingested.

Passive smoking has been investigated with respect to possible external contamination. Niedbala *et al.* [36] initially observed that the oral fluid of a passive smoker could contain up to a 26 ng/mL concentration of THC in the first 30 minutes after others had been smoking in an enclosed space. However, the same authors performed a subsequent study in 2005 [37] involving a vehicle and found that while some samples from the passive subjects were THC positive when sampled inside the vehicle, all of the samples taken from the subjects while outside the vehicle were negative. This indicated environmental contamination had been taking place and likely affected their previously published results. Niedbala *et al.* then concluded that passive smoking is not an issue with oral fluid collection. Conversely, Moore *et al.* [38] published a study in 2011 indicating that passive exposure did in fact lead to detectable levels of THC in oral fluid. Subjects were exposed to cannabis smoke inside a coffee shop, but as sampling was done outside the shop, ambient contamination was unlikely. The major metabolite, THC-COOH has been detected in very low concentrations in the oral fluid of cannabis smokers [39-41]. Moore *et al.* monitored the metabolite and noted that the passive smokers did not have any THC-COOH in their oral fluid and concluded that the presence of the metabolite can be used to avoid false positive results due to passive smoking [38].

Advantages of using oral fluid over other matrices for roadside testing has been outlined numerous times [31, 42-49], and in addition to its short window of detection, its collection is non-invasive and there is reduced risk of adulteration [44, 48, 50]. Privacy is not an issue with collection [43, 45, 47, 51] so sample manipulation is less likely and collection does not require any medical training or special facilities, making it a convenient sample to collect [52]. There is however, a significant issue with oral fluid collection for drugs of abuse testing since both THC and amphetamines are known to cause 'dry mouth' and due to this or other reasons, an adequate volume of oral fluid for analysis is not always collected [34, 53]. Many studies have investigated the performance of available oral fluid collection devices, which frequently fail to

collect an adequate volume of oral fluid, and many have significant recovery issues with respect to THC [43, 44]. Direct expectoration (spitting) can also result in a large amount of foam being collected and not enough actual fluid, which is exacerbated when 'dry mouth' is already a problem. It is therefore necessary to devise confirmatory analysis techniques that use a minimal volume of oral fluid, especially since some of the fluid collected initially, is used for screening tests and the remaining volume sent to the confirmatory testing laboratory may be small. As many people are uncomfortable spitting into a tube, collection devices utilising a swab and buffer, are more commonly used. Due to the wide variety of oral fluid collection methods and devices in use in the field, an ideal confirmatory analysis method should be suitable for both neat oral fluid samples and samples diluted with commercial buffers.

The same advantages of oral fluid testing may be applied to workplace testing and anti-doping programs since both are concerned with current impairment rather than past use. Point of care test (POCT) devices are preferred in workplace settings in order to immediately remove impaired employees from the workplace, at least until the effects of the drug wear off. However, these devices are not currently well-regulated and there is a higher probability of false positive and false negative results when compared with the well-established urine tests. Testing for anti-doping regulations is generally accomplished using urine or blood, but since only in-competition use is of interest, oral fluid may be used in the future for this purpose also.

1.3 Oral fluid testing procedures

Screening tests for cannabis and other drugs of abuse are generally immunoassay-type tests. Collection devices are sometimes used in conjunction with screening tests to obtain oral fluid samples for further confirmatory tests if the screening test returns a positive result. Many screening tests also incorporate a collection vessel to hold extra oral fluid in case subsequent confirmatory testing is required.

Screening test devices are developed for POCT and are evaluated based on their sensitivity and specificity. Early screening tests for THC were found to be acceptable in terms of specificity; however, sensitivity was unsatisfactory. Collection devices are evaluated based mainly on recovery. This is particularly important when considering the detection limits needed for confirmatory testing. Alternative confirmatory testing methods have also been investigated to replace the traditional GC–MS methods, such as LC–MS and tandem MS methods.

1.3.1 Screening tests

In the past few years, a number of screening test devices have been released and evaluated. Many studies were involved in the 2005 Roadside Testing Assessment 2 (ROSITA-2) project and in the more recent Driving Under the Influence of Drugs (DRUID) project in 2010 which evaluated different commercial devices used for oral fluid drug screening [54-58].

1.3.1.1 On–site drug screening devices

There are a number of on-site screening devices currently available in Australia that test for multiple drugs of abuse in oral fluid. Tests target drugs and drug classes including cannabis, cocaine, opiates, amphetamines, methamphetamine, 3,4-methylenedioxy-*N*-methylamphetamine (MDMA), benzodiazepines, phencyclidine and methadone [55, 59]. The sensitivity of on-site devices varies greatly between devices and also between different drug classes on the same device.

Screening devices are evaluated by calculating sensitivity, specificity and accuracy of the results for each drug class tested (Table 1-1). These parameters are calculated using the number of true positive (TP), false positive (FP), true negative (TN) and false negative (FN) results. True positive and true negative results are those screening results that are found to be true by confirmatory analysis. Conversely, false positive and false negative results are those where the confirmatory analysis disagree with the screening result. Sensitivity gives a percentage of positive samples successfully

screened out of the total number of confirmed positive results from laboratory analysis. Specificity indicates how well the device is able to distinguish the target compounds from others, and it is calculated using the false positive responses given by the device. Accuracy shows the overall percentage of correct screening results when confirmed by laboratory analysis. These three parameters are important for evaluating the performance of any on-site screening device. Prevalence is also useful as it gives an indication of how often a drug is present in any given study so results can be interpreted accordingly.

Table 1-1: Equations used to calculate statistical parameters for evaluation of device performance [60].

Parameter	Equation
Sensitivity	$\frac{TP}{TP + FN}$
Specificity	$\frac{TN}{TN + FP}$
Accuracy	$\frac{TP + TN}{TP + TN + FP + FN}$
Prevalence	$\frac{TP}{TP + TN + FP + FN}$

As there is no standardisation of screening tests it is up to the manufacturer which drug classes to test for, the specific compounds to target for each class, and the cut-off concentration levels to aim for. Most devices have a limited ability to detect cannabis reliably at any concentration below 100 ng/mL as there are often large overlaps observed between the ranges of concentrations found in true positive and false negative screening results. Table 1-2 summarises the cut-offs claimed by the manufacturers for the detection of cannabis in oral fluid.

Parent compound THC is the target analyte of the cannabis tests listed in Table 1-2, however the inactive metabolite, THC-COOH, has a high cross-reactivity with THC in the majority of devices. Regardless, the metabolite is normally present in oral fluid in

only picogram per millilitre concentrations which is unlikely to be detected by current on-site devices [61].

A major issue with on-site testing is the subjective interpretation of most of the available test devices. All are immunoassay based, and work by utilising stationary antibodies that bind to drug analytes to form visible lines on a test strip. Those tests without a digital reader must be read by the tester, and many false positive and false negative results are likely caused by misinterpretation of the appearance of the test strips. This may be due to the test itself, with reports of very faint lines that sometimes correspond to positive results. Also, the time frame in which the test result is read is important and reading the device outside of this time frame may lead to false results. Errors may also be due to inexperienced users as shown by Pehrsson *et al.* [52] where in roadside testing, there was a higher prevalence of failed tests (control lines failed) amongst testers who had performed 10 or less tests.

Of the devices listed in Table 1-2, four have the option of a digital reader that gives a simple positive or negative result, and which removes the subjectivity of reading the test result visually. These are the Cozart® DDS, the Dräger DrugTest® 5000, Rapid STAT® and OralScreen™ 4 with OSR reader. Of these four systems, the Cozart® DDS and Rapid STAT® both utilise a buffer solution into which the oral fluid is mixed, before analysis. All other devices use a swab to collect oral fluid, which is then used directly in the test. The use of buffer is problematic, not so much for the screening process, but for the subsequent confirmation analysis if the same samples are used. This is due to the uncertainty in the volume of oral fluid collected, and/or uncertainty in the volume of buffer included in the vials. This uncertainty makes it impossible to determine an exact concentration of any drug found and may be the difference between a result being above the cut-off value or below it. For screening purposes, the introduction of a buffer complicates the test as the device has to be much more sensitive in order to be able to detect drugs in diluted samples and to correspond with the cut-off value for the undiluted oral fluid. Conversely, screening tests and collection devices that do not use a dilution buffer may face issues of drug instability and poor recovery, especially for THC.

Table 1-2: Recently available on-site oral fluid test systems and their concentration cut-off levels for THC as stated by the manufacturer or distributer in Australia.

Name of device	Manufacturer	Manufacturer cut-off concentration for THC (ng/mL)	Ref.
DrugWipe® 5+	Securetec	30	[62]
Cozart® DDS	Concateno	31^	[63]
Dräger DrugTest® 5000	Dräger Safety	5	[64]
On-site® OraLab® 6	Varian	50	[65]
OrAlert®	Innovacon	100	[55]
OraTect® III	Branan Medical	40	[66]
Rapid STAT®	MAVAND	15	[62]
Oral7™	Oranoxis	25	[67]
First Sign™	AustraliaDrugTesting .com ‡	75	[68]
ToxSure®	CMM Technology™ ‡	25	[69]
OraLine®	CMM Technology™ ‡	25	[70]
On-site Oral 5	Onsite Diagnostics	25	[71]
Oral Screen™ OSR	Avitar	50	[72]
SalivaScan™	Express Diagnostics International	50	[73]

^*Approximation only as real subject samples were used. A THC concentration of 31 ng/mL was the lowest concentration detected.*
‡*Distributer in Australia. Manufacturer not known.*

1.3.1.2 Evaluations of on-site screening devices

In the last ten years, two large-scale projects have been undertaken to evaluate on-site screening devices. The ROSITA-2 project evaluated six on-site devices in six European

countries and five states in the United States between 2003 and 2005. They found a slight improvement in the general performance of on-site devices since the previous ROSITA study (1999); however cannabis detection was still a major issue [74]. As technologies have rapidly evolved, the results from the ROSITA-2 study are now obsolete as the devices evaluated are either no longer available or have since been updated several times. The more recent and relevant DRUID program completed in 2010, also evaluated a number of devices in three European countries [55]. Many of these devices have also been updated since this program completed, however the results still give a good indication of the current status of on-site devices.

Unfortunately, not all of the available devices have been evaluated in this way. The devices that have been evaluated in peer-reviewed journals within the last few years include the Cozart® DDS, Dräger DrugTest® 5000, Rapid STAT®, Drugwipe® 5+, BIOSENS® Dynamic, OraLab® 6, OrAlert® and OraTect® III [56, 75-77]. All of these studies were conducted outside Australia, and most have calculated sensitivity based on internationally agreed cut-off levels, for example, 1 ng/mL for THC in the DRUID evaluations conducted in Europe [55] (or 2 ng/mL for THC in the ROSITA-2 project [78]). These levels are much lower than the 25 ng/mL target concentration for screening devices suggested by the Australian Standard (AS) 4760:2006 [59] and therefore many of the reported sensitivities are much lower than they would be according to the Australian Standard and so are not currently relevant for Australia. Some publications have related sensitivity to the manufacturer's stated cut-off levels for the device and these results are much more helpful when trying to determine the reliability of the claims made by the manufacturers.

Manufacturer's claims for their products may be true within the confines of internal testing with spiked samples (which are sometimes performed in artificial oral fluid); however in the context of testing real subjects, they appear to be far from accurate. As demonstrated by the studies in the DRUID project, cut-offs for many devices are difficult to determine due to the overlapping of the concentration ranges that are observed with true positive and false negative results given by the same testing device in the same study, particularly with THC.

In New South Wales roadside drug testing, police first use a 'through-the-window' screening test (DrugWipe®). This takes only a couple of minutes, and if it returns a positive result for cannabis, a second screening test (Cozart® RapiScan) is performed in a specialised van (if available). If this subsequent test also returns a positive result, the remaining oral fluid collected is sent to a laboratory for confirmatory testing [79]. The DrugWipe® and Cozart® DDS will be analysed in more detail in the following sections since these were used for a study that forms part of this thesis.

1.3.1.3 DrugWipe®

The DrugWipe® II Twin (Securetec Detektions-Systeme AG) is currently used by some Australian state police forces in random roadside drug testing programs. The DrugWipe® comes in other forms including single drug tests and multiple drug tests, but all work via the same mechanism. The DrugWipe® 5+ tests for multiple drug classes: THC, cocaine, opiates, amphetamines, methamphetamine, and MDMA. The cut-off value as specified by Securetec for the detection of THC, is 30 ng/mL [62].

The DrugWipe® 5+ is an all-in-one test that includes a swab and the test device (Figure 1-5). The swab is simply wiped around the tongue and cheek and then reattached to the device. An ampoule inside the body of the device contains a buffer solution and must be broken to allow the oral fluid to mix and reach the test strips. Results can be read within 6 minutes, once the control lines have appeared on both strips.

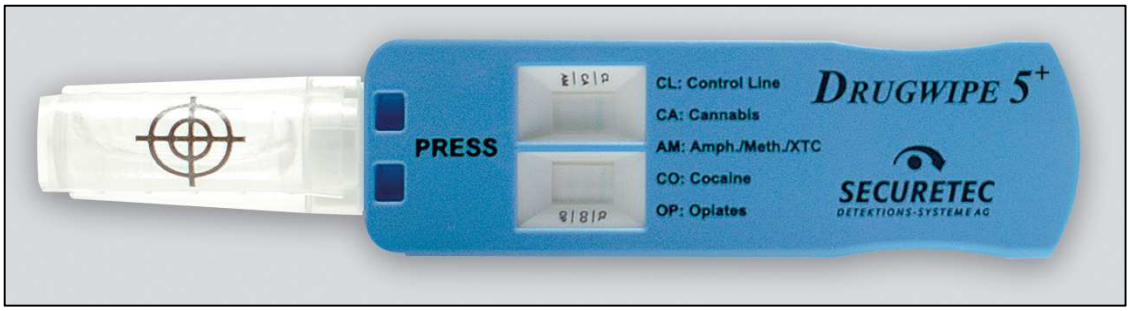

Figure 1-5: Image of the DrugWipe® 5+ all-in-one test cartridge for the detection of five drug classes including cannabis.

The DrugWipe® 5+ was evaluated in Finland as part of the DRUID project. According to this study, the test performed well for amphetamines with a sensitivity of 87% at the device's cut-off level of 50 ng/mL, which is the same as the target concentration suggested by AS4760. The sensitivity found for THC was only 63% using 30 ng/mL as the cut-off, however only 134 tests were conducted and the prevalence of cannabis was quite low (6.0%) and so the results may not be too meaningful [62].

In a larger scale study that evaluated the DrugWipe® 5+ among other on-site test devices, Strano-Rossi *et al.* [75] found the sensitivity of the test to be approximately 55% for THC, again quite poor. Wille *et al.* and more recently, Musshoff *et al.*, found the sensitivity for THC to be 71% when calculated based on confirmation using serum samples, indicating some improvement for the device [76, 77]. However, specificity was found to be quite low at respectively, 50% [76] and 29% [77].

Other earlier studies have noted a difficulty in the interpretation of the DrugWipe® device, particularly with reading the result for THC, since the control line was often faint. This was hypothesised to have resulted in a number of false positives where faint lines were anticipated by the testers [52]. It is unclear whether this is still an issue with the current devices, though Musshoff's results indicate that this may be the case.

1.3.1.4 Cozart® DDS

The Cozart® Drug Detection System or DDS (Concateno UK, formerly Cozart® Biosciences) is also used by police for roadside drug testing in some Australian states to test for cannabis and amphetamines including MDMA. The Cozart® DDS is one of only a few devices that require the oral fluid samples be added to a buffer solution before the screening procedure. This dilutes the oral fluid sample and therefore the sensitivity of the test must be much higher to detect any present drugs reliably. The cut-off level of 31 ng/mL for THC corresponds to the concentration of THC in the original undiluted oral fluid (see Appendix A). This cut-off is an approximation only, since real oral fluid samples were used. A confirmed THC concentration of 31 ng/mL was the lowest concentration successfully detected.

To conduct the test, the swab is moved around the tongue and cheeks of the subject until the indicator in the collector turns blue to indicate enough oral fluid has been collected (Appendix C). The swab is then inserted into a small bottle containing a buffer solution. The oral fluid is diluted in a 1:3 ratio of oral fluid: buffer. The bottle must be shaken for 30 seconds to allow the analytes to elute from the swab. Four to five drops of the buffer/oral fluid solution are then dropped onto a test cartridge, which is then inserted into a digital reader (Figure 1-6). The test can take between 90 seconds and 5 minutes to give a result depending on how many drug classes the instrument is programmed to test for [56].

The Cozart® DDS was evaluated in Belgium as part of the DRUID project. A total of 138 tests were performed. The test for cannabis only achieved 39% sensitivity relative to its 31 ng/mL cut-off [55]. All true positives were in high concentrations above 100 ng/mL. A large range of THC concentrations were found in the false negative samples, indicating the cut-off for the device might be much higher than estimated by the manufacturer.

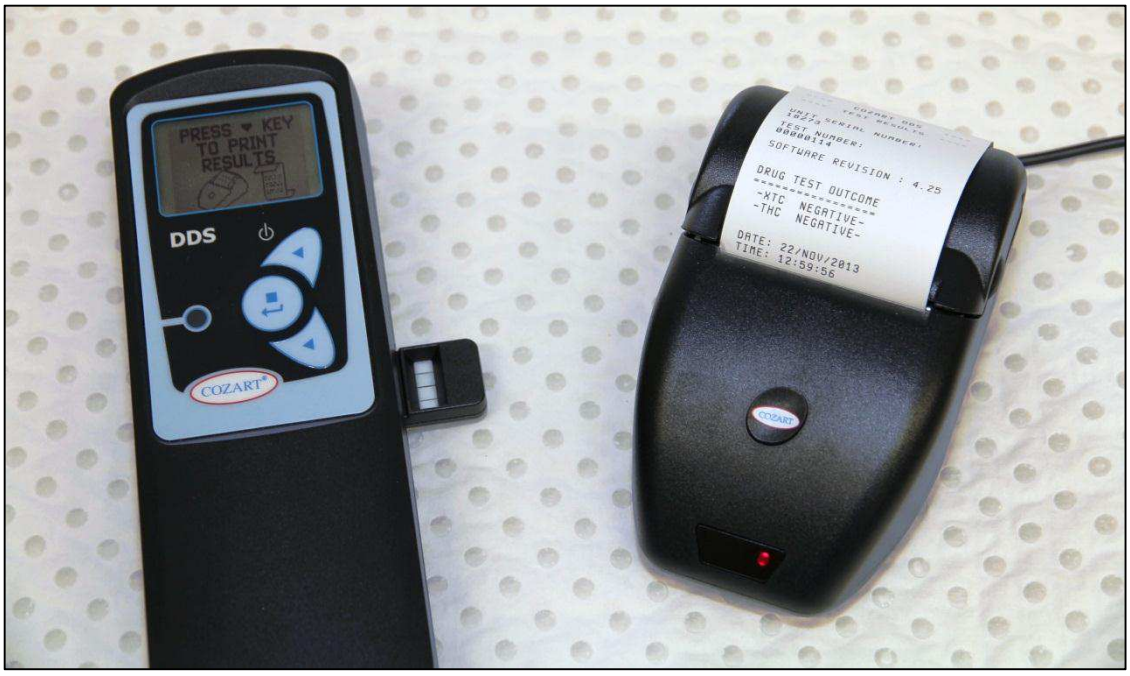

Figure 1-6: The Cozart® DDS digital reader with test cartridge inserted (left) and printer (right).

Strano-Rossi *et al.* [75] also evaluated the DDS. The sensitivity for THC was found to be better than that observed in the DRUID project; however, it was still quite low at a little over 60%.

Kintz *et al.* [80] evaluated the Cozart® DDSV device for the screening of oral fluid for THC. This device samples oral fluid in the same way as the DDS, however there is no digital reader and the cartridge is read visually with the appearance of the control lines. The manufacturer's specified cut-off for THC is 15 ng/mL in the oral fluid and buffer mix. Since the oral fluid is diluted in the buffer in a 1:3 ratio, this corresponds to 60 ng/mL in undiluted oral fluid. In this study a total of 25 subjects were tested. Of the 18 confirmed positive samples (by GC–MS), the samples that screened positive by the DDSV contained THC in the concentration range of 15.2–202.5 ng/mL. The samples that screened negative were found to have concentrations in the range of 0.7–14.0 ng/mL. According to these results, the DDSV had 100% sensitivity relative to its stated cut-off level. These are promising results; however the cut-off level is much higher than the AS4760 suggested target concentration. The dilution factor is also quite unreliable, as according to Kintz *et al.*, the manufacturer stated that oral fluid can be collected in the range of 340 μL ±60 μL [80]. This makes it impossible to determine the exact concentration of THC in the original oral fluid since the dilution factor is not accurately known.

Since these studies were reported, a new model of DDS with digital reader has been released under the new name, Alere® DDS2.

1.3.1.5 Australian Standard AS4760:2006

Australia was the first country to establish a standard for the testing of drugs in oral fluid for forensic purposes. The Australian Standard AS4760 released in 2006, outlines the recommended procedures for the collection and testing of oral fluid samples for THC, amphetamine-type stimulants (ATS), opiates and cocaine and its metabolites [59]. The standard suggests 'target' concentrations that on-site screening tests should adhere to wherever possible, instead of set 'cut-offs'. This was beneficial at the time as

none of the available tests were sensitive and reliable enough to warrant specific cut-off values, especially for THC. As a result, companies have widely varying 'cut-offs' for their devices.

Due to the inherent difficulties in the reliability of detecting drugs in oral fluid, the target concentrations suggested by AS4760 are much higher than the screening cut-offs proposed by various bodies such as the Substance Abuse and Mental Health Services Administration (SAMHSA) in the US (4 ng/mL for workplace testing [81]) and the ROSITA-2 and DRUID programs (2 ng/mL and 1 ng/mL respectively [55, 78]). However, if devices cannot meet the specified target concentration for a drug, it is acceptable under the Standard to use a different 'nominated' value, providing that the use of that value can be justified.

Drug screening in urine is well-established, which allows the Australian and New Zealand Standard for drug testing in urine (AS/NZS4308) to be much more definitive in its approach. On-site urine screening devices must be verified as specified by the Standard by an independent laboratory. Positive control samples must be spiked at 25% above the listed cut-off and negative control samples must be spiked at 30% below the cut-off [82]. There is no such requirement outlined in AS4760; however, the National Association of Testing Authorities (NATA) will require users of on-site oral fluid devices to have them verified in a similar manner. This is not so straight forward since AS4760 allows manufacturers to use nominated concentrations. Any nominated concentration between the confirmatory target concentration (10 ng/mL for THC) and 50% above the screening target concentration (25 ng/mL for THC) can be used as the positive control; therefore any concentration between 10 and 37.5 ng/mL can be used for THC. Additionally, negative controls are defined as drug-free specimens by the standard rather than being spiked at some concentration below the target. Therefore, even if a device can detect THC at less than 10 ng/mL, it would be deemed compliant to the standard if a drug-free negative control was used. Currently, there are no NATA accredited on-site testing devices. As of June 2015, there are ten laboratories in Australia accredited for confirmatory testing [83].

As it has been eight years since the release of AS4760:2006, a revision of the Standard is planned, which will hopefully provide clearer guidance and remove some of the ambiguities surrounding screening test performance requirements in Australia.

1.3.2 Collection devices and stability

When confirmation of a presumptive positive screening test is required, a sample of oral fluid is collected and stored until analysis. Oral fluid is commonly collected by either expectoration or through use of a specialised collection device. Expectoration is more cost effective and allows for increased sensitivity since the sample is undiluted. However, this method faces a number of issues including decreased stability of drugs during storage, possible adsorption to the containers and the sample being viscous and containing various types of debris from the mouth [10]. Additionally, reduced salivary flow can hinder collection and cause the oral fluid that is collected to contain more froth and less liquid [35]. This 'dry mouth' condition often affects those who have recently smoked cannabis. Commercial collection devices have been developed to overcome many of these issues.

Numerous products are currently available, such as the SalivaSampler™, Oral-Eze®, Salivette™, Quantisal™, Cozart® DDS and Intercept® devices. These devices make the process of collecting oral fluid easier, more hygienic and less embarrassing for the subject than expectoration. Many devices utilise a buffer that dilutes the oral fluid and reduces viscosity. This is particularly helpful when solid phase extraction (SPE) is performed prior to analysis since viscous samples can hinder flow and analyte binding within the SPE cartridges [35]. Buffers usually contain preservatives to reduce metabolic degradation of any drugs in the sample as microorganisms may also be present [33, 58, 84]. Surfactants may also be included to assist with elution of drugs from the absorbent pad [85]. The dilution of the sample in buffer allows multiple tests to be carried out later if required. 'Dry mouth' still poses a hindrance during the collection process, and frequently results in an inadequate volume of sample being collected that is unsuitable for laboratory analysis [44]. This lack of sufficient volume, in addition to dilution factors impacts heavily on the concentrations of THC present

in the samples. There is also wide variability in volumes collected [86-88] and it has been reported that THC can remain in the swab area of the device, significantly reducing recovery of the analyte [50, 86, 88], giving further reason for the need of much more sensitive confirmatory analyses.

Due to the unreliability of buffer volume and oral fluid volume collected using these devices, expectoration is still sometimes used, even in scientific studies [35, 56, 89, 90]. Nevertheless, the use of collection devices is clearly preferred and they are regularly used for research purposes. For example, the SalivaSampler™ was used in the DRUID program [56, 91, 92], and the Intercept® was used in the ROSITA-2 study [93, 94]. The Quantisal™ [38, 48, 95, 96] and Cozart® DDS [75] devices have also been utilised in research.

Many studies have reported the recoveries and stability of THC in oral fluid collected using the various collection devices available commercially. Speedy *et al.* [97] found very good recovery for THC from the Cozart® DDS device; 94.5% of the THC spiked was recovered at a concentration of 33.3 ng/mL. The manufacturer claims that the buffer used in this device extracts a larger amount of THC out of the swab when compared to other devices; which successfully increased its recovery. Quintela *et al.* [98] also found good recovery of THC from the Quantisal™ device (81.3–91.4%) after 12 hr storage at room temperature to simulate shipment time. After evaluating nine collection devices, Langel *et al.* [58] found good stability of THC in the collecting buffers of the Quantisal™, SalivaSampler™, Cozart® DDS, and Intercept® devices when stored frozen at –18 °C.

The Immunalysis Quantisal™ device has been evaluated a number of times in the literature. Moore *et al.* [84] found recovery of THC from the device to be 89.2%. They also determined the recovery of CBD, CBN and THCA-A to be 71.9%, 79.7% and 78.2%, respectively. Moore's study showed room temperature was not suitable for storage as they observed up to a 50% loss in THC over ten days. Lee *et al.* [99] performed a comparison study investigating the stability of a number of cannabinoids in the Quantisal™ device and neat expectorated oral fluid. The authors recommended

using the device, storing the samples at 4 °C and completing analysis within four weeks of collection. Milman *et al.* [40] found there was a good stability of cannabinoids in the Quantisal™ device after three freeze-thaw cycles (–6.3 to 12% concentration difference compared to freshly prepared controls). Storage for 18 hr at room temperature and 72 hr refrigerated gave a –15.1 to 4% difference when compared to the controls. Choi *et al.* [100] investigated the stability of THC and THC-COOH in the Quantisal™ device at 4 °C and at room temperature over a six day period. They observed good stability for THC under both conditions; however the average recovery of the drug from the device was <72%. THC-COOH was recovered at an even lower rate.

Laloup *et al.* [45] monitored the stability of THC collected and stored in the Intercept® device and found that at refrigerated and room temperatures, no significant effect was found over a period of 48 hr. The stability of samples that underwent three freeze/thaw cycles was tested and still no significant effect on the concentrations was observed. The recovery of the Intercept® device was determined to be 80.1% [33]. In a parallel study conducted by Crouch [86], it was suggested that THC positive oral fluid collected by the Intercept® device should be kept frozen for long term storage. The results indicated that after storage for six weeks at –20 °C, 4 °C and 21 °C, THC was recovered at 79%, 13% and 14% respectively. Lund *et al.* [101] also found THC to be stable in pooled real samples collected by the Intercept® device after one year of storage at –20 °C, whereas a decreasing trend was seen for the concentration of THC after just one week at both 4 °C and 20 °C.

The Intercept® device is FDA (US Food and Drug Administration) approved and is used widely in workplace drug testing in the United States [45]. The device reportedly provides good stability for refrigerated samples; however the salts and antibacterial agents in the device have been found to cause interferences, especially ion-suppression in the case of THC during LC–MS/MS analysis, and so a suitable clean-up method is required to eliminate this effect [102]. Wylie *et al.* [46] found that the buffer in the Cozart® OmniSal device also contained compounds that interfered in

the laboratory analysis of THC and so an alternate extraction method had to be used before analysis using GC–MS.

The addition of sodium azide to oral fluid to prevent the degradation of illicit drugs has been described in the literature [89, 103]. Ventura *et al.* demonstrated that in samples containing 0.1% sodium azide, degradation of multiple drugs was prevented for up to seven days in storage at 25 °C and 37 °C and up to two months at 4 °C and –20 °C [103]. Even so, the use of azide as an additive is generally avoided due to its toxicity.

The stability of THC when stored in plastic versus glass containers has also been investigated since it has been reported that THC often 'sticks' to glass and plastic surfaces due to its high lipophilicity [2]. It is a common practice to silanise glassware used in the sample preparation process for analysing THC in various biological matrices [20, 25, 84, 104-107]. Choi *et al.* [100] found that glass does not appear to cause any significant losses of THC in buffer diluted oral fluid when compared with polypropylene; although collection and storage in glass is impractical for this purpose. Langel *et al.* [58] examined the stability of THC in neat OF in polypropylene tubes stored in a freezer at –18 °C for up to four weeks. The study found that 86.4% of the THC was recovered after storage for two weeks and 82.0% after four weeks. In a separate study of THC in whole blood, Schwilke *et al.* [108] found a greater than 20% loss of THC from polypropylene tubes after two weeks of storage at either room temperature or frozen, but minimal losses at 4 °C. It appears the difference between the composition of blood and oral fluid may be the reason for these varied results and demonstrates that stability tests conducted in other matrices may not reflect the effects occurring in oral fluid. Regardless, Welsh *et al.* [109] successfully reduced adsorptive loss of THC-COOH and its glucuronide conjugate (THC-Glu) in urine samples by adding a non-ionic surfactant, Tergitol™, and this may well be effective against the losses encountered with THC in oral fluid. THC loss during storage may also be due to chemical reactions resulting from air exposure or other environmental factors such as fluorescent lighting as reported by Moore *et al.* [110].

1.3.3 Confirmatory analyses

The traditional confirmation and quantification methods involve GC–MS. The analysis of the metabolite THC-COOH in urine is done by GC–MS and now the technique is well-established for the confirmation of THC in oral fluid [105]. There have been a number of alternative methods put forward which give improved sensitivity and involve less sample preparation [44]. These are mainly LC–MS and LC–MS/MS methods that do not require a derivatisation step. GC–MS/MS methods have also been investigated for increased sensitivity along with two-dimensional (2D)–GC–MS methods.

A number of confirmatory methods have been published in peer-reviewed journals in the past decade involving the detection of THC in oral fluid using either GC–MS or LC–MS and tandem MS techniques (Table 1-3). Many of these methods are designed to analyse multiple drug analytes and metabolites simultaneously, while others have concentrated on quantifying THC only or cannabinoids and metabolites of THC. Table 1-3 summarises these methods, focussing on THC with respect to detection limits (entries in the table are listed by publication date; earliest first). As it is the parent compound and the most important analyte for oral fluid testing for cannabis, the methods discussed in this section will focus on THC.

Sensitive confirmatory testing methods are necessary to ensure reliable results are consistently achieved with real samples of small volume and low concentrations. The Australian Standard 4760:2006 specifies a target concentration for THC of 10 ng/mL [59] while the SAMHSA guideline for the limit of quantification (LOQ) of THC in neat oral fluid is 2 ng/mL [111]. Many collection devices dilute the oral fluid at the point of collection so lower limits of detection are necessary to accurately quantify THC in the neat oral fluid. Additionally, the oral fluid matrix and some commercial collection buffers can cause ion suppression issues in LC analyses; however this can be largely overcome by using a sample clean-up method such as liquid-liquid extraction (LLE) or solid phase extraction (SPE).

Generally, 500–1000 μL of oral fluid is required for GC–MS analysis methods [30, 35, 43, 46, 84, 112]. Concheiro *et al.* [44] developed an LC–MS method using 200 μL of oral fluid with a limit of detection of 2 ng/mL. Laloup *et al.* [33] used LC–MS/MS with multiple reaction monitoring (MRM) and developed a method using just 100 μL of oral fluid and determined an LOQ of 0.5 ng/mL; however this method utilised a 150 mm column resulting in a relatively long analysis time. Shorter analysis times are preferred since these methods are primarily designed for routine testing in high-throughput laboratories.

Tandem mass spectrometry is an even more sensitive detection technique gaining prominence in the analytical fields. The triple quadrupole (QQQ) mass spectrometer gives more sensitive results than the single MS system. It consists of three quadrupoles in series between where the analyte is introduced and the detector. The first quadrupole acts to only allow the ion of interest, or the 'precursor ion' to go through into the second quadrupole which is actually a collision cell. The collision cell contains either nitrogen or argon as a collision gas, which then fragments the precursor ion into product ions by collision-induced dissociation (CID). These product ions are then introduced into the third quadrupole, which only allows specific ions of interest to reach the detector. The benefits of the triple quadrupole system are its extreme sensitivity to an analyte and its ability to determine the structure of a molecule. The process of analysis using tandem MS is generally MRM. [113, 114].

Quadrupole time-of-flight (Q–TOF) mass spectrometers are another type of tandem mass spectrometry instrument. They have generally been used for the analysis of larger molecules such as proteins; however they are now being applied to smaller molecules such as THC. A main advantage of using Q–TOF compared to a triple quadrupole is the ability of the Q–TOF system to measure accurate mass. This allows for increased confidence when identifying target analytes without the need for reference standards. An additional factor is the reduced background noise which gives a better signal-to-noise ratio and hence, higher sensitivity for quantitative analyses and detection. [105].

Table 1-3: Summary of studies describing validated quantification methods for THC in oral fluid.

Separation	Detection	Analytes	Extraction technique[a]	Sample size (type)	Linear range[b]; R^2	LOQ[b,c]	LOD[b]	Ref.
LC	MS	THC only	LLE	200 µL neat	2–250; R^2>0.99	2	-	[44]
LC	MS	THC only	SPE	500 µL neat	2–100; R^2=0.999	2	1	[50]
LC	MS/MS	THC only	LLE	100 µL, 500 µL (1:2 OF:Intercept buffer)	0.5–100; 0.1–10; R^2=0.999 (both samples)	0.5 (100 µL) 0.1 (500 µL) diluted	-	[45]
LC	MS/MS	5 compounds inc. THC	SPE	1 mL (1:3 OF:Quantisal™ buffer)	0.5–50; R^2=0.999	0.5	-	[114]
GC	MS	THC only	LLE	500 µL (1:1 OF:Intercept. buffer)	1–200; R^2>0.99	1	-	[43]
LC	MS/MS	THC, THC-COOH	LLE	500 µL (artificial saliva:Milli Q 1:1)	0.1–100; R^2=0.9997	0.1	0.05	[105]
GC	MS	THC, CBD, CBN, THC-COOH	SPE	1 mL (1:3 OF:Quantisal™)	0.5–16; R^2=0.998	0.5	-	[84]

Table 1-3: Summary of studies describing validated quantification methods for THC in oral fluid.

Separation	Detection	Analytes	Extraction technique[a]	Sample size (type)	Linear range[b]; R^2	LOQ[b,c]	LOD[b]	Ref.
GC	MS	30 compounds inc. THC, CBD, CBN, 11-OH-THC, THC-COOH	SPE	~1 mL (w. Salivette buffer)	1.9–200; R^2=0.9919	1.9	0.6	[115]
GC	MS	3 compounds inc. THC	LLE	250 µL neat	2–1000	2	1	[116]
LC	MS	THC only	SPE	500 µL neat	5-2000; R^2=0.9991	5	2	[18]
GC	MS/MS	31 compounds inc. THC, CBD, CBN, 11-OH-THC, THC-COOH	SPE	300 µL (1:2 OF:Intercept buffer)	n/a	0.1 diluted	-	[117]
LC	MS/MS	23 compounds inc. THC	SPE	500 µL neat	1–200; R^2>0.99	1	-	[118]
LC	MS/MS	13 compounds inc. THC, THC-COOH	n/a	150 µL neat	3.7–100; R^2=0.9900	3.7	1.1	[119]
GC	MS	THC only	LLE	500 µL (1:3 OF:DDS buffer)	0.5–250; R^2>0.99	0.5	-	[80]

Table 1-3: Summary of studies describing validated quantification methods for THC in oral fluid.

