Further Research Concerning the Detection of Oxidation Products of THC-COOH Following Urinary Adulteration

PhD Thesis: Science

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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 requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Nathan Charlton

28/11/2014

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In closing, I offer to all those dear to me a quote from the animated television series "Metalocalypse", from the episode titled "Dethhealth" (Schnepp 2009). The main characters of this show humorously ponder how to return a false-negative drug test result:

Pickles: Dudes, we party too hard, so our bodies are in terrible shape. We gotta trick the doctor by making it seem like we're in really good shape. And there's only one way to do that. Bleach... Here, drink this Murderface...

Skwisgaar: Uhh, maybe this ams a stupid question, buts, why don'ts we just pours bleach into our cups of...urines?

Pickles: No! Drink the bleach!

Nathan: Bleach is healthy. It's mostly water. And we are mostly water. Therefore, we are bleach.

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List of Abbreviations

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APCI	Atmospheric Pressure Chemical Ionisation
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
cAMP	Cyclic Adenosine Monophosphate
CAN	Ceric Ammonium Nitrate
CB_1	Cannabinoid Receptor Type 1
CB_2	Cannabinoid Receptor Type 2
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
CEDIA	Cloned Enzyme Donor Immunoassay
CI	Chemical Ionisation
COSY	Correlation Spectroscopy
DEA	Drug Enforcement Administration
DNA	Deoxyribonucleic Acid
EIA	Enzyme Immunoassay
EIC	Extracted Ion Chromatogram
ELISA	Enzyme-Linked Immunosorbent Assay
EMIT	Enzyme Multiplied Immunoassay Technique
ESI	Electrospray Ionisation
FBI	Federal Bureau of Investigation
FPIA	Fluorescence Polarization Immunoassay
GC	Gas Chromatography
GC-MS	Gas Chromatography–Mass Spectrometry
HFBA	Heptafluorobutyric Anhydride
HMBC	Heteronuclear Multiple-Bond Correlation Spectroscopy

HPLC-MS	High-Performance Liquid Chromatography–Mass Spectrometry
HSCQ	Heteronuclear Single-Quantum Correlation Spectroscopy
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LLE	Liquid-Liquid Extraction
MRM	Multiple Reaction Monitoring
NIDA	National Institute on Drug Abuse
NMR	Nuclear Magnetic Resonance Spectroscopy
PAD	Programmable Absorbance Detector
PCC	Pyridinium Chlorochromate
PFPA	Pentafluoropropionic Anhydride
PFPOH	Pentafluoropropionic Alcohol
PVP	Polyvinylpyrrolidone
QToF-MS	Quadrupole Time-Of-Flight Mass Spectrometer
SAMHSA	Substance Abuse and Mental Health Services Administration
SRM	Selected Reaction Monitoring
THC	Tetrahydrocannabinol, Δ^9-Tetrahydrocannabinol
THC-COOH	11-nor-9-Carboxy-THC, 11-nor-9-carboxy-delta-9-tetrahydrocannabinol
THC-COOH-d9	Deuterated 11-nor-9-Carboxy-THC
THCV	Tetrahydrocannabivarin
TIC	Total Ion Chromatogram
UV-Vis	Ultraviolet–Visible Spectroscopy

Abstract

In Australia and throughout the world, cannabis is one of the most widely used recreational substances. Whilst the recreational use of cannabis remains widely controversial, and the detection of its use in a range of biological matrices is of vital importance for drug testing laboratories and law enforcement agencies. The detection of drugs of abuse is critical in various areas, including pre-employment and post-incident drug screening, and sports drug testing.

The use of cannabis by an individual may be ascertained by identifying the main metabolites of the major psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (THC), in biological matrices such as urine. The principal metabolite of THC is 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), and may be detected in urine in both its free and glucuronide-bound form, with detection of either regarded as compelling evidence for the use of cannabis by an individual.

Detection of THC-COOH by a range of instrumental techniques in drug testing laboratories is well established. However, this metabolite is known to be susceptible to reaction with certain adulterants. Adulteration of urine samples with oxidising adulterants has been shown to effectively mask cannabis use through reaction with THC-COOH. As such, the primary goals of this research are to assess the efficacy of a range of adulterants on the detection of THC-COOH in vitro, ascertain whether novel reaction products specific to the reaction of THC-COOH with selected adulterants form, and to assess the potential of these compounds to act as markers of both cannabis use and urine adulteration.

Successful detection of a range of reaction products of THC-COOH was achieved, and three adulterants selected for further research: pyridinium chlorochromate, Betadine and bleach. Structural elucidation of these reaction products was attempted, and validated methods were developed for the quantitative detection of THC-COOH and qualitative detection of the targed reaction products following urine adulteration. Kinetics, pH and stability studies demonstrated that these reaction products formed under a range of pH and sample storage conditions, and critically, remained detectable for at least twenty days following adulteration.

Detection of these potential markers of urine adulteration was also successfully achieved through the adulteration of authentic cannabis-positive urine specimens. This detection in authentic urine specimens is considered significant, as it highlights the potential for these novel compounds to be incorporated into current drug testing regimes employed by drug testing laboratories, and a potential means by which both cannabis use and urine adulteration may be conclusively identified.

Chapter 1

Introduction

1.1 – An Introduction to Cannabis

Cannabis is described in literature as the preparation of plants belonging to the *Cannabis* genus, with the intended use of the preparation as a psychoactive drug. Historically, the use of cannabis as a medicinal substance, source of hemp fibres, entheogen and recreational drug has been reported in both Asia and Europe over thousands of years (Joyne 2005; Paul & Jacobs 2002). The widespread adoption of cannabis as a recreational drug in Western countries is considered to have been a product of the counter-culture and anti-establishment movements that formed in the 1960s and 1970s (Musto 1991; Sandberg 2012; Zimmerman & Wieder 1977). From an Australian perspective, the use of cannabis is considered widespread, with the illicit production, supply and sale of cannabis estimated to rival the financial size of Australia's wine and gold industries (Wodak & Cooney 2004).

The major biologically active component of cannabis is Δ^9-tetrahydrocannabinol (THC), with an additional seventy cannabinoids present (ElSohly & Slade 2005). **Figure 1.1** illustrates five of the major cannabinoids present in cannabis. These cannabinoids provide the psychoactive effects of cannabis in humans, and may include feelings of euphoria, lethargy, alterations of perception and memory (Ashton 2001; Green *et. al.*, 2003). In humans, THC acts as both a central nervous system depressant and as a mild hallucinogen, and in combination with the other cannabinoids present in cannabis, are responsible for the psychoactive effects of this drug. Psychoactive effects are mediated by the binding of cannabinoids with the cannabinoid receptors present in the endocannabinoid system, which are associated with memory, mood, and perceptions of both pain and appetite (Iversen 2003). Agonism of the cannabinoid receptors directly affects concentrations of two endogenous neurotransmitters – dopamine and noradrenaline (Gessa *et. al.*, 1998). Currently, two cannabinoid type receptors are known; the Cannabinoid Receptor Type 1 (CB_1), commonly expressed in brain and spinal cord, and the Cannabinoid Receptor Type 2 (CB_2), which is expressed in the immune system, gastrointestinal system and peripheral nervous system (Pertwee 2008). Reported psychoactive effects of cannabis use vary in humans with dose, user physiology and frequency of use (Wachtel *et. al.*

2002). These psychoactive effects may also be affected by long-term use of cannabis, with potential reports of morphological alterations in the structure of the brain (Matochik *et. al.*, 2005; Yücel *et. al.*, 2005).

In Australia, cannabis is recorded as the most widely used recreational drug, with usage patterns considered steady over the past four decades (McLaren & Mattick 2007). Actual reports of usage patterns vary, with some reports estimating that one-third of Australians have tried cannabis within their lifetime, and that annually, approximately one in ten Australians will try and/or use cannabis. Despite being classified as an illicit substance in Australia, in terms of the prevalence of cannabis use, Australia ranks fifth internationally, behind the United States of America, Canada, New Zealand and Denmark (UNODC 2011).

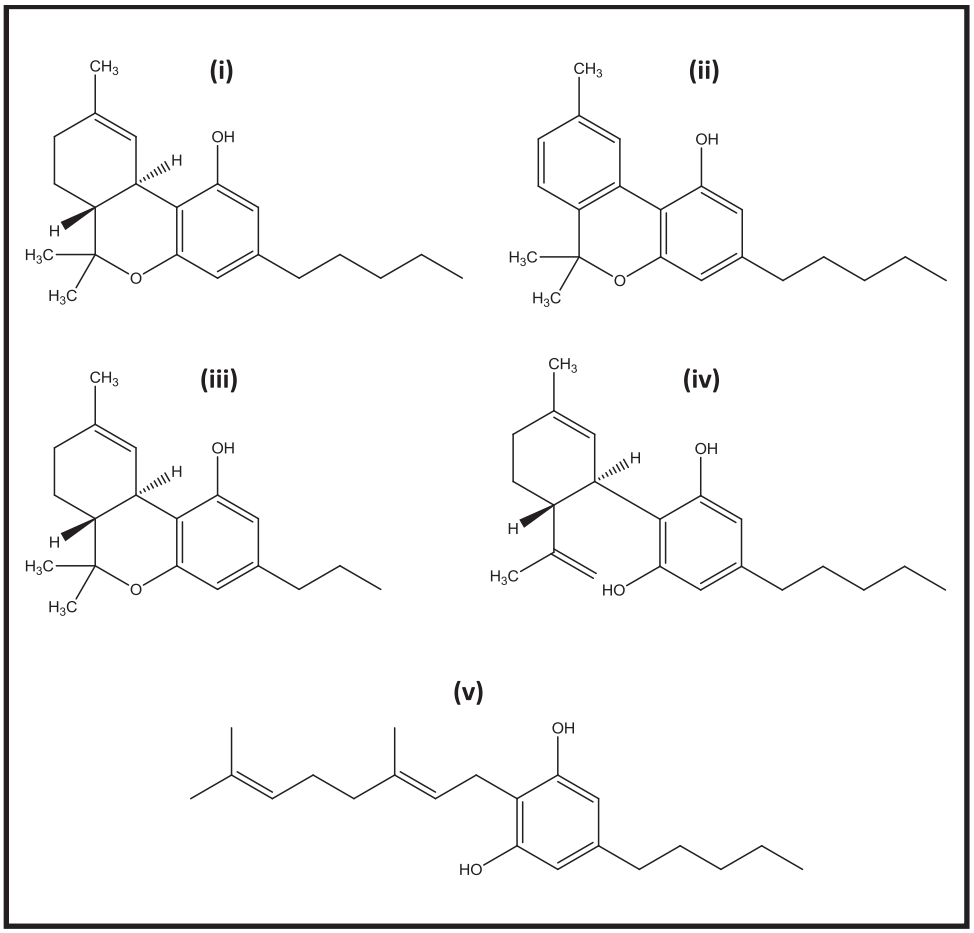


Figure 1.1 - Five common cannabinoids present in cannabis:
(i) Tetrahydrocannabinol (THC); (ii) Cannabinol (CBN); (iii) Tetrahydrocannabivarin (THCV);
(iv) Cannabidiol (CBD); (v) Cannabigerol (CBG).

Internationally, the legal status of cannabis is controversial, with challenges to the legality of the drug occurring in Europe (Gatto 1999), and the United States of America. With regards to the United States of America, the states of Colorado and Washington in 2012 passed amendments allowing for the recreational use and sale of preparations of cannabis (Ingold 2013; Martin 2012). Other states have also attempted to introduce laws changing the legal status of cannabis, including a successful vote in 2014 by the Council of the District of Columbia to decriminalise the recreational use of cannabis, and the replacement of prison sentences for those caught in possession of cannabis with civil fines (Newman & Smith 2014; Smith & McDonald 2014). A further challenge regarding the legal status of cannabis was voted on in November 2014, with Initiative 71 ("*Legalization of Home Cultivation and Possession of Minimal Amounts of Marijuana for Personal Use Act of 2014*") passed by voters (Chappell 2014). Based on the passing of this ballot initiative, residents of the District of Columbia aged 21 and over are now legally able to possess and sell small quantities of cannabis (less than two ounces / 56.7 grams) and use and sell drug paraphernalia associated with the use, growing and processing of cannabis.

In the United States of America, these challenges to the legal status of cannabis as a recreational drug are at odds with the federal government. Cannabis is recorded as a Schedule 1 drug in the *Comprehensive Drug Abuse Prevention and Control Act of 1970*, and as such, it is illegal to possess, use, buy, sell, and/or cultivate cannabis (Stolberg 2009). As such, agencies such as the Federal Bureau of Investigation (FBI) and the Drug Enforcement Administration (DEA) are, by federal law, obligated to consider the cultivation, possession, transport, supply and use of cannabis to be a criminal offence, regardless of changes made to the legal status of cannabis at the state level (Annas 2014; Hurley & Mazor 2013).

Despite international challenges to the legality of cannabis use, the detection of cannabis use remains important to law enforcement agencies, private and government employers, sporting authorities and drug testing laboratories. Due to alterations in mental state, memory, perception and response times associated with the use of cannabis, a significant risk is present for those that work in safety-sensitive environments, drivers, and individuals that operate heavy machinery, regardless of the legal status of cannabis and its preparations. Such testing programs are also necessary for the rehabilitation and treatment of recreational drug users, as well as the facilitation of harm minimisation within the healthcare and legal systems. Critically, it is reported that the relationship between the degree of cannabis-induced intoxication and the detection of THC metabolites in biological matrices is poor (Lewis 2010). As such, detection

of THC metabolites in biological matrices by quantitative methods displays a poor correlation with the degree of intoxication of a user. This potential disparity, in turn, may complicate the interpretation of a positive drug test result by drug testing laboratories and law enforcement agencies.

Further complicating the matter of urine drug testing is the issue of urine manipulation and adulteration. Due to the serious nature of urine drug testing, especially in cases of post-incident and workplace drug testing, it has become necessary for specific collection procedures to be put into effect, in order to ensure the accuracy, efficacy and legal grounding of the results of such tests. Despite this, reports of attempts, both successful and unsuccessful, at manipulating and adulterating urine drug samples are available. Samples may be tampered with by the individual supplying the sample or through the actions of corrupt doping control officers (AS/NZS 4308:2008). Similarly, in workplace drug testing, the individual providing the urine sample may be afforded a modicum of privacy, thereby allowing ample opportunities for the sample to be tampered with prior to it being handed over to a collection official (Thevis *et. al.*, 2012).

1.2 – Pharmacokinetics, Pharmacology and Metabolism

Multiple studies have been undertaken to ascertain the pharmacokinetics of THC and related cannabinoids (Ashton 2001). Use of cannabis can be achieved primarily through smoking this substance and related products, with the eating and drinking of cannabis preparations also presenting viable methods of administration (Lemberger *et. al.*, 1972). The bioavailability of THC varies significantly with the route of administration, with approximately 35% to 50% of the THC present in cannabis inhaled via smoking (Hall & Solowij 1998). In comparison, the eating or drinking of cannabis preparations results in a far lower THC bioavailability, with only 5 - 20% of the THC absorbed via these methods when compared to an equivalent dose that has been administered via smoking. THC and related cannabinoids are rapidly absorbed by the lungs, with the initial psychoactive effects of cannabis detected by the user within minutes of smoking.

Psychoactive effects are mediated through the binding of THC and other cannabinoids to the CB_1 and CB_2 receptors. As reported by Elphick and Egertová (2001), a number of the psychoactive effects of cannabis are the result of activation of the CB_1 receptors, whereby binding of THC to CB_1 indirectly increases the release of dopamine in the brain, and inhibits the action of the adenylate cyclase enzyme. Inhibition of this enzyme leads to an overall decrease of the molecule cyclic adenosine monophosphate (cAMP) within the brain (Bayewitcha *et. al.*, 1995). The binding of THC to these receptors and the effects of enzyme activity is considered to be connected to other neural pathways, including the dopaminergic, noradrenergic, serotonergic, cholinergic, glucocorticoid and prostaglandin systems (British Medical Association 1997).

The cannabinoids present in cannabis are highly lipophilic, and are rapidly distributed to fatty tissues present in the human body. Due to the solubility of cannabinoids in lipids, the elimination of cannabinoids and their metabolites from the body is slow compared to other common recreational drugs. The half-life of THC is reported as approximately 2 – 60 hours, and is excreted in both faeces and urine (Huestis & Cone 1998). The long half-life of THC is significant for the detection of cannabis use, as singular doses of this recreational drug may be effectively detected weeks after use.

In humans, THC is primarily metabolised in the liver by cytochrome P450 enzymes (Miners & Birkett 1998; Watanabe *et. al.*, 2007) – specifically, the CYP2C9, CYP2C19, and CYP3A4 enzymes. Metabolic products of THC are primarily formed through the oxidation and

hydroxylation of THC, with a total of eighteen metabolites for THC reported in the literature (Sharma, Murthy & Bharath 2012). Hydroxylation and oxidation of THC increases the water solubility of these products, and in combination with the conjugation of some THC metabolites with glucuronic acid, results in increased water solubility. Through this increase in water solubility, the excretion of these metabolites in urine and faeces is facilitated. The major metabolites reported for THC include 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC), and their respective glucuronide conjugates, and are shown in **Figure 1.2**. Literature reports that 11-OH-THC is primarily excreted in faeces, whilst THC-COOH and its glucuronide are primarily excreted in urine (Huestis 2007).

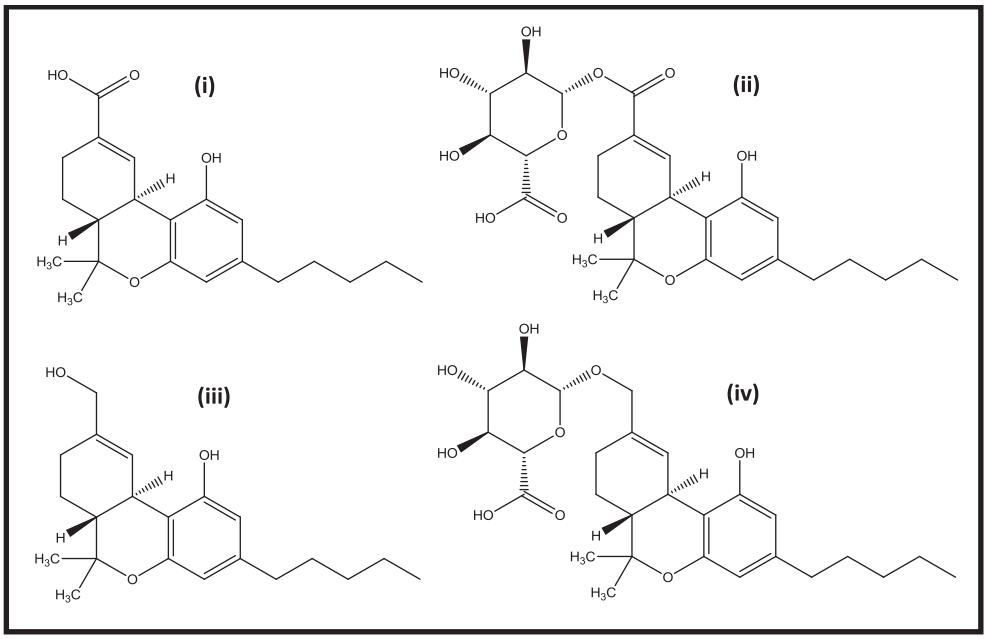


Figure 1.2 - Major metabolites of THC:

(i) 11-nor-9-Carboxy-THC (THC-COOH); (ii) THC-COOH Glucuronide;
(iii) 11-Hydroxy-THC (11-OH-THC); (iv) 11-OH-THC Glucuronide.

1.3 – Detection of Cannabis Use

1.3.1 – General Information Regarding Detection

Drug testing is designed to identify the use of both licit and illicit substances through the detection of drug metabolites in biological matrices submitted for analysis. Testing of samples may be undertaken by a range of groups and organisations, either as a part of a pre-employment screening process, post-incident screening, sports drug testing, roadside sobriety testing and for occupations where the intoxication of an individual may represent a significant risk to themselves, their co-workers, the general public, or to equipment and facilities.

In pre-employment drug screening procedures, an employer may be concerned as to whether a potential job candidate has recently or regularly used drugs of abuse, and whether this may influence job performance. In jurisdictions and occupations where allowed, additional tests may be requested throughout an employee's time at a company to monitor potential drug use by employees, and to ensure adequate productivity and employee safety within a workplace. Similarly, post-incident testing and roadside sobriety testing seeks to ascertain whether an individual was under the influence of drug/s of abuse either at the time of an incident or accident, or when they were driving a motor vehicle. In these scenarios, the presence or absence of drugs of abuse impacted on an incident or activity.

Detection of cannabis use by an individual may be identified through the detection of THC in saliva, and the detection of metabolites of THC in other biological matrices, including urine, blood, hair and faeces. Both THC-COOH and 11-OH-THC are routinely used in the detection of cannabis use. Current detection methods focus on the detection of both THC-COOH and its glucuronide in urine, and may be achieved through a range of instrumental techniques, including liquid chromatography – tandem mass spectrometry (LC-MS/MS), high-performance liquid chromatography-mass spectrometry (HPLC-MS), gas chromatography-mass spectrometry (GC-MS) and immunoassay-based techniques.

Urine is considered the most popular biological matrix to be tested for the detection of illicit drugs and their metabolites, as its collection is both considered non-invasive and simple, and allows for a relatively wide detection window (Caplan & Goldberger 2001; Moeller, Lee & Kissack 2008; Verstraete 2004). Instrumental detection methods for urine drug testing are well established, and have been appropriately validated, tested and documented. Urine samples are generally submitted initially for a confirmatory screening, typically by immunoassay, and in

the case of a positive test result, undergo confirmatory testing via mass spectrometric techniques.

It is important to note that the efficacy of the above listed techniques is severely limited by actions undertaken by users in order to obtain a false-negative test result. Though the collection of urine samples submitted to drug testing is tightly controlled and set out by standard operating procedures for drug testing laboratories and law enforcement agencies, it remains possible for urine samples to be diluted or have chemical agents to be introduced to a urine sample, thereby potentially invalidating the results, and leading to a false-negative test result for the sample in question. These attempts at urine manipulation and adulteration may be detected by laboratories through urine integrity tests, though in cases of urine substitution and urine adulteration, it may be highly difficult for a drug testing laboratory to ascertain which, if any, drugs of abuse and metabolites were present in the urine prior to tampering.

1.3.2 – Immunoassays and Presumptive Testing

Immunoassays are based on the principle of competitive binding between labelled and unlabelled antigens to specific antibodies (Tsai & Lin 2005). Antibodies for specific drugs of abuse are produced through the administration of a specific drug substance to an animal, with antibodies specific to the drug of abuse generated by the immune system of the animal in response to the presence of exogenous compounds in the body. These antibodies are isolated from a blood sample and integrated into an immunoassay. In the case of screening tests for cannabis use, the exogenous agent and antigen is the drug and/or drug metabolite.

Detection of specific analytes in immunoassay-based techniques may be achieved through a range of methods. Principally, labelling agents are bound to the antibodies used in the immunoassay. Labelling agents used in immunoassay procedures include enzymes, radioisotopes, and fluorescent molecules (Blake & Gould 1984; Cody & Schwarzhoff 1989; Melanson 2012). Labelling of the target antigen allows for the detection of the antigen through various techniques; for the detection of cannabis use, the labelling of antigens allows for drug metabolites present in a biological sample to be detected. The binding interaction between antibody-antigen pairs therefore allows for both the detection and quantification of drug metabolites in biological matrices.

Though immunoassays tend to display high sensitivity in their analyses, they are ultimately a presumptive testing method designed to detect a range of drugs and drug metabolites. Hence, these techniques often display poor specificity towards specific analytes. Despite issues related to the generally low specificity of immunoassay techniques, their use in reference laboratories and drug testing laboratories has been crucial in managing case loads and allowing for the rapid screening of samples submitted for analysis. A range of immunoassay-based screening methods are currently used by drug testing laboratories, and include *enzyme immunoassay* (EIA), *enzyme-multiplied immunoassay technique* (EMIT), *cloned enzyme donor immunoassay* (CEDIA), *enzyme-linked immunosorbent assay* (ELISA) and *fluorescence polarisation immunoassay* (FPIA).

As will be discussed in **Section 1.4.2**, the efficacy, validity and usefulness of these immunoassay-based techniques in the presumptive screening of samples for drugs of abuse can be invalidated through the manipulation or adulteration of urine. Specifically, the in vitro use of oxidising chemical agents and other adulterants has the potential for a drug-positive sample submitted for analysis to return a false-negative during this screening stage, thereby avoiding the later confirmatory stage of testing. Various mechanisms have been proposed for the interference of both oxidising and non-oxidising adulterants on immunoassay-based screening techniques, and are summarised in **Table 1.1**. The effect of various adulterants on the potential for a false-negative test result to be returned during presumptive screening has been extensively described in literature. **Table 1.2** summarises the findings of a number of authors, in terms of oxidising agents tested, drug class or metabolite targeted, and the immunoassay-based technique examined.

Table 1.1 – Proposed mechanisms for the interfering effects of in vitro adulterants on immunoassay-based screening tests.

Cause	Mechanism
Binding of adulterants to drug metabolites (Heard & Mendoza 2007)	Adulterants present in the specimen may lead to the formation of micelles or insoluble complexes that may not be detected with immunoassay-based techniques
Increase in ionic strength of the specimen (Cassells & Craston 1998; Ferslew, Nicolaides & Robert 2003)	An increase in the ionic strength of the specimen may alter the protein structure of the antibody-antigen pair, resulting in decreased immunoassay sensitivity
Alteration of the sample pH (Mikkelsen & Ash 1988)	Oxidising adulterants may increase or decrease the pH of the specimen, which may potentially alter the binding and reaction rates of the immunoassay. Changes in sample pH may also reduce the solubility of the drug or drug metabolite within the sample matrix
Direct interaction of the adulterant with the antibody protein or enzyme (Cassells & Craston 1998)	Interactions between oxidising adulterants and antibody proteins/enzymes may result in a significant decrease in the protein/enzyme binding capacity, and may also cause denaturation of the antibody/protein structures
Reaction of the drug metabolite with the oxidising adulterant (Luong, *et. al.* 2012; Mikkelsen & Ash 1988)	Oxidising adulterants may react extensively with the drug or drug metabolites present in the specimen. Major changes to the chemical structure may inhibit the enzyme or antibody-antigen binding

Table 1.2 – Summary of literature relating to the use of in vitro oxidising adulterants on the detection of cannabis use.

Adulterant	Detection Method	Observation
Hypochlorite	CEDIA	Decreased immunoassay sensitivity (Wu 1995)
	EIA	False-negative returned (Mikkelsen & Ash 1988)
Nitrite	EMIT	Decreased immunoassay sensitivity (Tsai *et. al.*, 2000)
	Roche Abuscreen OnLine	Decreased immunoassay sensitivity (Tsai *et. al.*, 1998)
Peroxide/Peroxidase	CEDIA	False-negative returned (Cody & Valtier 2001)
	Roche Abuscreen OnLine	False-negative returned (Cody & Valtier 2001)
Pyridinium Chlorochromate	EMIT	Major decrease in immunoassay sensitivity (Wu *et. al.*, 1999)

1.3.3 – Confirmatory-Based Techniques

In contrast to immunoassays, confirmatory detection methods based on mass spectrometry are capable of proving the presence of a drug or its metabolites in a biological matrix in a sensitive and specific manner. In the detection of drugs and their metabolites, drug testing laboratories may employ either gas chromatography or liquid chromatography in tandem with mass spectrometric techniques. Chromatographic separation of analytes is based on differences in the physical and chemical properties of target analytes and other compounds and their interaction with the stationary phase of the chromatographic column.

Two main choices are available for the separation and identification of both known and unknown compounds - gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS and LC-MS/MS). The former is routinely employed by drug testing laboratories for the detection of analytes of interest, with the application of this chromatographic technique well established. Liquid chromatography, in comparison, is used in drug testing laboratories, though it has generally not been widely adopted at the time of writing. The choice of chromatographic method of separation

ultimately depends on the instrumentation available in a laboratory, the training of the staff, and the availability of validated methods.

One advantage of LC-MS/MS over standard GC-MS techniques is that, for non-volatile analytes, the sample does not need to undergo a derivitisation step. A range of derivatising agents are recommended for use, including bis-(trimethylsilyl)-trifluoroacetamide (BSTFA), pentafluoropropionic anhydride (PFPA), pentafluoropropanol (PFPOH) (Stout, Horn & Klette 2001) and heptaflurobutyric anhydride (HFBA) (Sigma-Aldrich 2011). This derivatisation step adds additional time and cost to confirmatory testing procedures, as time is needed for samples to undergo derivatisation, as well as time for the sample to dry down prior to being reconstituted in a suitable solvent matrix. In comparison, LC-MS/MS and other chromatographic methods based on liquid chromatography forego this step, increasing sample throughput and potentially lowering the cost per analysis.

Further information relating to the use of confirmatory-based techniques is discussed in **Section 1.4.2**.

1.4 – Urine Manipulation and Adulteration

Urine manipulation is intended to allow an individual to conceal drug use, and represents an ongoing challenge to laboratories that undertake drug testing. In the United States of America, the National Institute on Drug Abuse (NIDA) and the Substance Abuse and Mental Health Services Administration (SAMHSA) define manipulation of urine under three broad categories (SAMHSA 2005; Scholer 2004):

- The dilution of urine externally or in vivo;
- The substitution of urine, and;
- The adulteration of urine through the use of interfering agents and/or oxidising adulterants.

The manipulation of urine for the purposes of masking drug use is widely reported in literature, with a range of commonly abused drugs targeted by these methods, and are summarised in **Table 1.2**, **Table 1.3** and **Table 1.4**.

1.4.1 – The External and In Vitro Dilution of Urine Samples

The dilution of urine samples may be achieved through both external and in vitro methods. External dilution of urine is achieved through the addition of a volume of water or other suitable liquid to the urine sample. Dilution of the sample is intended to dilute the drugs and drug metabolites present in the sample to below the limit of detection, and in the case of detecting cannabis use, has been reported as successful (Cone, Lange & Darwin 1998). In contrast, the in vitro dilution of urine samples may occur through either the consumption of a large volume of liquid prior to providing a urine sample, or through the consumption of diuretics to increase the volume of urine voided by an individual (Ventura & Segura 2010).

1.4.2 - Interferents

Interfering agents are utilised to reduce the efficacy of screening techniques, and thereby cause these techniques to report false positives for the presence drugs and drug metabolites in urine. Various mechanisms have been proposed for the effect of Interferents on screening techniques, and are summarised in **Table 1.1**. Interferents may be sourced from household chemicals and cleaning supplies, and includes ascorbic acid, vinegar and detergents (Dasgupta

2007). Alternately, other interferents may be purchased from online stores. One such product is UrinAid, a preparation of glutaraldehyde that reportedly is effective in masking the presence of a number of drugs and drug metabolites (Wu, Schmalz & Bennett 1994).

Another compound commonly employed as an interfering agent is benzalkonium chloride, a cationic surfactant used in eye drop preparations and as a cleaning agent (Pearson, Ash & Urry 1989). As with detergents and similar products, it forms lipophilic micelles when added to a urine sample. Due to the lipophilic properties of THC, THC-COOH and other metabolites and cannabinoids, partitioning of the cannabinoids between the aqueous urine sample and the benzalkonium chloride micelles occurs. This partitioning thereby apparently decreases the concentration of THC-COOH detected in the sample, and consequently enables the individual to successfully mask cannabis use.

1.4.3 – Urine Substitution

Urine substitution is achieved through the replacement of a urine sample with that of drug-free urine or synthetic urine. Various techniques exist for the substitution of urine, with a variety of products available through on-line stores. One specific brand of product, the "Whizzinator", has been the source of significant controversy amongst anti-doping officials, sporting authorities, and law enforcement agencies. This device, pictured in **Figure 1.3**, is comprised of a syringe or plastic reservoir for a clean or synthetic urine sample, a set of tubing, and a prosthetic penis designed to disguise the use of this device.

The manufacturer of the "Whizzinator", Alternative Lifestyle Systems, declares on the product's website that: "*This product is not intended for any illegal purpose. Nor is it to be used to defeat lawfully administered drug tests"* ('The Whizzinator Touch in White' 2014). Ostensibly, this product is intended for novelty purposes, or to simulate urolagnia, a paraphilia defined as "*sexual excitement associated with urine or with urination*" ('Urolagnia' 2014).

Figure 1.3 - Product image for "The Whizzinator Touch in White", accessed 28/08/2014 ('The Whizzinator Touch in White' 2014).

Despite the declaration on the product's website, recent news reports have detailed the use of the "Whizzinator" and similar devices in attempts by private individuals and athletes to avoid obtaining a positive drug test result (Symmonds 2014). Two high-profile reports detailing the use of the "Whizzinator" have involved professional boxer Mike Tyson (Erby 2013), and footballer Onterrio Smith (Joyner 2005). Similar news reports also note private individuals caught using this device in similar attempts (Smothers 2014).

Other techniques to substitute urine are also reported in literature. These techniques include swapping a urine sample with that of a drug-free sample, similar to the use of the "Whizzinator", and the introduction of a clean urine sample into the bladder via catheterisation (Casavant 2002).

1.4.4 – Oxidising Adulterants

Literature reports a range of oxidising agents that have been used in attempts to mask drug use. Oxidising adulterants may significantly alter the physical properties of a urine specimen submitted for analysis, and both oxidise and degrade target analytes. The latter issue is significant, as the chemical reaction between an oxidising adulterant and target analyte will potentially render the target analyte undetectable by confirmatory tests due to the altered chemical identity. Drug testing laboratories are also faced with the issue of the oxidising adulterant degrading the internal standard used for quantitative analysis and invalidating drug test results. **Table 1.3** summarises the findings of a number of authors in terms of the effects of oxidising adulterants on the detection cannabis-positive urine samples by drug testing laboratories.

At present, available oxidising agents may be separated into two categories: chemicals sold online for masking drug use, and household chemicals and other commercially available products. Household cleaning agents containing bleach represent the bulk of oxidising agents that may be sourced from a domestic residence (Paul & Jacobs 2002; Wong 2002). Papain, a non-oxidising adulterant and cysteine protease enzyme derived from the papaya represents another commonly available product, and is sold as a meat tenderising product (Ashie, Sorensen & Nielsen 2002; Tappel & Miyada 1956).

Oxidising agents sold online are often sold as pre-packaged kits that can be discretely added to a urine sample immediately after voiding. Due to changes undertaken by drug testing laboratories, the formulation of the following products has been noted to change, with last known formulations of a range of chemical products reportedly used in urine adulteration presented in **Table 1.4**. Other oxidising compounds known to be employed to mask drug use that would not be considered a commonly available household product include compounds that contain iodate, periodate, permanganate and chromate moieties (Dasgupta 2008). Many other chemicals and household products have the potential to invalidate presumptive and confirmatory testing procedures, with a selection of these chemicals and their reactions explored in **Section 1.7.**

It is important to note that in specific cases, bacterial infections, and more specifically, bladder and urinary tract infections, can result in nitrite and nitrate compounds in the urine (Lewis *et. al.* 1999; Luong *et. al.* 2012). With regards to the detection of urine adulteration, this presents an additional issue that may complicate the interpretation of results by drug testing

laboratories. In particular, the detection of nitrite in urine during presumptive screening of a urine sample may be interpreted as the urine donor adulterating or otherwise manipulating their urine sample, potentially in order to invalidate a urine test. However, as nitrite can be produced endogenously under certain conditions, interpretation of a nitrite-positive urine sample must be approached carefully in order to ascertain whether the source of this nitrite is endogenous or exogenous.

1.4.5 – Effect of Oxidising Adulterants on Detection of Cannabis Use

The effects of oxidizing adulterants on the detection of cannabinoids and related compounds have been researched extensively in literature. Screening and confirmatory testing of urine specimens to determine alleged cannabis use incorporates the metabolites of the main active component of cannabis. Confirmatory analysis by GC-MS or LC–MS/MS is routinely performed targeting THC-COOH after hydrolysis of the glucuronide conjugate (AS/NZS 4308:2008; Fu & Lewis 2008; Smith Barnes & Huestis 2009).

As summarized in **Table 1.2** and **Table 1.3**, a number of oxidizing agents such as hypochlorite, nitrite, pyridinium chlorochromate (PCC) and Stealth® commonly used to chemically adulterate urine specimens, are effective at decreasing the level of THC-COOH and related cannabinoids and, in some cases, may effectively render a false-negative test result. Schwarzhoff and Cody (1993) studied the effect of 16 adulterating agents including hypochlorite on analysis of urine by FPIA for drugs of abuse. The cannabinoid test was most susceptible to adulteration, affording false-negative test results. Baiker et. al. (1994) also reported that hypochlorite adulteration of urine caused a decrease in the concentration of THC-COOH as measured by GC-MS, accompanied by false-negative results with the FPIA screen and the Roche Abuscreen tests. Wu et. al. (1999) reported that PCC caused a decrease in the response rate for all EMIT II drug screens, as well as for the Abuscreen cannabinoid immunoassays and loss of THC-COOH in GC-MS confirmatory tests. Paul et. al. (2000) also studied the effect of 'Urine Luck' on testing for drugs of abuse. When urine specimens containing THC-COOH were treated with 2 mM of PCC, 58–100% of the THC-COOH was lost. The loss increased with decreasing pH and increasing time of incubation (0–3 days).

ElSohly et. al. (1997) first reported that nitrite led to the decomposition of THC-COOH and the deuterated internal standard THC-COOH-d6 measured by GC-MS. Tsai et al. (2000) further studied the effects of nitrite on immunoassay screening tests for illicit drugs and/or drug

metabolites including THC-COOH. Nitrite at a concentration of 1000 mM only moderately decreased the sensitivity of immunoassays to cannabinoids. When assayed by GC-MS, however, nitrite concentrations as low as 30 mM were effective at producing significant losses of THC-COOH.

Of particular note is the research undertaken by Lewis et al. (1999), in which THC-COOH was destroyed following adulteration with potassium nitrite under acidic conditions, and resulted in an unstable nitroso-derivative of THC-COOH being detected. As discussed by Paul and Jacobs (2002) and Tsai et. al. (2000), this reaction between nitrite and THC-COOH progresses rapidly with acidic urine specimens, and progresses marginally under neutral and basic pH conditions. Given that the pH range of urine in healthy individuals is between pH 4.5 and 8.0 (Cook *et. al.* 2000), it is interesting to note that, for persons attempting to subvert the results of a drug test through the adulteration with nitrite, the attempt at adulteration may not be effective if the individual in question provides a urine specimen with a neutral or basic pH reading. However, sample preparation procedurals usually involve an acidification step to aid extraction of the acidic THC-COOH; this acidification step may facilitate the destruction of the THC-COOH analyte by nitrite due to the lowered urine pH environment.

From the perspective of the drug testing laboratory, the use of hydrosulfite or bisulfite as a reducing agent for nitrite to be added to a nitrite-adulterated specimen at the beginning of sample preparation has been suggested as a countermeasure (ElSohly *et. al.* 1997; Tsai *et. al.* 2000). This countermeasure can reduce interference caused by high levels of nitrite during sample processing, but with only limited efficiency, and is unlikely to be routinely incorporated into sample preparation methods due to the additional cost, time required, and limited utility.

Table 1.3 – Summary of relevant literature regarding the use of in vitro oxidising adulterants and the detection of cannabis use through confirmatory testing methods.

Adulterant	Detection Method	Observation
Hypochlorite	GC-MS	Complete degradation of target analyte (Paul & Jacobs 2002)
Nitrite	LC-MS	Significant decrease in recovery of target analyte. Unstable reaction product detected (Lewis *et. al.* 1999)
	GC-MS	Complete degradation of target analyte (Paul & Jacobs 2002; Tsai & Lin 2005)
Potassium chlorate	GC-MS	Minor decrease in recovery of target analyte (Paul & Jacobs 2002)
Potassium chromate	GC-MS	Complete degradation of target analyte (Paul & Jacobs 2002)
Potassium permanganate	LC-MS	Complete degradation of target analyte (Paul & Jacobs 2002)
Pyridinium chlorochromate	GC-MS	Significant decrease in recovery of target analyte (Wu *et. al.* 1999)

Table 1.4 – A summary of common oxidising adulterants used for the in vitro adulteration of urine samples.

Product Name (Manufacturer, Location)	Active ingredient
Betadine® (Sanofi, NSW, Australia)	Iodine/Povidone-Iodine (Charlton & Fu 2012)
Bleach (hypochlorite-based, various)	Sodium hypochlorite (Uebel & Wium 2002)
Klear® (Klear Co., TX, USA)	Potassium nitrite (ElSohly *et. al.* 1997; Peace & Tarnai 2002)
Stealth® (unknown)	Peroxide + peroxidase (Valtier & Cody 2002)
Urine Luck (Spectrum Labs, OH, USA)	Pyridinium chlorochromate (Paul *et. al.* 2000; Wu *et. a.* 1999)
Whizzies® (unknown)	Sodium nitrite (Dasgupta *et. al.* 2004)

Table 1.5 (Continued) – A summary of common oxidising adulterants used for the in vitro adulteration of urine samples.

Product Name (Manufacturer, Location)	Active ingredient
Other chemicals (various)	Ceric ammonium nitrate (Charlton & Fu 2012; Kuzhiumparambil & Fu 2013) Potassium chlorate (Paul & Jacobs 2002) Potassium chromate (Paul & Jacobs 2002) Potassium dichromate (Kuzhiumparambil & Fu 2013) Potassium perchlorate (Kuzhiumparambil & Fu 2013; Paul & Jacobs 2002) Potassium permanganate (Kuzhiumparambil & Fu 2013; Paul & Jacobs 2002) Sodium iodate (Paul & Jacobs 2002) Sodium metaperiodate (Kuzhiumparambil & Fu 2013)

1.5 – Detecting Urine Adulteration

A significant challenge to of the attempts of an individual to adulterate their urine sample is through personnel at both specimen collection sites and drug testing laboratories. These personnel are responsible for visual inspection of the sample, as well as performing urine integrity tests by monitoring parameters associated with normal, human urine. The collection cup may also incorporate panels that monitor these parameters, though their efficacy is debatable.

In cases where household chemicals, such as bleach, Betadine and surfactants have been used to adulterate the sample, the odour of the sample may also provide strong evidence that a sample has been adulterated (Wu 2003). **Table 1.5** summarises the typical endogenous characteristics of freshly collected, normal human urine. Integrity testing and urinalysis are critical for cases where sample tampering is suspected, and general guidelines for detecting urine adulteration and substitution are outlined in **Table 1.6**.

The composition and properties of urine can be modified through four commonly employed techniques, with the goal of concealing a positive drug test result. As discussed above, these methods include the dilution of urine, the addition of interferents, the addition of oxidising adulterants, and the substitution of urine. Detecting the adulteration of urine can be achieved, with specific techniques outlined in literature for the four commonly used methods of sample manipulation and adulteration.

Table 1.6 - Physiological measurements of parameters in normal human urine for urine integrity tests.

Parameter	Expected Range
Creatinine Concentration	80 – 200 mg/dl (7.0 – 17.8 mM) (Edwards *et. al.* 1993; Murray 1989)
pH	4.7 – 4.8 (Cody 1990; Schumann & Schweitzer 1989)
Specific Gravity	1.003 – 1.035 g/mL (Cody 1990; Edwards *et. a.*, 1993)
Temperature	32.5 – 37.7 °C (Department of Health and Human Services Substance Abuse and Mental Health Services Administration 2004)

1.5.1 – Detecting Adulteration of Urine Samples

Urinalysis provides an effective means for detecting cases of urine dilution. As shown in **Table 1.5** and **Table 1.6**, a number of parameters are monitored during routine urinalysis, with abnormal results providing strong evidence of dilution. Currently, the presence of diuretics is not monitored by immunoassay techniques, though instrumental techniques including LC-MS/MS are capable of detecting and quantifying diuretics and their metabolites in samples submitted for analysis (Deventer *et. al.* 2002).

Table 1.7 - Guidelines for the detection of urine adulteration.

Test	Cut-Off Limits	Interpretation
Creatinine	<5 mg/dl (0.44 mM) and specific gravity <1.002 g/ml	Substituted
	≥5 mg/dl (0.44 mM) and <20 mg/dl (1.77 mM) and specific gravity <1.003 g/ml	Diluted
	≥5 mg/dl (0.44 mM) and specific gravity = 1.000 g/ml or ≥1.020 g/ml	Invalid result
pH	<3 or ≥11	Adulterated, pH outside of endogenous range
	≥3 and <4 or pH ≥10 and <11	Invalid result
Nitrite	≥11 mM	Adulterated
	≥4 mM and <11 mM	Invalid result
Chromate	> the laboratory's limit of detection	Adulterated, chromium (VI)
Halogen	> the laboratory's limit of detection	Adulterated, halogen-containing adulterant
Glutaraldehyde	> the laboratory's limit of detection	Adulterated, glutaraldehyde
Decreased GC-MS internal standard	≥ 70%	Invalid result

Data taken from (Chou & Giang 2007; Cody & Valtier 2001; Cook *et. al.* 2000; Department of Health and Human Services Substance Abuse and Mental Health Services Administration 2004)

1.5.2 – Detecting the Use of Interferents

As with the detection of cases of urine dilution, urinalysis and immunoassays may be employed to detect the use of interferents. In addition, specific techniques may be used by drug testing laboratories to detect and confirm the use of specific interfering agents.

The use of glutaraldehyde may be detected through fluorimetric analysis (Dasgupta 2005). In comparison with instrumental techniques including LC-MS/MS and GC-MS, fluorimetry acts as a rapid, sensitive and inexpensive method to determine if glutaraldehyde has been used to adulterate a urine sample.

Other interfering agents that may affect the efficacy of immunoassays include ascorbic acid, vinegar and benzalkonium chloride. Both vinegar and ascorbic acid are acidic substances, with their addition to a urine sample causing a marked decrease in pH, and hence are detected during routine urinalysis. For benzalkonium chloride, it has been noted in literature (Dasgupta 2007) that this compound is unable to be detected in routine specimen integrity testing. Chromatographic techniques in tandem with mass spectrometry, however, are capable of detecting this interferent. If detected, the presence of benzalkonium chloride in a urine sample is considered a strong indicator of urine manipulation, and needs to be considered when assessing the results of drug screening tests.

1.5.3 – Detecting Urine Substitution

Due to the potential difficulty of differentiating between substituted urine samples, current methods for detecting urine substitution have relied on an interdisciplinary approach. Currently, a combination of bioanalytical assays, chromatographic techniques and DNA typing has allowed laboratories to individualise and characterise urine samples (Junge, Steevens & Madea 2002). As such, cases of a drug positive urine sample being swapped with either synthetic urine or a drug negative urine sample may be identified.

1.5.4 – Detecting Oxidising Adulterants

A significant challenge to drug testing laboratories and detecting the use of oxidising adulterants has been studied at length in literature (Baiker, Serrano & Lindner 1994; Dasgupta 2008; Kuzhiumparambil & Fu 2013). Detection of these adulterants may be difficult, as their

use can escape detection even at relatively high concentrations when assessed solely by urine integrity tests. At low concentrations, the colour of urine may change slightly when exposed to pyridinium chlorochromate, betadine and other coloured oxidants. These variations in colour may not be apparent due to the natural variation of urine in the human population, with such variations readily attributed to medication, as well as dietary, physiological and pathologic conditions (Urry *et. al.* 1998).

Spot tests provide drug testing laboratories a rapid technique that may be used to detect the presence of oxidising adulterants. This method is limited by its poor specificity, with the potential for false-positive results with a number of chemicals (Moeller, Lee & Kissack 2008). Spot tests have been developed for a number of commonly used oxidants, including pyridinium chlorochromate, nitrite compounds, as well as Stealth.

Testing for the presence of PCC can be achieved through the addition of 1% solution (w/v) of 1,5-diphenylcarbazide in methanol to 1 ml of the urine. Development of a red/purple colour change may indicate the presence of hexavalent chromium ions, though it should be noted that this test displays cross-reactivity to other metal ions, including molybdenum, vanadium and mercury (Wu *et. al.* 1999). Other spot test reagents for PCC include the use of acidified potassium iodide or hydrogen peroxide (Dasgupta, Wahed & Wells 2002). A rapid colour change with these methods is indicative of the presence of hexavalent chromium ions in solution.

Samples adulterated with nitrite salts may be identified through the use of acidified solutions of either potassium iodide or potassium permanganate (Dasgupta 2005). In the case of the latter test, a nitrite-positive sample will turn pink and evolve gas. False-negative results may occur in cases where a high concentration of glucose is present in the sample, as the in vitro reaction between potassium permanganate and glucose will compete with the reaction between the acidified permanganate solution and nitrite (Ridgway 1931).

The peroxidase enzyme in Stealth can be detected using a buffered tetramethylbenzidine solution. In the presence of peroxidase, this test rapidly develops a dark brown colour change. In addition, an acidified potassium dichromate solution can also be used to detect the presence of Stealth in samples suspected of adulteration. The colour change for a positive test result is deep blue, and has been noted to slowly fade with time (Caitlin *et. al.* 1992; Dasgupta 2005).

Instrumental techniques represent another means by which the use of oxidising adulterants may be detected. These range from immunoassay-based tests, capillary electrophoresis, spectrophotometric methods, and chromatographic methods in tandem with mass spectrometry. These techniques can be considered specific and highly sensitive, though as these techniques are not commonly incorporated into routine testing procedures by drug testing laboratories, their utility is limited.

The immunoassay-based Microgenics DRI® General Oxidant-Detect® test is specifically used for the detection of urine adulteration by oxidizing compounds (Microgenics Corporation 2013). This assay can be performed on an automated clinical chemistry analyser, and is based on the reaction between tetramethylbenzidine reagent and the oxidant in the specimen, which forms a coloured complex that can be observed at 660 nm.

Capillary electrophoresis has been successfully used in the detection of the chromate and nitrite ions found in pyridinium chlorochromate and nitrite-containing salts (Alnajjar & McCord 2003; Ferslew, Nicolaides & Robert 2003). Other chromatographic methods, including GC-MS, LC-MS and ICP-MS, have also shown the ability to detect hexavalent chromium ions (Minakata *et. al.* 2008), as well as nitrite ions (Dasgupta 2007).

Detection of pyridinium chlorochromate, the active ingredient in previous formulations of Urine Luck, can be achieved through multiple techniques. A number of spectrophotometric techniques have been developed to detect this compound (Paul *et. al.*, 2000), with other spectrophotometric methods developed for other oxidising adulterants, such as chromate, nitrite, permanganate, hydrogen peroxide/peroxidase and sodium oxychloride (Cody & Valtier 2001; Paul & Jacobs 2002).

Dipstick detection devices may also be used in the screening of samples for the presence of oxidising adulterants. In comparison to other methods, dipstick devices offer portability, allowing for the screening of samples to take place at specimen collection sites. The use of these devices has been extensively reviewed in literature, with a number of products currently available for use by drug testing laboratories (Dasgupta 2007; Jaffee *et. al.* 2007).

The Bayer Multistix® and Combur-Test® dipsticks provide analysts with readings for a range of urinalysis parameters, including tests for nitrite, pH, specific gravity, and peroxidase activity. However, the efficacy and accuracy of the test for peroxidase activity is interfered with if the urine sample contains blood, glucose, or nitrite ions. These strips are also ineffective at

distinguishing between samples that have been diluted or substituted, as it has difficulty distinguishing specific gravity at the cut-off levels determined for these two types of adulteration. The test for nitrite is also inaccurate for screening samples that are suspected of being adulterated with nitrite, as the upper limit in a clinical range, whereas the concentration of nitrite-containing products, such as Klear, will far exceed this clinical range.

The Adultacheck® 4 and Adultacheck® 6 dipsticks are two other dipstick detection products, and are designed to be used for forensic toxicology testing purposes. Tests available with these products include creatinine, pH, nitrite, glutaraldehyde and PCC in urine specimens. The Adultacheck® 4 and Adultacheck® 6 dipsticks can detect a large range of creatinine and pH values at both ends of the physiological spectrum, including abnormally low and high levels that would signify manipulation or adulteration of the urine sample (Dasgupta *et. al.* 2004; Peace & Tarnai 2002). Reported limitations of these products include imprecise readings for creatinine and pH levels (King 1999).

Another dispstick detection device used for on-site detection of adulterants is the MASK Ultrascreen. Compared to the Adultacheck® 4 and Adultacheck® 6 dipsticks, this device can detect a larger range of potential adulterants, with assays for creatinine, pH, specific gravity, Stealth (peroxide/peroxidase), pyridinium chlorochromate, nitrite and glutaraldehyde are available on the testing panel. Low concentrations of the included adulterants, however, will yield a false-negative result (Peace & Tarnai 2002).

Finally, the Intect®7 has been evaluated to be the most sensitive and economical adulteration test strip on the market. A total of seven test pads are included on each strip, and allow for the assessment of the presence of creatinine, pH and specific gravity, in addition to exogenous hypochlorite, pyridinium chlorochromate, nitrite and glutaraldehyde. In comparison to the MASK Ultrascreen, the Intect®7 can detect adulterants at low concentrations, an advantage given that small amounts of oxidising adulterants are capable of returning a false-negative for a sample submitted for testing.

1.6 – Current Methods for Detecting Cannabis Use

The basis of urine drug testing for cannabis use is the detection of metabolites of THC in urine, with THC-COOH and its glucuronide typically monitored by drug testing laboratories. Due to the physical and chemical properties of THC and its metabolites, a variety of detection methods is available for the confirmation of the presence of cannabis metabolites in urine. Suitability of detection methods depends on a range of factors, including the availability of equipment, the separation technique used, as well as the mobile phase that has been selected. Detection methods described in literature include:

- UV-Vis spectrophotometry (Paul & Jacobs 2002)
- Mass spectrometry (Moeller, Lee & Kissack 2008)
- Tandem mass spectrometry (MS/MS) (Chiarotte & Costamagna 2000)
- Electrochemical detection (Krämer & Kovar 1999)

The presence of an aromatic ring within the molecule allows the use of UV-Vis spectroscopy as a detection method, as advocated by Bianchi and Donzelli (1996). This detection method is of particular interest as it requires no specialised interfaces between liquid chromatography systems and the detector. In addition, UV-Vis spectrophotometry may be used in tandem with mass spectrometry through use of an eluent flow splitter. In this scenario, information obtained from both the spectrophotometer and mass spectrometer may be combined to assist in the detection and analysis of specific analytes.

Multiple liquid-gas interfaces are available for when instruments such as HPLC and LC are used in tandem with mass spectrometry. These interfaces include electrospray ionisation (ESI), chemical ionisation (CI) and atmospheric pressure chemical ionisation (APCI). The selection of an interface depends on the separation technique being utilised, the resources available to a laboratory and the expertise of the available staff.

Tandem mass spectrometry represents another viable detection method that may be used in the detection of THC-COOH oxidation products. Used previously by both Chiarotte and Costamagna (2000) and Weinmann et. al. (2001), tandem mass spectrometry (MS/MS), also known as selected reaction monitoring, allows for the highly selective and sensitive analysis of ions produced by the target analyte. Specific ions, referred to as precursor ions, are selected in the first quadrupole based on their mass-to-charge ratio, and passed through consecutive

mass filters and a collision cell, producing product ions that can be analysed by a third quadrupole mass separator and detector (Harris 2007).

Tandem mass spectrometry has several advantages over traditional single-quadrupole mass spectrometry. Advantages include a markedly increased signal-to-noise ratio and sensitivity, and very high selectivity. Reported detection limits with these methods for THC-COOH have approached concentrations as low as picograms per millilitre. Low detection limits are particularly desirable in the analysis of biological matrices believed to contain drugs and/or drug metabolites, as these analytes are often found in very low concentrations in such samples. This sensitivity is also advantageous, as it may allow for the detection of both stable and unstable oxidation products of THC-COOH, in cases of adulteration with oxidising agents.

1.7 – Potential Reactions Involving THC-COOH and Related Compounds

The reported effects of oxidising adulterants on THC-COOH are likely through the reaction of THC-COOH to form different chemical compounds not currently incorporated into drug testing procedures. One of the main challenges facing drug testing laboratories is the variety of oxidising adulterants available by which an individual may mask a positive drug test result. **Figure 1.4** shows the structure of THC-COOH with functional groups highlighted. This figure reveals that THC-COOH may undergo a wide range of reactions that may target a variety of functional groups.

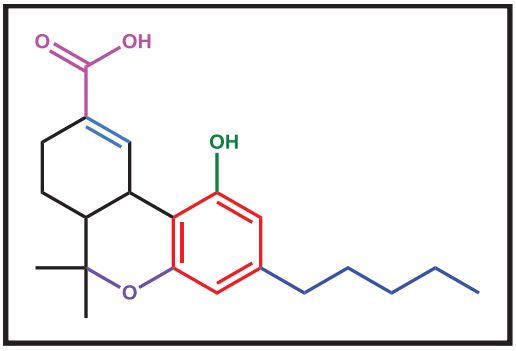


Figure 1.4 - Highlighted regions of THC-COOH that are likely to undergo chemical reactions. The phenol group (green) is highly activating. A number of electrophilic aromatic substitution (EAS) reactions may occur on the aromatic ring (red).

Table 1.7 explores a number of reactions that THC-COOH is expected to undergo when exposed to a several chemicals, with proposed reactions derived from "*March's Advanced Organic Chemistry: Reactions, Mechanisms and Structure*" (Smith & March 2007). It should be noted that this summary is incomplete, and that a number of proposed chemical reactions are not intended to represent viable means by which an individual may adulterate a urine sample. Rather, by exploring theoretical reactions of THC-COOH under a wide variety of conditions, it can be demonstrated that from the perspective of drug testing laboratories, the abundance of reagents and oxidising agents that may be used to invalidate a urine sample pose a significant challenge. Consequently, continual research will be required to detect the in vitro use of oxidising adulterants, determine their effect on drug metabolite detection, and in cases where these chemicals adversely affect detection, explore the possibility that stable markers of both drug abuse and urine adulteration exist.

Where possible, reactions occurring over multiple sites within the THC-COOH molecule have been noted, though this is by no means exhaustive. The presence of a highly activating phenol group in THC-COOH may also significantly alter the outcome of the proposed reactions. In particular, reactions with halogens or hypohalous acid salts, such as sodium hypochlorite, may not attack the cycloalkene ring, and are more likely to result in the substitution of halides on the aromatic ring through electrophilic aromatic substitution.

Table 1.8 - Proposed reactions of THC-COOH, demonstrating chemical versatility of this molecule.