Separation	Detection	Analytes	Extraction technique[a]	Sample size (type)	Linear range[b]; R^2	LOQ[b,c]	LOD[b]	Ref.
GC	MS	THC only	PMME	200 µL neat	3–300; R^2>0.999	2.26	0.68	[120]
LC	MS/MS	29 compounds inc. THC	SPE	200 mg (neat)	0.5–100 µg/mg; R^2=0.9960	0.5	-	[91]
GC	MS	THC, THC-COOH	SPE	1 mL (1:3 OF:phosphate buffer)	1–100; R^2=0.9994	2	1	[100]
2D GC	MS	THC, CBD, CBN, 11-OH-THC, THC-COOH	SPE	1 mL (1:3 OF:Quantisal™ buffer)	0.5–50; R^2=0.9971	0.5	0.5	[40]
LC	MS/MS	11 compounds inc. THC	LLE	400 µL (1:1 OF:SalivaSampler buffer)	1–200; R^2=0.998	1	-	[55, 57]
GC	MS	50 compounds inc. THC	LLE	1 mL (1:1 OF:SalivaSamper buffer)	1–50; R^2=0.9996	1	-	[55, 121]
2D GC	MS	THC, CBD, CBN, 11-OH-THC, THC-COOH	SPE	500 µL (neat)	0.25–50; R^2=0.9993	0.25	0.20	[35]

Table 1-3: Summary of studies describing validated quantification methods for THC in oral fluid.

Separation	Detection	Analytes	Extraction technique[a]	Sample size (type)	Linear range[b]; R^2	LOQ[b,c]	LOD[b]	Ref.
LC	MS/MS	32 compounds inc. THC	LLE	500 µL (collected using Intercept device)	0.16–1.0; R^2=0.983	0.16 diluted	-	[101]
LC	MS/MS	31 compounds inc. THC	LLE	200 µL (1:3 OF:Cozart® RapiScan buffer)	2.5–100	2.5	-	[122]
LC	MS/MS	32 compounds inc. THC, CBN	LLE	500 µL (1:3 OF:Quantisal™ buffer)	0.5–20; R^2>0.99	1.04	0.52	[96]
LC	MS/MS	44 compounds inc. THC	n/a	500 µL neat	1–25; R^2=0.9968	0.8	0.24	[89]
Microflow LC	MS/MS	THC, CBD, CBN, THC-COOH	SPE	1 mL (1:3 OF:Quantisal™ buffer); 750 µL (1:2 OF:Oral-Eze buffer)	0.5–50; R^2=0.9957 (Quantisal™); R^2=0.9937 (Oral-Eze)	0.5	0.5	[47]

[a]*n/a indicates centrifugation paired with either protein precipitation or filtration was employed; PMME refers to polymer monolith microextraction.*
[b]*Concentrations listed for linear range, LOQ and LOD refer specifically to THC and are given as ng/mL unless stated otherwise.*
[c]*LOQ refers to THC concentration in original undiluted oral fluid unless stated otherwise.*

Analytical methods are subjected to validation studies to determine the specificity, inter-day and intra-day precision (reproducibility and repeatability), accuracy, linearity and extraction recovery of each method. Limits of detection and quantification are reported and are an important key to finding the most ideal method for minimum volumes of oral fluid. Specificity in most cases is achieved by monitoring one ion for quantification purposes and a different fragment or product ion for qualification. Ion suppression and interference tests are also carried out on some methods to ensure the best sensitivity possible is achieved.

1.3.3.1 Matrix effects and sample clean–up

Suitable clean-up techniques result in low matrix effects in the sample analyses. Ion suppression is a common matrix effect that can greatly decrease the sensitivity of LC–MS analyses. GC–MS is not as prone to matrix effects but clean-up procedures are still preferred to help maintain instrument cleanliness and efficiency. The use of an appropriate extraction technique aims to reduce matrix effects by removing the analyte completely from the matrix. Matrix effects are typically determined experimentally by post-extraction addition. This involves extracting blank samples and spiking the analytes of interest into the final extracts. The resulting peak areas of the analytes in these samples are then compared to those of non-extracted samples spiked at the same concentration so a percentage increase or decrease in signal can be determined. Another method involves continuous post-column infusion of the analyte into the mass spectrometer while a sample of extracted blank matrix is injected. A spike or dip in the chromatogram signifies ion enhancement or suppression where the matrix elutes. This infusion method generally gives a qualitative result useful for method development as co-elution of the analyte and interferences can be avoided. The post-extraction addition method allows for a quantitative measurement of any matrix effects occurring and is useful for validation studies to ensure consistency between samples objectively [123].

Quintela *et al.* [105] found some ion suppression effects on THC responses in an LC–MS/MS instrument when non-extracted oral fluid diluted in water in a ratio of 1:1 was

injected, but this effect was eliminated after an LLE procedure. Concheiro *et al.* [44] evaluated the matrix effect on an LC–MS system after LLE. No suppressive effect was detected. These studies support the argument that sample clean-up methods successfully minimise matrix effects to a negligible level for neat oral fluid. Introduction of buffers such as those in many commercial collection devices, have proven to be an additional obstacle when overcoming matrix effects due to the presence of stabilisers, preservatives and surfactants in the samples [124].

Oral fluid can be quite viscous and sample preparation methods may be as simple as dilution, centrifugation and filtering, which serve to produce a less viscous sample [119]. A number of methods also involve protein precipitation using acetonitrile at –18 °C [89]. This is also utilised prior to SPE to minimise blockages in the extraction columns [35].

The most common extraction techniques used for either oral fluid or buffered oral fluid samples are LLE and SPE. LLE has the benefits of speed, ease of operation and high controllability through adjustment of the chemicals used and their concentrations. The major drawback of LLE is the difficulty of automating the process, making it tedious when large numbers of samples require analysis especially when multi-step extractions are required. Both LLE and SPE have been widely used, prior to instrument analysis by either GC–MS or LC–MS, though SPE is generally used before GC and LLE is more commonly performed for LC analyses. Since each method has its own advantages and disadvantages, the choice is based upon effectiveness and efficiency on a case-by-case basis. Extraction efficiency is an important point to consider. Analyte recoveries are optimised to maximise the amount of analyte remaining in the clean samples so the lowest limits of detection and quantification are possible. Polymer monolith microextraction (PMME) has also been found to be a suitable extraction procedure for THC in oral fluid with Luo *et al.* achieving a recovery of >89% [120].

A volume of 500 μL oral fluid sample was most commonly used in the literature for LLE methods [43, 45, 80, 96, 101, 105], however this was sometimes already diluted

with buffer from the collection device used, and so the actual volume of oral fluid required was much smaller. Achieving results with the lowest possible amount of oral fluid is preferred due the common 'dry mouth' effect of THC smoking. This can result in difficulty collecting an adequate volume of oral fluid required for analysis. Laloup *et al.* [45] performed two separate sets of extractions on neat oral fluid, firstly using 500 μL oral fluid and secondly, using only 100 μL of oral fluid and adding 400 μL water. Both experiments were successful, resulting in high sensitivity towards THC. Successful results have also been obtained with 250 μL neat oral fluid [116] and 200 μL of oral fluid diluted with collecting buffer (1:3 v/v) [44].

Most authors used the buffer mixture included in the collection device and did not add any additional buffer. Concheiro *et al.* [44] added Sørensen's phosphate buffer to neat oral fluid. Drummer *et al.* [116] treated the buffer/oral fluid mixture with 1 mL of 1 M ammonium sulfate solution at pH 4.5. Hexane was used to extract the drug in most studies reviewed, with some using a mixture of hexane and ethyl acetate at 90:10 v/v. Different volumes of hexane were used ranging from 4 to 7 mL. Where hexane/ethyl acetate was used, the volume varied between 3 and 5 mL. Øeistad *et al.* [85] extracted buffered samples with 1.3 mL ethyl acetate/ heptane at 4:1 v/v. Fabritius *et al.* [125] also used a mixture of heptane and ethyl acetate (4 mL; 4:1 v/v). Langel *et al.* [121] used 3 mL butyl acetate as the extraction solvent for multiple compounds including THC. After the addition of extraction solvent, samples were typically shaken for 10–30 min and then centrifuged for 5–15 min at 1400–4000 x *g* before transferring as much as possible of the organic phase into a clean tube. The extracts were then evaporated to dryness under a gentle nitrogen stream at 40–45 °C. GC methods included a further derivatisation step. Drummer *et al.* [116] used dry ethyl acetate to reconstitute extracts for GC analysis; isooctane has also been used for this purpose [43, 80]. For LC analysis, the dried extracts were reconstituted in 40–50 μL of the initial mobile phase composition.

Concheiro *et al.* [44] used a LLE procedure with hexane and a phosphate buffer at pH 6. The separated organic phase was evaporated under a gentle stream of nitrogen gas, (as is typical for these methods), at 45 °C and reconstituted in mobile phase before

injection into an LC–MS system. Recovery was found to be >84% and the LLOQ for the method reached 2 ng/mL. Laloup *et al.* [45] used a liquid-liquid extraction method using 4 mL hexane for their LC–MS/MS analysis and recovery was found to be >85%.

SPE can be automated and this is especially advantageous in forensic applications where there may often be a large volume of samples to be analysed. Choi *et al.* [100] used an automatic SPE method and found recovery of THC to be >65% and THC-COOH >79%. SPE is also beneficial over LLE due to its decreased amount of solvent waste in these automated procedures. SPE using cartridges that are co-polymeric (or mixed mode) are particularly useful when analytes in the same sample have vastly differing pH, e.g. neutral THC and acidic THC-COOH as these can be extracted simultaneously [19]. Positive pressure manifolds can be used for SPE to allow for uniform flow rates through the extraction columns [84].

The SPE process involves conditioning cartridges before loading the sample, which is followed by washing, drying, and finally, elution of the analytes of interest. Each step uses a fixed amount of various solvents that can be adjusted to suit the sample. The conditioning step for THC usually involves methanol, de-ionised water and/or acetic acid. Teixeira *et al.* [18] used phosphoric acid and methanol on mixed-mode Bond Elut LRC-Certify columns (10 mL; 300 mg) in their process. Pujadas *et al.* [115] also utilised Bond Elut Certify columns for their SPE method and used methanol and a phosphate buffer in the conditioning step. The sample is then added and allowed to drain with or without application of a low vacuum. Once dry, the wash stage consists of loading methanol, de-ionised water and/or acetic acid. Hydrochloric acid and acetonitrile have also been used as well as phosphoric acid and hexane [18]. SPE cartridges are then dried for 5–10 min with or without vacuum. Some methods include a pre-wash at this point with a small volume of hexane [18]. Then, elution of the sample is done using a hexane/ethyl acetate mix 80:20 v/v or hexane/acetic acid. For LC analyses, the eluted sample is then dried under a gentle nitrogen flow as with LLE procedures and reconstituted in 50–100 μL mobile phase. A method by Teixeira *et al.* [3] used SPE to achieve a recovery of 79%. A subsequent paper by Teixeira *et al.* [50] showed a similar method that gave a recovery of 76–83%.

Derivatisation of THC improves its volatility and ionisability, which increases sensitivity of detection. Reagents and methods must be chosen carefully to optimise derivatisation as it is an additional step where analyte loss can occur. *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with or without 1% trimethylchlorosilane (TMCS) is the most prevalent reagent used by researchers for cannabinoids in oral fluid and gives trimethylsilyl (TMS) derivatives of THC and other cannabinoids for electron impact ionisation methods. Typically, 20–30 μL of BSTFA is added to dried extracts with or without added ethyl acetate and samples are incubated at 60–90 °C for 15–30 min [35, 46, 84, 100, 117, 126]. Trimethylsilation has also been performed using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) [115, 121]. Methylation and perfluoroacylation derivatisation procedures have also been used [43, 80, 116]. Milman *et al.* [35] used trifluoroacetic anhydride (TFAA) and hexafluoroisopropanol (HFIP) to derivatise THC-COOH before analysis using 2D–GC–MS with negative chemical ionisation. Lee *et al.* [127] recently proposed using dansyl chloride (5-(dimethylamino)-1-naphthalenesulfonyl chloride) to improve sensitivity of a LC–ESI–MS/MS method for THC and THC-COOH. They achieved impressive limits of quantification of 25 pg/mL for THC and 10 pg/mL for THC-COOH.

1.3.3.2 Reported GC–MS methods

GC–MS is the most widely accepted method of performing analyses on oral fluid for the determination of THC and other drug concentrations. As can be seen in Table 1-3, many validated GC–MS methods exist and are used in forensic testing as well as in evaluation studies of screening devices [58, 62]. A major drawback of using gas chromatography methods is that derivatisation is always required. GC–MS typically involves the analysis of the methylated or TMS derivatives of THC using electron impact ionisation and SIM mode [30]. The ions commonly monitored are *m*/*z* 386 and 371 for the THC-TMS derivative and *m*/*z* 389 and 374 for the TMS derivative of d_3-THC, the internal standard [30, 84, 112]. Choi *et al.* [100] also monitored *m*/*z* 303 and 306 for further qualification of the THC-TMS and d_3-THC-TMS ions, respectively. Kauert *et al.* [30] described a method using GC–MS that achieved a limit of detection

(LOD) of 0.5 ng/mL and a lower limit of quantification (LLOQ) of 2.4 ng/mL. Moore *et al.* [84] developed a method that gave an LOQ of 0.5 ng/mL with $R^2 = 0.998$. Cirimele *et al.* [43] monitored ions *m/z* 328, 313 and 285 for methylated THC and *m/z* 331 and 316 for methylated d_3-THC. The authors determined an LOQ of 1 ng/mL with the signal-to-noise ratio of 10:1 and linearity over the range of 1–200 ng/mL with correlation, $R^2 > 0.99$. Langel *et al.* [121] also achieved an LOQ of 1 ng/mL for THC with a method developed to quantify 36 compounds simultaneously. Wylie *et al.* [46] optimised a GC–MS method, achieving linearity with $R^2 = 0.999$ over a concentration range of 10–400 ng/mL, an LOD of 0.3 ng/mL and LOQ of 0.5 ng/mL [46]. These are very good results, but due to the extra derivatisation step required, LC–MS with its minimised sample preparation is still preferred if detection limits can be matched.

While electron impact ionisation (positive or negative) is the most common ionisation mode employed with GC–MS, there have also been methods reported which use positive or negative chemical ionisation (PCI and NCI). Good results for THC in other matrices such as whole blood and plasma have been observed [19, 128]. When NCI is used for analysing THC, the trifluoroacetate derivative is normally the target analyte as it has a high electron affinity [128]. A main difference with CI is the lower fragmentation energies used which result in increased sensitivity of a specified ion mass, however the abundance of additional characteristic ions are reduced and a smaller number of prominent peaks are observed in the spectra [19]. Therefore, it is more difficult to identify a substance according to its ion fragments, compared to when EI is used. Due to the increased sensitivity possible, CI has been particularly useful for the detection and quantification of THC-COOH in oral fluid since it is present in very small concentrations.

GC–MS/MS methods have been investigated as well as two-dimensional GC, the latter of which employs a Dean's switch that allows most of the matrix to be removed before the analytes reach the second column. This greatly reduces any potential matrix interferences and increase sensitivity of the method [126]. Moore *et al.* [41] developed a method to quantify THC-COOH in oral fluid collected with the Quantisal™ device using a 2D–GC–NCI–MS and achieved an LOQ of 2 pg/mL. Later,

Milman *et al.* [40] developed 2D–GC–MS methods to quantify THC, CBD, CBN, 11-OH-THC and THC-COOH in oral fluid also collected by the Quantisal™ device. All analytes were extracted from the same sample by SPE, but THC-COOH was eluted separately and analysed on a 2D–GC–NCI–MS system (the other system utilised EI for the other analytes). The LOQ for THC was 0.5 ng/mL and for THC-COOH it was 7.5 pg/mL. More recently, Milman *et al.* [35] also successfully validated the method for neat, expectorated oral fluid (THC LOQ = 0.25 ng/mL; THC-COOH LOQ = 5 pg/mL).

1.3.3.3 Reported LC–MS methods

Many LC–MS methods quantifying THC in oral fluid have been developed in the last few years (Table 1-3). A validated method by Concheiro *et al.* [44] published in 2004, was the earliest LC study found detecting THC in oral fluid. The method required 200 μL of oral fluid and achieved an LOQ of 2 ng/mL for THC. This met the SAMHSA guidelines cut-off of 2 ng/mL for the detection of THC in neat oral fluid [111]. Deuterated THC (d_3-THC) was used as an internal standard for THC in all studies found. The mobile phase was 0.1% formic acid in H_2O/acetonitrile (15:85 v/v). The extract (15 μL) was injected into the LC system containing a C18 column (3.5 μm, 2.1 mm I.D. × 100 mm) and elution was isocratic. Concheiro *et al.* found that the ratio of the mobile phase was suitable for rapid analysis and gave a good analyte ionisation. Electrospray in positive ionisation mode was the ion source used in this study and in all others found [3, 18, 33, 44, 45, 50, 54, 85, 89, 91, 96, 101, 105, 114]. MS was performed in selected ion recording (SIR) mode monitoring *m*/*z* 315.4, the ion used for quantification and *m*/*z* 193.1, a fragment ion used as a qualifier to achieve specificity. Validation studies revealed the repeatability, reproducibility, precision and accuracy were all adequate. Linearity was achieved over the range of 2–250 ng/mL with R^2 = 0.995. This study showed that LC–MS could be used as an alternative to GC–MS for the detection and quantification of THC in oral fluid achieving adequate limits of detection.

Another analysis by LC–MS used 500 μL of oral fluid to achieve an LOD of 1.0 ng/mL and an LOQ of 2.0 ng/mL [3]. The mobile phase used by Teixeira *et al.* in this study

was acetonitrile and 0.05% ammonia (70:30 v/v) eluted in isocratic mode with a C18 reversed-phase 3.5 μm, 2.1 mm I.D. × 50 mm column. Detection was performed in SIR mode monitoring *m/z* 315 [THC + H^+] and *m/z* 318 [d_3-THC + H^+]. The standard concentration range for calibration was 2–100 ng/mL and linearity was "achieved", though no R^2 value was quoted by the authors. The method was described by Teixeira *et al.* [3] to be sensitive, specific and had a high recovery with the SPE method used. In a subsequent study by the same authors in 2005 [50], the same method is used with correlation, $R^2 = 0.999$. THC had a short retention time of 3.4 minutes [50]. THC and metabolite THC-COOH were detected in oral fluid by Teixeira *et al.* [18] using a procedure with similar conditions as in the previous studies, however a 150 mm column with 5 μm particle size was used. A much longer run time was necessary for the separation, with a retention time of 12.3 minutes for THC. For the purposes of routine drug testing, which generally involves a large volume of samples to be tested daily, a more rapid method would be preferred. Hence, a shorter column with a smaller particle size would be more suitable. C18 columns with 1.8 μm particle size have been used in a number of validated LC–MS/MS methods quantifying multiple drug compounds including THC [91, 96, 101, 114]. Di Corcia *et al.* used a column with 1.7 μm particle size [89]. Column lengths have varied between 50, 100 and 150 mm.

Mobile phases most commonly used for LC analyses of drug compounds including THC, involve acetonitrile or methanol as the organic component and formic acid (generally 0.1%), ammonium formate or ammonium acetate at concentrations of 2–10 mM for the aqueous component [89, 91, 96, 101, 114]. These are eluted either isocratically or with a gradient beginning with a lower organic content.

Laloup *et al.* [45] used the Intercept® device to collect 100 μL and 500 μL samples of oral fluid. The LOQs achieved were 0.5 ng/mL for the 100 μL samples and 0.1 ng/mL for the 500 μL samples. The much lower LOQs achieved in this study were possible with the use of tandem mass spectrometry. The column used was 150 mm in length and the mobile phase was 1 mM ammonium formate and methanol (10:90 v/v) eluted isocratically at 0.2 mL/min. A higher volume of 20 μL was injected into the LC instrument. Linearity for both sample volumes gave $R^2 = 0.999$ (0.5–100 ng/mL for 100

μL sample and 0.1–10 ng/mL for 500 μL sample). Precision and accuracy were found to be <6% and >93% respectively. The transitions monitored in MRM mode by Laloup *et al.* [45] were:

- *m/z* 315.2 → 193.1 as the quantification ion;
- *m/z* 315.2 → 259.3 as the qualifying ion;
- *m/z* 318.2 → 196.1 as d_3-THC internal standard.

Figure 1-7 shows the proposed fragment structures for THC. These ions are commonly used in LC–MS/MS analyses with positive electrospray ionisation [89, 91, 96, 101].

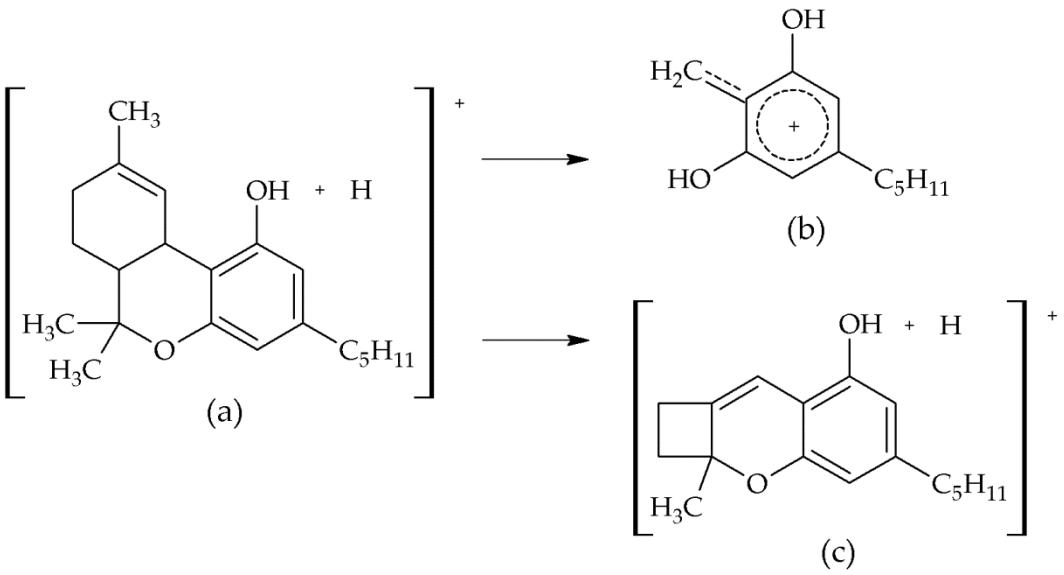

Figure 1-7: Proposed fragment structures of THC; (a) THC parent ion m/z 315; (b) product ion m/z 193; (c) product ion m/z 259 [45].

Laloup *et al.* also investigated the possibility of other cannabinoids interfering with the detection of THC. Cannabinol and cannabidiol were monitored in addition to THC and were found not to interfere with THC given the specificity of the method used [45]. Even though cannabidiol has the same precursor and product ions as THC due to their similar structures, they are separated by chromatography and do not cause any interferences with each other.

Agilent Technologies published a working paper in 2006 [114] outlining a method for the detection of THC and a number of other drugs in oral fluid collected by the Quantisal™ device, using LC coupled to a QQQ mass spectrometer. The column used was only 50 mm long with 1.8 μm particle size. MRM mode was utilised to make the separation highly selective. THC eluted at 4.2 minutes, resulting in a rapid run time. The LOQ was determined to be 0.5 ng/mL and linearity was good with correlation of $R^2 > 0.999$. A method validated using a LC–QTOF mass spectrometer to quantify THC and THC-COOH was published by Quintela *et al.* in 2007 [105]. They achieved an LOQ of 0.1 ng/mL for THC and 0.5 ng/mL for THC-COOH.

Novel LC systems that utilise capillary and nano flow pumps have recently been released. Companies such as Agilent Technologies and Thermo Scientific have developed LC instruments that contain a capillary pump that directs the analyte to a 'trapping' or 'enrichment' column first, and washes mobile phase through that column before the system switches configuration and a nano pump directs the analytes remaining in the first column onto the second, analytical column for separation of compounds before entering the MS via nano-spray ionisation. This extra step is effectively an in-line pre-concentration step that also attempts to remove matrix while 'trapping' the analytes of interest in the column. He *et al.* [104] developed a method to detect THC and quantify THC-COOH in oral fluid using a Thermo Scientific 300 RSLCnano system coupled to a Thermo Scientific Q Exactive Mass Spectrometer operated in negative ionisation mode. The LOQ for THC-COOH was 7.5 pg/mL. Ion suppression was still observed when using the 'trapping' column for further sample clean-up and pre-concentration. Concheiro *et al.* [47] developed a method on the same instrument to quantify THC, THC-COOH, CBD and CBN in oral fluid. They achieved an LOQ of 0.5 ng/mL for THC, CBD and CBN, and 15 pg/mL for THC-COOH.

1.4 Aims of this project

This study aims to fill a number of gaps in current knowledge relating to the detection of cannabis in oral fluid, for example, the stability of cannabinoids stored in neat oral fluid in various conditions over the short and long term. This study also discusses a new drug called Sativex® and its potential to give positive results for THC in roadside drug-testing programs. In this work, the focus is on oral fluid, as this matrix is gaining more and more interest in the field of forensic toxicology, workplace drug testing, and especially in roadside testing. Roadside testing methods are an important area of research since every effort should be made to minimise drug-driving incidents and deter people from taking the risk of driving whilst affected by drugs.

Simple, rapid and sensitive methods for the detection of THC, CBD, and CBN have been developed using specialised tandem mass spectrometry instruments to ensure reliability and robustness. Additionally, there was a need for procedures suitable for use with neat oral fluid as well as oral fluid that has been diluted in various commercial buffers since there is a wide variety available.

The specific aims of this thesis were to:

1. Evaluate the stability of THC in oral fluid in various storage conditions.

2. Address the adsorptive behaviour of THC by comparing the degree of adsorption to container surfaces under various conditions; and attempt to minimise this adsorption using a non-ionic surfactant. A non-ionic surfactant will also be utilised to try to differentiate THC loss due to degradation from adsorptive processes.

3. Validate a method for the quantification of THC, CBD and CBN in oral fluid using LC–QQQ–MS and investigate the implications of the possible release of the nabiximols drug, Sativex®, with regards to roadside drug testing procedures.

4. Evaluate the suitability of novel nano-spray chip LC–MS for detecting and quantifying cannabinoids in oral fluid and determining whether this technique can help reduce common matrix effects issues with LC–MS.

In all, this thesis aims to provide new information regarding the most efficient ways to confirm and quantify THC and its metabolites using tandem mass spectrometry methods.

Chapter 2: Preliminary study of THC loss associated with storage in oral fluid

Chapter 2: Preliminary study of THC loss associated with storage in oral fluid

2.1 Introduction

Key factors to consider when dealing with any biological matrix are the storage conditions. There will always be an interval between collection and testing, thus stability of the analyte must be determined if the measured concentration is to be reliable. Is the analyte stable in the matrix for extended time periods? Will refrigeration or freezing help or hinder its stability? What about the immediate recovery rates of the analytes from the collection vessel? Oral fluid is a complex matrix containing a multitude of components including proteins, food residues, mucous and microorganisms. These may all cause potential hurdles for the stability of cannabinoids contained within oral fluid as they may accelerate degradation, for example, by way of metabolic action by any microorganisms present in the sample.

Recovery is known to be a major issue with oral fluid collection devices, especially for THC, as it often 'sticks' to the absorbent pad used in the collection process. This requires confirmatory testing to be more sensitive to accommodate for lost THC in the pad, as well as compensating for the dilution factor due to included buffers. This unavoidably leads to complications with the interpretation of quantitative results. The buffer may also cause problems when performing confirmatory analysis with LC–MS, introducing interferences leading to ion suppression or enhancement [45, 46], and so a suitable clean-up procedure is required before analysis. Stability experiments also need to be carried out to determine the exact effects of different storage conditions so losses can be minimised.

Commercial oral fluid collection devices often utilise buffers containing preservatives and surfactants to minimise loss of analytes during storage [129, 130]. However, these buffers significantly dilute the oral fluid and previous studies have found that these

devices often have difficulty collecting consistent volumes of oral fluid making accurate quantification of THC challenging [43, 44].

Use of neat oral fluid is therefore the only viable way to analyse an accurate volume. There are a number of issues surrounding expectoration, such as reduced salivary flow, resulting in difficulties collecting a sufficient volume, an excess of froth being collected rather than liquid [35], or samples being highly viscous and containing various debris from the mouth [10]. However, due to the certainty of the volume collected using this method, it is still sometimes preferred.

The aims of this chapter are:

- To provide preliminary results investigating the stability of THC in neat oral fluid when stored in polypropylene containers;

- To conduct a long term stability of THC in the oral fluid/buffer mix of samples collected using a Cozart® device and to compare the stability of THC stored in a stabilising buffer with THC stored in neat oral fluid; and,

- To form an immediate recovery profile of THC in oral fluid from polypropylene containers in order to determine the degree to which THC may be lost to the polypropylene surfaces due to adsorptive processes.

2.2 Materials and methods

2.2.1 Materials

High-performance liquid chromatography (HPLC) grade methanol and acetonitrile were purchased from Chem-Supply (Gillman, SA, Australia). Analytical grade ethyl acetate was obtained from Fronine Laboratory Supplies (Australia). HPLC grade n-hexane was obtained from Ajax Finechem (Taren Point, NSW, Australia).

Concentrated formic acid was purchased from Sigma–Aldrich (Castle Hill, NSW, Australia), and filtered using 0.22 μL pore diameter filters before use. All water used was purified using a Sartorius Arium® Milli-Q system. Cerilliant® standards of THC (1 mg/mL in methanol) and d_3-THC (0.1 mg/mL in methanol) were purchased from Novachem (Collingwood, VIC, Australia). These reference standards were diluted with methanol to obtain working stock solutions for THC at 10 μg/mL and d_3-THC at 1 μg/mL. Di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and sodium dihydrogen orthophosphate (NaH_2PO_4), used to make 0.1 M Sørensen's phosphate buffer were sourced from Ajax Chemicals (Taren Point, NSW, Australia) and mixed to achieve a pH of approximately 6.

Real oral fluid specimens were collected from the Roadside Drug Testing Program (RDTP) conducted by the New South Wales (NSW) police. These samples were collected by a proprietary collection kit (Cozart® collector), part of the Cozart® RapiScan testing system. The oral fluid was mixed with a proprietary buffer that effectively diluted the oral fluid three-fold (1 mL oral fluid and 2 mL buffer). After initial analysis by the Division of Analytical Laboratories (DAL), (now called the Forensic and Analytical Science Service (FASS)) in Lidcombe, NSW, Australia, the residual samples were stored in a refrigerator at 4 °C for at least 12 months following the RDTP protocol. Therefore, all samples used in this study had been stored at 4 °C in the Cozart® proprietary dilution buffer for 13 to 18 months following sample collection. The residual samples were supplied by DAL after de-identification.

THC-free oral fluid was provided from volunteers and used for the study on the day of collection. The absence of THC in collected oral fluid was confirmed by following the sample preparation and analysis procedures, excluding the addition of any THC standards, as described in the following paragraphs.

2.2.2 Sample preparation – LLE

Aliquots (200 or 500 μL) of freshly collected drug-free oral fluid were transferred into 10-mL screw-cap glass test tubes and spiked with THC at various specified

concentrations. Deuterated internal standard solution (5 or 10 μL of 1 μg/mL d_3-THC) was added to the glass test tubes followed by Sørensen's phosphate buffer (1 mL). Hexane/ethyl acetate (5 mL; 9:1 v/v) was then added and the tubes placed on a roller mixer on high speed for 30 min. The organic upper layers (4.5 mL) were transferred into high recovery GC vials (1.5 mL; PM Separations, Brisbane, Australia) and evaporated under a gentle stream of N_2 gas at 40 °C. Residues were reconstituted in 100 μL 75% acetonitrile in water with 0.1% formic acid.

2.2.3 LC–MS/MS

A chromatographic method was developed and analyses were carried out using an Agilent 1200 series LC system with a Zorbax Eclipse XDB–C18 Rapid Resolution High Throughput column (2.1 mm × 50 mm × 1.8 μm). Isocratic elution using 75% acetonitrile in water with 0.1% formic acid was performed for injections of 5 μL of the sample extracts with a flow rate of 0.4 mL/min.

The LC system was coupled to an Agilent 6460 Triple Quadrupole mass spectrometer with an ESI source. MS was operated in MRM mode monitoring transitions *m*/*z* 315.2 → 193.1 and 259.1 for THC and *m*/*z* 318.3 → 196.1 and 262.1 for d_3-THC. Ions *m*/*z* 193.1 and 196.1 were used as quantifying ions and *m*/*z* 259.1 and 262.1 were the qualifying ions. The fragmentor voltage was 145 V and collision cell voltage was 21 V for both of the THC transitions and 17 V for the two d_3-THC transitions. The gas temperature was 340 °C, nebuliser pressure was 40 psi and the capillary and nozzle voltages were 4000 and 1500 V, respectively. Additional parameters, sheath gas temperature and sheath gas flow, were 400 °C and 12 L/min. Dwell time was 240 ms for each transition.

Data acquisition and analysis were performed using the software included in the Agilent MassHunter Workstation package and Microsoft Excel 2010.

2.2.4 Validation studies

The LC–MS/MS method was validated for the detection of THC in neat oral fluid. Serial dilution of the methanolic THC working stock solution gave six concentrations: 10, 2, 1, 0.2, 0.1 and 0.02 μg/mL. Ten microliter aliquots were spiked into 200 μL of drug-free oral fluid giving final concentrations of 500, 100, 50, 10, 5, and 1 ng/mL. Quality control (QC) samples were spiked at 8 and 25 ng/mL for intra- and inter-day precision and accuracy studies (n = 5). Five microliters of internal standard solution (1 μg/mL) was then added to each tube giving a concentration of 25 ng/mL of d_3-THC. After addition of 1 mL of 0.1 M Sørensen's phosphate buffer, the LLE procedure was followed as previously described. Linearity of the calibration curve was calculated using a line of best fit with the acceptable correlation factor set at >0.99. Accuracy was determined to be acceptable if the calculated concentrations fell within 15% of the concentration spiked (expressed as an MRE). Precision was deemed acceptable if the RSD was <15%. The LOD was the lowest concentration of analyte that still gave a signal-to-noise ratio (SNR) of 3. The LOQ was the lowest concentration of analyte that repeatedly gave a SNR of at least 15 and MRE of <20%. Precision and accuracy data was determined for the LOQ and the transition ion ratio was required to be within 20% of the determined value from higher concentration standard samples for the results to be valid.

2.2.5 Recovery and matrix effects

The overall recovery of the analyte was assessed by spiking drug-free oral fluid (n = 3) with THC at 25 ng/mL, then carrying out the LLE as previously described. Three non-extracted control samples were prepared by fortifying mobile phase with the equivalent concentration of drug analyte and internal standard. All samples were run on the LC–MS/MS in triplicate and the three values averaged. The peak areas of the quantifying ions of the extracted samples were compared directly to those of the fortified mobile phase samples and percentage recoveries were calculated.

Matrix effects were investigated by extracting both neat drug-free oral fluid samples (n = 3) and then diluted (with Cozart® collecting buffer) negative oral fluid samples from the RDTP (n = 9) and reconstituting with mobile phase fortified with drug and internal standard analytes at 50 ng/mL. Peak areas of the resulting MRM chromatograms were compared with those of samples where the analytes were spiked at the same concentration into mobile phase only.

2.3 Results and discussion

2.3.1 Method validation

Linearity was achieved over the range of 1–500 ng/mL with an average correlation coefficient of 0.9998. The relative standard deviations (RSD) of the QC samples were 7.1–9.4% and the mean relative error (MRE) ranged from 2.2% to 19.3%. The LOQ was 1 ng/mL with 1% RSD and 16% MRE. The LOD was 0.25 ng/mL. Specific validation data can be seen in Table 2-1.

Table 2-1: Validation data for the LC–MS/MS method (n = 5).

Concentration level (ng/mL)	Intra-day precision (RSD%)	Intra-day accuracy (MRE%)	Inter-day precision (RSD%)	Inter-day accuracy (MRE%)
8	8.6	19.3	9.4	4.0
25	7.1	7.3	8.3	2.2

2.3.2 Recovery and matrix effects

A recovery of 75% of THC was achieved (17.7% RSD) when the acetonitrile (75 µL) component was added first when reconstituting and vortex mixing this before adding the aqueous component (25 µL 0.1% formic acid in water) into the extract.

Minimal matrix effects were observed as average peak areas of THC and d_3-THC in extracted samples including 3 neat drug-free oral fluid samples from 3 donors and 9 negative diluted oral fluid samples from the RDTP showed little difference to the 3 non-extracted samples. The extracted samples fortified with standards compared to mobile phase fortified with the same volume of analyte, resulted in 103.6% and 106.3% peak area responses for THC and d_3-THC, respectively (RSD was <10% for all sample populations).

2.3.3 Preliminary stability study – neat oral fluid

Schwilke *et al.* [108] had reported >20% loss of THC in whole blood after two weeks storage in polypropylene tubes at both room temperature and in the freezer (–18 °C), but minimal losses at 4 °C. It is therefore critical to establish the stability of THC in oral fluid under similar storage conditions.

Stability of THC in neat oral fluid in 12-mL polypropylene tubes was investigated in this study. Samples (n = 5) of neat oral fluid (200 µL) were spiked with 5 µL of 1 µg/mL THC to a concentration of 25 ng/mL and left either at room temperature (22 °C), refrigerated (4 °C) or frozen (–18 °C). Samples were removed from storage and extracted after the addition of 5 µL of 1 µg/mL d_3-THC at t = 0, 1, 8 and 14 days. Responses were compared to the t = 0 samples and calculated as percentage losses.

This stability experiment showed storage of 200 µL of spiked neat oral fluid at room temperature for just one day resulted in losses over 50%. THC concentrations in both the refrigerated and frozen samples declined to 70–85% after one day and appeared to have stabilised at around 65% of the original concentration after two weeks (Figure 2-1). These results indicate drastic losses occur even in the short term at room temperature, however it becomes apparent in future experiments that other forces may be at play. The use of larger 12-mL tubes resulted in a relatively large headspace area that may have allowed for more oxidative functions to occur, although the timeframe may be too short for this to have had a significant impact. Another factor to consider is the contact area between sample and container surface. This was not

controlled in this experiment and may also have affected the results. Refer to Section 3.3.7 for the results of a longer stability study utilising smaller 2-mL tubes that minimise the available headspace and control contact between sample and polypropylene surface. The losses at room temperature in that experiment are not as severe after one day, as seen here. This highlights the importance of considering all variables in the storage of oral fluid to maximise the stability of THC.

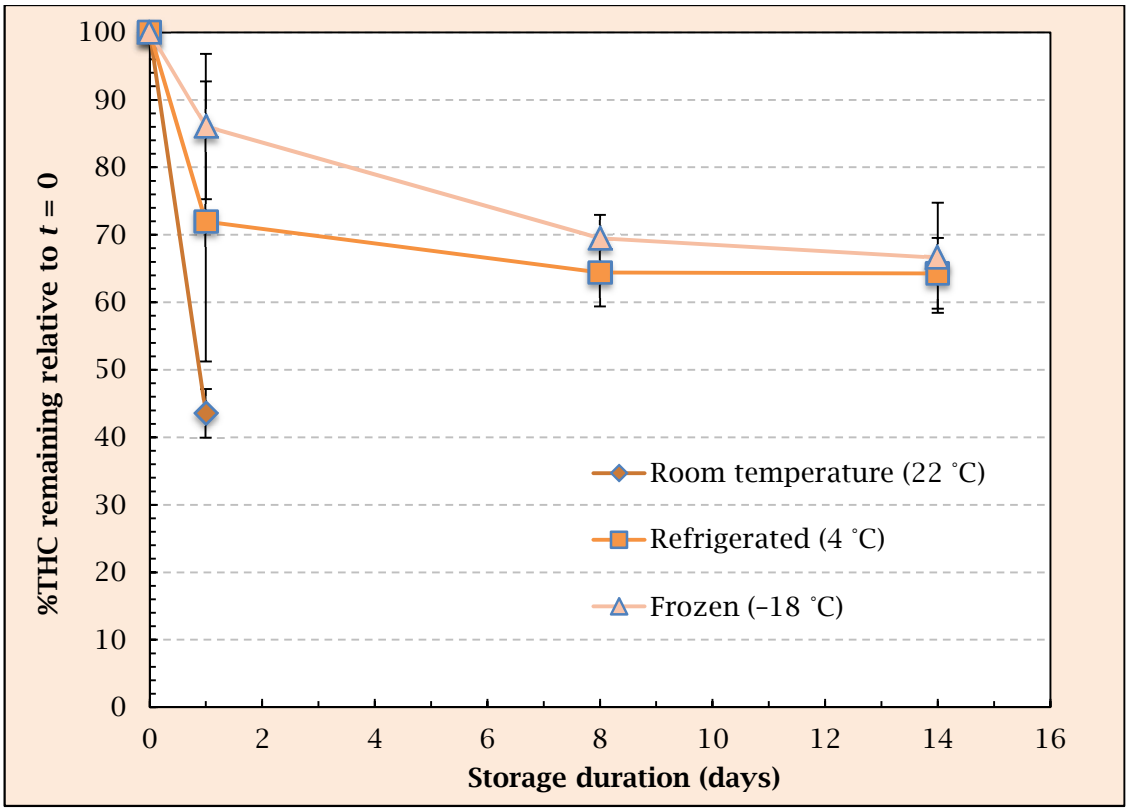

Figure 2-1: Loss of THC from oral fluid samples over a two-week period under different storage conditions.

It should be stated, this is the first study in which stability of THC in neat oral fluid under various storage conditions was investigated. Only one other published work was found, (Langel *et al.*) [58] in which THC stability in neat oral fluid stored frozen in polypropylene tubes was investigated. The study by Langel *et al.* found that the recovery of THC in oral fluid was 84% and 82% after storage at –18 °C for two weeks and four weeks, respectively. Although the current study found no differences in recovery from freezing or refrigeration, it was clear that both are superior to storage at room temperature. Due to likely differences in collection procedures, freezing

appears to be the most appropriate storage condition for THC in oral fluid. The different composition between blood and oral fluid must be responsible for the different behaviour of THC when stored frozen in polypropylene tubes, although the exact reason remains unknown.

THC is a highly lipophilic substance and its strong interaction with the plastic surface might be responsible for its loss when plastic containers are used. This is supported by the findings from Choi *et al.* [100], that minimal THC losses were observed when THC in oral fluid diluted with buffer was stored in glass tubes. Degradation of THC in oral fluid may also be attributable to metabolic action of microorganisms present in the oral fluid specimens. This may form the basis for many suppliers of commercial oral fluid collecting devices to add preservatives in their collecting buffers [58]. Ventura *et al.* [103] reported that adding sodium azide (0.1%) to oral fluid prevented degradation of several illicit drugs during up to seven days of storage at 25 °C and 37 °C and up to two months at 4 °C and –20 °C. Another contributing factor for THC loss during storage may be due to chemical reactions of THC induced by oxygen or other environmental factors such as fluorescent lighting as reported by Moore *et al.* [110]. Due to the importance of oral fluid testing in forensic toxicology, the stability of THC in oral fluid under various storage conditions warrants further investigation

2.3.4 Preliminary stability study – oral fluid in Cozart® buffer

In another experiment looking at THC stability, real oral fluid samples (n = 48) were analysed. These samples, originally collected by police as part of the RDTP, had been previously analysed at DAL, a National Association of Testing Authority (NATA) accredited and NSW government appointed drug testing laboratory, using an in-house method. The de-identified samples were diluted at the point of collection in a buffer (oral fluid: buffer, 1:2) so results determined from the calibration curve using the current method were back-calculated to account for the dilution factor. The reliability of this calculation was tested experimentally using neat oral fluid samples spiked at 25 and 250 ng/mL (n = 5). These neat oral fluid samples were diluted 1:2 with the Cozart® collection buffer and extracted and analysed as described previously.

Results showed that back-calculating to find the original concentrations gave an MRE of 8.4% and 13.7% for the 25 and 250 ng/mL samples respectively, with an RSD of 8.1% and 1.0%.

THC concentrations in these 48 samples were compared to their initial results. No false positives or false negatives were encountered and 21 of the 22 positive samples fell within the linear range of the calibration curve. Concentrations were determined to be in the range of 0–710 ng/mL after correcting for the three-fold dilution with buffer. One positive sample fell outside the calibration range as it was calculated to have approximately 3400 ng/mL THC (DAL reported the sample to contain 4100 ng/mL THC). The levels found using the validated LC–MS/MS method were all below those determined previously by DAL at 67–99% (Figure 2-2). The average THC concentration obtained from this reanalysis was 89.2% of the initial value determined by DAL with an RSD of 9.3%.

When various commercial oral fluid collection devices were evaluated by Langel *et al.* [58], good stability of THC in the collecting buffers was achieved for Quantisal™, Statsure®, Cozart®, and Intercept® when stored at –18 °C. Crouch [86] reported recoveries of THC from Intercept® collecting buffers at 79%, 13% and 14% after storage for six weeks at –20 °C, 4 °C and 21 °C, respectively, indicating that THC positive oral fluid collected by Intercept® devices ought to be kept frozen for long term storage. On the other hand, data presented in this current study indicates that THC in oral fluid collected by the Cozart® collecting device has excellent stability with an average recovery of 89% when stored in a refrigerator (4 °C) for 13–18 months (Figure 2-2). This value is merely an estimate since the reanalysis of samples was performed in a different laboratory using a different method; however it is still clear that THC stored in the Cozart® buffer has not been subject to the drastic losses observed in other studies when refrigerated for an extended period of time.

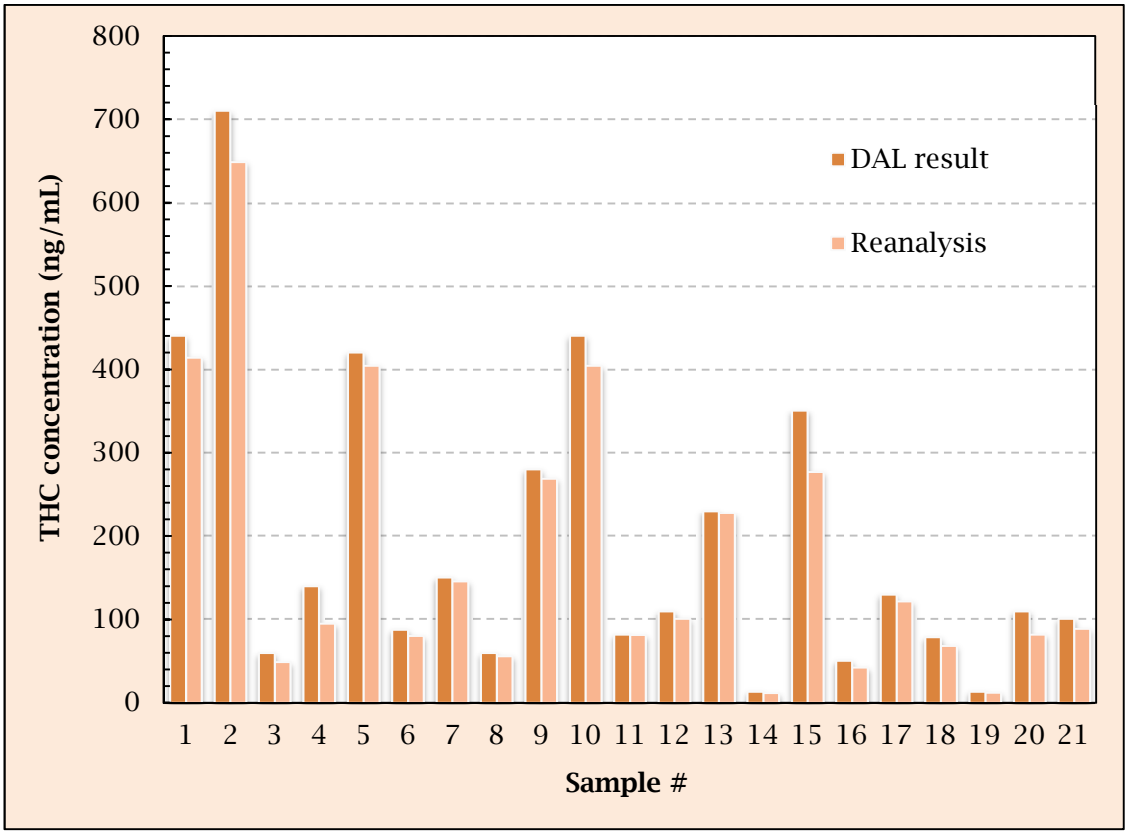

Figure 2-2: THC levels (ng/mL) found by DAL and from reanalysis 13–18 months later using the validated LC–MS/MS method. DAL utilised an LLE with 1-chlorobutane followed by analysis using an Agilent 1100 High Performance LC coupled to an Applied Biosystems API4000 Triple Quadrupole mass spectrometer. LC was performed on a Zorbax Eclipse SB-C18 column (4.6 mm × 50 mm × 3.5 µm) with a gradient elution of 5% acetonitrile in water containing 0.1% formic acid and 5 mM ammonium formate (solvent A) and methanol (solvent B). Total run time was 9 min. MRM mode was used to detect multiple drug analytes including THC (m/z 315 → 193 and 123 for THC; m/z 318 → 196 and 123 for internal standard d_3-THC).

2.3.5 Immediate recovery of THC from plastic storage containers

In order to determine if THC in neat oral fluid is being lost immediately on contact and during transfer stages of sample preparation, an experiment involving rinses of the containers was carried out. Three sets of extractions were run. Firstly, 500 µL of oral fluid was spiked with THC (200 ng/mL) in 25-mL polypropylene containers, the entire volume of which was transferred via Pasteur pipettes into extraction tubes containing internal standard and extracted as previously outlined, but with a reduced 1.5 mL of extraction solvent. Phosphate buffer (500 µL) was then added to the plastic tubes, swirled around and transferred to another set of tubes for extraction. Finally, 500 µL methanol was added to the plastic tubes, which were swirled again and transferred to a third set of glass tubes for extraction.

Initial recovery of THC from the oral fluid samples was found to be 46.8% (Figure 2-3). Following the buffer rinse, 1.9% more of the spiked THC was recovered, and following the methanol rinse a further 24.5% of the THC was recovered. These results clearly show the hydrophobicity of THC was preventing sufficient transfer of THC from the storage vial to the extraction tubes since the buffer rinse also failed to recover the large portion of THC still left in the container.

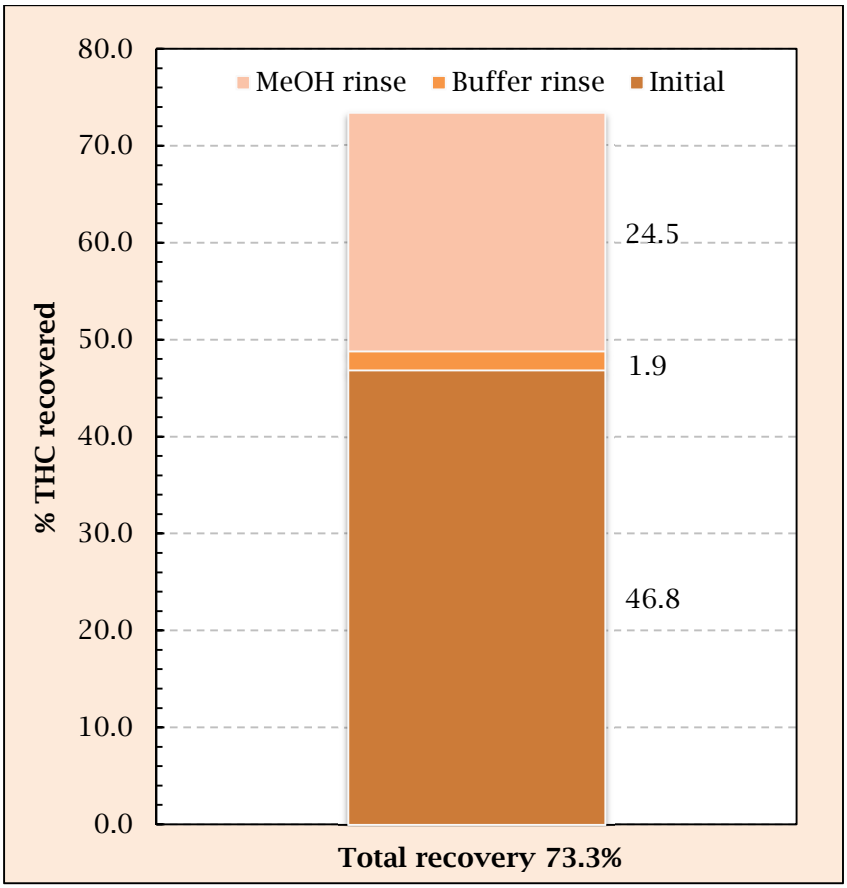

Figure 2-3: Average per cent recoveries of THC after initial transfer of oral fluid samples and subsequent rinses of the plastic containers (n = 3).

This result indicates that a significant amount of loss of THC observed from neat oral fluid samples may be due to immediate losses to the container surface, rather than degradation of the compound. In order to avoid adsorptive losses, it was felt that additives such as those used in commercial buffers may be appropriate. Of course, these must be added at a specific volume to a known volume of oral fluid, otherwise the same uncertainty issues will be faced as with the use of the commercial devices [43, 44].

2.4 Conclusions

THC has been observed to be poorly stable in the short term when stored in neat oral fluid in polypropylene containers, while excellent long term stability is seen in commercial buffer-stabilised samples. These preliminary results indicate that degradation and adsorption may both contribute to the losses observed. Using known volumes of additives in pre-measured volumes of oral fluid may be an alternative to current commercial collection devices that have large variations in the volume of oral fluid collected. The interactions between THC and polypropylene surfaces should be further investigated since a large proportion of losses appear to be related to this activity.

Chapter 3: Adsorptive losses of THC in oral fluid to polypropylene surfaces

Chapter 3: Adsorptive losses of THC in oral fluid to polypropylene surfaces

3.1 Introduction

Following from the conclusions made in chapter 2, it is clear that further investigation into the absorptive behaviour of THC to container surfaces is essential for the development of suitable protocols for sample collection and storage of neat oral fluid. THC is known to be highly lipophilic and poorly water soluble, having a high octanol/water partition coefficient (log P = 6.97) [131]. It is therefore generally accepted that THC can interact with non-polar plastic and glass materials via non-covalent interactions and adsorb to container surfaces, introducing another source of loss. This is especially of concern when dealing with biological matrices including oral fluid due to the aqueous nature of the sample.

Loss of THC via adsorption and metabolic degradation can be overcome by use of buffers containing surfactants and preservatives, a practice followed by many commercial manufacturers supplying oral fluid collecting devices. However, as mentioned previously, the uncertainties of the volumes of oral fluid collected by these devices gives rise to difficulties in interpreting quantitative results.

Since many commercial collection devices utilise stabilising buffers and preservatives in an effort to maximise analyte stability, it is hypothesised that the addition of such additives to an already known volume of oral fluid may be a better way to stabilise a sample without the drawback of unknown dilution factors.

In this chapter, the use of non-ionic surfactants is tested as a means to prevent THC from adsorbing onto the container surfaces. Non-ionic surfactants are generally long chain alcohol molecules that have a hydrophilic part and a hydrophobic part. This allows the molecules to form micelles and lift dirt and oils from surfaces and disperse them in the solution. Triton® X-100 and Tergitol® 15-S-30 were chosen to be

investigated for their potential in minimising the adsorption of THC to container surfaces. These surfactants were selected since they are both mild detergents, are suitably water soluble and have low critical micelle concentrations (CMC) that are required to achieve detergency [109]. Additionally, Tergitol® 15-S-30 has been successfully used to minimise adsorptive losses of urinary THC-COOH [109]. Furthermore, as sodium azide is used as a preservative to minimise microbial action [103], it was also investigated as a potential stabilising agent in conjunction with surfactant to observe degradation trends without interference from any adsorptive effects.

Sample containers are commonly made from polypropylene and such containers have been used in recent studies involving oral fluid [35, 50, 58, 100]. Polypropylene was therefore chosen again for these experiments to investigate the adsorptive properties of THC to plastic surfaces when in the oral fluid matrix and also to observe any losses that occur during storage for up to four weeks.

The aims of this chapter are:

- To investigate factors governing the interaction of THC with polypropylene, including the effect of silanisation, concentration of THC in the sample, surface area contact between sample and container and;

- To explore the potential use of non-ionic surfactants and preservatives to overcome such interactions and degradation so as to improve THC recovery from neat oral fluid samples.

3.2 Materials and methods

3.2.1 Materials

All solvents and chemicals used were analytical grade or higher. Methanol, toluene, n-hexane and ethyl acetate were obtained from Chem-Supply (Gillman, SA, Australia). Triton® X-100, Tergitol® Type 15-S-30 and dichlorodimethylsilane (DCMS) were obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). All water used was purified using a Sartorius Arium® Milli-Q system. Cerilliant® standards of THC (1 mg/mL in methanol) and d_3-THC (0.1 mg/mL in methanol) were purchased from Novachem (Collingwood, VIC, Australia). These reference standards were diluted with methanol to obtain working stock solutions for THC at 50 µg/mL and d_3-THC at 25 µg/mL. Na_2HPO_4 and NaH_2PO_4, used to make 0.1 M Sørensen's phosphate buffer were sourced from Ajax Chemicals (Taren Point, NSW, Australia) and mixed to achieve a pH of approximately 6. BSTFA with 1% TMCS was sourced from PM Separations (Capalaba, QLD, Australia). Sodium azide was obtained from BDH Laboratory Supplies (Poole, England), and Cozart® DDS buffer was obtained from Alere (Brisbane, QLD, Australia).

THC-free oral fluid was provided from volunteers and used for the study on the day of collection. The absence of THC in collected oral fluid was confirmed by following the sample preparation and analysis procedures without the addition of any THC standards, as described in the following paragraphs.

3.2.2 Sample preparation

Aliquots of freshly collected drug-free oral fluid were transferred into 2-mL polypropylene graduated micro tubes (Figure 3-1) manufactured by Scientific Specialties Inc. (Lodi, CA, USA) (Item number 1310-00).

Figure 3-1: Polypropylene graduated micro tubes used for all experiments involving plastic surface contact.

The oral fluid was then spiked with THC standards in methanol at various specified concentrations depending on the experiment performed. The introduction of methanol to the samples was unavoidable for these experiments; however, the absolute concentration of methanol in these spiked oral fluid samples was generally less than 4%. After capping, the sample tubes were vortex-mixed to ensure complete contact between sample and plastic surface. Samples thus prepared were pipetted or decanted into 10-mL screw-cap glass test tubes. Where 2-mL centrifuge tubes were used and samples transferred by decanting, each tube was weighed before and after sampling to account for liquid loss from the decanting process. Deuterated internal standard solution (d_3-THC) was added to the glass test tubes followed by addition of 0.1 M Sørensen's phosphate buffer (1 mL). For LLE, hexane/ethyl acetate (2.5 mL; 9:1 v/v) was then added and the tubes placed on a roller mixer on moderate speed for 60 min. The organic upper layers (2 mL) were transferred in 1 mL aliquots into high recovery GC vials (1.5 mL; PM Separations, Brisbane, Australia) and evaporated under a gentle stream of N_2 gas at 40 °C. Residues were reconstituted in 75 µL ethyl acetate and 50 µL BSTFA with 1% TMCS and heated at 75 °C for 20 min before analysis by GC–MS.

3.2.3 GC–MS

Chromatographic analyses were carried out using an Agilent 7890A/5975C GC–MS system with an Agilent 19091S capillary column (30 m × 250 µm × 0.25 µm). Splitless mode was used for injection and 2 µL was injected. The injector temperature was

280 °C. The oven was operated at an initial temperature of 140 °C for 1 min, then increased at 40 °C/min to 300 °C and held for 2 min. MS was operated in SIM mode monitoring the TMS derivatives of THC and d_3-THC at *m*/*z* 386, 371 and 303 for THC and *m*/*z* 389, 374 and 306 for d_3-THC. Ions *m*/*z* 371 and 374 were used for quantification purposes and the others were used as qualifiers. All calibration standards and samples were run with triplicate injections.

Data acquisition and analysis were performed using the included Agilent MSD ChemStation software package and Microsoft Excel 2010.

3.2.4 Validation studies

Analyses were conducted using a GC–MS for all experiments presented in this chapter as the LC–MS/MS utilised for the previous studies in chapter 2 was unavailable. Had this not been the case, there would have been a potential risk of the non-ionic surfactants presenting contamination issues and increased matrix effects with the LC–MS/MS system [132]. Therefore, the LLE method was again fully validated for measuring THC in oral fluid by GC–MS.

Serial dilution of the working stock solution of THC gave eight concentrations of THC in methanol: 50, 25, 10, 5, 2.5, 1, 0.5, and 0.25 μg/mL. Ten microlitres of each of these eight solutions was spiked into a separate tube containing 500 μL of drug-free oral fluid resulting in concentrations of 1000, 500, 250, 100, 50, 25, 10, and 5 ng/mL in the neat sample. Ten microliters of internal standard solution (25 μg/mL) was then added to each tube giving a concentration of 500 ng/mL of d_3-THC. After addition of 1 mL of 0.1 M Sørensen's phosphate buffer, the LLE procedure was followed as previously described. Linearity of the calibration curve was calculated using a line of best fit with the acceptable correlation factor set at >0.99. Inter- and intra-day precision and accuracy studies were carried out using QC samples spiked at 15, and 75 ng/mL (n = 5). Accuracy was determined to be acceptable if the calculated concentrations fell within 15% of the concentration spiked (expressed as an MRE). Precision was deemed acceptable if the RSD was <15%. The LOD was the lowest concentration of analyte

that was observed as a peak in the chromatograms at all monitored ion fragments. The LOQ was the lowest concentration of analyte that could be quantified with an RSD of <20% and an MRE of <20%.

3.3 Results and discussion

3.3.1 Method validation

Following the development of the method, validation was performed. Linearity of the GC–MS method was achieved over the range of 5–1000 ng/mL with an average correlation coefficient of 0.9990. Figure 3-2 is an exemplar calibration curve obtained using this method.

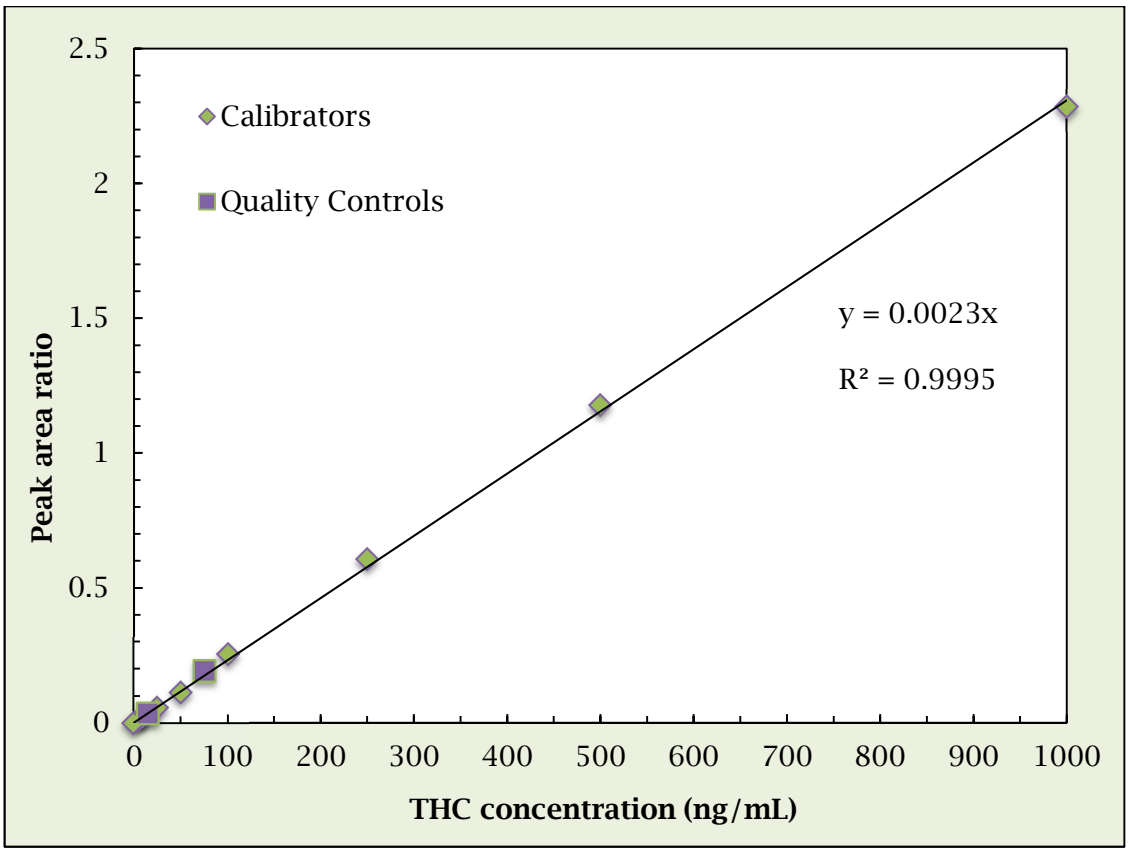

Figure 3-2: An example calibration curve for the GC–MS method showing calibrators and QC samples.

The intra- and inter-day precision and accuracy of the method were satisfactory and are summarised in Table 3-1. The RSD values found for the QC samples were 2.52–8.57% and the MRE ranged from 1.38% to 6.48%.

Table 3-1: Intra- and inter-day precision and accuracy results for the LOQ and QC samples (n = 5).

Concentration level (ng/mL)	Intra-day precision (RSD%)	Intra-day accuracy (MRE%)	Inter-day precision (RSD%)	Inter-day accuracy (MRE%)
5	3.14	9.73	1.81	8.32
15	8.57	6.48	8.18	1.99
75	2.52	1.38	6.37	5.37

The LOD was determined to be 1 ng/mL. The LOQ was found to be 5 ng/mL; precision and accuracy at this concentration level was determined to be 3.14% and 9.73% respectively.

3.3.2 Silanisation of glassware

Silanising glassware is a common practice to minimise interactions between sample analytes and glass surfaces. Some publications have indicated that silanised glassware is utilised in analyses involving THC to minimise adsorbance to glass walls [84, 104, 105]. An experiment designed to assess the benefit of silanising glassware used in the extraction process of THC from oral fluid was performed and found that no distinction could be made between the treated and untreated glassware (Figure 3-3).

Silanisation of glassware was performed by filling extraction tubes with, and submersing GC vials in the silanising solution (5% DCMS in toluene) and leaving refrigerated overnight. Tubes and vials were then emptied and rinsed once with toluene and then twice with methanol and allowed to dry. Eight extractions were performed, four using glassware that had not been treated, and four using glassware that had been silanised. Triplicate injections were run by GC–MS and found an average peak area response of 5638 for extractions using untreated glassware and 5608 for extractions using the treated glassware.

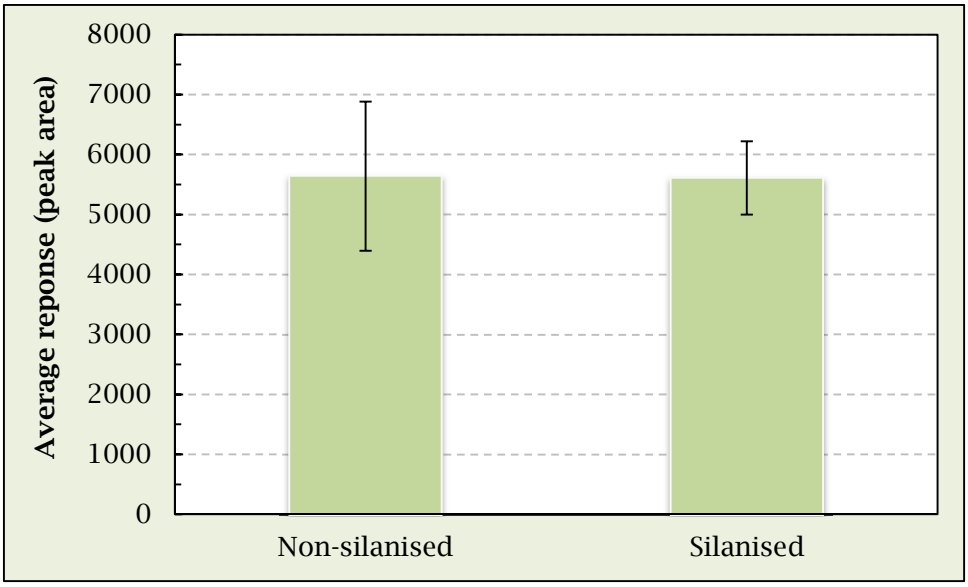

Figure 3-3: Average response of THC after extraction using either untreated glassware or silanised glassware (n = 4).

The lack of improvement in recovery when using silanised glassware may be explained by the fact that all processes involving glassware also involve organic solvents, i.e. the extraction solvent in the extraction tubes and the acetonitrile in the GC vials, which already effectively minimise THC adsorption to the glass surfaces. Therefore it appears that silanisation is unnecessary for such procedures and focus should be on minimising adsorption to the plastic surfaces the THC is exposed to whilst in a mostly aqueous environment.

3.3.3 Concentration of THC on rate of adsorbance

It was hypothesised that lower concentrations of THC in oral fluid may result in a higher rate of adsorbance to container walls relative to the concentration present due to less competition for any active sites where the THC may bind.

To test this hypothesis, THC was spiked into 1.5 mL of neat oral fluid at six concentration levels: 25, 50, 100, 250, 500 and 1000 ng/mL (n = 3) in 2-mL capped centrifuge tubes. After thorough mixing using a vortex mixer, 1 mL of each sample was withdrawn by pipette and analysed.

All samples experienced a similar degree of THC loss to the polypropylene tubes, ranging from 22.8% to 29.3% (Figure 3-4). The concentration of THC in oral fluid samples of equal volume did not appear to affect the degree of adsorption to the plastic surface.

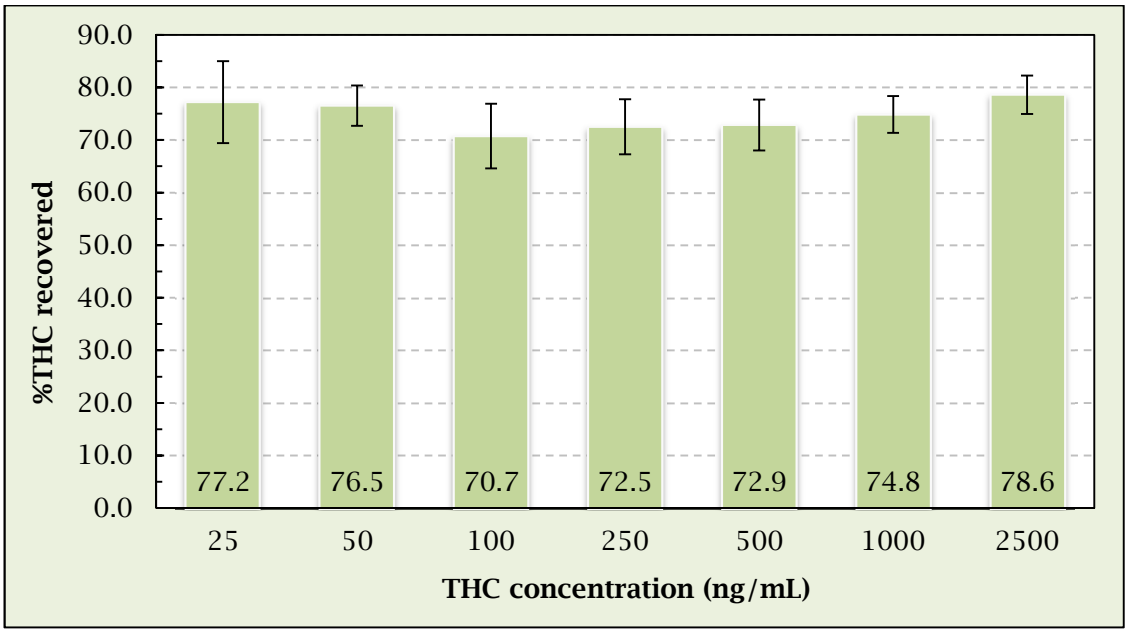

Figure 3-4: Recovery of THC from polypropylene tubes at various concentrations in 1.5 mL oral fluid. Data values represent the mean; error bars represent the standard deviation (n = 3).

It was anticipated that lower concentrations of THC would lead to a higher loss as there might be less competition for adsorbing space on the plastic surface; however this was not observed under these experimental conditions. This may be due to the presence of proteins or other materials in oral fluid that bind with THC and help prevent it from adsorbing to the container surface. This idea is further investigated in Section 3.3.6.