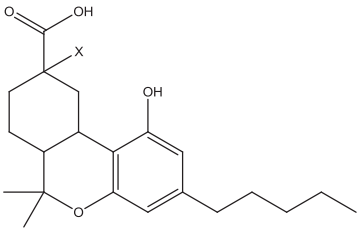
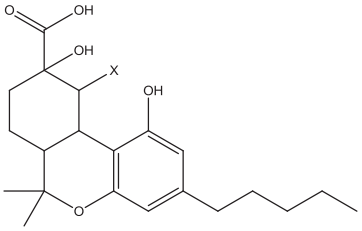
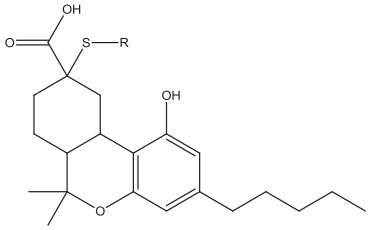
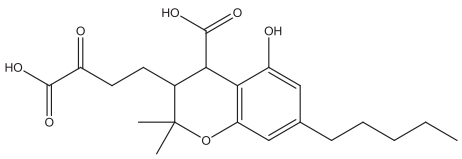
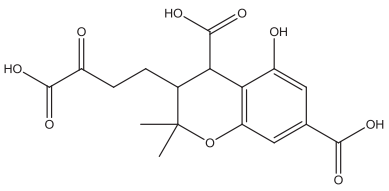
Reaction Type	Reagents/Conditions	Proposed Product/s
Addition	**Hydrogen halides** X = F, Br, I	
	Hypohalous acids X = Cl, Br, I Note: may preferentially substitute aromatic ring	
	Thiols, H_2S R = H, alkyl, etc.	
Alkene Cleavage	**Various oxidising agents** (I) Permanganate, dichromate (neutral, acidic conditions), chromium trioxide (II) Further oxidation may occur on aliphatic sidechain	(I)  (II) 

Table 1.7 (Continued) - Proposed reactions of THC-COOH, demonstrating chemical versatility of this molecule.

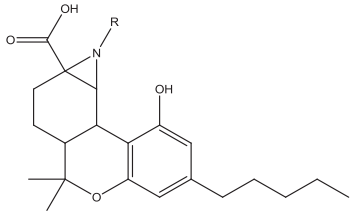
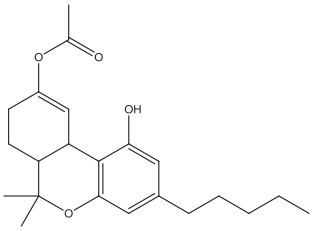
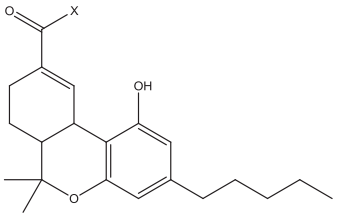
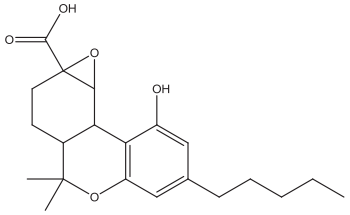
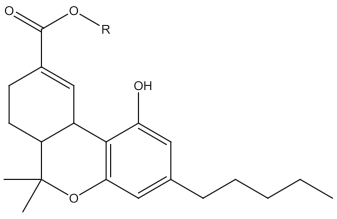
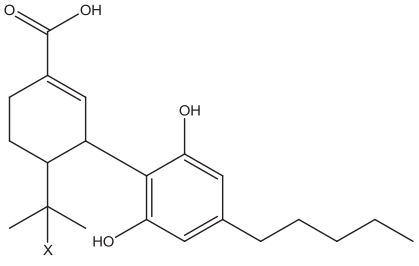
Reaction Type	Reagents/Conditions	Proposed Product/s
Aziridination	**Substituted azide** R = aryl, cyano, etc.	
Decarboxylation	**Lead Tetraacetate**	
Dehydroxylation	**Halogenating agent** E.g. $SOCl_2$, $SOBr_2$, PCl_3, $POCl_3$, etc X = halogen	
Epoxidation	**Peroxyacid** E.g. peracetic acid, peroxybenzoic acid, etc.	
Esterification	**Alcohol (R-OH)** Under acidic conditions	
Ether Cleavage	**Hydrogen Halides** HI, HBr, strongly acidic conditions X = halogen	

Table 1.7 (continued) - Proposed reactions of THC-COOH, demonstrating chemical versatility of this molecule.

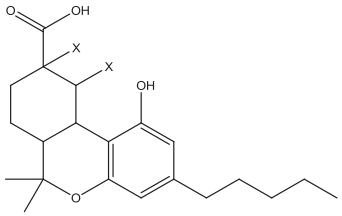
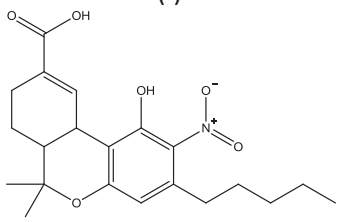
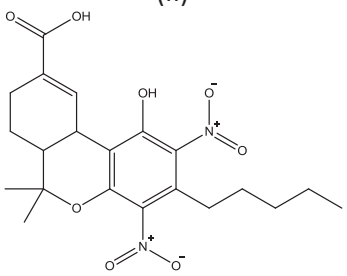
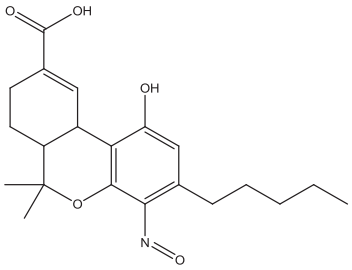
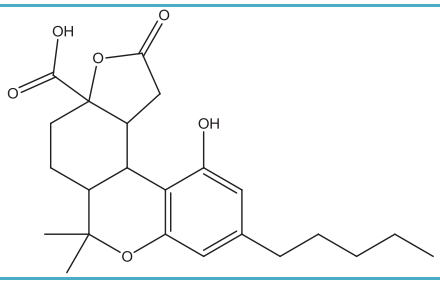
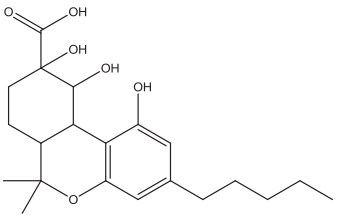
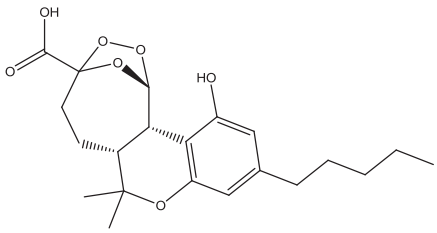
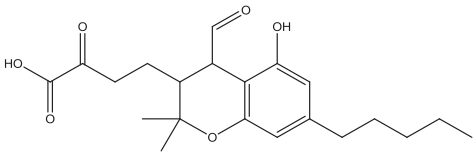
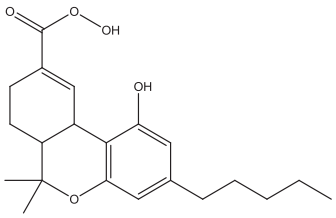
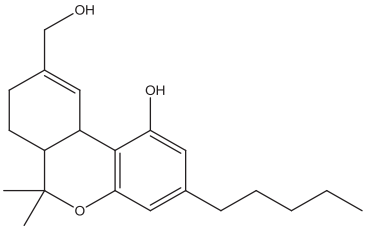
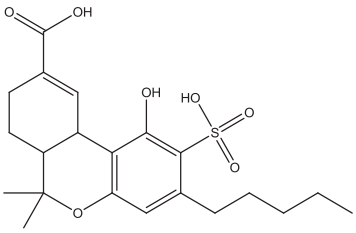
Reaction Type	Reagents/Conditions	Proposed Product/s
Halogenation	**Br_2, Cl_2, inter-halogen compounds** X = halogen Note: additional reactions may occur on aromatic ring.	
Nitration	**Nitric acid** (I) With dilute nitric acid, substitution may occur at either ortho or para position. (II) With concentrated nitric acid, nitration may occur at both available positions. Note: under highly acidic conditions, alkyl sidechain may undergo oxidation	(I)  (II) 
Nitrosylation	**Nitrous Acid** Acidic conditions. Substitution may occur at ortho and para positions relative to phenolic OH group.	
Oxidation	**Manganese (III) Acetate**	

Table 1.7 (continued) - Proposed reactions of THC-COOH, demonstrating chemical versatility of this molecule.

Reaction Type	Reagents/Conditions	Proposed Product/s
Oxidation	**Osmium tetroxide** **Alkaline potassium permanganate**	
Ozonolysis	**Ozone** (I) Further reaction in presence of zinc / acetic acid	 (I) 
Peroxidation	**Hydrogen Peroxide** Reaction in presence of acid catalyst	
Reduction	**Lithium Aluminium Hydride**	
Sulfonation	**Sulfuric acid** Ortho-substituted product forms preferentially at 25°C. Para-substituted product forms preferentially at 100°C	

1.8 – Search for Reaction Products of THC-COOH

The use of oxidising adulterants represents a major challenge to drug testing laboratories. Though multiple procedures are in place to detect the presence of these adulterants, this does not identify the drug/s being masked, nor does it rectify the action of urine adulteration. It is important to note that the act of adulterating or tampering with a urine sample may represent a serious criminal offence, once this act has been undertaken it remains highly difficult for drug testing laboratories to determine which drugs were concealed.

It is evident that oxidising adulterants will potentially oxidise the drugs and/or drug metabolites present in a urine sample. As it is not possible, nor feasible, to reverse this reaction, the reactions themselves can be studied to determine if they produce novel reaction products specific for the drug present in the urine sample, and the adulterant used. The detection of any stable oxidation products will provide clear evidence for drug testing laboratories that both the sample has been adulterated, and that specific drug metabolites were masked by this action.

Previously, Lewis et al. (1999) investigated the reaction of THC-COOH with potassium nitrite and reported formation of an unstable nitroso derivative of THC-COOH. This nitroso-derivative was detected through HPLC fractionation and subsequent negative ion electrospray tandem MS analysis of the collected fractions. This represents the very first and the only early attempt in identification of oxidation products of a drug metabolite by oxidizing urine adulterant. Given the unstable nature, the identified nitroso product is not suitable to be used as a marker for routine monitoring of cannabis use when urine specimens are adulterated by nitrite. As will be discussed in Chapter 2, further study of this reaction by LC-MS both confirmed the existence of the unstable nitroso-THC-COOH. Further analysis of this reaction also yielded the formation of an additional, stable product, that appears to be a nitro-substituted analogue of THC-COOH.

It is interesting to note that González-Mariño, I., et. al. (2013) reported that stable oxidation products of THC-COOH were detected in surface water samples obtained from the Santiago de Compostela region of Spain. These samples were produced through the disinfection of sewer effluent by chlorine. In this case, the detection of these compounds provides an alternate means of assessing the use of cannabis in nearby areas, even when the metabolite THC-COOH has undergone reactions with highly reactive chlorine species.

In terms of the research presented in this thesis, **Table 1.8** provides a summary of the oxidising adulterants and other chemicals studied for their potential for reaction with THC-COOH, and where a reaction with THC-COOH or degradation of the sample is noted, whether any stable oxidation products form. Though papain is a non-oxidising adulterant, as discussed previously, it is included in this research due to ready availability, and that it has been explored as a potential urine adulterant in previous literature (Ashie, Sorensen & Nielsen 2002; Tappel & Miyada 1956). In Chapter 2 the analysis of these reactions, and detected oxidation products, will be further examined.

Table 1.8 - Summary of oxidising adulterants tested in this research, with effect on target analyte concentration and formation of reaction products noted.

Oxidant	Effect Noted on THC-COOH in Water and Urine	Reaction Product/s Detected?
Betadine®	Major decrease in concentration	**Yes**
Ceric ammonium nitrate	Significant decrease in concentration	**Yes**
Papain	Slight decrease in concentration (water only)	No
Potassium Nitrite	Major decrease in concentration (water only)	**Yes**
Potassium Perchlorate	Moderate decrease in concentration	No
Potassium Permanganate	Moderate decrease in concentration	No
Pyridinium Chlorochromate	Major decrease in concentration	**Yes**
Sodium Hypochlorite (Bleach)	Major decrease in concentration	**Yes**
Sodium Iodate	Significant decrease in concentration	**Yes**
Sodium Metaperiodate	Major decrease in concentration	**Yes**

Currently, urine specimens are deemed invalid if they fail integrity tests (e.g., pH, creatinine, specific gravity , etc.), or if the presence of oxidising adulterants is suspected and/or detected. In cases where a urine sample has been deemed invalid, it is standard protocol for individuals to be requested to supply another urine specimen for testing. This represents a significant

limitation for drug testing laboratories, employers, sporting authorities and law enforcement agencies, as this delay not only allows for drugs to be metabolised and excreted from the system, but also results in time consuming and expensive laboratory practices.

Identification of unique oxidation products of a drug analyte represents a challenging but promising alternative approach to address the problem of urine manipulation by oxidizing chemicals. Oxidation products identified and elucidated will provide useful leads in developing new and unique markers for monitoring drug abuse. The markers would not only indicate the type of drug used, but also reveal the nature of the adulterants added to the urine specimens, thus potentially eliminating the need for the extra screening and confirmatory tests for adulterants currently employed in many drug testing facilities. As such, every specimen would remain valuable, providing information as to drug use regardless whether it has been adulterated by these popular and effective adulterants.

Additional research will need to be undertaken to make use of the oxidation products as markers. There is currently no commercial supply of the certified reference materials for these oxidation products. Certified reference materials are needed for drug testing laboratories to optimise chromatographic (e.g., retention time, peak shape and resolution) and MS parameters (e.g., ionization energy, collision energy and selection of MRM transitions) in order to develop and validate analytical methods for quantitative analyses of the marker analytes. In addition, the variety of oxidising adulterants and other chemical agents that may react with drugs and drug metabolites targeted by drug testing laboratories will require significant and continuing research to study these potential reactions, detect viable reaction products, and assess their suitability for incorporation into drug testing procedures. As shown in **Section 1.7**, a significant number of potential reactions may occur between THC-COOH and other chemical species.

For the detection of oxidation products of THC-COOH, and other drugs and metabolites, LC–MS/MS represents a core confirmatory detection method. Through the identification and study of novel oxidation products of known drug metabolites, it is possible to develop validated methods incorporating these novel compounds, and thereby adapt, where possible, current testing regimes to allow for the presence of these compounds to be monitored. This may allow drug testing laboratories to take a proactive approach with regards to the issue of urine adulteration, as in-depth investigations of these compounds and their presence in urine samples will become possible.

From this, the primary aims of this research are to assess the effectiveness of known oxidising agents on the detection of drug abuse, and in cases where apparent degradation of THC-COOH is noted, determine if stable markers of both cannabis use and urine adulteration can be detected. For novel reaction products detected under these adulteration regimes, it will therefore become necessary to elucidate the structure of these compounds, and produce a validated LC-MS/MS method that can detect these compounds in a sensitive and specific manner. Though GC-MS has been long used for the detection of drug abuse, the development of suitable LC-MS/MS methods has the potential to increase sample throughput for laboratories, and remove the time-consuming need for derivitisation of target compounds prior to analysis.

Chapter 2 will deal with the initial testing of a range of oxidising adulterants on the detection of THC-COOH in spiked water and spiked urine samples, and assess the potential of these reactions for the formation of stable oxidation products. Building on this initial search, Chapter 3 will explore the large-scale synthesis of two key major oxidation products of THC-COOH, and in Chapter 4, the structural elucidation of these products by Quadrupole Time-of-Flight mass spectrometry (QToF-MS) and nuclear magnetic resonance spectroscopy (NMR). In the remaining chapters of this thesis, the development and validation of suitable LC-MS/MS methods for the detection of reaction products from three oxidising agents will be examined in detail, as will the monitoring of these reactions in real, drug-positive urine samples and assessing if cross-reactivity of these novel products occurs during presumptive screening.

Chapter 2

Detection of Reaction Products

Detection of THC-COOH is known to be affected by the in vitro adulteration of urine specimens with a range of oxidising adulterants. This effect of oxidising adulterants, and specifically, the potential for reaction products to form under these conditions, has not been widely studied. Research undertaken by two groups, Lewis et. al. (1999) and González-Mariño et. al. (2013) has generated the only literature that has successfully discovered reaction products of THC-COOH to date.

Expansion of this search, both in terms of currently employed oxidising adulterants, and other possible oxidising agents, may yield the discovery of novel products of THC-COOH. Such discoveries may allow for the eventual incorporation of novel reaction products of drug metabolites into current testing procedures, thereby giving drug testing laboratories the unambiguously detecting both the in vitro adulteration of urine samples, and the use of specific drug metabolites that have been masked in this process. This chapter details the initial studies into a range of oxidising adulterants and the eventual fate of THC-COOH in spiked water and spiked urine samples. Adulterants were selected based on availability and their presence in literature detailing the use of in vitro oxidising adulterants.

A total of three main studies were undertaken, and are as follows:

1. Initial Study – Preliminary study testing the effects of three oxidising adulterants on the detection of THC-COOH: sodium hypochlorite, potassium nitrite, pyridinium chlorochromate;
2. Main Study – General study assessing the effects of sodium hypochlorite, pyridinium chlorochromate and Betadine (povidone-iodine);
3. Further Study – A general study of a range of oxidising adulterants to assess potential future research avenues in the study of urine adulteration.

Selected oxidising adulterants were prepared at a number of concentrations, detailed in their respective sections, and their effect on the detection of THC-COOH was examined. Initial tests of these potential reaction conditions was initially undertaken in ultrapure water spiked with a

methanolic solution of THC-COOH, as ultrapure water provided an interference-free matrix suitable for both examining whether the selected adulterants decreased the apparent concentration of THC-COOH, and allowing for the monitoring of the sample to ascertain whether oxidation products of THC-COOH formed. In later studies, selected reaction conditions that produced oxidation products were repeated in a pooled urine matrix. Urine represents a complex matrix due to potential variation of physiological parameters and the presence of a range of endogenous compounds. It was proposed that under these conditions, side-reactions between the oxidising adulterants with endogenous compounds, along with parameters such as pH, may inhibit the reactions observed between THC-COOH and the selected adulterants. By repeating these reactions in urine, it would be possible to observe whether these novel products are capable of forming in a urine matrix. Analysis of these reaction mixtures was undertaken by LC-MS/MS, due to the inherently high specificity and selectivity of this instrumental technique, increasing the likelihood of detection reaction products of THC-COOH.

2.1 – Experimental

2.1.1 – Drug Standards and Reagents

THC-COOH stock solution (1 mg/mL in methanol) was obtained from Cerilliant (Round Rock, Texas, USA). Ultrapure water was obtained from the Sartorius Arium® 611 Laboratory Water Purification System equipped with a Sartopore 0.2 µm membrane filter. Ceric ammonium nitrate, potassium nitrite, potassium permanganate, pyridinium chlorochromate, alkaline sodium hypochlorite solution (≥ 4% available chlorine), sodium iodate, sodium metaperiodate, sodium perchlorate, peroxidase enzyme (from horseradish), hydrochloric acid (30-35%) and hydrogen peroxide (30% w/w) were sourced from Sigma Aldrich (Castle Hill, NSW, Australia). Betadine, a topical antiseptic produced by Sanofi (NSW, Australia) was sourced from a local pharmacy. Papain (1.5 – 10 units/mg) derived from papaya latex was sourced from Sigma Aldrich.

Ammonium formate and formic acid (> 98% purity) were obtained from British Drug Houses Laboratory Supplies Ltd. (London, England), ammonium acetate from Ajax Finechem (NSW, Australia), acetonitrile (isocratic HPLC grade) from Scharlau (Barcelona, Spain) and methanol

(HPLC grade) from Burdick and Jackson (South Australia, Australia). All solvents and chromatographic materials were of analytical or HPLC grade.

2.1.2 – Urine Specimens

Urine specimens were collected from healthy individuals (n= 12) and stored in polypropylene urine specimen containers at 4°C to create a representative blank urine matrix. Volunteers were selected randomly, with an age range from 18-58, and both males (n=6) and females (n=6) equally represented. The primary qualification factor for selected donors was that they had not used cannabis, or had been in contact with cannabis products, for the past three months. Individual samples were analysed by using LC-MS/MS methods developed in this research in order to ensure that it was negative for cannabinoids prior to use. The combination of urine specimens to form pooled urine for research was not used for more than one experiment.

2.1.3 – Instrumentation

2.1.3.1 – Initial Study

The initial study into the effects of oxidising adulterants on the detection of THC-COOH were undertaken on a Perkin Elmer Sciex API 365 LC-MS/MS system (Massachusetts, USA), coupled with an Alltech 530 column heater (Illinois, USA) and Applied Biosystems 785A Programmable Absorbance Detector (Victoria, Australia). Ionisation of the analytes was achieved with the use of an electrospray ionisation interface in negative ionisation mode. The HPLC column was kept in an Alltech 530 column heater maintained at 30 °C. The eluent was also monitored with an Applied Biosystems 785A Programmable Absorbance Detector (PAD) set at 210 nm, and the Perkin Elmer Mass Spectrometer. A flow splitter was used to direct 60% of the eluent to the programmable absorbance detector, with the remaining 40% directed to the mass spectrometer. System parameters for LC-MS are shown in **Table 2.1**.

Table 2.1 - Instrument parameters for Perkin Elmer LC-MS/MS system for initial study of oxidising adulterants.

LC-MS System Parameters		Setting
Gas Flow Rate	Nebuliser Gas (NEB)	10
	Curtain Gas (CUR)	10
	Collision Gas (CAD)	0
Control Settings	Ion Spray Voltage (IS)	-4000 V
	Temperature (TEM)	350°C
	Orifice Voltage (OR)	-40.0
	Focus Ring Voltage (RNG)	-140.0
	Q0 Rod Offset (Q0)	5.0
	Q2 Entrance Lens (IQ2)	19.0
	Q0 Rod Offset – Collision Energy	80.0
	CEM Deflection Plate	400.0
	Channel Electron Multiplier (CEM)	2000.0
Analysis Settings	Number of Scans	500
	Method Runtime – Initial Study	60 mins
	Method Runtime – Papain Study	13 mins
	Mass Range (*m/z*) – Initial Study	100 – 500
	Mass Range (*m/z*) – Papain Study	250 – 700
	Absorbance Wavelength (PAD)	210nm
	Injection Volume	10 µL

Chromatographic separation of the samples was achieved through the use of a Zorbax® XDB-C8 HPLC column (150 mm × 4.6 mm, 5 micron particle size, Agilent Technologies), with an injection volume of 10 µL and the solvent flow rate set at 0.5 mL/minute. Mobile phase A consisted of ammonium formate (pH 6.5, 2 mM) prepared in water, and mobile phase B was 100% acetonitrile. Mobile phase A was diluted from a 2M ammonium formate stock solution prepared by dissolving solid ammonium formate in water. The gradient condition used in these analyses is shown in **Table 2.2**.

Table 2.2 - Elution condition for initial assessment on the effect of oxidising adulterants on the detection of THC-COOH.

Time (minutes)	Solvent A (%)	Solvent B (%)
0	100	0
30	30	70
35	10	90
36	10	90
55	100	0
60	100	0

Identification of THC-COOH in standards and reaction mixtures was based on the comparison of chromatograms obtained from blank samples, standards and reaction mixtures and mass spectra obtained for THC-COOH and the detection of specific significant product ions, as described by Weinmann et. al. (2001). Potential reaction products were identified through the appearance of new peaks in the chromatogram compared to the standards and reagent blanks.

2.1.3.2 – Main Study into Detection of Oxidation Products

All analyses were undertaken on a 1290 LC system coupled to a 6490 triple quadrupole (QQQ) mass spectrometer. These instruments were from Agilent Technologies (Forest Hills, Victoria, Australia). As these studies were undertaken over a period of time, two different chromatographic columns were used: a Supelco C_{18} HPLC column (25 cm x 4.6 mm, 5 micron, Sigma Aldrich) and a Phenomenex Luna C_5 HPLC column (150 mm x 5.6 mm, 5 micron, Phenomenex Incorporated). Mobile phase A consisted of 100% ultrapure water, and mobile phase B was 100% acetonitrile. The elution condition used for all studies was isocratic, though as with the column used in these experiments was changed, the elution conditions used did vary (**Table 2.3**). General instrument parameters are shown in **Table 2.4**.

Table 2.3 – Elution condition parameters for studies on the effect of selected oxidising adulterants on water and urine samples spiked with THC-COOH.

Study	Solvent A (%)	Solvent B (%)	Column[a]
PCC – Water	10	90	Zorbax XDB-C_8
PCC – Urine	15	85	Supelco C_{18}
Hypochlorite – Water	20	80	Supelco C_{18}
Hypochlorite - Urine	15	85	Supelco C_{18}
Betadine – Water	10	85	Zorbax XDB-C_8
Betadine – Urine	10	85	Zorbax XDB-C_8
Iodine – Water	15	85	Supelco C_{18}
[a] Refer to Section 2.1.3.2 for full column parameters and properties			

Table 2.4 - Instrument parameters for main study into the effects of oxidising adulterants on the detection of THC-COOH.

LC-MS System Parameters		Setting
LC Parameters	Solvent Flow Rate	0.5 mL/min
	Injection Volume	5 µL
	Column Temperature	35°C
	Method Runtime – PCC (Water)	8.0 minutes
	Method Runtime – PCC (Urine)	15.0 minutes
	Method Runtime – Hypochlorite	15.0 minutes
	Method Runtime – Betadine	12.0 minutes
	Method Runtime – Iodine	20.0 minutes
QQQ Parameters	Mass Range (*m/z*) – PCC	100 – 700
	Mass Range (*m/z*) – Hypochlorite	50 – 1000
	Mass Range (*m/z*) – Betadine	200 – 700
	Mass Range (*m/z*) – Iodine	150 – 650
	Fragmentor Voltage	380 V
	Sheath Gas Temperature	11 L/min
	Sheath Gas Flow Rate	250°C
	Scan Mode	MS2 Scan

2.1.3.3 – Further Studies into Detection of Oxidation Products

A total of six additional oxidants were selected for study: ceric ammonium nitrate, papain, peroxide/peroxidase, potassium permanganate, sodium iodate, sodium metaperiodate and sodium perchlorate. Reactions studying the effects of papain on detection of THC-COOH were studied on the Perkin Elmer Sciex API 365 LC-MS/MS system (Massachusetts, USA), coupled with an Alltech 530 column heater (Illinois, USA), with instrument parameters as per **Table 2.5**. Remaining oxidants were analysed on a 1290 LC system coupled to a 6490 triple quadrupole (QQQ) mass spectrometer. These instruments were from Agilent Technologies (Forest Hills, Victoria, Australia).

Chromatographic separation of analytes was undertaken on a ZORBAX Rapid Resolution Eclipse XDB-C18 column (4.6 mm × 50 mm, 3.5 µm). Mobile phase A consisted of 20 mM ammonium formate (pH 6.2) prepared in water, and mobile phase B was 100% acetonitrile. Mobile phase A was diluted from a 2M ammonium formate stock solution, prepared by dissolving solid ammonium formate in water. **Table 2.5** notes the instrument parameters used in this study. The gradient elution condition used in these analyses is shown in **Table 2.6**.

Table 2.5 - Instrument parameters for Agilent LC-MS/MS system for further study of the effects of oxidising adulterants on the detection of THC-COOH.

LC-MS System Parameters		Setting
LC Parameters	Solvent Flow Rate	0.3 mL/min
	Injection Volume	5 µL
	Column Temperature	35°C
	Method Runtime	12.0 minutes
QQQ Parameters	Mass Range (*m/z*)	200 - 1000
	Fragmentor Voltage	380 V
	Sheath Gas Temperature	250°C
	Sheath Gas Flow Rate	11 L/min
	Scan Mode	MS2 Scan

Table 2.6 - Elution condition for expanded assessment on the effect of oxidising adulterants on the detection of THC-COOH.

Time (minutes)	Solvent A (%)	Solvent B (%)
0.0	26	74
6.0	15	85
7.5	15	85
9.5	26	74
12.0	26	74

2.1.4 – Experimental Procedures

2.1.4.1 – Initial Search for Oxidation Products

Eight samples were prepared to examine the effects of sodium hypochlorite, potassium nitrite and pyridinium chlorochromate on ultrapure water samples spiked with THC-COOH. The final THC-COOH concentration in the THC-COOH standard and reaction mixtures was 2 µg/mL. Reaction mixtures and reagent blanks were made to 200 µL total volumes with MilliQ water in Agilent 100 µL glass vial inserts with polymer feet. Potassium nitrite reactions were acidified to pH 5 with 0.2M hydrochloric acid. The hypochlorite solution was reported as having 4% w/v available chlorine, corresponding to a concentration of 0.54M.

Table 2.7 provides information relating to the preparation of the reaction mixtures analysed, including reagent blanks, reaction mixtures and the THC-COOH standard solution. Replicates of the successful reaction conditions were prepared and analysed over four time points to assess reaction progress and the stability of products formed in these reactions: immediately after preparation (T=0h), one hour after preparation (T=1h), five hours after preparation (T=5h) and twelve days after preparation (T=12d). Following analysis, samples were sealed and refrigerated at 4°C.

Table 2.7 - Sample preparation for the initial study of the effects of three selected oxidising adulterants on the detection of THC-COOH in spiked water samples.

Sample Type	THC-COOH Stock (µL)	Water (µL)	Hypochlorite (0.54M) (µL)	Potassium Nitrite (0.95M) (µL)	HCl (0.20M) (µL)	PCC Stock (0.40M) (µL)
THC-COOH Standard	4	196	0	0	0	0
Hypochlorite Blank	0	100	100	0	0	0
Nitrite Blank	0	125	0	50	25	0
PCC Blank	0	150	0	0	0	50
Hypochlorite Reaction	4	186	10	0	0	0
Nitrite Reaction	4	161	0	10	25	0
PCC Reaction	4	186	0	0	0	10

Stock solutions for potassium nitrite and pyridinium chlorochromate were prepared through the addition of the solid oxidant to water, with both stock solutions having a final concentration of 2M. Working solutions were prepared through the dilution of the stock solutions. The working hypochlorite solution was prepared through the dilution of the stock alkaline sodium hypochlorite solution. For the THC-COOH standard, and the initial condition of the reaction mixtures, the concentration of THC-COOH was 2 µg/mL.

Prior to analysis, samples were allowed to react at room temperature for a total of 120 minutes. Analysis of samples was achieved through two injections of each standard, blank and reaction mixture, with a total analysis time for each sample of 60 minutes. The eluent was monitored by mass spectrometry in negative ionisation mode and by a programmable absorbance detector set at 210 nm.

An additional consideration for the detection of reaction products is their chemical properties, particularly their partitioning between two immiscible phases for the purposes of liquid-liquid extraction (LLE). For this separation procedure, the sample is acidified and basified in order to

alter the solubility of THC-COOH and the detected reaction products. The workflow prepared for liquid-liquid extraction of the compounds into two separate organic fractions is illustrated in **Figure 2.1**. This experiment assessed whether the compounds produced through the reaction of THC-COOH maintained the acidic functional groups (phenolic and carboxylic acid moieties) present on the parent molecule.

Liquid-liquid extraction techniques reported by Zhang et. al. (2000) note that these procedures allow for the detection and extraction of drugs and drug metabolites from biological samples. Methods used in the liquid-liquid extraction of samples include both manual and semi-automated methods, and have been used effectively in extracting drug metabolites from complex biological matrices, and present a method by which the large-scale synthesis and subsequent purification of the reaction products may be achieved.

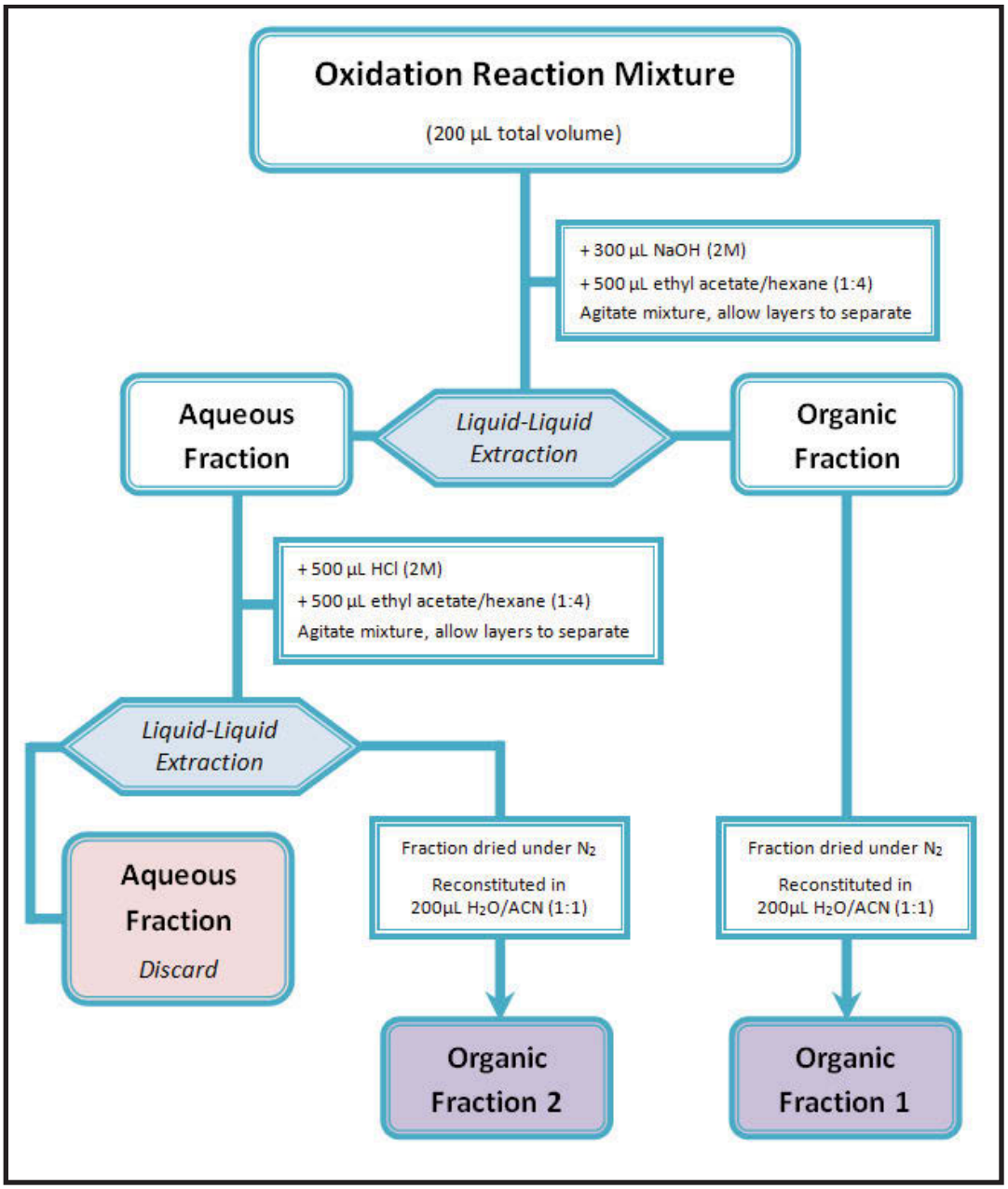


Figure 2.1 - Workflow for liquid-liquid extraction method.

2.1.4.2 – Main Study into Detection of Oxidation Products

The main study into the detection of oxidation products was designed as an extension of the initial study concerned with the effect of oxidising adulterants on the detection of THC-COOH by LC-MS. Sodium hypochlorite (bleach) and pyridinium chlorochromate were tested previously. Further study of these oxidants was undertaken due to the availability of bleach to the average consumer, and for pyridinium chlorochromate, its presence in prior formulations of the commercial urine adulterant Urine Luck. A third oxidant was selected for the main study: Betadine. As with bleach, Betadine is available to the average consumer from pharmacies and supermarkets, and based on the prior results obtained from exposure of bleach to samples spiked with THC-COOH, was considered a likely candidate to form reaction products following exposure to THC-COOH. Both spiked water and urine samples were tested for the potential formation of oxidation products of THC-COOH. The final concentration of THC-COOH in these studies was set at 2 µg/mL.

Urine Samples – Sample Cleanup

For the reaction samples prepared in urine, a sample clean-up procedure was instituted to ensure removal of all particulate matter from the samples:

1. Following addition of oxidising agent, samples sealed and vortexed for sixty seconds;
2. Samples left to react for five hours, followed by addition of a 25 µL aliquot of acetonitrile was added in order to precipitate proteins from urine;
3. Samples refrigerated overnight to aid in the precipitation of proteins;
4. Centrifuging at 8000 rpm for 30 minutes;
5. Supernatant from samples collected, and filtered through 0.2µm MilliPore syringe filters;
6. Filtered extract transferred to new vial and sealed prior to analysis.

Sodium Hypochlorite (Bleach)

Hypochlorite reaction samples were prepared in spiked water and urine samples. Concentration of the hypochlorite stock solution was determined spectrophotometrically as per the guidelines set by Morris (1966); the molar absorbance coefficient (ε) of 350 $M^{-1}cm^{-1}$ for the absorbance of the hypochlorite ion at 293 nm was used. The final hypochlorite stock

solution concentration was determined to be 0.391 M. Hypochlorite working solutions were prepared through the serial dilution of the hypochlorite stock solution, with 1:10, 1:100 and 1:1000 working solutions used in the hypochlorite reactions. For both the spiked water and spiked urine samples, a total of fifteen reaction samples were prepared respectively, with a final oxidant concentration range of 0.8 µM – 0.8 mM. Both sets of reactions were analysed in negative ionisation mode by MS2 Scan.

Pyridinium Chlorochromate

For the pyridinium chlorochromate reaction test in water, a 4.0 µM working solution of pyridinium chlorochromate was prepared. A total of fifteen reactions were prepared for this test, with a final in vitro oxidant concentration range of 0.4 – 2.0 µM. These samples were left to react at room temperature for a total of five hours prior to analysis by LC-MS/MS. Urine samples followed a similar preparation, with a total of fifteen samples prepared. The pyridinium chlorochromate working solution was prepared at 40 mM due to the possibility of competing reactions between pyridinium chlorochromate and the endogenous compounds present in urine. The final oxidant concentration range for the prepared urine samples was 4.0 – 20.0 mM. Analysis of the spiked water samples was undertaken in MS2 Scan mode, and the urine samples were analysed in negative ionisation mode by Selected Reaction Monitoring (SRM) for THC-COOH (*m/z* 343) and the PCC product (*m/z* 313).

Betadine

Testing of a Betadine working solution on the potential formation of reaction products with THC-COOH in spiked water and urine samples was also undertaken. Two Betadine working solutions were prepared through dilution of the neat Betadine (10% w/v povidone/iodine, equivalent to 1% w/v iodine) stock solution with water, providing 1:10 and 1:100 working solutions. A total of ten samples were prepared in water, and a further fifteen prepared in water. Analysis of the spiked water and spiked urine samples was undertaken in negative ionisation mode by MS2 Scan, with extracted ion chromatograms (EIC's) generated for the proposed products.

Comparison of Betadine Reaction with Methanolic Iodine Solution

Further experiments were undertaken to explore whether the exposure of THC-COOH to working solutions of iodine prepared in methanol would be equivalent to the exposure of THC-COOH to Betadine. The active component in Betadine is povidone-iodine, a complex between the water-soluble polymer polyvinylpyrrolidone (PVP) and elemental iodine. Inactive components include glycerine and C12-15 ethoxylated alcohols. Planned large-scale synthesis of reaction products formed between Betadine and THC-COOH, discussed in Chapter 3, required synthesis and isolation of relatively pure products. Presence of the inactive compounds and PVP in Betadine was considered to represent a serious source of contamination for the large-scale synthesis and purification of the desired reaction products. As such, additional experiments were planned to assess the effects of iodine solutions on THC-COOH, and whether reaction products formed would be the equivalent of those formed in the reaction between THC-COOH and Betadine.

Comparison of the Betadine reaction with the proposed iodine tincture reaction was carried out through preparation of a total of fifteen iodine reaction samples, which were subsequently compared with the successful Betadine reactions. The iodine stock solution was prepared through dissolving 50 mg of iodine in 10 mL of methanol. Two working solutions were prepared from the iodine stock solution through serial dilution, with dilution factors of 1:10 and 1:100. The iodine reaction mixtures were analysed by Selected Reaction Monitoring in negative ionisation mode, with THC-COOH (*m/z* 343), the proposed mono-iodo-THC-COOH products (*m/z* 469) and proposed di-iodo-THC-COOH product (*m/z* 595) targeted.

2.1.4.3 – Further Studies into Detection of Oxidation Products

Exposure of Papain to THC-COOH

Eight samples were prepared to examine the effect of papain on the detection of THC-COOH in spiked water samples. The final THC-COOH concentration in the THC-COOH standard and reaction samples was 10 µg/mL, and all samples were made up to a total volume of 500 µL with MilliQ water. Papain was obtained from Sigma Aldrich as a crude powder, and was reported to contain 1.5 – 10.0 units per milligram papain. A papain working solution as prepared by dissolving 5 mg of the crude papain powder in 10 mL of ultrapure water, yielding a solution containing 0.75 – 5.0 units/mL. **Table 2.8** lists the samples prepared for this study.

Following preparation, samples were allowed to react at room temperature for three hours. Prior to analysis of the samples, they were transferred to centrifuge vials and centrifuged at 8000 rpm for 15 minutes. A 250 µL aliquot of the supernatant was collected and transferred to clean amber-coloured glass GC vials prior to analysis. Analyses were undertaken on the Perkin Elmer Sciex API 365 LC-MS/MS system in negative ionisation mode, with a total method runtime of 13 minutes. Sample injection volume was set at 10 µL, with injections done in triplicate. Following analysis, samples were sealed and refrigerated at 4°C.

Table 2.8 - Samples prepared for exposure of THC-COOH to papain.

Sample	THC-COOH Stock (250 µg/mL)	Papain Working Solution (µL)	Water (µL)	Papain Concentration (units/mL)
Water Blank	0	0	500	0
Papain Blank 1	0	25	475	0.375 - 0.250
Papain Blank 2	0	50	450	0.075 - 0.500
Papain Blank 3	0	100	400	0.150 - 1.000
THC-COOH Standard	20	0	480	0
Papain Reaction 1	20	25	455	0.375 - 0.250
Papain Reaction 2	20	50	430	0.075 - 0.500
Papain Reaction 3	20	100	380	0.150 - 1.000

Analysis of the effects of papain on detection of THC-COOH was achieved through comparison of the chromatograms of the THC-COOH standard with the reagent blanks and reaction mixtures. Assessment of the effect of papain on detection of THC-COOH was achieved through identification of the THC-COOH peak by mass spectrometry, integrating the peak area and identifying if exposure to papain lead to a noticeable decrease in THC-COOH peak area. Chromatograms obtained from the papain reaction mixtures were visually assessed in order to determine the potential formation of novel peaks not present in the THC-COOH standard and reagent blanks had formed.

Exposure of Selected Additional Oxidising Adulterants to THC-COOH

Water and urine samples were spiked with a known quantity of THC-COOH. For reaction mixtures, an aliquot of the respective oxidising agents of known concentration were spiked into the sample. Due to issues with oxidant solubility, and formation of an insoluble precipitate in the potassium permanganate reaction, samples were subjected to the following clean-up procedure:

1. Following addition of oxidising agent, samples sealed and vortexed for thirty seconds;
2. Samples left to react for two hours;
3. For urine samples, a 100µL aliquot of acetonitrile was added in order to precipitate proteins from urine. Spiked water samples the same volume of acetonitrile was added for consistency;
4. Samples refrigerated for at least one hour to aid in the precipitation of proteins;
5. Centrifuging at 8000 rpm for 15 minutes;
6. Supernatant from samples collected, and filtered through 0.2µm MilliPore syringe filters;
7. Filtered extract transferred to new vial and sealed prior to analysis.

To monitor the effects of the selected oxidants on THC-COOH in aqueous environments, a series of samples were prepared and monitored by LC-MS. Two blank matrix samples (water and urine) were prepared, as were standards of THC-COOH (5000 ng/mL). Reagent blanks were prepared for all the oxidants, with the 0.01 M working solution pipetted into the sample matrix, giving a total volume of 400 µL. Final oxidant concentration in the reagent blanks was 2.25 mM.

Oxidant stock solutions (0.1 M) were prepared by dissolving the respective oxidants in ultrapure water. Two working oxidant solutions were prepared for each adulterant (0.01 M, 0.001 M) by serial dilution. THC-COOH was spiked into water, with a final concentration of 5000 ng/mL in the final 400 µL sample. A parallel sample set was created for the oxidising agents with the samples replicated in blank urine. Following analysis, samples were refrigerated at 4°C for storage. **Table 2.9** shows the reaction mixtures prepared for this study. Sets were prepared for the five selected oxidising adulterants, with a parallel sample set prepared in blank urine. Samples were prepared in triplicate for each reaction condition and matrix.

Table 2.9 -General guideline used for the preparation of reaction mixtures for the selected oxidising adulterants.

Sample Identifier	THC-COOH Stock (100 µg/mL) (µL)	Oxidising Agent (1 mM) (µL)	Oxidising Agent (10 mM) (µL)	Matrix (µL)	Final Oxidant Concentration (mM)
Reaction 1	20	20	0	360	0.050
Reaction 2	20	50	0	330	0.125
Reaction 3	20	100	0	280	0.250
Reaction 4	20	0	20	360	0.500
Reaction 5	20	0	50	330	1.250
Reaction 6	20	0	100	280	2.500

2.2 – Results and Discussion

2.2.1 – Initial Study

Detection of THC-COOH

Control samples of THC-COOH spiked into water were analysed by LC-MS to establish that this starting material would be detected by the method used, and that expected product ions of THC-COOH would be detected in negative ionisation mode ($[M-H]^-$ at *m/z* 343). In comparison to water and methanol solvent blanks, a single major peak was detected at 33.43 minutes. **Figures 2.2 and 2.3** provide the pertinent section of the THC-COOH standard and the mass spectrum generated for the THC-COOH peak at 33.43 minutes. Three main fragmentation patterns were detected for the peak at 33.43 minutes: *m/z* 299.4, 311.2 and 325.2. An additional ion (*m/z* 365.2) was detected, corresponding to a sodium adduct of THC-COOH.

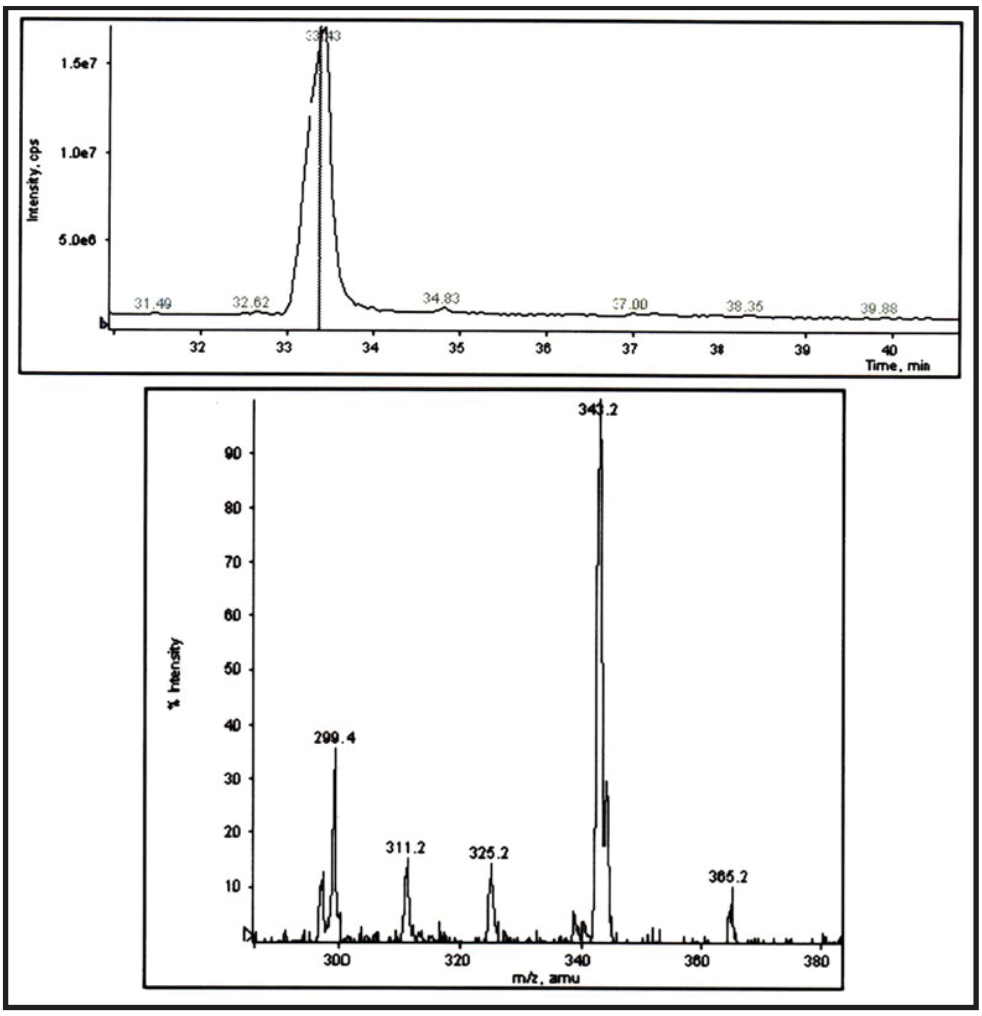


Figure 2.2 - Chromatogram and mass spectra obtained for THC-COOH standard at 2 µg/mL. Deprotonated molecule for THC-COOH is present at *m/z* 343.2.

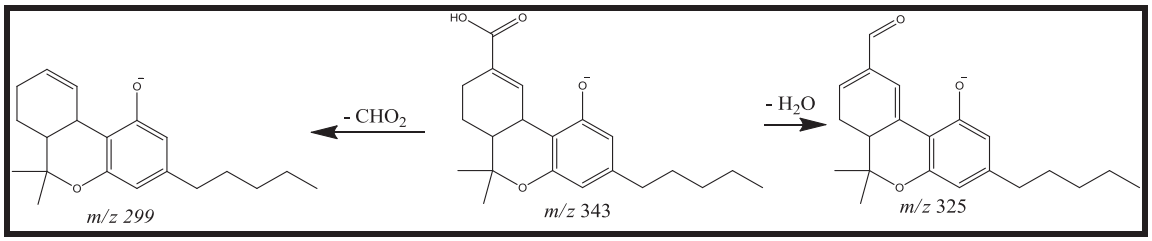


Figure 2.3 – Proposed fragmentation pattern for THC-COOH

Exposure of THC-COOH to Sodium Hypochlorite

Samples of THC-COOH were prepared in water and spiked with a 0.54 M solution of sodium hypochlorite. Analyses were undertaken to assess whether exposure of THC-COOH to sodium hypochlorite would result in a relative loss of THC-COOH from the sample, and whether the reaction condition trialled would result in the formation of oxidation products.

Comparison of the hypochlorite reaction mixture with the THC-COOH standard and reagent blanks indicated that three additional peaks were detected, with deprotonated molecules $[M-H]^-$ at *m/z* 377.0, 377.0 and 411.0 respectively. Both peaks detected at 34.83 and 36.27 minutes with deprotonated molecules at *m/z* 377.0 indicated the presence of chlorine atoms in the respective molecules, with the characteristic isotope profiles for the deprotonated molecules of the mono-chlorinated products (3:1, $^{35}Cl/^{37}Cl$) and the di-chlorinated product (9:6:1 - $^{35}Cl/^{35}Cl$, $^{35}Cl/^{37}Cl$, $^{37}Cl/^{37}Cl$), indicating chlorination of THC-COOH. These results strongly suggesting that the reaction with sodium hypochlorite results in the electrophilic aromatic substitution of the aromatic ring present in THC-COOH by chlorine.

The mass spectra of the three products detected in this reaction also contain two product ions in common with THC-COOH, at *m/z* 325.2 and 311.2, indicating that the THC-COOH structural base of these products remains intact. **Figure 2.4** shows the chromatogram indicating the presence of the three detected products relative to THC-COOH, and the mass spectra of the products formed in the reaction with sodium hypochlorite. These chromatograms show the effect of time on the formation of the detected reaction products. At T=0h, no apparent loss or degradation of THC-COOH is noted. Over the three remaining time periods, formation of the three reaction products is apparent, even after storage of the sample for twelve days at 4°C. Mass spectra of these analytes are shown in **Figure 2.5.** Proposed fragmentation patterns for the products detected in the reaction between THC-COOH spiked water and sodium hypochlorite are found in **Figure 2.11**.

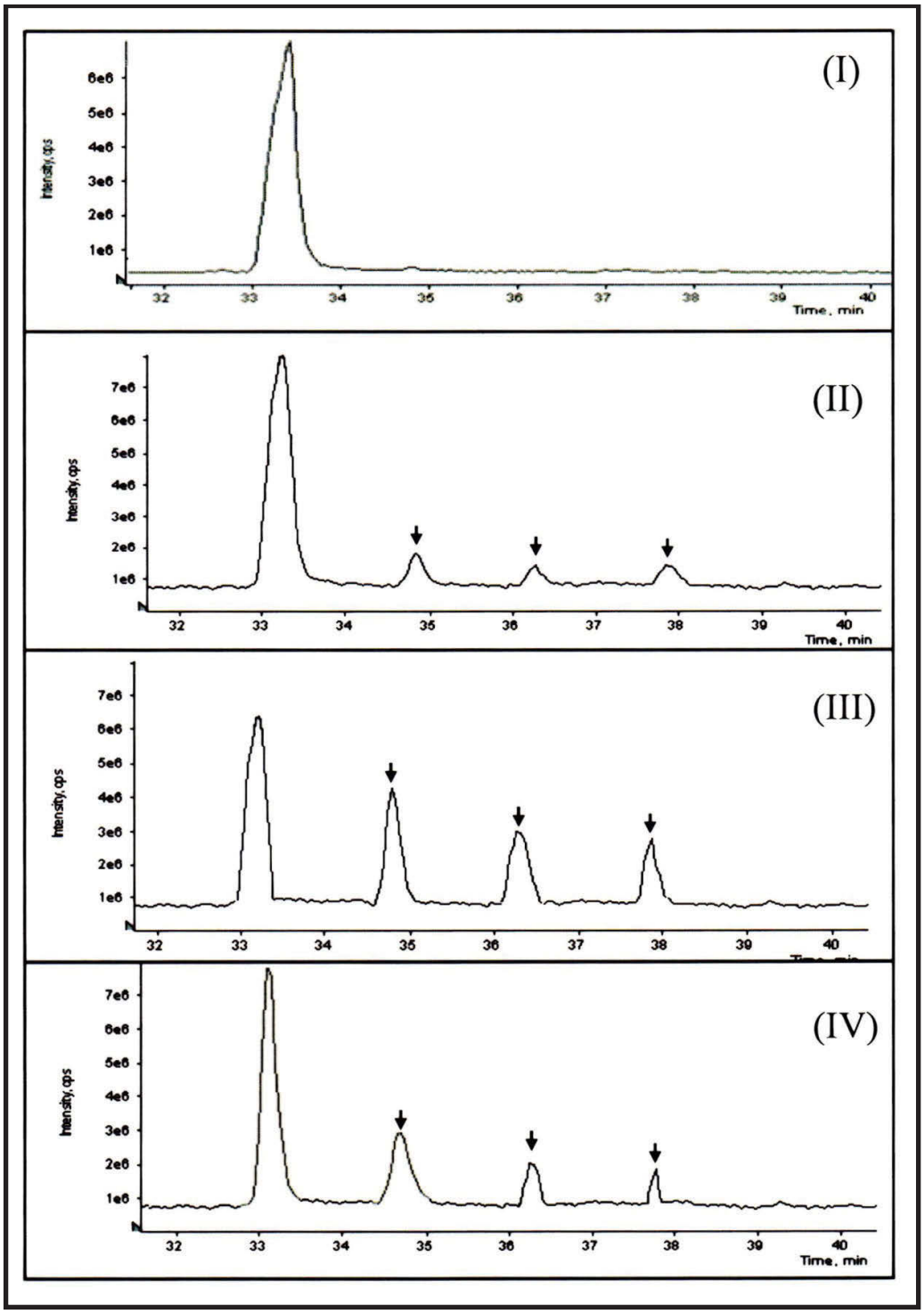


Figure 2.4 - Comparison of chromatograms obtains from analysis of the effect of sodium hypochlorite solution on the detection of THC-COOH. Chromatograms were generated: (I) Immediately after sample preparation; (II) One hour after sample preparation; (III) Five hours after sample preparation; (IV) Twelve days after sample preparation. Peaks indicating presence of possible reaction products marked with arrows.

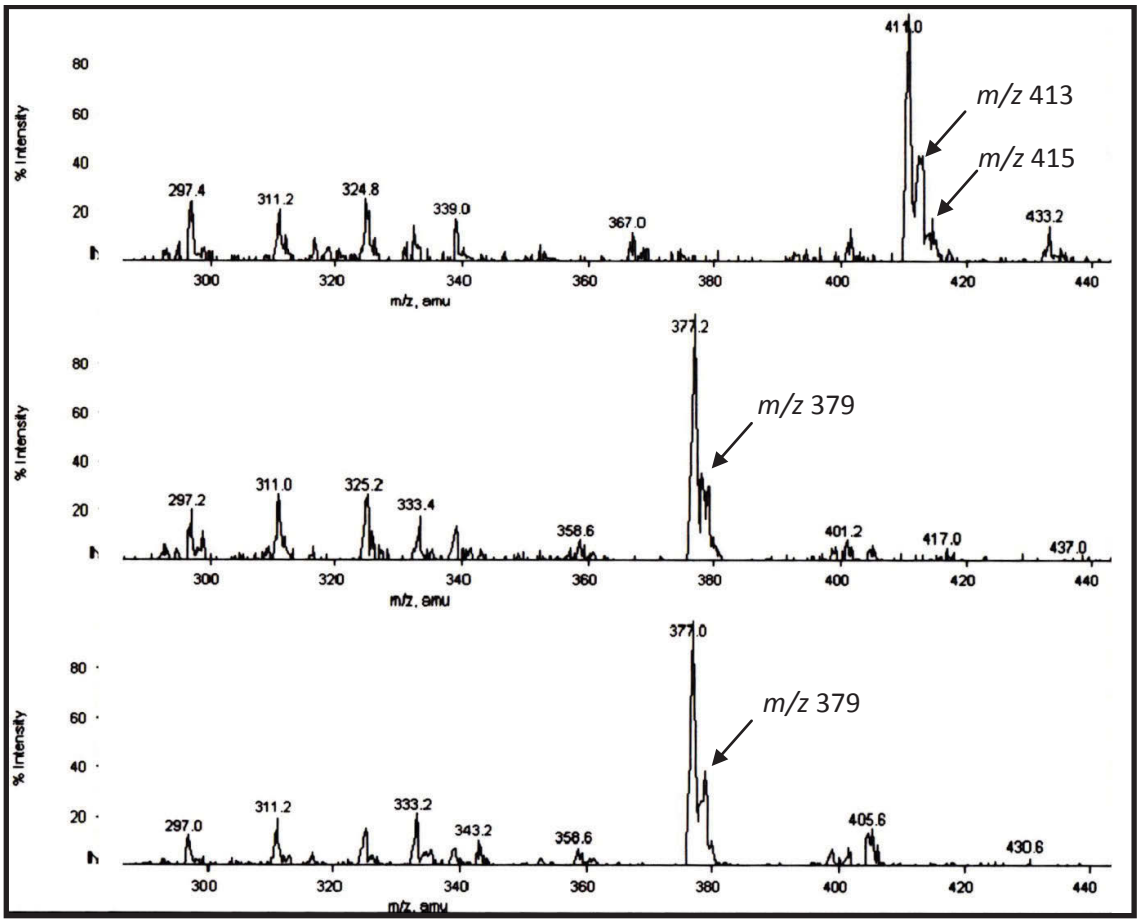


Figure 2.5 - Mass spectra of reaction product peaks from reaction between THC-COOH and sodium hypochlorite solution. Mass spectra of: (Top) Peak at 34.83 minutes ($[M-H]^-$ *m/z* 377); (Middle) Peak at 36.27 minutes ($[M-H]^-$ *m/z* 377); (Bottom) Peak at 37.85 minutes ($[M-H]^-$ *m/z* 411). Note the deprotonated molecules showing the characteristic 9:6:1 and 3:1 isotope ratios in the mass spectra, which are indicative of chlorination of THC-COOH. (Top) includes the 9:6:1 ($^{35}Cl/^{35}Cl, ^{35}Cl/^{37}Cl, ^{37}Cl/^{37}Cl$) isotope ratio expected for a dichlorinated molecule. (Middle) and (Bottom) includes deprotonated molecules for the mono-chlorinated products.

Exposure of THC-COOH to Potassium Nitrite

Exposure of THC-COOH to potassium nitrite was carried out in water acidified with hydrochloric acid, as research by Lewis et. al. (1999) indicated that in a neutral or basic reaction environment, the proposed reaction would occur slowly, if at all. In Lewis' research, a nitrosylated derivative of THC-COOH was detected through HPLC-tandem mass spectrometry (HPLC-MS/MS).

Though this nitrosylated derivative was found to be unstable, degrading over a period of hours in solution, the following reaction under acidic conditions was proposed:

I. $KNO_2 + H_2O \leftrightarrow HNO_2 + KOH$

II. $2HNO_2 \leftrightarrow N_2O_3 + H_2O$

III. $THC\text{-}COOH + N_2O_3 \leftrightarrow NO\text{-}THC\text{-}COOH + HNO_2$

Samples of THC-COOH were prepared in acidified water (pH 5.0) and spiked with 0.95 M potassium nitrite. As with the analysis of the proposed reaction between sodium hypochlorite and THC-COOH, the goal of this analysis was to assess whether exposure to potassium nitrite under acidic conditions would lead to a relative loss of THC-COOH from the sample, and whether the condition tested would result in the formation of oxidation products.

A comparison of the potassium nitrite reaction mixture with the THC-COOH standard and reagent blanks indicated the presence of two additional peaks following reaction of the sample, at 34.61 and 35.60 minutes. Deprotonated molecules for these peaks were $[M\text{-}H]^-$ *m/*z 372 and *m/*z 388, respectively. The deprotonated molecule at *m/*z 372 strongly corresponds to the nitrosylated derivative of THC-COOH detected by Lewis et. al. The deprotonated molecule at *m/*z 388 also suggests the formation of a nitro-derivative of THC-COOH in this reaction.

Two significant product ions were present in the mass spectrum of the *m/*z 372 deprotonated molecule, at *m/*z 328 and *m/*z 313. For the *m/*z 388 deprotonated molecule, a total of three significant product ions were detected at *m/*z 372, 325 and 265. For both products, each has a product ion that supports the formation of the nitroso- and nitro- derivatives of THC-COOH. For the nitroso product, the product ion detected at *m/*z 328 suggests that fragmentation of the deprotonated molecule has occurred through the loss of the formic acid. Similarly, the *m/*z 372 product ion suggests loss of the phenolic OH group from the aromatic ring. These results and the fragmentation patterns from the products of this reaction indicate that these products form through the electrophilic aromatic substitution of the aromatic ring present in THC-COOH. **Figure 2.6** shows the chromatogram indicating the presence of the two detected products relative to THC-COOH, and the mass spectra of the products formed in the reaction with potassium nitrite. Proposed fragmentation patterns for these compounds are found in **Figure 2.13**.

The effect of potassium nitrite on the detection of THC-COOH and the formation of the two detected reaction products over time was also assessed. Four time points were selected in this initial study: T=0h (immediately after preparation), T=1h (one hour after preparation), T=5h (five hours after preparation) and T=12d (twelve days after preparation). **Figure 2.6** reveals that, over the first three time points, both product peaks are present. At T=12d, however, the

peak corresponding to the nitrosylated product is no longer visible. In addition, at T=12d, the loss of the nitrosylated product coincides with an increase in the relative peak area of the nitro-derivative of THC-COOH. This suggests that the nitrosylated product may undergo hydrolysis or further oxidation, leading to the formation of the nitro-derivative of THC-COOH. In this scenario, the nitrosylated THC-COOH may represent a chemical intermediate between THC-COOH and the nitro-THC-COOH derivative.

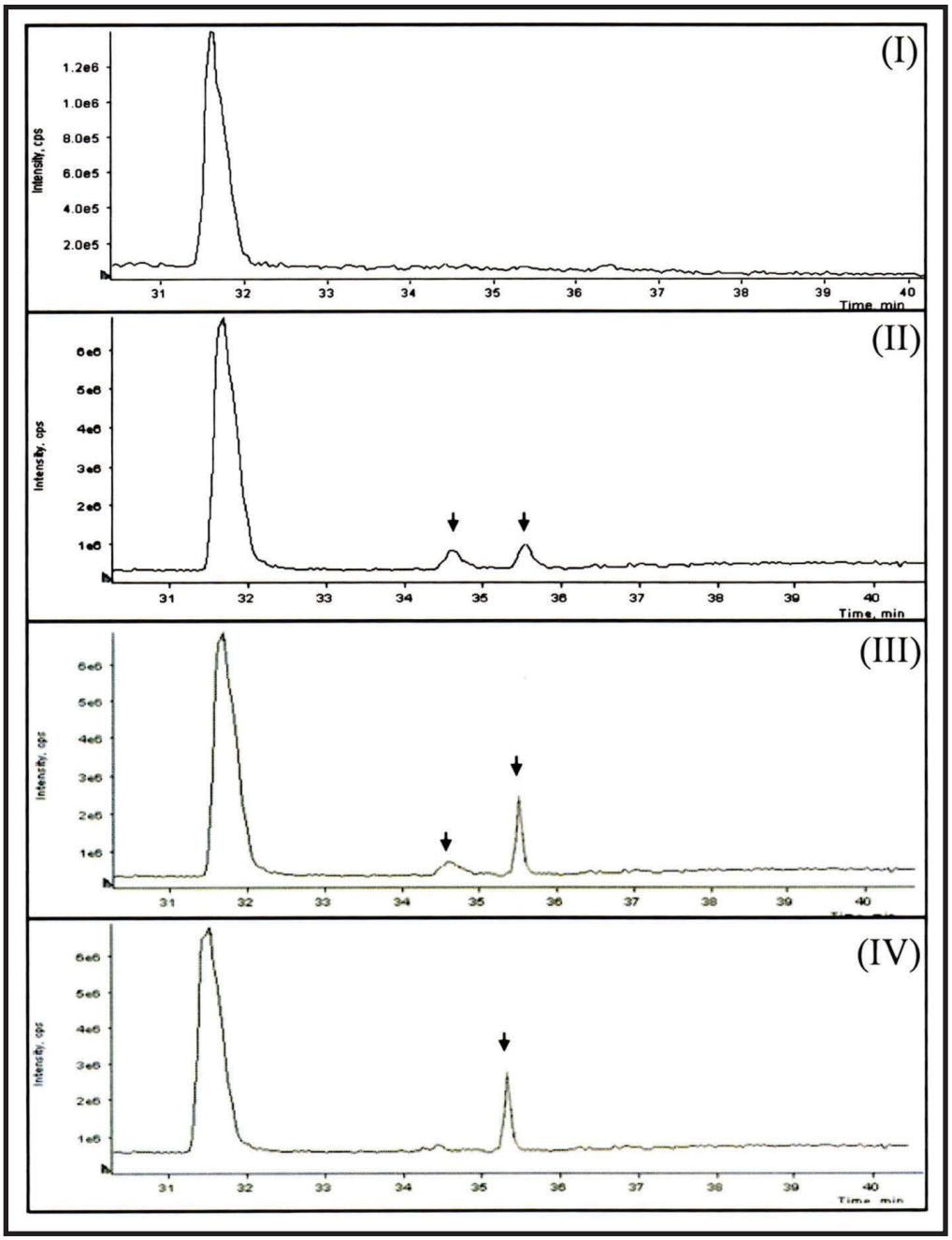


Figure 2.6 - Comparison of chromatograms obtains from analysis of the effect of acidified potassium nitrite solution on the detection of THC-COOH. Chromatograms were generated: (I) Immediately after sample preparation; (II) One hour after sample preparation; (III) Five hours after sample preparation; (IV) Twelve days after sample preparation. Peaks indicating presence of possible reaction products marked with arrows.

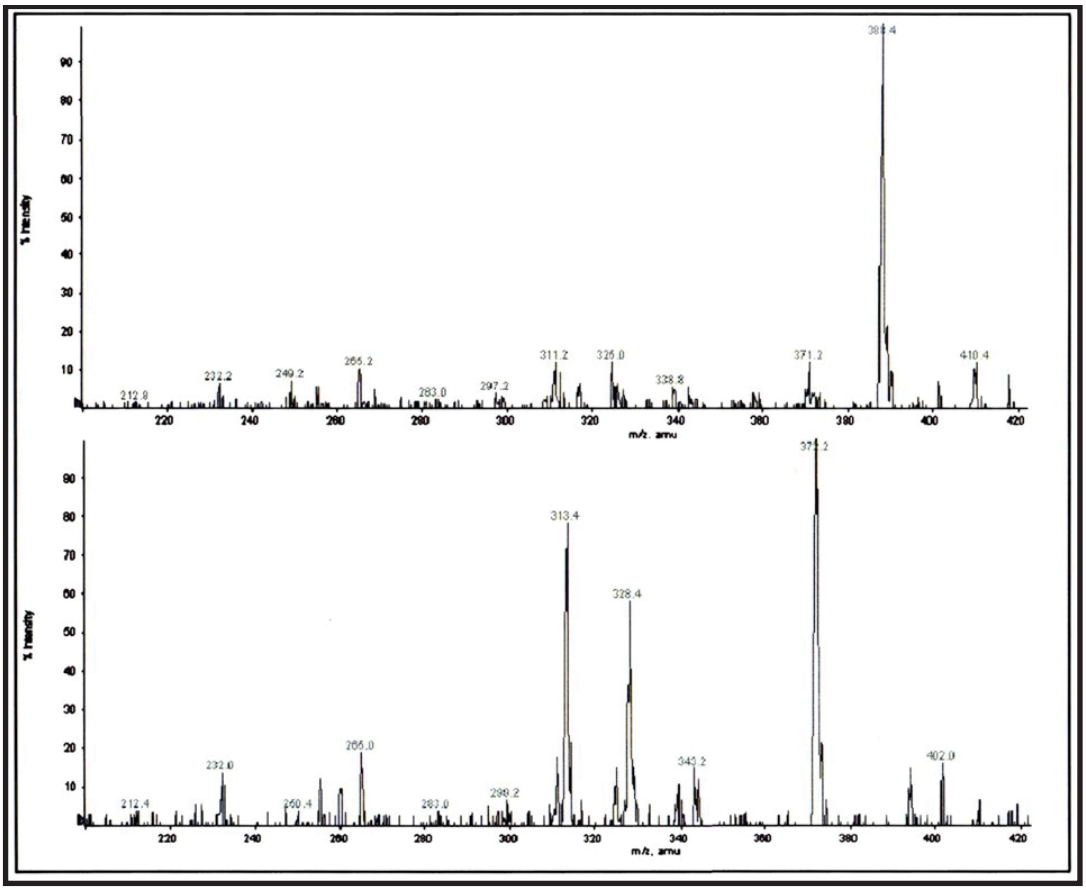


Figure 2.7 - Mass spectra of reaction product peaks from the reaction between THC-COOH and acidified potassium nitrite solution recorded in negative ion mode. Mass spectra of: (Top) Peak at 34.61 minutes ($[M-H]^-$ *m/z* 388); (Bottom) Peak at 35.46 minutes ($[M-H]^-$ *m/z* 372)

Exposure of THC-COOH to Pyridinium Chlorochromate

Pyridinium chlorochromate was added to water samples spiked with THC-COOH. Analyses were undertaken to assess whether exposure of THC-COOH to pyridinium chlorochromate would result in a relative loss of THC-COOH from the sample, and whether the reaction condition trialled would result in the formation of oxidation products.

A comparison of the chromatograms of pyridinium chlorochromate reaction mixture with the THC-COOH standard and reagent blanks indicated the presence of a new peak at 34.70 minutes (**Figure 2.8**). The deprotonated molecule of this peak was $[M-H]^-$ *m/z* 313 (**Figure 2.9**), with poor fragmentation of this deprotonated molecule under these instrumental conditions. Retention time of this analyte was noted to vary, and may be attributed to issues regarding equilibration of the LC system.

Based on **Figure 2.9**, it is important to note that the compound formed in the reaction between THC-COOH and pyridinium chlorochromate has a lower mass than the starting material. It is expected that this loss of mass is a result of the oxidising adulterant in question reacting with THC-COOH, and as a result, causing cleavage of chemical bonds to remove a section of the molecule. This is further examined in **Chapter 4**.

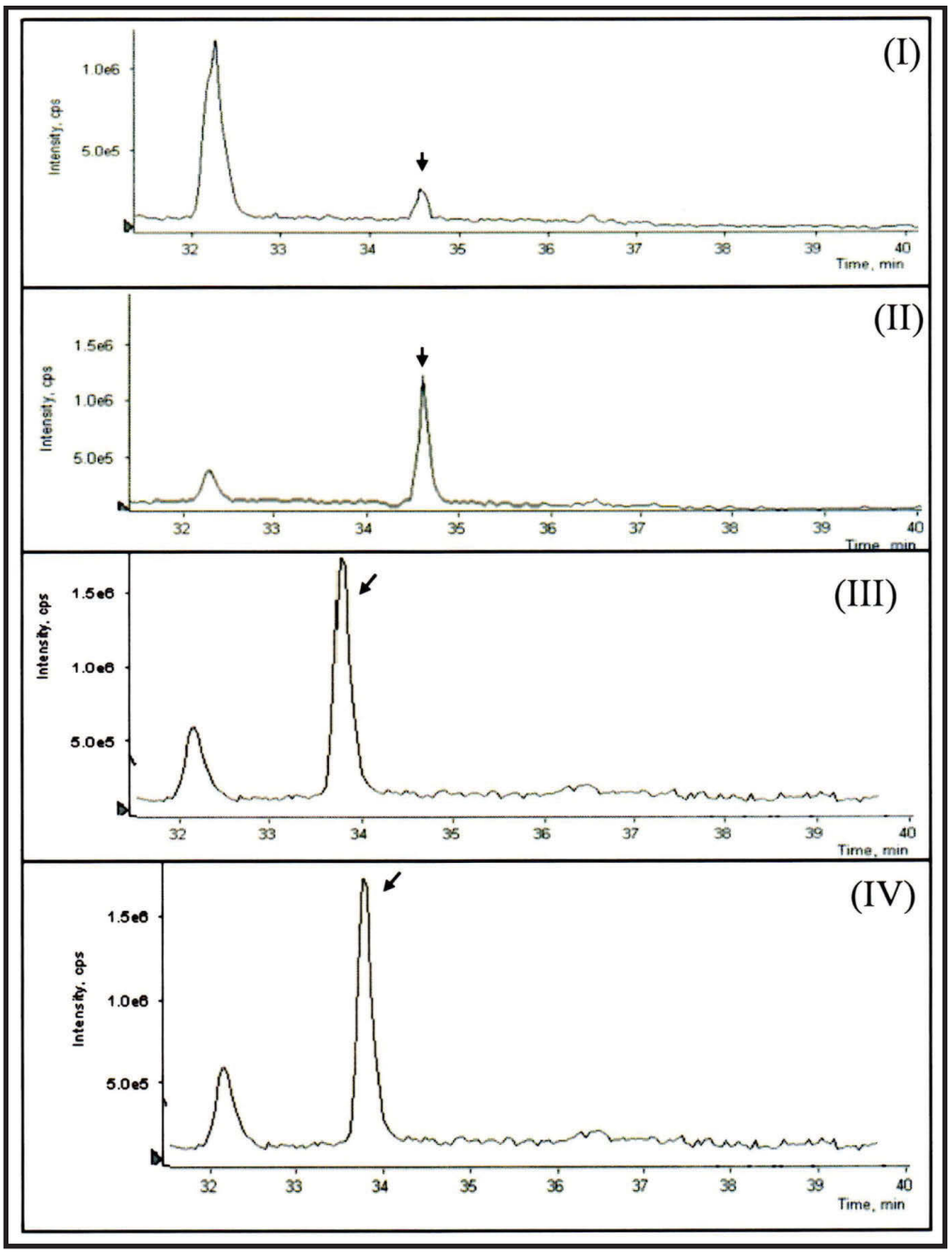


Figure 2.8 - Comparison of chromatograms obtains from analysis of the effect of pyridinium chlorochromate solution on the detection of THC-COOH. Chromatograms were generated: (I) Immediately after sample preparation; (II) One hour after sample preparation; (III) Five hours after sample preparation; (IV) Twelve days after sample preparation. Peaks indicating presence of possible reaction products marked with arrows.

Changes in analyte position are due to equilibration of the LC system between injections.

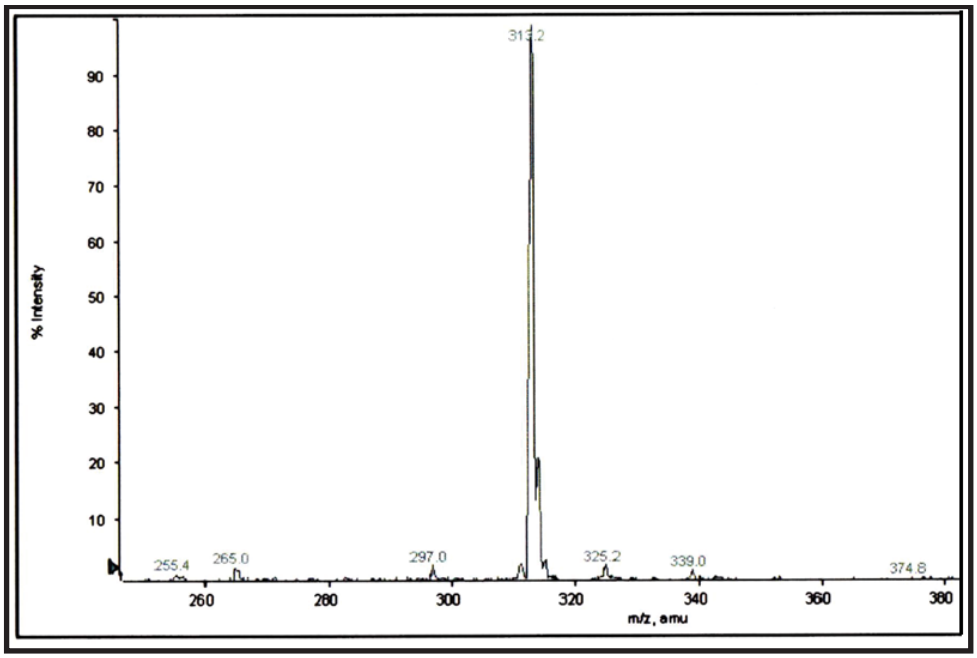


Figure 2.9 - Mass spectrum of reaction product detected in reaction between THC-COOH and pyridinium chlorochromate. Peak detected at 34.70 minutes ($[M-H]^-$ *m/z* 313).

Detected Reaction Products

In this initial study, a total of six reaction products were detected through the in vitro adulteration of water samples spiked with THC-COOH. Of these six products, five were found to be stable over a period of twelve days when stored at 4°C. Mass fragmentation data was able to provide a tentative structural elucidation of five of the products detected. Under the tested experiment conditions, the detected PCC product did not undergo significant fragmentation. **Table 2.10** provides a summary of the data obtained from the mass spectra of the products detected in the reactions carried out.

Table 2.10 - Summary of reaction products detected during initial study of oxidising adulterants. Retention times, deprotonated molecules and major product ions shown.

Sample	Peak (Minutes)	Deprotonated Molecule $[M-H]^-$ (*m/z*)	Product Ions Detected (*m/z*)
Hypochlorite Reaction	34.88	377	333, 311, 297
	36.32	377	333, 311, 265
	37.88	411	367, 297, 265
Acidified Potassium Nitrite Reaction	34.61	372	328, 313
	35.60	388	372, 325, 265
Pyridinium Chlorochromate Reaction	34.70	313	-

Products formed in the reaction between THC-COOH and sodium hypochlorite appear to have formed through the electrophilic aromatic substitution of chlorine on to the aromatic ring present in THC-COOH. **Figure 2.10** provides tentative structures proposed for these products. For the two proposed mono-chlorinated products, it should be noted that the exact substitution position cannot be definitively assigned without NMR spectroscopy. **Figure 2.10** shows the proposed fragmentation of the *m/z* 373 deprotonated molecule to the *m/z* 333 product ion, and the proposed fragmentation of the *m/z* 411 deprotonated molecule to the *m/z* 367 product ion.

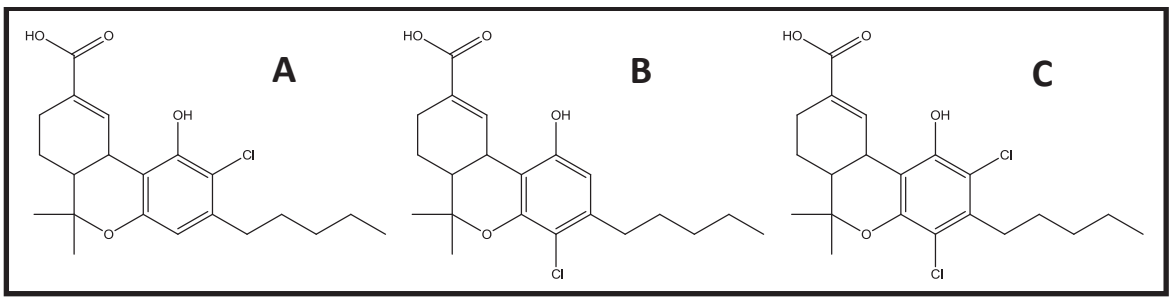


Figure 2.10 – Tentative structures of reaction products formed following exposure of THC-COOH in spiked water samples to sodium hypochlorite solution. (Left) and (Middle) mono-chlorinated species with $[M-H]^-$ *m/z* 377; (Right) di-chlorinated species with $[M-H]^-$ *m/z* 411.

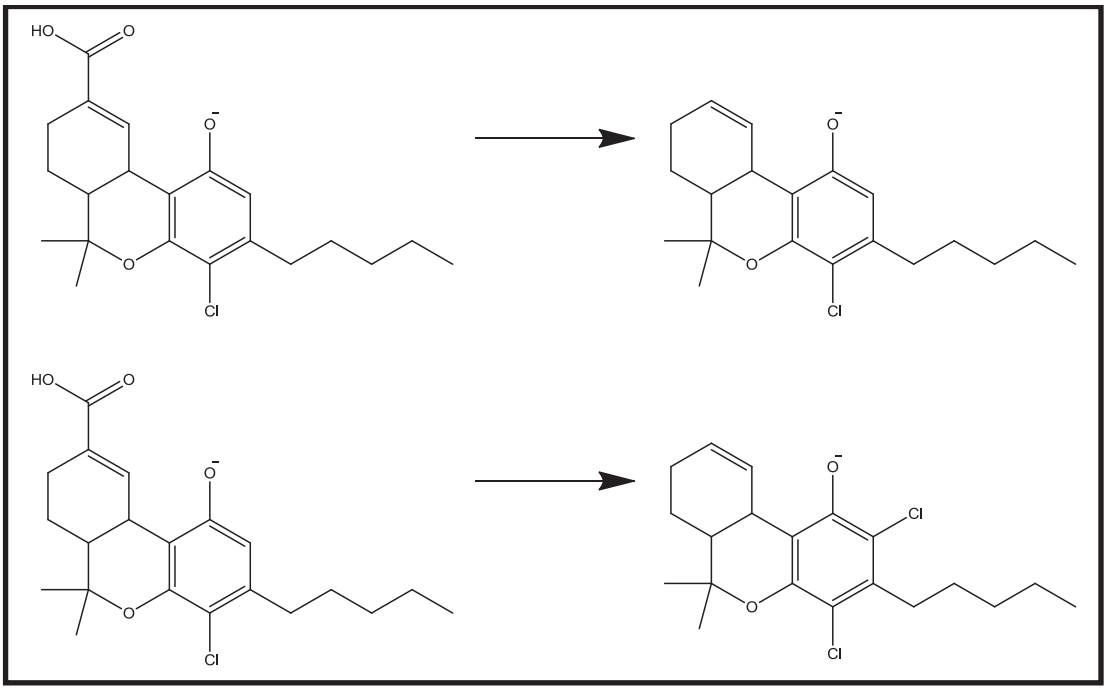


Figure 2.11 - Proposed fragmentation pathway of mono-chlorinated and di-chlorinated THC-COOH to form product ions at *m/z* 333 and *m/z* 367, respectively.

The observed reaction between THC-COOH and potassium nitrite under acidic conditions yielded two products. The product detected at 34.61 minutes appears to have formed through the electrophilic aromatic substitution of the aromatic ring, forming an unstable nitroso-THC-COOH complex. This unstable product appears to further react to form a stable, nitro-derivative of THC-COOH. As with the hypochlorite reaction, due to limited data, the exact substitution pattern for the aromatic ring present in THC-COOH cannot be assigned. **Figure 2.12** shows the proposed reaction products for this reaction, and **Figure 2.13** shows the proposed fragmentation of the parent molecule to provide the major product ion produced from the proposed products.

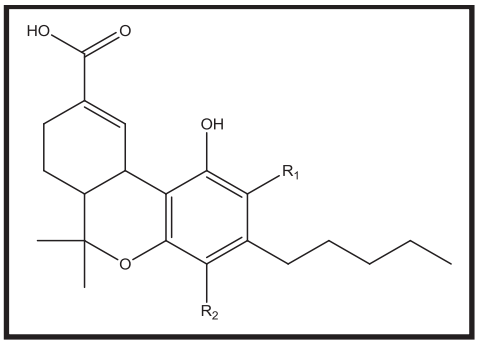


Figure 2.12 - Proposed reaction products for nitrite reaction.
Nitroso reaction product: R_1 = NO and R_2 - H, or R_1 = H and R_2 = NO.
Nitro product of THC-COOH, where R_1 = NO_2 and R_2 = H, or R_1 = H and R_2= NO_2.

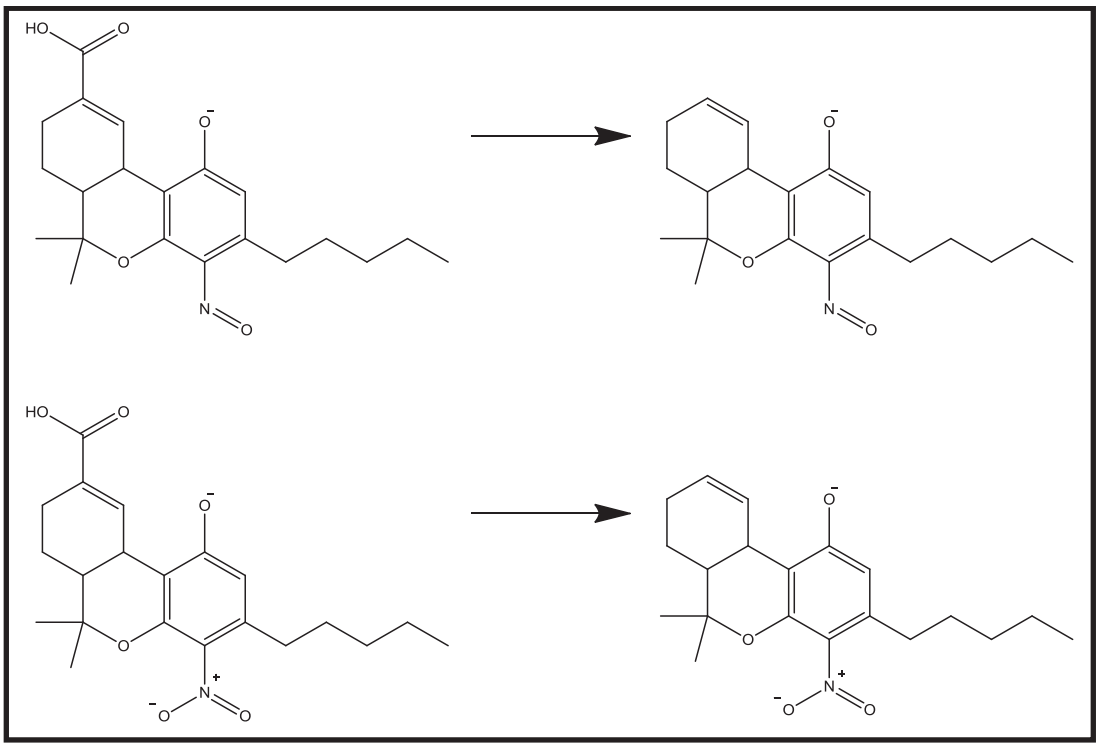


Figure 2.13 – Proposed fragmentation pathway of nitrosylated THC-COOH and nitro-THC-COOH to form ions at *m/z* 328 and *m/z* 372, respectively.

Liquid-Liquid Extraction

Definitive structural elucidation of the products formed in the reaction between THC-COOH and the selected oxidising adulterants may be achieved through Nuclear Magnetic Resonance (NMR) Spectroscopy. For appropriate signal-to-noise (S/N) ratios and generation of suitable spectra, significant quantities of the reaction products are required. The required amount of reaction products required for effective NMR analysis varies in relation to model/strength of the NMR Spectrometer employed, as well as the analyses undertaken. As a result, a minimum of 1 mg of the reaction products is required, and furthermore, need to be produced with relatively high purity. Liquid-liquid extraction (LLE) provides a possible method by which contaminants present in the reaction mixtures, including remaining traces of oxidising adulterants, can be removed from the sample.

THC-COOH contains two acidic functional groups: a carboxylic acid group and a phenolic group. Both functional groups are capable of losing a proton under basic conditions to form salts that are more likely to partition into an aqueous phase. Oxidation products containing similar functional groups will therefore be found in the same phase as would be expected for the THC metabolite. Shown previously, **Figure 2.1** shows the workflow proposed for use in LLE, where the reaction mixture initially undergoes basification, with subsequent acidification. **Table 2.11** displays the results of the attempt to separate the detected reaction products through LLE. Attempted extraction of the nitroso-THC-COOH product was not attempted due to its unstable nature.

Table 2.11 - General findings from trial of liquid-liquid extraction of reaction products detected during initial study.

Compound/s	Compound/s Present in Fraction?	
	Organic Fraction 1	Organic Fraction 2
Mono-chlorinated Products	No	Yes
Di-chlorinated Product	No	Yes
Nitro-THC-COOH	No	Yes
PCC Product	Yes	Yes

Based on the preliminary structural elucidation assigned to the products detected in the hypochlorite and nitrite reactions, it was expected that they contain the carboxylic acid and phenolic functional groups. Detection of these compounds in Organic Fraction 2 suggests these

products are acidic, and provides evidence that these compounds likely contain the aforementioned acidic functional groups. For the PCC reaction product, detection in both organic fractions may indicate that this compound is neutral, or that it displays poor solubility in the aqueous fraction.

2.2.2 – Main Study

Exposure of THC-COOH to Sodium Hypochlorite

Spiked samples of THC-COOH were prepared in water and urine, with a final THC-COOH concentration of 2 µg/mL. These samples were spiked with the three prepared hypochlorite working solutions and allowed to react for a period of five hours. Following sample clean-up of the spiked urine samples, these were analysed by LC-MS/MS in MS2 Scan Mode. These analyses were undertaken to confirm the results obtained in the initial study involving exposure of THC-COOH to sodium hypochlorite, and to confirm that the detected reaction products would form in spiked urine samples. **Figure 2.14** shows the chromatogram and EIC obtained for the hypochlorite reaction carried out in water, with the mass spectra of the respective peaks shown in **Figure 2.15**.

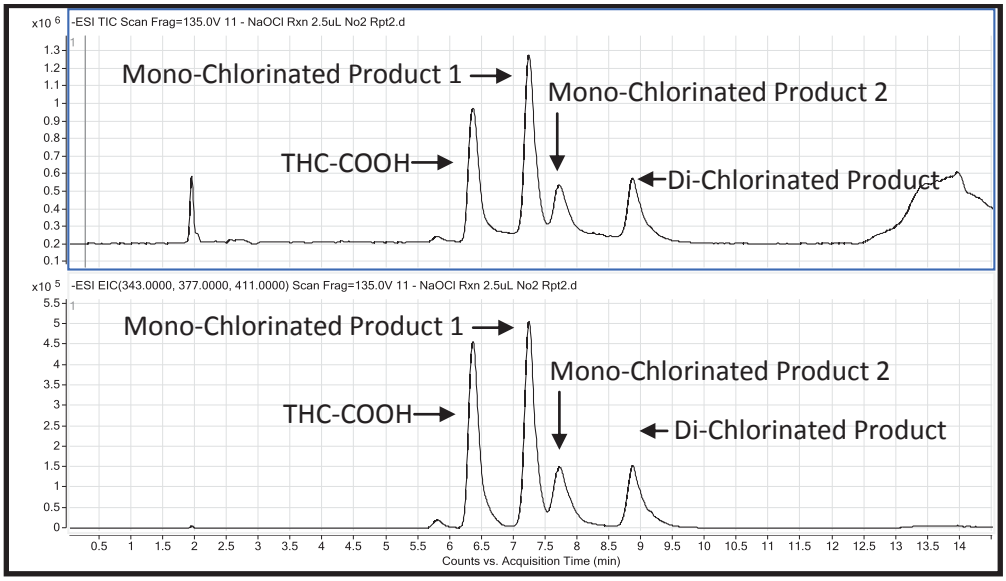


Figure 2.14 – (Top) Chromatogram and (Bottom) EIC of hypochlorite reaction in water. Final hypochlorite concentration is 0.02 mM. THC-COOH ($[M-H]^-$ *m/z* 343) elutes at 6.36 minutes; first mono-chloro product ($[M-H]^-$ *m/z* 377) elutes at 7.23 minutes; second mono-chloro product ($[M-H]^-$ *m/z* 377) elutes at 7.72 minutes; and the di-chloro product ($[M-H]^-$ *m/z* 411) elutes at 8.86 minutes).

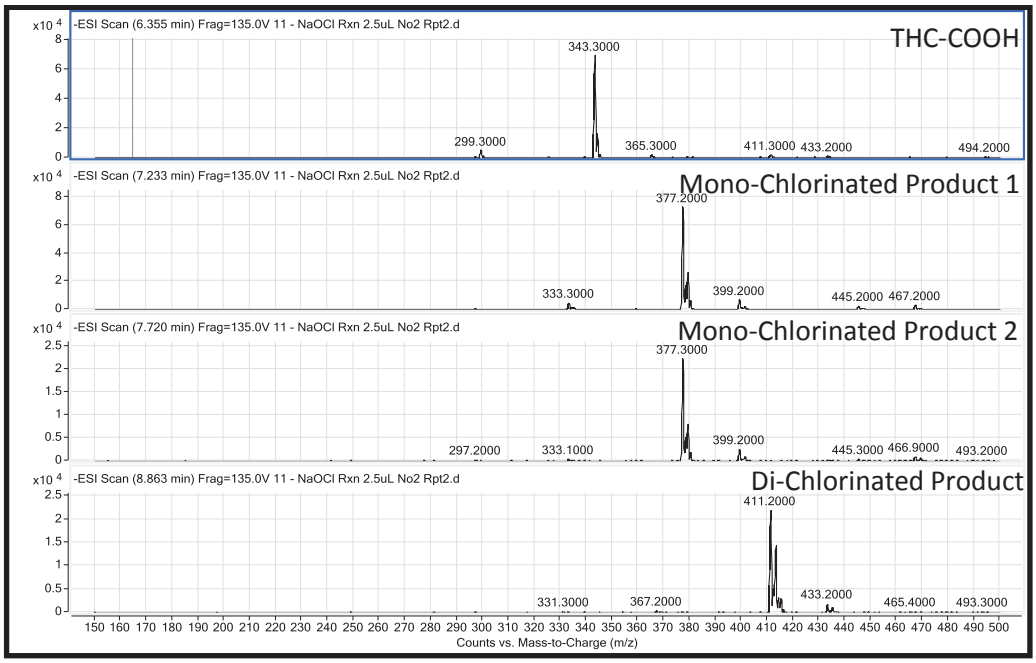


Figure 2.15 - Mass spectra (*m/z* 150 - 500) of targeted analytes for hypochlorite reaction in urine (final hypochlorite concentration 0.02 mM): THC-COOH at 6.36 minutes; mono-chloro product 1 at 7.23 minutes; mono-chloro product 2 at 7.72 minutes; and di-chloro product at 8.86 minutes. Note that as in the initial hypochlorite study, the mass spectra show deprotonated molecules with peaks characteristic of chlorination of THC-COOH.

The results obtained for the sodium hypochlorite reaction reveals a total of four peaks, and bears a strong similarity to the results obtained in the initial study (**Figure 2.3**), with the formation of the same products, with two potential mono-chloro-THC-COOH species produced alongside a di-chloro-THC-COOH compound. In **Figure 2.16**, the chromatogram obtained for the reaction in urine is shown. It is important to note that due to the elution condition employed in this portion of the study, poor resolution between THC-COOH and the first proposed mono-chlorinated product is found. Despite co-elution of the THC-COOH and the first product, mass spectra can be obtained for both compounds (**Figure 2.17**).

Comparison of **Figure 2.15** and **Figure 2.17** reveals that the proposed products form both in spiked water and spiked urine samples, as evidenced by the similarities in the mass spectra. Of particular note is the presence of isotope peaks for chlorine, with the expected 3:1 $^{35}Cl/^{37}Cl$ isotope ratio for a molecule containing a single chlorine atom. Similarly, for the di-chlorinated product, three peaks are present, and with a 9:6:1 ratio of peaks for the deprotonated molecules is typical of a dichlorinated compound. As with the initial study of the hypochlorite reaction undertaken previously, the ratio of the isotope peaks is consistent with the presence of chlorine atoms in the proposed products.

The required concentration of hypochlorite required for the reaction in urine can be seen to be significantly higher than the equivalent reaction in water. In both reaction sets, the final concentration of THC-COOH was set at 2 µg/mL. The required final concentration of hypochlorite in the shown spiked water sample was 0.02 mM, whilst the final concentration in the spiked urine sample was 3.91 mM. It is expected that the oxidant undergoes extensive reactions with the endogenous compounds present in urine, hence the significantly higher hypochlorite concentration required in the spiked urine samples.

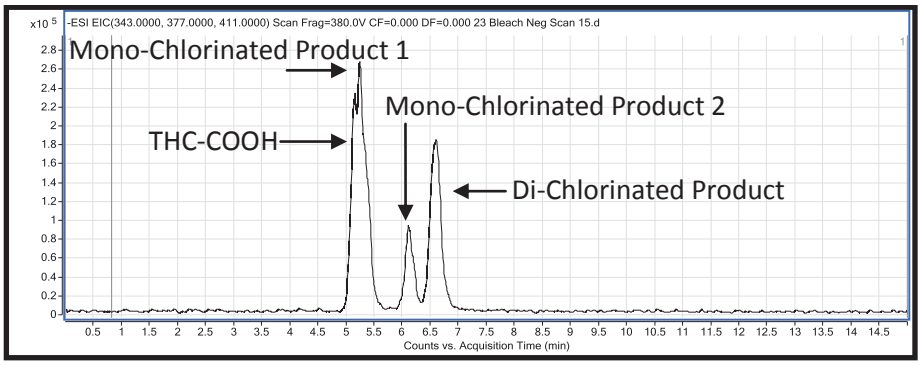


Figure 2.16 - EIC of hypochlorite reaction in urine. Final hypochlorite concentration is 3.91 mM. THC-COOH ([M-H]$^-$ *m/z* 343) elutes at 5.15 minutes, and partially co-elutes with the first mono-chloro product ([M-H]$^-$ *m/z* 377) at 5.25 minutes. The second mono-chloro product ([M-H]$^-$ *m/z* 377) elutes at 6.10 minutes; and the di-chloro product ([M-H]$^-$ *m/z* 411) elutes at 6.60 minutes).

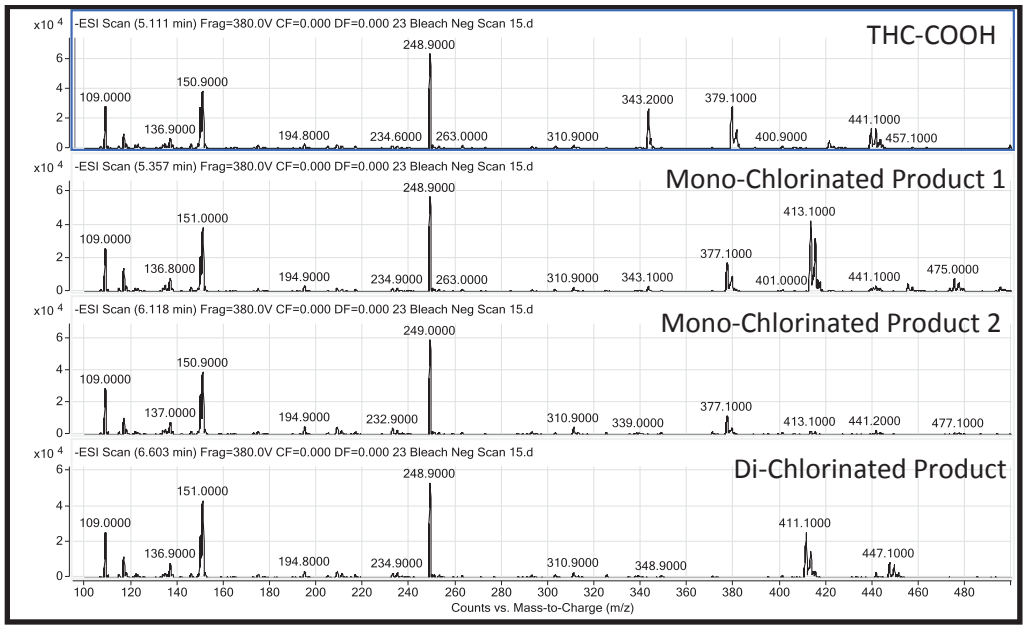


Figure 2.17 - Mass spectra (*m/z* 100 - 500) of targeted analytes for hypochlorite reaction in urine (final hypochlorite concentration 3.91 mM): THC-COOH at 5.11 minutes; mono-chloro product 1 at 5.36 minutes; mono-chloro product 2 at 6.12 minutes; and di-chloro product at 6.60 minutes.

Exposure of THC-COOH to Pyridinium Chlorochromate

As with the exposure of spiked THC-COOH samples to hypochlorite, the final concentration of THC-COOH in these samples was 2 µg/mL. For the spiked water samples, the final oxidant concentration range was 0.4 – 2.0 µM, and for the spiked urine samples, this range was set at 4.0 – 20.0 mM. The reaction samples prepared for this study were allowed to react for a total of five hours. Following sample clean-up of the spiked urine samples, analysis of the samples was undertaken in MS2 Scan mode.

For the spiked water sample, formation of the expected pyridinium chlorochromate product was confirmed. **Figure 2.18** provides the total ion chromatogram for the spiked water reaction containing a final oxidant concentration of 0.2 µM. The unreacted THC-COOH ($[M-H]^-$ *m/z* 343) elutes at 4.80 minutes, whilst the PCC product ($[M-H)^-$ *m/z* 313) is found to elute at 5.10 minutes. The mass spectra for these two peaks are found in **Figure 2.19**, and confirm formation of the previously detected PCC product in spiked water samples.

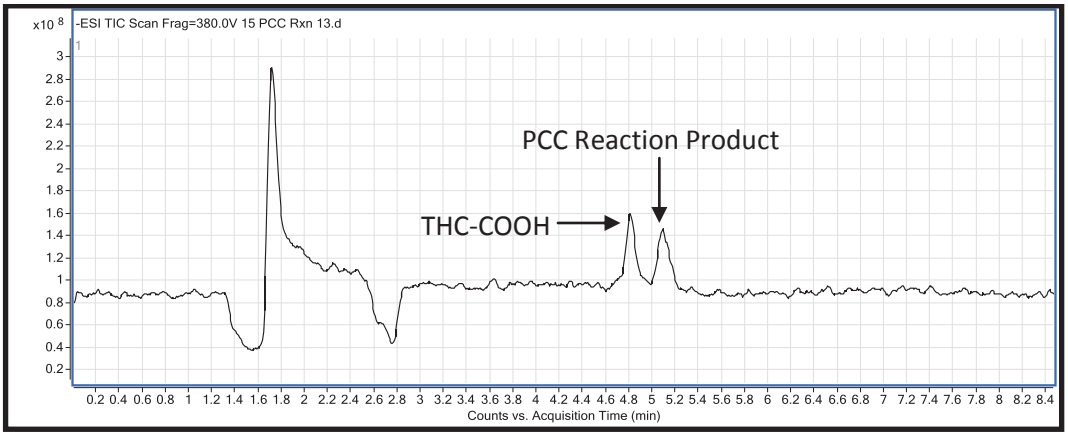


Figure 2.18 - Chromatogram of pyridinium chlorochromate reaction in water. Final oxidant concentration is 0.2 µM. THC-COOH ($[M-H]^-$ *m/z* 343) elutes at 4.80 minutes, and the pyridinium chlorochromate product ($[M-H]^-$ *m/z* 313) elutes at 5.10 minutes.

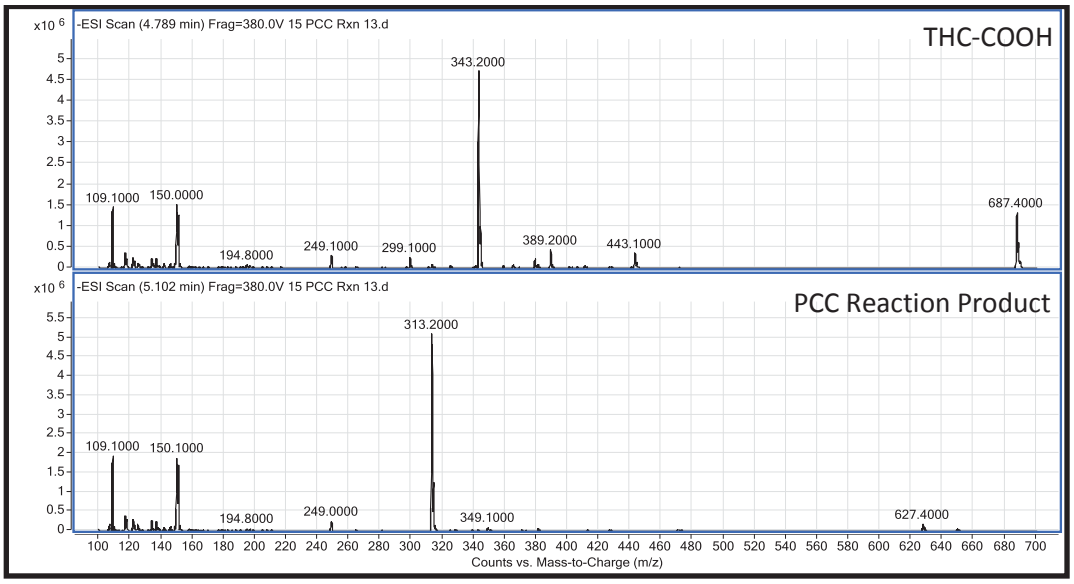


Figure 2.19 - Mass spectra (*m/z* 100 - 700) of targeted analytes for pyridinium chlorochromate reaction in water (final oxidant concentration 0.2 µM): THC-COOH at 4.80 minutes; PCC product at 5.10 minutes.

Similar results are found in the reaction prepared in spiked urine. At a final oxidant concentration of 2.0 mM, the proposed PCC product dominates the chromatogram, eluting at 5.01 minutes. The remaining unreacted THC-COOH elutes at 4.09 minutes. The chromatogram and mass spectra obtained for this reaction are shown in **Figure 2.20** and **Figure 2.21**, respectively. As with the hypochlorite reaction, a considerably higher oxidant concentration is required for this reaction to occur.

It is also interesting to note that, as with the initial study into the effects of pyridinium chlorochromate on the detection of THC-COOH, poor fragmentation of the detected PCC product is found. Though the reasons for this poor fragmentation in comparison with THC-COOH and other detected reaction products is unknown, the detection of a characteristic deprotonated molecule for this product confirms the formation of this product in both spiked water and spiked urine samples. In **Chapter 4**, dealing with structural elucidation, and in **Chapter 5**, dealing with the optimisation of detection parameters and method validation, extensive fragmentation of the PCC product was achieved.

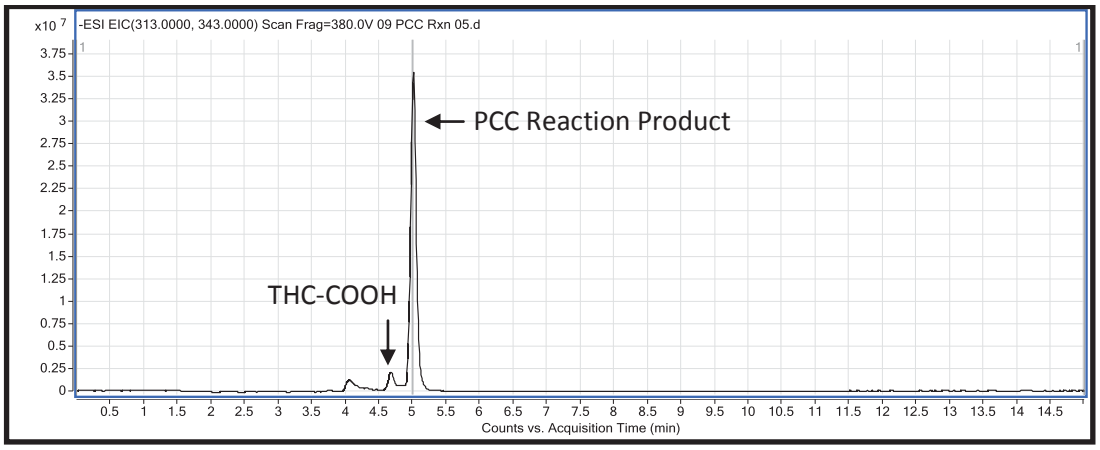


Figure 2.20 - Chromatogram of pyridinium chlorochromate reaction in urine. Final oxidant concentration is 2.0 µM. THC-COOH ($[M-H]^-$ *m/z* 343) elutes at 4.09 minutes, and the pyridinium chlorochromate product ($[M-H]^-$ *m/z* 313) elutes at 5.01 minutes.

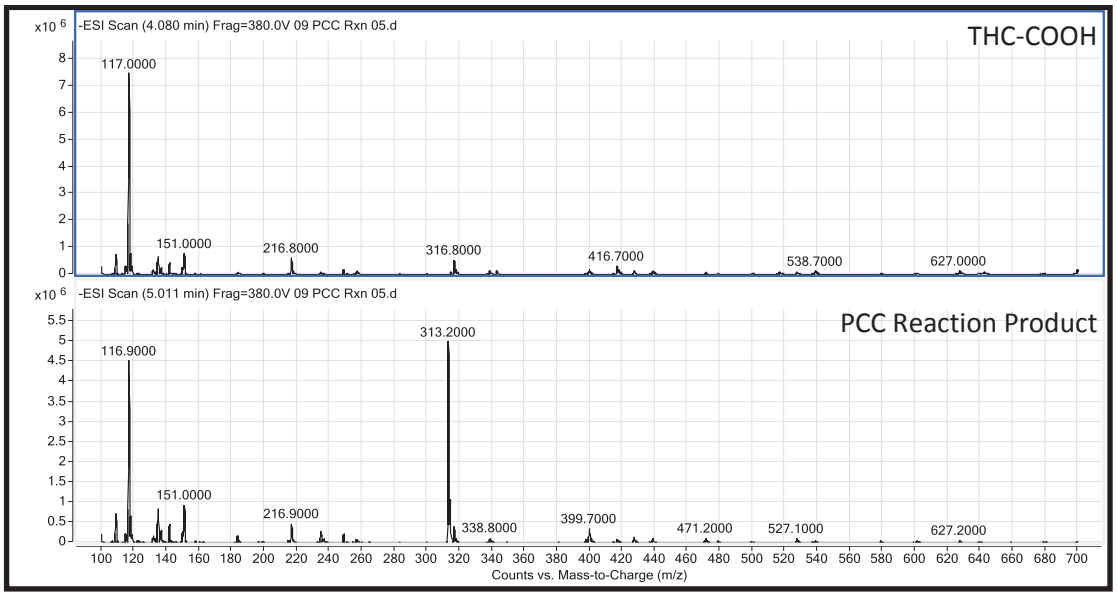


Figure 2.21 - Mass spectra (*m/z* 100 - 700) of targeted analytes for pyridinium chlorochromate reaction in urine (final oxidant concentration 2.0 mM): THC-COOH elutes at 4.09 minutes; the PCC product elutes at 5.01 minutes. Due to low concentration following reaction with pyridinium chlorochromate, the deprotonated molecule for THC-COOH is difficult to observe. Deprotonated molecule position for THC-COOH is therefore marked with an arrow.

Exposure of THC-COOH to Betadine

The effect of Betadine on the detection of THC-COOH and potential formation of reaction products has not been explored previously in this research. Therefore, a range of samples were prepared over a range of Betadine concentrations, with ten and fifteen samples prepared in spiked water and spiked urine samples, respectively. Based on the results obtained from the effect of sodium hypochlorite on THC-COOH, it is expected that a reaction between THC-COOH and Betadine will result in the formation of iodinated THC-COOH products.

Betadine is reported to have 1% w/v available iodine present. Determination of the final concentration of iodine present in the stock Betadine solution was not undertaken – as such, reported iodine concentrations for the reaction samples prepared are based on the concentration provided by the manufacturer. As such, the reported iodine concentration range for the spiked water and spiked urine samples is 0.002 – 0.100 ‰ w/v available iodine. **Figure 2.22** shows the chromatogram obtained for the spiked water reaction containing a final iodine concentration of 0.010 ‰ w/v available iodine, and **Figure 2.23** provides the mass spectra obtained for the peaks detected.

In this reaction, a major peak dominates the chromatogram. Eluting at 3.23 minutes, the deprotonated molecule recorded for this peak is $[M-H]^-$ *m/z* 595.0. Three additional peaks are detected at 1.03 minutes ($[M-H]^-$ *m/z* 343.0), 1.56 minutes ($[M-H]^-$ *m/z* 469.0), and 1.95 minutes ($[M-H]^-$ *m/z* 469.0). It is interesting to note that the deprotonated molecules generated for the two minor product peaks (1.56 minutes, 1.95 minutes) are similar to the two mono-chlorinated products found in the hypochlorite reaction, in that in the respective reactions, two peaks with identical deprotonated molecules are detected. This provides strong evidence that in the Betadine reaction, two mono-iodinated products are formed in the reaction with THC-COOH. The molecular weight of iodine is reported at 126.9 Da – the total mass increase for the detected products. Both minor product peaks are therefore expected to represent the electrophilic aromatic substitution of THC-COOH to form mono-iodinated products. In addition, the major peak, detected at 3.23 minutes has a deprotonated molecule of *m/z* 595; taking into account the loss of hydrogen from the aromatic ring during electrophilic aromatic substitution, this deprotonated molecule is therefore associated with the di-iodination of THC-COOH.

In the reaction with hypochlorite, the substitution of chlorine onto the aromatic ring results in the presence of peaks in the mass spectra for these compounds with the peak ratios

characteristic of chlorination. In contrast, for the Betadine reaction, iodine is found in nature as a single stable isotope. As such, no such isotope peaks are observed for the three products found in the reaction between THC-COOH and Betadine in spiked water samples.

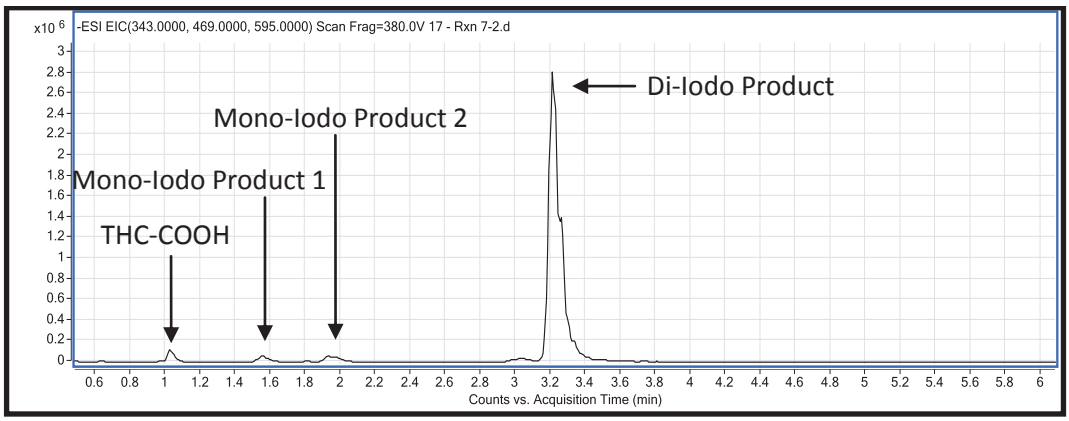


Figure 2.22 - Chromatogram of Betadine reaction in water. Final estimated adulterant concentration is 0.010 ‰ w/v available iodine. THC-COOH ([M-H]$^-$ *m/z* 343) elutes at 1.56 minutes. Two additional minor peaks elute at 1.56 minutes ([M-H]$^-$ *m/z* 469) and 1.95 minutes ([M-H]$^-$ *m/z* 469). A single major peak is also detected at 3.23 minutes ([M-H]$^-$ *m/z* 594).