3.3.4 Surface area ratio on rate of adsorbance

In contrast to the previous experiment, when different volumes (0.5, 1.0 and 1.5 mL) of oral fluid containing the same concentration of THC (100 ng/mL) were tested in the same 2-mL tubes, there was an apparent trend of increasing loss in lower oral fluid volumes (p = <0.001) (Figure 3-5).

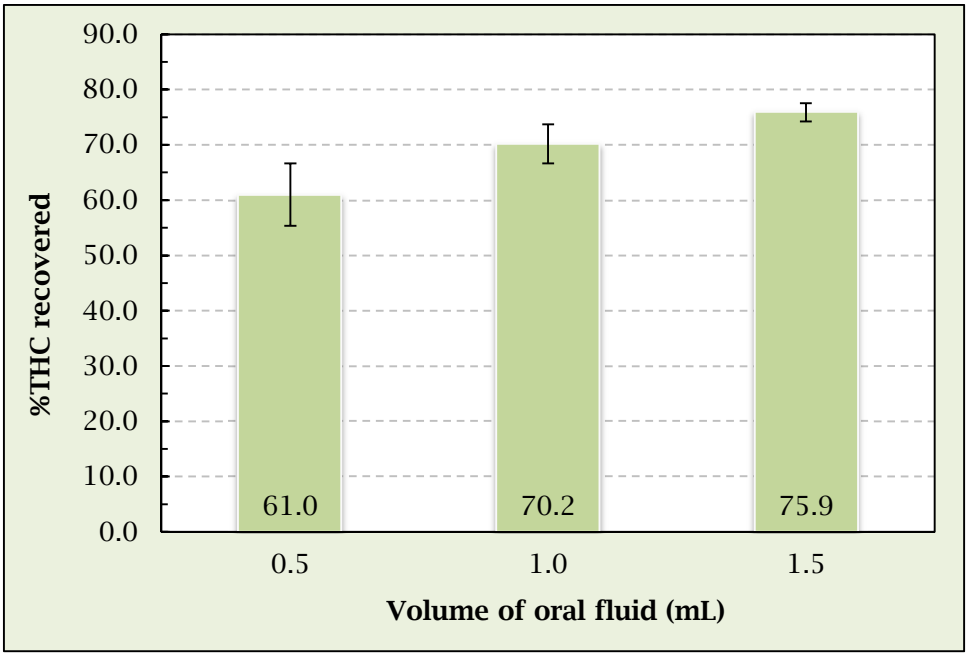

Figure 3-5: Recovery of THC from oral fluid volumes of 0.5, 1.0 and 1.5 mL all spiked at 100 ng/mL THC in polypropylene tubes. Data values represent the mean; error bars represent the standard deviation (n = 5).

These results demonstrate that the loss of THC to plastic surfaces when in the neat oral fluid matrix is relative to the surface area to volume ratio with larger ratios resulting in a greater loss. Choi *et al.* [100] made a similar observation when they found that a higher THC loss over six days occurred in polypropylene containers of a larger internal diameter. This can be explained by the larger surface area to volume ratio of the containers with larger internal diameter.

3.3.5 Non–ionic surfactant as a desorbing agent

Triton® X-100 (Triton) and Tergitol® 15-S-30 (Tergitol) were sourced to investigate whether recovery of THC from container walls can be improved by adding a non-ionic surfactant to the sample. Tergitol is a white solid at room temperature and has a CMC of 558 ppm [109, 133]. A 1000 ppm solution of Tergitol was prepared; however this was found to be time consuming as extensive vortex mixing was required despite the material being highly water soluble. Eventually the surfactant dissolved and left overnight to settle. Due to the difficulty in dissolving Tergitol in a concentration barely twice its CMC, this surfactant was not investigated any further as additional

dilutions reducing its concentration to below the CMC would have been unavoidable when added to samples.

Triton is a viscous liquid at room temperature with a lower CMC than the Tergitol at 189 ppm or ~0.02% by weight [134]. The structure of Triton is shown in Figure 3-6; where *n* = approximately 9.5 ethylene oxide units. A stock solution of 5% Triton was prepared by adding 1 mL Triton to 19 mL water. The solution was mixed using a vortex and left to settle overnight.

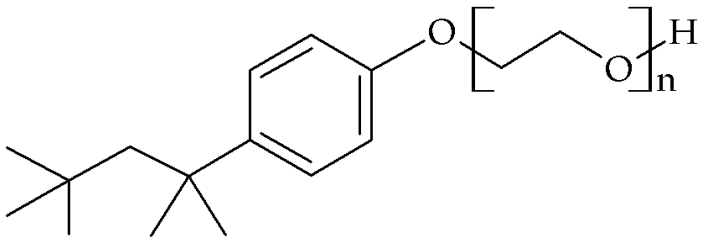

Figure 3-6: Chemical structure of Triton® X-100 non-ionic surfactant. Subscript "n" signifies an average of 9.5 ethylene oxide units per molecule.

The effects of adding Triton to oral fluid containing 100 ng/mL THC were trialled at different concentrations of the surfactant. Concentrations of Triton were trialled above its CMC at 0.02%, 0.5% and 1% (v/v) in oral fluid. It was found that in comparison to samples extracted directly from glass tubes, samples made in plastic tubes containing Triton and then transferred to glass tubes for extraction returned 52.3%, 81.7% and 81.2% of the spiked THC respectively. It was clear that 0.02% Triton was not sufficient to effectively increase THC recovery; however there appears to be a plateau effect occurring by 0.5% Triton. Higher concentrations of Triton cause interferences in the GC–MS, hence, a second experiment was carried out to determine the lowest concentration of Triton that still gives a suitable improvement in recovery. This experiment investigated the efficiency of the surfactant to desorb THC at Triton concentrations of 0.05, 0.1, 0.2 and 0.5% in oral fluid samples (n = 3). Recovery of THC improved by 14, 28, 31, 52 and 57% respectively for the concentrations tested when compared to untreated samples. Taking into account the standard deviation, improvement in recovery was insignificant between the 0.2% and 0.5% samples. It

was therefore decided that for all future experiments involving Triton, a concentration of 0.2% (approximately 10 × CMC) would be used.

THC loss to the plastic was found to be significantly minimised during repeated experiments in which Triton was mixed with the THC-spiked oral fluid samples before transfer to the glass test tubes for LLE. As shown in Figure 3-7, the use of Triton resulted in >96% recovery of THC from the polypropylene containers when compared to the untreated samples at all oral fluid volumes tested.

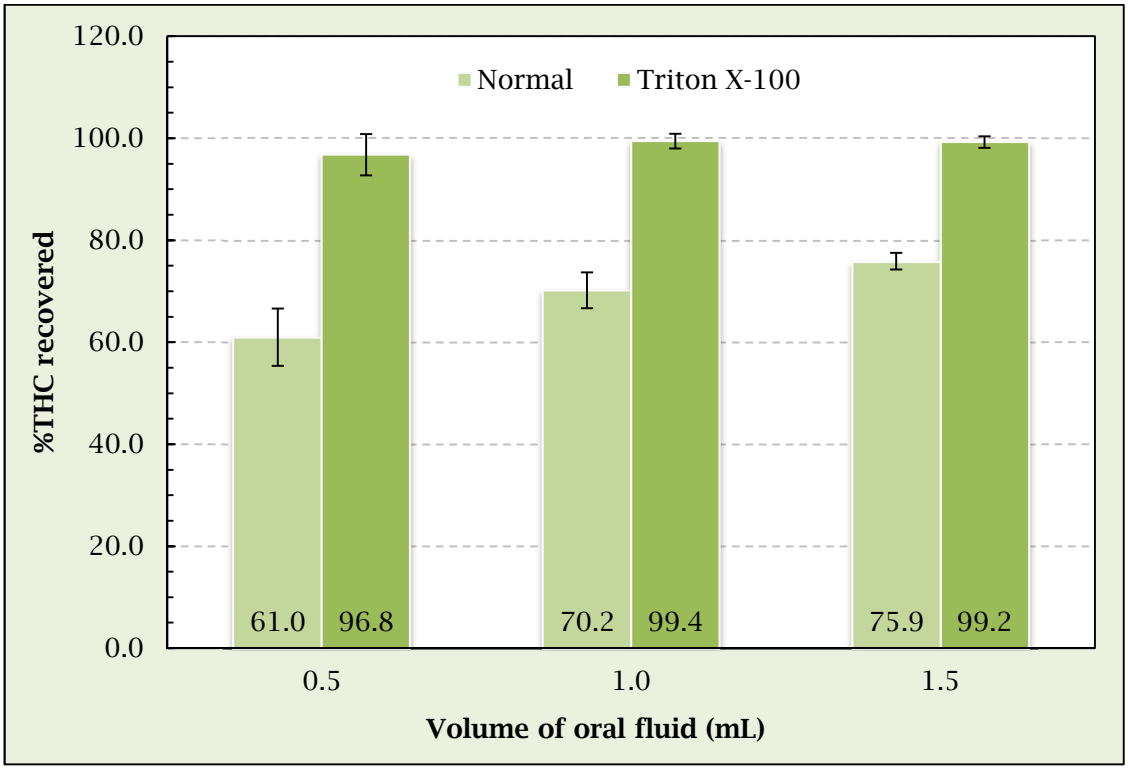

Figure 3-7: Recovery of THC from oral fluid volumes of 0.5, 1.0 and 1.5 mL not treated (normal) and treated with 0.25% Triton, all spiked at 100 ng/mL THC in polypropylene tubes. Data values represent the mean; error bars represent one standard deviation (n = 5).

It was also noted that the absolute signal intensities of d_3-THC following extraction from the glass test tubes increased significantly when Triton was present compared to the control experiments in which the surfactant was not utilised (Figure 3-8).

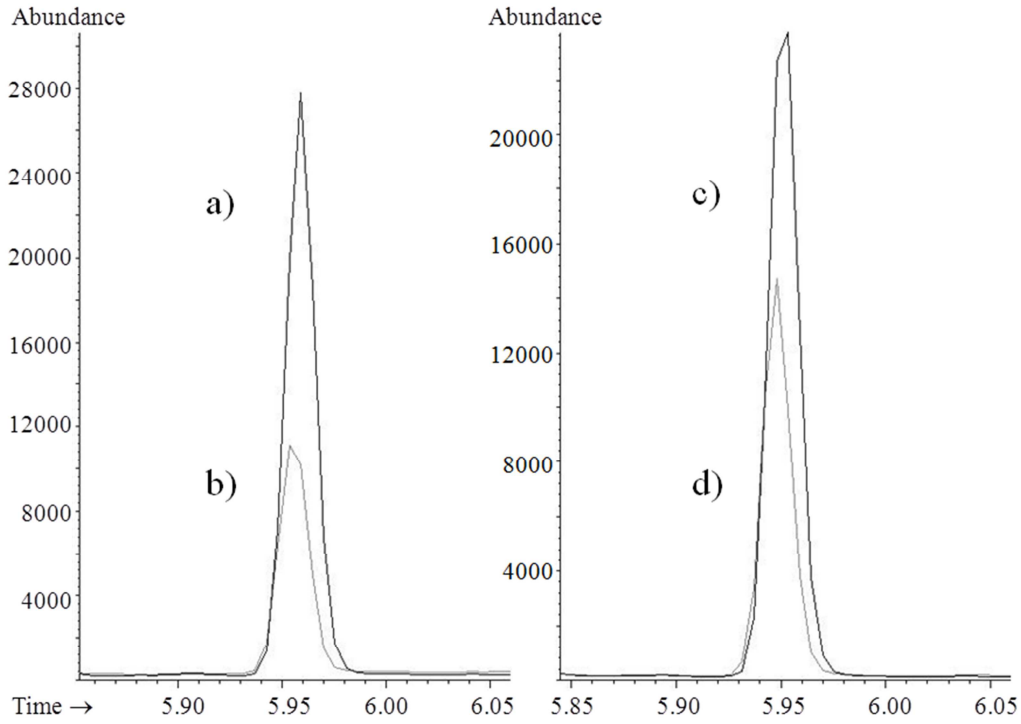

Figure 3-8: GC–MS chromatograms showing increased signal intensity from 1 mL samples spiked with 100 ng/mL THC and internal standard after transfer and extraction; a) m/z 371 (THC) from a Triton-treated sample; b) m/z 371 from an untreated sample; c) m/z 374 (d_3-THC) from a Triton-treated sample; d) m/z 374 from an untreated sample.

During the course of this investigation, it was demonstrated that the THC had not degraded in the short term since the addition of non-ionic surfactant Triton increased recovery of the spiked THC to almost 100%. Other researchers have previously published studies describing loss of THC over time [41, 58], but none have given data on immediate losses thus far.

3.3.6 THC distribution in stored neat oral fluid

The homogeneity of oral fluid with regards to THC distribution was evaluated by taking multiple aliquots from a single spiked sample and comparing the concentration of THC found in each aliquot. Two samples were prepared with THC spiked into 12 mL of oral fluid in a 25-mL polypropylene container at 100 ng/mL. After mixing, four aliquots of 500 µL each were transferred from the first sample into separate tubes for extraction. The second sample was refrigerated overnight. After refrigeration, the second sample appeared to have a viscous, more opaque material at

the bottom of the container. Without disturbing the sample too much, three aliquots of 500 µL were taken from the less viscous liquid nearer the top and one aliquot was taken from the bottom including the more viscous portion. The eight samples were extracted and analysed and the resulting concentrations measured are shown in Figure 3-9.

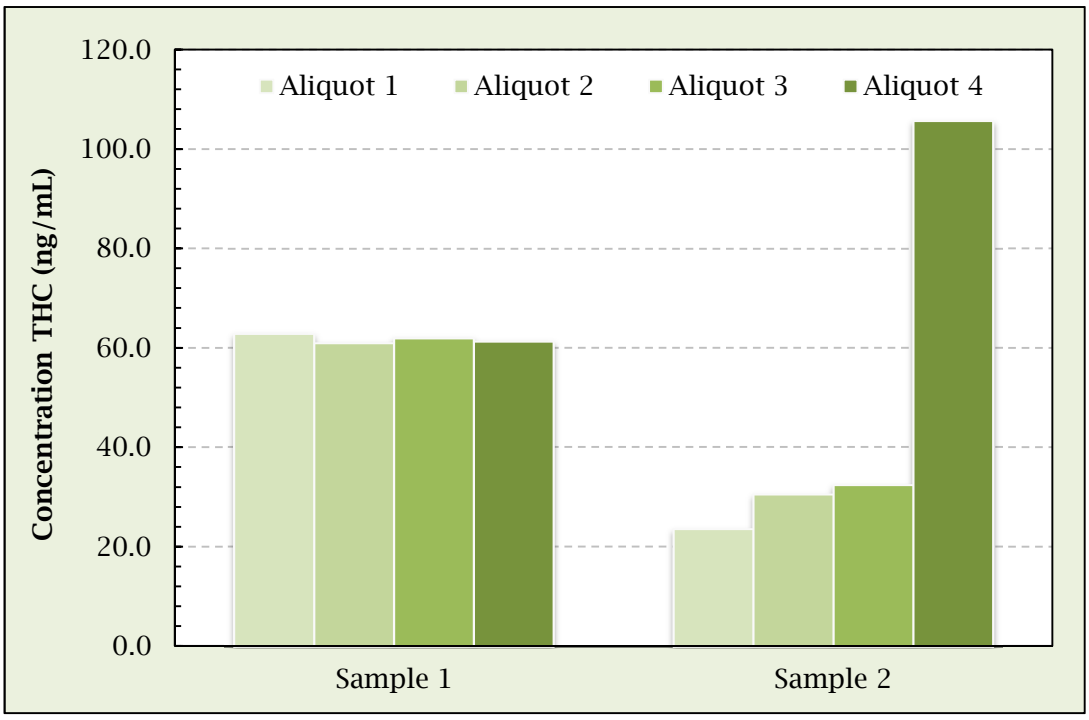

Figure 3-9: THC concentration in each aliquot taken from two samples. Sample 1 shows good homogeneity while sample 2 shows much more THC was recovered from the more viscous part of the sample after settling.

The THC concentrations estimated for sample 1 indicate good homogeneity in the freshly mixed sample. Sample 2 however, showed that significantly more THC was found in the more viscous part that had been allowed to settle in the container, and conversely, a reduced amount was found in the less viscous portion of the sample. These findings suggest that THC may be interacting with the proteinaceous part of oral fluid. To further test this hypothesis, the two samples were returned to the refrigerator and a further five aliquots of 500 µL each were removed for testing after 16 days. Aliquots 1 and 2 were taken from the top of the sample without disturbing the viscous part that had settled at the bottom. Aliquot 3 was then taken from the bottom. The samples were then mixed by shaking until the more viscous portion

dispersed and aliquots 4 and 5 were then taken. As expected, aliquot 3 which contained the bulk of the more viscous material showed high concentrations of THC present, even higher than the original concentration spiked in sample 1 (Figure 3-10). Clearly, THC interacts with the proteinaceous component of oral fluid, but this interaction may also be providing protection from adsorption to container surfaces. Regardless, these results highlight the importance of sufficiently homogenising neat oral fluid samples before taking aliquots for analysis, as resulting concentrations may be over- or underestimated if samples have not been properly mixed after a period of storage.

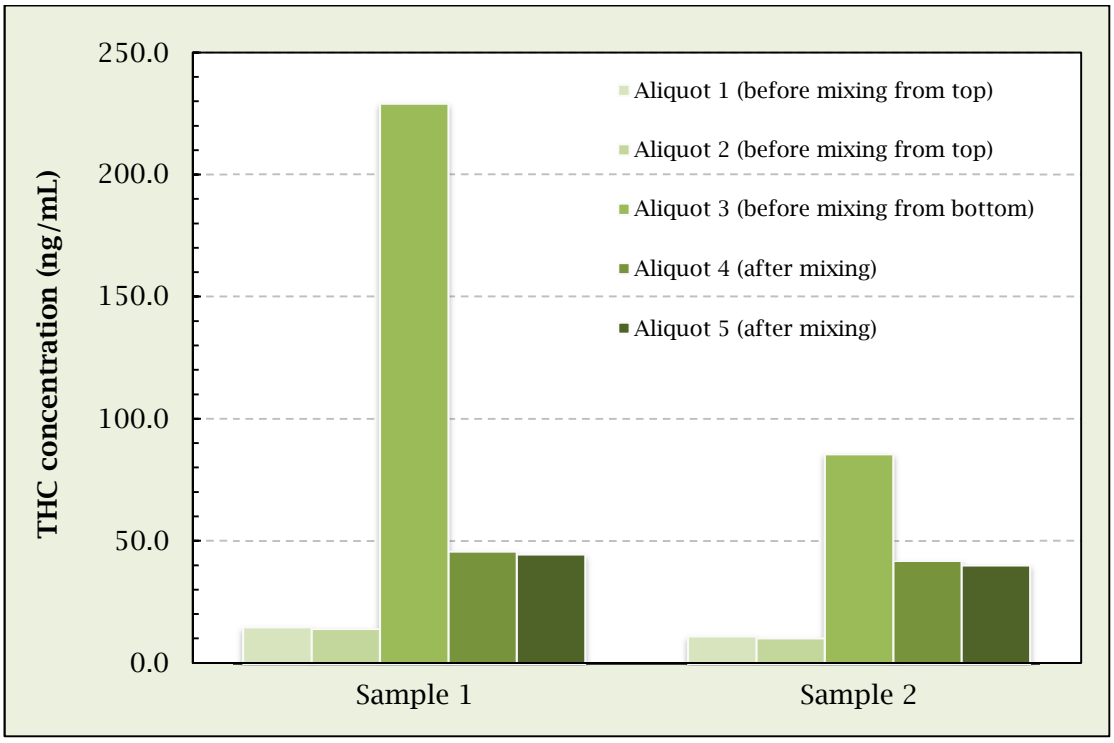

Figure 3-10: THC aliquots taken from two samples that had been stored refrigerated for 16 days. Aliquots 1 and 2 taken from the top of the sample show minimal recovery of THC; aliquot 3 taken from the settled material at the bottom contained a significantly higher amount of THC; aliquots 4 and 5 show recovery after the samples were mixed.

In order to investigate the role of oral fluid proteins in THC recovery from the plastic surface, the workflow shown in Figure 3-11 was followed.

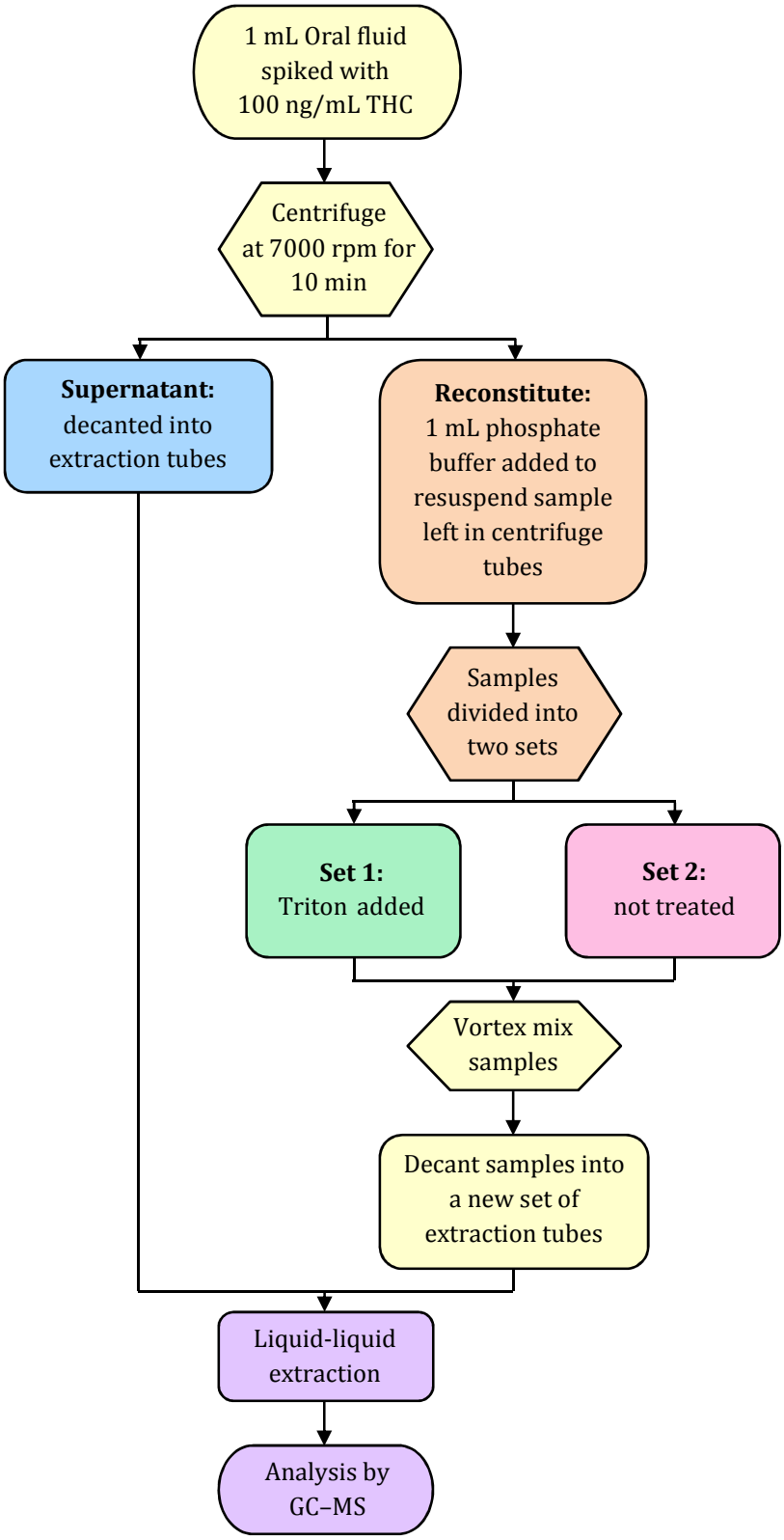

Figure 3-11: Flow diagram showing the treatment method for centrifuging and separating samples.

THC-fortified oral fluid samples (1.5 mL in 2-mL tubes) were centrifuged at 7000 rpm for 10 minutes. The supernatant was decanted into extraction tubes for analysis and

the resulting protein pellets were reconstituted in phosphate buffer. The reconstituted samples were either treated with Triton or left untreated before extraction. The three sets of samples were compared with regards to THC concentration.

It was found that 51.7% of the spiked THC was recovered from the protein pellet fraction, while 28.8% of THC was recovered from the supernatant, giving a total THC recovery of 80.5%. When Triton was added into the polypropylene tube that contained the protein pellet an additional 14.7% of THC was recovered, raising the total THC recovery to 95.2% (Figure 3-12). This addition of Triton resulted in a significant effect on the recovery of THC from the supernatant (p <0.001).

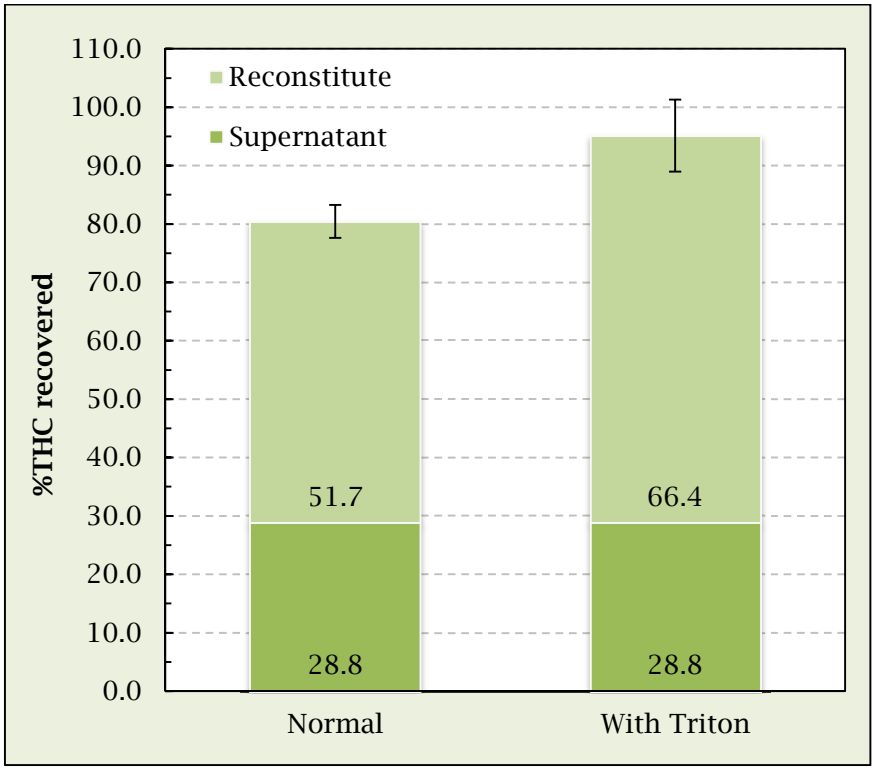

Figure 3-12: Recovery of THC from the supernatant and reconstitute of protein pellets after centrifugation of oral fluid samples. THC was spiked at 100 ng/mL in polypropylene tubes. Samples without treatment of Triton (normal) are represented on the left, on the right are the samples treated with Triton after supernatant was removed. Data values represent the mean; error bars represent one standard deviation (n = 5).

To further investigate the role of proteins in THC recovery from plastic surfaces, THC was spiked into Sørensen's phosphate buffer and its recovery was compared with that fortified in oral fluid. When THC (100 ng/mL) was spiked into phosphate buffer (0.5

and 1.5 mL), a very low recovery was observed: 24.5% in 0.5 mL buffer and 48.1% in 1.5 mL buffer (Figure 3-13).

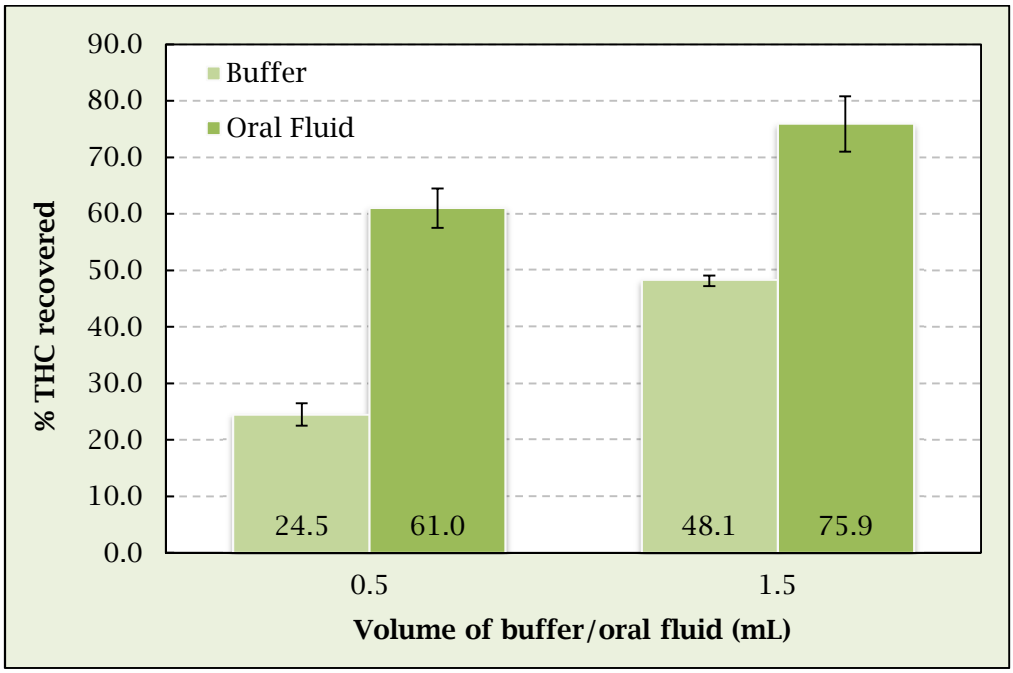

Figure 3-13: Comparison of THC recovered from phosphate buffer and oral fluid spiked into 0.5 and 1.5 mL at 100 ng/mL concentration in polypropylene tubes. Data values represent the mean; error bars represent one standard deviation (n = 3).

The extraction efficiency of the LLE method was also determined for THC spiked into phosphate buffer with and without the addition of Triton. These extractions were carried out directly in the glass test tubes and did not involve any transfer from plastic tubes. The results showed a high recovery of THC even without the additive. From the plain phosphate buffer extractions approximately 90% of THC was recovered; when Triton was present, the recovery increased to 106% when compared to control samples where THC was spiked directly into mobile phase.

The supernatants of centrifuged samples had such a low recovery of THC compared to the protein-rich reconstitute (Figure 3-12) supporting the theory that THC is bound in some way to constituents of oral fluid which helps keep it from adsorbing to the container surface. Additionally, a very low recovery was found from plain phosphate buffer when compared to oral fluid spiked at the same THC concentration in the same volumes (p <0.001), which further supports the possible role of proteins in binding

THC in oral fluid. Protein binding of THC also explains the low absolute recovery of both THC and d_3-THC observed from oral fluid during the LLE process conducted in glass test tubes compared to the much higher recovery when extracting from plain phosphate buffer. It is noteworthy that at this stage it is unknown what specific component or components in the protein-rich fraction of oral fluid are responsible for binding THC.

3.3.7 Stability of treated samples over one month

Sodium azide (NaN_3) is commonly used as a preservative for biological specimens [2, 129]. Additives such as this can inhibit bacterial growth that can be helpful in preventing degradation of THC due to microbial action. Ventura *et al.* [103] observed reduced degradation in samples treated with 0.1% sodium azide. Furthermore, as the Cozart® DDS buffer is known to contain sodium azide (see Appendix B) this additive was chosen to be investigated for its usefulness on THC recovery in oral fluid in various storage protocols over one month. Oral fluid (250 μL) fortified with THC (50 ng/mL) in 2-mL polypropylene centrifuge tubes was mixed with 750 μL of either Sørensen's phosphate buffer alone, Sørensen's phosphate buffer with 1% sodium azide (resulting in a concentration of 0.75% azide in the samples), or the Cozart® DDS buffer containing 0.1% azide. This 1:3 dilution was chosen to be in line with the dilution factor of the buffer in the DDS device. These samples were stored either at 4 °C in a refrigerator or at 20 °C in a cabinet for up to four weeks. After the addition of 50 μL of 5% Triton to eliminate adsorption as a cause of loss, samples were mixed and 500 μL aliquots were taken for analysis of THC content.

As shown in Figure 3-14, samples treated with the DDS buffer suffered only a minimal loss of THC over the four week period while refrigerated, but even at room temperature the losses were small. More loss was observed in samples treated with phosphate buffer and azide; however these losses were still minimal up to three weeks into storage in a refrigerator. The samples kept at room temperature however, showed a larger loss and both treatments resulted in 40–50% loss of THC by the end of the four weeks. Whilst the phosphate buffer treated samples lost more THC by the

fourth week, up until then, there was minimal difference between the concentrations of THC found in these samples and the samples treated with azide.

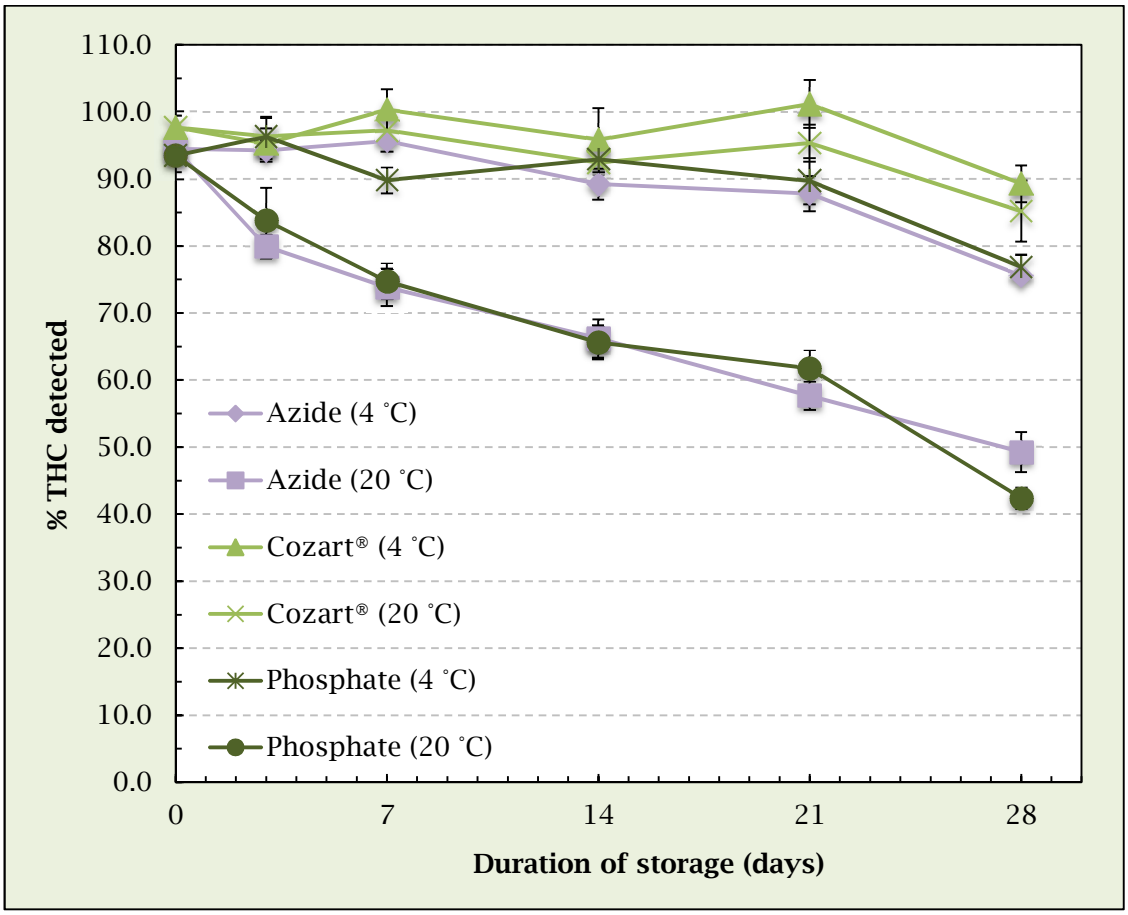

Figure 3-14: Percentage of THC remaining in oral fluid during storage from three to 28 days in the presence of 0.1 M phosphate buffer, 0.75% sodium azide solution and Cozart® DDS buffer solution. Results are means from replicate samples (n = 4) with standard deviations represented by the error bars.