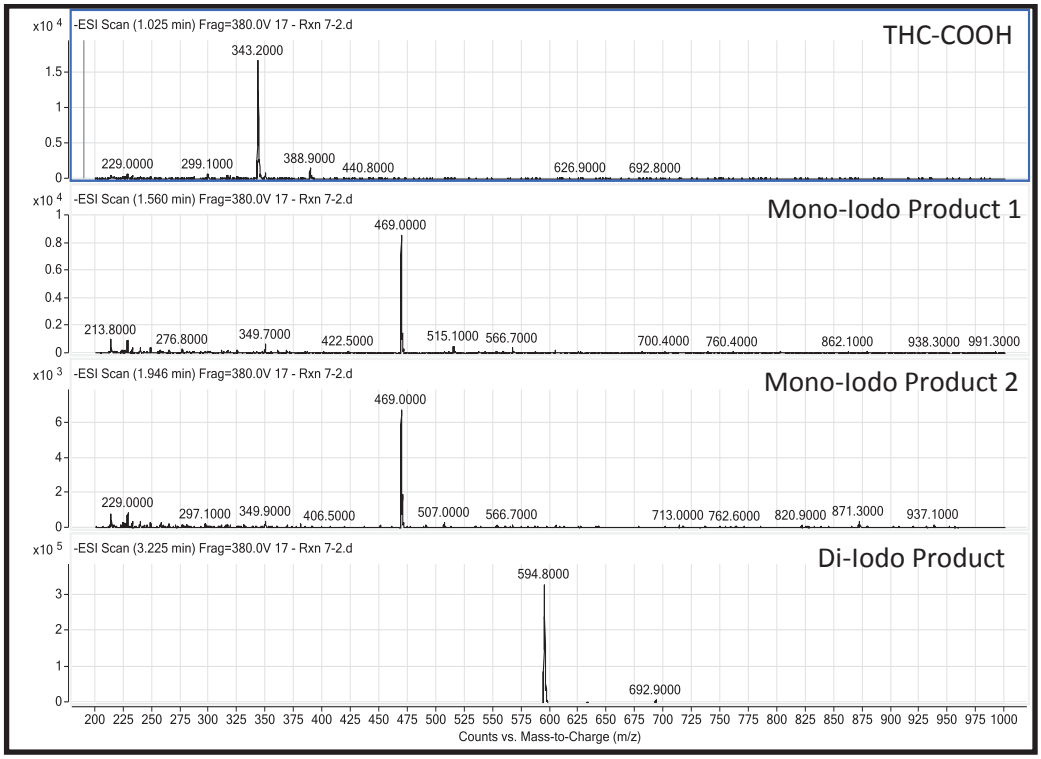


Figure 2.23 - Mass spectra (*m/z* 150 - 1000) of targeted analytes for Betadine reaction in water (final oxidant concentration 0.010‰ w/v available iodine): THC-COOH elutes at 1.03 minutes; the first Betadine product elutes at 1.56 minutes; the second Betadine product elutes at 1.95 minutes; and the final detected Betadine product elutes at 3.23 minutes. Poor fragmentation of the deprotonated molecules of the three iodinated products is observed.

Figure 2.24 and **Figure 2.25** present the extracted ion chromatogram and mass spectra obtained from the reaction of Betadine with spiked urine samples. As can be seen, the resulting reaction at an estimated 0.100‰ w/v available iodine results in a very similar chromatogram to the reaction carried out in spiked water. Traces of the remaining, unreacted THC-COOH are detected in the chromatogram, as are two minor peaks corresponding to the proposed mono-iodinated THC-COOH products ($[M-H]^-$ *m/z* 469). In addition, a major peak corresponding to the proposed di-iodinated THC-COOH product ($[M-H]^-$ *m/z* 595) is also detected, indicating that the reaction of Betadine with THC-COOH is possible in spiked urine samples.

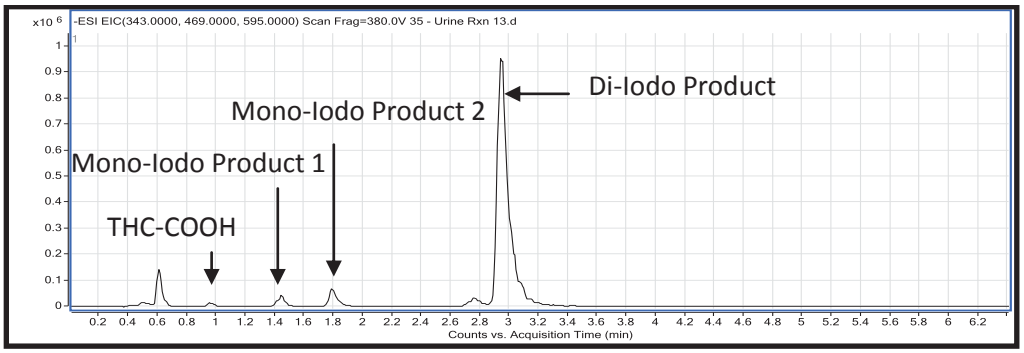


Figure 2.24 - Chromatogram of Betadine reaction in urine. Final estimated adulterant concentration is 0.100 ‰ w/v available iodine. THC-COOH ($[M-H]^-$ *m/z* 343) elutes at 1.00 minutes. Two additional minor peaks elute at 1.44 minutes ($[M-H]^-$ *m/z* 469) and 1.79 minutes ($[M-H]^-$ *m/z* 469). A single major peak is also detected at 2.96 minutes ($[M-H]^-$ *m/z* 495).

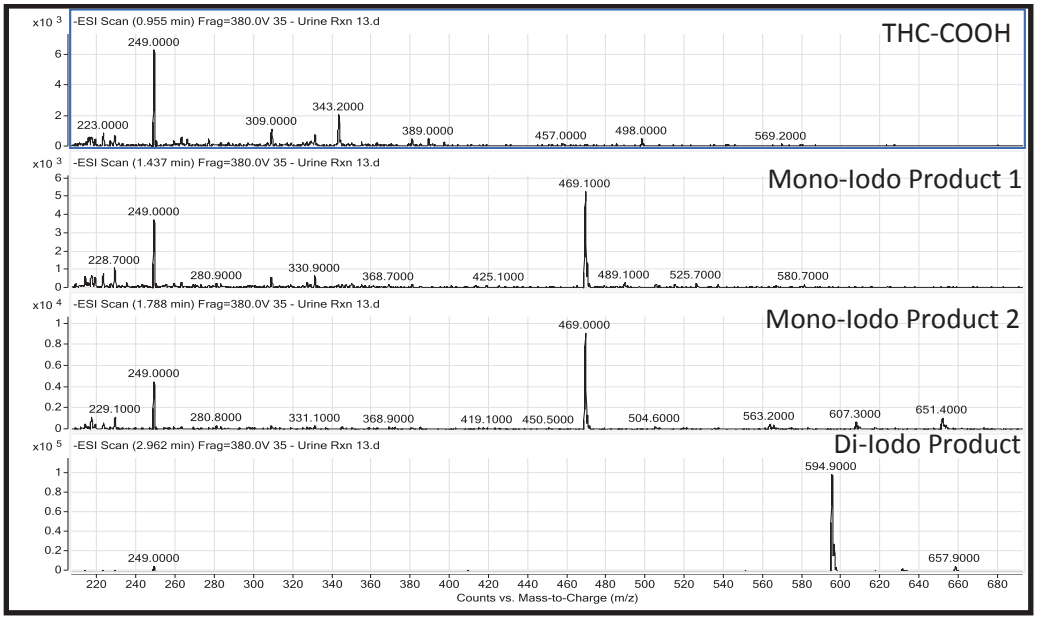


Figure 2.25 - Mass spectra (*m/z* 100 - 700) of targeted analytes for Betadine reaction in urine (final oxidant concentration 0.100‰ w/v available iodine): THC-COOH elutes at 1.00 minutes; the first Betadine product elutes at 1.44 minutes; the second Betadine product elutes at 1.79 minutes; and the final detected Betadine product elutes at 2.96 minutes.

Exposure of THC-COOH to Methanolic Iodine Solution

As discussed previously, an important consideration for the large-scale synthesis of the major product detected in the Betadine reaction is the presence of additional compounds in Betadine that may hinder purification of the targeted products. As such, an additional study was organised to explore whether a methanolic solution of iodine (or iodine tincture) would be capable of reproducing the reaction observed between Betadine and both spiked water and spiked urine samples. As with the previous experiments, the final concentration of THC-COOH was set at 2 µg/mL.

Figure 2.26 and **Figure 2.27** presents the chromatogram and mass spectra, respectively, obtained in the reaction between a prepared iodine tincture and a water sample spiked with THC-COOH. The iodine stock solution prepared in methanol had a final concentration 0.5% w/v, with two working solutions prepared through serial dilution of the stock solution (1:10, 1:100). In the successful reaction shown **in Figure 2.26**, the final concentration of available iodine in the sample was calculated to be 0.005 ‰ w/v. In this reaction, remaining unreacted THC-COOH is found to elute at 5.06 minutes. Similar to the Betadine reactions undertaken in spiked water and spiked urine samples, three other peaks are detected in this chromatogram: a peak corresponding to the first mono-iodinated product (6.070 minutes, $[M-H]^-$ *m/z* 469), the second mono-iodinated product (7.100 minutes, $[M-H]^-$ *m/z* 469), and the proposed di-iodinated product (9.678 minutes, $[M-H]^-$ *m/z* 595).

A major difference between this reaction and the similar successful reaction by Betadine in the spiked water sample is that the peak associated with the proposed di-iodinated product no longer dominates the chromatogram, as larger peaks are recorded for THC-COOH and the first mono-iodinated product, and a significant peak is detected for the second mono-iodinated product. As the final concentration of available iodine is half that of the Betadine reaction, it is expected that the lower oxidant concentration has limited the reaction with THC-COOH. As the Betadine reaction results in a major peak for the di-iodinated product, it is expected that at lower oxidant concentrations, the extent of reaction is limited, with the mono-iodinated products potentially representing stable intermediates of the reaction. At higher final iodine concentrations, it is possible that the mono-iodinated products undergo further reaction to form the di-iodinated product.

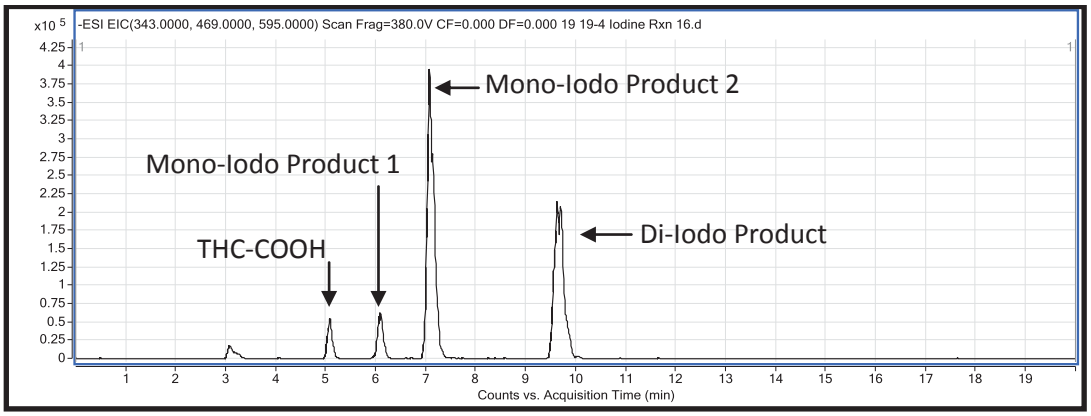


Figure 2.26 - Chromatogram of iodine reaction in water. Final estimated adulterant concentration is 0.005 ‰ w/v available iodine. THC-COOH ([M-H]$^-$ *m/z* 343) elutes at 5.06 minutes. Two additional minor peaks elute at 6.0700 minutes ([M-H]$^-$ *m/z* 469) and 7.10 minutes ([M-H]$^-$ *m/z* 469). A single major peak is also detected at 9.68 minutes ([M-H]$^-$ *m/z* 495).

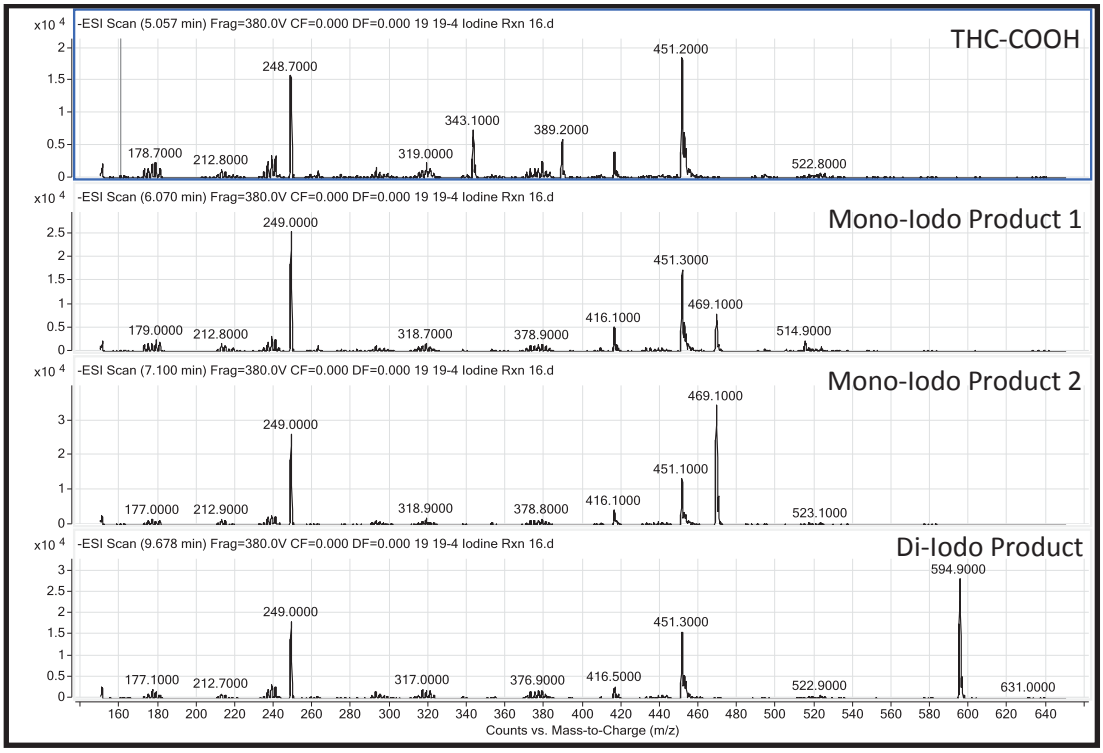


Figure 2.27 - Mass spectra (*m/z* 100 - 700) of targeted analytes for iodine reaction in water (final oxidant concentration 0.050‰ w/v available iodine): THC-COOH elutes at 5.06 minutes; the first iodine product elutes at 6.07 minutes; the second iodine product elutes at 7.10 minutes; and the final detected iodine product elutes at 9.68 minutes.

2.2.3 – Further Study

Exposure of THC-COOH to Papain

Papain is a cysteine protease enzyme derived from the latex of the papaya plant. Use of this enzyme to mask positive drug test results has been reported in literature previously (Burrows *et. al. 20*05; Larson *et. al.* 2008). In this research, the effect of papain on the detection of THC-COOH and the potential for detection of reaction products were studied. Samples were prepared in spiked water samples as opposed to urine to simplify both detection of possible reaction products and assessment of any apparent decreases in THC-COOH concentration (**Table 2.8**). Previous studies of the effect of papain in drug-positive urine samples have focused on detection by immunoassay-based techniques and GC-MS. Results from these studies concluded that the effect of papain is due to a chemical interaction between this enzyme and THC-COOH, as the apparent decrease in THC-COOH concentration is uniform between the screening and confirmatory testing procedures.

Samples of THC-COOH were prepared in water and spiked with a working solution of papain (0.75 – 5.00 units/mL). Analyses were undertaken to assess whether the exposure of THC-COOH to papain would result in a relative loss of THC-COOH from the reaction samples, and if so, whether the apparent reaction would produce viable oxidation products. Comparison of the papain reaction mixtures with the THC-COOH standard and reagent blanks indicated that a slight loss of THC-COOH occurred following this exposure. **Figure 2.28** shows the chromatogram and mass spectrum obtained from analysis of the 10 µg/mL THC-COOH standard (retention time 4.00 minutes, $[M-H]^-$ *m/z* 343). Integration of target peaks was used to obtain the THC-COOH peak area.

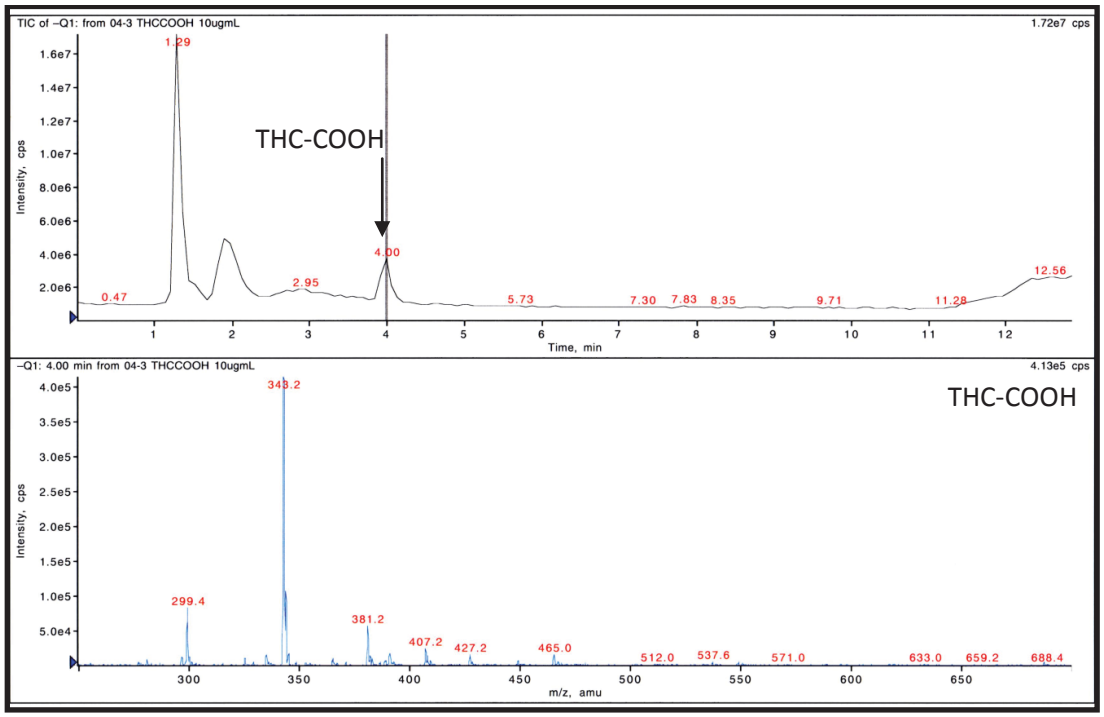


Figure 2.28 - Chromatogram and mass spectra of 10 µg/mL THC-COOH standard ([M-H]$^-$ *m/z* 343, R_t 4.00 minutes).

Three reaction samples were prepared by pipetting papain working solution into spiked water samples. Over the tested reaction conditions, it was found that exposure of the sample to papain resulted in a noticeable decrease in the peak area of THC-COOH (retention time 3.47 minutes, [M-H]$^-$ *m/z* 343). The shift in retention time for THC-COOH is attributed to issues relating to equilibration of the solvent pump module. Chromatograms for the three tested reactions are shown in **Figure 2.29**. At the lowest concentration of papain, an apparent 60% decrease of the THC-COOH peak area was noted. At the highest tested concentration, exposure of THC-COOH to papain resulted in an 80% reduction in peak area.

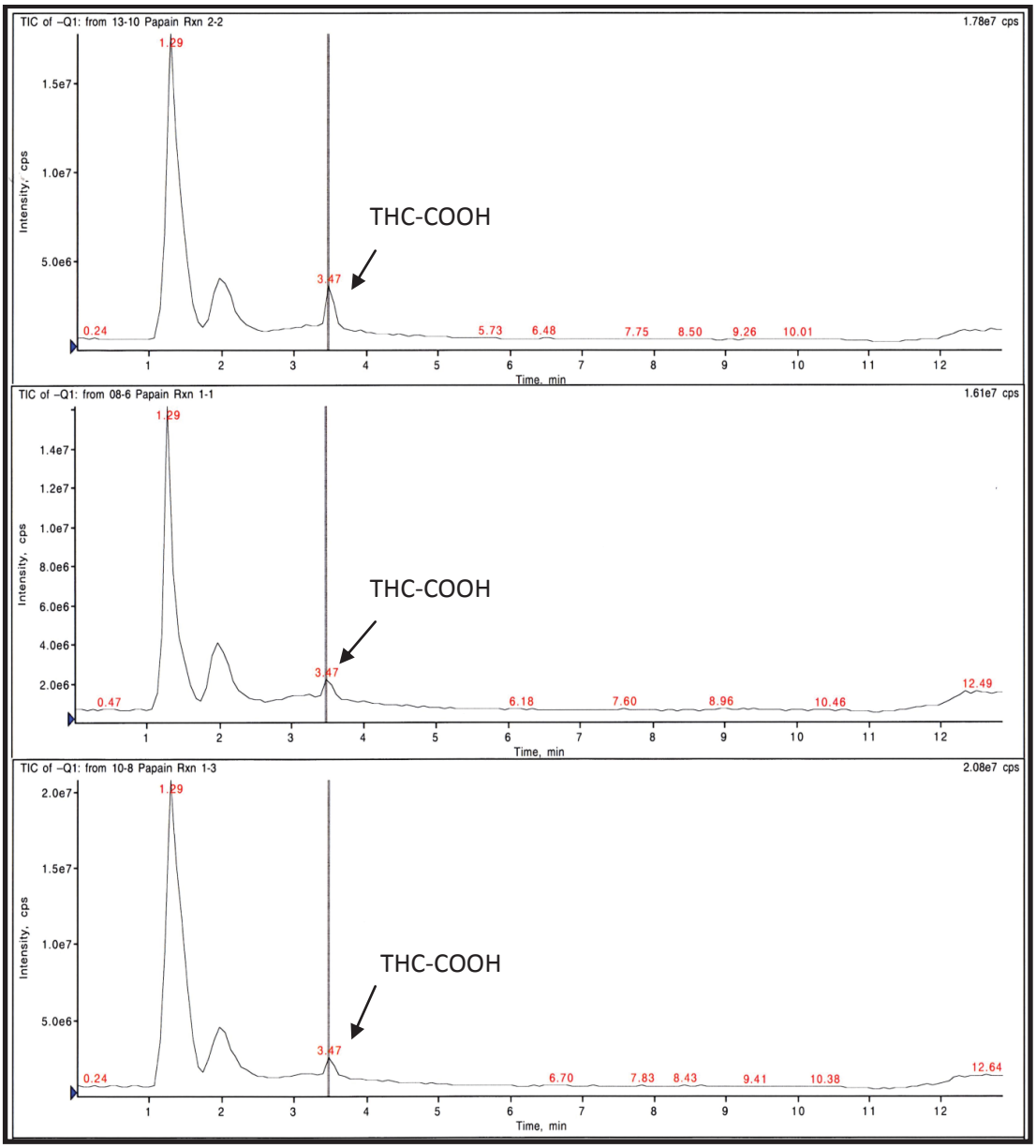


Figure 2.29 - Chromatograms from exposure of THC-COOH to papain at three concentrations; (Top) Lowest concentration; (Middle) Middle concentration; (Bottom) Highest concentration. THC-COOH peak at 3.47 minutes, $[M-H]^-$ *m/z* 343.

The data collected in this experiment suggests that papain represents a potentially effective means by which an individual may mask a drug-positive test result. As shown in **Table 2.12**, complete loss of THC-COOH from the samples was not observed, though it is expected that at higher papain concentrations a total loss of THC-COOH may be observed. Analysis of the chromatograms and mass spectra from the papain reaction samples did not indicate the presence of any reaction products over the analysis parameters (13 minutes, mass range *m/z* 250 – 750). Lack of detection may indicate that no stable reaction products exist for this

reaction, or that the products formed have deprotonated molecules outside of the mass range tested.

Table 2.12 - Summary of results from exposure of THC-COOH to papain.

Sample	THC-COOH Retention Time (Minutes)	Calculated Peak Area	Decrease in Peak Area (%)
THC-COOH Standard	4.00	1.260	-
Papain Reaction 1	3.47	0.500	60.31
Papain Reaction 2	3.47	0.313	75.20
Papain Reaction 3	3.47	0.250	80.16

Previous studies into the effects of papain have concluded that the effect of this enzyme on detection of THC-COOH is mediated through a chemical reaction, as opposed to an interfering effect (Burrows 2005, Larson 2008). Consequently, future studies may focus on confirming the mechanism of action as a chemical reaction. If the effects of papain on detection of cannabis use are through a chemical reaction, future studies may also determine if compounds formed in the reaction between THC-COOH and papain may be detected and studied.

Exposure of Selected Additional Oxidising Adulterants to THC-COOH

An additional study was undertaken to assess the effects of exposure of five alternate oxidation agents in both spiked water and urine samples. As with the previous studies explored in this chapter, the goals of this study were twofold: to assess the effects of the selected oxidising agents on the detection of THC-COOH by LC-MS, and to determine if potential markers of both cannabis-positive urine and subsequent urine adulteration could be identified. Ceric ammonium nitrate (CAN), potassium perchlorate, potassium permanganate, sodium iodate and sodium metaperiodate were selected for this study based on their properties as oxidising agents. In the case of ceric ammonium nitrate, it represents one of the strongest shelf-stable oxidising agents available, with an oxidising potential rivalling that of chlorine. Commonly used in chemical synthesis, it is unlikely to enter into the marketplace as an adulterant readily available for purchase, though this may also be alleged for pyridinium chlorochromate. Out of the five oxidising agents, potassium permanganate is readily available to the average consumer, available from pharmacies and farming product suppliers as a disinfectant and anti-fungal agent.

The effects of these oxidising agents were studied in water and urine samples spiked with THC-COOH (4 µg/mL). Solvent and reagent blanks were prepared, as were two THC-COOH standards, prepared in water and blank urine, respectively. **Figure 2.30** presents the chromatograms and mass spectra of the target analytes of the THC-COOH standards in both matrices. Two working solutions were prepared for each oxidant (1 mM, 10 mM), with three oxidant solution volumes tested for each working solution. For the reaction samples, these five oxidant solution volumes represented a final in vitro oxidant concentration of 0.02, 0.04, 0.10, 0.20, 0.40 and 1.00 mM.

For the spiked water sample, elution of THC-COOH is at 0.99 minutes, with the $[M-H]^-$ deprotonated molecule for this compound confirmed at *m/*z 343. Expected product ions for THC-COOH were present in the obtained mass spectra, including *m/z* 248.9 and *m/*z 299.1, as well as the presence of the THC-COOH dimer at 687 *m/*z. Retention time of THC-COOH in the spiked urine sample was close to that of the spiked water sample, with the retention time recorded at 0.98 minutes. To assess the effect of the oxidants on the peak area of THC-COOH, the extracted ion chromatograms for THC-COOH were generated, with the subsequent peaks integrated. The integrated peak area for THC-COOH was calculated as 31,827,268 and 27,725,120, for water and urine respectively. The slight differences in peak area between the

two matrices may be attributed to a mild ion suppression effect, or variation in preparation of the standard.

Reagent blanks for the oxidising agents were prepared at a final oxidant concentration of 10 mM in water and urine. Analysis of these samples by LC-MS did not indicate the presence of any significant peaks in the chromatograms, and in addition, preparation of the reagent blanks also allowed for the effect of the selected oxidising agents on the physical properties of the water and urine samples to be established. Potassium perchlorate, sodium iodate and sodium metaperiodate did not alter the colour of the reagent blanks prepared in the two matrices, and as such may not be readily noticed in a laboratory setting. In contrast, ceric ammonium nitrate contributed a pale yellow to the reagent blank prepared in water, and did not have an observable effect on the colour of the urine reagent blank. Potassium permanganate provided the most striking colour change to the two reagent blanks, with both taking on a dark purple colour immediately following addition of the permanganate working solution. In the water sample, this colour persisted for several hours before fading. In urine, the initial colour change faded over two hours, turning the sample a dark brown colour. This colour change is due to the reduction of the permanganate ion to form the brown solid manganese dioxide. Upon standing, the dark brown colour of the permanganate reagent blank prepared in urine slowly faded, with the precipitation of manganese dioxide on the bottom of the sample vial noted.

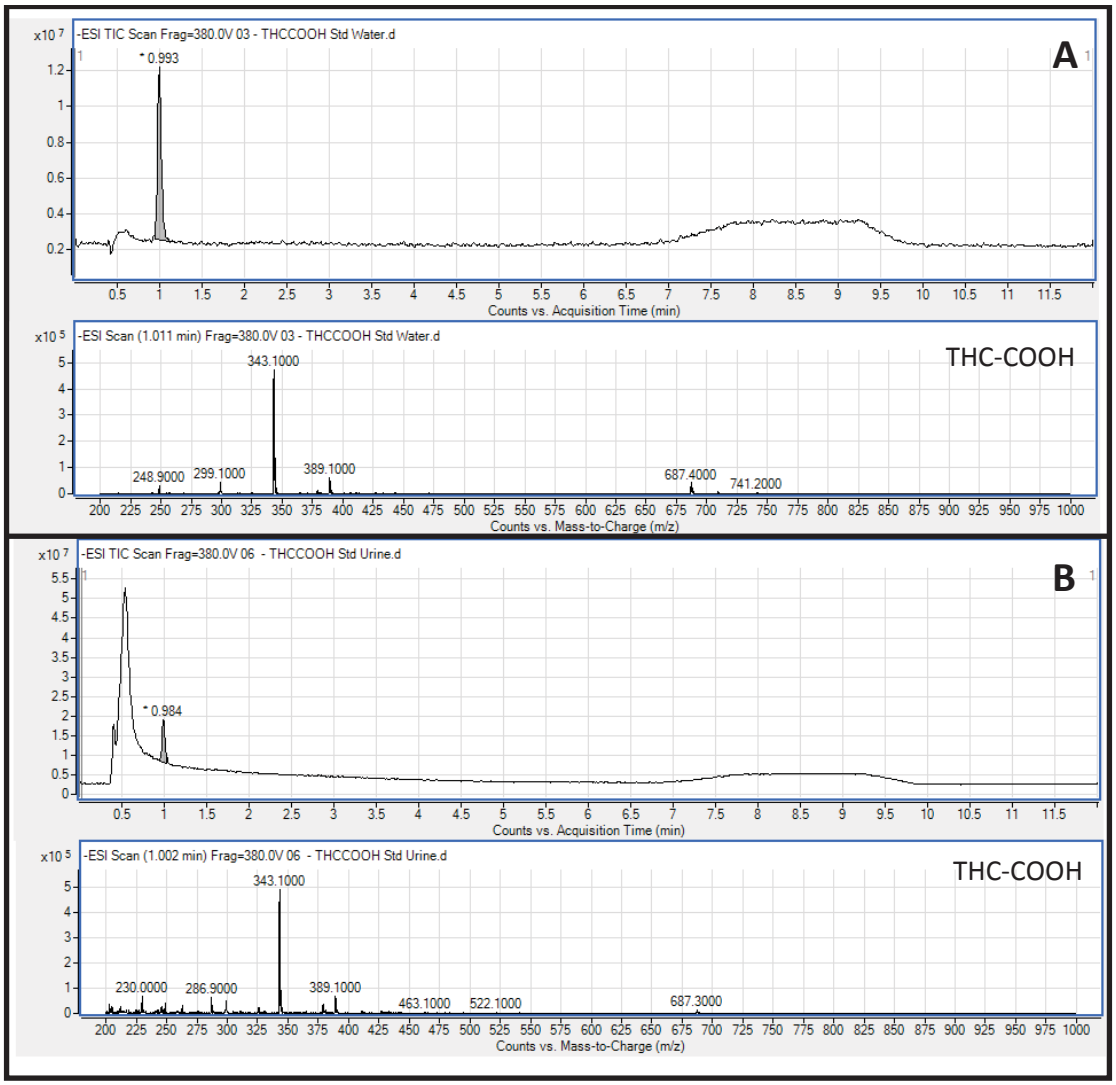


Figure 2.30 - Chromatograms and mass spectra of THC-COOH in: (A) Spiked water sample (R_t = 0.99 minutes, $[M-H]^-$ *m/z* 343*)*, and (B) Spiked urine sample (Rt = 0.98 minutes, [M-H]- *m/z* 343).

Initial analyses of the reaction mixtures were focussed on any possible reduction in the apparent peak area of THC-COOH. Each reaction mixture was analysed by LC-MS, and from the generated chromatograms, the THC-COOH peak was detected where possible. Extracted ion chromatograms for THC-COOH at *m/z* 343 were prepared, and the subsequent peaks integrated. Assessment of the effects of the oxidants was based on a comparison of the peak areas for THC-COOH in the respective matrices, and the calculated peak area of THC-COOH in the reaction mixtures. **Figure 2.31** depicts the recorded peak areas over the twelve reactions tested for each oxidant.

For reactions prepared in both matrices, increasing oxidant concentration coincided with a decrease in the peak area for THC-COOH. In water, the greatest reductions in peak area were observed with ceric ammonium nitrate and potassium permanganate, with a total loss of THC-COOH occurring at a final oxidant concentration of 0.20 and 0.40 mM respectively. In urine, the greatest total reduction in peak area was recorded with sodium iodate and sodium metaperiodate. Relative loss of THC-COOH peak area for the selected oxidants in both matrices is included in **Table 2.13**. Calculation of relative peak area reduction was through comparison of the THC-COOH standards in their respective matrices with the final peak area for each oxidant.

Table 2.13 - Calculated relative reduction in peak area for selected oxidants at highest oxidant concentration (1.00 mM)

Oxidant	Percent Loss THC-COOH (%)	
	Water	Urine
Ceric ammonium nitrate	100	61.75
Potassium Perchlorate	64.98	74.77
Potassium Permanganate	100	66.95
Sodium Iodate	58.92	84.37
Sodium Metaperiodate	83.48	90.7

Variations in the percent loss of THC-COOH peak area varied over both matrices. For ceric ammonium nitrate and potassium permanganate, relative peak area loss was lower in the urine samples when compared to the samples prepared in water. For these two oxidants, it is likely that competing side-reactions with endogenous compounds present in urine limited the oxidising capacity of these reagents. Conversely, for the remaining oxidants, a higher relative loss of peak area was found in the spiked urine samples. This variation may be accounted for by the effect of urine pH on the reactivity of the oxidants. Prior to sample preparation, the pH of the blank urine sample was measured at pH 5.5. As such, it is proposed that under acidic conditions certain oxidants may possess a higher oxidising capacity. It is possible that in samples prepared at a neutral or alkaline pH, further differences in the effectiveness of these oxidants may be discovered.

However, for potassium perchlorate, sodium iodate and sodium metaperiodate, this trend was reversed, with a greater loss of THC-COOH observed in the urine samples. This reversal can be

accounted for, specifically by the effect of urine pH on the reactivity of the oxidants. Prior to sample preparation, the pH of the blank urine sample was measured at pH 5.5, hence, it is proposed that under acidic conditions, certain oxidants may possess a higher oxidising capacity when compared to spiked water samples, and possibly, urine at a neutral or alkaline pH.

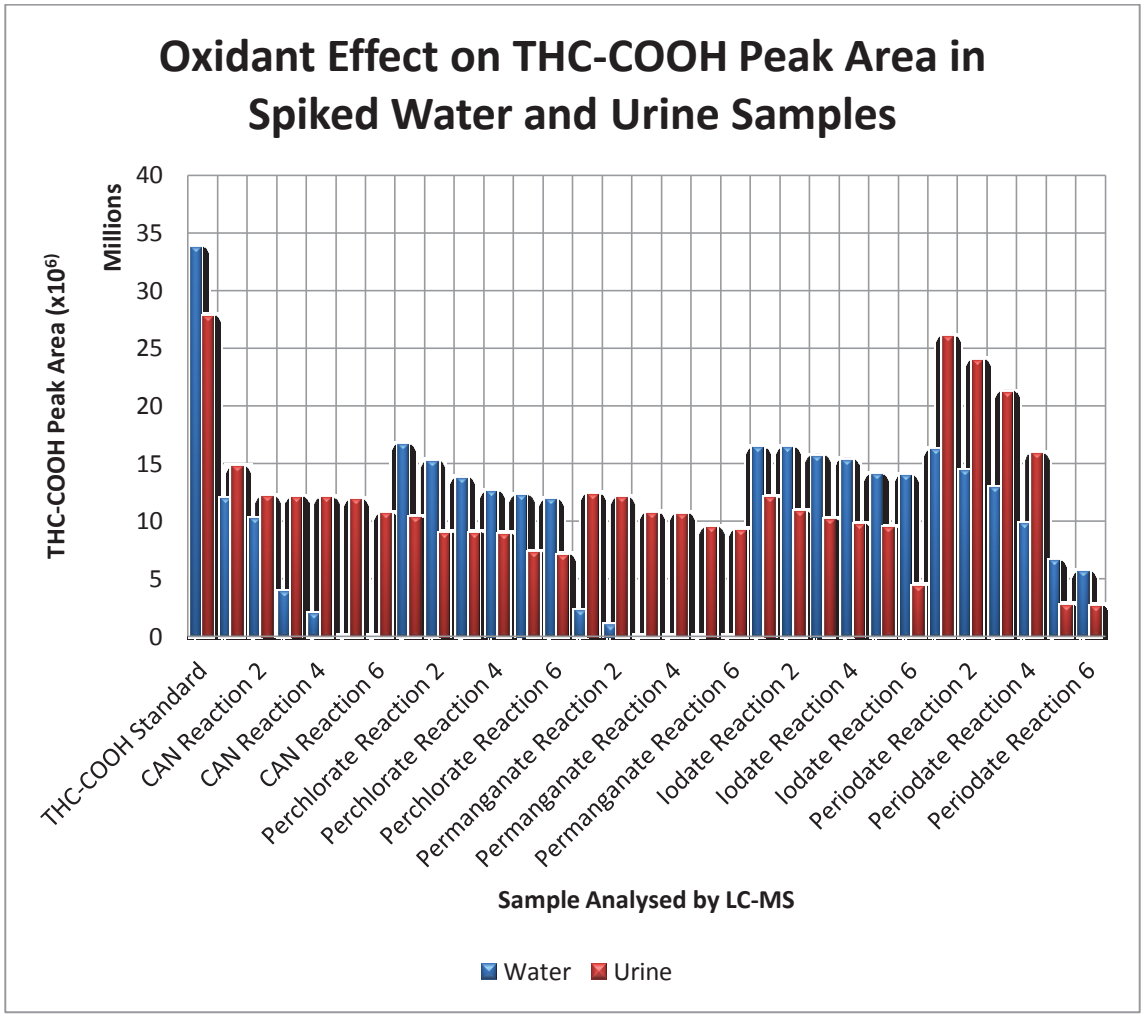


Figure 2.31 – Chart illustrating changes in THC-COOH peak area in water and urine samples following exposure to oxidising adulterants.

The second goal of this study was to determine if exposure of THC-COOH to the selected oxidants would result in the formation of reaction products. Initial analyses were performed through comparison of the THC-COOH standards and the reagent blanks in order to identify any major peaks. **Figure 2.32** and **Figure 2.33** provide the extracted ion chromatograms for the reactions in water and urine, respectively. Further discussion of the results for each oxidising adulterant is included below.

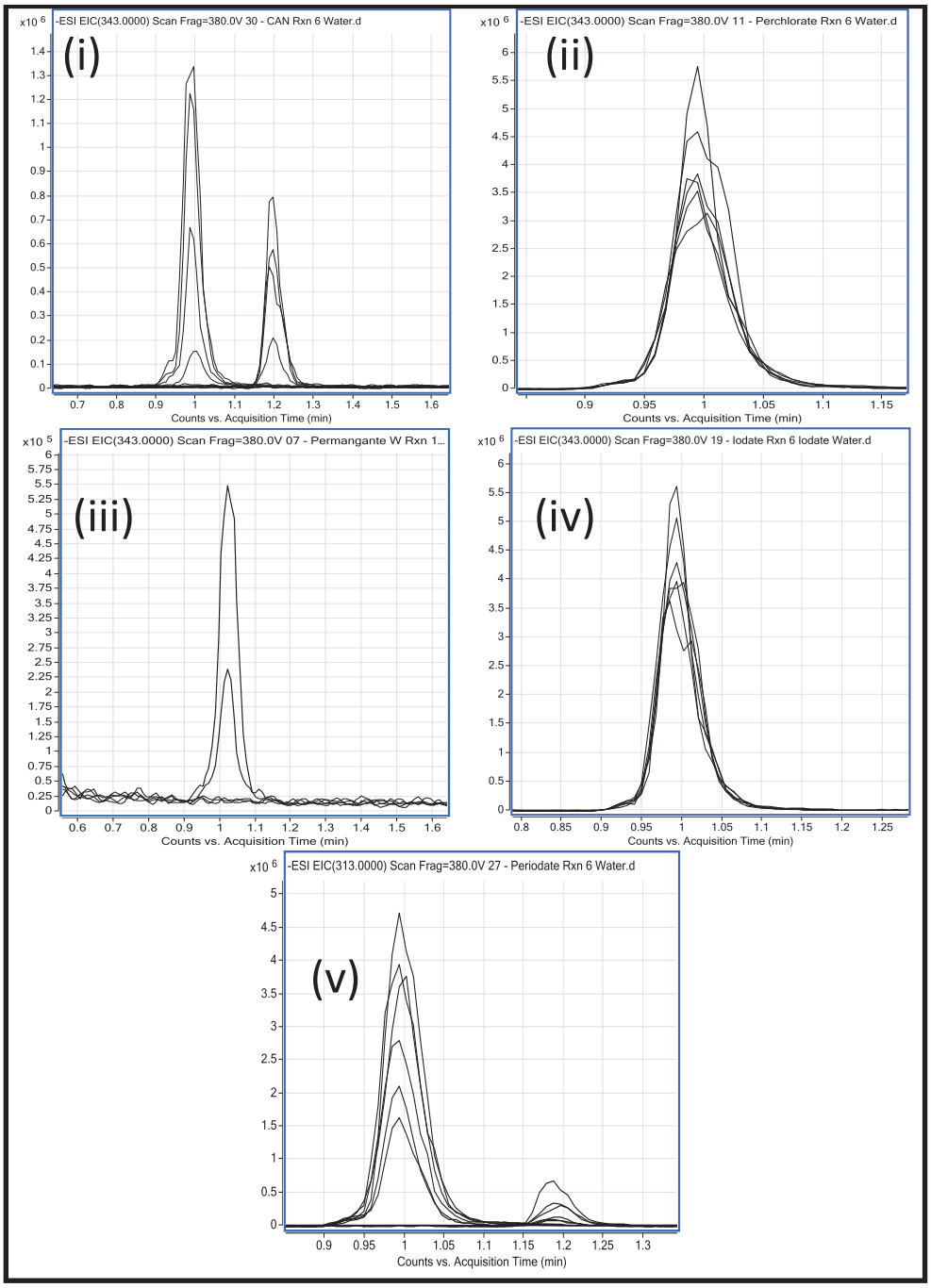


Figure 2.32 -Extracted ion chromatograms generated for the tested oxidants in spiked water samples. Highest peak for THC-COOH at 0.02 mM oxidant concentration, smallest peak for THC-COOH at 1.00 mM oxidant concentration.

(i) Ceric ammonium nitrate – THC-COOH (R_t 0.99 minutes, $[M-H]^-$ *m/z* 343) and reaction product (R_t 1.19 minutes, $[M-H]^-$ *m/z* 313);
(ii) Potassium Perchlorate – THC-COOH (R_t 0.99 minutes, $[M-H]^-$ *m/z* 343);
(iii) Potassium Permanganate – THC-COOH (R_t 1.02 minutes, $[M-H]^-$ *m/z* 343);
(iv) Sodium Iodate – THC-COOH (R_t 0.93 minutes, $[M-H]^-$ *m/z* 343);
(v) Sodium Metaperiodate – THC-COOH (R_t 0.99 minutes, $[M-H]^-$ *m/z* 343) and reaction product (R_t 1.19 minutes, $[M-H]^-$ *m/z* 313).

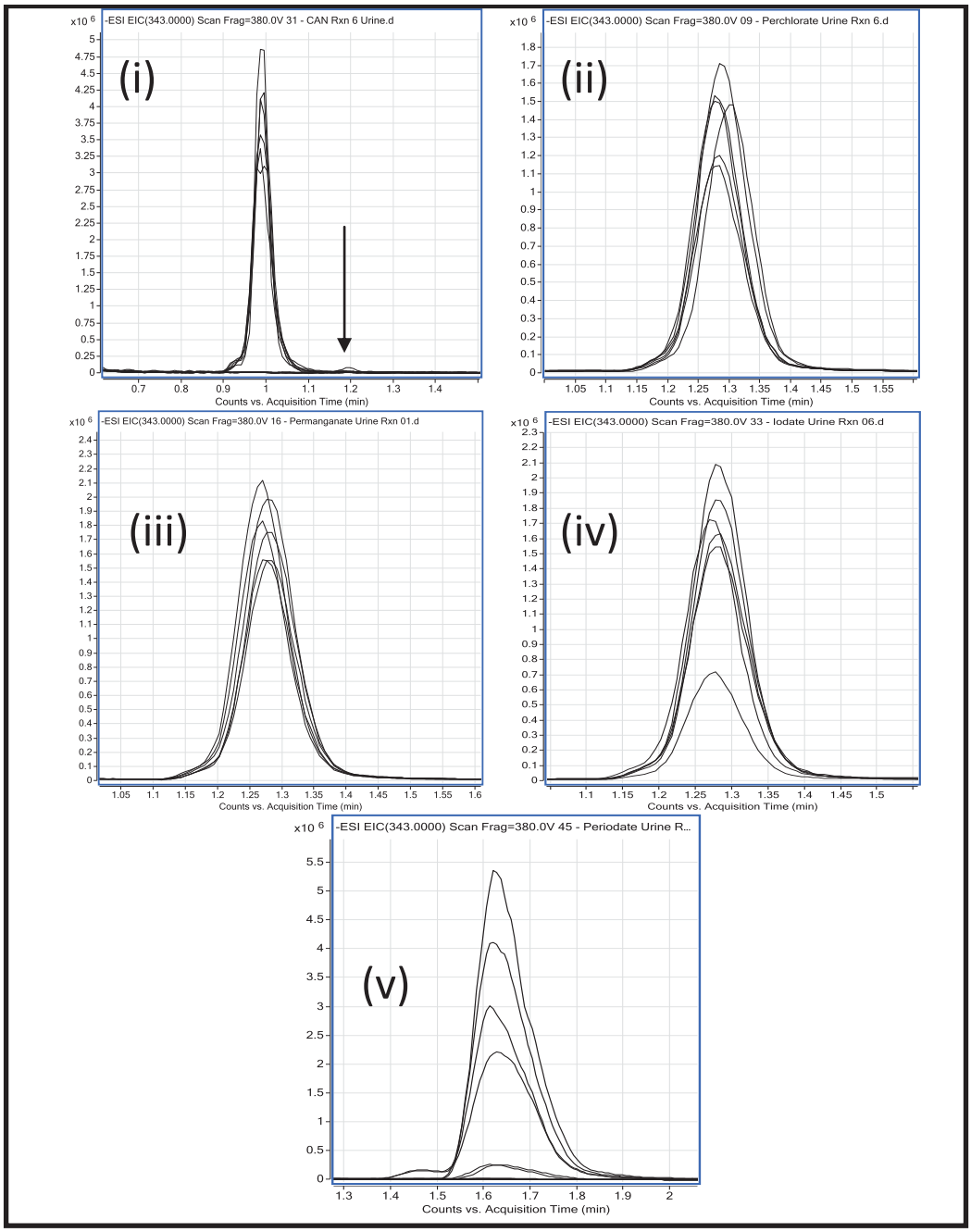


Figure 2.33 - Extracted ion chromatograms generated for the tested oxidants in spiked urine samples. Highest peak for THC-COOH at 0.02 mM oxidant concentration, smallest peak for THC-COOH at 1.00 mM oxidant concentration

(i) Ceric ammonium nitrate – THC-COOH (R_t 0.98 minutes, $[M-H]^-$ *m/z* 343) and reaction product (R_t 1.20 minutes, $[M-H]^-$ *m/z* 313, peak marked with arrow);
(ii) Potassium Perchlorate – THC-COOH (R_t 1.28 minutes, $[M-H]^-$ *m/z* 343);
(iii) Potassium Permanganate – THC-COOH (R_t 1.27 minutes, $[M-H]^-$ *m/z* 343);
(iv) Sodium Iodate – THC-COOH (R_t 1.28 minutes, $[M-H]^-$ *m/z* 343);
(v) Sodium Metaperiodate – THC-COOH (R_t 1.62 minutes, $[M-H]^-$ *m/z* 343).

Ceric ammonium nitrate exhibited a strong effect on the reduction of THC-COOH peak area, especially in the spiked water samples. Analysis of the reaction samples prepared in water yielded an additional peak not observed in the standards or reagent blanks. Eluting at 1.19 minutes (**Figure 2.32**), this peak was found to have a $[M-H]^-$ deprotonated molecule present at *m/z* 313.2 (**Figure 2.34**). This peak was found in Reactions 1 – 4 prepared in spiked water. At oxidant concentrations above 0.4 mM, total loss of this peak was observed along with THC-COOH. Analysis of the reaction samples prepared in the spiked urine matrix also revealed the presence of this additional peak. In Reaction 6, a small peak was detected at 1.20 minutes (**Figure 2.34**), and displayed the previously detected deprotonated molecule. This potential reaction product was not observed in the urine samples at lower oxidant concentration, and is likely due to the competing reaction of this oxidant with the compounds typically present in urine. The mass spectrum of the reaction product in urine was convoluted due to the presence of a significant amount of background noise due to the small peak area of this compound. Removal of the background noise allowed for a clean mass spectrum of the potential reaction product to be produced (**Figure 2.35**). Comparison of the mass spectra prepared for the product peaks in water and urine indicates that the potential reaction product was formed in both matrices.

Repetition of experiments was undertaken in order to determine if this product provisionally detected with a deprotonated molecule of *m/z* 313 could be confirmed. Two new batches of the samples prepared for this experiment with ceric ammonium nitrate did not reveal the existence of this peak. It is unknown if this alleged product was the result of background noise, contamination of the sample, or some other artefact related to analysis of these samples.

Exposure of spiked THC-COOH samples to working solutions of potassium perchlorate and potassium permanganate resulted in extensive reductions in THC-COOH peak area (**Table 2.13**). Despite this reduction in peak area, the chromatograms obtained for these reaction mixtures did not reveal the formation of possible reaction products. The lack of reaction products may be due to the complete degradation of THC-COOH in vitro, and may signify that both oxidants represent effective urine adulterants that can both mask the presence of THC-COOH and not provide an indication of urine adulteration. Alternately, it is possible that both of these reactions did form reaction products, with $[M-H]^-$ deprotonated molecules below *m/z* 200. As the mass range observed in this study was *m/z* 200 – 1000, further studies may seek to repeat these studies with a lower mass range. Extracted ion chromatograms for the potassium

perchlorate and potassium permanganate reactions in both water and urine are shown in **Figure 2.32** and **Figure 2.33**, respectively.

Sodium iodate exhibited a moderate effect on THC-COOH peak area in spiked water samples, with a more pronounced effect apparent in the spiked urine samples. Analysis of the reaction samples prepared in water showed an additional peak present in Reactions 3 – 5 not observed in the standards or reagent blanks. This peak eluted at 2.60 minutes, and indicates the formation of a possible reaction product. The mass spectrum generated for this peak is unclear; subtraction of a background spectrum did not improve yield an obvious $[M\text{-}H]^-$ deprotonated molecule (**Figure 2.34**). Significant ions were detected at *m/z* 249 and *m/z* 369, though these are not necessarily associated with a deprotonated molecule for the potential reaction product. At oxidant concentrations above 0.40 mM the peak associated with the potential reaction product was no longer observed. In the spiked urine samples sodium iodate exhibited a very strong effect on THC-COOH peak area, with a calculated peak area reduction of 84.37%, with these increased oxidant effect attributed to sample pH. Chromatograms obtained from the exposure of sodium iodate to spiked urine samples did not indicate the formation of the reaction product detected in the spiked water samples. This lack of product formation may be due to both the sample matrix and matrix pH.

The exposure of sodium metaperiodate working solutions to spiked water samples over the six oxidant concentrations revealed a major decrease in THC-COOH peak area. At the highest oxidant concentration (1.0 mM) an 84.38% decrease in the target analyte peak area was calculated. Analysis of the reaction samples prepared in water yielded an additional peak not observed in the standards or reagent blanks. This additional peak was observed in Reactions 4 – 6, and was found to elute at 1.19 minutes (**Figure 2.32**). The $[M\text{-}H]^-$ deprotonated molecule for this peak was observed at *m/z* 313 (**Figure 2.35**). This potential reaction product was not observed in the spiked urine samples; the extensive reaction between sodium metaperiodate and THC-COOH in urine may have lead to degradation of this possible reaction product. This potential degradation of reaction products was also observed in the ceric ammonium nitrate reactions in water, where increased oxidant concentration resulted in the degradation of THC-COOH and the respective reaction product.

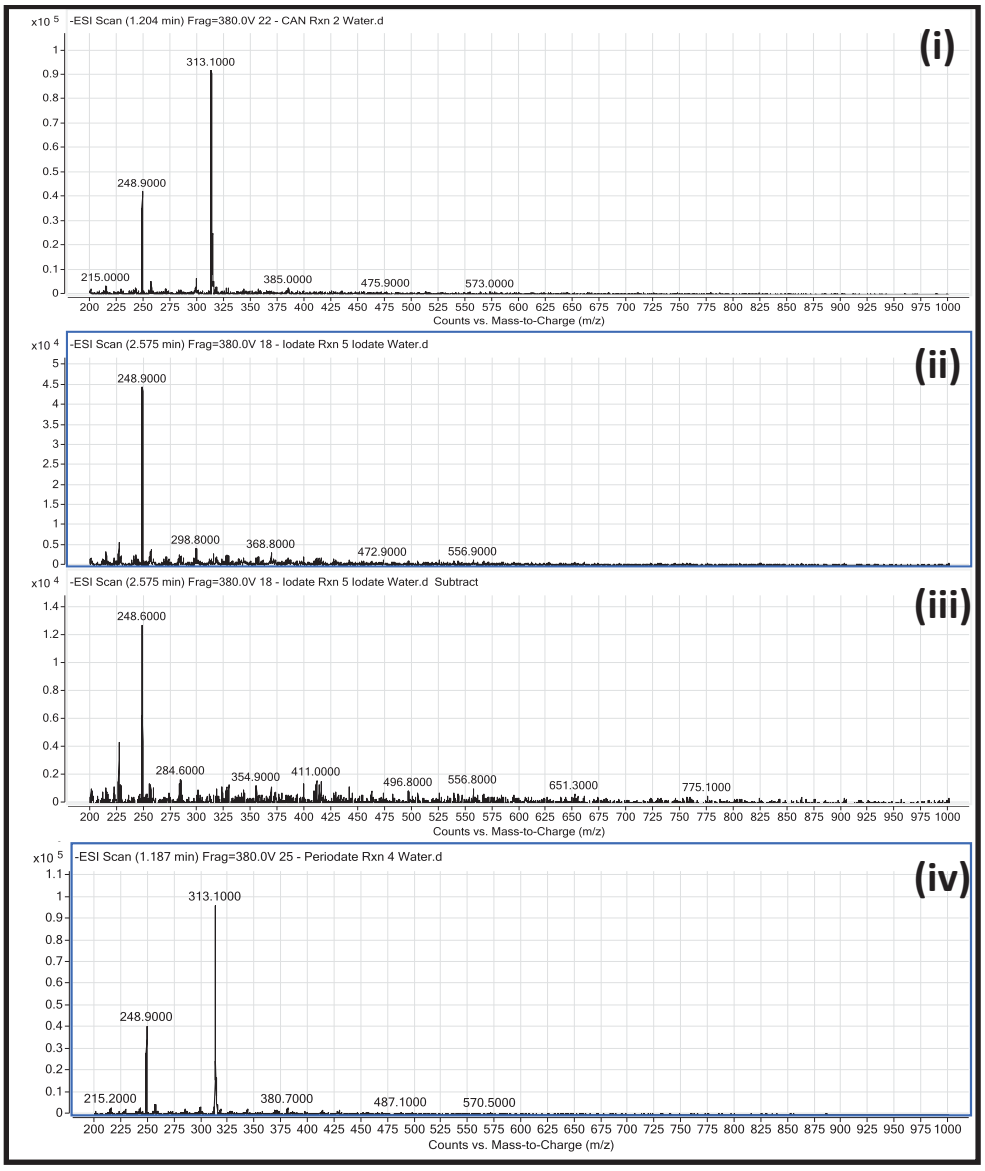


Figure 2.34 – Mass spectrum of reaction products detected in spiked water samples:

(i) Ceric ammonium nitrate – Peak at 1.19 minutes, $[M\text{-}H]^-$ *m/z* 313; (ii) Sodium Iodate – Peak at 2.60 minutes, unknown $[M\text{-}H]^-$; (iii) Background-subtracted mass spectrum for sodium iodate reaction product; (iv) Sodium Metaperiodate – Peak at 1.19 minutes, $[M\text{-}H]^-$ *m/z* 313.

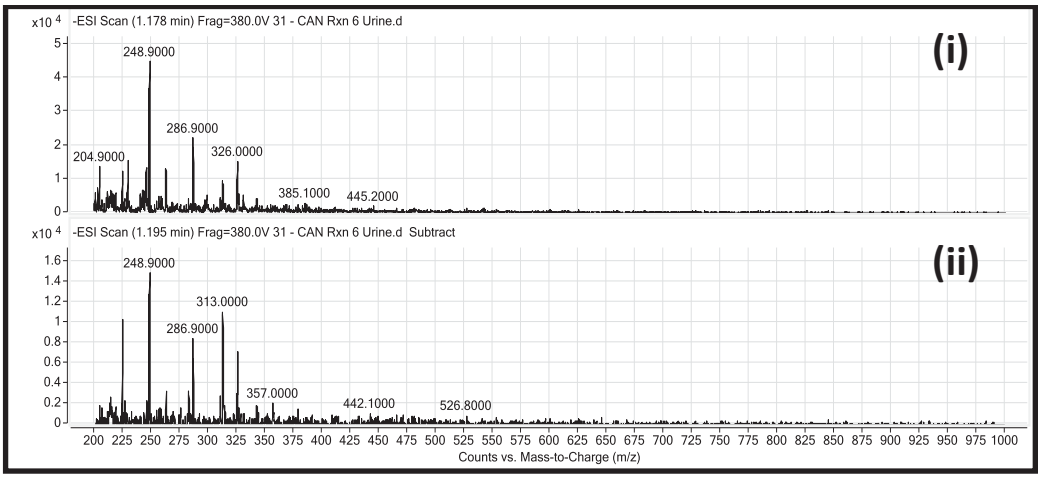


Figure 2.35 – Mass spectrum of: (i) Reaction product peak detected in ceric ammonium nitrate reaction in urine; (ii) Background-subtracted mass spectrum of reaction product peak, revealing characteristic $[M\text{-}H]^-$ at *m/z* 313.