From the results of the preliminary stability study presented in Section 2.3.4, it was found that THC has excellent stability in the Cozart® buffer with an average recovery of 89% when stored in a refrigerator (4 °C) for 13–18 months. The current study found that even at room temperature (20 °C, in the dark), there is minimal loss over a four week period. Ventura *et al.* [103] reported that adding sodium azide (0.1%) to oral fluid helped to prevent degradation of several illicit drugs during up to seven days of storage at 25 °C and 37 °C and up to two months at 4 °C and –20 °C. Sodium azide is also present in many commercial oral fluid collecting buffers including the Cozart® DDS buffer which contains 0.1% sodium azide. In this study, azide did not seem to give any benefit over phosphate buffer as an additive to minimise loss, as both sets of

samples showed a loss of almost 25% from refrigerated samples and over 50% from the room temperature samples after a four-week period, even though an excess of azide was added. These results show that azide had no protective effect on THC in oral fluid, at least not over the four-week period studied, although its longer-term effect remains to be further studied. Conversely, the samples stored in Cozart® buffer were only observed to lose 10% of added THC when refrigerated and 15% at room temperature after four weeks of storage. Unlike previously published stability studies involving THC in oral fluid [58, 100, 103], this study reduced the possibility of losses occurring due to adsorptive processes by adding Triton during sample analysis, since this was shown to significantly increase recovery of THC from plastic surfaces. Therefore it can be assumed that the losses seen in this present study are due to the degradation of THC and not adsorption. It can also be assumed that since the addition of sodium azide in large excess would have prevented any microbial growth, the degradation losses of THC observed under these experimental conditions are not due to microbial action either, but to other factors such as oxidative degradation. Moore *et al.* [110] previously reported that THC loss during storage may be exacerbated by chemical reactions induced by oxygen or other environmental factors such as fluorescent lighting. Exposure to light and oxygen was minimised during the current study by storing the samples in the dark and using smaller tubes to limit the available headspace above the samples during storage. This may explain the difference between these results and the previous finding shown in Section 2.3.3 that more than 50% of the spiked THC was lost in just one day (Figure 2-1) after storage at room temperature when larger 12-mL tubes were used and left on the bench exposed to fluorescent lighting. These results suggest that addition of anti-oxidants rather than azide into oral fluid may be more beneficial in THC preservation.

3.4 Conclusions

It has been demonstrated that THC has the tendency to bind to polypropylene surfaces, particularly in aqueous phases, leading to poor overall recovery from neat oral fluid. The recovery of THC is dependent on the oral fluid volume to inner surface area ratio; smaller oral fluid volume in larger containers suffered from a higher loss of THC regardless of the concentration of THC in the sample. Use of a non-ionic surfactant such as Triton can significantly increase the THC recovery from polypropylene containers and thus reduce adsorptive losses. The addition of Triton or another suitable non-ionic surfactant can also be used as a tool in future studies to investigate the individual factors affecting stability and recovery of THC in oral fluid. THC binds with the proteinaceous components in oral fluid, so thorough mixing is required to ensure a representative sample is obtained after a period of storage. This binding helps to reduce THC adsorption to container surfaces; however, it is also responsible for reducing the extraction recovery rate. Addition of sodium azide did not appear to provide any further benefit, suggesting THC is not being lost to microbial action in the first month of storage. It is further suggested that perhaps anti-oxidants may be a helpful additive instead of, or combined with, azide. Given the current importance of detecting THC in oral fluid to society, further research into understanding other factors that govern the behaviour of THC in this biological matrix is warranted.

Chapter 4: Sativex® and roadside drug testing

Chapter 4: Sativex® and roadside drug testing

4.1 Introduction

Sativex® (nabiximols) is an oromucosal spray containing THC and CBD, indicated for the treatment of spasticity in multiple sclerosis in the UK and a number of other countries [135]. Sativex® has recently been registered by the Therapeutic Goods Administration (TGA) in Australia as the first cannabis-based medicine [136], however, changes to the Poisons Schedule are still outstanding so it is not yet available for prescription. Since THC is the target analyte of roadside oral fluid testing for illicit cannabis use in Australia, the introduction of Sativex® to the market may have implications for patients who drive.

The oromucosal delivery of the Sativex® medicine results in lower and later peaking of blood concentrations of THC compared to when cannabis is smoked. A dose of Sativex® containing 21.6 mg THC was observed to give a peak plasma concentration of 5.40 ng/mL at 60 min [135], while smoking cannabis with 33.8 ng/mL THC was shown to give a peak plasma concentration of 193 ng/mL at 12 min [29]. The lower concentration occurs due to slower absorption coupled with fast redistribution into fatty tissues. This results in patients generally experiencing less potent effects from THC than those who smoke cannabis. GW Pharmaceuticals advise patients in the UK that Sativex® may cause dizziness and somnolence and that they should not attempt to drive if they are experiencing "significant CNS effects" [135]. The law in the UK allows individuals to drive whilst taking prescription medicines providing they are not "under the influence" of the medicine [137]. Recently, blood concentration limits for eight controlled drugs were enforced in the UK with the limit for THC being 2 ng/mL [138].

Although the blood concentrations of THC are lower in Sativex® patients, and they are perhaps not significantly impaired whilst on the medication, oral fluid concentrations may still be quite high due to the deposits left in the mouth from the spray. This is relevant since in Australia, roadside drug testing of oral fluid is used

solely to identify drug use and does not take impairment (or lack thereof) into consideration.

Cannabinoid levels in oral fluid following Sativex® dosing have recently been published by Lee *et al.* [95]. It was found that THC and CBD concentrations peaked at well over 1000 ng/mL and both were still detectable (1.0–60.0 ng/mL THC and 0.5–67.8 ng/mL CBD) up to 10.5 h after a single two-spray dose. Due to the physical nature of an oromucosal spray and the high concentrations of THC present, it is hypothesised that a recent dose of Sativex® may result in positive test results for THC using roadside screening tests currently used in NSW, Australia.

Two different screening tests are utilised in New South Wales. The tests target THC, methamphetamine and MDMA. The first screening test employed is the DrugWipe® II Twin. Following a positive result, an oral fluid sample is taken and analysed using the Cozart® RapiScan device. If this test also gives a positive result, the sample of oral fluid collected by the RapiScan device is stored for further confirmatory analysis [139]. Other Australian states employ a similar testing program, however some utilise updated instrumentation such as the Cozart® DDS as used in this study, which has lower reported limits of detection than the Cozart® RapiScan (31 ng/mL versus 150 ng/mL for THC in undiluted oral fluid [63, 116]).

This chapter aims to determine whether the use of Sativex® will trigger a positive result for THC in two oral fluid screening tests employed by the police in random roadside drug testing programs in NSW, Australia, and to quantify THC and CBD levels in the oral fluid of patients in the two hours following high and low doses of Sativex®, using LC–MS/MS.

4.2 Materials and methods

4.2.1 On–site and laboratory materials

Vials of Sativex® and placebo were obtained from GW Pharmaceuticals, UK. DrugWipe® II Twin tests (Figure 4-1a) were obtained from Pathtech (Preston, VIC, Australia). Cozart® DDS oral fluid collection kits and the handheld screening device (Figure 4-1b,c) were obtained from Alere™ (Brisbane, QLD, Australia) as were the standalone Concateno Certus® Oral Fluid Collection Kits. The DDS kits contain 1.8 mL buffer and the pad collects 0.6 mL oral fluid resulting in a 1:3 dilution of the oral fluid. The DDS device and kit is an updated version of the Cozart® RapiScan device currently used in NSW roadside drug testing.

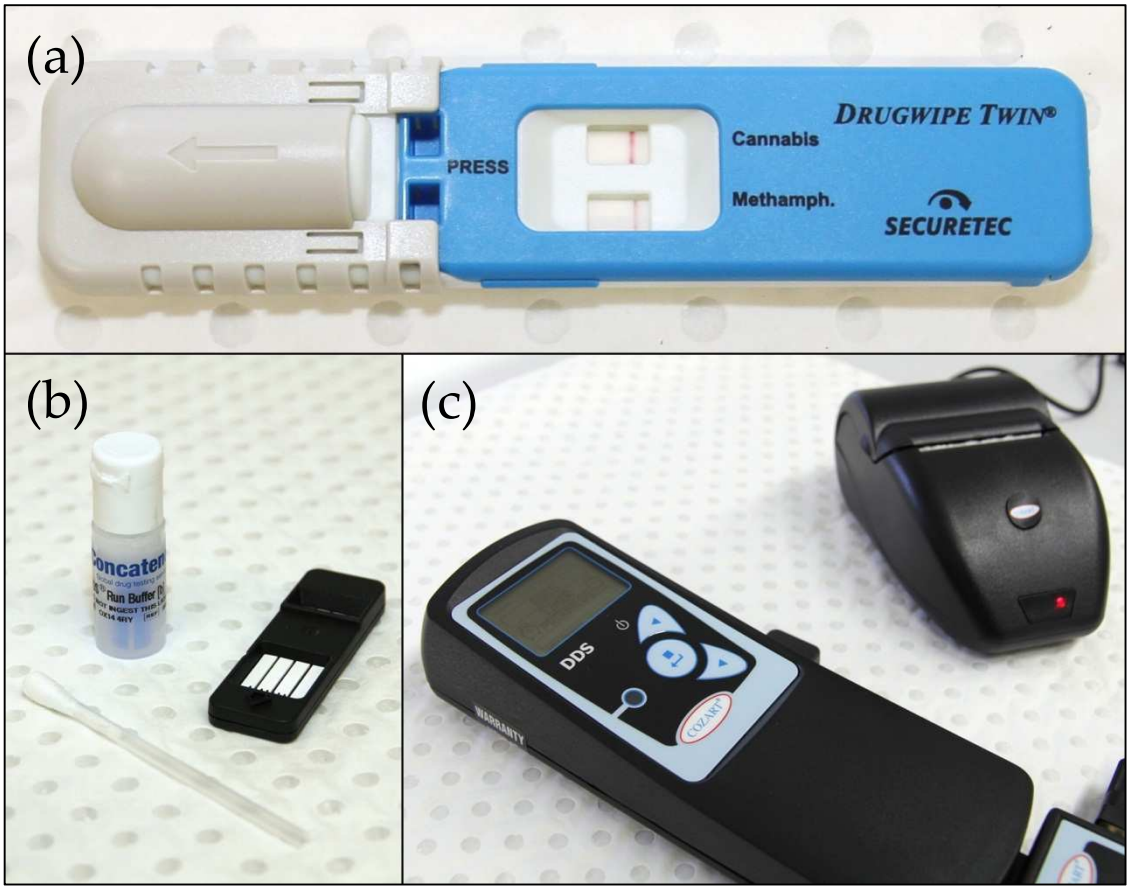

Figure 4-1: Screening test devices used in this study, (a) DrugWipe® II Twin; (b) Cozart® DDS swab, buffer vial and test cassette; and (c) Cozart® DDS digital reader and printer.

The standalone collection devices contain 2 mL buffer and the pad collects 1 mL of oral fluid resulting in a 1:2 dilution.

THC-free oral fluid was provided from volunteers and used to make calibration samples on the day of collection. HPLC grade acetonitrile and n-hexane were purchased from Chem-Supply (Gillman, SA, Australia). Analytical grade ethyl acetate was obtained from Ajax Finechem (Taren Point, NSW, Australia). All water was purified using a Sartorius Arium® Milli-Q system. Na_2HPO_4 and NaH_2PO_4, used to make 0.1 M Sørensen's phosphate buffer were sourced from Ajax Chemicals (Taren Point, NSW, Australia) and mixed to achieve a pH of approximately 6. Concentrated formic acid was purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Cerilliant® standards of THC, CBD and CBN (1 mg/mL in methanol) and d_3-THC, d_3-CBD and d_3-CBN (0.1 mg/mL in methanol) were purchased from Novachem (Collingwood, VIC, Australia). These reference standards were mixed and diluted with methanol to obtain working stock solutions for THC/CBD/CBN at 20 mg/mL and d_3-THC/d_3-CBD/d_3-CBN at 2 mg/mL. Cozart® DDS buffer solution used to make calibration standards and for method validation was obtained from Alere™ (Brisbane, QLD, Australia).

4.2.2 Participants and Sativex®

The study was run as an inpatient trial at Sydney Hospital in parallel with another study by the University of New South Wales (UNSW) trialling Sativex® as a withdrawal aid for chronic cannabis smokers. Dosing of Sativex® was conducted by hospital staff according to Poisons Schedule 8 requirements. Ethics approval for the Sativex® trial was obtained from the relevant committee for each site (HNEHREC: 10/12/15/3.03; NSW HRECEL HREC.10/HNE/355; and NSW SSA: SSA/11/HNE/84). Participants provided written informed consent to undergo screening tests and sample collection during the double-blind, placebo controlled study. Recruitment of patients was done by UNSW researchers. The inclusion and exclusion criteria they used in this process were as follows:

Inclusion criteria were:

(1) aged 18–65;
(2) meets criteria for current DSM-IV-TR (Diagnostic and statistical manual of mental disorders [140]) for cannabis dependence with no other current alcohol or drug dependence except for nicotine and/or caffeine;
(3) experienced withdrawal during previous quit attempts; and
(4) a desire to reduce or quit cannabis use.

Exclusion criteria were if the patient presented with:

(1) unstable medical or psychiatric conditions;
(2) medications commenced or changed dose in the previous month;
(3) pregnancy;
(4) urine negative for cannabinoids (THC-COOH);
(5) a positive urine test for other illicit substances or benzodiazepines; or
(6) formal drug or alcohol treatment in the previous month (excluding treatment for nicotine dependence).

Overall, thirteen participants were sampled during the trial. Twelve participants provided pre-admission samples and all had self-reported cannabis use within the 30 h preceding the first sample collection. One participant (the first) did not provide pre-admission samples due to the unavailability of the collection devices at that time. Ten participants were sampled during the Sativex® dosing period. Three participants were not sampled during Sativex® dosing due to one being discharged on day 1 of the trial, and the absence of the test conductor when the remaining two were admitted. After the blind was broken, two female and three male participants were deemed to have been administered with the Sativex® treatment and the other five participants were given the placebo medication. Participant demographics and previous cannabis use are outlined in Table 4-1.

Table 4–1: Participant demographics and previous cannabis use. "SD" indicates standard deviation.

Variable	Subjects given Sativex® (*n* = 5)	Subjects given placebo (*n* = 5)	Statistical analysis (ANOVA or Fisher's exact test)
Age – years (SD)	32.8 (12.83)	34.2 (8.49)	F1,9=0.04, P=0.8
Age at first cannabis use – years (SD)	14.6 (2.41)	16.4 (2.3)	F1,9=1.46, P=0.3
Days per week using cannabis in previous three months – days (SD)	7 (0)	6.6 (0.55)	F1,9=2.67, P=0.1
Gender – no. (%) male	3 (60%)	5 (100%)	Fishers Exact Test: P=0.4
Body weight – kg (SD)	71.72 (16.24)	65.6 (37.94)	F1,8=0.09, P=0.8
Height – cm (SD)	175 (13.6)	174 (1.52)	F1,7=1, P=0.36

The standalone oral fluid collection devices were employed upon admission on the first day before any Sativex® or placebo doses were given. Three oral fluid samples were collected from each participant over a period of 3–6 h following patient arrival. A bottle of Sativex® contains 27 mg/mL THC and 25 mg/mL CBD dissolved in ethanol, propylene glycol and peppermint oil. The spray delivers approximately 100 μL per pump [135]. Ten participants, eight male and two female were given the trial medications according to the following schedule: The first dose was administered at 4 pm on day 1 (eight sprays, a total of 21.6 mg THC and 20.0 mg CBD) and again at 10 pm (eight sprays). A maximal dose (eight sprays QID (four times a day) = 86.4 mg THC and 80.0 mg CBD per day) was administered on days 2 and 3. The dose was tapered to six sprays QID on day 4 (64.8 mg THC and 60.0 mg CBD per day), four sprays QID on day 5 (43.2 mg THC and 40.0 mg CBD per day), and two sprays QID (21.6 mg THC and 20.0 mg CBD per day) on day 6 (Table 4-2).

Table 4-2: Sativex® dosing and screening test schedule.

Time/Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
08:00	--	8 sprays	8 sprays	6 sprays	4 sprays	2 sprays
12:00	--	8 sprays[b]	8 sprays[c]	6 sprays	4 sprays	2 sprays[d]
18:00	8 sprays[a]	8 sprays	8 sprays	6 sprays	4 sprays	2 sprays
22:00	8 sprays	8 sprays	8 sprays	6 sprays	4 sprays	2 sprays

[a] *Dose given at 16:00 instead of 18:00 on day 1.*
[b] *High dose testing – 8 sprays = 21.6 mg THC and 20.0 mg CBD.*
[c] *For one subject high dose sampling occurred on day 3 instead of day 2.*
[d] *Low dose testing. – 2 sprays = 5.4 mg THC and 5.0 mg CBD.*

4.2.3 Screening tests

Screening tests using the DrugWipe® II Twin and Cozart® DDS were performed on days 2 and 6 of the inpatient trials, focussing around the midday dose. These days were selected because they represent high and low dosing for comparisons (see Table 4-2). On the high dose day (day 2), subjects received eight sprays of the trial medication at the midday dose, equating to 21.6 mg THC and 20.0 mg CBD. On the low dose day (day 6), subjects received only two sprays of trial medication at midday (5.4 mg THC and 5.0 mg CBD). Both screening tests were performed before dosage

and at approximately t = 10, 30, 60 and 120 min after dosage. Participants were given lunch at various times during the testing period, typically within a 50 min window.

At each test point, the patient was asked to move their tongue around the mouth before the DrugWipe® sampler was swabbed over the tongue three times (or more if needed) and returned to the device. The test was conducted according to instructions provided by the supplier. Briefly, the ampoule containing a buffer solution was broken whilst the device was held vertically for 15 s to allow the oral fluid to mix and reach the test strips; the device was then set down horizontally and the result was read after 5 min once the control lines appeared. While waiting for the result of the DrugWipe®, the DDS swab was given to the patient and swabbed around the mouth and then held in the mouth until the volume adequacy indicator turned blue. The swab was then snapped off into the buffer vial, which was then shaken for 30 s. After which, five drops were transferred to the test cassette and analysed by the digital reader. Results were printed and stored with the samples. Full instructions for the use of both screening devices and the Concateno Certus® Oral Fluid Collection Kits can be found in Appendix B (over-the-phone training was also completed for the use of the screening devices by their respective suppliers before any patient testing was attempted). Results of both tests were read in a separate room to the patients to protect the blind. All oral fluid samples collected by the DDS kit regardless of screening results were stored at 4 °C for further quantitative analysis.

4.2.4 Sample preparation for confirmatory analysis

The LLE method presented in Chapter 2, Section 2.2.2 was adapted to include the detection and quantification of CBD and CBN in addition to THC in an oral fluid/buffer mix. Freshly collected drug-free oral fluid was mixed with DDS buffer in a 1:3 ratio. Aliquots of 200 μL were transferred into 10-mL glass extraction tubes and spiked with mixed standard containing THC, CBD and CBN in methanol at various specified concentrations for calibration. The absence of THC in collected oral fluid was confirmed by incorporating a blank sample that was not spiked but subjected to the same sample preparation and analysis procedures as follows. Mixed deuterated

internal standard solution (5 μg/mL) was added to the glass test tubes followed by the addition of 1 mL of 0.1 M Sørensen's phosphate buffer (pH ~6). Hexane/ethyl acetate (2.5 mL; 9:1 v/v) was then added and the tubes placed on a roller mixer on moderate speed for 60 min. The organic upper layers (2 mL) were transferred into clean vials and evaporated under a gentle stream of N_2 gas at 40 °C. Residues were reconstituted in 100 μL of 75% acetonitrile in water with 0.1% formic acid before analysis by LC–MS/MS.

4.2.5 LC–MS/MS

Chromatographic analyses were carried out using an Agilent 1290 series LC system with a Zorbax Eclipse XDB–C18 column (2.1 mm × 50 mm × 3.5 μm). Isocratic elution using 75% acetonitrile in water with 0.1% formic acid was performed for triplicate injections of 5 μL of each sample at a flow rate of 0.4 mL/min. Total run time was 10 min per sample. The LC system was coupled to an Agilent 6490 Triple Quadrupole mass spectrometer with an ESI source run in positive ionisation mode. MS was operated in MRM mode monitoring two transitions per analyte and respective internal standard (Table 4-3). Dwell time was 60 ms for each transition. The same transitions were monitored for THC and CBD due to their similar molecular structure so these compounds were separated by chromatography. The fragmentor voltage was fixed at 380 V and the collision cell voltage was set at 20 V for all compounds. The gas temperature was 290 °C, nebuliser pressure was 40 psi and the capillary and nozzle voltages were 4000 and 1500 V, respectively. Sheath gas temperature and sheath gas flow were set at 400 °C and 12 L/min.

Data acquisition and analysis were performed using the Agilent MassHunter Workstation package and Microsoft Excel 2010.

Table 4-3: Ion transitions monitored for analytes and internal standards.

Analyte(s)	Quantitative transition (*m*/*z*)	Qualitative transition (*m*/*z*)
THC/CBD	315.2 → 193.1	315.2 → 259.1
d_3-THC/d_3-CBD	318.3 → 196.1	318.3 → 262.1
CBN	311.2 → 222.8	311.2 → 241.0
d_3-CBN	314.0 → 222.9	314.0 → 240.9

4.2.6 Validation studies

Serial dilution of the working stock solution of THC, CBD and CBN gave 11 concentrations of the cannabinoids in methanol. Of each of these, 10 µL was spiked into a separate tube containing 200 µL drug-free oral fluid/DDS buffer mix (1:3 v/v) resulting in concentrations of 500, 400, 300, 200, 100, 50, 10, 5, 1, 0.5 and 0.25 ng/mL for each analyte in solution. These concentrations corresponded to 2000, 1600, 1200, 800, 400, 200, 40, 20, 4, 2 and 1 ng/mL in undiluted oral fluid. Internal standards were then added to each tube (5 µL) giving a concentration of 50 ng/mL of d_3-THC, d_3-CBD and d_3-CBN in solution. After addition of Sørensen's phosphate buffer, LLE was performed as previously described in Section 4.2.4. Linearity of the calibration curve was calculated using a line of best fit with acceptable correlation factor set at >0.99. Inter- and intra-day precision and accuracy studies were carried out using QC samples spiked at 2, 25 and 250 ng/mL in solution corresponding to 8, 100 and 1000 ng/mL in undiluted oral fluid (n = 5). Accuracy was determined to be acceptable if the calculated concentrations fell within 15% of the concentration spiked (expressed as an MRE). Precision was deemed acceptable if the percentage RSD was <15%. The LLOQ was the lowest concentration of analyte that could be quantified with an RSD of <20% and an MRE of <20% [123]. To determine if the calibration curve can be successfully extrapolated to measure higher concentrations, standards at 1, 2, 3, 4 and 5 µg/mL (4, 8, 12, 16 and 20 µg/mL in undiluted oral fluid) were prepared and analysed as samples. Inter-day accuracy and precision was determined at each of these five concentrations (n = 5). Matrix effects (ion suppression) of the oral fluid/DDS buffer mix were investigated by extracting non-spiked samples (n = 3) and patient samples

found negative for all cannabinoids (n = 10) and reconstituting at the final stage with mobile phase fortified with the mixed drug and internal standard analytes at 100 ng/mL. Matrix effects using 100% oral fluid were also investigated for comparison. Peak areas of the resulting MRM chromatograms were compared with those of samples where the analytes were spiked at the same concentration into mobile phase only, expressed as a percentage loss. Recovery was also investigated from the oral fluid/DDS buffer mix. Drug-free oral fluid (n = 10) was fortified with 50 ng/mL of each analyte and internal standard and subjected to the same LLE and LC–MS/MS analysis. Resulting peak areas were compared to those of samples extracted without analyte and reconstituted with mobile phase fortified with the mixed drug and internal standard analytes at 50 ng/mL.

4.2.7 Stability of THC and CBD in oral fluid/DDS buffer solution

The stability of THC and CBD stored in the oral fluid/buffer mix over 70 days was investigated. THC and CBD were spiked at 40 ng/mL into an oral fluid/DDS buffer solution (1:3 v/v). Aliquots of 300 µL were stored either at room temperature (20 °C) or refrigerated (4 °C) in 2-mL polypropylene centrifuge vials. Samples were taken out of storage and 200 µL withdrawn for analysis at t = 3, 7, 14, 21, 29, 49 and 70 days and compared to samples analysed at t = 0 (n = 5). Five positive patient samples were also re-analysed after a further period of 11–12 weeks in refrigerated storage to observe any loss of THC and CBD.

4.3 Results and discussion

4.3.1 Confirmatory testing method validation

Linearity of the LC–MS/MS method for THC was achieved over the range of 0.25–500 ng/mL for the oral fluid/DDS buffer mixture (equivalent to 1–2000 ng/mL in undiluted oral fluid) with a correlation coefficient of 0.9993. The curve was also determined to successfully extrapolate to higher concentrations up to 5000 ng/mL (or 20,000 ng/mL in undiluted oral fluid). The inter- and intra-day precision and accuracy of the method for all analytes were deemed satisfactory and are summarised in Table 4-4. The RSD values for THC were 1.9–5.6% and the MRE ranged from 2.1% to 10.0%. The LLOQ was 1 ng/mL for THC and 2 ng/mL for CBD and CBN in the undiluted oral fluid. Precision at the LLOQ for THC, CBD and CBN were determined to be 11.6%, 11.8% and 9.0%, respectively. Accuracy at the LLOQ was found to be 10.2% for THC, 10.0% for CBD, and 7.6% for CBN. Ion suppression of the oral fluid/buffer mix was found to be minimal for THC and CBN and their respective deuterated compounds as a 7–14% loss in peak area was observed in samples containing the extracted matrix. CBD and d_3-CBD had a higher degree of suppression in this matrix (26–30% loss observed). When evaluating the matrix effect of 100% oral fluid, no significant suppression or enhancement effects were observed for any of the analytes, which is in agreement with the matrix effect results of THC in neat oral fluid seen in Section 2.3.2. The suppression observed in the oral fluid/buffer matrix was consistent between the analyte and internal standard and therefore the method was deemed suitable, albeit the sensitivity was affected. Recovery of THC was found to be 70% while the recovery of CBD and CBN were both only 50%. Although far from ideal, these results were deemed satisfactory for this study since the method still adhered to the target concentration of 10 ng/mL for THC as suggested by the Australian Standard 4760:2006 [59] and also the 2 ng/mL cut-off for THC as specified by SAMHSA [81].

Table 4-4: Validation data for the confirmatory LC–MS/MS method.

	THC	CBD	CBN
LLOQ (ng/mL)	1	2	2
ULOQ (ng/mL)	20000	20000	20000
R^2 (LLOQ to 2000 ng/mL)	0.9993	0.9981	0.9993
QC concentrations	8, 100 and 1000 ng/mL		
Intra-day precision (%RSD)	1.87-2.75	2.12-5.68	1.07-4.89
Intra-day accuracy (%MRE)	2.34-10.0	4.67-9.06	4.93-10.6
Inter-day precision (%RSD)	2.88-5.63	3.86-6.58	3.08-6.74
Inter-day accuracy (%MRE)	2.13-4.25	5.20-6.65	5.00-5.42

4.3.2 Stability of THC and CBD in oral fluid/buffer mix

As previously seen in the results of Chapter 2 Section 3.3.7, THC again remained stable over the whole time period studied when stored at 4 °C and 20 °C in the oral fluid/Cozart® buffer mix (Figure 4-2). CBD showed similar stability, with no significant losses (<20%) from refrigerated samples over the 70 day period. When left at room temperature, CBD remained stable for at least 29 days, but by 49 days in storage all five samples analysed suffered loss in the range of 20–26%. After 70 days, loss of CBD was found to be 21–34%. CBN concentration levels were also monitored over the course of the study. Concentrations remained below quantifiable levels for the duration of the study with the exception of one refrigerated sample, which after 49 days in storage was found to have a concentration of CBN at 0.55 ng/mL. While CBN is a known degradation product of THC in stored plant material [141], the concentration of CBN found was too low to correlate with loss of THC.

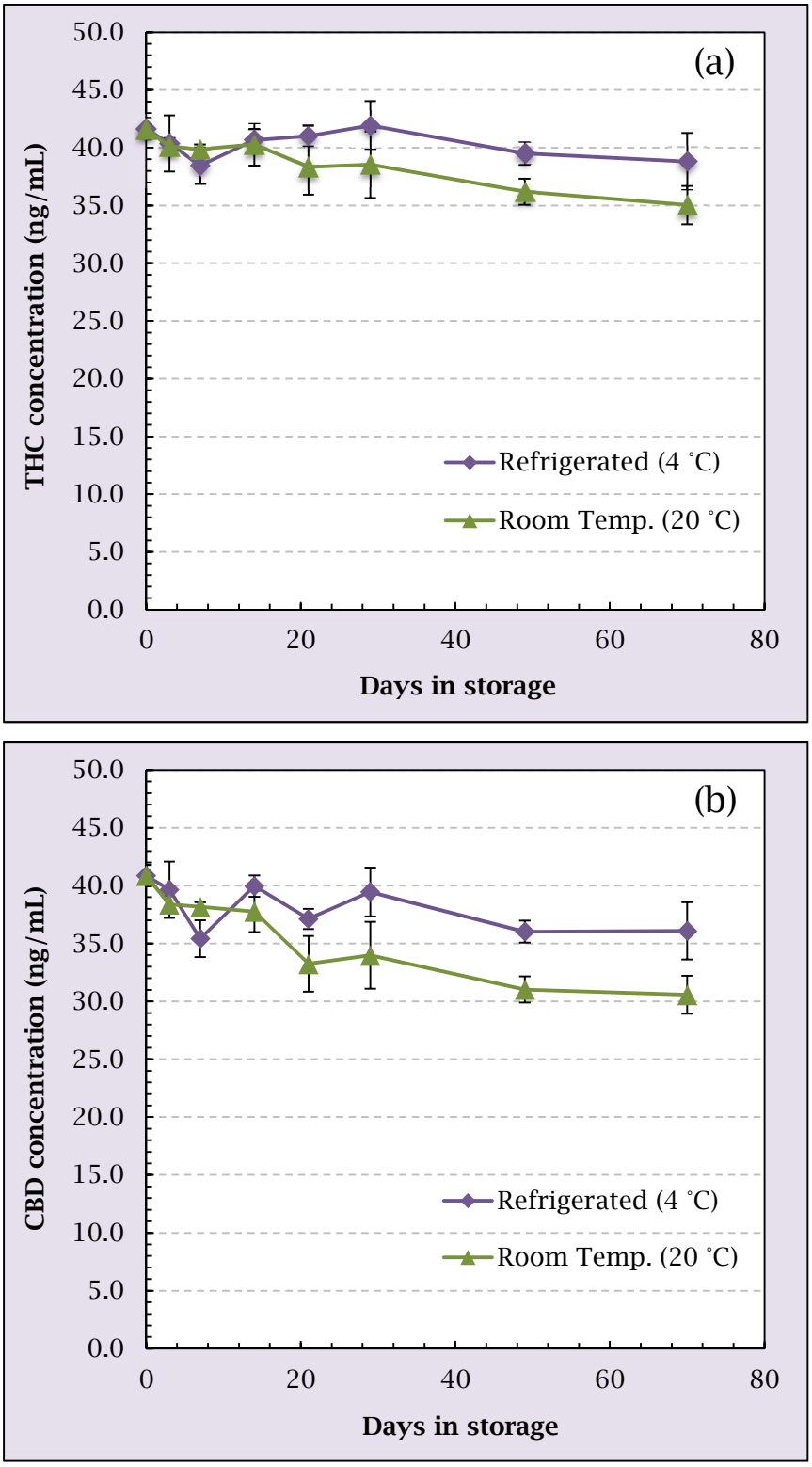

Figure 4-2: Stability of (a) THC and (b) CBD in oral fluid stored in Cozart® DDS buffer solution (1:3) v/v over a period of 70 days refrigerated and at room temperature.

The stability of THC in the oral fluid/Cozart® RapiScan and oral fluid/Cozart® DDS solutions have been previously evaluated in Chapter 2 Sections 2.3.4 and 3.3.7, and it

has been concluded that THC is stable in these mixtures when refrigerated and at room temperature. This present study corroborates the previous findings as no significant loss of THC was observed over the 70 day study. CBD is an analyte of interest when detecting cannabinoids in oral fluid [38, 40]; however, since there is no requirement for it to be tested for by law, it has not been examined as thoroughly as THC. Moore *et al.* [84] found that THC and CBD stored in the Quantisal™ device suffered a loss of 50% when stored at room temperature for 10 days but showed no degradation when refrigerated for the same time period. The stability of CBD in neat oral fluid and oral fluid collected using the Quantisal™ device after controlled cannabis smoking has been recently investigated by Lee *et al.* [99]. CBD was found to be stable for four weeks at 4 °C when collected and stored in the Quantisal™ device [99]. Following a literature search, it seems that the stability of CBD in the oral fluid/Cozart® DDS buffer solution has not yet been evaluated. Since samples were stored in a refrigerator awaiting confirmatory analysis, it was pertinent to investigate whether any significant losses would be incurred between sampling and analysis. The results show that CBD is similarly stable to THC in the mixture when refrigerated, since any observed changes to concentration over a 70 day period were limited to within 20% of the original spiked concentration. Further to this, while stability in authentic oral fluid samples was not investigated, five samples that were THC and CBD positive with concentrations in the range of 73–1879 ng/mL (diluted concentration) were re-analysed after a period of 11–12 weeks. The cannabinoid levels detected were consistent with those previously measured showing no significant loss of either THC or CBD as concentrations detected were ≤13.7% for THC ($t(4) = 1.56$, $p = 0.19$) and ≤18.0% for CBD ($t(4) = 1.21$, $p = 0.29$) when compared to the original analyses (Figure 4-3). These results provide confidence that there was no significant loss of CBD (or THC) from the Sativex® patient samples that were analysed after a period of refrigerated storage.

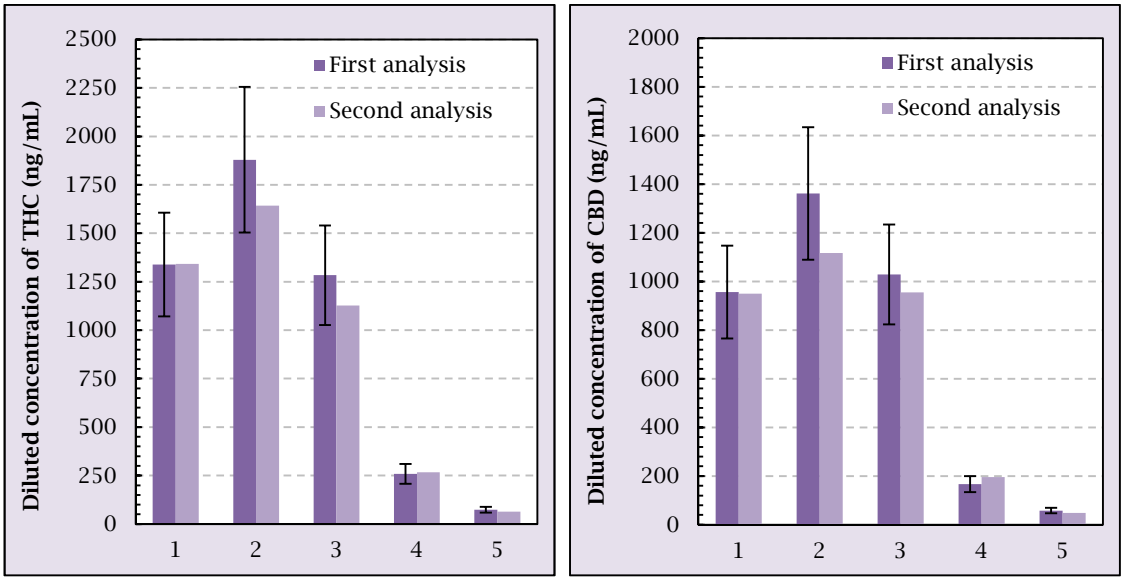

Figure 4-3: Stability of THC and CBD in five Sativex® samples re-analysed after a further 11–12 weeks of refrigerated storage. Error bars represent acceptable ±20% threshold for analyte stability in subsequent analyses.

4.3.3 Screening test performance

A total of 72 of each of the two screening tests were performed. For each of the ten subjects, 5–10 paired tests (one DrugWipe® and one DDS) were performed. Only one of the DrugWipe® tests failed (control line did not appear) and no failed tests were observed using the DDS. The DrugWipe® did not give any false positive results, however there were many false negatives leading to a very low sensitivity for the device in this study (Table 4-5). The small sample population makes it difficult to draw any firm conclusions from this observation. Confirmed concentrations in these negatively screened oral fluid samples were in the range of 52–11,624 ng/mL. Samples that screened positive were in the range of 166–15,468 ng/mL. There is a large overlap in these ranges, showing no clear cut-off for a detection limit for the device in this instance.

The primary screening test, the DrugWipe® II Twin, gave variable results with true positives and false negatives. It is difficult to confirm with such a small sample range, however, a trend appeared to emerge among three of the test subjects. This trend showed the DrugWipe® giving a positive result for the test immediately following the dose in four out of five testing sessions (Table 4-5).

In two of these sessions a positive result for the subsequent test was also found and a third positive test was observed for one of these. So although the concentration ranges for true positive and false negative results overlapped, it appeared that for each subject, the device could detect the THC at its peak concentrations regardless of the actual levels present. This indicates that the reliability of the DrugWipe® result varies, based on the individual. Note that with the exception of one subject for whom the DrugWipe® device consistently gave a distinct line indicating a positive result, the test lines were almost always quite faint, so perhaps other positive results were missed. This illustrates the well-known issue of the subjectivity of result interpretation for many on-site immunoassay screening devices. It should also be noted that lunch was served during the two hour testing window, usually between the 10 min and 1 h tests and occasionally, tests had to be conducted within 10 min of the subject consuming either food or drink. This may have had an effect on the sensitivity of the device, as although no advice was received for the DrugWipe® specifically, the supplier of the Cozart® DDS recommended that tests should not be conducted within 10 min of the subject consuming food or drink [97].

Table 4-5: Screening test results for all subjects for the DrugWipe® device. Dose of Sativex® or placebo was given after test 1 and before test 2 was conducted; n/a indicates a test was not performed due to the unavailability of the subject.

		Day 2					Day 6				
		Dose ↓					Dose ↓				
Subject	Treatment	Test 1	Test 2	Test 3	Test 4	Test 5	Test 1	Test 2	Test 3	Test 4	Test 5
1	Sativex®	+	+	+	+	+	+	+	+	+	+
2	Placebo	–	–	–	–	–	–	–	n/a	–	–
3*	Placebo	–	–	–	–	–	n/a	n/a	n/a	n/a	n/a
4	Sativex®	–	+	–	–	–	–	+	–	–	–
5	Placebo	–	–	**	–	–	–	–	–	n/a	n/a
6	Sativex®	–	–	–	–	–	–	–	–	–	–
7	Sativex®	–	+	+	–	–	–	–	–	n/a	n/a
8	Placebo	–	–	n/a	–	–	n/a	n/a	n/a	n/a	n/a
9	Placebo	–	–	n/a	–	n/a	n/a	n/a	n/a	n/a	n/a
10	Sativex®	–	+	+	+	–	n/a	n/a	n/a	n/a	n/a

** High dose sampling for patient 3 occurred on day 3 instead of day 2.*

*** DrugWipe® test device failed validity test.*

Secondary screening test, Cozart® DDS gave true positive results for all test subjects receiving Sativex® and true negative results were found for all samples taken from patients receiving the placebo (Table 4-6). All positive samples were found to contain >52.4 ng/mL THC, which is above the stated cut-off concentration of the DDS device (31 ng/mL). It should be noted that there is no cross-reactivity caused by CBD (Appendix A).

Table 4-6: Screening test results for all subjects for the Cozart® DDS device. Dose of Sativex® or placebo was given after test 1 and before test 2 was conducted; n/a indicates a test was not performed due to the unavailability of the subject.

		Day 2					Day 6				
		Dose ↓					Dose ↓				
Subject	Treatment	Test 1	Test 2	Test 3	Test 4	Test 5	Test 1	Test 2	Test 3	Test 4	Test 5
1	Sativex®	+	+	+	+	+	+	+	+	+	+
2	Placebo	–	–	–	–	–	–	–	n/a	–	–
3*	Placebo	–	–	–	–	–	n/a	n/a	n/a	n/a	n/a
4	Sativex®	+	+	+	+	+	+	+	+	+	+
5	Placebo	–	–	–	–	–	–	–	–	n/a	n/a
6	Sativex®	+	+	+	+	+	+	+	+	+	+
7	Sativex®	+	+	+	+	+	+	+	+	n/a	n/a
8	Placebo	–	–	n/a	–	–	n/a	n/a	n/a	n/a	n/a
9	Placebo	–	–	n/a	–	n/a	n/a	n/a	n/a	n/a	n/a
10	Sativex®	+	+	+	+	+	n/a	n/a	n/a	n/a	n/a

**High dose sampling for patient 3 occurred on day 3 instead of day 2.*

Consumption of food or drink within the 10 min preceding the sample collection did not affect the accuracy of the tests in these instances. Consumption of food and drink would likely have a wash-off effect on the cannabinoids deposited in the oral cavity, and thus reduce the concentration of cannabinoids present. However, it was not possible to observe this independently since all subjects showed a significant decrease in cannabinoid concentration after the tests that immediately followed the dose of Sativex® and lunch may or may not have been served by the time of the second dose. Additionally, due to the taste of the medications, all subjects had a beverage two minutes after the dose, and so it can be assumed there was some wash-off from the very beginning, thus affecting even the first test taken 10 min after the dose.

Cannabis smokers may experience inhibition of oral fluid production that may result in difficulty producing enough oral fluid for a screening test sample. During the present study, all subjects were able to give adequate oral fluid specimens using the

collection devices employed. Some subjects took longer than others to produce the specified volume of oral fluid however, this occurred across both the Sativex® and placebo patients and as there was no reference to compare to, it is unknown whether or not there was any inhibition of oral fluid production caused by the Sativex® spray.

Patients prescribed Sativex® are advised that they should not drive or operate heavy machinery whilst on the medication [135]. Due to the method of administration, high concentrations of THC in oral fluid as a result of taking Sativex® do not necessarily equate to such high concentrations in blood, and so the patient may not be as affected cognitively in the same way as a cannabis smoker with a lower level of THC in their oral fluid. Therefore, even if patients do not feel impaired, they should be aware that they may test positive for THC at a roadside drug test and in turn face legal implications, since Australian law does not require proof of impairment for conviction.

4.3.4 Confirmatory testing results

Three pre-admission tests from each of the 12 participants showed relatively low amounts of THC in the oral fluid; 1.45–369 ng/mL (median 17.3 ng/mL; mean 45.2 ng/mL). CBD was not detected in any of the subjects prior to taking Sativex®. CBN was only quantifiable in two of the pre-admission samples at 3.57 and 4.78 ng/mL, which correlate to the two samples with the highest measured THC concentrations. LC–MS/MS analysis confirmed all DDS screening results. Participants 1, 4, 6, 7 and 10 were found to have been given the Sativex® treatment, as THC and CBD were detected in all samples collected (Table 4-6). The remaining five participants were confirmed to have been given the placebo medication as neither THC nor CBD were detected in any of the samples collected from those participants. Both THC and CBD were detected in very high amounts 10 to 20 min after dosage in all subjects receiving Sativex® on both high and low dose days (Figure 4-4). Peak concentrations varied greatly between subjects. THC concentrations on either day peaked between 5356 and 15,468 ng/mL. CBD had a slightly wider range with 3826 to 17,233 ng/mL. The oral fluid consistently remained positive with 91.6–1166 ng/mL THC and 67.6–1175 ng/mL

CBD detected at two hours after dosage and even the pre-dose samples were positive for all analytes. The average concentration ratio of THC/CBD across all positive samples was 1.10 (%RSD 19.9) reflecting the composition of the Sativex® spray. CBN was also detected in smaller amounts (2.08–593 ng/mL) in all Sativex® positive samples with peak concentrations correlating with peak THC and CBD concentrations shortly after dosage. The average THC/CBN ratio was 30.6 (%RSD 14.3). Participants on the placebo consistently gave negative results for THC, CBD and CBN throughout the trial.

Results indicate that shortly after Sativex® administration, very high concentrations of both THC and CBD are detected in the oral fluid along with elevated levels of CBN. Since peak concentrations of CBN correlated with peak concentrations of THC and CBD shortly after dosage, it is reasonable to assume that CBN is also present in the spray, most likely resulting as an impurity from the extraction process from the *Cannabis sativa* L. plant or as an oxidative degradation product of THC from extended storage of the drug. Cannabinoid concentrations declined rapidly within 30 min but remained at readily detectable concentrations two hours after dosage. All baseline samples taken 10 to 55 min before the midday dose were positive for cannabinoids in patients receiving the treatment. Since the previous dose of Sativex® was given at 8 am, this suggests that THC and CBD are detectable in oral fluid at least 4 h after dosage. This correlates with the work of Lee *et al.* [95] who found that residual THC and CBD were detectable 10.5 h after either a low dose (2 sprays) or a high dose (6 sprays) of Sativex®. The daily schedule of dosing in the current trial was intense and it is unsurprising that residual THC and CBD were detected before subsequent doses on both the high and low dose days. Higher peak concentrations were observed on the low dose day for four out of the five patients receiving the treatment compared to the high dose. This shows some correlation with findings from a clinical study where variability in concentrations of THC and CBD following repeat dosing over a number of days was evident as the concentration increased for some subjects and decreased for others [135].

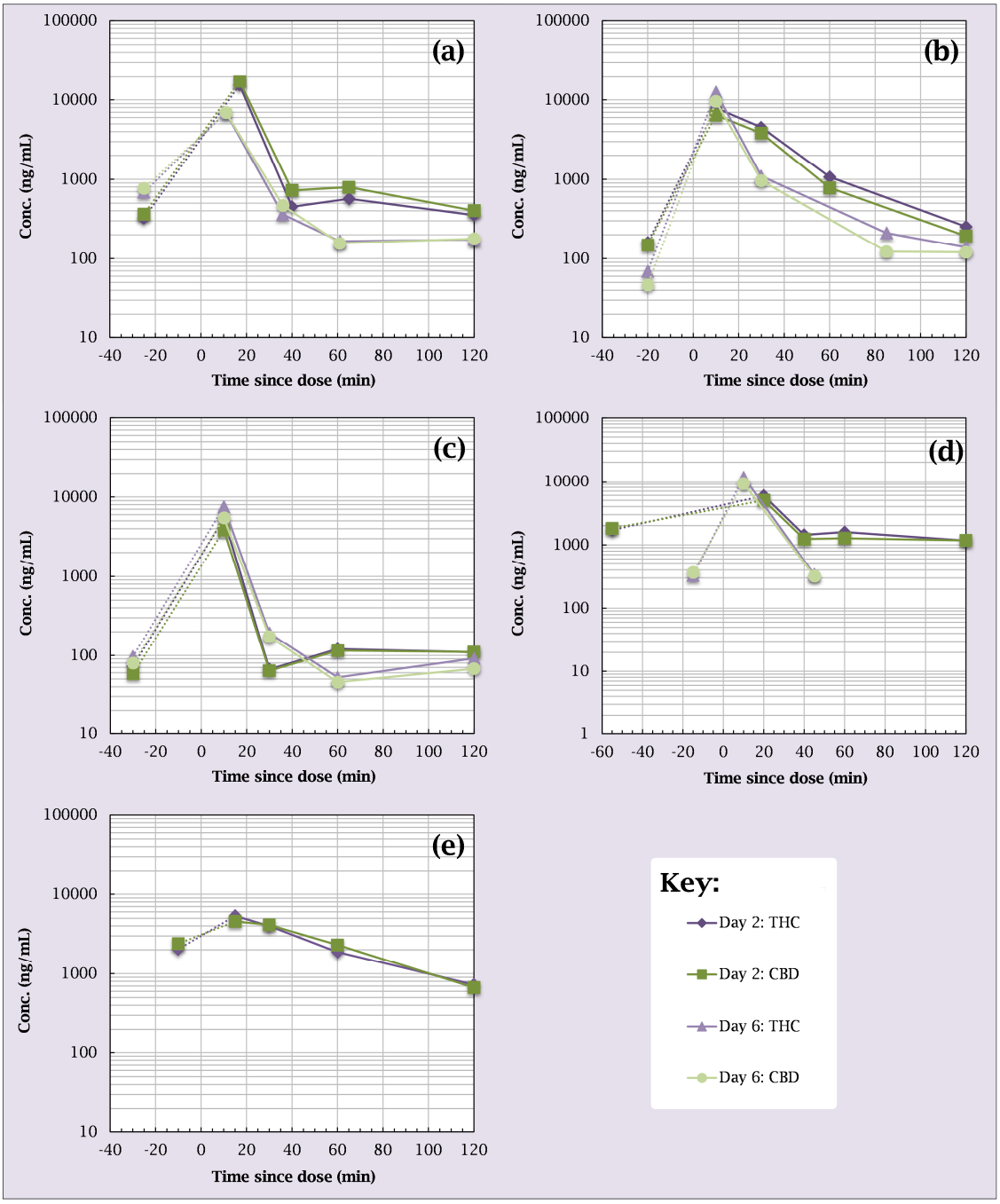

Figure 4-4: THC and CBD levels confirmed by LC–MS/MS in the oral fluid of the five patients on the Sativex® treatment; (a) subject 1; (b) subject 4; (c) subject 6; (d) subject 7; (e) subject 10. Two data points are missing in (d) as the last two tests for day 6 were not completed. Tests were only conducted on day 2 for (e). The dose for each subject was given at t = 0. CBN concentrations not shown in figure; peak concentrations of CBN for days 2 and 6 respectively were 593 and 213 ng/mL (a); 319 and 395 ng/mL (b); 160 and 256 ng/mL (c); 163 and 353 ng/mL (d); 183 ng/mL for day 2 (e).

There is a possibility that accumulation of doses is causing this phenomenon; however baseline (pre-12 pm dosing) levels were often lower on day 6 (low dose day) than day 2 (high dose day) and the samples taken at the two hour post dosing point frequently gave a lower concentration than the baseline sample. Due to the small sample population of this study, no conclusions can be drawn from this result. As previously mentioned, a bottle of the Sativex® solution contains approximately 27 mg/mL THC and 25 mg/mL CBD [135]. If required, presence of THC as a result of Sativex® medication or smoking cannabis can be distinguished using the THC/CBD ratio which is almost 1:1 in the oral fluid of Sativex® patients. This reflects the composition of the spray itself. CBD is only present in very low amounts in cannabis seized in New South Wales, Australia [142] and from the analysis of the initial samples collected before any Sativex® administration in this study, CBD is not detectable in the oral fluid of local cannabis smokers. However, if a patient taking Sativex® decides to smoke cannabis concurrently, it may be difficult to distinguish this use from the prescribed Sativex®. Further research may show that THC/CBD ratios are in fact useful for this purpose. Another possible way of differentiating between Sativex® use and non-medicinal cannabis use, may be to analyse samples for the THC precursor, Δ^9-tetrahydrocannabinolic acid A (THCA-A). THCA-A is found in the plant material and is only partially decarboxylated to THC during smoking [143]. A study by Fabritius *et al.* [125] found relatively high concentrations of THCA-A were present in oral fluid after smoking cannabis joints. Oral fluid samples were collected from five subjects up to 4 h after smoking and all samples were found to contain both THC and THCA-A. Peak THCA-A concentrations of 44–2031 ng/mL correlated with peak THC concentrations in the oral fluid shortly after commencing smoking indicating that some THCA-A is not decarboxylated to THC during the smoking process and is also deposited into the oral cavity [125]. If Sativex® contains only a small amount of THCA-A or none at all, presence of this cannabinoid at elevated levels in the oral fluid could indicate use of non-medicinal cannabis.

4.4 Conclusions

It was determined that THC and CBD are present in high concentrations in the oral fluid of patients taking high doses of Sativex® (eight sprays in a single dose) for at least two hours following the dose using confirmatory LC–MS/MS analysis. However, in Australian roadside drug testing, a large proportion of Sativex® patients following this dosage pattern would likely pass a DrugWipe® II Twin test within the two hours following the dose and the subsequent Cozart® DDS analysis would not be performed. For those who do test positive, the high level of CBD relative to THC should indicate that Sativex® is the source of the cannabinoids, since CBD is rarely detected in the oral fluid of cannabis smokers in Australia. However concurrent use of cannabis cannot be excluded. Further research is recommended to expand the study to a larger sample size for more conclusive results and to determine how long after a dose the screening tests can return positive results for THC. Additionally, any new testing devices introduced to roadside testing should be evaluated for their response to Sativex® in the same way. In conclusion, any patients prescribed with Sativex® in Australia must be made aware that driving is illegal whilst on the medication since they will have some unknown and possibly detectable amount of THC in their oral fluid for some time after each dose.

Chapter 5: The analysis of cannabinoids in oral fluid using nanospray LC–chip–Q–TOF–MS

Chapter 5: The analysis of cannabinoids in oral fluid using nanospray LC–chip–Q–TOF–MS.

5.1 Introduction

Microfluidic nanospray LC–MS is an emerging technology that has the advantages of low sample consumption and sample pre-concentration capabilities. Pre-concentration is achieved through the use of an enrichment column which results in higher sensitivity and reduced matrix effects since the matrix is flushed away before the sample is introduced onto the separation column.

The Thermo Scientific Ultimate 3000 RSLCnano system has been used to analyse cannabinoids in oral fluid and can achieve very high sensitivities. He *et al.* [104] used this system to quantify THC-COOH down to an LOQ of 7.5 pg/mL in oral fluid while Concheiro *et al.* [47] analysed a number of cannabinoids in oral fluid and found an LOQ of 500 pg/mL for THC, CBD and CBN and 15 pg/mL for THC-COOH.

HPLC–chips have been developed by Agilent which integrate the enrichment column, separation column, and sprayer tip all in one. This reduces the amount of user interaction with the system and claims to be more rugged because of this. Previous to this study, Agilent's LC–chip system had not been used to analyse illicit drugs in biological fluids. It has been used mostly for the analysis of proteins and biomarkers in blood; however they also produce a chip suited to small molecule analysis that has been used to analyse pharmaceutical drugs in blood [144, 145].

The aim of this chapter was to see if this novel device would be effective for the detection of cannabinoids in oral fluid. The cannabinoids of interest in this study are THC, CBD, CBN, THC-COOH and THCA-A. It will be of particular interest to see whether the use of a system with an in-line clean-up capability will effectively decrease matrix effects and increase sensitivity for the detection of these compounds.

5.2 Materials and methods

5.2.1 Standards, solvents and samples

HPLC grade acetonitrile, isopropyl alcohol and n-hexane were purchased from Chem-Supply (Gillman, SA, Australia). Analytical grade ethyl acetate and glacial acetic acid were obtained from Ajax Finechem (Taren Point, NSW, Australia). Concentrated formic acid, HPLC grade methanol, and analytical grade ammonium acetate were sourced from Sigma-Aldrich (Castle Hill, NSW, Australia). All water was purified using a Sartorius Arium® Pro VF system. Cerilliant® standards of THC, CBD and CBN (1 mg/mL in methanol), THC-COOH, d_9-THC-COOH, d_3-THC, d_3-CBD and d_3-CBN (100 μg/mL in methanol) were obtained from Novachem (Collingwood, VIC, Australia). Lipomed AG standard, THCA-A (1 mg/mL in isopropanol) was obtained from PM Separations (Capalaba, QLD, Australia).

Drug-free oral fluid was collected fresh on each day of analysis by expectoration from healthy volunteers.

5.2.2 Sample preparation

Freshly collected drug-free oral fluid (500 μL) was transferred into 10-mL glass extraction tubes and spiked with mixed standard containing THC, CBD, CBN, THCA-A and THC-COOH in methanol at various specified concentrations for calibration. The absence of THC in collected oral fluid was confirmed by incorporating a blank sample that was not spiked but subjected to the same sample preparation and analysis procedures as follows. Mixed deuterated internal standard solution (2 μg/mL) was added to the glass test tubes followed by the addition of 1 mL of 0.1 M ammonium acetate buffer (pH ~4.5). Hexane/ethyl acetate (1.5 mL; 9:1 v/v) was then added and the tubes placed on a roller mixer on moderate speed for 30 min. The organic upper layers (1 mL) were transferred into clean vials and evaporated under a gentle stream of N_2 gas at 40 °C. Residues were reconstituted in 200 μL of 35% acetonitrile: methanol (1:1) in water with 5 mM ammonium acetate before analysis by

LC–chip–MS. For normal flow LC–MS analysis, residues were reconstituted in 200 µL of 75% acetonitrile in water with 0.1% formic acid.

5.2.3 Instrumentation

All MS analyses were performed using an Agilent 6510 Q–TOF–MS (the QQQ–MS used in previous studies was unavailable for this study). For normal flow LC analyses, an Agilent 1290 Infinity LC system was used with a Zorbax Eclipse XDB–C18 column (2.1 mm × 50 mm × 3.5 µm). Experiments involving nano flow LC were performed using an Agilent 1260 Infinity LC system that included a nano pump, capillary loading pump, micro-well plate sampler (auto sampler), cooler and chip cube (Figure 5-1).

Figure 5-1: Instrument set-up for nano flow LC–chip–Q–TOF–MS and (inset) chip is inserted into slot in chip cube. Sitting on a trolley on the left is the capillary loading pump (also in picture is a DAD and regular LC column compartment not used in these studies). Next to this are the nano pump, autosampler and cooler. Each pump has its own set of mobile phase bottles seen on top of each stack. On the bench is the chip cube mounted on the Q–TOF–MS. A capillary from the autosampler and a capillary from the nano pump connect the LC system to the chip cube.

The LC system also included a column compartment for normal flow LC use and a diode array detector (DAD); neither of these was used during this study.

Separation was achieved using the Ultra High Capacity Chip (UHC Chip II, Part No. G4240-65010) shown in Figure 5-2. It has a 150 mm × 75 μm analytical column and a 25 mm, 500 nL enrichment column, both packed with Zorbax 80SB-C18 5 μm stationary phase. The chip includes a nanospray emitter that protrudes from the chip casing when in operation (Figure 5-2 inset). EZ Grabber software version 3.0 was used to monitor the camera feed to view the nanospray inside the chip cube.

Figure 5-2: Agilent UHC Chip II, Part No. G4240-65010 and inset, showing the nanospray emitter tip.

The majority of the results presented in this chapter were completed using one chip (serial number: DE54F002266). Some early experiments during method development were conducted on different chips (serial numbers (1) DE53G02239 and (2) DE54B02262). Both of these chips suffered irreversible damage due to (1) a blockage and (2) an issue with lamination, and were subsequently replaced by Agilent. Chips contain an RF tag that stores information about the chip (Figure 5-3). This is useful for

monitoring the number of injections made since the chip is a consumable item and will only last for around 500–1000 injections, much less than a regular LC column.

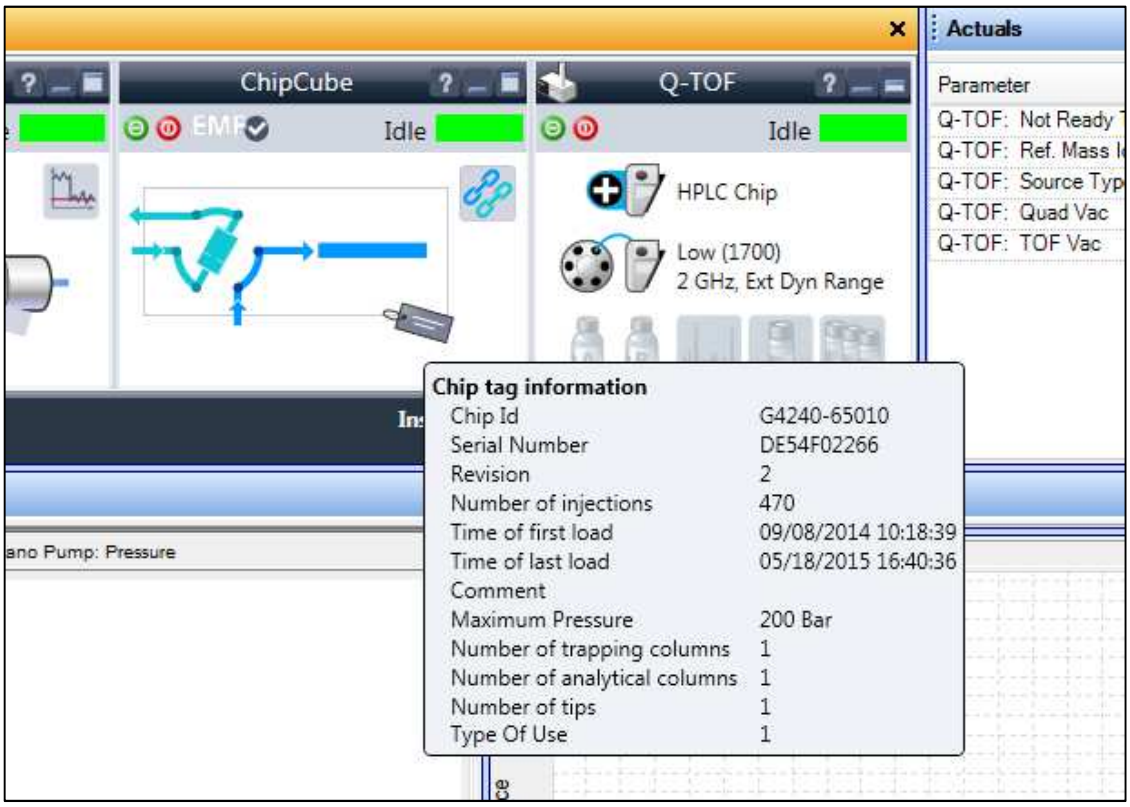

Figure 5-3: Chip tag information accessible when chip is loaded. Image is from a screenshot taken from the Agilent MassHunter Workstation Acquisition Software.

MassHunter Workstation software was used for all acquisition and analyses. Acquisition was run using LC/MS Data Acquisition version B.05.01, and analysis was carried out using Qualitative Analysis version B.06.00. Personal Compound Database and Library (PCDL) Manager version B.04.00 was also used for analysis for database matching and identification of compounds. Microsoft Excel 2010 was used to construct calibration curves from the data obtained through MassHunter.

5.2.3.1 Chip HPLC

The chip itself contains an enrichment or "trapping" column, an analytical column and the nanospray emitter (Figure 5-4). The capillary pump flow runs through the auto sampler and then to the chip cube. The nano pump flow runs directly to the chip cube.

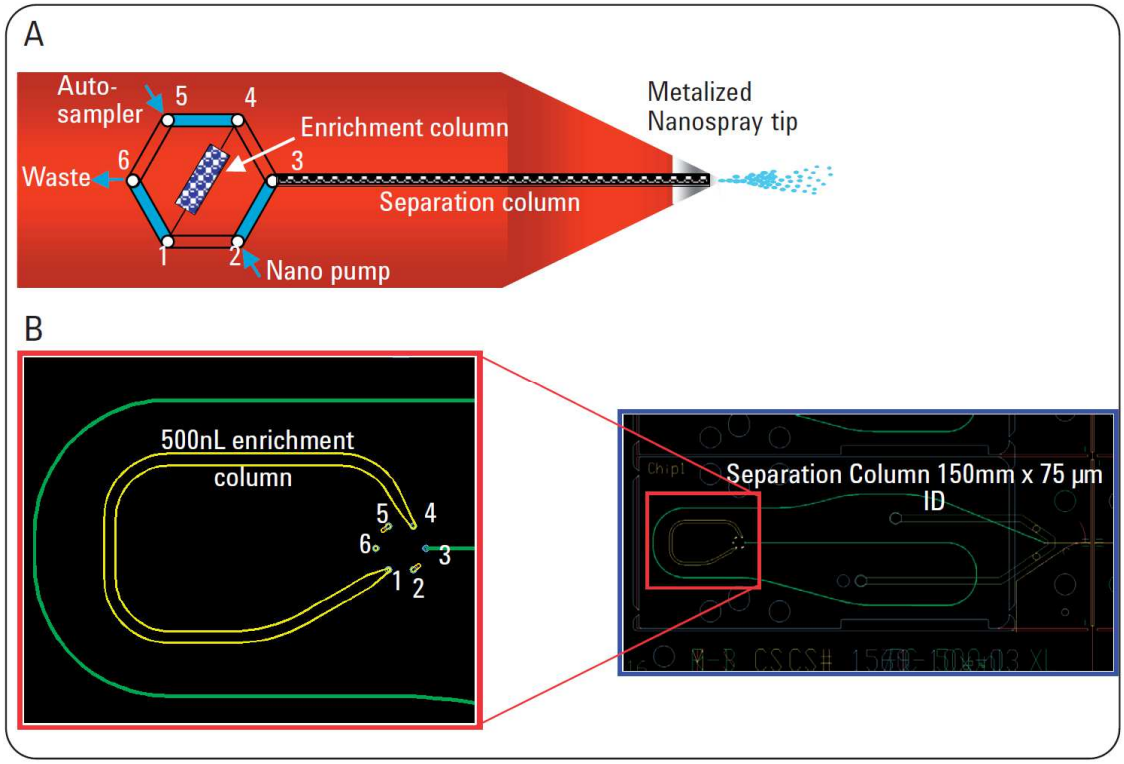

Figure 5-4: Chip schematic (A) and blueprint of UHC small molecule chip (B) like the one used in this study. This image is taken from an Agilent application note [146].

When the chip cube is configured to enrichment mode, the capillary pump flow is directed through the enrichment column and out to waste and the nano pump flow is directed through the analytical column to the MS. In analysis mode, the capillary pump flow is directed straight to waste and the nano pump flow is directed through the enrichment column and then onto the analytical column and through to the MS (Figure 5-5).

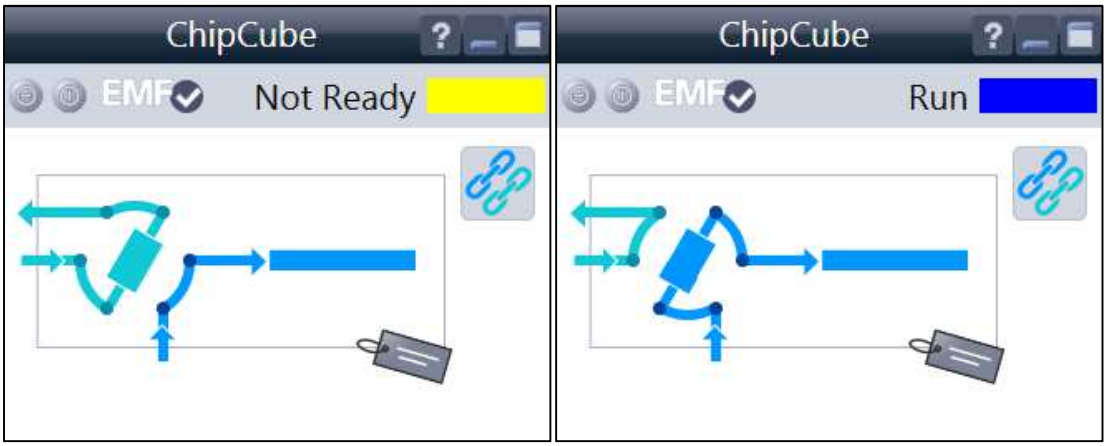

Figure 5-5: Enrichment mode (left); analysis mode (right). The teal arrows indicate flow coming from the capillary pump; the blue indicates flow from the nano pump. These images are screenshots taken from the Agilent MassHunter Workstation Acquisition Software.

When an injection is made, the chip cube is set to enrichment mode and the capillary flow runs through the auto sampler and loads the sample onto the enrichment column. The analytes are then "trapped" and concentrated in the column while the highly aqueous mobile phase washes the sample matrix compounds through to waste. The acquisition software calculates the time taken to complete this step and then switches the chip cube into analysis mode. The flow from the nano pump then pushes the analytes onto the analytical column for chromatographic separation before entering the MS.

The chip can be operated in either forward-flush or back-flush modes. The diagrams in Figure 5-5 show the operation of the chip in forward-flush mode. In this mode, the flow from the nano pump enters the enrichment column from the same end that the capillary pump loaded the sample, so the sample is washed all the way through the enrichment column before being moved onto the analytical column. This can allow for more separation to occur but can also result in peak broadening. In back-flush mode, the nano pump flow enters the enrichment column from the opposite end to where the sample was loaded by the capillary column. This should result in the sample being eluted faster onto the separation column and give sharper peaks; however operating in this mode can increase the risk of a blockage occurring in the enrichment column.

5.3 Results and discussion

5.3.1 Method development

5.3.1.1 Chromatographic separation

The capillary pump must be run in isocratic mode through the enrichment column, while the nano pump may be run in gradient mode through the analytical column for separation of the analytes. The default capillary pump setting is to run at 3% organic in order to ensure the analytes are not washed straight through the enrichment column to waste. Unfortunately, a lot of carryover was observed using only 3% acetonitrile as the loading mobile phase. The chip was excluded as the source of the carryover, so after various needle wash compositions and procedures were trialled, it was realised that the cannabinoids were not being washed through the lines sufficiently at this low level of organic content. A number of higher organic compositions were trialled, and it was found that 35% acetonitrile was the highest organic content that would avoid any carryover issues and still allow for adequate analysis. Higher organic compositions resulted in analytes not being detected and it appeared that they were being lost off the enrichment column.

It was noted that the starting composition of the nano pump should be the same as the capillary pump so that there is a smooth transition between mobile phase when the valve switches to analysis mode. Poor peak shape was found when the nano pump began with a higher organic content than the capillary pump.

A number of mobile phases were trialled to achieve separation of the analytes, THC, CBD, CBN, THC-COOH and THCA-A. These included using acetonitrile, methanol, and both together at various ratios as the organic phase, and water with 0.1% formic acid, 5 mM ammonium formate, or 5 mM ammonium acetate as the aqueous phase. Finally, it was found that a 1:1 mix of acetonitrile:methanol as the organic phase (B) and 5 mM ammonium acetate as the aqueous phase (A) gave the best overall response

and peak shape across all analytes. Carryover was not observed to be an issue at the loading mobile phase composition of 35% B.

It was found that the capillary pump required a long equilibration time (at least one hour) before starting analysis otherwise shifts in retention time of all analytes would be seen in subsequent runs. Analysis was attempted in both forward-flush and back-flush modes. Peak shape was found to be very poor in forward-flush mode, with broad bands and low sensitivity and so back-flush mode was used.

Flow rates of each mobile phase were chosen to be 3 µL/min for the capillary pump and 0.3 µL/min for the nano pump. The maximum pressure recommended for the chip is 200 bar, and these flow rates produced pressures that sat comfortably below this threshold.

The optimum gradient found for the separation of the five analytes was as follows: Initial 35% B, increase to 75% B by 0.10 min, increase to 90% B by 13.00 min, hold for two min, then reduce back to 35% B by 15.10 min. Stop time was set at 17 min. The chip was switched back to enrichment mode at 15 min. This gradient achieved the separation shown in Figure 5-6.

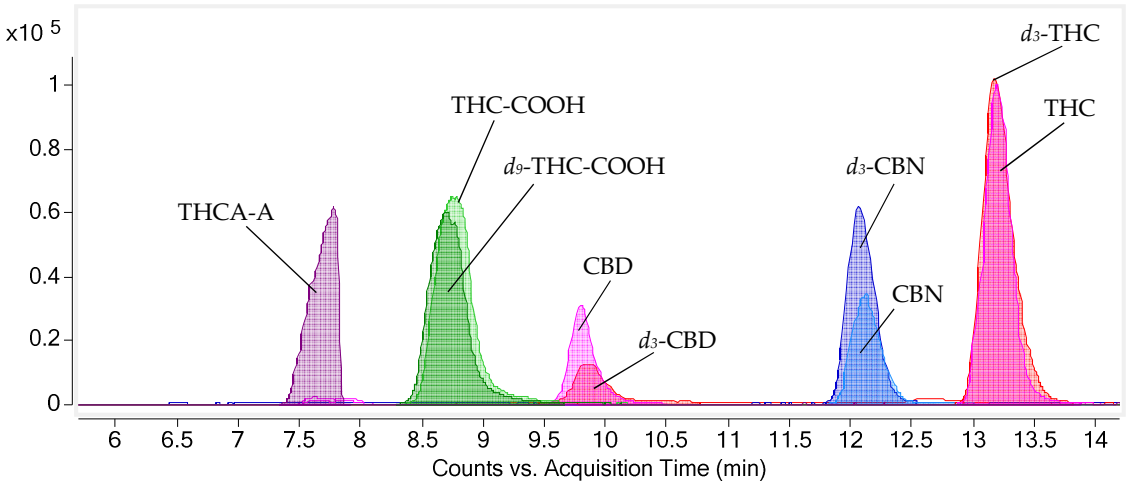

Figure 5-6: Chromatographic separation of the compounds achieved using the LC–chip system.

Injection volumes of one to five microlitres were trialled. There did not appear to be any improvement in sensitivity beyond two microlitres and so this volume was set for

all remaining analyses. As the enrichment column has a volume of 0.5 μL, samples were being concentrated four-fold in the column.