A total of five oxidants were tested in this study, and analysis of reaction samples by LC-MS indicates that the oxidants were generally effective at reducing the total peak area of THC-COOH. For spiked urine samples, it is expected that increased oxidant concentration would result in the complete loss of THC-COOH from the samples, as seen with the ceric ammonium nitrate and potassium permanganate reactions in spiked water samples. Though potassium permanganate is the most readily available of the tested oxidants, the potential range of various oxidants that can be used to invalidate a drug-positive test result is significant for drug testing laboratories. As the potential effectiveness of less common oxidants becomes more widely known, it is possible that manufacturers of urine adulteration products may incorporate additional oxidants to their product lines, as seen with the presence of pyridinium chlorochromate in prior formulations of Urine Luck. Consequently, it may become critical for research to be undertaken in testing the effects of a range of oxidising adulterants in vitro to both determine their effectiveness at masking the presence of drug metabolites, and for their potential to form characteristic reaction products.

In terms of the oxidants tested in this study, three were found to reliably produce peaks in spiked water samples not detected in the THC-COOH standard or reagent blanks. Potential reaction products of water samples spiked with THC-COOH were detected in the ceric ammonium nitrate, sodium iodate and sodium metaperiodate reactions. Of these potential products, only the ceric ammonium nitrate product was detected in the spiked urine reaction samples, though under different reaction conditions it may be possible for the iodate and metaperiodate products to form in drug-positive urine samples.

2.3 – General Discussion

The goal of the three studies explored in this chapter was twofold: to assess the effects of a range of oxidising adulterants on the detection of THC-COOH; and to determine if exposure of THC-COOH to any of these adulterants would result in the formation of reaction products. Though it is useful to know that the use of oxidising adulterants may effectively mask a cannabis-positive urine sample, the detection of potentially stable reaction products can provide the means by which a drug testing laboratory can identify both drug-positive samples and cases of urine adulteration.

Initial studies into the effects of oxidising adulterants dealt with three selected oxidising agents: sodium hypochlorite (bleach), pyridinium chlorochromate, and potassium nitrite. Tested in spiked water samples, all three oxidants were found to produce reaction products that could potentially link attempts at urine adulteration with the masking of a cannabis-positive urine sample. For sodium hypochlorite, a total of three oxidation products were detected, with deprotonated molecules at *m/z* 377, 377 and 411. For the potassium nitrite reaction under acidic conditions, two products were formed, an unstable compound with a deprotonated molecule at *m/z* 372, and a stable product with a deprotonated molecule at *m/z* 388. For the pyridinium chlorochromate reaction, a single reaction product was detected, with a deprotonated molecule at *m/z* 313.

Investigation of the mass spectra for these products yielded five possible structures. For the hypochlorite reaction, it is expected that the three products represent two mono-chlorinated compounds, and one di-chlorinated compound. For the potassium nitrite reaction, an unstable nitroso-substituted THC-COOH product is observed, and based on a preliminary stability study over twelve days, appears to undergo further reaction to form a nitro-substituted THC-COOH compound. At this stage of the research, the structure of the PCC product is unable to be elucidated due to the limited fragmentation of the deprotonated molecule. Further information regarding the structural elucidation of the PCC product is present in Chapter 4.

Papain, a non-oxidising adulterant, was found to readily decrease the apparent peak area of THC-COOH in spiked water samples. Under the experimental conditions, no apparent reaction products are observed. This may be attributed to either complete degradation of the target analyte, or the formation of products that are not detected under the detection parameters used. As discussed previously, it does not appear that the effects of papain are mediated through an interference effect, as this is far more likely to affect screening-based techniques.

For the remaining oxidising adulterants, it is clear that these reagents are potentially effective in masking cannabis-positive urine samples, resulting in a false-negative test result. Though complete loss of THC-COOH was not observed in the spiked urine samples, significant decreases in peak area were detected. It is important to note that for these oxidants, a total of three potential reaction products were detected. Two of the detected compounds were determined to have a mass of 314 Da, with the mass of the remaining product formed in the sodium iodate reaction currently unknown due to the poor mass spectrum generated for the respective product peak.

From these studies dealing with the potential effects of adulterants on the detection of THC-COOH in spiked urine and/or spiked water samples, it is apparent that the potential for a range of oxidising and non-oxidising adulterants to mask a positive drug test result in significant for drug testing laboratories. At present, a number of products are available and ostensibly marketed to drug-using individuals that intend to mask a positive drug test result. This range of products is likely to change with changes in drug testing techniques and the detection of urine adulteration. As a consequence, drug testing laboratories may be faced with an expanding list of products allegedly capable of masking a positive drug test. Though a number of the chemicals tested during these studies discussed in this chapter are considered to be difficult to obtain from the perspective of the average consumer, many remain available. Potassium permanganate, Betadine and bleach are commonly available from pharmacies, and in the case of sodium iodate, it is present in iodised salt to combat iodine deficiency.

An added difficulty regarding the detection of urine adulteration is the issue of adulterants and their potential interfering effect on immunoassay-based screening techniques. In cases where an adulterant may form a stable reaction product with a drug metabolite, remaining traces of the adulterant may result in a false-negative screening test result, or alternately, the reaction products may not display cross-reactivity in the immunoassay screening. As such, it remains possible for, and hence the urine sample, containing stable reaction products, may not be submitted to confirmatory testing. Despite this, the detection of characteristic reaction products formed from drug metabolites may allow for drug testing laboratories to detect both cases of urine adulteration and drug use.

Chapter 3

Synthesis and Purification

In Chapter 2, three oxidants were selected for their ability to simultaneously degrade THC-COOH in vitro and reliably produce oxidation products of THC-COOH. Structural elucidation of the reaction products, detailed in Chapter 4, was undertaken by a combination of high-resolution mass spectrometry and nuclear magnetic resonance spectroscopy. For the purposes of structural elucidation by NMR, it is necessary to scale up the reactant ratios to produce milligram quantities of the major reaction products.

As noted in Chapter 2, the reaction between Betadine and THC-COOH is identical to the reaction that occurs between a methanolic solution of iodine and THC-COOH. The active ingredient in Betadine is a povidone-iodine complex. Other compounds present in this antiseptic include glycerine, a buffering agent and mixture of long-chain ethoxylated alcohol compounds. To minimise the effects of these additional components on the desired reaction with THC-COOH and to simplify product isolation and purification, synthesis of the Betadine reaction products was performed using the equivalent reaction with a methanolic iodine solution.

The Betadine and pyridinium chlorochromate reactions were assessed for their suitability for large-scale synthesis, as was the sodium hypochlorite reaction. The hypochlorite reaction was found through initial trials to be effectively unsuitable for large-scale synthesis due to the sensitivity of this reaction to slight variations in oxidant concentration. This sensitivity resulted in samples where formation of the three detected hypochlorite products was minimal and samples where a slight excess of hypochlorite lead to significant degradation of the reaction products. For the Betadine and pyridinium chlorochromate reactions, this apparent sensitivity to slight variations in oxidant concentration was not noted.

3.1 – Experimental

3.1.1 – Drug Standards and Reagents

THC-COOH stock solution (1 mg/mL in methanol) was obtained from Cerilliant (Round Rock, Texas, USA). Ultrapure water was obtained from the Sartorius Arium® 611 Laboratory Water Purification System equipped with a Sartopore 0.2 µm membrane filter. Pyridinium chlorochromate and iodine were obtained from Sigma Aldrich (Castle Hill, NSW, Australia). Betadine, a topical antiseptic produced by Sanofi (NSW, Australia) was sourced from a local pharmacy. Acetonitrile, hydrochloric acid (0.5M), methanol, n-hexane and ethyl acetate were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). All solvents were of analytical or HPLC grade.

3.1.2 – Instrumentation

Small-Scale Synthesis and Large-Scale Synthesis Tests

Analyses of synthesis reaction mixtures were undertaken on Agilent Technologies 1290 LC system coupled to a 6490 triple quadrupole (QQQ) mass spectrometer (Forest Hills, Victoria, Australia). Chromatographic separation of analytes was undertaken on a Phenomenex Luna C_5 HPLC column (150 mm x 4.6 mm, 5 micron, Phenomenex Incorporated). Mobile phase A consisted of 100% ultrapure water, and mobile phase B was 100% acetonitrile. The elution condition in this study was isocratic (15% Solvent A, 85% Solvent B). **Table 3.1**, below, notes the instrument parameters used in this study. Monitoring of the small scale reactions and large-scale reactions was undertaken in Selected Reaction Monitoring mode (SRM) to increase instrument sensitivity and to focus solely on reaction progression, degradation of THC-COOH, and formation of reaction products. For the Betadine reaction, analyses were performed in negative ion mode, and the pyridinium chlorochromate reaction, analyses were performed in positive ion mode.

Table 3.1 - Instrument parameters for Agilent Technologies 1290 LC system and 6490 QQQ system.

LC-MS System Parameters		Setting
LC Parameters	Solvent Flow Rate	0.5 mL/min
	Injection Volume	2.5 µL
	Column Temperature	40°C
	Method Runtime – Betadine Samples	10.0 minutes
	Method Runtime – PCC Samples	16.0 minutes
QQQ Parameters	Mass Range (*m/z*) – Betadine Samples	50 – 750
	Mass Range (*m/z*) – PCC Samples	50 – 1000
	Fragmentor Voltage	380 V
	Sheath Gas Temperature	250°C
	Sheath Gas Flow Rate	11 L/min
	Scan Mode	SRM

Isolation of Reaction Products

Method development for the isolation of reaction products was performed on a Perkin Elmer Sciex API 365 LC-MS/MS system (Massachusetts, USA), coupled with an Alltech 530 column heater (Illinois, USA) and Applied Biosystems 785A Programmable Absorbance Detector (Victoria, Australia). Ionisation of the analytes was achieved with the use of an electrospray ionisation interface in negative ionisation mode. Chromatographic separation of analytes was undertaken on the Phenomenex Luna C_5 column, as per the small-scale and large-scale synthesis tests, and was maintained at 30°C in an Alltech 530 column heater. During development of the product isolation method, the eluent was directed post-column through a flow splitter (1:1 split). Equal volumes of eluent were directed to the LC-MS/MS system and to the Applied Biosystems 785A Programmable Absorbance Detector (PAD), which was set to monitor the eluent at 210 nm. Following development of the isolation method, isolation of the products was performed by removing the flow splitter and directing the eluent solely through the Applied Biosystems 785A Programmable Absorbance Detector. The elution condition used for the isolation of the reaction products was isocratic (15:85); mobile phase A consisted of 100% ultrapure water and mobile phase B was 100% acetonitrile. System parameters for the LC-MS system are shown in **Table 3.2**.

Table 3.2 - Instrument parameters for the Perkin Elmer LC-MS/MS system and Programmable Absorbance Detector.

LC-MS System Parameters		Setting
Gas Flow Rate	Nebuliser Gas (NEB)	10
	Curtain Gas (CUR)	10
	Collision Gas (CAD)	0
Control Settings	Ion Spray Voltage (IS)	-4000 V
	Temperature (TEM)	300°C
	Orifice Voltage (OR)	-40.0
	Focus Ring Voltage (RNG)	-140.0
	Q0 Rod Offset (Q0)	5.0
	Q2 Entrance Lens (IQ2)	19.0
	Q0 Rod Offset – Collision Energy	80.0
	CEM Deflection Plate	400.0
	Channel Electron Multiplier (CEM)	2000.0
Analysis Settings	Number of Scans	500
	Method Runtime – Iodine Reaction	20 mins
	Method Runtime – PCC Reaction	14 mins
	Mass Range (*m/z*) – Iodine Reaction	300 – 600
	Mass Range (*m/z*) – PCC Reaction	100 – 400
	Absorbance Wavelength (PAD)	314nm
	Injection Volume	20 µL

3.1.3 – Experimental Procedures

Small-Scale Reaction Tests

Small-scale tests of the reaction between THC-COOH and methanolic iodine solutions and pyridinium chlorochromate were trialled to confirm that adjustment of these reactions to accommodate 10 mg of THC-COOH would produce sufficient quantities of the reaction products detected in these reactions. Major reaction products were defined as the analytes produced in the highest relative proportion in a sample following reaction at room temperature for a total of seven days. For the purposes of this experiment, and for all other experiments, room temperature was determined through regular monitoring of the experimental space at various points throughout the day, and was determined to be, on average, 23°C. In addition, it is important to note that, unless specified otherwise, all samples were stored in the dark when not undergoing analysis. For the iodine reaction, the major reaction product was defined as the proposed di-iodo-THC-COOH ($[M-H]^-$ *m/z* 595). For the pyridinium chlorochromate reaction, the sole reaction product ($[M-H]^-$ *m/z* 313) was identified as the major product.

The iodine test reaction was prepared by the addition of 8.0 mL of a methanolic iodine working solution (3.152 mM) to a spiked water solution containing THC-COOH. The final volume of the small-scale test reaction was 10 mL, and the final concentration of THC-COOH in this reaction mixture was 40 µg/mL (400 µg of THC-COOH).

The pyridinium chlorochromate test reaction was prepared by the addition of 4.5 mL of a PCC working solution (40 µg/mL, 0.185 mM) to a spiked water solution containing THC-COOH. The final volume of the small-scale test reaction was 10 mL, and the final concentration of THC-COOH in this reaction mixture was 40 µg/mL (400 µg of THC-COOH).

Following preparation and subsequent reaction of the small-scale test samples at room temperature for seven days, samples were transferred to 15 mL glass centrifuge vials and centrifuged at 8000 rpm for 30 minutes to separate out any precipitates or particulate matter present in the reaction mixtures. An aliquot of the supernatant (500 µL) was transferred to an amber-coloured glass GC vial, sealed and refrigerated at 4°C for storage prior to analysis. The remaining supernatant from these reactions was collected and transferred to separate round bottom flasks for storage.

Large-Scale Reaction – Iodine Reaction

Following analysis of the small-scale reaction mixtures by LC-MS/MS, samples were prepared for large-scale synthesis. Due to the limited availability of THC-COOH, the reactant ratios were not scaled up. Synthesis of products was achieved by producing replicates of the samples tested in the small-scale synthesis. Twenty-five replicates of the 400 µg reactions were prepared for the iodine and pyridinium chlorochromate reactions, with a total of 10 mg of THC-COOH present in each reaction set. Samples were allowed to react at room temperature for a total of seven days, with random samples of these reactions tested throughout the reaction period to monitor the progress of the reaction.

Following the seven day reaction period, the respective reaction mixtures were decanted into two 500 mL separation funnels. The reaction mixtures were acidified with 0.5 M hydrochloric acid to pH 5. Liquid-liquid extraction of the mixtures was undertaken with an organic phase prepared from a 1:4 mixture of ethyl acetate and n-hexane. Extraction of the products was done in triplicate, with 150 mL of the organic phase used in each extraction. The organic fractions from the two reactions were recovered after each extraction step, and transferred to separate 500 mL round-bottom flasks. Extracted organic fractions were dried down under a gentle stream of nitrogen gas at 30°C. The remaining residues from both reactions were reconstituted in 10 mL of methanol, transferred to scintillation vials and dried overnight in a desiccator under vacuum.

Isolation of Reaction Products

The dried residues prepared via the large-scale syntheses were separately reconstituted with 1.0 mL of methanol and transferred to amber-coloured glass GC vials and stored prior to purification of the reaction products present. Isolation of the products was achieved through liquid chromatography, using the Perkin Elmer Sciex API 365 LC system. Detection of the reaction products and other compounds present was achieved through the use of mass spectrometry and the Applied Biosystems 785A Programmable Absorbance Detector.

Isolation of the reaction products was achieved through chromatographic separation followed by drop-wise collection of the eluent post-programmable absorbance detector. Development of this purification method was achieved through preparation of dilute samples of the reconstituted reaction mixtures (1:100 dilution), and post-column, were monitored by both mass spectrometry and UV-Vis spectroscopy. Peaks detected in the chromatograms were

matched with the mass spectra and UV-Vis spectra, and retention times of major peaks identified (**Figure 3.1, Figure 3.2**).

Matching of the major peaks in both data sets allowed for the mass spectrometer to be decoupled from the flow splitter, thereby directing 100% of the eluent to the programmable absorbance detector. The collection method for the identified fractions for each reaction mixture is shown in **Table 3.3**, and notes the timing system used for the drop-wise collection of the targeted fractions containing the desired reaction products.

Table 3.3 - Timing method for purification of reaction products.

Reaction	Fraction	Time Period (min)
Betadine/Iodine	1	0.00 – 4.50
	2	4.50 – 7.00
	3	7.00 – 8.50
	4	8.50 – 11.00
	5	11.00 – 14.00
	6	14.00 – 17.00
Pyridinium Chlorochromate	1	0.00 – 5.00
	2	5.00 – 7.50
	3	7.50 – 9.00
	4	9.00 – 10.00
	5	10.00 – 15.00

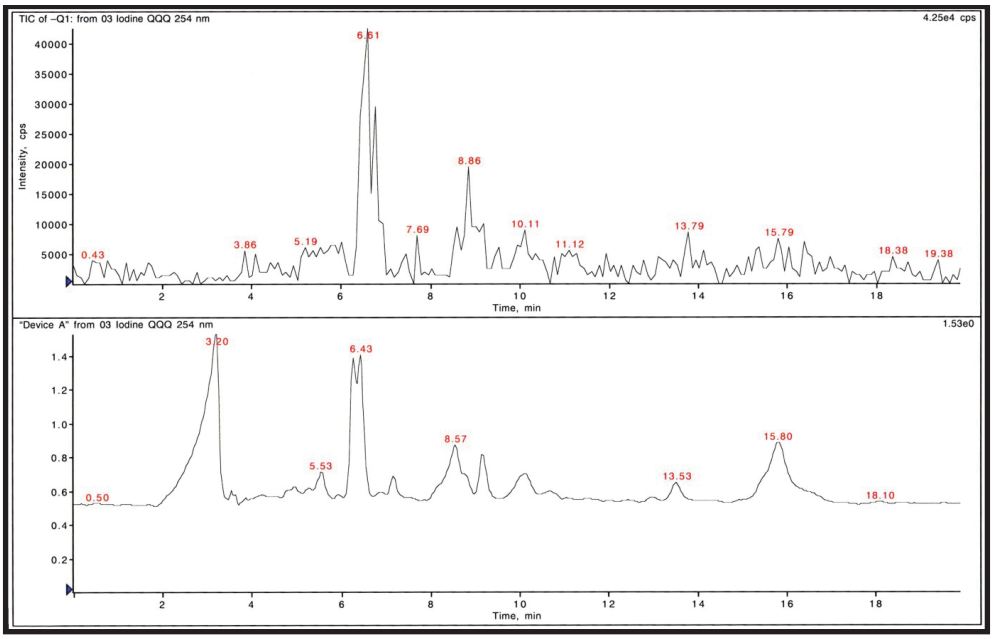


Figure 3.1 - Iodine Reaction. (Top) Chromatogram obtained via LC-MS; (Bottom) UV-Vis spectrum recorded at 314 nm. Traces of THC-COOH elute at 4.23 minutes, the mono-iodo substituted analogues of THC-COOH elute at 6.43 and 8.57 minutes respectively, and the di-iodo substituted analogue of THC-COOH elutes at 15.80 minutes

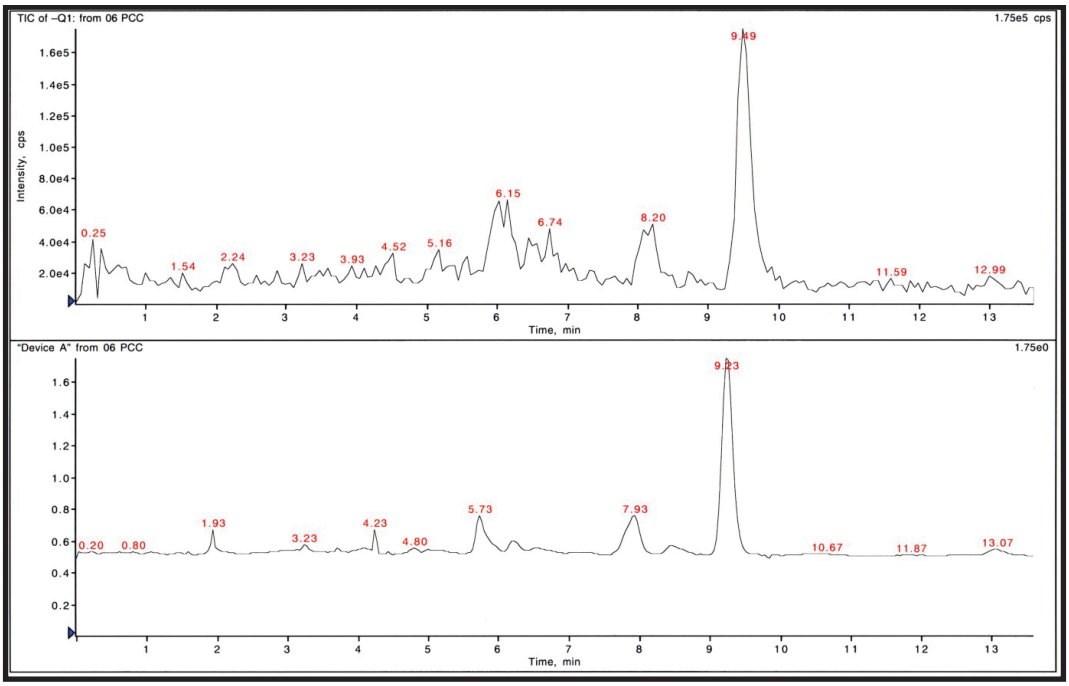


Figure 3.2 - PCC Reaction. (Top) Chromatogram obtained via LC-MS; (Bottom) UV-Vis spectrum recorded at 314 nm. Unreacted THC-COOH elutes at 5.73 minutes, and the sole reaction product elutes at 9.23 minutes.

Each injection of the reaction mixtures into the LC system was set at 20 µL. The eluent for each fraction was collected in a labelled scintillation vial and dried down under a gentle stream of nitrogen gas at 35°C. Following the injection of the total volume of the reconstituted reaction product mixtures, 500 µL of methanol was pipetted into each vial. The vials were sealed, agitated, and then subjected to the same drop-wise collection procedure outlined above, in order to maximise recovery of the reaction products. Fractions obtained during this second round of chromatographic separation were combined with their respective fractions obtained from the initial chromatographic separation. The combined fractions were dried down under nitrogen gas at 30°C and then placed in a desiccator under vacuum overnight.

Assessing Fraction Purity

The relative purity of the major products from the iodine reaction and PCC reaction were assessed by LC-MS in negative ionisation mode. Diluted samples of the fractions for analysis were prepared by first reconstituting the dried fractions from the isolation method with 1.0 mL of methanol. From this, 10 µL aliquots were transferred to amber-coloured glass GC vials and made up to a final volume with 490 µL of methanol. Following analysis, major peaks detected in the chromatograms of these samples were integrated and the relative purity calculated.

3.2 – Results and Discussion

Synthesis of the reaction products detected in the Betadine/iodine and PCC reactions represents a critical step towards structural elucidation of the major products by NMR. Monitoring of the reaction between THC-COOH and the selected oxidising agents in a small-scale test of this synthesis confirmed the production of the major reaction products in a reliable manner. Ordinarily, large-scale synthesis of the reaction products following successful small-scale testing would be achieved through scaling of the reaction to proportionally increase the concentration of the reactants. In the case of this study, issues pertaining to the availability of THC-COOH presented a significant problem. Preparation of a series of replicates of the successful small-scale tests was seen as a suitable alternative to the scaling of the reactant concentrations, as this would allow for the monitoring of the reactions over a period of time and minimise the risk of poor product yields due to variations in oxidant concentration. In **Figure 3.3** and **Figure 3.5** a comparison of the small-scale and large-scale tests of the iodine reaction reveals slight changes in the proportion of the products formed. This variation is expected to arise due to changes in oxidant concentration.

Chromatographic separation of the desired products from unreacted starting material and contaminants formed in the reactions was achieved via LC-MS of the reaction mixtures. Repeated injections of the reaction mixtures were passed through a chromatographic column and through drop-wise collection of the eluent after passing through the programmable absorbance detector, reasonably pure fractions containing the desired products was achieved.

Small-Scale Reaction Tests

Small-scale synthesis of the products detected in reactions between THC-COOH and Betadine/iodine and PCC was achieved through the exposure of aqueous solutions of THC-COOH to concentrated methanolic iodine and aqueous PCC solutions. The reaction mixtures were allowed to react at room temperature for a total of seven days. Following reaction of the samples, 500 µL aliquots of the two reactions were subjected to analysis by LC-MS/MS. Analysis was undertaken in Selected Reaction Monitoring (SRM) mode to specifically monitor degradation of THC-COOH in the sample, and the subsequent formation of the desired reaction products. The Betadine/iodine reaction produced three reaction products: two mono-iodinated analogues of THC-COOH (mono-iodo-THC-COOH, $[M-H]^-$ *m/z* 469) and a major reaction product designated as the di-iodo analogue of THC-COOH (di-iodo-THC-COOH, $[M-H]^-$

m/z 595). The reaction between pyridinium chlorochromate and THC-COOH produced a single major product ([M-H]$^-$ *m/z* 313). **Figure 3.3** and **Figure 3.4** depict the chromatograms and SRM data obtained from the analysis of the small-scale reaction tests of Betadine/iodine and PCC, respectively. **Table 3.4** sets out the retention times and relative proportion of compounds present in the small-scale synthesis reactions. The relative proportion of compounds present was calculated based on the total peak area of all major peaks present in the chromatogram.

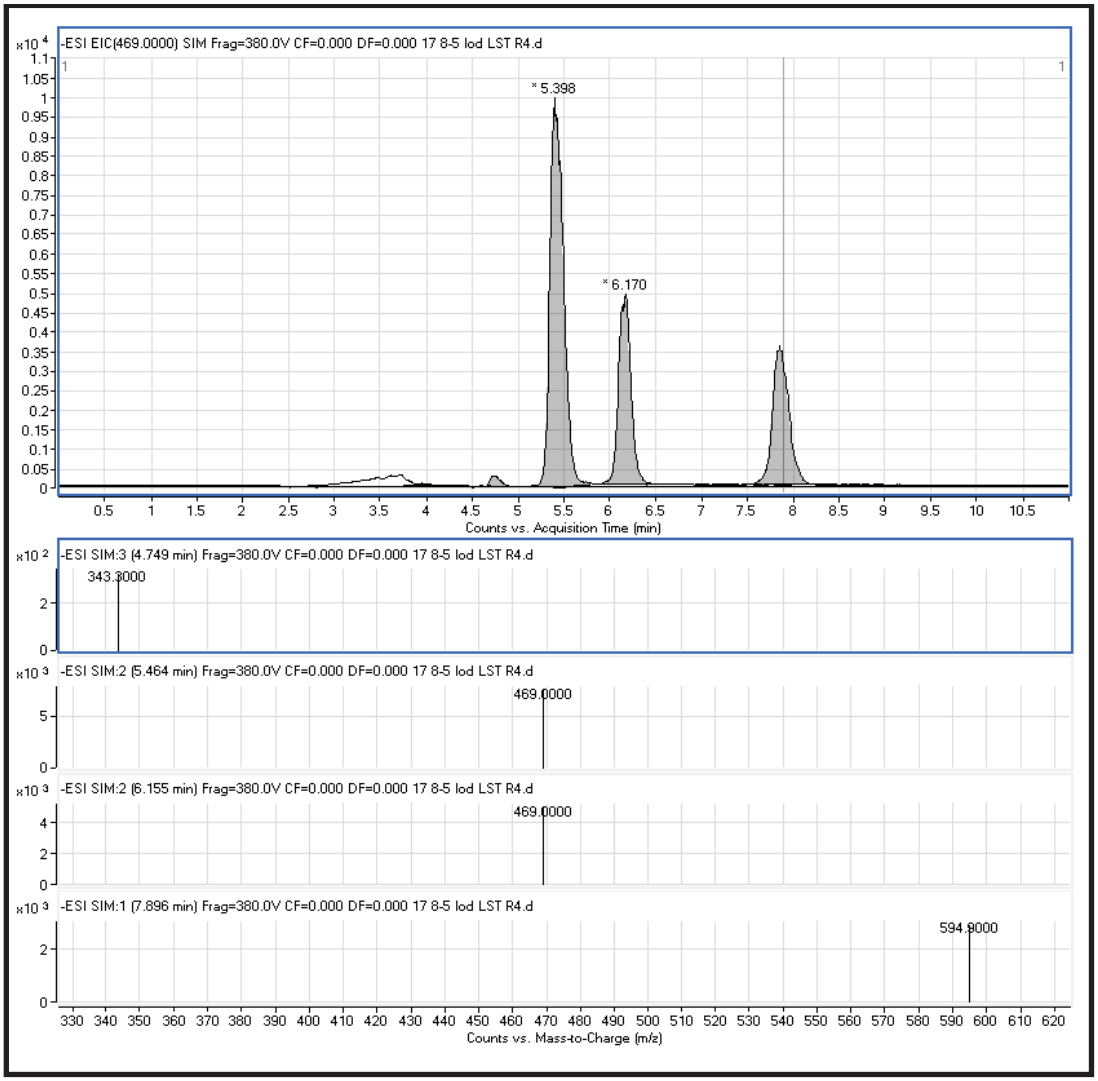


Figure 3.3 - Chromatogram and SRM data for small-scale Betadine/iodine synthesis test. THC-COOH ([M-H]$^-$ *m/z* 343) elutes at 4.75 minutes, mono-iodo-THC-COOH product 1 ([M-H]$^-$ *m/z* 469) elutes at 5.50 minutes, mono-iodo-THC-COOH product 2 ([M-H]$^-$ *m/z* 469) elutes at 6.16 minutes, and di-iodo-THC-COOH ([M-H]$^-$ *m/z* 469) elutes at 7.90 minutes.

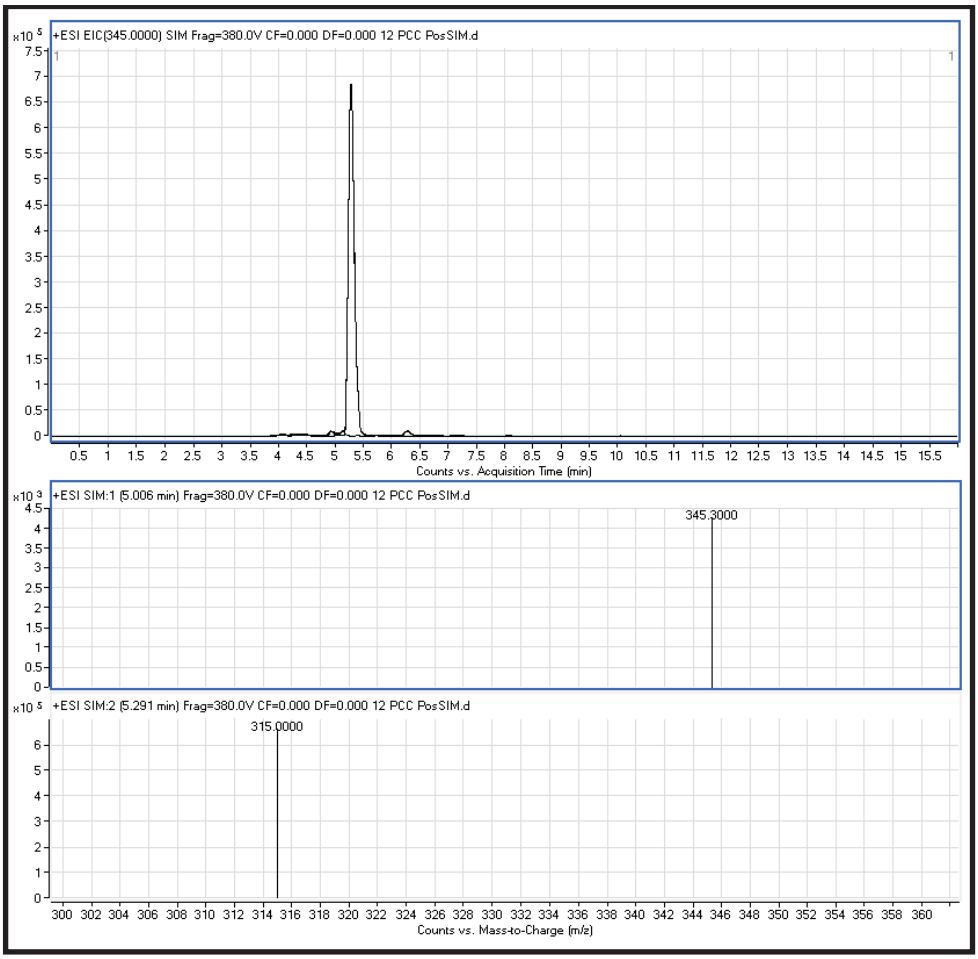


Figure 3.4 - Chromatogram and SRM data for small-scale pyridinium chlorochromate synthesis test. Unreacted THC-COOH ($[M+H]^+$ *m/z* 345) elutes at 5.00 minutes, and the PCC reaction product ($[M+H]^+$ *m/z* 315) elutes at 5.29 minutes.

Table 3.4 - Relative formation of desired reaction products in the small-scale Betadine/iodine and PCC reactions.

Reaction	Compound	Retention Time (minutes)	Proportion – Peak Area (%)
Betadine/Iodine	THC-COOH	4.75	1.13
	Mono-iodo product 1	5.50	52.73
	Mono-iodo product 2	6.16	23.43
	Di-iodo product	7.90	22.70
PCC	THC-COOH	5.00	0.05
	PCC Product	5.29	99.50

Large-Scale Reaction Tests

Large-scale synthesis of the major reaction products was achieved through the preparation of replicate samples based on the reactant ratios from the successful small-scale synthesis tests. Analysis of aliquots obtained from the reaction mixtures following the reaction period were found to be in agreement with the results of the small-scale synthesis tests. Analysis was undertaken in Selected Reaction Monitoring (SRM) mode, as the goal of these analyses was solely to monitor the progress of the reaction and to confirm formation of the reaction products. **Figure 3.5** and **Figure 3.6** depict the total ion chromatograms (TIC) and SRM spectra of the major peaks detected in the analyses of the iodine and PCC reactions, respectively. **Table 3.5** outlines the retention times and relative proportion of compounds present in the large-scale synthesis reactions.

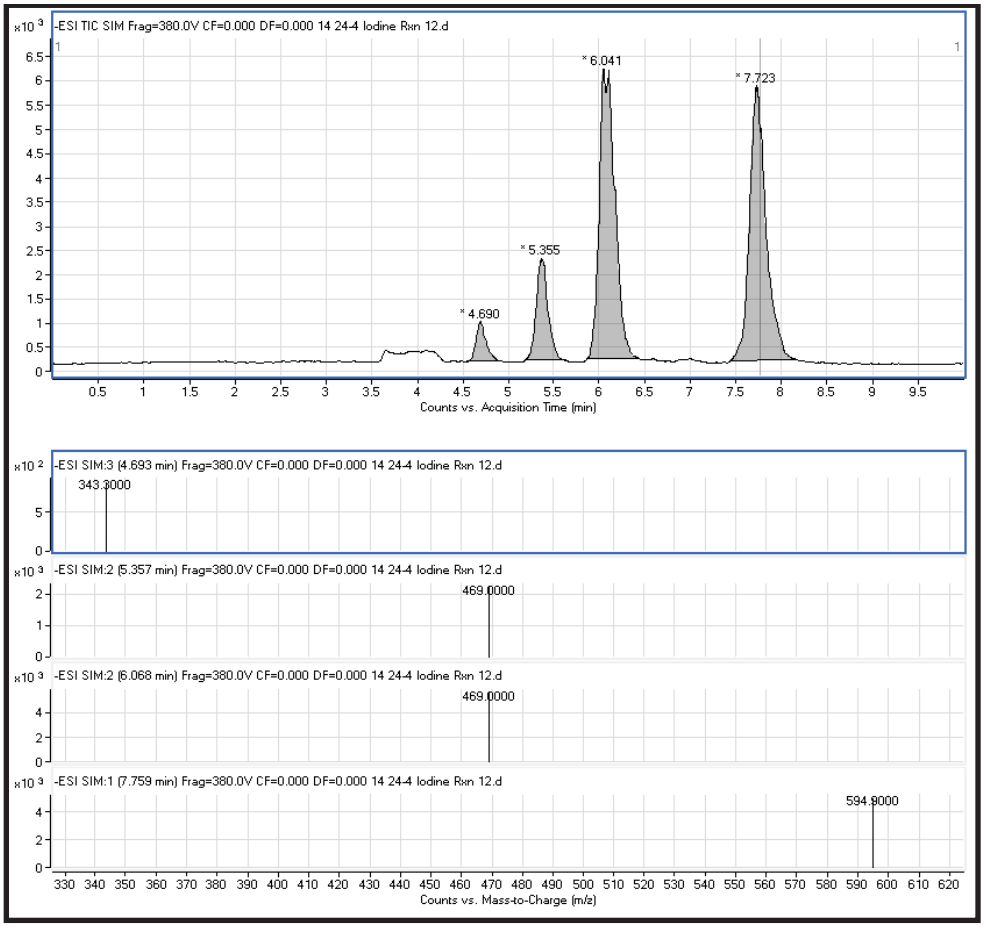


Figure 3.5 – Chromatogram and SRM data for large-scale Betadine/iodine synthesis test. THC-COOH ($[M-H]^-$ *m/z* 343) elutes at 4.70 minutes, mono-iodo-THC-COOH product 1 ($[M-H]^-$ *m/z* 469) elutes at 5.36 minutes, mono-iodo-THC-COOH product 2 ($[M-H]^-$ *m/z* 469) elutes at 6.07 minutes, and di-iodo-THC-COOH ($[M-H]^-$ *m/z* 469) elutes at 7.80 minutes.

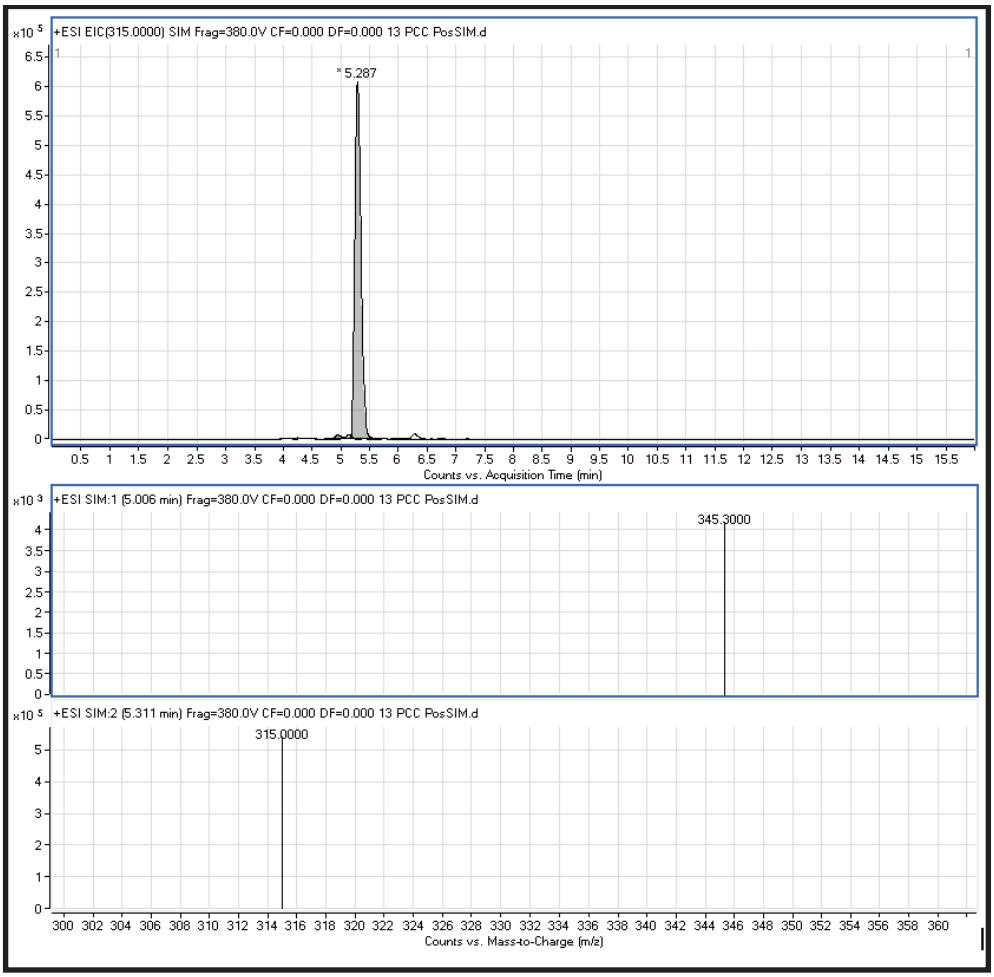


Figure 3.6 – Chromatogram and SRM data for large-scale pyridinium chlorochromate synthesis test. Unreacted THC-COOH ($[M+H]^+$ *m/z* 345) elutes at 5.00 minutes, and the PCC reaction product ($[M+H]^+$ *m/z* 315) elutes at 5.31 minutes.

Table 3.5 - Relative formation of desired reaction products in the large-scale Betadine/iodine and PCC reactions.

Reaction	Compound	Retention Time (minutes)	Percent of Total Peak Area (%)
Betadine/Iodine	THC-COOH	4.70	3.61
	Mono-iodo Product 1	5.36	11.14
	Mono-iodo Product 2	6.07	42.71
	Di-iodo Product	7.80	42.54
PCC	THC-COOH	5.00	1.91
	PCC Product	5.31	98.09

Isolation

Initial tests of the isolation method (**Figure 3.7 and Figure 3.8**) indicate that careful collection of fractions via this method would allow for the successful purification of the major reaction products from the iodine and PCC reactions. Analysis of the chromatograms of the reconstituted reaction mixtures indicated the presence of several major peaks indicating the presence of impurities in these samples. Analysis of the mass spectra of these peaks allowed for the identification of the desired reaction products, and as shown in **Table 3.6**, also allowed for the identification of contaminants present in the reconstituted reaction mixtures. In addition, for the iodine reaction, Fraction 1, collected between 0.00 and 4.50 minutes, revealed the presence of excess, unreacted iodine from the initial reaction, and was confirmed due to Fraction 1 exhibiting a strong yellow colouration during fractionation. During collection, no other fractions exhibited noticeable colouration.

Table 3.6 – Guidelines for collection of fractions from reaction mixtures, with unreacted starting material, reaction products and contaminants detected.

Reaction	Fraction	Time Period (min)	Detected Compounds
Betadine/Iodine	1	0.00 – 4.50	Excess iodine
	2	4.50 – 7.00	Excess THC-COOH (*m/z* 343)
	3	7.00 – 8.50	Iodine product 1 (*m/z* 469)
	4	8.50 – 11.00	Iodine product 2 (*m/z* 469)
	5	11.00 – 14.00	Contaminant
	6	14.00 – 17.00	Iodine product 2 (*m/z* 595)
Pyridinium Chlorochromate	1	0.00 – 5.00	No detected compounds
	2	5.00 – 7.50	Trace THC-COOH (*m/z* 343)
	3	7.50 – 9.00	Contaminant
	4	9.00 – 11.00	PCC Product (*m/z* 313)
	5	11.00 – 15.00	No detected compounds

Following collection of the fractions, each fraction was dried down under nitrogen gas, as discussed previously. Visual inspection of the dried fractions was undertaken, with observations recorded for each fraction. Post-drying, Fraction 2 from the iodine reaction revealed the presence of approximately 2 – 3 milligrams of an off-white powder. This powder

was determined to be unreacted THC-COOH. For the remaining fractions obtained from the iodine reaction, Fraction 3 yielded a small quantity of a brown resinous substance. Only a small quantity of material was recovered from Fraction 4, and like Fraction 3, consisted of a brown resinous substance. The dried extract from Fraction 6 contained approximately 3 – 4 milligrams of a yellow/brown oily substance that was found to be readily soluble in methanol.

Of the fractions obtained from purification of the PCC reaction, two were identified as containing unreacted THC-COOH and the expected major reaction product. Post-drying, Fraction 2 was found to contain a sub-milligram quantity of an off-white powder, which was positively identified as the unreacted THC-COOH. Fraction 4 yielded approximately 4 – 5 milligrams of a dark brown resinous material, and was identified as the PCC reaction product. Solubility of this resinous material was tested during reconstitution of this fraction in 1.0 mL of methanol, with the resinous material readily dissolved.

Estimated Purity of the Major Reaction Products

Chromatograms, extracted ion chromatograms (EIC) and mass spectra for the tested fractions are shown in **Figure 3.7** and **Figure 3.8**. Retention times and peak areas of the major peaks and relative purity of the two fractions is outlined in **Table 3.7**. Extraction ion chromatograms were used for the calculation of the purity of the fractions due to the highly dilute nature of the samples. Analysis of major peaks present in the chromatograms obtained from these samples not associated with the expected compounds is attributed to baseline noise.

The main fraction obtained from the isolation of the iodine reaction products was expected to contain the proposed compound di-iodo-THC-COOH ($[M-H]^-$ *m/z* 595). Analysis of this fraction confirmed the presence of this major product, as well as a significant quantity of one of the proposed mono-iodinated products ($[M-H]^-$ *m/z* 469). The presence of this mono-iodinated product may be attributed to either contamination of the major product during the isolation procedure or possible degradation of the di-iodo-THC-COOH product during sample storage to form one of the mono-iodinated THC-COOH species.

Analysis of the main product fraction obtained through purification of the PCC reaction indicated that a high yield of the PCC reaction product ($[M-H]^-$ *m/z* 313) was obtained. Traces of THC-COOH were detected in the tested fraction, with slight co-elution occurring between

the THC-COOH and reaction product peaks. Presence of THC-COOH in the PCC product fraction is attributed to contamination during the isolation procedure.

Table 3.7 – Retention times and calculated relative purity of compounds detected in the major product fractions collected following large-scale synthesis.

Fraction	Compound	Retention Time (minutes)	Percent of Total Peak Area (%)
Iodine Fraction	Mono-iodo product	6.97	25.63
	Di-iodo product	11.46	74.37
PCC Fraction	THC-COOH	5.50	6.37
	PCC Product	5.68	93.63

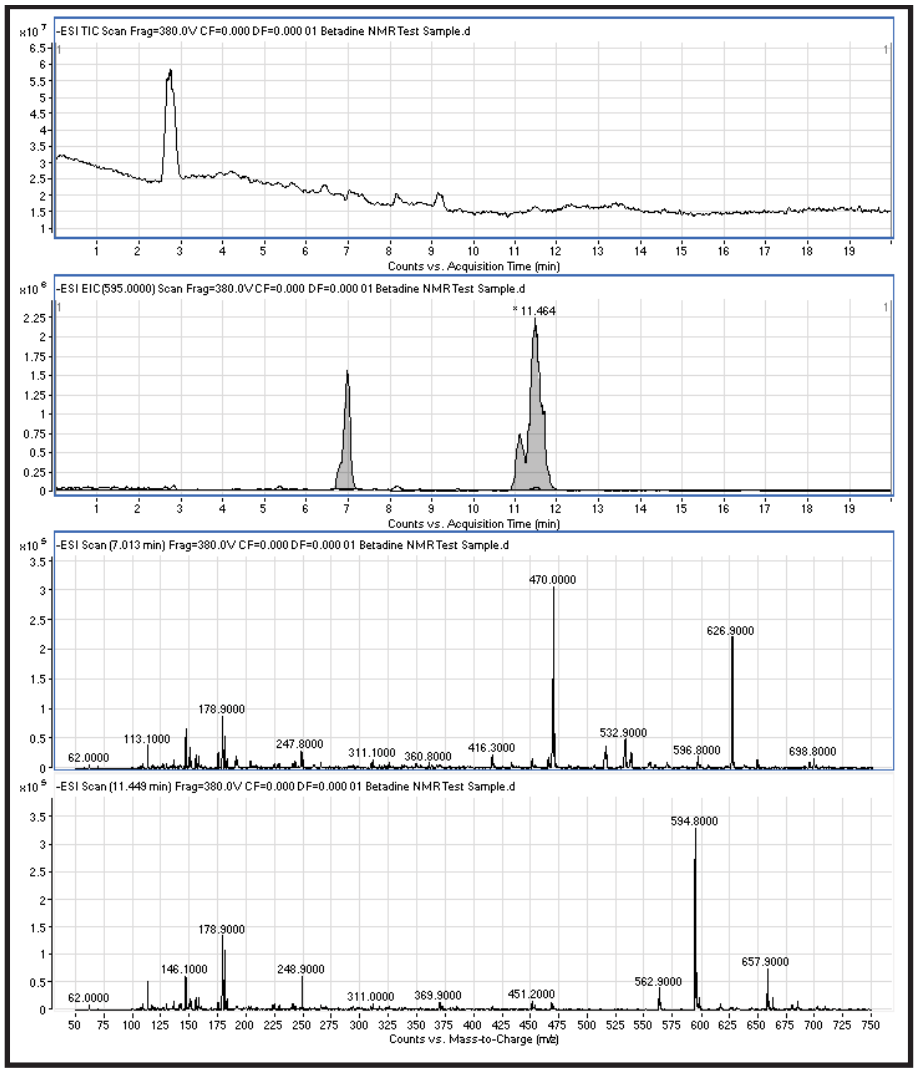


Figure 3.7 – Chromatogram, extracted ion chromatogram and mass spectra for purity testing of fraction containing the major iodine reaction product. Mono-iodo-THC-COOH product 2 ($[M-H]^-$ *m/z* 469) elutes at 7.01 minutes, and di-iodo-THC-COOH ($[M-H]^-$ *m/z* 595) elutes at 11.50 minutes.

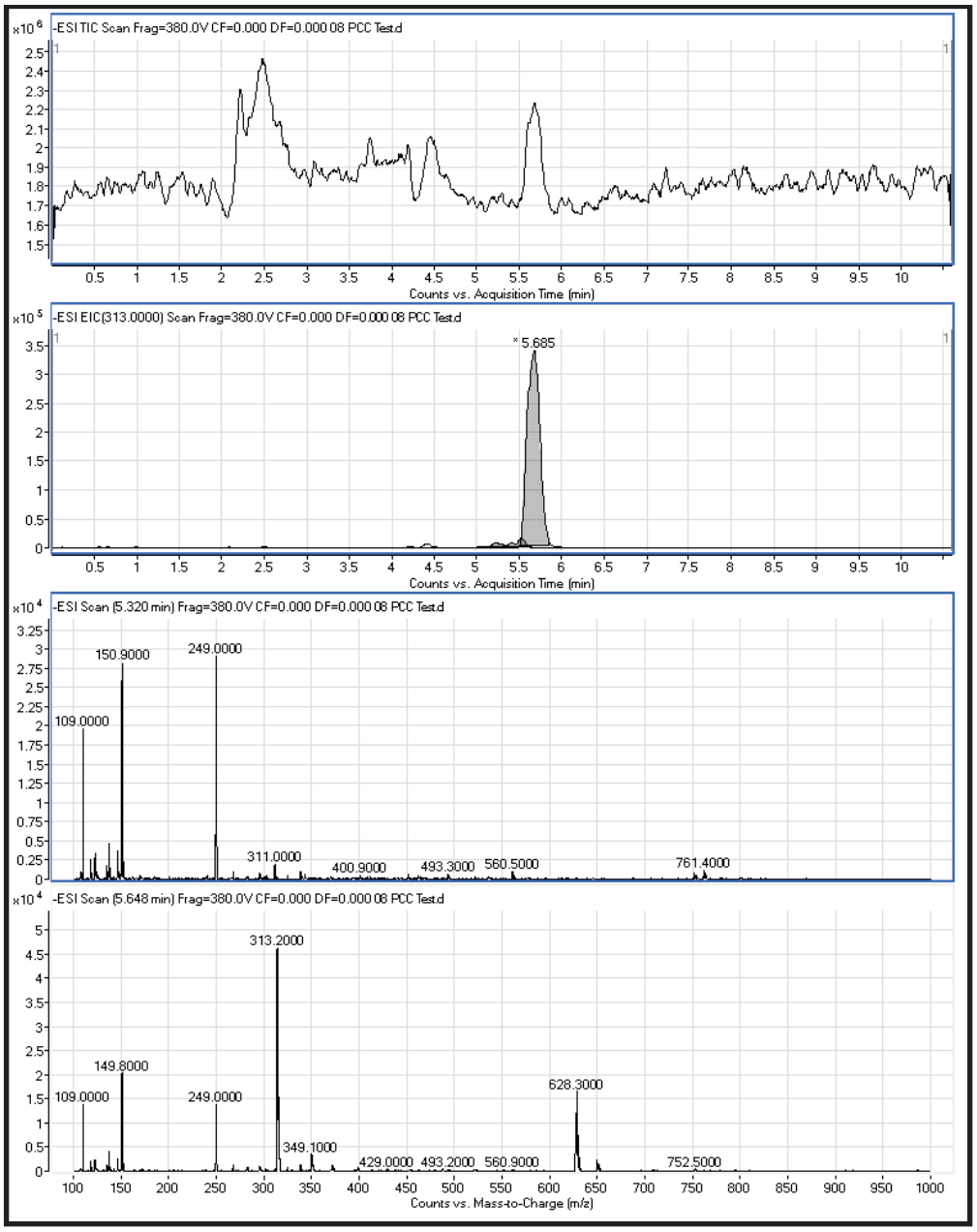


Figure 3.8 – Chromatogram, extracted ion chromatogram and mass spectra for purity testing of fraction containing the major PCC reaction product. Unreacted THC-COOH ($[M+H]^+$ *m/z*345) elutes at 5.32 minutes, and the PCC reaction product ($[M+H]^+$ *m/z* 315) elutes at 5.65 minutes.

General Discussion

The aim of this study was to assess the potential for large-scale synthesis of the major reaction products formed in the reactions between THC-COOH and both pyridinium chlorochromate and Betadine/iodine. Successful small-scale synthesis confirmed the potential of these reactions to form the desired products in water. As mentioned previously, the low quantity of THC-COOH available for these reactions meant that increasing the concentration of the reagents in the reaction mixture was not viable, as an error in preparation could result in a poor product yield. The chosen alternative was to produce replicate samples of the successful small-scale reaction mixtures. Consequently, reaction progress was monitored over both reactions throughout the reaction period by transferring aliquots of the reaction mixtures to GC vials for analysis by LC-MS.

Isolation of the products was carried out via the drop-wise collection of fractions following chromatographic separation on a liquid chromatography column. Purity of the isolated fractions was reasonable, though contamination issues were noted. It is likely that the source of the contamination was the stainless steel tubing from the photodiode array from which the fractions were collected. Traces of the previous fractions may have remained present in the tubing, contributing to the presence of unwanted compounds in specific fractions.

Further evidence of this contamination is the presence of compounds from preceding fractions being present in the fractions of the major reaction products. In the case of the pyridinium chlorochromate reaction, this resulted in unreacted THC-COOH being found in the major product fraction. Similarly, one of the mono-iodinated THC-COOH products was detected in the di-iodo product fraction in a significant quantity. The proportion of contaminants appears to be related to the time between fractions, as with the pyridinium chlorochromate reaction, a three minute period existed between elution of the unreacted starting material and the desired product. In the case of the Betadine/iodine reaction, this delay was two minutes.

It is important to note that in the above experiments, analyses were undertaken in both negative ionisation mode and positive ionisation mode. This was carried out to assess whether positive ionisation mode would provide additional information relating to the fragmentation of the THC-COOH and the desired reaction products, and in turn, provide further details in terms of structural elucidation. Though peak areas and instrument response for THC-COOH were favourable and provided acceptable fragmentation data, this was found to not be the case for the bulk of the reaction products. Indeed, in the case of the iodinated reaction products, poor

instrument response and limited fragmentation of the reaction products resulted in positive ionisation mode being disregarded as a useful method of analysis. In addition, though other reaction products did undergo extensive fragmentation in positive ionisation mode, no additional significant fragmentation patterns were found, and as was the case with the iodinated reaction products, generally resulted in low sensitivity to the aforementioned products.

As the next chapter deals with structural elucidation, the solubility of the isolated products was tested in both chloroform and methanol, as the deuterated analogues of these solvents are commonly used in NMR. Both products were noted to be dark brown resinous materials, with both found to be readily soluble in methanol, forming yellow/brown coloured solutions. In contrast, solubility of the reaction products in chloroform was found to be poor, with both products failing to fully dissolve in 0.5 mL of chloroform.

Chapter 4

Structural Elucidation

In Chapter 2 the potential for oxidising adulterants to form reaction products with THC-COOH was explored, with a number of viable candidates discovered that may serve as markers of both cannabis use and subsequent urine adulteration. In Chapter 3, two reaction products from the reactions of pyridinium chlorochromate and Betadine with THC-COOH were selected for large-scale synthesis. These compounds were selected based on their relative ease of synthesis compared to the other reaction products found in the reaction of THC-COOH with Betadine and bleach. In the case of bleach, large-scale synthesis was not attempted due to difficulties arising from subtle variations in sodium hypochlorite concentration, and though the three bleach products were detectable in both spiked water and urine samples, yields of these compounds were poor.

In the case of the proposed di-iodinated reaction product formed in the Betadine reaction, this compound is proposed to form through the electrophilic aromatic substitution of two iodine atoms on to the two free aromatic positions present in THC-COOH. It is also expected that the proposed di-chlorinated bleach product and the four mono-halogenated products from the Betadine and bleach reactions also form through this same mechanism. As such, characterisation of the di-iodinated product is expected to serve as a proof of concept for the reaction mechanisms of the products formed through exposure of these two adulterants to THC-COOH.

The goal of the studies discussed in this chapter is to propose viable chemical structures for the reaction products detected in the main study found in Chapter 2, and may be achieved through a variety of instrumental techniques. Specifically, through the use of high-resolution accurate mass spectrometry (LC-QToF), Nuclear Magnetic Resonance spectroscopy (NMR) and the tentative identification of major product ions by LC-MS/MS, viable candidates for the structural formulae of the reaction products are to be proposed.

4.1 – Experimental

4.1.1 – Drug Standards and Reagents

Deuterated methanol (methanol-d_4) and deuterated THC-COOH (THC-COOH-d_9) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Dried and purified fractions of the pyridinium chlorochromate reaction product and the di-iodinated THC-COOH product were synthesised and purified as per Chapter 3. All other drug standard and reagents are as per Chapter 2.

4.1.2 – Instrumentation

High-Resolution Accurate Mass Spectrometry

High-resolution accurate mass spectrometry was carried out on an Agilent 6510 quadrupole time-of-flight (QToF) mass spectrometer coupled with an Agilent 1290 LC system (Forest Hills, Victoria, Australia). Analysis of compounds was through direct injection, bypassing the chromatographic column. System parameters are presented in **Table 4.1**. Solvent composition was ultrapure water (15%) and acetonitrile (85%). Mass correction was carried out using the *m/z* 121.0509 and *m/z* 922.0098 reference ions.

Table 4.1 - LC-QToF system parameters for high-resolution accurate mass spectrometry.

LC-QToF System Parameters		Setting
LC Parameters	Solvent Flow Rate	0.5 mL/min
	Injection Volume	2.5 µL
	Column Temperature	N/A
	Method Runtime	3 minutes
QQQ Parameters	Mass Range (*m/z*)	180 - 600
	Fragmentor Voltage	380 V
	Collision Energy	30 eV
	Sheath Gas Temperature	250°C
	Sheath Gas Flow Rate	11 L/min
	Scan Mode	SRM
	Scan Time (ms)	200 – 500

LC-MS/MS

Detection of product ions and ion transitions for THC-COOH, THC-COOH-d_9 and the seven reaction products formed following exposure to the three selected adulterants was achieved through the Agilent MassHunter Optimizer software suite. Further details regarding instrument parameters and the detection of ion transitions for the targeted compounds are found in Chapter 5 (**Tables 5.1, 5.4 and 5.5**).

NMR

NMR spectroscopy was undertaken on an Agilent Technologies 500 MHz / 54 Premium Shielded NMR Spectrometer coupled with a 7510-AS autosampler NMR Spectrometer (Forest Hills, Victoria, Australia).

A total of five NMR experiments were undertaken for each THC-COOH, the pyridinium chlorochromate reaction product and di-iodinated reaction product. Key acquisition parameters for these experiments are outlined in **Table 4.2**.

- ^{1}H NMR: Assignment of proton environments;
- ^{13}C NMR: Assignment of carbon environments;
- COSY: Homonuclear (^{1}H) proton correlations over multiple bonds;
- HSCQ: Single ^{1}H-^{13}C bond correlations;
- HMBC: Heteronuclear (^{1}H-^{13}C) correlations over multiple bonds.

Table 4.2 - Key NMR acquisition parameters for the analysis of THC-COOH, the PCC product and di-iodinated THC-COOH product.

Compound	Parameters	NMR Experiment				
		^{1}H NMR	^{13}C NMR	COSY	HSCQ	HMBC
THC-COOH	Spectral Width (Hz)	8012.8	31250.0	5296.6	8012.8	8012.8
	Number of Scans	512	2000	8[a]	16[a]	32[a]
	Relaxation Delay (s)	1.000	1.000	2.000	1.000	0.800
	Pulse Angle (°)	60	45	-	-	-
	Acquisition Time (s)	4.089	2.097	0.250	0.250	0.250
	t1 Increments	-	-	512	2 x 400	2 x 512
	F1 Nucleus	-	^{13}C	-	^{13}C	^{13}C
	^{13}C Spectral Width (ppm)	-	0 - 200	-	0 - 220	0 - 220
PCC Product	Spectral Width (Hz)	8012.8	31250.0	4595.6	8012.8	N/R
	Number of Scans	1024	10000	16[a]	8[a]	
	Relaxation Delay (s)	1.000	1.000	2.000	1.500	
	Pulse Angle (°)	60	45	-	-	
	Acquisition Time (s)	4.089	2.097	0.250	0.150	
	t1 Increments	-	-	512	2 x 512	
	F1 Nucleus	-	^{13}C	-	^{13}C	
	^{13}C Spectral Width (ppm)	-	0 - 220	-	0 – 160	
Di-iodo Product	Spectral Width (Hz)	8012.8	N/R	N/R	N/R	N/R
	Number of Scans	1024				
	Relaxation Delay (s)	1.000				
	Pulse Angle (°)	60				
	Acquisition Time (s)	4.089				

[a] per t1 increment
N/R – Experiment not run due to insufficient quantities of products to produce acceptable signal.

It should be noted that for the PCC product and the di-iodinated reaction product, the full number of experiments were not able to be run. This is due to insufficient quantities of the pure reaction products being available for analysis by NMR. In particular, for the di-iodinated reaction product, data was only able to be acquired from the ^{1}H NMR experiment.

4.1.3 – Experimental Procedures

High-Resolution Accurate Mass Spectrometry and LC-MS/MS

Samples for the collection of accurate mass data were prepared by spiking methanol with the respective target analytes (THC-COOH and the seven reaction products). Sample preparation for the generation of product ions in negative ionisation mode by LC-MS/MS were prepared in a similar manner, by spiking methanol with THC-COOH, THC-COOH-d_9 and the seven reaction products.

For the pyridinium chlorochromate and the di-iodinated reaction product, test samples were prepared through a 1:10 dilution of purified fractions obtained in the large-scale synthesis study, as outlined in Chapter 3. Samples of the mono-iodinated reaction products were prepared as per the large-scale synthesis test discussed in Chapter 3, and the bleach reaction products were prepared as per Chapter 2, Section 2.1.3.2.

Samples for both experiments were injected into the respective analytical instruments and the data recorded. Generation of product ions for the selected compounds was carried out as per the optimisation procedure discussed in Chapter 5.

NMR

Three samples were prepared for analysis by NMR for the purposes of structural elucidation. A 5 mg sample of THC-COOH was prepared in a glass NMR tube with 0.4 mL of deuterated methanol. Samples of the pyridinium chlorochromate reaction product and di-iodinated reaction product obtained from the large-scale synthesis of these compounds, discussed in Chapter 3, were prepared in the same manner.

Dissolved samples of the selected compounds were visually observed to ensure that no particulate matter, debris, or undissolved residues from the purified reaction products were present in the sample before analysis. Following analysis, all samples were sealed, covered in a layer of aluminium foil, and transferred to a refrigerator to be stored at 4°C.

4.2 – Results and Discussion

4.2.1 – High-Resolution Mass Spectrometry

Analysis of the protonated molecule ($[M+H]^+$) yielded accurate mass data for all the selected compounds. The mass spectra results obtained from this experiment were then analysed with the Spectrum Identification tool present in the Agilent MassHunter Qualitative Analysis suite to yield suggested chemical formulae for these compounds. Suggested formulae were also provided with a "score" to indicate the relative likelihood that the suggested chemical formula for a given compound is correct. The difference in the *m/z* of the suggested and detected protonated molecule is also provided as a part-per-million (ppm) difference to indicate the degree of difference between these expected and actual *m/z* values of the analysed compounds. **Table 4.3** lists the results obtained for the targeted compounds, including the suggested chemical formulae, protonated molecule *m/z* and the reported mass difference.

Table 4.3 – Results of high-resolution accurate mass spectrometry for the selected compounds.

Compound	Protonated Formula	Chemical Formula	Protonated Molecule *m/z*	*m/z* Difference (ppm)
THC-COOH	$C_{21}H_{29}O_4$	$C_{21}H_{28}O_4$	345.2055	-4.730
PCC Reaction Product	$C_{20}H_{27}O_3$	$C_{20}H_{26}O_3$	315.1961	-1.586
Mono-iodo Products	$C_{21}H_{28}O_4I$	$C_{21}H_{27}O_4I$	471.1033	-1.011
Di-iodinated Product	$C_{21}H_{27}O_4I_2$	$C_{21}H_{26}O_4I_2$	597.0088	7.709
Mono-chloro Products	$C_{21}H_{28}O_4Cl$	$C_{21}H_{27}O_4Cl$	379.1676	-0.034
Di-chlorinated Product	$C_{21}H_{27}O_4Cl_2$	$C_{21}H_{26}O_4Cl_2$	413.1286	-0.099

It should be noted that for the pyridinium chlorochromate reaction product, the suggested formula was ranked 11th out of the 14 suggested formulae. All other formulae suggested for this compound were discarded as they contained an unusual number of nitrogen and/or sulfur atoms in various combinations that are considered highly unlikely given the starting material (THC-COOH) and the adulterant used in this reaction.

From the accurate mass data obtained for these compounds, we are able to obtain useful information pertaining to the elemental composition and chemical formulae of these compounds. With regards to THC-COOH, the obtained accurate mass data and chemical

formula are in line with expectations. For the products formed through reaction of THC-COOH with Betadine and bleach, we observe mono-halogenated and di-halogenated compounds that have formed through the loss of hydrogen and the subsequent substitution of a halogen. By referring to **Figure 4.1**, we can see that for these two reactions, the likely reaction sites are present on the aromatic ring. For these two adulterants, it is proposed that the reaction with THC-COOH is via the electrophilic aromatic substitution of the aromatic ring at the ortho and para positions relative to the phenolic hydroxyl functional group.

For the pyridinium chlorochromate reaction product the chemical formula obtained is $C_{20}H_{26}O_3$, and indicates that this compound has formed through the loss of CH_2O from THC-COOH. Possible candidates for the reaction site resulting in this product are proposed to be the carboxylic acid and ether functional groups. This conclusion is drawn from the likelihood that these sites are more likely to undergo reaction with THC-COOH and result in the loss of an oxygen atom than the phenolic hydroxyl functional group. However, further data is required to provide a tentative structure for this reaction product, and is further explored in the NMR experiments undertaken on this compound.

4.2.2 – Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy provides a powerful method by which the chemical environments present within a molecule can be identified. In conjunction with complementary experiments involving ^{13}C NMR and two-dimensional NMR techniques such as COSY, HSQC and HMBC, it may be possible to ascribe a chemical structure for the two reaction products analysed by NMR. In order to facilitate analysis and interpretation of the reaction products, it is first necessary to interpret the data obtained for THC-COOH.

4.2.2.1 - NMR Analysis of THC-COOH

The chemical structure of THC-COOH is known, and has been encountered previously in this thesis. **Figure 4.1** illustrates the different proton and carbon environments present in this molecule. For the assignment of protons, a total of 18 environments are seen, and for the assignment of carbon atoms, 21 separate chemical environments are also observed. **Figure 4.2** illustrates that the 16 of the total 18 proton environments present in THC-COOH were able to be assigned. Protons associated with the carboxylic acid (H-11) and the phenol (H-8) functional

groups were not visible due to the exchange of deuterium between the deuterated solvent and THC-COOH.

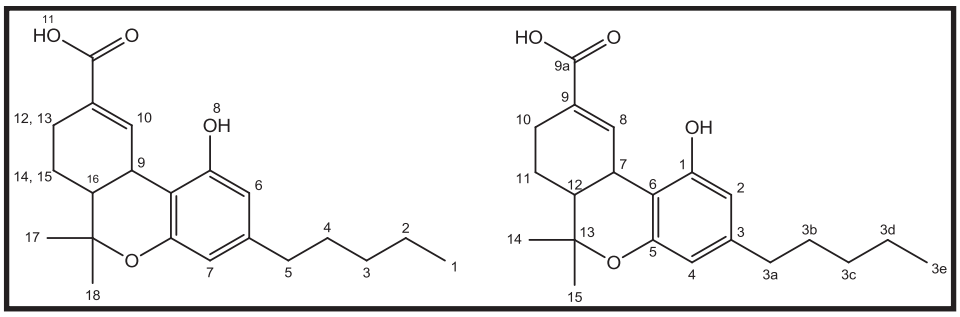


Figure 4.1 - (Left) Numbered proton environments of THC-COOH; (Right) Numbered carbon environments of THC-COOH

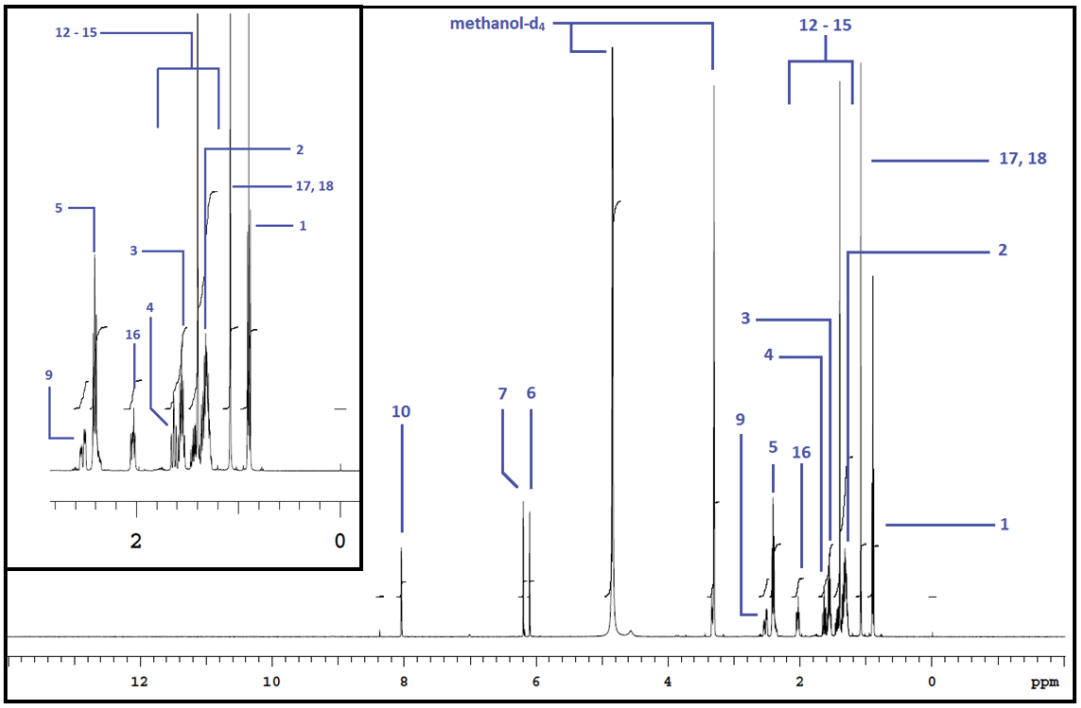


Figure 4.2 - 1H NMR spectrum of THC-COOH 0 - 12 ppm with assigned protons. Refer to Figure 4.1 for proton numbering system.
INSET: Expanded ^{1}H NMR spectra 0.0 - 2.8 ppm.

Table 4.4 provides further information regarding the assignment of the proton environments within the THC-COOH molecule. It can be seen that multiple environments can be conclusively identified, with these assignments given further credence through the detection of expected splitting patterns based on the presence of neighbouring protons. In particular, it can be

observed that two doublet peaks corresponding to the aromatic ring are evident, and in addition, a number of peaks associated with the alkyl sidechain display characteristic splitting patterns that confirm the presence of an alkyl sidechain in the THC-COOH molecule, and furthermore, that this alkyl sidechain contains five separate proton environments.

Table 4.4 – Data obtained from the ^{1}H NMR analysis of THC-COOH. Refer to Figure 4.1 for proton numbering for THC-COOH.

Assigned Proton Number	Chemical Environment	Number of Protons	Chemical Shift (ppm)	Splitting Pattern
1	Alkyl sidechain – methyl	3	0.914 - 0.886	Triplet
2	Alkyl sidechain – methylene	2	1.350 - 1.286	Sextet
3	Alkyl sidechain – methylene	2	1.591 - 1.532	Quintet
4	Alkyl sidechain – methylene	2	1.663 - 1.613	Quintet
5	Alkyl sidechain – methylene	2	2.431 - 2.400	Triplet
6	Aromatic	1	6.109 - 6.106	Doublet
7	Aromatic	1	6.212 - 6.209	Doublet
8	Phenol	1	n.d.	-
9	Ring Bridge	1	2.560 - 2.506	Skewed Quartet
10	Alkene (Cyclic)	1	8.047	Singlet
11	Carboxylic Acid	1	n.d.	-
12	Cycloalkene Section	1	1.449 - 1.282	Complex Multiplet
13	Cycloalkene Section	1	1.449 - 1.282	
14	Cycloalkene Section	1	1.449 - 1.282	
15	Cycloalkene Section	1	1.449 - 1.282	
16	Ring Bridge	1	2.062 - 2.022	Quartet
17	Methyl group	3	1.086	Singlet
18	Methyl group	3	1.086	Singlet

^{13}C NMR was also able to provide important information relating to the structure of THC-COOH, as well as the chemical environments present in this molecule. **Figure 4.3** provides the spectrum of THC-COOH obtained through ^{13}C NMR analysis. This data confirms the presence of of the carboxylic acid functional group (C-9a), as well as the aromatic ring with associated

phenolic hydroxyl group (C-1). **Table 4.5** provides the results obtained from this analysis, and also summarises the results obtained through the 2-dimensional HSQC experiment. Data obtained from the HSQC experiment is provided in the Appendix.

A comparison of the assigned carbon atoms to the HSQC data reveals multiple instances of correlation between ^{1}H and ^{13}C atoms. Importantly, this data confirms the assignments obtained through analysis of the ^{1}H and ^{13}C experiments. In particular, correlations are found between the proton and carbon atoms associated with the alkyl sidechain, aromatic ring and other major chemical environments present in this molecule. In addition, in cases where carbon atoms are not bonded to protons, the ^{13}C spectrum reveals the presence of the carboxylic acid and phenol functional groups, confirming the known structure of THC-COOH.

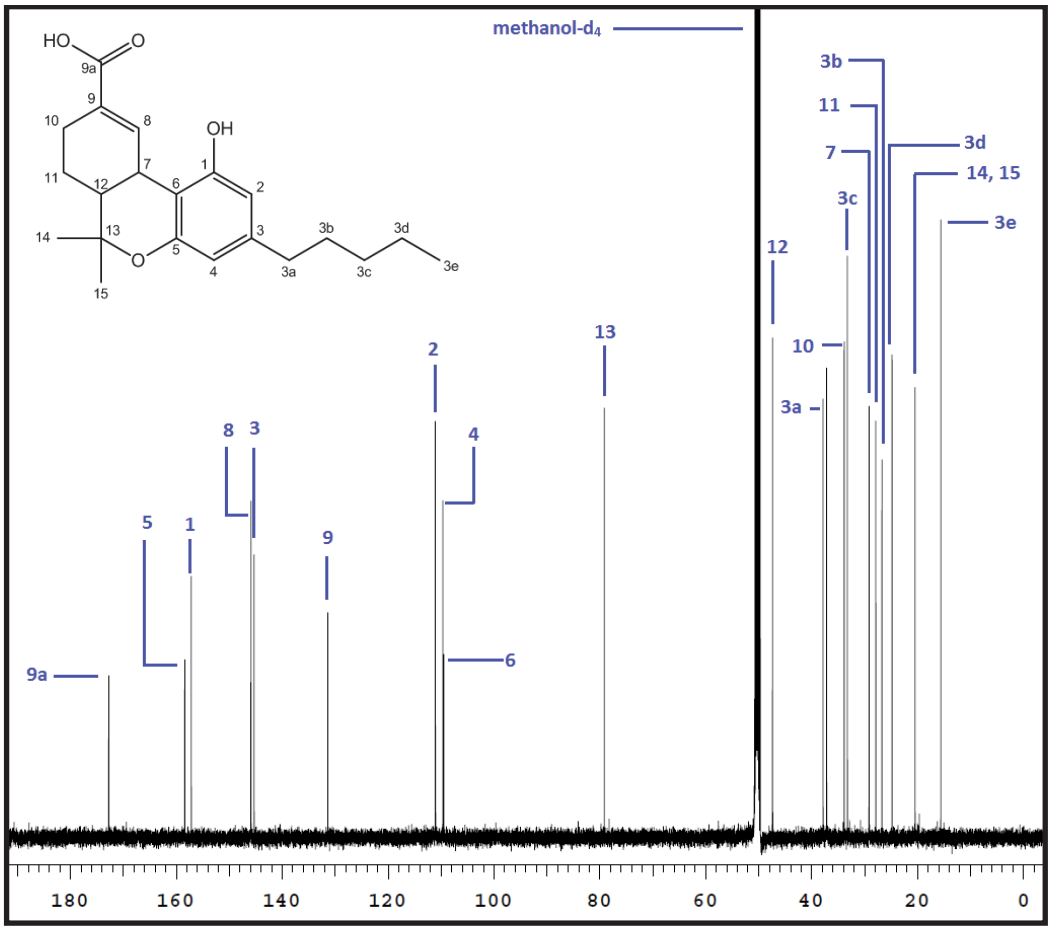


Figure 4.3 - ^{13}C spectrum of THC-COOH with assigned carbon atoms. Refer to Figure 4.1 for carbon numbering system.

Table 4.5 – Summary of data obtained through ^{13}C and HSQC experiments for THC-COOH. Refer to Figure 4.1 for carbon numbering for THC-COOH. Note that a number of single-bond correlations are found between the protons and carbons present in this molecule.

Assigned Carbon Number	Chemical Environment	Chemical Shift (ppm)	HSQC Results: Correlation to Assigned Proton Number
1	Aromatic (adjacent to phenol)	157.0	-
2	Aromatic	109.5	6
3	Aromatic (adjacent to sidechain)	145.0	-
3a	Alkyl Sidechain – methylene	38.0	5
3b	Alkyl Sidechain – methylene	26.5	4
3c	Alkyl Sidechain – methylene	33.5	3
3d	Alkyl Sidechain – methylene	24.5	2
3e	Alkyl Sidechain – methyl	15.0	1
4	Aromatic	111.0	7
5	Aromatic (adjacent to ether)	158.0	-
6	Aromatic	109.0	-
7	Ring Bridge	29.0	9
8	Cycloalkene	145.5	10
9	Cycloalkene (adjacent to 9a)	131.0	-
9a	Carboxylic acid	173.0	-
10	Cycloalkene Section	34.0	12 – 15
11	Cycloalkene Section	27.5	12 – 15
12	Ring Bridge	47.0	-
13	Quaternary (adjacent to ether)	79.0	-
14	Methyl (adjacent to ether)	20.5	17
15	Methyl (adjacent to ether)	20.5	18

Further information was also obtained from the COSY and HMBC experiments (**Table 4.6**), where long-range correlations over multiple bonds were successfully detected, and providing further confidence with regards to the assignment of protons and carbon atoms in the ^{1}H and ^{13}C NMR experiments, respectively. Coupling is observed both between protons and between protons and carbon atoms present in the alkyl sidechain. Coupling is also observed between the H-9 and H-16 protons, where these protons are coupled across the carbon-carbon bridge

between the two of the six-membered rings present in THC-COOH. In addition coupling is observed between the H-9 and H-10 protons, and also between H-5 and both H-6 and H-7 protons, providing a clear indication of coupling between the alkyl sidechain and the aromatic ring.

Table 4.6 – Results obtained from the COSY and HMBC experiments for THC-COOH. Long-range coupling is observed between protons and between protons and carbon atoms. Refer to Figure 4.1 for proton and carbon assignment for THC-COOH.

Assigned Proton Number	COSY Coupled Proton (Distance)	HMBC Coupled Carbon (Distance)
1	2 (3)	6 (3), 9a (3), 11 (3), 12 (3)
2	1 (2), 3 (3)	3a (4), 3c (2), 3e (2)
3	2 (3), 4 (3), 5 (4)	3 (4), 3d (2)
4	3 (3), 5 (3)	3 (3), 7 (4)
5	4 (3), 6 (4), 7 (4)	3 (2), 3b (2), 3c (3), 4 (3)
6	7 (4)	3a (3), 4 (3), 5 (4)
7	6 (4)	3a (3), 5 (2), 6 (3)
8	-	-
9	10 (3), 16 (3)	8 (2), 12 (2)
10	9 (3)	6 (3), 9a (3), 11 (4), 12 (3)
11	-	-
12	N/A	N/A
13	N/A	N/A
14	N/A	N/A
15	N/A	N/A
16	9 (3)	9 (4), 11 (2), 13 (2)
17	nc	7 (4), 12 (3), 13 (2)
18	nc	7 (4), 12 (3), 13 (2)
N/A – Not assigned (complex multiplet region) nc – No coupling observed		

Based on the NMR analysis of THC-COOH, assignment of the proton and carbon signals obtained from the respective ^{1}H NMR and ^{13}C NMR experiments is possible. Based on the data obtained, it is possible to confirm the presence of the aromatic ring, alkyl sidechain, carboxylic

acid and the two methyl groups adjacent to the ether functional group. Furthermore, this data provides critical information that will facilitate interpretation of the NMR data obtained for the pyridinium chlorochromate reaction product, and the di-iodinated reaction product.

4.2.2.2 - NMR Analysis of Di-Iodo-THC-COOH

As mentioned previously, only the ^{1}H NMR experiment was carried out for the di-iodinated reaction product due to the low yield of this compound following large-scale synthesis and purification. Information obtained through high-resolution mass spectrometry suggests that this compound forms through the electrophilic aromatic substitution of iodine on to the aromatic ring present in THC-COOH. If this is the case, it is expected that the aromatic region apparent in the ^{1}H NMR data of THC-COOH will no longer be present. **Figure 4.4** provides the ^{1}H NMR spectrum obtained for this compound.

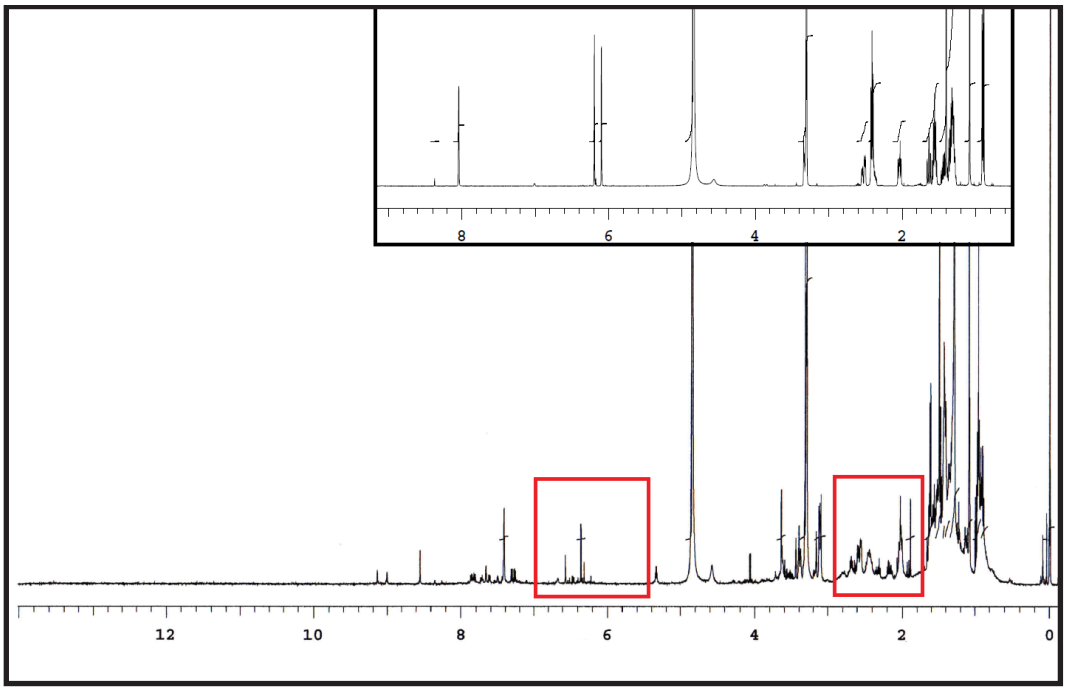


Figure 4.4– ^{1}H NMR spectrum of di-iodo-THC-COOH, with key differences highlighted in red. INSET: Equivalent ^{1}H NMR region from THC-COOH

The first major difference noted between the ^{1}H NMR spectrum of THC-COOH is in the 6 – 7 ppm region, previously assigned to the aromatic protons present in THC-COOH. In the ^{1}H NMR spectrum of the di-iodinated product, it is seen that the H-6 and H-7 protons are no longer detected in this spectrum, strongly suggesting substitution of these protons with iodine in the

reaction of THC-COOH with Betadine. In addition, it is observed that in the 2 – 4 ppm region in the spectrum of the di-iodinated product another major difference to the ^{1}H NMR spectrum of THC-COOH is present. Peaks previously observed in the THC-COOH spectrum in this region are distorted and shifted downfield. Due to this change in chemical shift for these protons, it is proposed that iodine, as an electronegative atom, has reduced the local diamagnetic shielding of the H-5 proton and possibly the H-4 proton, resulting in a decreased electron density around these protons. As a result, the chemical shift of these protons has moved downfield towards 3 ppm relative to their previous position observed in the ^{1}H NMR spectrum of THC-COOH.

Table 4.7 shows the tentative assignment of the protons in di-iodo-THC-COOH in comparison with the results from the ^{1}H NMR analysis of THC-COOH. In this table the extent of this reduction in local diamagnetic shielding is clear, with the chemical shifts of the H-2, H-3, H-4 and H-5 protons all shifted downfield. It is also interesting to note that the chemical shift of the H-10 proton has moved upfield, perhaps due to increased shielding of this proton as a result of the presence of the electronegative iodine atoms on the aromatic ring.

Table 4.7 - Comparison of ^{1}H NMR spectra of THC-COOH and di-iodinated THC-COOH. Significant changes in chemical shift between THC-COOH and this product are indicated with bold text. Refer to Figure 4.1 for THC-COOH proton numbering and assignment.

THC-COOH Proton Number	Chemical Shift (ppm)	Present in Product?	Chemical Shift (ppm)
1	0.914 - 0.886	Yes	0.850
2	1.350 - 1.286	Yes	**2.300**
3	1.591 - 1.532	Yes	**2.500**
4	1.663 - 1.613	Yes	**2.650**
5	2.431 - 2.400	Yes	**3.100**
6	6.109 - 6.106	No	-
7	6.212 - 6.209	No	-
8	-	No	-
9	2.560 - 2.506	Yes	2.500
10	8.047	Yes	**7.400**

Table 4.7 (Continued) - Comparison of ^{1}H NMR spectra of THC-COOH and di-iodinated THC-COOH. Significant changes in chemical shift between THC-COOH and this product are indicated with bold text. Refer to Figure 4.1 for THC-COOH proton numbering and assignment.

THC-COOH Proton Number	Chemical Shift (ppm)	Present in Product?	Chemical Shift (ppm)
11	-	No	-
12	1.449 - 1.282	Yes	1.449 - 1.282
13	1.449 - 1.282	Yes	1.449 - 1.282
14	1.449 - 1.282	Yes	1.449 - 1.282
15	1.449 - 1.282	Yes	1.449 - 1.282
16	2.062 - 2.022	Yes	2.042
17	1.086	Yes	1.100
18	1.086	Yes	1.100

4.2.2.3 - NMR Analysis of the Pyridinium Chlorochromate Product

Due to relatively low yield of the pyridinium chlorochromate reaction product from the large-scale synthesis of this compound, outlined in Chapter 3, interpretation of the NMR data obtained through analysis of this compound is complicated. Acceptable results are obtained for the ^{1}H NMR experiment, and allows for 16 proton environments to be identified and tentatively assigned. Significant noise was encountered in the ^{13}C NMR analysis of this reaction product, though through analysis of the HMBC and HSQC data, additional peaks in the ^{13}C spectra were able to be identified through their correlation with protons.

Figure 4.5 shows the ^{1}H NMR data obtained for the pyridinium chlorochromate reaction product. For the pyridinium chlorochromate product proton numbers have been assigned in their order of appearance, from 0 ppm through to 8 ppm. Confirmation of peaks associated with the reaction product was undertaken through use of 2-dimensional NMR experiments. HSQC allowed for correlation between protons and carbon atoms to be established, and thereby determine which peaks are associated with the product. Data from the COSY experiment for this product was also used to ascertain which peaks present in the ^{1}H NMR spectrum are from the pyridinium chlorochromate reaction product. Data from the 2-dimensional NMR experiments can be found in the Appendix.

In the highlighted regions of **Figure 4.5**, a number of differences are found in comparison with the ^{1}H NMR spectrum of THC-COOH. In particular, two small peaks are observed between 7.5 – 7.8 ppm, as is an additional peak at 4.05 ppm. Further differences are also observed in the 1.0 – 3.0 ppm region. Several key similarities are also observed in comparison with the ^{1}H NMR spectrum of THC-COOH. The aromatic region corresponding to H-6 and H-7 protons in THC-COOH remains present, as do the peaks associated with H-1, H-2, H-3, H-4, H-5, H-17 and possibly H-18 protons. The presence of these peaks in the ^{1}H NMR spectrum of the pyridinium chlorochromate product provides strong evidence that the alkyl sidechain, aromatic ring, and potentially one or both of the methyl groups attached to C-13 remain present in this molecule.

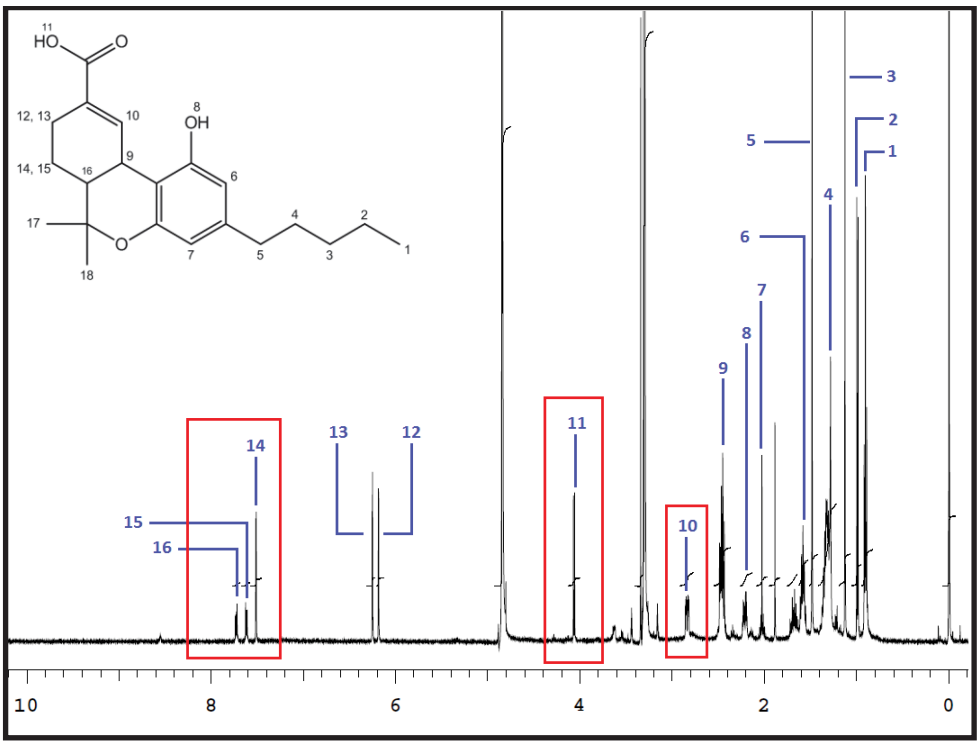


Figure 4.5 - ^{1}H NMR spectrum of pyridinium chlorochromate reaction product, with key differences to THC-COOH highlighted in red.

Table 4.8 presents the data obtained from the ^{1}H NMR analysis of the pyridinium chlorochromate reaction product, as well as the data obtained from the COSY experiment. Further interpretation of **Figure 4.5** also allows for potential equivalent proton environments present in the ^{1}H NMR spectrum of THC-COOH to be identified, shown in **Table 4.8**. Though it is possible to identify correlation between multiple bonds via COSY, as the exact structure of

this reaction product is unknown, it is not possible to effectively determine the distance between these coupled protons.

Table 4.8 – Summary of ^{1}H NMR and COSY data obtained for the pyridinium chlorochromate reaction product. Where possible, equivalent proton environments present in THC-COOH have been identified.

PCC Product Proton Number	Chemical Shift (ppm)	COSY Coupled Proton	Equivalent Proton Environment in THC-COOH
1	0.9	4	H-1
2	1	4	-
3	1.14	5	-
4	1.26 - 1.39	1	-
5	1.48	3	-
6	1.61	9	H-4
7	2.45	-	H-5
8	2.2	10, 9	-
9	2.45	6	-
10	2.85	14, 8	-
11	4.05	7	-
12	6.15	13	H-6
13	6.25	12	H-7
14	7.5	-	H-10
15	7.6	16	-
16	7.7	15	-

The pyridinium chlorochromate reaction product analysed through a ^{13}C NMR experiment (**Figure 4.6**). As with the ^{1}H NMR experiment, the HSCQ experiment was used to assist in identifying peaks associated with the reaction product. Through this, it was possible to identify peaks in the ^{13}C spectrum that were difficult to resolve from the spectrum baseline.

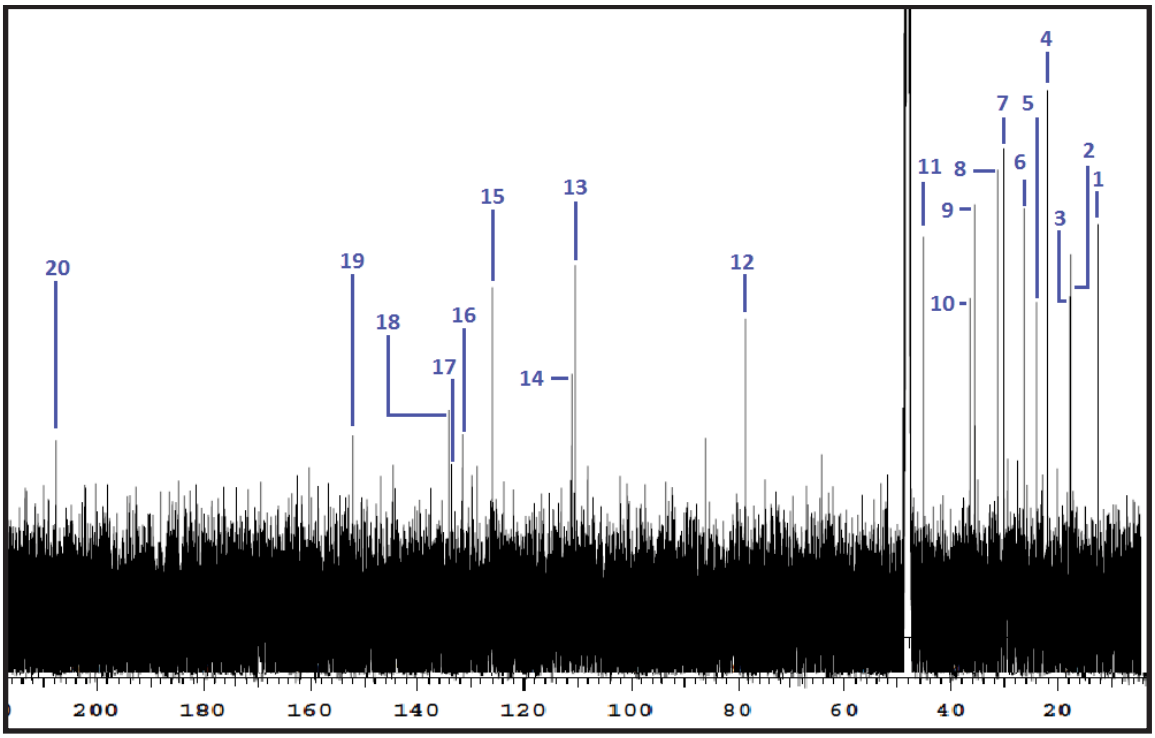


Figure 4.6 - ^{13}C spectrum of pyridinium chlorochromate product with assigned carbon atoms. Possible correlation associated with C-20 was found in the HSQC experiment, though this finding may be due to a visual artefact in this data.

Table 4.9 lists the findings from the ^{13}C experiment, and in particular, whether equivalent carbon atoms are found in THC-COOH. This table also contains the results of the HSQC experiment, and shows the correlation between protons and carbon atoms found in this molecule, and a tentative identification of equivalent carbon environments present in THC-COOH.

Table 4.9 – Summary of the results obtained for the ^{13}C NMR and HSQC experiments on the pyridinium chlorochromate product. Where possible, equivalent carbon atoms present in THC-COOH have been suggested, and correlation between carbon atoms and proton environments in this product have also been assigned.

PCC Product Carbon Number	Chemical Shift (ppm)	THC-COOH Equivalent Carbon	HSQC: Coupled Proton
1	15	C-3e	H-1
2	19	-	H-3
3	20	-	H-2
4	22	-	H-4
5	24	C-3d	H-6
6	26	C-3b	H-5

Table 4.9 (Continued) – Summary of the results obtained for the ^{13}C NMR and HSQC experiments on the pyridinium chlorochromate product. Where possible, equivalent carbon atoms present in THC-COOH have been suggested, and correlation between carbon atoms and proton environments in this product have also been assigned.

PCC Product Carbon Number	Chemical Shift (ppm)	THC-COOH Equivalent Carbon	HSQC: Coupled Proton
7	30	-	H-6
8	32	C-3c	H-4
9	35	-	H-9
10	36	-	H-9
11	46	-	H-10
12	80	-	-
13	110	C-4	H-13
14	112	C-2	H-12
15	126	-	H-14
16	131	C-9	-
17	134	-	H-16
18	136	-	H-15
19	150	C-9a	-
20	208	-	-

As can be seen in the above table, a total of 20 carbon environments have been assigned for the pyridinium chlorochromate reaction product. A number of equivalent carbon environments present in THC-COOH are also observed, and correspond to the carbon atom environments present in the carboxylic acid functional group (C-9a), two positions in the aromatic ring (C-2 and C-4), and four members of the alkyl sidechain (C-3b through C-3e). Based on this data, areas of this molecule are proposed to be highly similar to that of THC-COOH, and provide a strong indication of possible sites where the reaction of THC-COOH and pyridinium chlorochromate is thought to have occurred. Despite this, the data obtained from the NMR studies on the pyridinium chlorochromate reaction product do not provide enough information to assign a definitive structure to this compound.

4.2.3 – Structural Elucidation of Product Ions

Another means by which the structure of the reaction products can be elucidated is through the product ions they produce. In Chapter 5 a number of product ions will be proposed for the reaction products, as well as THC-COOH and THC-COOH-d_9. **Table 4.10** presents the ions generated from these compounds in negative ionisation mode:

Table 4.10 – Product ions formed from the selected compounds in negative ionisation mode.

Compound	Product Ions (*m/z*)
THC-COOH	325.2 299.3 297.2 245.2 179.1
THC-COOH-d_9	334.2 308.2 306.3 254.2
Mono-chloro-THC-COH	359.1 333.2 331.1 279.2
Di-chloro-THC-COOH	393.1 367.2 365.2 331.2 275.0

Table 4.10 (Continued) – Product ions formed from the selected compounds in negative ionisation mode.

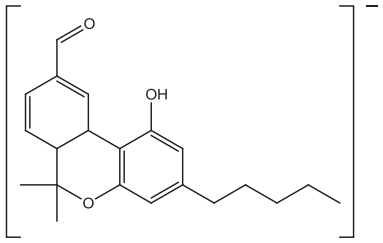
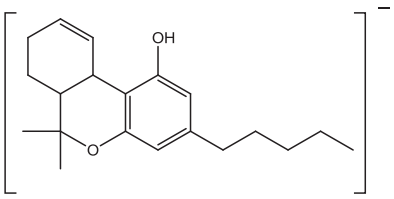
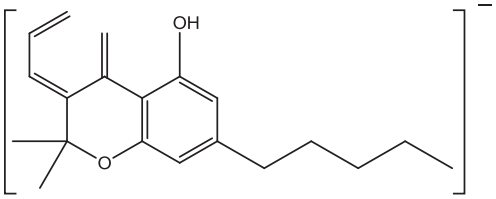
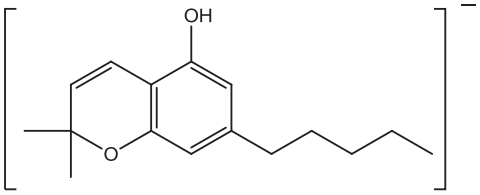
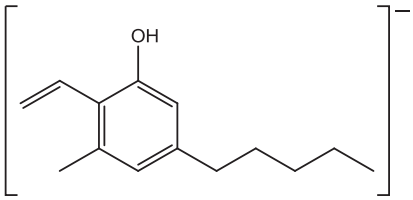
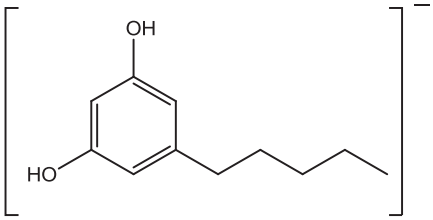
Compound	Product Ions (*m/z*)
Mono-iodo-THC-COOH	425.1 400.8 126.9
Di-iodo-THC-COOH	576.8 126.9
PCC Product	297.1 269.2 256.1 212.0 175.1 133.1

Based on the proposed structure of these compounds it is possible to propose tentative structures for these product ions, and may provide further information for the structural elucidation of the reaction products.

4.2.3.1 – Fragmentation of THC-COOH

Table 4.11 illustrates the detected product ions and their proposed structures. By referring to **Table 4.10**, it can be seen that all six of the main product ions detected for THC-COOH can be assigned structures.

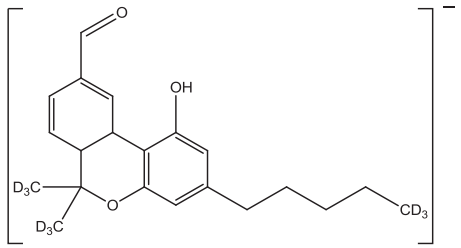
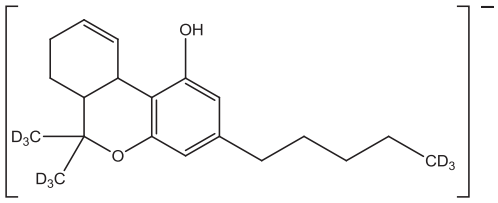
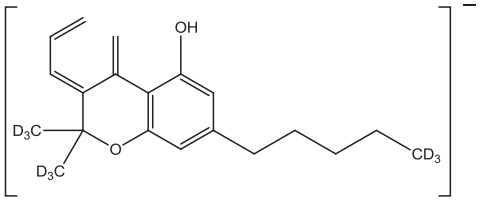
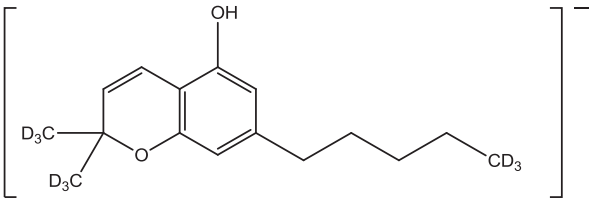
Table 4.11 - Proposed structures for the main product ions of THC-COOH.

Product Ions	*m/z*
	325.2
	299.3
	297.2
	245.2
	203.1
	179.1

4.2.3.2 – Fragmentation of THC-COOH-d_9

The observed product ions of THC-COOH-d_9 were also assigned structures based on their likely formation pathways. **Table 4.12** illustrates the detected product ions and their proposed structures. As can be seen, all of the observed product ions are able to be assigned tentative structures. It is important to note the product ion that occurs at *m/z* 308.2, which arises through loss of $–CO_2H$, and corresponds to the fragmentation of the carbon-carbon bond connecting the carboxylic acid functional group to the remainder of the molecule. It is this type of fragmentation that provides additional evidence regarding the possible structure of the pyridinium chlorochromate reaction product.

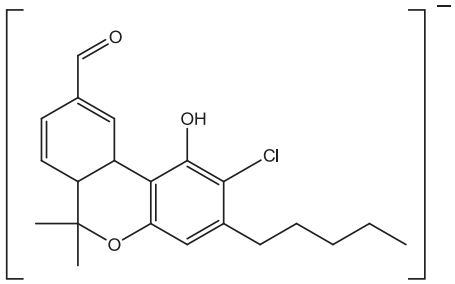
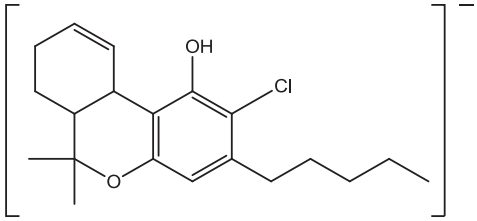
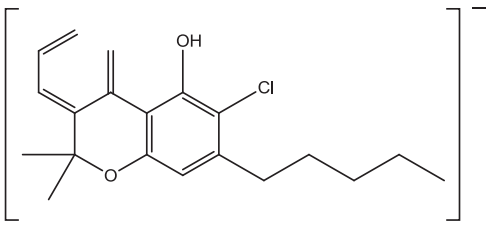
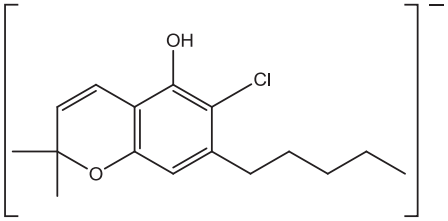
Table 4.11 - Proposed structures for the main product ions of THC-COOH-d_9.

Product Ions	*m/z*
	334.2
	308.2
	306.3
	254.2

4.2.3.3 – Fragmentation of the Mono-Chlorinated Products

For simplicity the mono-chlorinated reaction products are thought of as a single compound, with the substitution position of chlorine on the aromatic ring considered interchangeable for the purposes of assigning structures to the product ions. This has been done as fragmentation of the aromatic ring is unlikely under the given fragmentation conditions. **Table 4.13** illustrates these proposed structures of these product ions. As can be seen, all of the observed product ions for the mono-chlorinated reaction products are able to be assigned suitable structures. As with THC-COOH-d_9 and THC-COOH, it is observed that one of the main fragmentation pathways is via the loss of $–CO_2H$, and corresponds to the loss of the carboxylic acid functional group.

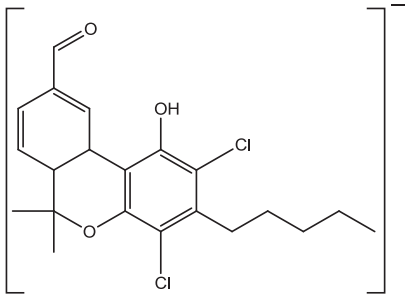
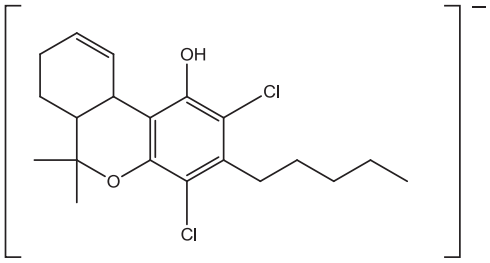
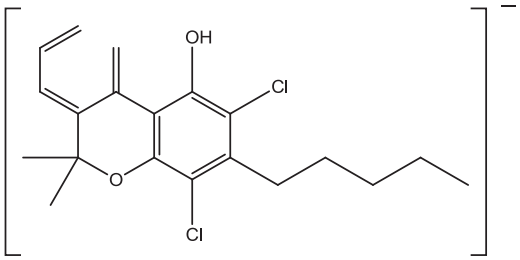
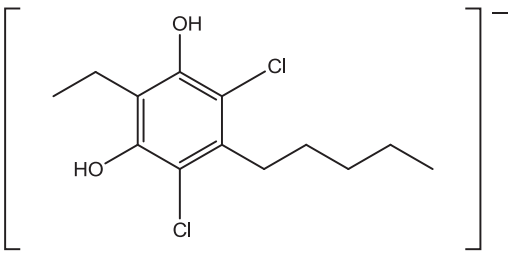
Table 4.13 - Proposed structures for the main product ions of the mono-chlorinated reaction products.

Product Ions	*m/z*
	359.1
	333.2
	331.1
	279.2

4.2.3.4 – Fragmentation of the Di-Chlorinated Reaction Product

Table 4.14 illustrates the detected product ions for the di-chlorinated THC-COOH product. Unlike the other compounds where tentative structures for the product ions have been assigned, two of the five observed product ions for this reaction product were not able to be assigned. Despite this, it can be seen that of the three ions assigned potential structures, similar fragmentation pathways are observed for THC-COOH, THC-COOH-d_9 and the mono-chlorinated reaction products.

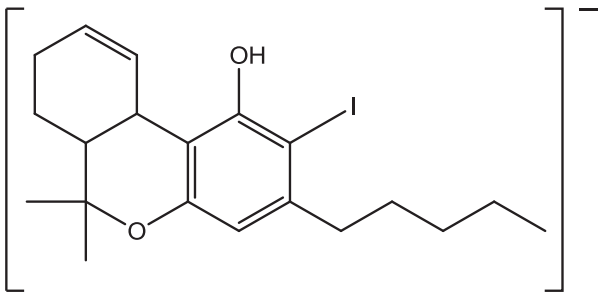
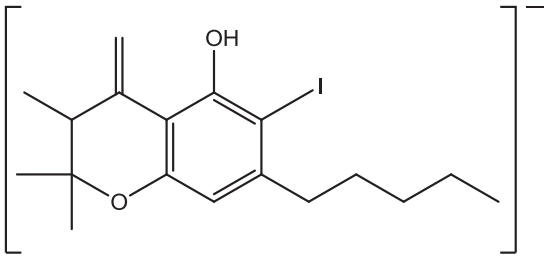
Table 4.14 - Proposed structures for the main product ions of the di-chlorinated reaction product.

Product Ions	*m/z*
	393.1
	367.2
	365.2
Not Assigned	331.2
	275.0

4.2.3.5 – Fragmentation of the Mono-Iodinated Products

For simplicity the mono-iodinated reaction products are thought of as a single compound, with the substitution position of iodine on the aromatic ring considered interchangeable for the purposes of assigning structures to the product ions as fragmentation of the aromatic ring is unlikely. **Table 4.15** illustrates these proposed structures of these product ions. As will be discussed in Chapter 5, fragmentation of the Betadine reaction products is generally poor in both positive and negative ionisation mode, as the primary fragmentation pathway for these molecules occurs through the loss of a charged iodine ion. Although fragmentation of the iodinated THC-COOH products is limited overall, in negative ionisation mode three main product ions are observed for the mono-iodinated reaction products, and fragment in a similar fashion to THC-COOH and the bleach reaction products.

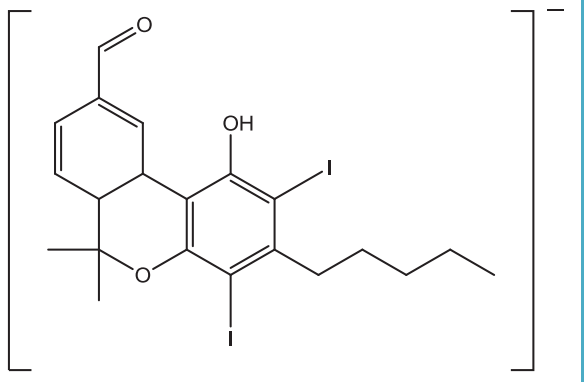
Table 4.15 - Proposed structures for the main product ions of the mono-iodinated reaction products.

Product Ions	*m/z*
	425.1
	400.8
I^-	126.9

4.2.3.6 – Fragmentation of the Di-Iodinated Reaction Product

Table 4.16 illustrates the detected product ions for the di-iodinated THC-COOH product. As with the mono-iodinated reaction products, fragmentation of this compound typically occurs through the loss of an iodine anion. This results in the remainder of the molecule remaining uncharged, and therefore undetected by the LC-MS/MS method used.

Table 4.16 - Proposed structures for the main product ions of the mono-iodinated reaction products.

Product Ions	*m/z*
	576.8
I^-	126.9

4.2.3.7 – Fragmentation of the Pyridinium Chlorochromate Reaction Product

As seen with the NMR data obtained for the pyridinium chlorochromate reaction product, it was not possible to accurately assign a structure to this molecule. Analysis of this compound by NMR and interpretation of the data was hindered due to the limited quantity of this product available for analysis. Despite this, the accurate mass data in combination with the NMR data allow for certain structural features of this molecule to be identified.

The accurate mass data provides us with a chemical formula for this compound: $C_{20}H_{26}O_3$. In addition, the NMR data strongly suggests that several regions present in the THC-COOH molecule have not reacted with pyridinium chlorochromate. **Figure 4.8** highlights the regions of the THC-COOH molecule that do not appear to have undergone reaction. It is proposed that the aromatic ring, alkyl sidechain and carboxylic acid functional group have not undergone a reaction with this adulterant. Selected assigned carbon atoms from **Figure 4.1** are reproduced in **Figure 4.7**, and from this, the likely reaction sites for this adulterant appear to be the C-7

and C-12 through C-15 carbon environments, and the ether functional group. Based on the proposed structures for the product ions of THC-COOH, the *m/z* 313 → *m/z* 212 transition for the pyridinium chlorochromate product likely corresponds to the loss of $–CO_2H$, providing further evidence that the carboxylic acid functional group present in THC-COOH is not lost or altered by the reaction with pyridinium chlorochromate.

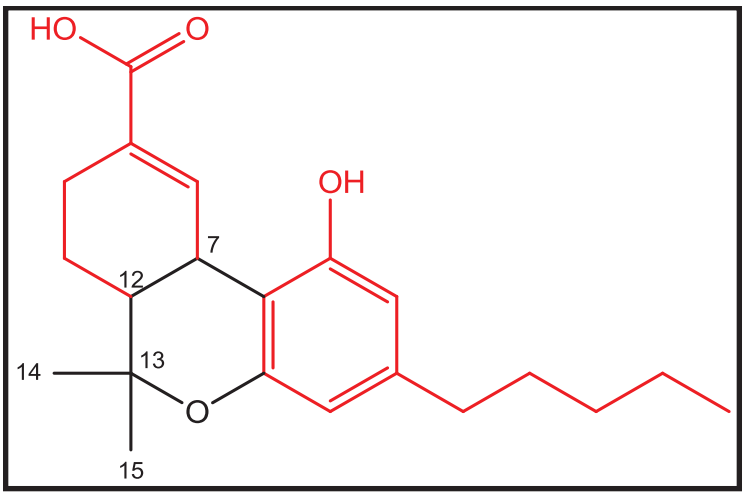


Figure 4.8 – Structure of THC-COOH with regions unlikely to have reacted with pyridinium chlorochromate highlighted in red. Selected assigned carbon atoms from ^{13}C NMR analysis of THC-COOH are included to show possible candidates for the reaction site.

Based on this information, it is likely that the pyridinium chlorochromate reaction product will contain an aromatic ring, the untouched alkyl sidechain, and the carboxylic acid. As such, a number of theoretical reaction products that may or may not reflect the actual structure of this compound are proposed, and despite the results from the NMR experiments undertaken on this compound, include structures where the carboxylic acid and aromatic ring have underwent a reaction with pyridinium chlorochromate. **Table 4.17** shows these proposed products, and indicates the number of viable structures that could be assigned to the six product ions found in **Table 4.10**.

Table 4.17- Theoretical proposed structures of the pyridinium chlorochromate reaction product. Structures ordered by highest number of structural assignments for observed product ions for this compound.