5.3.1.2 Troubleshooting – Blockages

After an unexpectedly high number of blockages occurring during use that required the replacement of capillaries (from both the capillary and nano pumps), it became apparent that the continual attachment and detachment of the LC-chip system for use with the Q–TOF–MS were detrimental to the capillaries, so one-day uses of the instrument were ceased. Experiments were then planned for multiple days at a time (and over the weekend in order to minimise disruption to the other Q–TOF–MS users). It appears that although user interaction is minimised through the use of the chip, the system is still not as robust as regular LC systems due to the fragility of the capillaries.

5.3.1.3 Ionisation

Most ESI sources have a window built-in so the spray can be viewed from the outside. There is no such window on the chip cube; instead, a mini digital camera is mounted inside so the spray chamber can be viewed on the computer screen. This is important for the development of a method since a number of settings are required to be optimised in order to achieve a stable spray. If the voltage is too low, liquid will accumulate at the tip of the needle and drip intermittently. If the voltage is too high, the spray will become erratic. The spray shape also varies based on mobile phase composition, which creates some difficulties when using a gradient elution. The lower the organic content of the mobile phase, the higher the voltage is needed to give an adequate spray. The optimum capillary voltage for these analyses was found to be 1750 V with the needle positioned as shown in Figure 5-7.

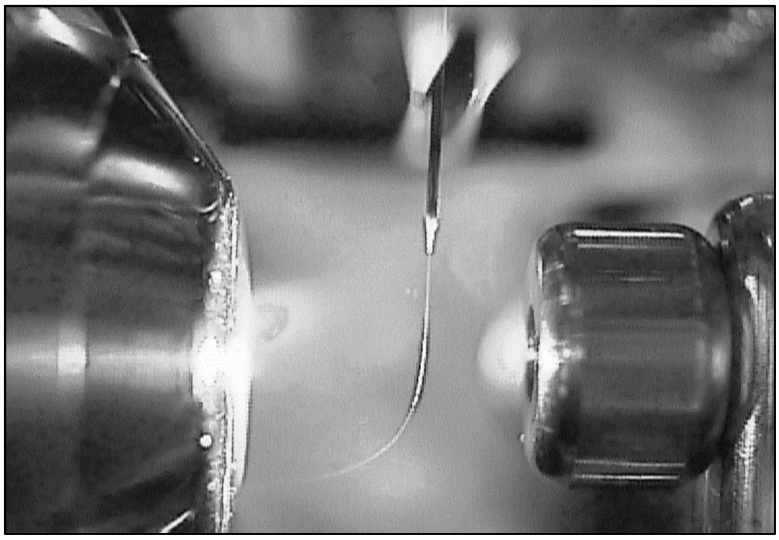

Figure 5-7: Nano spray emitter positioned with ideal spray shape viewed via the camera mounted inside the chip-cube.

Since the optimum mobile gradient began with a higher aqueous content but the analytes eluted later with a higher organic content, this voltage was usually insufficient to produce a spray at the beginning of the run. However, a stable spray was formed by the time the analytes were eluted from the column and these were successfully ionised and detected in the MS.

A reference mix including two reference compounds was made up and applied according to instructions, but unfortunately these were not sufficiently detected in the MS. The high mass (*m*/*z* 922.009798) was detected for a short while, but the low mass (*m*/*z* 322.048121) could never be found, even when ten times the recommended amount was added to the wick.

5.3.1.4 Mass spectrometry

Analysis was trialled in auto MS/MS and targeted MS/MS modes, however since the reference masses were not being detected, auto MS/MS was the better option to avoid analytes being missed because they were out of the targeted mass accuracy range. All analytes were listed in the preferred ion list with their respective optimal collision energies (Table 5-1). A ±1000 ppm mass threshold was set for all analytes.

Table 5-1: Auto MS/MS preferred ion masses and their respective collision energies.

Compound	Mass (*m*/*z*)	Collision energy (V)
CBN	311.2006	20
d_3-CBN	314.2194	23
CBD/THC	315.2319	23
d_3-CBD/d_3-THC	318.2507	27
THC-COOH	345.2061	25
d_9-THC-COOH	354.2625	27
THCA-A	359.2217	10

5.3.1.5 Data analysis

After trialling a number of methods of analysis using the Qualitative Analysis software, data was ultimately analysed using the find by formula function. A spectral library of the relevant cannabinoids and internal standards was created in the PCDL software and this was used to find matches within the acquired data. Matches were sometimes poorly scored, but this was generally due to a large mass error which was unavoidable as a result of the lack of reference masses found during analysis. A smoothing function was also programmed into the analysis method before peaks were integrated.

5.3.2 Validation studies

5.3.2.1 Spiked mobile phase

Calibration was first attempted with analytes in mobile phase only. This was performed via a serial dilution of the working stock solution of THC, CBD, CBN, THC-COOH and THCA-A that gave ten concentrations of the analytes in methanol. Three QC samples were also prepared. Mixed deuterated internal standard was added to each vial. These samples were gently dried under N_2 gas at 40 °C before being reconstituted with 500 μL of mobile phase, resulting in analyte concentrations

of 500, 400, 300, 200, 100, 50, 25, 10, 5 and 2 ng/mL for calibration and 360, 75, and 7.5 ng/mL as QC samples. Internal standards were present at 40 ng/mL in each vial.

Calibration was run in this way three times, and from this, validation data was obtained (Table 5-2). Linearity was calculated using the line of best fit and a correlation coefficient >0.99 was achieved for CBN, CBD, THC and THC-COOH (Figure 5-8). Results for THCA-A did not produce suitably linear curves, so further validation was not possible for this analyte. LOQs were determined as being the lowest concentration calibrator that gave %MRE and %RSD values <20. The method was most sensitive towards THC-COOH which had an LOQ of 5 ng/mL. The LOQ for THC was 10 ng/mL and CBD and CBN both has an LOQ of 25 ng/mL. Specific LODs were not calculated; however peaks were often visible at concentration points below the determined LOQs, even sometimes at 2 ng/mL for THC and THC-COOH.

Table 5-2: Sensitivity and linearity data for the detection of cannabinoids by LC-chip-MS.

Compound	LOQ (ng/mL)	Linear range (ng/mL)	R^2
CBN	25	25-500	0.9987
CBD	25	25-500	0.9980
THC	10	10-500	0.9996
THC-COOH	5	5-500	0.9995

The peak areas of THCA-A were normalised using the peak areas of d_9-THC-COOH as a deuterated version of THCA-A was unavailable. This use of d_9-THC-COOH was chosen since it eluted closest to the analyte. Unfortunately, this seems to have been unsuitable as calibration was unsuccessful. The other deuterated internal standards were also tested to normalise the THCA-A peak areas, but all were unsuccessful. THCA-A was the first analyte eluting off the column, appearing shortly after the stabilisation of the nanospray. This may have had an impact on the consistency of its response seen in the MS. THCA-A also gave a much narrower peak resulting in considerably smaller areas compared to the internal standard. Perhaps modifying the

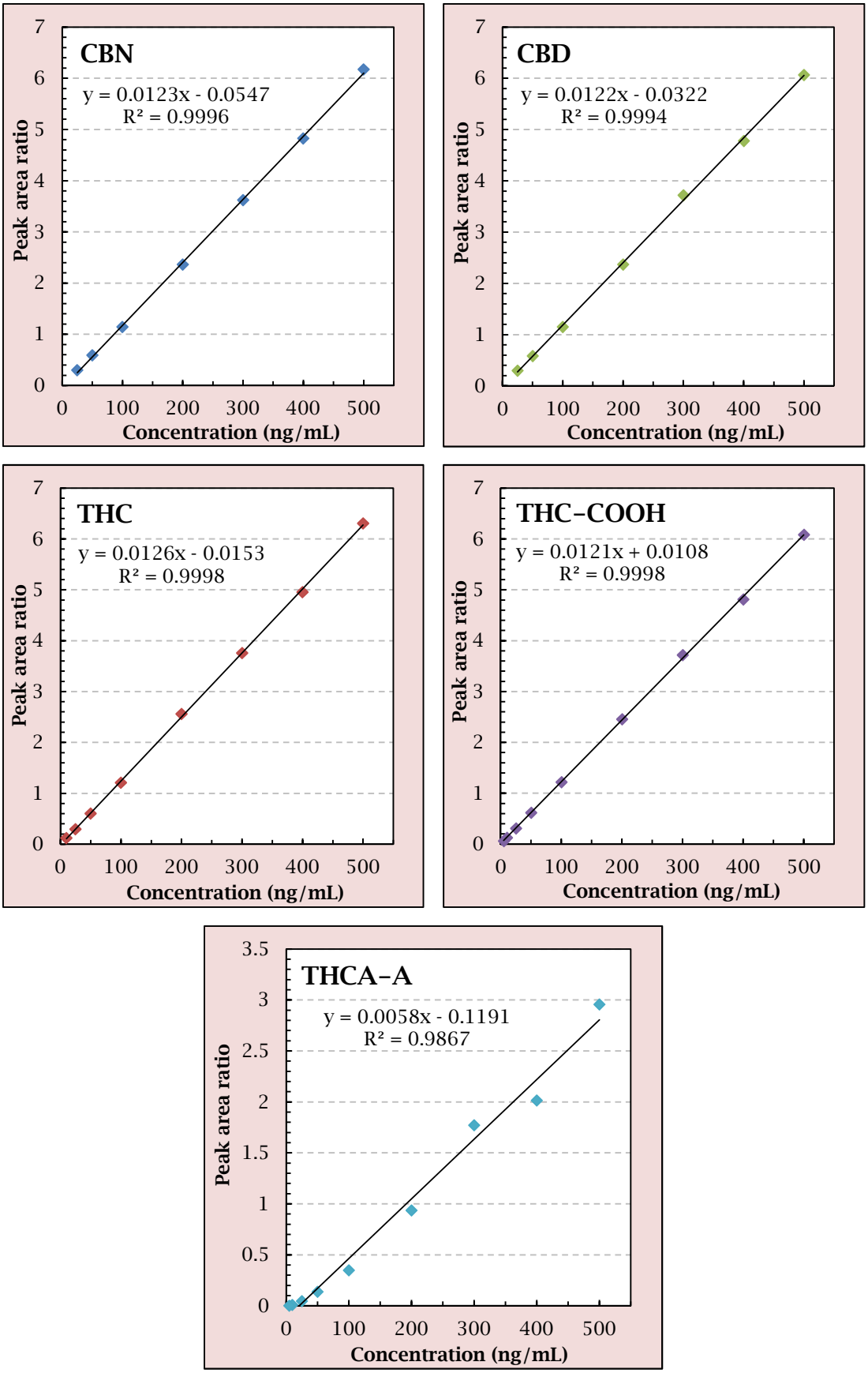

Figure 5-8: Example standard calibration curves of CBN, CBD, THC, THC-COOH, and THCA-A in mobile phase by LC-chip-Q-TOF-MS.

mobile phase to allow THCA-A to elute later and much closer to another internal standard may allow for an acceptable calibration curve. A deuterated form of THCA-A would be ideal if it becomes available in the future.

Although the method is not sensitive, validation was successful as precision and accuracy data for the QC samples gave values for %MRE and %RSD at <15% (Table 5-3). As 7.5 ng/mL is below the LOQ for CBN, CBD and THC, this QC was excluded for these analytes.

Table 5-3: Precision and accuracy data for intra-assay calibration using spiked mobile phase (n = 3).

		CBN	CBD	THC	THC-COOH
360 ng/mL	%MRE	6.6	4.2	0.8	5.5
	%RSD	3.5	2.5	0.9	1.5
75 ng/mL	%MRE	10.1	4.4	0.4	1.3
	%RSD	2.0	1.6	0.5	1.7
7.5 ng/mL	%MRE	-	-	-	12.6
	%RSD	-	-	-	14.9
LOQ*	%MRE	11.4	4.0	4.0	15.9
	%RSD	9.2	4.5	4.5	19.7

**LOQ concentration levels are 25 ng/mL for CBN and CBD, 10 ng/mL for THC, and 5 ng/mL for THC-COOH.*

5.3.2.2 Extracted neat oral fluid samples

Following the calibration in mobile phase, the same procedure was carried out for neat oral fluid samples in triplicate. As before, serial dilution of the working stock solution of THC, CBD, CBN, THC-COOH and THCA-A gave ten concentrations of the analytes in methanol. Of each of these, 25 µL were spiked into a separate tube containing 500 µL of drug-free oral fluid resulting in concentrations of 500, 400, 300, 200, 100, 50, 25, 10, 5 and 2 ng/mL for each analyte in solution. Internal standard (2 µg/mL) was then added to each tube (25 µL) giving a concentration of 100 ng/mL of d_3-THC, d_3-CBD, d_3-CBN and d_9-THC-COOH in solution. This higher concentration was chosen to ensure adequate response was achieved, given expected losses due to

extraction recovery and matrix interferences. LLE was performed as previously described in Section 5.2.2.

Linearity was again achieved for CBN, CBD, THC and THC-COOH with calibration curves producing correlation coefficients >0.99 (Table 5-4 and Figure 5-9).

Table 5-4: Sensitivity and linearity data for the detection of cannabinoids in oral fluid by LC-chip-Q-TOF-MS (n = 3).

Compound	LOQ (ng/mL)	Linear range (ng/mL)	R^2
CBN	25	25-500	0.9985
CBD	100	100-500	0.9979
THC	25	25-500	0.9972
THC-COOH	10	10-500	0.9992

An LOQ of 10 ng/mL was achieved for THC-COOH. The LOQ of CBN and THC was 25 ng/mL. THC consistently produced peaks at concentrations lower than the calculated LOQ; however, matrix peaks eluting near the internal standard interfered and affected the peak area ratios at lower concentrations. Likewise, both CBD and d_3-CBD suffered from interfering peaks resulting in an LOQ of 100 ng/mL. Validation was not performed for THCA-A due to the poor results seen in the spiked mobile phase calibrations. Example calibration curves for CBN, CBD, THC and THC-COOH can be seen in Figure 5-9.

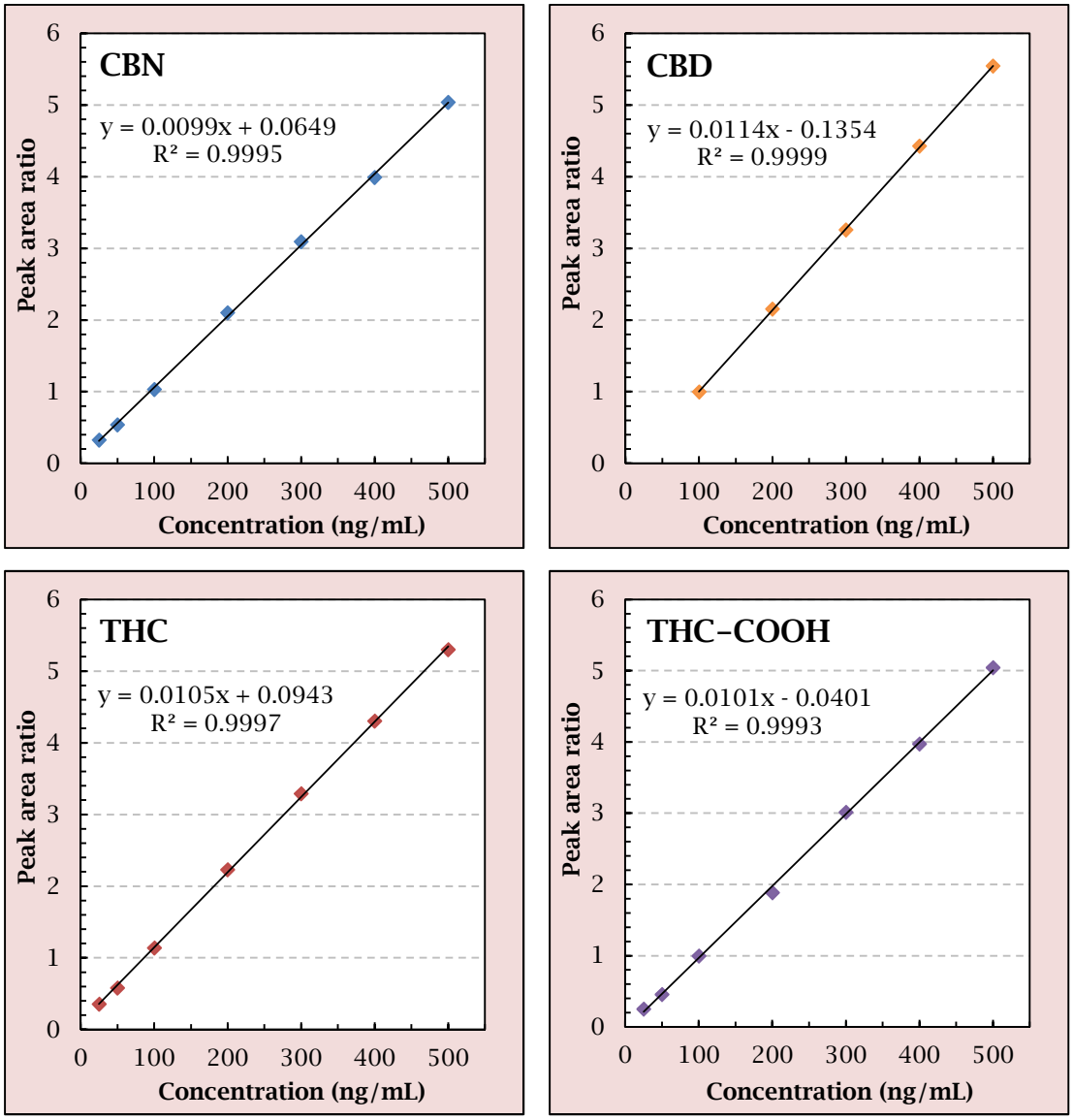

Figure 5-9: Example calibration curves for THC, CBD, CBN, and THC-COOH extracted from oral fluid and analysed by LC-chip-Q-TOF-MS.

Precision and accuracy data at 360 and 75 ng/ml and at the LOQ of each analyte can be seen in Table 5-6. CBD does not have data for the 75 ng/mL QC as the LOQ was above this level at 100 ng/mL. Validation was again successful, despite the poor sensitivity as all relevant QCs achieved %MRE and %RSD values <15.

Table 5-5: Precision and accuracy data for intra-assay calibration of extracted oral fluid samples (n = 3).

		CBN	CBD	THC	THC-COOH
360 ng/mL	%MRE	6.7	5.0	9.2	10.4
	%RSD	10.3	6.3	11.5	11.5
75 ng/mL	%MRE	9.3	-	6.0	5.1
	%RSD	10.0	-	9.3	6.9
LOQ*	%MRE	12.7	4.3	4.4	10.5
	%RSD	16.5	4.7	4.1	9.0

**LOQ concentration levels are 25 ng/mL for CBN and THC, 100 ng/mL for CBD, 10 ng/mL for THC-COOH.*

5.3.2.3 Matrix effects

Matrix effects were assessed by extracting blank samples and spiking with analytes and internal standards at 100 ng/mL at the last stage before reconstituting with mobile phase. These samples (n = 5) were run alongside samples of analytes spiked into mobile phase at the same concentration. Only a single set of injections was used for the calculations as there were issues with repeat injections not being particularly consistent; responses were observed to decline over time. The measurements used were alternate injections between extracted samples and mobile phase samples.

While there was minimal ion enhancement or suppression seen for CBN, THC, THC-COOH, their respective internal standards, and THCA-A (0–18% reduction in response compared to spiked mobile phase), ion enhancement appeared to affect CBD (23% increase in response) and even more so, d_3-CBD (87% increase). These results are preliminary approximations only, due to the inconsistency of responses seen between injections.

Regardless, the matrix introduced a large number of peaks which interfered with the resolution of the analyte and internal standard peaks. Figure 5-10 shows an example of overlaid extracted ion chromatograms (EICs) of an extracted 100 ng/mL sample with internal standards also present at 100 ng/mL. While the find by formula function worked well to integrate the analyte peaks, comparing with the chromatograms of

spiked mobile phase in Figure 5-6, it is clear that there are a number of other compounds now appearing from the matrix and this has greatly affected the sensitivity of the method.

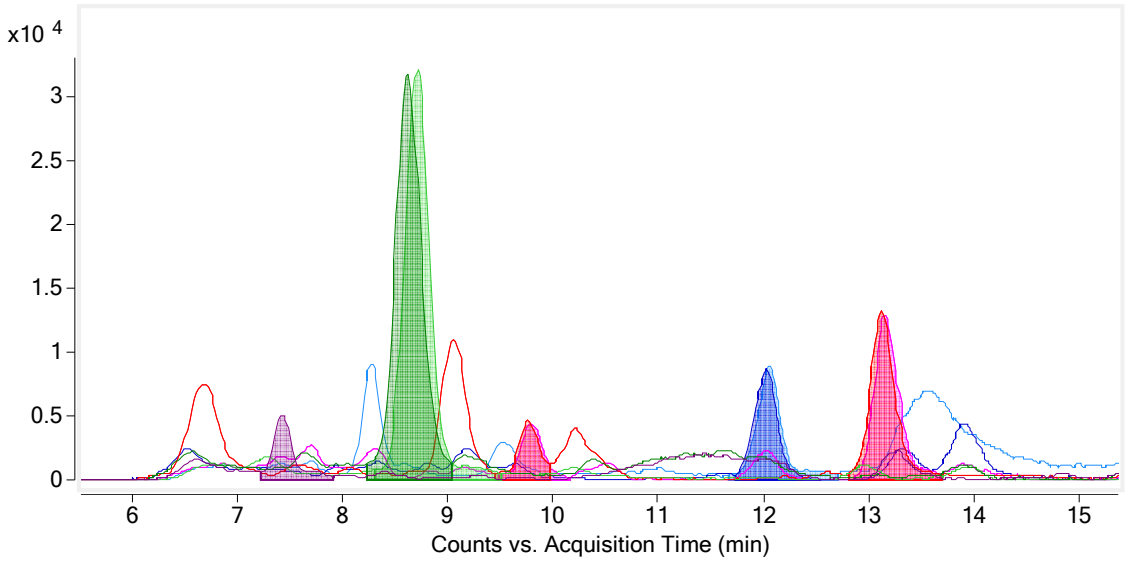

Figure 5-10: Example overlaid EICs of an extracted 100 ng/mL sample. Shaded peaks represent the analytes of interest. Left to right: THCA-A, (d_9-)THC-COOH, (d_3-)CBD, (d_3-)CBN, and (d_3-)THC.

It was thought that the use of an enrichment column would greatly reduce the number and concentrations of endogenous compounds from the matrix of the sample, but it appears to not have been the case. It seems these compounds were also retained in the column and were not washed through at 65% aqueous mobile phase.

5.3.3 Comparison with normal flow LC–Q–TOF–MS

The sensitivity seen with the LC–chip–Q–TOF–MS method was quite poor; unfortunately it was not possible to attain the detection limits achieved by He *et al.* [104] and Concheiro *et al.* [47] using the Thermo Scientific nano flow LC system. However, the MS instrumentation used in this study may be contributing to this, as it is not as sensitive as the QQQ–MS used in the previous studies outlined in chapters 1 and 3 of this thesis. A method was set up on the Q–TOF–MS using a normal flow LC system to compare the resulting sensitivities with those found using the LC–chip system. Recovery of the extraction procedure was also carried out using this instrumentation.

5.3.3.1 Instrument parameters

Chromatographic elution was isocratic with 75% acetonitrile in water with 0.1% formic acid. Twenty microlitres of sample was injected for analysis. The MS parameters remained the same, except for the capillary voltage which was set at 3500 V.

All analytes eluted within 5 min; total run time was 9 min to allow for matrix compounds to elute for the oral fluid extracted samples. An example overlaid chromatogram of a spiked mobile phase sample with 50 ng/mL of each analyte and internal standard can be seen in Figure 5-11.

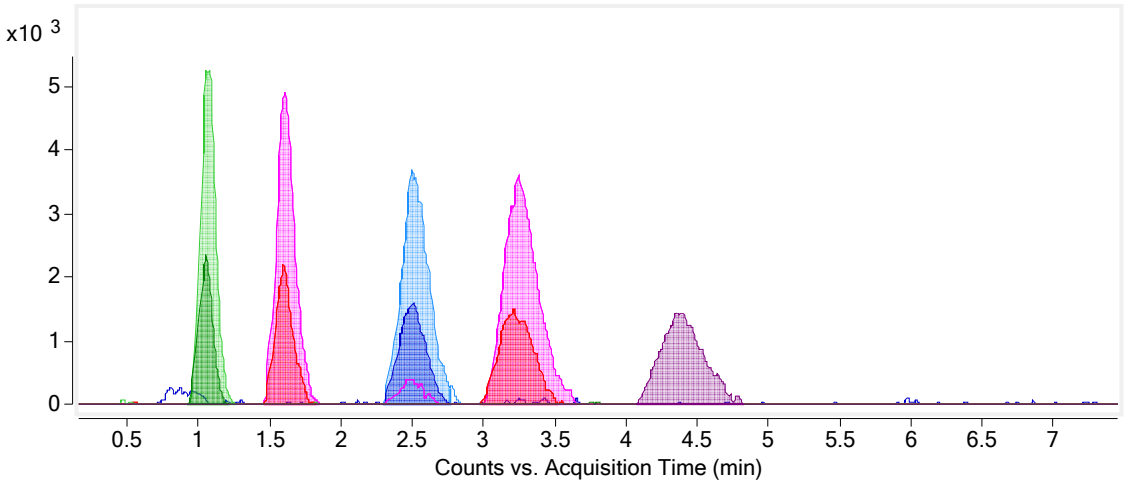

Figure 5-11: Example of overlaid EICs of all analytes (100 ng/mL) and internal standards (50 ng/mL) spiked into mobile phase and analysed by LC–Q–TOF–MS. Left to right: (d_9-)THC-COOH, (d_3-)CBD, (d_3-)CBN, (d_3-)THC, and THCA-A.

THCA-A elutes last, shortly after THC. Therefore, deuterated THC was used as the internal standard for THCA-A for calibration and validation purposes.

5.3.3.2 Validation

Calibration samples were made up in mobile phase according to the same procedure as outlined in Section 5.3.2 for the LC-chip method. Concentrations were 2, 5, 10, 50, 100, 200, 300, 400 and 500 ng/mL.

Linearity was achieved for all analytes, including THCA-A, with correlation coefficients all >0.999 (Table 5-6). An LOQ of 5 ng/mL was achieved for CBD, THC and THC-COOH. An LOQ of 10 ng/mL was achieved for CBN and THCA-A. While these are an improvement on the LC-chip method, they are far from the standard expected for the analysis of cannabinoids by LC–MS/MS. Additionally, a high volume of twenty microlitres was injected to achieve this level of sensitivity.

Table 5-6: Sensitivity and linearity data for the detection of cannabinoids by LC-Q-TOF-MS (n = 3).

Compound	LOQ (ng/mL)	Linear range (ng/mL)	R^2
CBN	10	10-500	0.9995
CBD	5	5-500	0.9999
THC	5	5-500	0.9996
THC-COOH	5	5-500	0.9996
THCA-A	10	10-500	0.9996

Precision and accuracy data was calculated for the QC samples at 360, 75 and 7.5 ng/mL (Table 5-7). The 7.5 ng/mL concentration level was excluded from the data for CBN and THCA-A as their LOQ was higher than this at 10 ng/mL. The majority of the %MRE and %RSD values fell within the acceptable 15%. Only the %RSD for THC at 7.5 ng/mL was found to be outside this range at 15.85%.

Clearly, the sensitivity of the Q–TOF–MS instrument used in these studies has contributed to the poor sensitivity of the LC–chip–MS method. Better results may be achieved if a more sensitive MS is used. Unfortunately, this was not able to be pursued for this project as an additional set of equipment was required for the LC–chip system to be set up with the QQQ–MS.

Table 5-7: Precision and accuracy data for intra-assay calibration of normal flow LC-Q-TOF-MS using spiked mobile phase (n = 3).

		CBN	CBD	THC	THC-COOH	THCA-A
360 ng/mL	%MRE	1.69	0.47	2.33	3.06	0.91
	%RSD	9.37	10.13	9.86	6.25	12.01
75 ng/mL	%MRE	0.96	2.51	0.13	0.04	3.36
	%RSD	9.84	11.52	12.17	11.35	10.37
7.5 ng/mL	%MRE	-	4.19	4.77	6.89	-
	%RSD	-	9.33	15.85	5.55	-
LOQ*	%MRE	0.41	6.23	2.35	5.72	8.69
	%RSD	19.25	3.10	17.02	18.17	9.16

**LOQ concentration levels are 10 ng/mL for CBN and THCA-A, and 5 ng/mL for CBD, THC, and THC-COOH.*

5.3.3.3 Matrix effects and recovery

Matrix effects were assessed, similarly to the LC–chip method, by extracting blank samples and spiking with analytes and internal standards at 100 ng/mL before reconstituting with mobile phase (n = 5). Peak areas of the analytes and internal standards were compared to those of samples of mobile phase with the analytes spiked at the same concentration.

Matrix effects in terms of ion enhancement and suppression were minimal, with only a 1–4.6% change in response observed between the samples containing matrix and spiked mobile phase. This was expected, as similar results were seen with the QQQ–MS in previous studies involving neat oral fluid (Sections 2.3.2 and 4.3.1). As some ion suppression has been observed when analysing samples that have been diluted with commercial buffers such as the Cozart® DDS buffer (see Section 4.3.1), it was hoped that the LC–chip system could overcome this issue by utilising the in-built enrichment column. However, as the results for neat oral fluid samples are already problematic with respect to matrix interferences, it is unlikely that the LC–chip system can provide any improvement for matrix effects for buffer-diluted samples, and so this was not further investigated.

As the extracted ion chromatographs show in Figure 5-12, it is clear that there is much less interference from the matrix as the traces are much cleaner than those obtained from the LC–chip method. Out of all the analytes and internal standards, only d_3-CBN suffered from a small co-eluting peak from the matrix using the regular flow LC system. The response at 100 ng/mL was not adversely affected by this interference.

Poor extraction efficiency may also be contributing to the high LOQs seen with the oral fluid extracted samples run by the LC-chip system. The recovery of the extraction method was determined by comparing the peak areas of each analyte and internal standard of oral fluid extracted samples with those of samples prepared by extracting blank oral fluid that was then spiked with analytes before reconstitution in mobile phase, at the same concentration of 100 ng/mL (n = 5). As 1.5 mL of extraction solvent was added to the samples and only 1 mL aliquots were transferred to vials, the calculated recoveries were multiplied by 1.5 to estimate the extraction efficiency of the method.

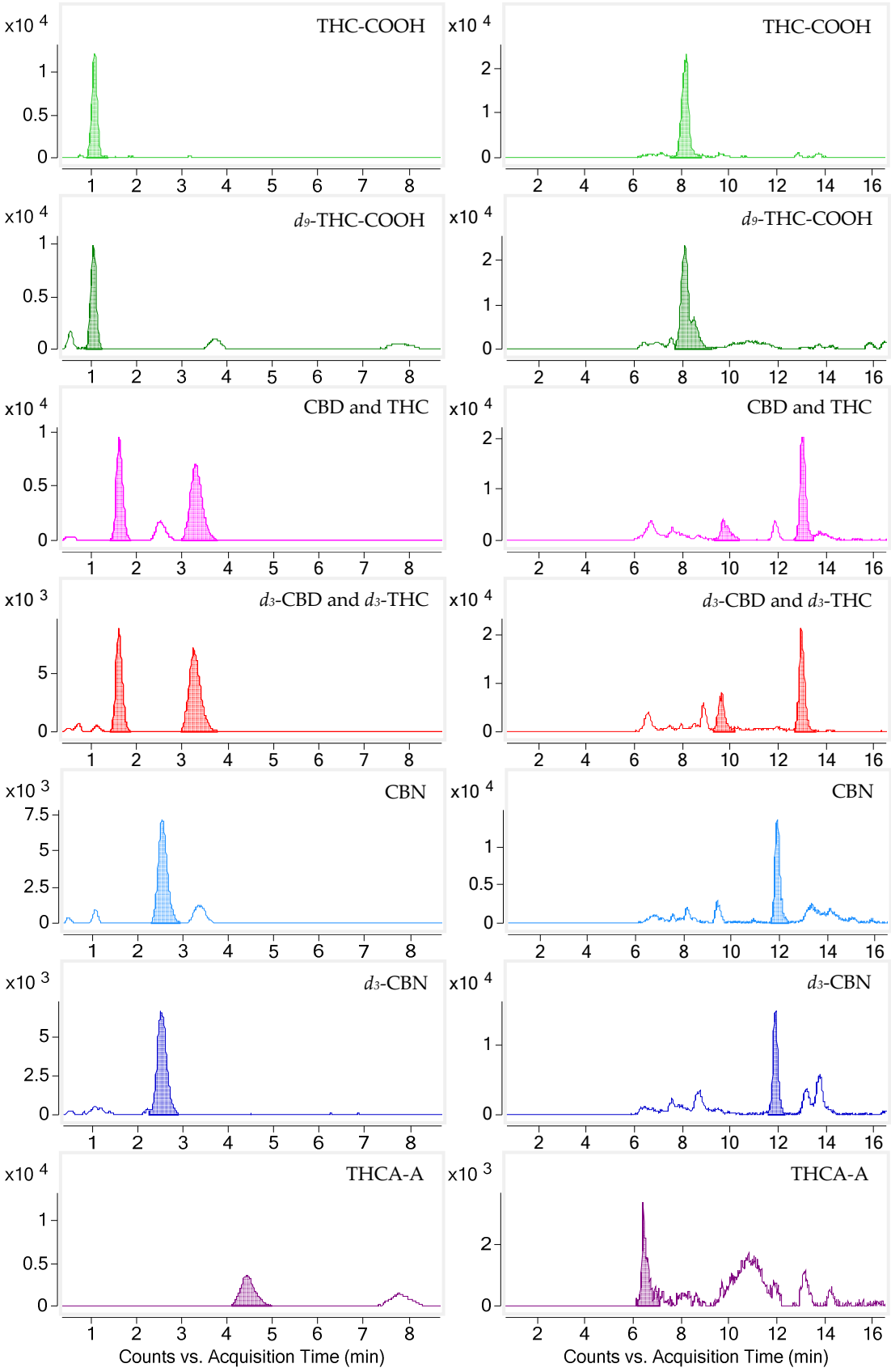

Figure 5-12: EICs for each analyte and internal standard for a 100 ng/mL extracted sample with analytes added prior to reconstitution in mobile phase. Shaded peaks represent the analytes of interest. There is much less noise and interfering peaks seen in the normal flow LC results (left column) compared to the chromatograms obtained using the LC–chip system (right column).

As shown in Figure 5-13, the extraction efficiency varied between the analytes with approximately 100% of THC-COOH and its deuterated standard being transferred into the extraction solvent while 55.7–74.4% of the other analytes was transferred. These results correlate with those found in previous studies using similar extraction procedures (refer to Sections 2.3.2 and 4.3.1). Optimising the extraction procedure may significantly improve the LOQs found for the extracted oral fluid samples analysed by the LC–chip method. The use of non-ionic surfactant Triton, would greatly increase the extraction efficiency as seen in the results of Section 3.3.5; however, this additive is not suitable for use with LC–MS. Regardless, an optimised recovery could only improve the method to the point of the LOQs seen in the spiked oral fluid samples, which was still quite high for both the LC–chip and normal flow LC methods.