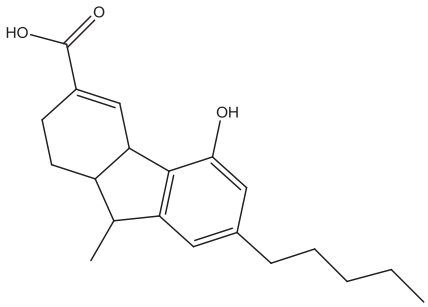
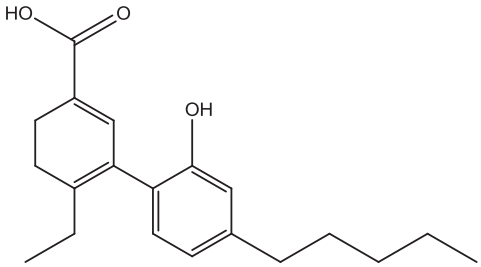
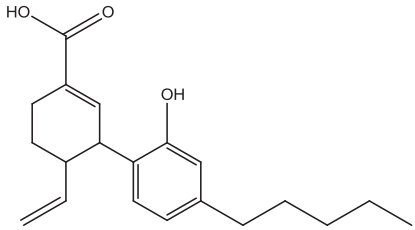
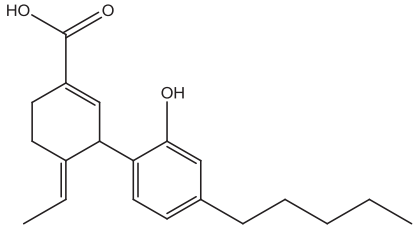
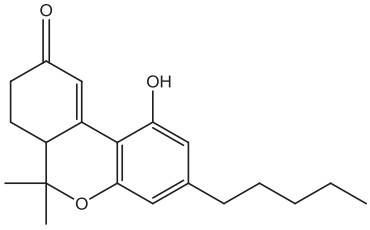
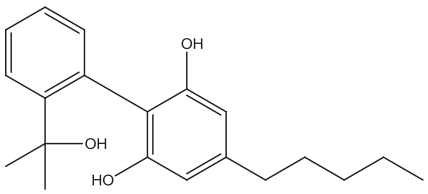
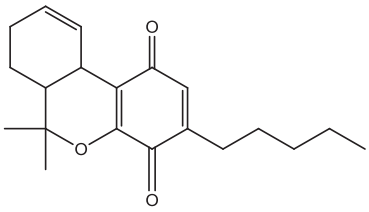
Proposed PCC Product Structure	Number of Product Ion Structures Assigned
	**6**
	**3**
	**3**
	3
	**2**

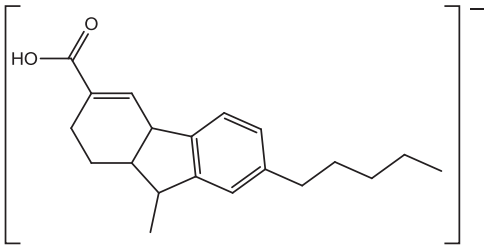
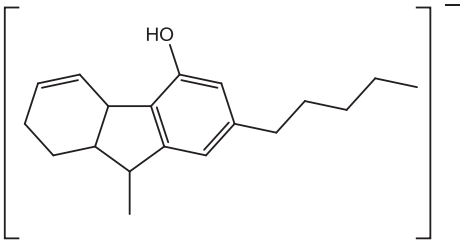
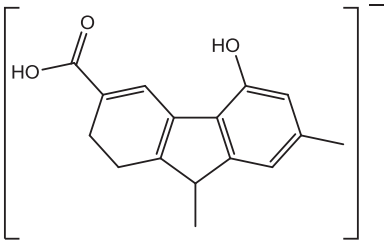
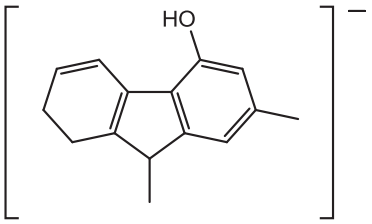
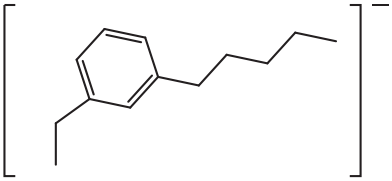
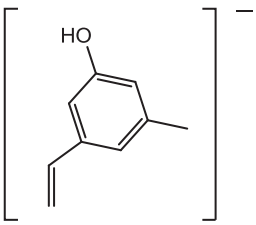
Table 4.17 (Continued) - Theoretical proposed structures of the pyridinium chlorochromate reaction product. Structures ordered by highest number of structural assignments for observed product ions for this compound.

Proposed PCC Product Structure	Number of Product Ions Structures Assigned
	**2**
	**1**

As can be seen from **Table 4.17**, a wide range of proposed structures may represent the actual chemical structure of the pyridinium chlorochromate product, though whether these compounds form in the reaction between THC-COOH and pyridinium chlorochromate is unclear, as this oxidising agent typically reacts with primary and secondary alcohols to form the respective aldehydes and ketones (Smith, M.B. & March, J., 2007). It is noted that of the proposed product ion structures observed for the pyridinium chlorochromate product, in conjunction with the proposed structures in **Table 4.17**, several fragmentation pathways are similar to those predicted for THC-COOH. Namely, the loss of $–CO_2H$, corresponding to the carboxylic acid, and $–C_2H_9$, corresponding to fragmentation of the alkyl sidechain, are frequently found. This may provide further evidence that the product formed from this reaction still contains these functional groups.

From **Table 4.17**, one proposed structure is capable of generating all six product ions observed for the pyridinium chlorochromate product. **Table 4.18** illustrates the tentative structures of these product ions, assuming that this proposed structure does represent the pyridinium chlorochromate product.

Table 4.18 - Proposed structures of the main product ions for one tentative structure proposed for the pyridinium chlorochromate reaction product.

Product Ions	*m/z*
	297.1
	269.2
	256.1
	212.0
	175.1
	133.1

4.2.4 – General Discussion

Accurate masses generated for THC-COOH and the seven reaction products provided critical information for the structural elucidation of the reaction products. For the halogenated reaction products formed in the respective reactions with Betadine and bleach, it is expected that these compounds form through the electrophilic aromatic substitution of the aromatic ring present in THC-COOH. Based on the ^{1}H NMR data obtained for the di-iodinated reaction product, this hypothesis seems likely, and it is expected that this result is applicable to the other halogenated reaction products that have been detected.

Proposed structures for the observed product ions for these halogenated products also support the proposed reaction mechanism. As these compounds contain a THC-COOH backbone, fragmentation of these compounds was overall found to be similar to that of THC-COOH. Indeed, loss of $–CO_2H$ and C_4H_9 corresponded to the loss of the carboxylic acid and alkyl sidechain functional groups, respectively. Though it should be noted that structures were not able to be proposed for some of the observed product ions, interpretation of the available data strongly suggests the following structures for the compounds formed in the Betadine and bleach reactions (**Figure 4.8**).

Analysis of the pyridinium chlorochromate reaction product has provided some information relating to the possible chemical structure of this compound. Though at present the structure of this compound remains unknown, accurate mass data, in conjunction with the NMR data and the product ions observed for this molecule, provides general details relating to the structure of this molecule. The chemical formula of this compound is known, and the NMR data suggests that this compound retains its alkyl sidechain, aromatic ring and carboxylic acid following the reaction between THC-COOH and pyridinium chlorochromate.

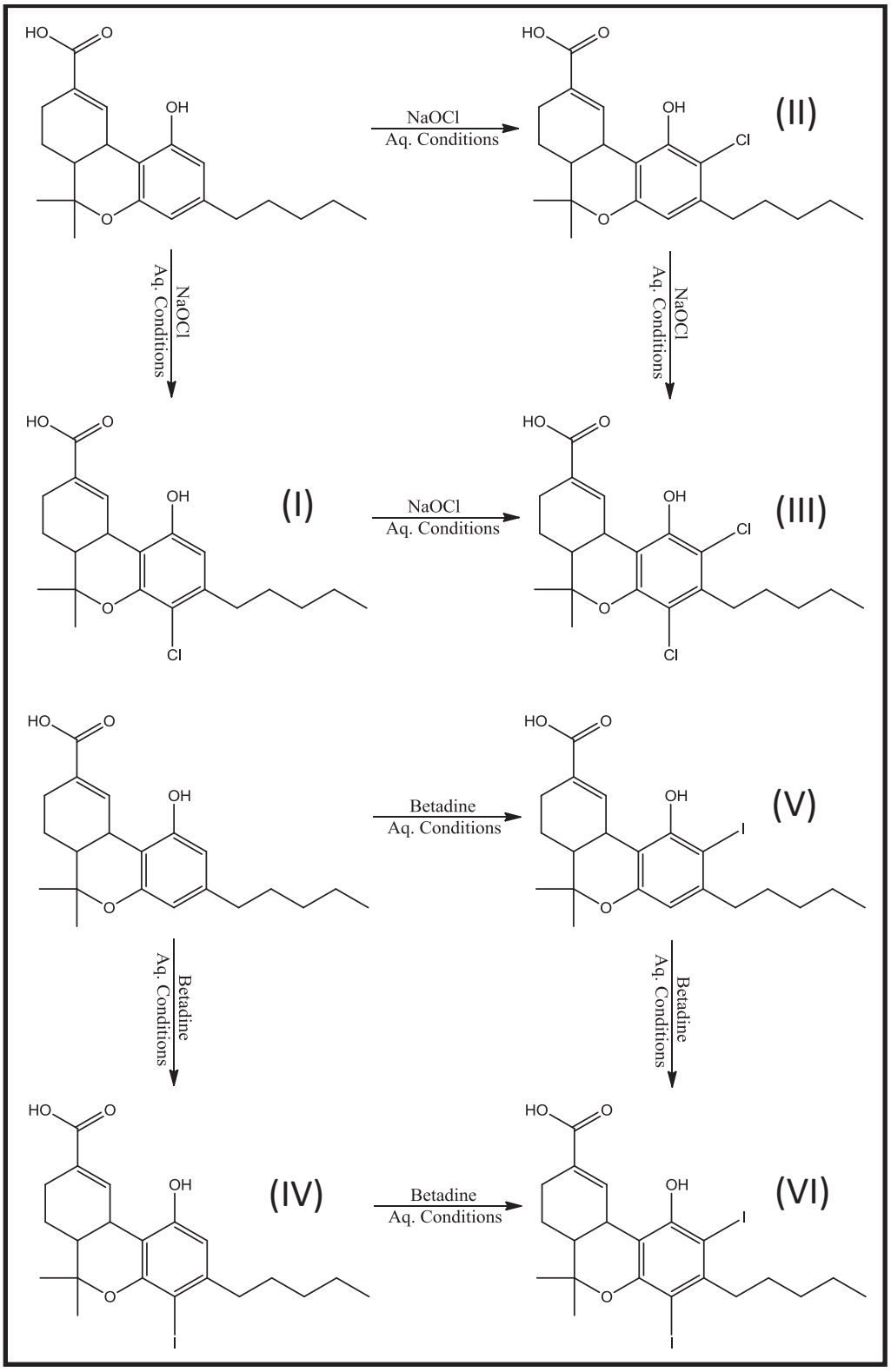


Figure 4.8 - Proposed reaction products from the reactions between THC-COOH and bleach and Betadine.

(I) and (II) Mono-chloro-THC-COOH; (III) Di-chloro-THC-COOH; (IV) and (V) Mono-iodo-THC-COOH; and (VI) Di-iodo-THC-COOH.

Overall, structural elucidation of the majority of the reaction products was possible. Assignment of potential structures of the observed product ions serves to confirm the ability of instrumental techniques such as LC-MS/MS to detect these compounds in a variety of matrices, including urine. Chapter 5 will discuss optimisation of the detection parameters for these compounds, explain the creation of suitable LC-MS/MS methods in MRM mode and the validation of these methods for the detection of THC-COOH and these targeted seven reaction products in urine.

Chapter 5

Optimisation of Detection Parameters and Method Validation

Previous chapters have outlined the detection, synthesis and structural elucidation of the reaction products formed between THC-COOH and the three selected oxidants. For the selected oxidants, validated LC-MS/MS Multiple Reaction Monitoring (MRM) methods were developed to ensure that THC-COOH and the targeted reaction products could be detected at low concentrations in a urine matrix. Due to the lack of certified reference materials for the targeted reaction products, validation was undertaken for THC-COOH specifically. As such, analysis of reaction mixtures allows for the quantification of THC-COOH and qualitative detection of the reaction products. Selection of suitable ion transitions was carried out through the preparation of samples of THC-COOH, the internal standard (THC-COOH-d_9) and the selected reaction products, and optimisation of the detection parameters by LC-MS/MS with the Agilent MassHunter Optimizer.

Validation of the three methods was carried out as per the recommendations of the *International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use* (ICH):

- **Linearity:** Determined through correlation coefficient of the calibration curves. This includes the correlation coefficient (R^2) and linear equation;
- **Linear Range:** Based on calibration curves and the concentration range of the calibration standards, with acceptable values based on reported concentrations 80-120% of the expected THC-COOH concentration;
- **Accuracy:** Determined through inter-day testing (n=8) of quality control samples, and reported as % MRE;
- **Precision:** Determined through intra-day analysis of quality control samples through repeated injections (n = 6), and reported as % RSD;
- **Limit of Quantitation (LOQ):** A signal-to-noise ratio of 10:1 for THC-COOH;

- **Limit of Detection:** A signal-to-noise ratio of 3:1 for THC-COOH. This was not directly assessed during method validation, and was based on the least concentrated calibration standard and the limit of quantitation.

Two sets of methods were validated, with the first set not involving hydrolysis of the samples prior to analysis, and were intended for assessment of reaction pH, kinetics and stability (Chapter 6). The second set of methods, which involved the sample hydrolysis step, were intended for analysis of authentic urine samples following adulteration (Chapter 7).

An additional consideration for method validation for the three methods not involving sample hydrolysis was robustness testing. As with the above parameters, this was based on the recommendations of the ICH for validation of analytical procedures. Though not typically assessed during method validation, robustness was assessed to ensure the validity of results obtained during later studies due to differences in instrument calibration and variations in operational parameters. Three system parameters were deliberately changed to assess method robustness: sample injection volume, column temperature and solvent condition. Results from robustness testing were analysed by the Student's *t* test with a 95% confidence interval to determine the statistical significance of changes in THC-COOH concentration and retention time. Robustness testing was not carried out on the methods involving sample hydrolysis, as the robustness testing of the methods not involving sample hydrolysis was considered proof of concept.

Matrix effects were also assessed through a comparison of quality control samples prepared in water and urine samples. Direct injection of these samples was undertaken, with decreases in absolute peak area of THC-COOH and the internal standard interpreted as evidence of ion suppression.

5.1 – Experimental

5.1.1 – Drug Standards and Reagents

Drug standards and reagents used for method validation are found in Chapter 2. Sodium hypochlorite concentration of the hypochlorite solution was determined spectrophotometrically as per Chapter 3. The final hypochlorite stock solution concentration was determined to be 0.255 M.

5.1.2 – Urine Specimens

Collection and storage of urine specimens was as per Chapter 3.

5.1.3 – Instrumentation

All analyses were undertaken on a 1290 LC system coupled to a 6490 triple quadrupole (QQQ) mass spectrometer. These instruments were from Agilent Technologies (Forest Hills, Victoria, Australia). Chromatographic separation of analytes during method validation was undertaken on a Phenomenex Luna C_5 HPLC column (150 mm x 4.6 mm, 5 micron, Phenomenex Incorporated). The mobile phase was composed of 100% ultrapure water and 100% acetonitrile (15:85). Instrument parameters for optimisation of MRM conditions for the target analytes and method validation are outlined in **Table 5.1** and **Table 5.2**, respectively.

Table 5.1 - Instrument parameters for Agilent LC-MS/MS system for optimisation of analyte detection.

LC-MS System Parameters		Setting
LC Parameters	Solvent Flow Rate	0.7 mL/min
	Injection Volume	2 µL
	Column Temperature	35°C
	Method Runtime	3 minutes
QQQ Parameters	Mass Range (*m/z*)	200 - 1000
	Fragmentor Voltage	380 V
	Cell Accelerator Voltage	4 V
	Collision Energy	5 – 80 eV
	Ionisation Mode	Negative, Positive
	Sheath Gas Temperature	250°C
	Sheath Gas Flow Rate	11 L/min
	Scan Mode	MS2 Scan, Product Ion Scan, Multiple Reaction Monitoring

Table 5.2 - Instrument parameters for Agilent LC-MS/MS system for validation of three detection methods.

LC-MS System Parameters		Setting
LC Parameters	Solvent Flow Rate	0.7 mL/min
	Injection Volume	2 µL
	Column Temperature	35°C
	Method Runtime	15 minutes
QQQ Parameters	Fragmentor Voltage	380 V
	Cell Accelerator Voltage	4 V
	Collision Energy	5 – 80 eV
	Ionisation Mode	Negative
	Sheath Gas Temperature	250°C
	Sheath Gas Flow Rate	11 L/min
	Scan Mode	Multiple Reaction Monitoring

5.1.4 – Preparation of Samples for Optimisation

Samples for parameter optimisation were prepared by spiking water samples with the respective target analytes: THC-COOH, THC-COOH-d_9 and the seven reaction products. For the pyridinium chlorochromate and major Betadine reaction product, test samples were prepared through 1:10 dilution of purified fractions obtained in the large-scale synthesis study, as outlined in Chapter 3. As large-scale synthesis of the mono-iodinated Betadine reaction products was not successful, samples of these analytes were prepared through a replicate of the large-scale reaction test for the Betadine reaction as per Chapter 3. Similarly, large-scale synthesis of the bleach reaction products was not considered viable; therefore optimisation of the detection parameters for the three bleach reaction products was achieved through preparation of a test sample per the water-matrix bleach reaction outlined in Section 2.1.3.2.

5.1.5 – Preparation of Samples for Method Validation

Calibration standards and quality control samples were prepared in pooled urine, and refrigerated at 4°C prior to use. Prior to analysis, all samples were filtered through 0.2µm MilliPore syringe filters to remove particulate matter. Samples prepared for method validation are outlined in **Table 5.3**. The concentration range of THC-COOH was over a wide concentration range in order to allow for the effective detection and quantitation of this analyte at both very low and very high concentrations. Quality control specimens were prepared at 20.0, 650.0 and 2500 ng/mL, providing low, medium and high quality control specimens. Calibration standards were prepared in triplicate for generation of the calibration curves for the three final detection methods, and the final sample volume for all samples was set at 1000 µL.

Table 5.3 - Matrix blank, calibration standards and quality control samples prepared for method validation. Internal standard (THC-COOH-d_9) concentration was set at 1000 ng/mL for all samples.

Sample	THC-COOH Concentration (ng/mL)
Blank	0
Calibration 1	5
Calibration 2	10
Calibration 3	25
Calibration 4	75
Calibration 5	175
Calibration 6	500
Calibration 7	800
Calibration 8	1100
Calibration 9	2250
Calibration 10	5000
Quality Control 1	20
Quality Control 2	650
Quality Control 3	2500

5.1.6 – Sample Hydrolysis

Alkaline hydrolysis of the urine samples was undertaken to recover unconjugated THC-COOH, and if they had formed from glucuronidated THC-COOH, the targeted reaction products. The tested samples had a volume of 1 mL, and were basified with 25 µL of 6 M sodium hydroxide, as per earlier research by Breindahl and Andreasen (Breindahl, T. & Andreasen, K., 1999). Following addition of sodium hydroxide, samples were heated at 50°C for 30 minutes, and upon cooling, were acidified with 0.5 M hydrochloric acid to pH 4.

After acidification of the samples, extraction of the unconjugated THC-COOH and desired reaction products was achieved through the use of liquid-liquid extraction. The samples were extracted with a 1:5 ethyl acetate/n-hexane solution in triplicate, with the organic layer transferred to a labelled vial. The organic fractions were then dried under a gentle stream of nitrogen at 25°C, spiked with deuterated THC-COOH, and made up to a final volume of 1 mL

with methanol. The concentration of the internal standard following reconstitution was 1000 ng/mL. Prior to sampling the samples were stored at 4°C.

Recovery of the samples following hydrolysis and extraction was also assessed. In comparison with the three methods generated for the selected oxidants, recovery of the three methods involving sample hydrolysis was determine to be approximately 98%.

5.1.7 – Optimisation of Product ions and Detection Parameters

Selection of product ions and optimisation of detection parameters for THC-COOH, the selected internal standard and the reaction products was undertaken on the Agilent G3793AA MassHunter Optimizer Automated MS Method Development Software. During optimisation of THC-COOH, internal standard and the seven reaction products, the chromatographic column was bypassed to minimise total experiment runtime. Optimisation of parameters was tested in both positive ionisation and negative ionisation modes, with a Cell Accelerator Voltage (CAV) of 4 volts and a collision energy range of 5 – 80 eV. An injection volume of 2 µL was set for all samples. Final selection of the product ions for all compounds was determined through highest detected abundances.

5.1.8 – Development of Validated Methods

Development of validated methods for the three selected reactions was undertaken through preparing calibration standards, quality control standards and blanks for THC-COOH by serial dilution in pooled urine. Deuterated THC-COOH (THC-COOH-d_9) was selected as the internal standard, with a final concentration of 1 µg/mL. A ten-point calibration was performed with the selected calibration standards prepared in triplicate.

As mentioned, validation of the three analytical methods was based on the recommendations of the *International Conference for Harmonization* (ICH). Calibration curves were generated for the three methods over the 5 – 5000 ng/mL range, and used to determine the linear equation for the calibration curves, as well as the correlation coefficients and the limits of detection and quantitation. Inter-day and intra-day precision (% RSD) and accuracy (% MRE) were calculated through repeated injections of the three quality control samples. Intra-day variations were

assessed through a total of 6 injections on a single day, and inter-day variations were assessed through 8 replicate injections over 8 separate days.

Robustness of the three detection methods was determined through statistical analysis of the quality control samples and tested the following parameters: changes in column temperature (± 5%), changes in elution condition (organic solvent B ± 5%) and changes in injection volume (± 5%). The Student's *t* test was also used to provide an additional statistical analysis of the acquired data at a 95% confidence interval with values of $P<0.05$ considered statistically significant. The null hypothesis considered for the Student's *t* test was that for values of $P > 0.05$, variations in recorded retention times and calculated THC-COOH concentration were due to random variations in instrument sensitivity, instrument equilibration and sample preparation. The ion transitions selected for monitoring are outlined in **Table 5.5**.

Matrix effects were assessed through preparation of two sets of the quality control samples, one prepared in water and the other in pooled urine. These samples were analysed for the three methods, and the absolute peak areas of THC-COOH and the internal standard recorded. The absolute peak areas were compared between the quality control samples and matrices in order to determine whether ion suppression would occur in the pooled urine samples.

5.2 – Results and Discussion

5.2.1 – Analyte Optimisation

Optimisation of the target analytes for analysis by LC-MS/MS was carried out in positive and negative ionisation modes to assess their suitability. **Table 5.4** and **Table 5.5** outline the results of the optimisation method for positive and negative ionisation mode respectively. Ion transitions selected for development of an LC-MS/MS method were based on the three highest ion transition abundances detected for each target analyte.

Singular results were obtained for the two mono-iodo-THC-COOH and mono-chloro-THC-COOH compounds. Though these mono-halogenated species represent a total of four separate compounds, as analogues differing by the site of electrophilic aromatic substitution, a single optimisation experiment was done for the mono-chlorinated and mono-iodinated species. Results for these compounds suggested that the optimised parameters are suitable for both sets of these halogenated THC-COOH analogues.

Table 5.4 - Optimised parameters for target analytes in positive ionisation mode. Blank cells indicate no detected ion transitions during optimisation.

Compound	Mass (Da)	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (eV)	Abundance[a]
THC-COOH	344.2	345.21	327.00	17	113,010
			299.10	21	88,614
			193.10	29	29,682
THC-COOH-d_9	353.26	354.27	74.90	41	6,217
			312.30	25	4,783
			74.00	61	1,183
PCC Product	314.00	315.01	273.10	25	1,860,860
			193.10	33	765,653
			55.10	45	657,675

[a] Abundance derived from the absolute peak area of the samples

Table 5.4 (Continued) - Optimised parameters for target analytes in positive ionisation mode. Blank cells indicate no detected ion transitions during optimisation.

Compound	Mass (Da)	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (eV)	Abundance[a]
Mono-chlorinated Products	378.16	379.17	-	-	-
Di-chlorinated Product	412.12	413.13	141.00	29	1,912
Mono-iodinated Products	470.10	471.11	-	-	-
Di-iodinated Product	595.99	597	-	-	-

[a] Abundance derived from the absolute peak area of the samples

Table 5.4 provides the detected ion transitions, collision energies and ion transition abundance in positive ionisation mode. As can be seen for the mono-chlorinated, mono-iodinated and di-iodinated products, fragmentation of the precursor ions in positive ionisation mode was nonexistent. This is likely due to the presence of carboxylic acid and phenolic functional groups present in these molecules which will preferentially undergo ionisation through loss of hydrogen to form a negatively ionised species. Relatively low abundances were also recorded for THC-COOH, the internal standard, and the di-chlorinated product, indicating that positive ionisation mode is not suitable for detection of the target analytes by LC-MS/MS.

In comparison with the results obtained for optimisation in positive ionisation mode, the results obtained in negative ionisation mode provide three ion transitions for all target analytes. Overall, acceptable abundances were recorded for all targeted ion transitions, with the exception of the two final transitions recorded for the di-iodinated product. It is interesting to note that for the PCC product, ion transition abundance was highest in positive ionisation mode, whilst for the remaining target analytes, specifically THC-COOH and the internal standard, the highest ion transition abundances were recorded in negative ionisation mode.

Table 5.5 - Optimised parameters for target analytes in negative ionisation mode.

Compound	Mass (Da)	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (eV)	Abundance[a]
THC-COOH	344.20	343.20	299.30	21	914,670
			245.20	29	224,814
			191.10	37	149,369
THC-COOH-d_9	353.26	354.27	308.20	21	139,516
			254.20	29	37,864
			334.20	21	22,497
PCC Product	314.00	312.99	175.1	37	407,332
			256.10	33	374,211
			269.2	37	235,601
Mono-chlorinated Products	378.16	377.15	333.20	17	253,938
			359.10	21	59,661
			279.20	29	26,682
Di-chlorinated Product	412.12	411.11	331.20	25	114,298
			367.20	21	77,685
			275.00	29	43,283
Mono-iodinated Products	470.1	469.09	126.90	45	37,787
			188.70	53	6,899
			400.80	17	6,481
Di-iodinated Product	595.99	594.98	127.00	53	133,318
			576.80	21	1,136
			362.80	57	87

[a] Abundance derived from the absolute peak area of the samples

This, combined with the successful generation of three ion transitions for the halogenated products suggests that due to the presence of acidic functional groups in these compounds, loss of an acidic hydrogen atom from the carboxylic acid or phenol groups is preferential, leading to the formation of negative ions.

A significant issue was noted with the optimised fragmentor parameters for the iodinated THC-COOH products. Though optimisation of the parameters was successful, generating multiple

ion transitions for each compound, repeated optimisation tests typically resulted in a single ion transition being detected. This ion transition, detected at *m/z* 129, represents the loss of a single negatively charged iodine ion from the product structures. This result is attributed to the transfer of electric charge from the deprotonated molecule to iodine, resulting in a single detected ion transition.

The potential for charge transfer to limit fragmentation of the iodinated products was tested in an additional experiment. Two aliquots of the prepared iodine products were spiked with acetone, and were separately acidified with 0.1 M hydrochloric acid and basified with 0.1 M sodium hydroxide. Analysis of the acidified sample resulted in single ion transitions being detected for the iodinated products, and corresponded to the loss of iodine from the reaction products. Under alkaline conditions, the previously detected ion transitions listed in Table 5.5 were found for the three Betadine reaction products. It is therefore likely that the addition of the negative hydroxide ion limits the charge transfer to iodine, allowing for the full range of ion transitions to be detected.

Though basification of samples containing the iodine products assisted in formation multiple product ions, the utility of this sample preparation step is limited. For the studies discussed in Chapter 6 and Chapter 7, this step is not considered viable as it alters sample pH and represents a factor that will complicate both quantitative detection of THC-COOH and the study of the effects of sample pH on product formation in vitro. As the abundances of the three ion transitions for the mono-iodinated reaction products were detectable outside of the charge transfer experiment, these compounds may be detected and identified through the selected ion transitions. In contrast, the poor abundance of the second ion transition for the di-iodinated product will complicate monitoring of this compound in cannabis-positive urine samples adulterated with Betadine.

From the optimised detection parameters, three LC-MS/MS methods utilising Multiple Reaction Monitoring (MRM) were developed, and are outlined in **Table 5.6**. The goal of these three methods is to provide a highly sensitive, specific and robust method for both the quantitative detection of THC-COOH, and for the qualitative detection of compounds formed following adulteration of THC-COOH positive urine samples with Betadine, bleach and pyridinium chlorochromate. Though GC-MS is typically used in drug testing laboratories for the purposes of sample analysis, this research has focussed solely on LC-MS/MS due to its versatility and lack of a necessary and time-consuming derivatisation step. It is expected,

however, that the methods developed in this research may be transferred in part to gas chromatography methods with development of a suitable derivatisation step for the targeted reaction products. For the parameters listed in **Table 5.6**, it is noted that the parameters associated with THC-COOH and the internal standard remains consistent across the validated methods regardless of whether the sample hydrolysis step is incorporated into sample preparation.

Table 5.6 - LC-MS/MS method parameters for detection of THC-COOH and compounds formed following adulteration of urine samples with the selected adulterants. Quantifier ion used for analysis of targeted compounds is italicised. Ion transition dwell time is set at 20 ms for all targeted transitions.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)
THC-COOH	343.20	*299.30*	21
		245.20	29
		191.10	37
THC-COOH-d_9	354.27	*308.20*	21
		254.20	29
		334.20	21
Mono-chloro THC-COOH Products	377.15	333.20	17
		359.10	21
		279.20	29
Di-chloro THC-COOH	411.11	*331.20*	25
		367.20	21
		275.00	29
Mono-iodo THC-COOH Products	469.09	*126.90*	45
		188.70	53
		400.80	17
Di-iodo THC-COOH	594.98	127.00	53
		576.80	21
PCC Product	312.99	*175.1*	37
		256.10	33
		269.2	37

For the methods listed in **Table 5.6**, other parameters common to these methods are listed in **Table 5.2**. The following section will detail validation of these methods.

5.2.2 – Method Validation

Validation was undertaken for the two sets of methods prepared for the three selected oxidising adulterants, with analysis undertaken by LC-MS/MS. The quantitative detection of THC-COOH and compounds formed following urine adulteration required several key parameters and statistical analyses to be undertaken. Validation of the methods was achieved through the use of a ten-point calibration range (5 – 5000 ng/mL) and analysis of quality control standards prepared at 20 ng/mL, 650 ng/mL and 2500 ng/mL. Concentration of THC-COOH in the samples was assessed through analytical software, with the presence of THC-COOH confirmed via analyte retention time and ion transition ratios.

Calibration curves were generated for six total methods, through triplicate injections of the calibration samples. **Figures 5.1** through **5.3** provide the calibration curves generated for the three methods not involving sample hydrolysis. Calibration curves for the three methods involving the sample hydrolysis step were observed to be very similar to the calibration curves shown in **Figures 5.1** through **5.3**. R^2 values for the six calibration curves were above 0.999, with a linear range from 5 ng/mL to 5000 ng/mL. As the six respective analytical methods monitor different numbers of ion transitions, it is expected that the instrument response to THC-COOH in terms of absolute peak area will vary between these methods. As such, the calibration curves for the three methods are shown to confirm that similar calibration curves were generated. Further validation results are displayed in **Table 5.7** and will be discussed further in the following sections. Raw data for calculations of robustness and intra-day and inter-day precision and accuracy are included in the Appendix.

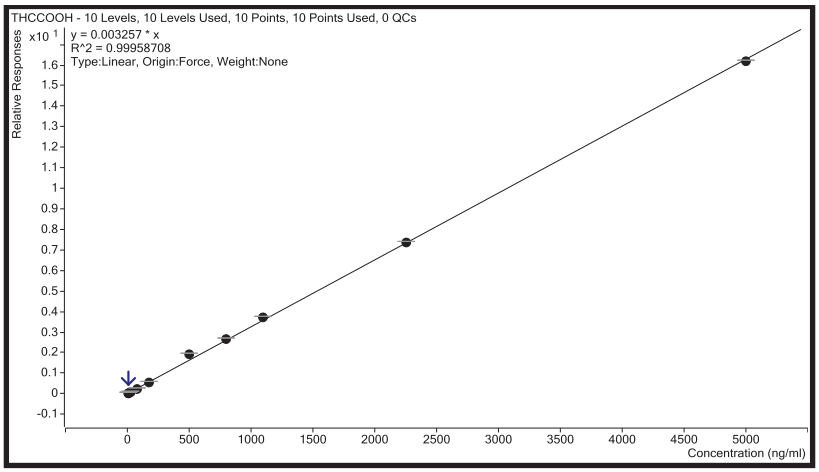


Figure 5.1 - Calibration curve generated for THC-COOH concentration for the validated bleach detection method that does not incorporate sample hydrolysis.

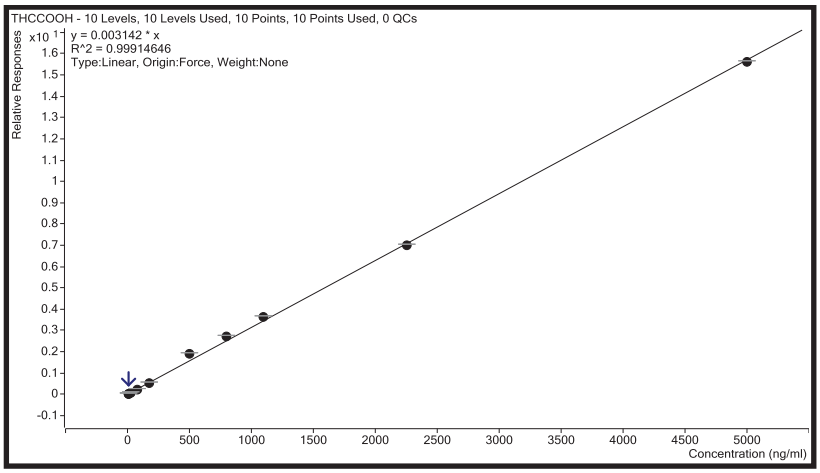


Figure 5.2 - Calibration curve generated for THC-COOH concentration for the validated Betadine detection method that does not incorporate sample hydrolysis.

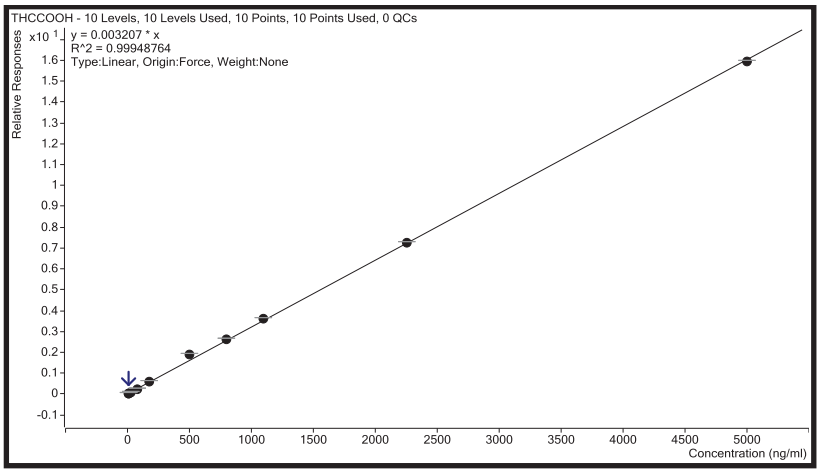


Figure 5.3 - Calibration curve generated for THC-COOH concentration for the validated PCC detection method that does not incorporate sample hydrolysis.

The calibration curves generated for the three methods display a high degree of linearity over the tested concentration range, with correlation coefficients greater than 0.9990 recorded. The calculated THC-COOH concentration from the calibration standards and quality control samples were found to be in close agreement with the expected concentrations for the respective detection methods. **Table 5.7** and **Table 5.9** provide the general results from the validation of the three methods not involving sample hydrolysis, and **Table 5.8** and **Table 5.10** provide the general results from the three methods that incorporated a sample hydrolysis step. The limit of quantitation for the six methods was based on the 5 ng/mL calibration standard, with a signal-to-noise ratio above 10:1 considered acceptable for reporting this value. The limit of detection was not directly assessed for the three methods, and was based on the determined limit of quantitation. As such the limit of detection is expected to be below 5 ng/mL.

Table 5.7 - LC-MS/MS MRM method validation results for methods not incorporating sample hydrolysis.

Method	Calibration Range (ng/mL)	Linearity (R^2)	Quantifier Ion (*m/z*)	Qualifier Ions (*m/z*)	LOD (ng/mL)	LOQ (ng/mL)	S/N (at 5 ng/mL)
Bleach	5 - 5000	0.9996	299.3	245.2 191.1	< 5.0	5.0	24.07
Betadine	5 - 5000	0.9991	299.3	245.2 191.1	< 5.0	5.0	10.79
PCC	5 - 5000	0.9995	299.3	245.2 191.1	< 5.0	5.0	11.45

Table 5.8 - LC-MS/MS MRM method validation results for methods incorporating sample hydrolysis.

Method	Calibration Range (ng/mL)	Linearity (R^2)	Quantifier Ion (*m/z*)	Qualifier Ions (*m/z*)	LOD (ng/mL)	LOQ (ng/mL)	S/N (at 5 ng/mL)
Bleach	5 - 5000	0.9995	299.3	245.2 191.1	< 5.0	5.0	25.33
Betadine	5 - 5000	0.9991	299.3	245.2 191.1	< 5.0	5.0	26.08
PCC	5 - 5000	0.9994	299.3	245.2 191.1	< 5.0	5.0	11.48

Table 5.9 - LC-MS/MS MRM method validation results regarding intra-day and inter-day precision and accuracy, for the three methods not requiring the sample hydrolysis step.

Method	QC Concentration (ng/mL)	Intra-day accuracy (% MRE)	Intra-day precision (% RSD)	Inter-day accuracy (% MRE)	Inter-day precision (% RSD)
Bleach	20	-1.122	1.447	2.037	6.511
	650	-2.576	4.496	-9.813	8.548
	2500	-5.731	4.841	-8.428	10.300
Betadine	20	-2.191	2.757	-3.279	4.266
	650	-2.581	3.513	-2.253	10.259
	2500	-6.786	8.777	-10.158	10.717
PCC	20	-2.639	3.519	0.490	7.055
	650	-1.860	5.372	-5.133	10.461
	2500	-3.182	10.064	-0.276	12.968

Table 5.9 illustrates the intra-day and inter-day variation of the calculated THC-COOH of the three quality control samples analysed by the three selected methods. Intra-day accuracy and precision for the lowest concentration quality control sample (20 ng/mL) was acceptable over

the three MRM methods. At higher quality control sample concentrations, large values were reported for the precision and accuracy of the methods. Similar statistical results were obtained for the inter-day tests, with statistically significant variations attributed to instrument sensitivity and instrument calibration. Due to the fluctuations in precision and accuracy of the tested methods, a Student's *t* test (with two tails and two degrees of freedom) comparing the reported THC-COOH retention times and calculated THC-COOH concentration for the quality control samples was undertaken at the 95% confidence interval to assess the significance of these results (**Table 5.11**).

Table 5.10 - LC-MS/MS MRM method validation results regarding intra-day and inter-day precision and accuracy, for the three methods involving the sample hydrolysis step.

Method	QC Concentration (ng/mL)	Intra-day accuracy (% MRE)	Intra-day precision (% RSD)	Inter-day accuracy (% MRE)	Inter-day precision (% RSD)
Bleach	20	-1.089	1.439	2.104	6.481
	650	-1.551	4.470	-7.371	8.509
	2500	-1.797	4.814	0.853	10.253
Betadine	20	-2.517	2.742	-3.193	4.246
	650	-1.566	3.493	0.161	8.509
	2500	-2.851	8.727	-0.871	10.253
PCC	20	-2.604	3.499	0.563	7.023
	650	-0.836	5.342	-2.709	10.414
	2500	0.748	10.007	8.975	12.909

As can be seen from **Table 5.10**, the intra-day and inter-day precision and accuracy results are generally similar to those found for the three methods not requiring the sample hydrolysis step. As with the results from **Table 5.9**, a Student's *t* test (with two tails and two degrees of freedom) comparing the reported THC-COOH retention times and calculated THC-COOH

concentration for the quality control samples was undertaken at the 95% confidence interval to assess the significance of these results (**Table 5.12**).

The null hypothesis proposed ($P > 0.05$) is that variations in the intra-day and inter-day values for retention time and calculated THC-COOH concentration are due to random variations in instrument sensitivity and calibration, and do not signify a statistically significant effect. **Table 5.1** displays the Student's *t* test results for the data generated for THC-COOH retention time and concentration over the intra-day and inter-day tests, with similar calculations for the methods involving sample hydrolysis found in **Table 5.12**. The results contained in **Tables 5.11** and **5.12** indicate that the variations in THC-COOH retention time and peak area from the intra-day and inter-day precision and accuracy tests all fall outside of the $P < 0.05$ confidence interval. The precision and accuracy results obtained through method validation are critical for method validation, with the observed changes in retention time and peak area over the intra-day and inter-day studies are due to random variations in instrument performance, and are not due to deliberate changes in instrument sensitivity, operation, or method parameters.

Table 5.11 - Results for the Student's *t* test for the three methods not requiring sample hydrolysis. THC-COOH retention time and calculated concentration derived from the inter-day (n=8) accuracy and precision tests.

Method	Quality Control Sample	Student's *t* Test Result – Retention Time (*P*)	Student's *t* Test Result – THC-COOH Concentration (*P*)
Bleach	1	0.580	0.270
	2	0.724	0.085
	3	0.126	0.566
Betadine	1	0.500	0.598
	2	0.110	0.942
	3	0.718	0.542
PCC	1	0.187	0.341
	2	0.612	0.500
	3	0.089	0.658

Table 5.12 - Results for the Student's *t* test for the three methods involving sample hydrolysis. THC-COOH retention time and calculated concentration derived from the inter-day (n=8) accuracy and precision tests.

Method	Quality Control Sample	Student's *t* Test Result – Retention Time (*P*)	Student's *t* Test Result – THC-COOH Concentration (*P*)
Bleach	1	0.385	0.724
	2	0.941	0.061
	3	0.078	0.249
Betadine	1	0.653	0.589
	2	0.098	0.927
	3	0.716	0.270
PCC	1	0.341	0.345
	2	0.706	0.410
	3	0.330	0.992

As can be seen, the statistical analysis of the retentions times and calculated concentrations of THC-COOH in both sets of methods are generally similar. Based on the results of the Student's *t* Tests for both retention time and concentration (n=8), it can be seen that the variations in these values arise from random variations in instrument performance.

Accuracy of the methods was also assessed through comparing the expected THC-COOH concentration for the calibration standards and quality control samples to the actual calculated values. **Table 5.13** and **Table 5.14** illustrate the accuracy of the calculated concentration of the quality control samples for the two sets of methods. This data was acquired during construction of the calibration curves via triplicate injections of the quality control samples for both sets of methods. Accuracy of the calculated concentrations of the tested samples was generally acceptable, with most reported concentrations in close agreement with the expected THC-COOH concentration.

Table 5.13 - Comparison of expected THC-COOH concentration, calculated concentration and accuracy for the calibration standards and quality control samples following triplicate injections for each method, without sample hydrolysis.

Sample (No Hydrolysis)	Expected Concentration (ng/mL)	Average Concentration (ng/mL) and Accuracy (%)		
		Bleach Method	Betadine Method	PCC Method
Quality Control 1	20.0	19.79 (99.0)	20.93 (104.6)	19.55 (97.7)
Quality Control 2	650.0	672.56 (96.5)	667.76 (102.7)	644.16 (99.1)
Quality Control 3	2500.0	2451.50 (98.1)	2569.10 (102.8)	2525.62 (101.0)

Table 5.14 - Comparison of expected THC-COOH concentration, calculated concentration and accuracy for the calibration standards and quality control samples following triplicate injections for each method, with sample hydrolysis.

Sample (Hydrolysis)	Expected Concentration (ng/mL)	Average Concentration (ng/mL) and Accuracy (%)		
		Bleach Method	Betadine Method	PCC Method
Quality Control 1	20.0	18.65 (93.3)	20.53 (102.7)	19.08 (95.4)
Quality Control 2	650.0	645.09 (99.2)	665.0 (102.3)	637.66 (98.1)
Quality Control 3	2500.0	2489.03 (99.6)	2487.98 (99.5)	2489.05 (99.6)

A further consideration in validation of the three selected methods was the peak shape observed for THC-COOH. Reproducible, consistent and symmetrical peaks with minimal apparent fronting or tailing were considered to be representative of an effective and reliable elution condition and detection method. Three samples were tested for each method not requiring sample hydrolysis to assess peak shape, and are outlined in **Figure 5.4**. As can be seen, for the low and high calibration standards, and quality control sample 1 (20 ng/mL), peak shape for all samples is consistent, and provides evidence that the elution condition of the target analyte is acceptable. Visually similar results were also obtained for the equivalent samples that underwent sample hydrolysis.

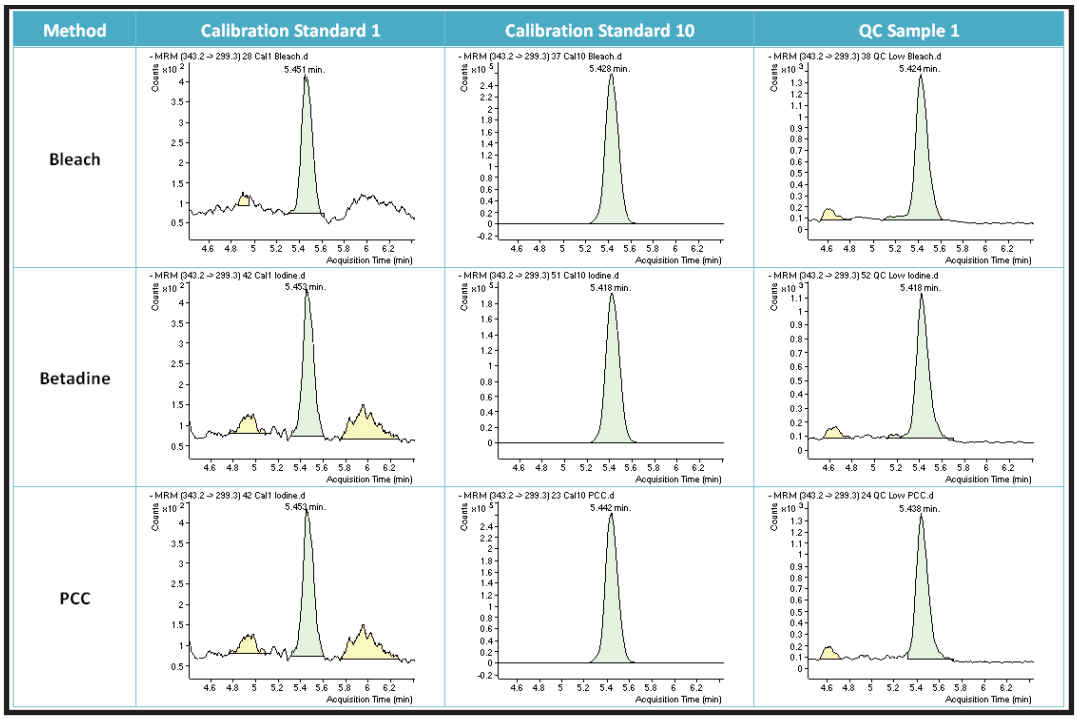


Figure 5.4 - LC-MS/MS chromatograms obtained for THC-COOH (*m/z* 343.2 → *m/z* 299.3 transition) for selected samples over the three methods not incorporating sample hydrolysis. Note that the peak shape and retention time are consistent over all nine tested samples. Also note that for Calibration Standard 1 (5 ng/mL) an acceptable signal-to-noise ratio can be seen.

Analysis of the quality control samples for intra-day and inter-day testing of method validation indicate that the selected method are consistent across multiple analyses. Precision and accuracy were reported for the intra-day tests, and though statistically significant variations in target analyte concentration were reported in the inter-day precision and accuracy tests, a Student's *t* test show that these variations on THC-COOH concentration are due to random variations in instrument performance.

Three key instrument parameters were used to assess method robustness both in terms of target analyte retention time and calculated concentration: chromatographic column temperature, elution condition and injection volume. The parameters were selected on the basis that they represent common parameters that may be subjected to variation due to operator error or changes in detection method and instrument performance. The three tested parameters were subjected to a ± 5% variation, corresponding to:

- Column temperature range of 38 – 40°C;
- Solvent B ratio of 0.8075 – 0.8925 %;
- Injection volume range of 1.9 – 2.1 µL.

The ± 5% change in these parameters was selected as it provides a small but potentially significant source of variation in target analyte retention time and calculated concentration. **Table 5.15** provides the calculated standard deviation for THC-COOH concentration and retention time in response to the deliberately modified parameters for the three quality control samples over the three tested methods.

Table 5.15 - Robustness testing: Calculated standard deviations in calculated THC-COOH concentration and retention time following changes in injection volume, column temperature and elution condition.

Parameter	Method	Adjustment	Standard Deviation of Calculated Concentration			Standard Deviation of Reported Retention Time		
			QC1	QC2	QC3	QC1	QC2	QC3
Injection Volume	Bleach	+5%	0.930	8.650	11.501	0.010	0.020	0.009
		0%	0.506	0.732	2.230	0.021	0.019	0.007
		-5%	0.674	2.045	6.569	0.014	0.013	0.012
	Betadine	+5%	0.863	2.401	6.151	0.020	0.012	0.004
		0%	1.158	1.078	3.940	0.013	0.010	0.006
		-5%	0.465	3.681	14.369	0.007	0.002	0.011
	PCC	+5%	0.676	7.436	4.878	0.008	0.014	0.010
		0%	0.159	3.001	3.773	0.008	0.005	0.007
		-5%	0.766	2.981	5.137	0.010	0.005	0.007
Column Temperature	Bleach	+5%	0.793	1.170	3.584	0.002	0.003	0.004
		0%	0.506	0.732	2.230	0.021	0.019	0.007
		-5%	1.661	0.841	3.878	0.005	0.019	0.008
	Betadine	+5%	0.771	2.381	3.740	0.005	0.004	0.007
		0%	1.158	1.078	3.940	0.013	0.010	0.006
		-5%	0.841	1.744	2.577	0.004	0.006	0.006
	PCC	+5%	0.614	1.186	1.483	0.002	0.007	0.008
		0%	0.159	3.001	3.773	0.008	0.005	0.007
		-5%	1.501	1.209	1.468	0.002	0.008	0.006

Table 5.15 (Continued) - Robustness testing: Calculated standard deviations in calculated THC-COOH concentration and retention time following changes in injection volume, column temperature and elution condition.

Parameter	Method	Adjustment	Standard Deviation of Calculated Concentration			Standard Deviation of Reported Retention Time		
			QC1	QC2	QC3	QC1	QC2	QC3
Elution Condition	Bleach	+5%	1.139	2.338	6.430	0.010	0.007	0.012
		0%	0.506	0.732	2.230	0.021	0.019	0.007
		-5%	0.285	2.451	3.654	0.041	0.023	0.038
	Betadine	+5%	1.637	1.950	8.573	0.006	0.027	0.010
		0%	1.158	1.078	3.940	0.013	0.010	0.006
		-5%	0.601	4.481	5.348	0.010	0.009	0.004
	PCC	+5%	0.957	4.140	9.115	0.006	0.005	0.012
		0%	0.159	3.001	3.773	0.008	0.005	0.007
		-5%	1.255	2.023	5.959	0.005	0.007	0.013

Table 5.15 provides the calculated standard deviations for changes in method parameters in order to assess the statistical significance of recorded data within a sample set. The calculated standard deviations for retention time through the robustness testing indicate that the recorded retention times of THC-COOH are highly consistent within a sample, and are subject to extremely minor variations. It is interesting to note that in terms of calculated THC-COOH concentration, the standard deviations (% RSD) recorded for the changes in column temperature and elution condition are similar to those recorded for the intra-day and inter-day precision tests, and suggest that the fluctuations in apparent THC-COOH concentration noted during method validation can be attributed to regular variations in instrument performance and sensitivity.

Low standard deviations were generally found for the calculated THC-COOH concentration, indicating that within a sample set the three tested samples resulted in similar reported concentrations. Outliers were found during this experiment, particularly with the repeated injections of the quality control samples at the highest concentration. For the PCC method a standard deviation of 9.115 was recorded for the elution condition test at + 5% organic solvent ratio. However, it is important to note that despite this high value, the reported THC-COOH

concentration was close to the expected value of 2500 ng/mL, with a calculated concentration range of 2489.6 – 2507.0 ng/mL.

The recorded retention times and calculated THC-COOH concentration are consistent within a sample set. **Table 5.16** provides the reported Student's *t* test values for the robustness tests for each method. As previously, a 95% confidence interval was used, with values of $P < 0.05$ considered statistically significant. Calculations were performed through comparison of the reported retention times and concentrations of THC-COOH under the standard, unmodified method, with each of the modified parameters. *P* values below 0.05 will indicate whether a change in method parameter results in a statistically significant effect on retention time or target analyte concentration.

Table 5.16 - Robustness testing: Results of the Student's *t* test following changes in injection volume, column temperature and elution condition. Statistically significant results (*P < 0.05*) in bold.

Parameter	Method	Adjustment	Reported Retention Time Student's *t* Test (*P*)			Calculated Concentration Student's *t* Test (*P*)		
			QC1	QC2	QC3	QC1	QC2	QC3
Injection Volume	Bleach	+5%	0.243	0.578	0.156	**0.001**	**0.027**	**0.001**
		-5%	0.827	0.478	0.368	0.510	**0.001**	**0.001**
	Betadine	+5%	0.232	0.891	**0.006**	**0.016**	**0.001**	**0.000**
		-5%	0.748	0.425	0.339	**0.014**	**0.019**	**0.002**
	PCC	+5%	0.476	0.913	0.626	**0.004**	**0.020**	**0.000**
		-5%	0.421	0.699	0.791	**0.003**	**0.008**	**0.002**

Table 5.16 (continued) - Robustness testing: Results of the Student's t test following changes in injection volume, column temperature and elution condition. Statistically significant results (*P < 0.05*) in bold.

Parameter	Method	Adjustment	Reported Retention Time Student's *t* Test (*P*)			Calculated Concentration Student's *t* Test (*P*)		
			QC1	QC2	QC3	QC1	QC2	QC3
Column Temperature	Bleach	+5%	**0.005**	**0.005**	**0.000**	0.078	0.585	0.974
		-5%	**0.044**	0.081	**0.002**	0.111	0.363	0.709
	Betadine	+5%	**0.001**	**0.001**	**0.000**	0.949	0.296	0.431
		-5%	**0.018**	**0.003**	**0.001**	0.998	0.754	0.770
	PCC	+5%	**0.000**	**0.000**	**0.001**	0.303	0.432	0.985
		-5%	**0.002**	**0.003**	**0.002**	0.577	0.938	0.353
Elution Condition	Bleach	+5%	**0.043**	**0.037**	**0.018**	**0.049**	0.970	0.858
		-5%	0.879	0.722	0.977	**0.005**	0.492	0.858
	Betadine	+5%	**0.004**	0.158	**0.018**	0.683	0.597	0.637
		-5%	0.217	**0.023**	**0.001**	0.063	0.165	0.837
	PCC	+5%	**0.001**	**0.000**	**0.030**	0.270	0.584	0.961
		-5%	**0.012**	**0.004**	**0.050**	0.889	0.953	0.672

From the reported values in **Table 5.16**, variations in injection volume results in a statistically significant variation in reported target analyte concentration. Deliberate changes in sample injection volume did not have a significant effect on the retention time of THC-COOH, and in contrast, changes in column temperature and elution condition, major effects on retention time of THC-COOH were reported. Changes in column temperature and elution condition did not have a significant effect on the calculated concentration of THC-COOH.

The above statistical analyses provide strong evidence for the potential of deliberate changes in instrument parameters to affect the resultant data. In **Table 5.17** and **Table 5.18**, the robustness of the three methods is reported in terms of the average percent difference. This statistical value was calculated through a comparison of the average retention time for each

method and quality control samples under the deliberately modified instrument parameter, and was then compared with the average of the baseline results obtained in the unmodified method. This value provides a direct assessment of robustness to be established based on the deliberate variation of method parameters on the retention time of THC-COOH. The average percent difference was also calculated for the reported peak area of THC-COOH and the effect of deliberate changes in method parameters, and is shown in **Table 5.18**.

A clear indication of the effects of modified instrument parameters on how changing parameters affect obtained results can be seen in **Table 5.17** and **Table 5.18**. It can be seen that in terms of the retention time of THC-COOH, an increase in column temperature or organic solvent proportion in the elution condition can results in a decreased retention time for the target analyte. Of the parameters tested during robustness, column temperature had the most significant effect. Conversely, decreasing the column temperature or proportion of organic solvent in the mobile phase both resulted in minor changes in the retention time of THC-COOH.

Table 5.17 - Robustness testing: Reported retention times for quality control samples following changes in column temperature, elution condition and injection volume.

Method	QC Sample	Average Retention Time (minutes)	Average Retention Time (minutes)					
			Column Temperature		Elution Condition		Injection Volume	
			+5 %	- 5%	+5 %	- 5%	+5 %	- 5%
Bleach	1	5.47	5.40	5.51	5.43	5.47	5.46	5.47
	2	5.47	5.41	5.51	5.44	5.48	5.461	5.46
	3	5.46	5.41	5.50	5.43	5.46	5.55	5.47
Betadine	1	5.480	5.41	5.51	5.43	5.49	5.46	5.48
	2	5.47	5.41	5.52	5.44	5.50	5.47	5.48
	3	5.46	5.41	5.51	5.44	5.50	5.47	5.47
PCC	1	5.47	5.41	5.51	5.43	5.50	5.47	5.48
	2	5.48	5.42	5.51	5.43	5.51	5.48	5.48
	3	5.47	5.41	5.52	5.45	5.50	5.48	5.48
Average % Difference			98.86	100.70	99.35	100.31	99.92	100.04

For the concentration of THC-COOH it is important to note the differences that exist between the expected THC-COOH concentration and the actual concentration of the tested samples. As discussed previously in regards to assessment of accuracy, this variation is due in part to factors involved during sample preparation and instrument sensitivity. Regarding the data present in **Table 5.18**, the trend observed for retention time is reversed: both column temperature and elution condition have a negligible effect, and injection volume has a significant effect. In addition, the effect of injection volume is most apparent with the quality control samples at the lowest concentration, and remains apparent at the higher concentrations. In terms of target analyte concentration, the trend observed for retention time is reversed, whereby injection volume has the largest effect on analyte peak area.

Table 5.18 - Robustness testing: Calculated THC-COOH concentration for quality control samples following changes in column temperature, elution condition and injection volume.