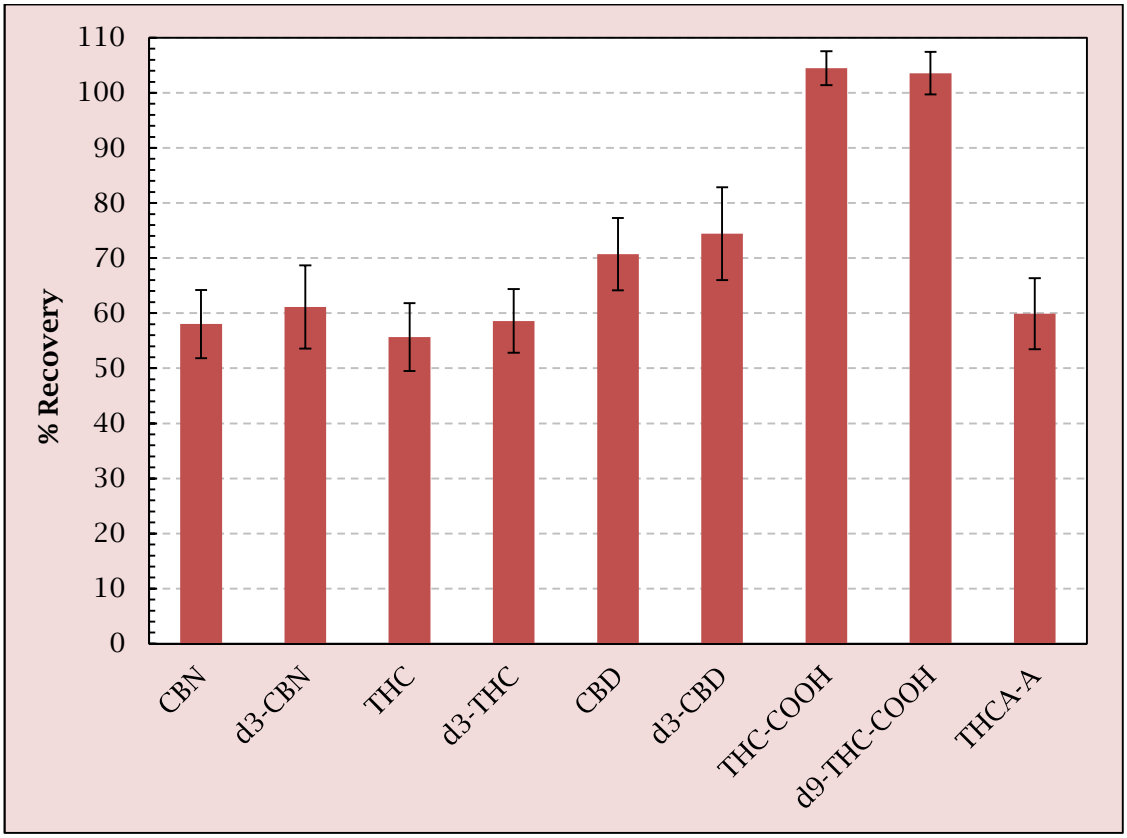

Figure 5-13: Extraction efficiency of the LLE method used to extract CBN, THC, CBD, THC-COOH, their respective internal standards, and THCA-A from neat oral fluid for LC–Q–TOF–MS analyses.

The low sensitivity of the Q–TOF–MS has undoubtedly impacted on the potential of the LC–chip system. Even with the reduction in matrix interferences using the

normal-flow LC system, the sensitivity of the method was not much better than with LC–chip. These results are all preliminary, and although more experiments are necessary to improve the use of the chip for the analysis of cannabinoids, it would be pointless to pursue these on the current instrumentation. Ideally, it would have been best to use the available QQQ–MS that was utilised in previous chapters and is known to have superior sensitivity. However, this was not possible due to the need to purchase additional equipment in order to have it set up which, unfortunately was not practicable for these studies. Q–TOF–MS does have its benefits in qualitative screening methods with its capability of measuring exact masses; and so if the reference mass detection issues with the chip system are resolved, it may be useful for such applications.

5.4 Conclusions

Chip–HPLC has the potential to be used to analyse oral fluid samples for cannabinoids as chromatographic separation was achieved. However, the lipophilic nature of cannabinoids gives rise to difficulties when using an enrichment column, as a high aqueous phase is necessary, which resulted in carryover issues in the instrument. Additionally, as the enrichment column resulted in an increase in matrix effects, more effective clean-up procedures are required before sample analysis by this technique. These are, however, preliminary results and further trials may show improvements. Any future studies should utilise a more sensitive MS detector than was used in this study. The LC–chip system, while useful for its ability to analyse very small volumes of a sample, does not seem to provide any real advantage over the current routine testing systems for cannabinoids in oral fluid due to its high consumable cost and larger physical footprint. This study has shown that the small molecule chip can be used to analyse cannabinoids in oral fluid, so perhaps the system may be more useful for looking for the presence of drugs in trace biological fluids such as those sometimes found at crime scenes.

Chapter 6: Conclusions and recommendations for further work

Chapter 6: Conclusions and recommendations for further work

Oral fluid testing for cannabis has become popular due to its ease of collection, minimal invasiveness and relatively short window of detection. While oral fluid has been used widely for drug testing purposes for a number of years, there are a number of gaps in knowledge relating to the detection of cannabinoids, particularly the major analyte THC, in the oral fluid matrix.

THC has been observed to be poorly stable in the short term of a couple of weeks when stored in neat oral fluid in polypropylene containers, while excellent long term stability is seen in commercial buffer-stabilised samples stored for over a year. Preliminary results indicated that degradation and adsorption may both contribute to the losses observed. Further experiments revealed the tendency of THC to bind to polypropylene surfaces, particularly in aqueous phases. This adsorption of THC to surfaces was observed to lead to a poor overall recovery from neat oral fluid samples. The recovery of THC was shown to be dependent on the oral fluid volume to inner surface area ratio, as a smaller oral fluid volume in larger containers suffered from a higher loss of THC. The concentration of THC in the sample did not have any effect on the recovery rate. The interactions between THC and polypropylene surfaces should be further examined since a large proportion of losses appear to be related to this activity. The use of the non-ionic surfactant, Triton, significantly increased the THC recovery from polypropylene containers and thus reduced adsorptive losses. The addition of Triton or another suitable non-ionic surfactant may be used as a tool in future studies using GC–MS to investigate the individual factors affecting the stability and recovery of THC in oral fluid. LC–MS is subject to contamination from surfactants such as Triton. Investigating alternative additives that can decrease adsorption and have a greater compatibility with LC–MS would be useful, given the prevalence of this instrumentation in toxicological laboratories.

Sodium azide did not appear to provide any benefit as an additive in these studies which indicates THC is not being lost to microbial action in the first month of storage. Azide is a common anti-microbial additive in storage solutions, acting as a bacteriostatic. It is used to minimise microbial degradation in buffers such as the Cozart® DDS and perhaps has a more noticeable effect on samples stored over a longer period of time. According to the results presented in this thesis, anti-oxidants may be a more helpful additive to be used instead of, or combined with, azide. Further work could involve monitoring the bacterial profile of neat oral fluid samples in the presence of either azide or another anti-microbial agent, and investigating the effectiveness of these additives to inhibit microbial growth in these samples. Oxidative degradation products of THC could also be monitored with and without the presence of anti-oxidants. These could then be correlated with any THC losses seen in samples to determine whether microbial action or oxidative degradation contributes most to the degradation of THC, or if it is an equal combination of both. Using known volumes of additives in pre-measured volumes of oral fluid may be an alternative to current commercial collection devices that have large variations in the volume of oral fluid collected, and even the volume of buffer included in the vials. Finding an optimal balance between surfactant, anti-microbial and anti-oxidant would be ideal for such purposes. In the meantime, currently available commercial buffers such as the Cozart® DDS buffer can be added to neat oral fluid samples after the collection of a known volume, to stabilise samples whilst ensuring certainty in the calculated concentrations of THC.

The interactions between THC and oral fluid itself are not fully understood. THC was observed to bind with the proteinaceous components in oral fluid. These components typically begin to settle to the bottom of the storage containers almost immediately following the collection of neat oral fluid samples. Therefore thorough mixing is required to ensure a representative sample of the oral fluid is obtained after a period of storage. This binding was shown to help reduce the degree of THC adsorption to plastic container surfaces; however, it was also responsible for the reduction of the LLE recovery rate. It is unclear whether this effect also applies to SPE methods.

Further research into understanding the other factors that govern the behaviour of THC in oral fluid is necessary, as the matrix gains more and more popularity in the field of drug testing.

THC and CBD were shown to be present in high concentrations in the oral fluid of patients taking high doses of Sativex® for at least two hours following the dose. Despite this, a large proportion of Sativex® patients following this heavy dosage pattern would likely pass a DrugWipe® II Twin test within the two hours following the dose, even with high concentrations of THC present in their oral fluid. The Cozart® DDS device correctly identified all instances of Sativex® use; however, following the current procedures on the roadside, this test would not be performed after a negative DrugWipe® result. For those who do give a positive result in both tests, the high CBD to THC ratio found by confirmatory testing should indicate that Sativex® has been used, since CBD is rarely detected in the oral fluid of Australian cannabis smokers; however, concurrent use of cannabis cannot be excluded. These conclusions are based on only five participants receiving Sativex® treatment, and so repeating the study with a larger sample size will give more conclusive results. Further studies should also aim to determine how long after a dose the screening tests can return positive results for THC. Smaller doses should be trialled, as the doses given to the participants in this study were excessive.

The DrugWipe® devices have previously been shown to have poor sensitivities, and even recently, Wille *et al.* [147] found the newer, DrugWipe® 5S to still have a low sensitivity of 50% using a 10 ng/mL cut-off level with tests taken 80 min after dosing. The confirmed concentrations of these oral fluid samples were found to be 34–281 ng/mL. However, at only 5 min after dosing, and with confirmed concentrations in the range of 77.7 to 12 360 ng/mL, a sensitivity of 90% was recorded for the device. The authors concluded that the device is useful if the aim of testing is to determine (very) recent use, though detection limits should be improved if the intent is to police zero tolerance.

If attempting to determine the risk of impairment, there are always arguments against zero tolerance based on issues such as passive exposure and chronic use [147]. Add to this, relatively high doses of Sativex® that can be detected by screening devices may not actually result in intoxication [148, 149]. This can have significant implications for roadside drug testing if cannabis becomes decriminalised in Australia. Decisions regarding cut-off levels will have to be made, especially in light of the recent plans to fund research into medicinal cannabis in NSW and Victoria. As clinical trials continue, medicinal cannabis is likely to become increasingly available. Pharmaceutical formulations are more likely to be made available for prescription since botanical forms are difficult to standardise and have highly variable effects on individuals [150, 151]. While any form of cannabis is currently illegal in Australia, many individuals are already using cannabis for medical purposes, with cannabis oil being administered in hospitals as part of clinical trials in NSW [152]. These developments highlight the need for further reform regarding roadside drug testing objectives. The AS4760 is currently undergoing revision and changes to the current testing procedures may be required in order to adhere to the new guidelines for oral fluid testing procedures. With the ever-changing situation regarding oral fluid testing, evaluations of screening tests and improved laboratory testing procedures are an ongoing part of this field of research.

Chip–HPLC showed some potential to be used to analyse oral fluid samples for cannabinoids. However, the lipophilic nature of cannabinoids resulted in difficulties overcoming carry-over in the instrumentation due to the high aqueous phase required for sample loading. Additionally, the in-built enrichment column appeared to enhance matrix effects, which is likely a result of the endogenous compounds also being concentrated in the column. The results presented are preliminary, and further trials may show improvements. A more sensitive MS/MS detector should be used in any future quantitative studies. The LC–chip system has a high consumable cost and larger physical footprint than regular, normal flow LC systems. Based on this, even with improvements in sensitivity, the LC–chip system does not appear to provide any real advantage over the current testing systems for cannabinoids in oral fluid.

However, this study provided a proof of concept for the detection of drugs by LC–chip–MS/MS as the detection of cannabinoids was achieved using the small molecule chip. With improved sensitivity, the system may be more useful in other applications requiring analysis of drugs in small sample volumes.

Appendices

Appendix A

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Technical Specifications

Cozart® DDS Drug Detection System

Intended Use: For the detection of drugs of abuse in oral fluid (saliva) samples. Samples are collected with the Cozart oral fluid collection swab. This is then applied via a dropper bottle on to a disposable cartridge.

Kit Contents: Each box contains 25 test cartridges and oral fluid collection swabs to perform 25 individual drug tests on the DDS.

Sample Type: Oral fluid (saliva)

Catalogue No.: **DDS805** **DDS 2 Drug Test Kit for:**
MDMA/Cannabis
Cartridge run time: 3 minutes

Shelf Life : 18 months from date of manufacture, when stored at 15-25°C

Cut-off Levels: Neat oral fluid cut-off levels for this drug panel were established by fortifying negative saliva samples with the following drug levels;

Drug Class	Target drug	Cut-off (ng/mL)
Cannabis*	Δ^9THC*	*
MDMA	MDMA	150
Methamphetamine	Methamphetamine	50

*Fortifying samples with Δ^9THC is difficult due to losses of material to surfaces and degradation. Validation using real samples was employed to illustrate the levels of Δ^9THC found in DDS positive samples.

Fifty-five poly-drug positive samples were obtained at a drug clinic. Twenty six samples gave a DDS positive response for cannabis and were further tested by GCMS for the presence of Δ^9THC in oral fluid.

All DDS screen positives were found to contain Δ^9THC. Six of the twenty six samples had Δ^9THC concentrations between **31 and 150 ng/mL**. The remaining twenty samples had concentrations of Δ^9THC ranging from **174 to 3006 ng/mL**.

This illustrates that the DDS system is able to detect at least 31 ng/mL of Δ^9THC.

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Figure A-1: Cozart® DDS technical specifications, page 1.

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Technical Specifications

Specificity and Cross Reactivity

The following compounds show a negative response at 100,000 ng/mL. Concentrations that are highlighted in yellow will produce a positive result.

	Compound	THC	Methamphetamine
1	Codeine	100,000	100,000
2	6-Acetyl Morphine	100,000	100,000
3	Oxycodone	100,000	100,000
4	Heroin	100,000	100,000
5	Dihydrocodeine	100,000	100,000
6	Morphine	100,000	100,000
7	MDA	100,000	100,000
8	MDMA	100,000	150
9	MDEA	100,000	2500
10	MBDB	100,000	100
11	(+) Ephedrine	100,000	100,000
12	(+) Pseudoephedrine	100,000	100,000
13	Methamphetamine	100,000	50
14	Amphetamine	100,000	100,000
15	Diazepam	100,000	100,000
16	Temazepam	100,000	100,000
17	Cocaine	100,000	100,000
18	Cocaethylene	100,000	100,000
19	Ecgonine methyl ester	100,000	100,000
20	Benzoylecgonine	100,000	100,000
21	Cotinine	100,000	100,000
22	Amobarbital	100,000	100,000
23	11-Hydroxy- Δ9-THC	1000	100,000
24	Δ9-THC	*	100,000
25	Cannabidiol	100,000	100,000
26	11-nor-9-Carboxy- Δ9-THC	10	100,000
27	Methadone	100,000	100,000
28	Amitriptyline	100,000	100,000
29	Chlorpheniramine Maleate	100,000	100,000
30	Paracetamol	100,000	100,000
31	Aspirin	100,000	100,000
32	β Phenylethylamine	100,000	100,000

Other drugs and drug metabolites that are not tested here may cause a positive result. Compounds for testing are under constant review, please contact Technical Services (techserv@cozart.co.uk) for any drugs not listed.

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Figure A-2: Cozart® DDS technical specifications, page 2.

Appendix B

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Material Safety Data Sheet

1. Product Identification

Product name:	Cozart® DDS Buffer
Chemical name:	Cozart® DDS Buffer containing 0.1% sodium azide
Product description:	Cozart® DDS Buffer
Manufacturer:	Concateno UK Ltd, 92 Milton Park, Abingdon, Oxon OX14 4RY, UK
Telephone:	+44 (0)1235 861483

2. Hazardous Ingredients

Hazardous component	Percent	CAS number	EEC classification	Symbol
Sodium Azide	0.1%	26628-22-8	247-852-1	T+ N

3. Hazard Identification

None: due to the exceptionally low levels of sodium azide it is believed that there is no hazard posed.

4. First Aid Measures

Eye contact:	Irrigate thoroughly with water for at least 10 minutes. OBTAIN MEDICAL ATTENTION IF IRRITATION PERSISTS.
Ingestion:	Provided person is conscious, wash out mouth with copious amounts of water. Do not swallow rinse water.
Inhalation:	Remove from exposure, rest and keep warm. In severe cases, OBTAIN MEDICAL ATTENTION.
Skin contact:	Wash skin with mild soap and water.

If warranted, seek medical attention. If possible, save sample of material that caused the reaction for use in determination of appropriate treatment.

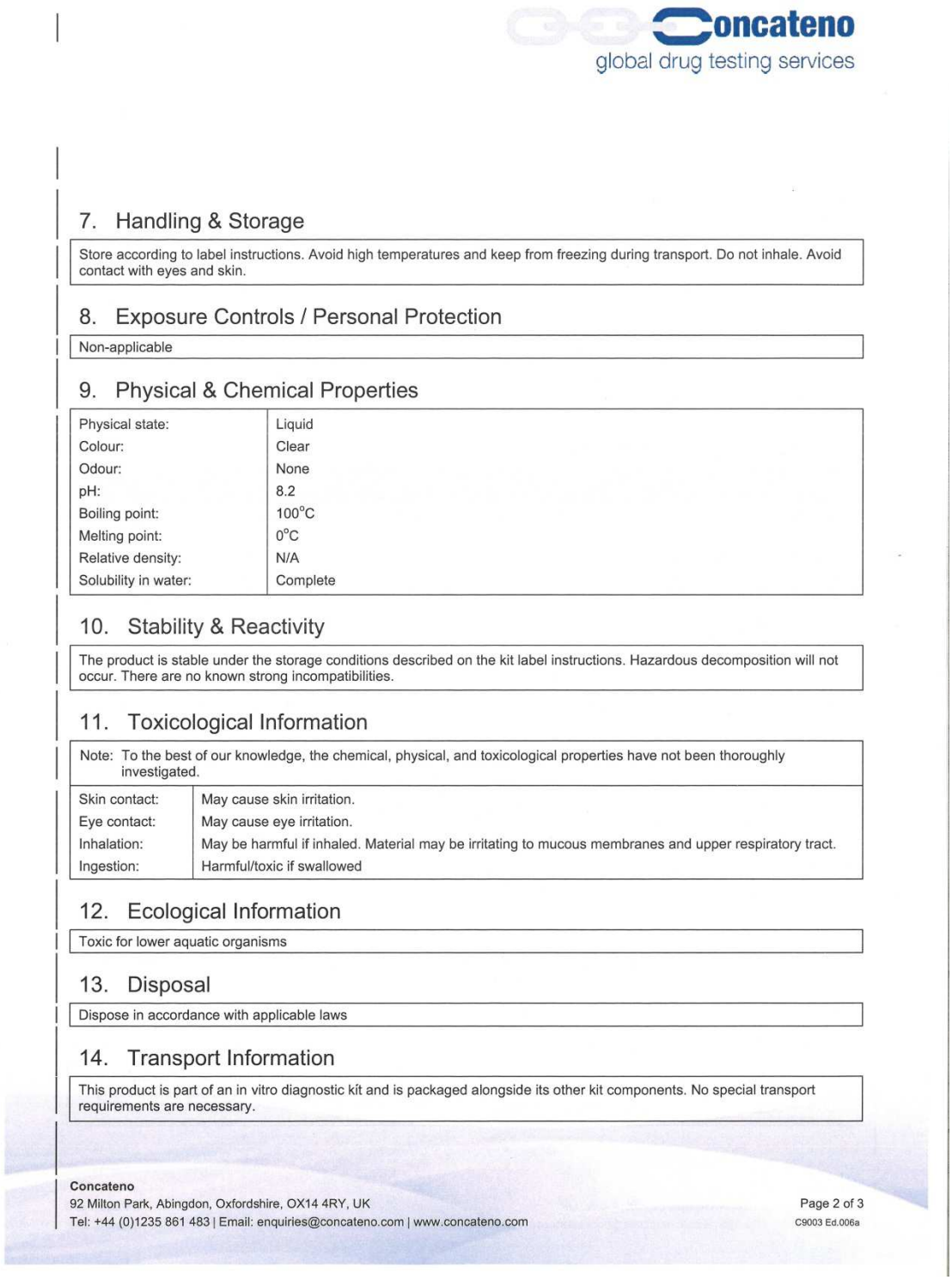
5. Fire Fighting Measures

Flash point:	Not known.
Fire extinguishing media:	Dry powder. Foam and water spray are effective but may cause frothing.
Fire fighting procedures:	Standard procedures for Class A fires. Use self contained breathing apparatus (SCBA) and protective clothing for structural fire fighting and protective clothing to prevent contact with skin and eyes. Emits toxic fumes under fire conditions.
Explosion hazards:	N/A

6. Accidental Release Measures

Avoid contaminating the water supply. Do not allow the material to enter drains or water courses. Wash site of spillage thoroughly with water and detergent. As with non-hazardous materials, wear protective gloves, eyewear and outer clothing. Caution: Products can present a slipping hazard, if spilled

Figure B-1: MSDS of Cozart® DDS buffer, page 1.



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7. Handling & Storage

Store according to label instructions. Avoid high temperatures and keep from freezing during transport. Do not inhale. Avoid contact with eyes and skin.

8. Exposure Controls / Personal Protection

Non-applicable

9. Physical & Chemical Properties

Physical state:	Liquid
Colour:	Clear
Odour:	None
pH:	8.2
Boiling point:	100°C
Melting point:	0°C
Relative density:	N/A
Solubility in water:	Complete

10. Stability & Reactivity

The product is stable under the storage conditions described on the kit label instructions. Hazardous decomposition will not occur. There are no known strong incompatibilities.

11. Toxicological Information

Note: To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Skin contact:	May cause skin irritation.
Eye contact:	May cause eye irritation.
Inhalation:	May be harmful if inhaled. Material may be irritating to mucous membranes and upper respiratory tract.
Ingestion:	Harmful/toxic if swallowed

12. Ecological Information

Toxic for lower aquatic organisms

13. Disposal

Dispose in accordance with applicable laws

14. Transport Information

This product is part of an in vitro diagnostic kit and is packaged alongside its other kit components. No special transport requirements are necessary.

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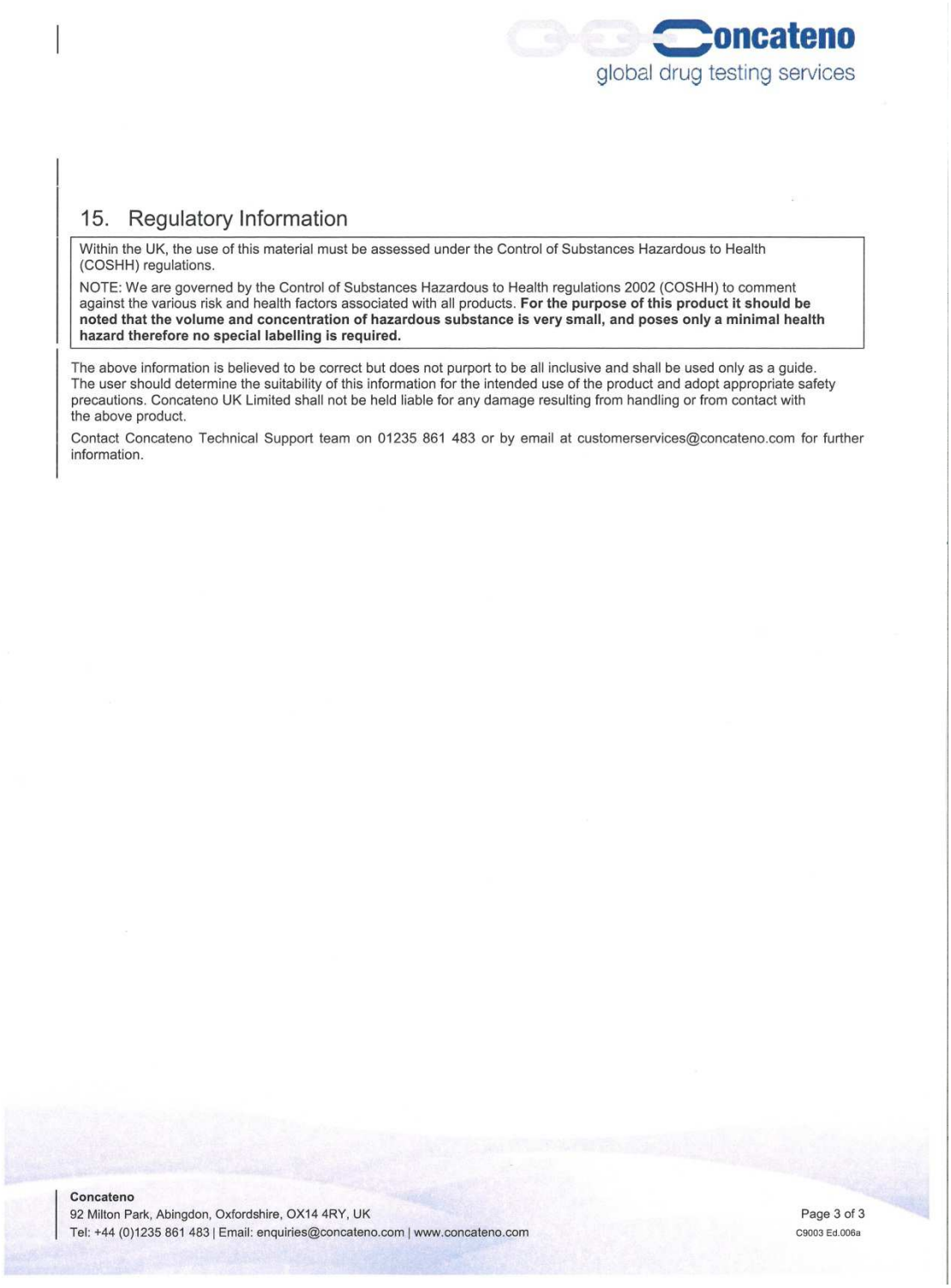
Figure B-2: MSDS of Cozart® DDS buffer, page 2.



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15. Regulatory Information

Within the UK, the use of this material must be assessed under the Control of Substances Hazardous to Health (COSHH) regulations.

NOTE: We are governed by the Control of Substances Hazardous to Health regulations 2002 (COSHH) to comment against the various risk and health factors associated with all products. **For the purpose of this product it should be noted that the volume and concentration of hazardous substance is very small, and poses only a minimal health hazard therefore no special labelling is required.**

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The user should determine the suitability of this information for the intended use of the product and adopt appropriate safety precautions. Concateno UK Limited shall not be held liable for any damage resulting from handling or from contact with the above product.

Contact Concateno Technical Support team on 01235 861 483 or by email at customerservices@concateno.com for further information.

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Figure B-3: MSDS of Cozart® DDS buffer, page 3.

Appendix C

REF COF304 AUS

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Concateno Certus® Oral Fluid Collection Kit – Laboratory Testing

Instructions for use:

Intended use:

The **Concateno Certus® Oral Fluid Collection Device** is intended for the collection and transport of oral fluid specimens for laboratory analysis of drugs of abuse. This medical device is intended for professional use only.

Kit Contents:

Each box of **Concateno Certus® Oral Fluid Collection Kits** contains:

- 25 x Concateno Certus® devices
- 25 x Oral fluid collection tubes containing buffer solution
- 25 x Empty oral fluid collection tubes
- 25 x Clear transit bags
- 25 x Transfer pipettes
- 50 x tamper evident seals
- 1 x Instructions for use leaflet

Precautions:

- To obtain an appropriate sample, the Sample Collection procedure must be followed
- Unreasonable force during sampling may cause swab shaft to break
- Do not reuse the swab or place the swab in the mouth after it has been in contact with the oral fluid collection buffer
- Disposable gloves should be worn when handling samples
- Do not use if the pouch is damaged or punctured
- Do not use the kit after the stated expiry date
- Do not ingest the liquid contained within the oral fluid collection tube
- Handle the swab by the plastic stem only
- The device must not be in contact with the mouth for more than 25-30 minutes. If an oral fluid sample is not obtained within 25-30 minutes use an alternative testing method (urine)

Storage:

The **Concateno Certus® Oral Fluid Collection Kits** must be stored between 2ºC-30ºC. Do not use after the stated expiry date.

The Material Safety Data Sheet (MSDS) is available for the professional user on request.

Limitations:

- The collection device provided should be used for the collection of oral fluid samples only
- False results may be obtained if the collection procedure is not followed correctly

Sample Collection:

Important – Please read prior to collecting a sample:

- The donor should not eat or drink ten minutes prior to collecting the sample
- Do not allow the donor to suck or chew the Concateno Certus® device during the collection procedure
- Allow the donor to collect the sample

CE

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Figure C-1: Instructions for use of the Concateno Certus® Oral Fluid Collection Kit used to collect the pre-admission samples for the Sativex® study, page 1.

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Concateno Certus® Oral Fluid Collection Kit – Laboratory Testing

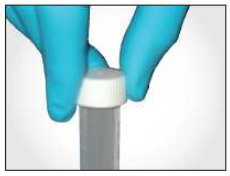
Instructions for use continued:

Sample collection:

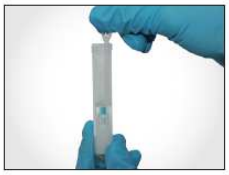
1. Ask the donor to remove the Concateno Certus® device from the packaging, ensuring they hold the plastic stem of the swab, then place in the mouth.

2. The sample donor must actively swab the Concateno Certus® device around gums, tongue and inside the cheek, and then hold inside the mouth until the sample presence indicator turns completely blue, as shown above.

3. Remove the white cap from the collection tube containing the **buffer solution** and gently insert the Concateno Certus® device into the tube ensuring the swab end goes in first.



4. Replace the cap onto the tube and turn it clockwise to tighten. **Mix by shaking the tube for 30 seconds.** Label both tubes in accordance with local guidelines.



5. Unscrew the caps, and using the pipette transfer half of the mixed sample into the **empty collection** tube. Replace both caps.

6. Place a red tamper evident seal over the cap and down the sides of each of the collection tubes, ensuring the collection tube labels are not obscured. The donor must initial the top of each sealed tube.

7. Place the sealed and labelled tubes into the front pouch of the clear transit bag with the absorbent pad. Place any completed laboratory forms in to the back pouch of the clear transit bag and seal.

Advice:

For further information, please contact Alere Customer Service on 1800 622 642.

Figure C-2: Instructions for use of the Concateno Certus® Oral Fluid Collection Kit used to collect the pre-admission samples for the Sativex® study, page 2.

DrugWipe® II TWIN short instructions

Please follow instructions carefully!

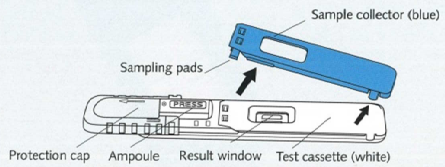
Preliminary comments

Check the expiration date

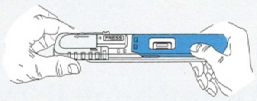
Check that package is not damaged

Tear open foil packet at the cut, remove DrugWipe® II TWIN

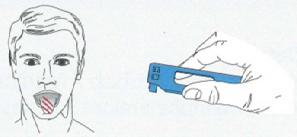
1. Remove the sample collector (blue) from the test cassette (white).



2. Move cap in the direction of the arrow, until the word PRESS is entirely visible.

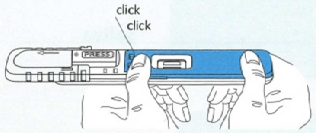


3. Advise the test person to move the tongue around the cheek 3 times. Request the test person to wipe the sampling pads of the sample collector directly on the tongue in a downward motion.

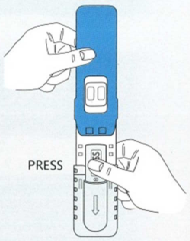


4. Check for color change of the sampling pads from pink to yellow.

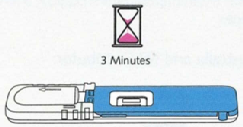
5. Place the sample collector back onto the test cassette and click together.



6. Hold the test cassette vertically, ampoule at the bottom. Squeeze your thumb thoroughly on the word PRESS in order to break the ampoule. Hold the test cassette with the broken ampoule vertically for 10 to 15 seconds.



7. Hold DrugWipe® II TWIN horizontally for 5 minutes.



8. Reading and interpretation of the test result:

- One result window for each drug THC and MET
- Each window with control line and test line
- 1 line = negative result
- 2 lines = positive result

REMARK:

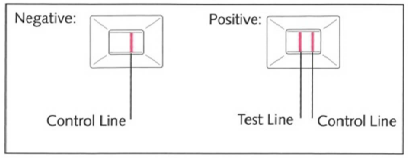
Protect result window from rain in bad weather conditions! Do not freeze DrugWipe®! Do not store DrugWipe® in car below 0 °C and above 40 °C.

Interpretation of the results

1 drug parameter (TL) and 1 functional control (CL) can be detected in each read out window.

The drug parameters are:

Cannabis (THC/Marijuana/Hashish)
Methamphetamines (including Ecstasy)



The positions of the control lines are visible as pale blue lines in the read out window of an unused DrugWipe® test. During the test development the blue lines disappear. The functional control position must always show a red control line after test development.

Valid: The test result is valid when the red control line appears.
Negative: The test result is negative when the red control line appears and no additional red test lines developed.
Positive: The test result is positive when the red control line and the red test line appear.

Preliminary negative results require at least 5 minutes of test development before final read out. All control and test lines indicated at 5 minutes of test development have to be interpreted. Lines appearing later than 5 minutes must not be interpreted.

REMARK: Interpret a test result as positive when the test line is faint, interrupted or irregular. Irregular sample transfer may lead to interrupted/irregular test lines. Defective color vision may prevent from correct interpretation.

This product is sold to law enforcement, security, medical and corporate professionals for use in accordance with internal policies and in conformance with Federal, State and Local laws. The manufacturer, agents and distributors are not liable for misuse or abuse of this product.

Figure C-3: Instructions for use of the DrugWipe® II Twin screening device supplied with the devices.

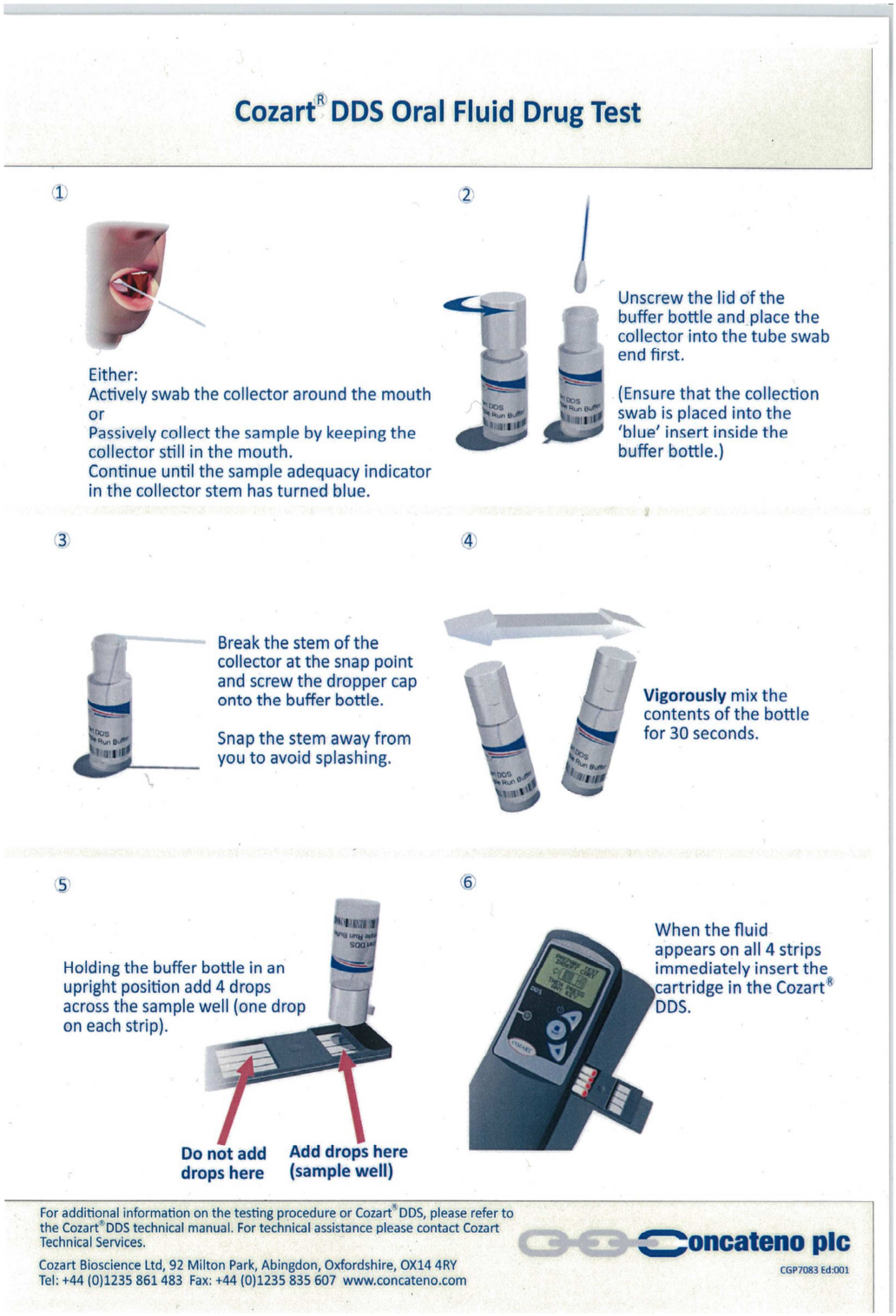


Figure C-4: Instructions for use of the Cozart® DDS screening device supplied with the device.

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