Method	QC Sample	Expected THC-COOH Concentration (ng/mL)	Average THC-COOH Concentration (ng/mL	Average Calculated Concentration (ng/mL)					
				Column Temperature		Elution Condition		Injection Volume	
				+5 %	- 5%	+5 %	- 5%	+5 %	- 5%
Bleach	1	20	17.7	19.0	19.7	19.7	19.5	23.6	17.3
	2	650	652.8	653.3	653.5	652.9	651.7	669.9	642.9
	3	2500	2501.9	2501.7	2502.9	2501.2	2501.4	2567.9	2461.6
Betadine	1	20	19.7	19.7	19.7	20.2	21.6	23.0	16.7
	2	650	649.7	651.5	650.1	650.4	654.2	661.6	641.3
	3	2500	2501.2	2502.2	2499.2	2504.0	2502.1	2576.9	2442.3
PCC	1	20	19.8	20.3	20.4	20.5	19.7	22.2	16.8
	2	650	650.4	648.8	650.6	648.6	650.3	667.6	638.2
	3	2500	2500.2	2500.2	2502.6	2499.9	2502.0	2584.4	2475.3
Average % Difference – Expected Concentration		QC1		98.4	99.8	100.9	101.6	115.0	85.0
		QC2		100.1	100.2	100.1	100.3	102.5	98.6
		QC3		100.0	100.0	100.0	100.0	103.0	98.4
Average % Difference – Actual Concentration		QC1		103.0	101.3	101.1	100.6	113.1	73.9
		QC2		100.0	100.0	99.9	100.1	102.3	98.4
		QC3		100.0	100.0	100.2	100.0	103.0	98.4

Matrix effects were also assessed through the preparation of two sets of quality control samples in water and urine. Direct injection of the two sets of quality control samples was undertaken for the three methods. Assessment of matrix effects was achieved through a comparison of the absolute peak area for THC-COOH and the internal standard in both matrices, and it was found that the peak area for the two compounds was consistently lower in the urine matrix. This decrease in peak area is associated is attributed to ion suppression, and resulted in an average 10% decrease in peak area for both THC-COOH and the internal standard.

Throughout the method validation testing it has been possible to generate a total of three validated methods for the simultaneous quantitative detection of THC-COOH and the qualitative detection of selected compounds formed through adulteration of cannabis-positive urine samples. With the exception of the di-iodo-THC-COOH compound, optimisation of the detection and instrument parameters has allowed for a total of three ion transitions to be selected for all target analytes – one quantifier ion and two qualifier ions, with collision energies optimised. At present, certified reference standards are not available for the detected reaction products. Despite this, should these reference materials become available in the future, it will be possible to design methods that will allow for the quantitative detection of the targeted reaction products. In addition, should additional oxidation products of THC-COOH be found for other adulterants, it may be possible to design a mixed method that will allow for the simultaneous detection of the use of a range of oxidising adulterants in attempts to invalidate the confirmatory testing of cannabis-positive urine samples.

Validation of the three methods has allowed for the suitability of these methods for pH, kinetics, stability and real urine studies to be assessed. Linearity range and correlation coefficients indicate that the three methods are suitable for the quantitative detection of THC-COOH over a wide concentration range, allowing for the analysis of low and high concentration samples. Intra-day and inter-day precision and accuracy were also found to be generally acceptable, with weaker precision and accuracy discovered in the inter-day tests of the three methods. It is interesting to note that though variations in retention time and THC-COOH concentration was apparent over the intra-day and inter-day studies, a Student's *t* test with a 95% confidence interval indicates that these variations are ultimately due to slight changes in instrument stability, equilibration and calibration, and did not impugn prior aspects of the method validation. Based on the method validation results it is possible for a consolidated

method to be developed that is capable of detecting the ion transitions for the target analytes present in the three methods.

The largest fluctuations in retention time and concentration were discovered during the robustness testing of the three methods. In general the results during robustness were as expected considering that changes in retention time are associated with elution condition and column temperature, and calculated concentration will vary with changing injection volumes. Overall it can be stated that with regards to THC-COOH, the three generated methods for the three selected oxidising adulterants have been validated. Consistent results for target analyte concentration are obtained through replicate injections, as evidenced by the results of the intra-day and inter-day precision and accuracy studies. In addition, the retention time of THC-COOH is consistent, an important consideration for the reliable and reproducible detection of the target analyte.

The following chapter will deal with pH, kinetics and stability studies for the three selected oxidising adulterants. Though the validated methods are only quantitative for THC-COOH, these studies will assess the formation of the targeted reaction products in vitro, and will determine the effects of sample temperature and sample pH on product formation and stability. It is important to note that one major limitation of these methods for the quantitative detection of THC-COOH and the qualitative detection of the targeted reaction products is the internal standard selected. Though THC-COOH-d_9 is commonly used for the quantitative analysis of cannabis-positive drug samples, in cases where oxidising agents have been used to mask potential drug use, traces of the oxidant remaining within the sample may result in degradation of the internal standard. This issue will be discussed further in Chapter 6.

Chapter 6

Product Formation and Stability in Spiked Urine

As outlined in previous chapters, the detection of potential markers of both drug abuse and subsequent urine adulteration may allow for laboratories to effectively test samples that would otherwise return a false-negative test result. Though the formation of these reaction products in both spiked water and urine samples has been demonstrated, the formation and stability of the targeted reaction products in urine needs to be ascertained to determine their suitability as potential markers of urine adulteration. As such, additional studies were undertaken to test the effects of urine pH and storage temperature on the formation and stability of these compounds, and were achieved through use of the three validated methods from Chapter 5 that did not include a sample hydrolysis step, and whether they remain detectable following prolonged periods of storage.

The chemical properties of the adulterants are considered as a potential source of differences in both the in vitro loss of THC-COOH and reaction product formation. Both pyridinium chlorochromate and Betadine antiseptic solution are acidic, and it is therefore expected that these reactions will progress more readily under acidic conditions. Conversely, bleach is an alkaline solution, and it is therefore considered likely that the reaction between THC-COOH and sodium hypochlorite will be more extensive under alkaline conditions.

Sample storage is also considered to have an effect on the formation and stability of the reaction products. At lower temperatures, the reaction between THC-COOH and the selected adulterants may be inhibited, and may result in the preferential formation of the mono-halogenated products found in the sodium hypochlorite and Betadine reactions. Alternately, the di-halogenated reaction products may form preferentially at higher temperatures due to a more extensive reaction the mono-halogenated reaction products and THC-COOH to form the di-halogenated products.

6.1 – Experimental

6.1.1 – Drug Standards and Reagents

Drug standards and reagents used for method validation are found in Chapter 2. Sodium hypochlorite concentration of the hypochlorite solution was determined spectrophotometrically as per Chapter 3. The final hypochlorite stock solution concentration used in these studies was determined to be 0.240 M.

6.1.2 – Urine Specimens

Urine specimens were collected from healthy individuals (n= 14) and stored in polypropylene urine specimen containers at 4°C to create a representative blank urine matrix. Volunteers were selected randomly, with an age range from 21-62, and both males (n=7) and females (n=7) equally represented. As with previous studies using pooled urine, the primary qualification factor for selected donors was that they had not used cannabis, or had been in contact with cannabis products, for the past three months. The combination of urine specimens to form pooled urine for research was not used for more than one experiment. Pooled urine specimens were stored at 4°C, and following analysis samples were sealed and stored under the same conditions.

6.1.3 – Instrumentation

Instrumentation for these studies is as per the three validated methods not incorporating a sample hydrolysis step found in Chapter 5. Chromatographic separation was undertaken on a Phenomenex Luna C_5 HPLC column (150 mm x 4.6 mm, 5 micron, Phenomenex Incorporated).

6.1.4 – Preparation of Samples for pH, Kinetics and Stability Studies

Pooled urine samples were spiked with THC-COOH to provide a final THC-COOH concentration of 2000 ng/mL following adulteration of the samples. For the pH studies, aliquots of the pooled urine had their pH adjusted with 0.05 M hydrochloric acid and 0.05 M sodium hydroxide, resulting in two pH-adjusted pooled urine specimens at pH 5 and pH 8, and were intended to represent the variability of pH in typical human urine. For the kinetics studies, the pH of the pooled urine was determined to be 6.5, and was not adjusted prior to sample preparation.

Working solutions of the selected adulterants were prepared for use in these studies. The pyridinium chlorochromate working solution was prepared by dissolving pyridinium chlorochromate in water to a final concentration of 0.370 M. Betadine and sodium hypochlorite were used undiluted in these studies, with a final stock solution concentrations of 1% w/v iodine and 0.240 M sodium hypochlorite.

Four sets of samples were prepared in triplicate for each of the oxidants to assess the effects of two pH conditions and two sample storage conditions, with the final sample volume set at 500 µL. The pyridinium chlorochromate reactions were prepared through the addition of 50 µL of the pyridinium chlorochromate working solution, with a final oxidant concentration of 37.0 mM. Reaction samples for sodium hypochlorite were prepared through the addition of 50 µL of the sodium hypochlorite stock solution, resulting in a final hypochlorite concentration of 24.0 mM. Betadine reaction samples were prepared through the addition of 100 µL to the spiked urine sample, resulting in a final iodine concentration of 0.2% w/v.

Following sample preparation, the samples were filtered through 0.2 µm Millipore syringe filters into a new vial, sealed, and immediately submitted for analysis. Between analyses all samples were sealed prior to storage. For the storage condition study, one set of samples were refrigerated at 4°C, with the other set stored at room temperature away from any light sources. Samples prepared for the pH study were also refrigerated at 4°C between analyses.

Further details concerning the preparation of the samples for these studies are present in the Appendix.

6.1.5 – Effect of Oxidants on Internal Standard

The potential effect of the selected adulterants on the internal standard was also assessed. This was carried out through analysis of the samples prepared for the pH, kinetics and stability studies, and was compared to the peak area and peak height recorded for the internal standard in the calibration standards and quality control samples. Degradation of the internal standard will have an adverse effect on the quantitation of THC-COOH, and requires an alternate means of obtaining an estimate of the in vitro THC-COOH concentration. This is further explored in **Section 6.2.1**.

6.2 – Results and Discussion

6.2.1 – Effects of Oxidants on Internal Standard

A major issue to consider for the quantitative analysis of THC-COOH is the effect of the oxidising adulterants on the internal standard. As the internal standard selected for these studies is deuterated THC-COOH, it is expected to also react with the oxidants in vitro, thereby hindering accurate quantitation of the target analyte. A subset of the pH, kinetics and stability studies was therefore an analysis of the effect of oxidising adulterants and reaction conditions on potential degradation of the starting material over time.

Figure 6.1 shows the effect of pyridinium chlorochromate on the peak area of the internal standard over the selected urine pH and storage conditions over time. The results of this study were compared with the average internal standard peak area obtained from the quality control samples collected throughout the studies presented in this chapter. As can be seen, pyridinium chlorochromate has a major effect on the peak area of the internal standard, with the apparent loss of the internal standard attributed to its reaction with the oxidant.

In terms of the kinetics studies, at the lower sample storage temperature (4°C) loss of the internal standard is slower in comparison with the sample stored at room temperature. Similarly, for the pH studies, an acidic reaction condition leads to a rapid loss of the internal standard. As pyridinium chlorochromate is an acidic compound, it is expected that under alkaline conditions, the reaction with the internal standard is slowed temporarily.

Overall it can be seen that a near-total loss of the internal standard occurs between 100 minutes and 120 minutes, with traces of the internal standard remaining after these time points. In addition, no apparent regeneration of the internal standard is apparent over the 24 days of the study. Due to the extensive loss of the internal standard for the pyridinium chlorochromate studies, an accurate determination of the concentration of THC-COOH is not likely, and will therefore require an estimate of THC-COOH concentration in these studies.

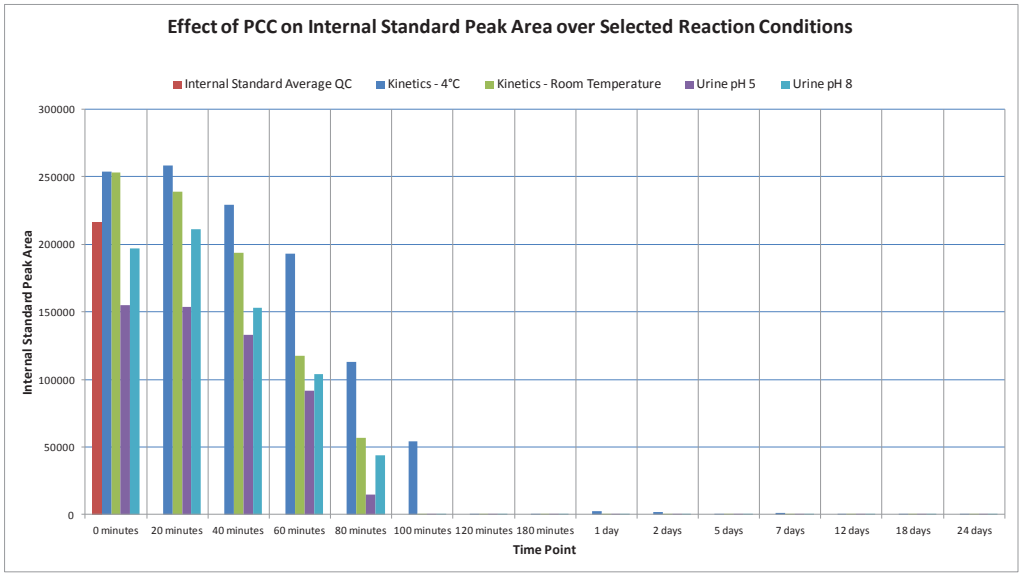


Figure 6.1 - Effect of pyridinium chlorochromate on internal standard peak area over a period of 24 days under the four tested reaction conditions.

In the studies concerning the effect of sodium hypochlorite on the detected peak area of the internal standard, it can be seen that most reaction and sample storage conditions do not lead to a near-total loss of the internal standard. **Figure 6.2** reveals that the most significant loss of the internal standard occurs at an acidic urine pH, whilst an alkaline urine pH results in a significant amount of the internal standard remaining. Under alkaline conditions, it is interesting to note that at the initial time point (0 minutes), a major decrease in internal standard peak area is apparent. After this time point, the peak area for the internal standard begins to increase, and may indicate regeneration of the internal standard under these specific pH conditions, or alternately, formation of a complex that temporarily decreases the apparent concentration of the internal standard.

The cause of this potential regeneration of the internal standard is unclear, as it is expected that under alkaline conditions, addition of the alkaline sodium hypochlorite solution should not result in regeneration of the internal standard. Were the internal standard to undergo a reversible reaction with sodium hypochlorite, it would be expected that this would be evident at room temperature, where the higher sample temperature would accelerate the regeneration of the internal standard. Such an apparent increase in internal standard peak area at room temperature does appear to occur approximately 100 minutes after addition of the oxidant, with a slight increase in internal standard peak area reaching a maximum peak area at 160 minutes.

In comparison with the pyridinium chlorochromate studies, storage of the hypochlorite samples at the last time point (20 days) still allows for detection of the internal standard. Though the internal standard remains detectable at this time point, the apparent losses of the internal standard will likely lead to an incorrect calculation of the in vitro concentration of THC-COOH. As such, an alternate method will be devised to provide an estimate of the THC-COOH concentration in these studies at the set time points.

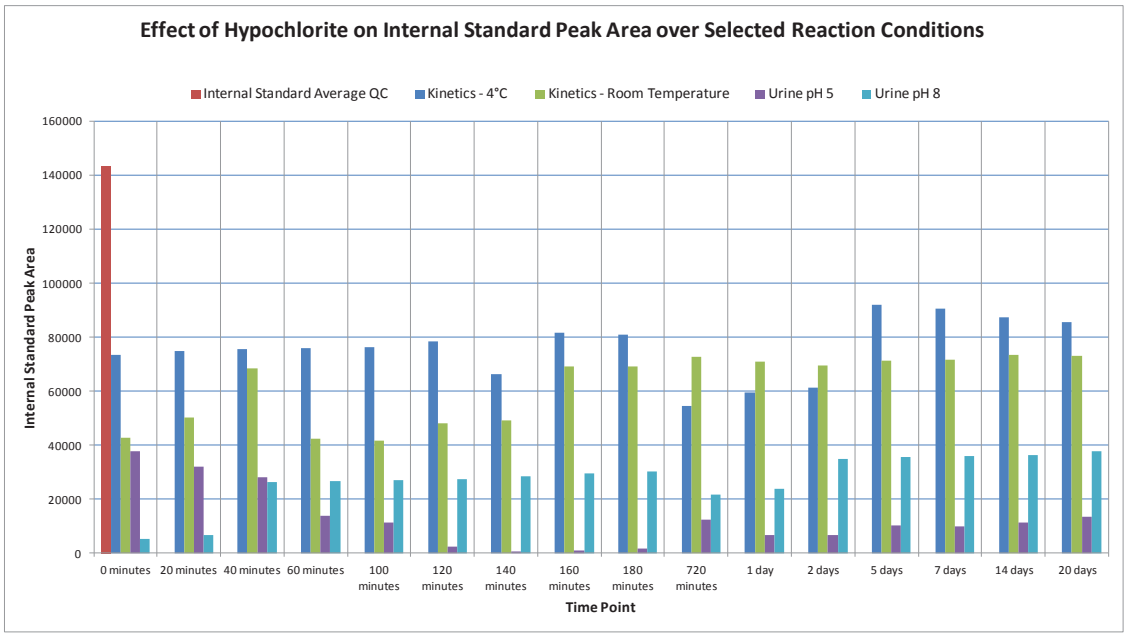


Figure 6.2 – Effect of alkaline sodium hypochlorite (bleach) on internal standard peak area over a period of 20 days under the four tested reaction conditions.

For the studies on the effect of Betadine on the peak area of the internal standard (**Figure 6.3**), the results obtained bear a resemblance to those found in the pyridinium chlorochromate studies. Overall, a near-total loss of the internal standard occurs under three of the reaction and sample storage conditions at approximately 60 minutes; however, at 4°C, traces of the internal standard remain after 24 days of storage. For the kinetics studies loss of the internal standard is slower under the cooler sample storage condition and stops at approximately 100 minutes, with the internal standard remaining detected subsequently. In comparison, at room temperature the loss of the internal standard progresses quickly, and leads to effectively total loss of the internal standard following 100 minutes.

In terms of the pH studies, it appears that the loss of the internal standard progresses rapidly under acidic conditions. For these studies it is noted that like studies on the effect of sodium hypochlorite on the peak area of the internal standard, an apparent regeneration of the

internal standard occurs. For Betadine this apparent regeneration occurs between 0 minutes and 40 minutes, following which degradation of the internal standard reoccurs and results in a near-total loss of the internal standard.

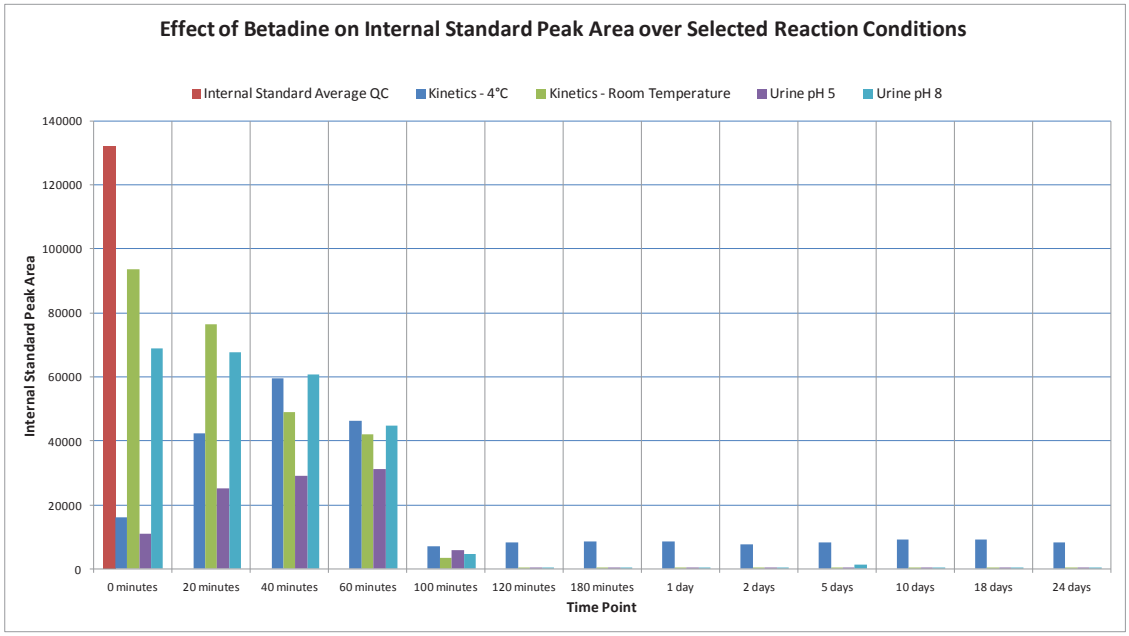


Figure 6.3 - Effect of Betadine on internal standard peak area over a period of 20 days under the four tested reaction conditions.

It is important to note that for drug testing laboratories, the effect of the oxidising adulterants on the internal standard is less likely to present a major issue. For pyridinium chlorochromate and Betadine, near-total degradation of the internal standard occurs around 120 minutes. In comparison, the bleach studies indicate that under most reaction conditions, a significant amount of the internal standard will remain even at the 20 day mark, though under acidic conditions this loss is more extensive. From the perspective of a drug testing laboratory, addition of the internal standard will occur well before sample derivatisation, extraction, and reconstitution of the sample prior to analyses undertaken with GC-MC. In spite of this, the period between collection of the sample and it being received by the drug testing laboratory may result in sufficient time for adulterants to extensively react with the target analytes. Indeed, at higher oxidant concentrations, it is likely that the oxidant will have been exhausted through reaction with THC-COOH and the endogenous compounds present in the urine. As a consequence, this may limit the ability of a drug testing laboratory to determine the pre-adulteration concentration of THC-COOH, although qualitative detection of the reaction

products at a lower oxidant concentration may remain possible. However, in the studies undertaken in this chapter, it should be noted that the analysis of the effects of the oxidising adulterants occurs immediately following addition of the oxidant to a spiked urine sample, and is followed by the addition of the internal standard. Though this is a departure from what would be expected in drug testing laboratories, this step was undertaken primarily to maximise the number of time points immediately after sample adulteration. A secondary consideration is that later addition of the internal standard significantly later than the addition of the adulterant would ultimately dilute the sample, decrease the peak areas for the targeted analytes, and therefore complicate interpretation of the data obtained in these studies.

As it is apparent that the loss of the internal standard occurs quickly and to a major extent with the bulk of the tested reaction and sample storage conditions, an accurate determination of THC-COOH is not possible. Consequently, it is necessary to develop a method by which a suitable estimation of the concentration of THC-COOH can be obtained, and will be discussed below.

6.2.2 – Estimation of THC-COOH Concentration Following Internal Standard Degradation

Due to the effects of the oxidising adulterants on the internal standard, it is necessary to devise a suitable means by which an estimation of THC-COOH concentration may be calculated, with the THC-COOH peak representing a viable means by which such estimations may be made. For the purposes of estimating THC-COOH concentration, the peak area of the target analyte was selected for analysis, with the equation of the calibration curves for the three detection methods used for these estimations. In order to determine the accuracy of these calculations, samples with a known THC-COOH concentration were prepared, namely the calibration standards and quality control samples prepared for method validation, and were subsequently tested.

Updated calibration curves were generated based on the absolute peak area of THC-COOH in order to assess the relative accuracy of the estimated THC-COOH concentration in cases where the internal standard is not available due to the effect of the adulterants. It was found that with the updated calibration curve that the estimated concentration of samples of known concentration would routinely be under-estimated and over-estimated. As such, a correction factor was proposed to allow for a good estimation of THC-COOH concentration to be

calculated. This correction factor was based on the difference between the known and estimated THC-COOH concentrations in the calibration standards and quality control samples as per the following equation:

$$\text{Correction Factor} = \frac{\text{True THC} - \text{COOH Concentration}}{\text{Estimated THC} - \text{COOH Concentration}}$$

This correction factor was subsequently incorporated into the line equation of the calibration curves without the internal standard. The correction factors for the PCC method, bleach method and Betadine method were 0.645, 0.622, and 0.712, respectively. Application of the correction factor to the estimation of THC-COOH concentration significantly increased the accuracy of the reported estimations, and is considered a suitable means of estimating THC-COOH concentration in the remainder of this study where the original calibration curves cannot be effectively used due to loss of the internal standard.

Table 6.1 shows the estimation of the concentration of the calibration standards and initial quality control samples for the pyridinium chlorochromate method. Following use of a correction factor based on the accuracy of the initial estimates of THC-COOH concentration based on peak area, the average post-correction accuracy for the thirteen samples is 99.9%. Accuracy for individual samples was found to generally fall in a ±5% range, with an outlier found at the highest calibration standard concentration, where the estimated concentration was underestimated by the calculation following incorporation of the correction factor.

Table 6.1 - Estimation of THC-COOH concentration for calibration standards and quality control samples for the PCC method. Based on initial accuracy of the estimated THC-COOH concentration, a correction factor of 0.645 is proposed.

Sample	Accurate Concentration (ng/mL)	Peak Area Estimate (ng/mL)	Accuracy (%)	Updated Estimate (ng/mL)	Post-Correction Accuracy (ng/mL)
QC1	19.5	31.1	159.0	20.0	102.5
QC2	644.2	1035.2	160.7	667.7	103.7
QC3	2525.6	3752.2	148.6	2420.2	95.8
Calibration 1	4.7	6.9	145.5	4.4	93.8
Calibration 2	10.9	17.5	160.4	11.3	103.4
Calibration 3	23.7	36.6	154.6	23.6	99.7
Calibration 4	75.6	120.0	158.7	77.4	102.4
Calibration 5	176.6	284.4	161.1	183.4	103.9
Calibration 6	596.9	930.0	155.8	599.9	100.5
Calibration 7	819.5	1310.3	159.9	845.1	103.1
Calibration 8	1132.2	1823.0	161.0	1175.8	103.9
Calibration 9	2254.5	3489.2	154.8	2250.5	99.8
Calibration 10	4978.1	6692.3	134.4	4316.6	86.7
Average Accuracy			154.9		99.9

For the reactions involving sodium hypochlorite a similar estimation of the concentration of the calibration standards and quality control samples is made for the sodium hypochlorite method. Based on the initial estimates of THC-COOH concentration and the accuracy calculated for these results, a correction factor of 0.622 is proposed. **Table 6.2** shows the results obtained through these calculations. As can be seen, incorporation of the correction factor provides an acceptable estimate of THC-COOH concentration. Accuracy for individual samples was found to generally fall in a ±5% range. It should be noted at the lowest calibration standard concentrations, overestimation of the THC-COOH concentration was found, and as with the pyridinium chlorochromate calculations, underestimation of the THC-COOH concentration occurred at the highest calibration standard concentration.

Table 6.2 - Estimation of THC-COOH concentration for calibration standards and quality control samples for the sodium hypochlorite method. Based on initial accuracy of the estimated THC-COOH concentration, a correction factor of 0.622 is proposed.

Sample	Accurate Concentration (ng/mL)	Peak Area Estimate (ng/mL)	Accuracy (%)	Updated Estimate (ng/mL)	Post-Correction Accuracy (ng/mL)
QC1	19.3	31.1	161.0	19.3	100.1
QC2	626.9	1045.7	166.8	650.4	103.8
QC3	2446.9	3768.3	154.0	2343.9	95.8
Calibration 1	0.5	0.9	170.5	0.6	106.1
Calibration 2	0.7	1.1	174.4	0.7	108.4
Calibration 3	24.9	38.3	153.9	23.8	95.7
Calibration 4	74.3	122.6	165.1	76.3	102.7
Calibration 5	170.9	280.9	164.3	174.7	102.2
Calibration 6	583.8	932.0	159.7	579.7	99.3
Calibration 7	818.5	1350.5	165.0	840.0	102.6
Calibration 8	1136.6	1856.2	163.3	1154.6	101.6
Calibration 9	2261.4	3552.9	157.1	2209.9	97.7
Calibration 10	4975.6	6787.0	136.4	4221.5	84.8
Average Accuracy			160.9		100.1

Table 6.3 shows the results of the calculations related to estimation of THC-COOH concentration for the Betadine method. As with the other oxidants, estimates of THC-COOH concentration were based on the calibration standards and quality control samples prepared for this method. Based on the initial accuracy calculations, a correction factor of 0.712 is proposed. Incorporation of the correction factor leads to an average THC-COOH concentration accuracy of 99.9%. For most individual samples, the calculated post-correction accuracy fell in a ±10% range, with several instances where the estimated THC-COOH concentration was above or below the expected concentration. Despite this, incorporation of the correction factor provides a far better estimate of the target analyte concentration, and will therefore be used in the pH, kinetics and stability studies to estimate THC-COOH concentration.

Table 6.3 - Estimation of THC-COOH concentration for calibration standards and quality control samples for the Betadine method. Based on initial accuracy of the estimated THC-COOH concentration, a correction factor of 0.712 is proposed.

Sample	Accurate Concentration (ng/mL)	Peak Area Estimate (ng/mL)	Accuracy (%)	Updated Estimate (ng/mL)	Post-Correction Accuracy (ng/mL)
QC1	20.9	26.1	124.8	18.6	88.8
QC2	667.8	828.5	124.1	589.9	88.3
QC3	2569.1	3660.1	142.5	2606.0	101.4
Calibration 1	4.7	7.6	161.8	5.4	115.2
Calibration 2	11.5	18.7	162.4	13.3	115.7
Calibration 3	26.2	36.8	140.6	26.2	100.1
Calibration 4	76.8	116.3	151.5	82.8	107.9
Calibration 5	173.8	278.2	160.0	198.1	113.9
Calibration 6	604.1	896.5	148.4	638.3	105.7
Calibration 7	858.0	1089.0	126.9	775.4	90.4
Calibration 8	1159.9	1454.0	125.4	1035.2	89.3
Calibration 9	2231.2	3383.9	151.7	2409.3	108.0
Calibration 10	4975.6	5209.8	104.7	3709.3	74.6
Average Accuracy			140.4		99.9

Overall, analysis of the accepted and estimated THC-COOH concentrations allowed for a suitable method to be devised for an acceptable estimation of THC-COOH to be made following the addition of the oxidising adulterants and subsequent degradation of the internal standard. Though these estimations will not necessarily provide a true value of THC-COOH concentration to be determined, incorporation of the correction factor will regardless improve the accuracy of the estimated target analyte concentrations.

6.2.3 – pH Studies

Urine pH is likely to have a significant effect on both the in vitro reaction of THC-COOH and the subsequent formation of reaction products and their stability. As discussed, both pyridinium chlorochromate and Betadine solution are slightly acidic, whilst the sodium hypochlorite solution is alkaline. Accordingly, addition of these adulterants to a urine sample will result in a subsequent increase or decrease in sample pH. For the pH studies, the pooled urine specimens were separated into two groups. Pooled urine samples for the study at pH 5 were acidified

with dilute hydrochloric acid; and pooled urine samples for the study at pH 8 were basified with a dilute solution of sodium hydroxide.

For all three adulterants, THC-COOH and the reaction products were monitored over time to assess the effects of urine pH on the reaction of THC-COOH, formation of products, and the stability of the products over time. As the separate methods were validated only in terms of THC-COOH concentration due to the lack of reference standards for the reaction products, retention time, peak area, peak height, calculated concentration based on the calibration curves and estimated concentration due to loss of the internal standard were monitored for THC-COOH. For the reaction products, the key data monitored was the retention time, peak area and peak height of the products. For all analyses the retention times, peak areas and peak heights were also recorded for the internal standard where possible.

6.2.3.1 – Urine pH and Effect on THC-COOH Peak Area and Concentration

The first aspect of exploring the effects of oxidising adulterants on the in vitro reaction of THC-COOH and subsequent formation of products is to analyse the effects of the oxidants and urine pH on the loss of THC-COOH. As such, the peak areas and calculated THC-COOH concentrations were monitored for the three oxidants at both urine pH conditions over an extended period of time.

For the adulteration of spiked urine samples with pyridinium chlorochromate, a total of fifteen data points were collected over a period of 24 days. **Figure 6.4** provides the estimated THC-COOH concentration over the fifteen data points for the two different pH conditions. As can be seen, the initial loss of THC-COOH was highest under the acidic condition and under alkaline condition progresses more slowly. The initial THC-COOH concentration in all spiked urine samples was set at 2000 ng/mL. At the first time point (T = 0 minutes) at pH5, an approximate 50% decrease in THC-COOH concentration is recorded. At the same time point for the urine sample at pH 8, a 16% decrease in THC-COOH concentration is observed. At the second time point (T = 20 minutes) a slight increase in THC-COOH concentration is recorded, with the cause of this apparent concentration increase unknown.

At the final time point (T = 24 days) the final THC-COOH concentration for the pH 5 and pH 8 urine samples is 64 ng/mL and 84 ng/mL, respectively. This corresponds to a total decrease in THC-COOH concentration above 95%, with only a small amount of THC-COOH remaining.

Though the remaining amount of THC-COOH is relatively low, these concentrations still fall into the detection range of immunoassay-based screening techniques, potentially allowing for a positive test result to be returned, and will be discussed further in Chapter 7.

The increased rate of loss of THC-COOH at the initial time point (T = 0 minutes) is likely due to the acidic nature of pyridinium chlorochromate, with a similar effect seen with the degradation of the internal standard. With regards to the loss of THC-COOH from the spiked urine sample, under both pH conditions it appears that the reaction ceases after 100 minutes, with no additional significant losses of THC-COOH noted.

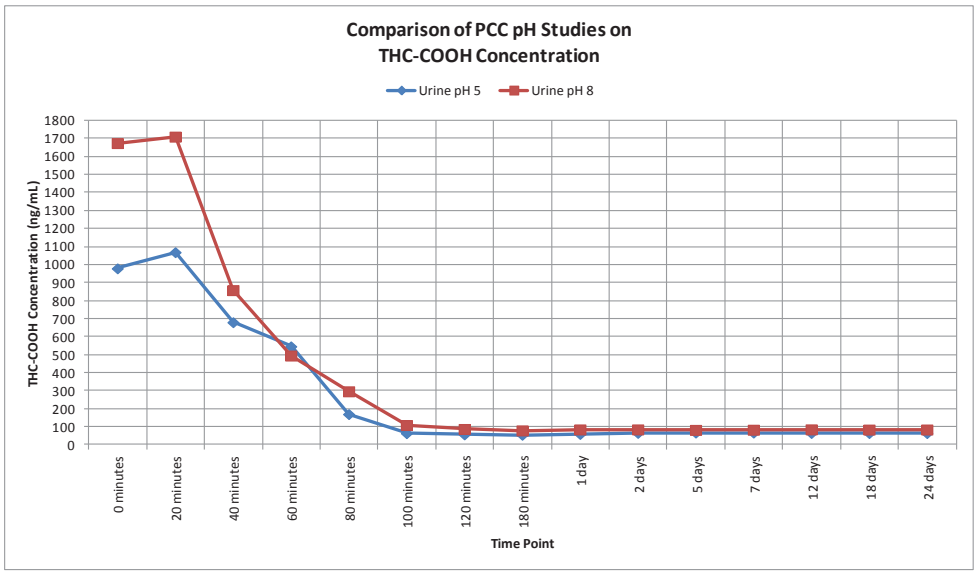


Figure 6.4 - Comparison of the PCC pH studies on the corrected estimated THC-COOH concentration over 24 days.

Similar analyses were undertaken for the urine samples spiked with sodium hypochlorite solution. For the tests involving adulteration with sodium hypochlorite, a total of sixteen data points were recorded for both pH conditions, corresponding to an analysis of the samples over a period of 20 days. **Figure 6.5** reports the calculated concentration of THC-COOH over the two urine pH conditions over the sixteen time points. A comparison with the results obtained for the pH studies with pyridinium chlorochromate reveals key differences. The concentrations recorded for the hypochlorite reactions, when compared to the equivalent pyridinium chlorochromate reactions, reveals a rapid and significant decrease in THC-COOH peak area 0 minutes after addition of the oxidising adulterant.

At pH 5 the decrease in THC-COOH concentration continues over time, with an apparent halt to the reaction occurring after 120 minutes. However, at 720 minutes (12 hours after addition of sodium hypochlorite), an increase in THC-COOH concentration can be seen. This may indicate that the reaction of sodium hypochlorite with THC-COOH may indeed be reversible under certain conditions, or that sodium hypochlorite was able to mask the presence of THC-COOH through the formation of a sodium adduct of THC-COOH that eventually decayed to release THC-COOH back in to the sample. A similar result is also seen at pH 8, whereby a major decrease in THC-COOH concentration is recorded at the first time point. 40 minutes after addition of sodium hypochlorite the same type of apparent increase in THC-COOH concentration is observed. Under the alkaline conditions the recorded concentration of THC-COOH is relatively stable 40 minutes after adulteration, and appears somewhat consistent over the remaining time periods. In later time periods the variations in target analyte concentration may be attributed to changes in instrument sensitivity and response, an effect that may not be observed if the internal standard was still appreciably present in the samples.

For the pH 5 urine sample, the initial decrease in target analyte concentration is significant, especially when compared to the equivalent pyridinium chlorochromate sample. The percentage decrease in THC-COOH concentration at the first time point is calculated to be approximately 87%, and after 20 days, ultimately results in a 96% decrease in THC-COOH concentration. For the pH 5 urine sample, this corresponds to a final calculated concentration of 75 ng/mL.

At pH 8 the reaction progresses rapidly, and at the first time point a 97.7% decrease in THC-COOH concentration is recorded. As a consequence, at the first time point it is apparent that the reaction between THC-COOH and sodium hypochlorite progresses rapidly under alkaline conditions when compared with acidic conditions. However, this initial decrease in THC-COOH concentration in short-lived, with a 610% increase in concentration between the data points recorded at 20 minutes and 40 minutes.

Over the remaining time points it can be seen that the THC-COOH concentration remains consistent up until the 1 day mark, where a decrease in THC-COOH concentration is noted. However, with the remaining data points consistently above the recorded concentration at 1 day, it is possible that this result is an outlier and may not be significant. At the remaining time point at pH 8, the total decrease in THC-COOH concentration relative to the initial THC-COOH

concentration of 2000 ng/mL is approximately 90%, and corresponds to a concentration of 204 ng/mL.

Overall, it can be seen that in spiked urine samples, sodium hypochlorite represents a highly effective and readily available urine adulterant, and it is expected that at higher oxidant concentrations, or conversely, lower THC-COOH concentrations, a total apparent loss of THC-COOH may occur in a drug-positive urine sample.

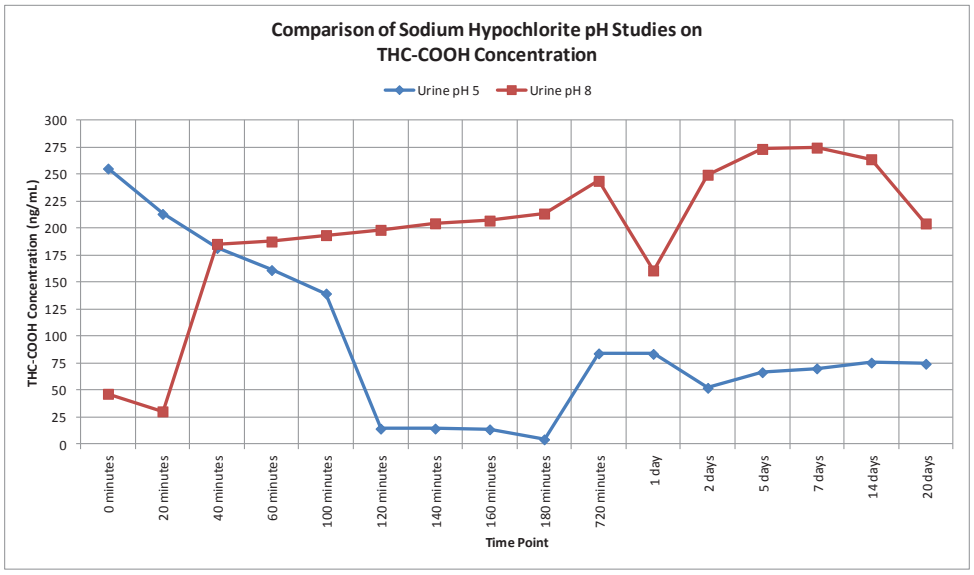


Figure 6.5 - Comparison of the sodium hypochlorite pH studies on the corrected estimated THC-COOH concentration over 20 days.

The effect of urine pH on the loss of THC-COOH following adulteration with Betadine was also explored. A total of thirteen data points were collected for the effect of urine pH on urine adulteration with Betadine, and covered a 24 day period. **Figure 6.6** provides the reported concentration of THC-COOH under both pH conditions over the thirteen time points. It is interesting to note that, similar to the samples adulterated with sodium hypochlorite and pyridinium chlorochromate, urine pH had a major effect on the reaction between Betadine and THC-COOH. As can be seen, the Betadine reaction progresses rapidly under acidic conditions, with a significant decrease in THC-COOH concentration recorded at the first data point. Conversely, under alkaline conditions, the reaction is initially slowed. In the following time points, it can be seen that under both pH conditions the decrease in THC-COOH concentration is significant, and appears to stabilise after approximately 100 minutes.

Under acidic conditions at the first time point, a 97.5% decrease in THC-COOH concentration occurs, in comparison with the initial 2000 ng/mL concentration prior to addition of the adulterant. In comparison, under basic conditions the first time point reveals only a 26.4% decrease in THC-COOH concentration. This strongly suggests that, like pyridinium chlorochromate and sodium hypochlorite, the effectiveness of this adulterant is partly linked with urine pH.

Over the remaining time points for both pH conditions, the apparent loss of THC-COOH from the sample is significant. At 24 days, the pH 5 and pH 8 samples display a decrease in THC-COOH concentration of 99% and 98%, respectively. At the final time point the estimated concentration of THC-COOH in the samples is 22.6 ng/mL and 39.6 ng/mL, and highlights the effectiveness of this adulterant at potentially masking cannabis use. As with the other adulterants, the final THC-COOH concentration is within the detection range of immunoassay-based screening tests, though it is expected that an increase in oxidant concentration relative to the initial THC-COOH concentration would potentially result in a reduction in THC-COOH concentration below the limit of detection.

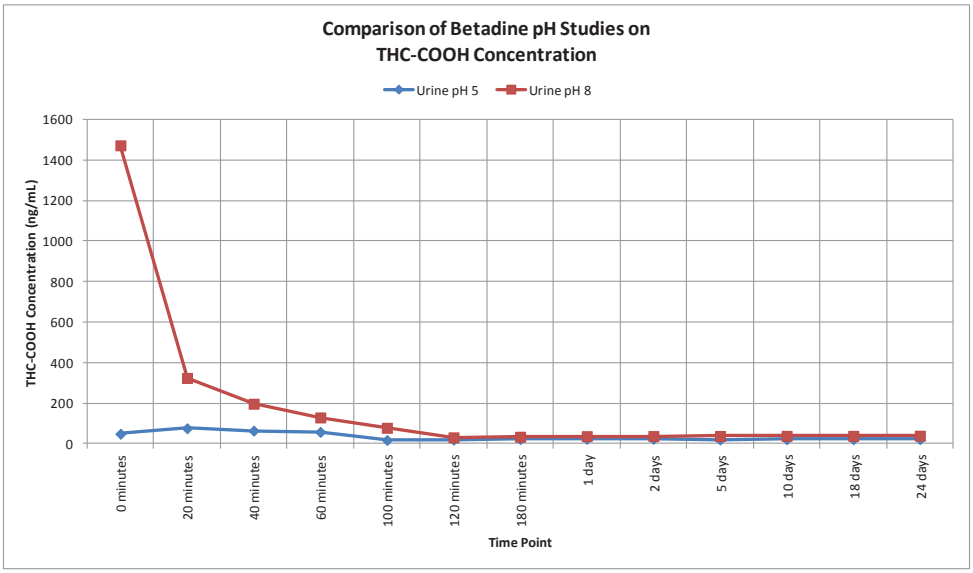


Figure 6.6 - Comparison of the Betadine pH studies on the corrected estimated THC-COOH concentration over 24 days.

In general it can be suggested that the three oxidising adulterants represent highly effective means by which an individual may mask a cannabis-positive urine test result. Though decreasing the in vitro concentration of THC-COOH below the limit of detection is a significant

result in itself, it is also necessary to explore the effects of sample storage conditions on the reaction between THC-COOH and the selected oxidants, and furthermore, whether it remains possible to detect reaction products of THC-COOH in urine under the tested conditions for an extended period of time.

6.2.3.2 – Urine pH and Effect on Product Formation

The previous section demonstrates the relative effectiveness of the selected adulterants at masking the presence of THC-COOH in spiked urine samples at two pH conditions. It is also necessary to explore the effect of urine pH on the formation of the selected reaction products. As the detection methods used for these analyses are quantitative only for THC-COOH, product formation is reported in terms of the product peak area.

For these analyses, the two mono-iodo-THC-COOH products and the two mono-chloro-THC-COOH products are considered as a single product in their respective methods for the sake of convenience. As such, reported retention times are for the first mono-halogenated product to elute, and the peak areas reported for the mono-halogenated products are combined to give a singular result. This step was undertaken due to the fact that, as seen for the initial studies of the Betadine reaction, one mono-halogenated product forms preferentially, and with complications arising in the Agilent Quantitative Analysis software resulting from the mono-halogenated products having identical deprotonated molecules and different retention times. Though this does remove some information relating to the relative formation of the two separate products, the subsequent data and interpretation still allows for general information relating to the mono-halogenated product formation to be obtained.

The results obtained for the pyridinium chlorochromate reaction in spiked urine are shown in **Figure 6.7**, and displays the peak area recorded for the reaction product and the estimated THC-COOH concentration over 24 days. The loss of THC-COOH from the sample stabilises 100 minutes following addition of the adulterant. It is interesting to note that the formation of the sole reaction product progresses slowly in comparison with the loss of THC-COOH, with the reaction reaching completion approximately 120 minutes after addition of the oxidant. It is also evident that for a urine sample at pH 5 the reaction product remains readily detectable after 24 days of storage, and indicates that if it can be confirmed that the pyridinium chlorochromate reaction product forms in authentic urine samples, this compound may represent a suitable marker of both cannabis use and urine adulteration.

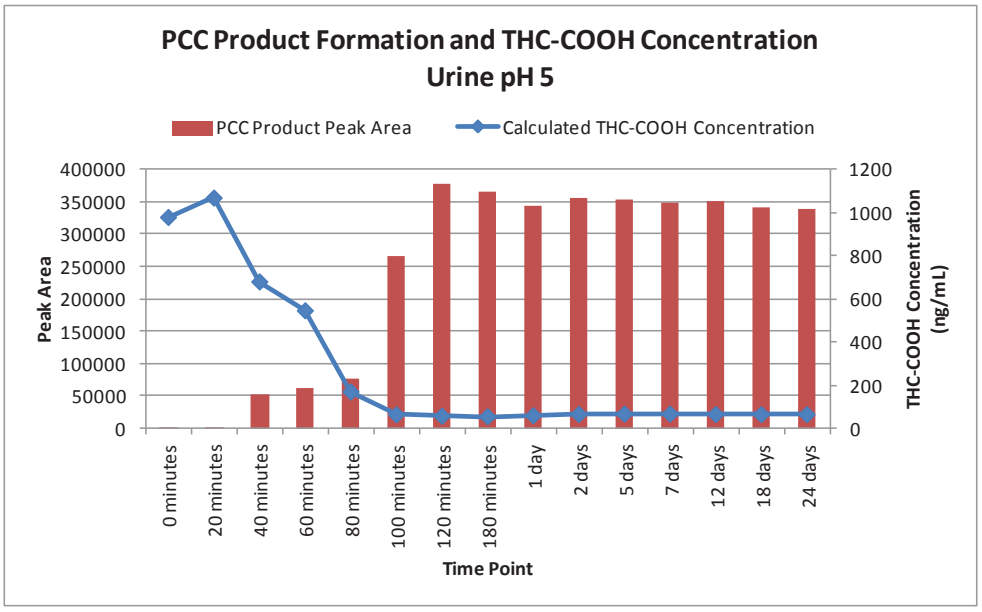


Figure 6.7 – Assessment of PCC product formation in a spiked urine sample at pH 5 over 24 days. PCC product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period.

The effectiveness of pyridinium chlorochromate as a urine adulterant was also assessed with spiked urine sample adjusted to pH 8 (**Figure 6.8**). In comparison with the equivalent sample at pH 5, it can be seen that under alkaline conditions, loss of THC-COOH still stabilises at approximately 100 minutes following addition of the adulterant. Furthermore, it appears that the formation of the reaction product reaches completion at this time point, and is slightly earlier than the reported reaction completion time for the pH 5 sample. Overall, peak area for the reaction product is lower with the pH 8 sample, and is likely due to the effect of the alkaline reaction environment on the reactivity of the acidic pyridinium chlorochromate molecule. A potentially aberrant result is reported 1 day after addition of the adulterant, with a significant increase in peak area for the reaction product. The following data points do not reflect this sudden increase in product peak area, and as such, this result is not considered to be indicative of the pyridinium chlorochromate reaction in spiked urine at pH 8.

Despite the unusual reported peak area at the 1 day mark, it is noted that the reaction product remains detectable in the alkaline urine sample over the 24 day testing period. As with the acidic urine sample, the data obtained from this experiment indicates that the reaction product formed between THC-COOH and pyridinium chlorochromate may prove useful as a marker of urine adulteration. Furthermore, it is likely that the pH of a urine sample adulterated with pyridinium chlorochromate will not have a significant effect on reaction product

formation and the subsequent decrease in THC-COOH concentration required to mask a cannabis-positive urine test.

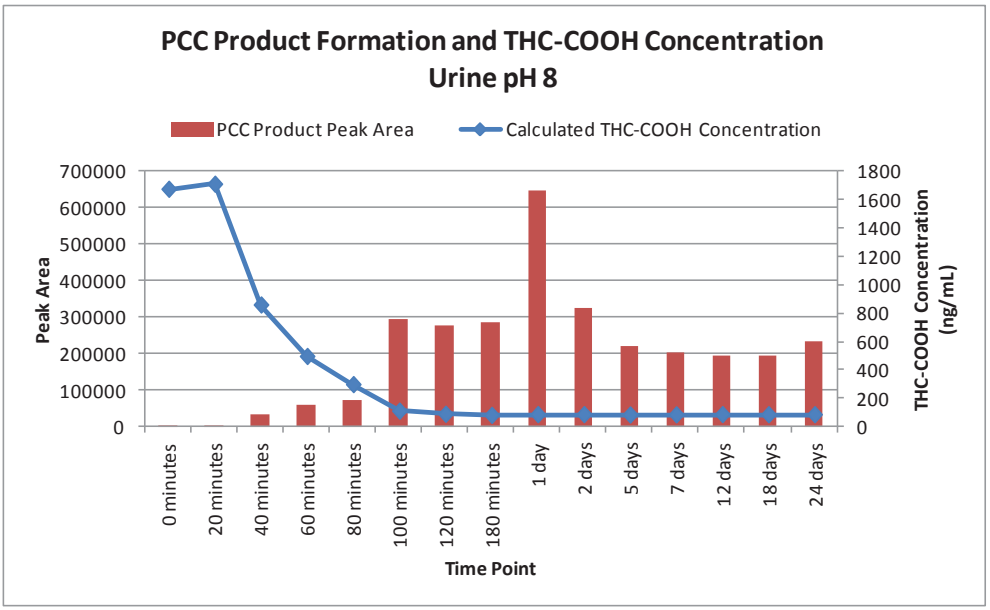


Figure 6.8 - Assessment of PCC product formation in a spiked urine sample at pH 8 over 24 days. PCC product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period.

The formation of reaction products for the sodium hypochlorite reaction in an acidified urine sample (pH 5) was also studied over a 20 day period (**Figure 6.9**). In this sample, it was found that the mono-chlorinated products continued to form up to 720 minutes following addition of sodium hypochlorite. The recorded peak areas for these products is interesting, with individual data points where the peak area suddenly decreases before increasing at a subsequent time point. This result is unusual, and it is unclear as to why sudden decreases and increases in the peak areas of the reaction products occurred. Despite this, it can be seen that the mono-chlorinated reaction products remain detectable in urine following storage of the sample for 20 days.

In comparison with the mono-chlorinated products, formation of the di-chlorinated product at pH 5 was generally slow, with small peak areas recorded for the 20 day period. However, it should be noted that after 180 minutes following addition of the adulterant, the di-chlorinated product remained detectable in solution over the sample testing period.

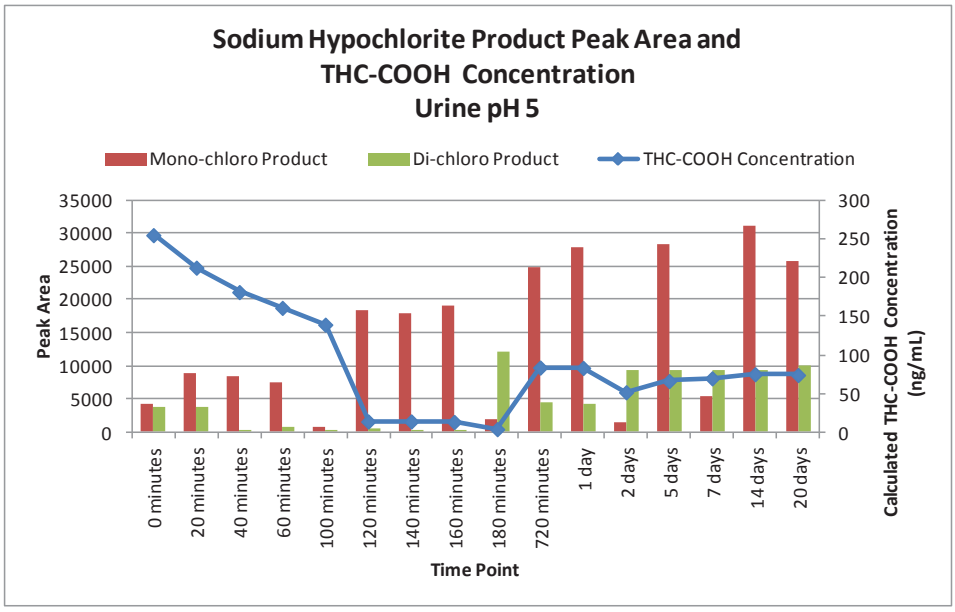


Figure 6.9 - Assessment of sodium hypochlorite product formation in a spiked urine sample at pH 5 over 20 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-chlorinated product is the sum of the recorded peak areas for both mono-chlorinated products.

The results obtained for the adulteration of a spiked urine sample at pH 8 with sodium hypochlorite (**Figure 6.10**) contain key differences when compared to the equivalent sample at pH 5. It can be seen that unlike the pH 5 sample, the di-chlorinated product dominates the sample, and is attributed to a more complete reaction occurring under alkaline conditions. Indeed, it appears that under alkaline conditions, sodium hypochlorite will continue to react with THC-COOH, and potentially with the mono-chlorinated products, to preferentially form the di-chlorinated product. It is also expected that the converse of this will be true, in that under acidic conditions the extent of the reaction between sodium hypochlorite and THC-COOH will be limited, thereby resulting in the preferential formation of the mono-chlorinated reaction products.

It is also interesting to note that for both pH conditions that the recorded peak areas for the reaction products undergo periods of sudden decreases and increases over time. As mentioned previously, the mechanism for these variations is unclear, and depending on the in vitro concentration of THC-COOH prior to adulteration with sodium hypochlorite and the time in which the sample is submitted to confirmatory testing, these variations may potentially have an adverse effect on the quantitative detection of THC-COOH and the qualitative detection of the targeted reaction products. Despite this potential complication regarding detection of the reaction products under certain conditions, the results obtained from this pH

study confirm that the reaction products remained detectable under both pH conditions for a total of 20 days. Though the formation of the specific reaction products appears to be pH dependent, successful detection of these products in spiked urine samples indicates that these compounds, like the pyridinium chlorochromate product, represent potential markers of both cannabis use and adulteration of a urine specimen with sodium hypochlorite.

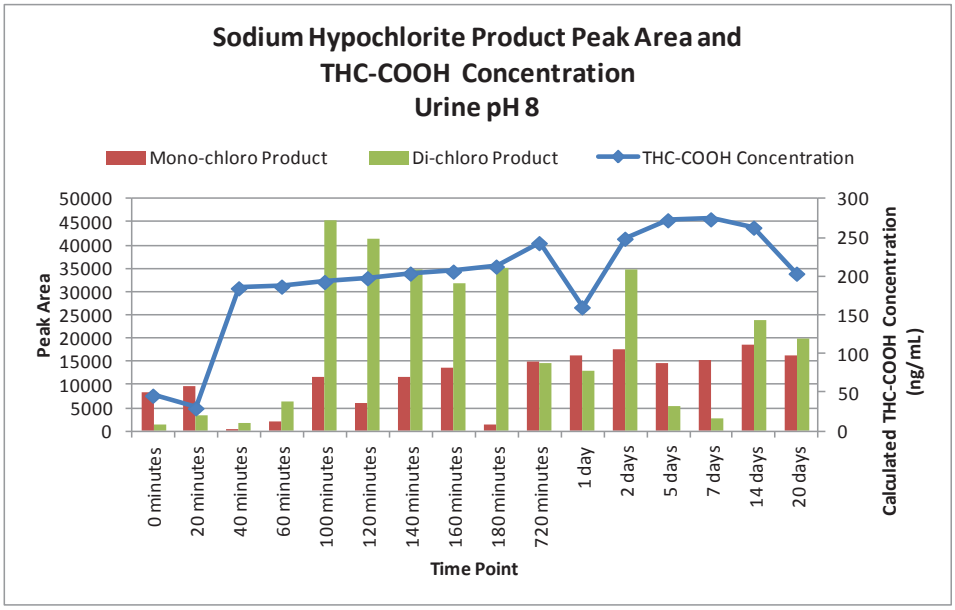


Figure 6.10 - Assessment of sodium hypochlorite product formation in a spiked urine sample at pH 8 over 20 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-chlorinated product is the sum of the recorded peak areas for both mono-chlorinated products.

The formation of reaction products in spiked urine samples following adulteration with Betadine was studied over 24 days. **Figure 6.11** provides the results of the pH study under acidic conditions (pH 5). In the time-based study of this sample, it can be seen that the in vitro loss of THC-COOH stops at approximately 100 minutes following adulteration of the sample, with the estimated THC-COOH concentration remaining relatively consistent subsequently. Under acidic conditions it can be seen that formation of the di-iodo-THC-COOH product dominates, with traces of the mono-iodinated products detected throughout the study, with both products remaining detectable over the 24 day period of study. Formation of the reaction products is observed 20 minutes after addition of Betadine to the urine sample, and it can be seen that the peak areas recorded for the reaction products stabilise approximately 100

minutes following addition of Betadine. Unlike the pH studies undertaken for sodium hypochlorite, significant decreases and increases in product peak area were not observed.

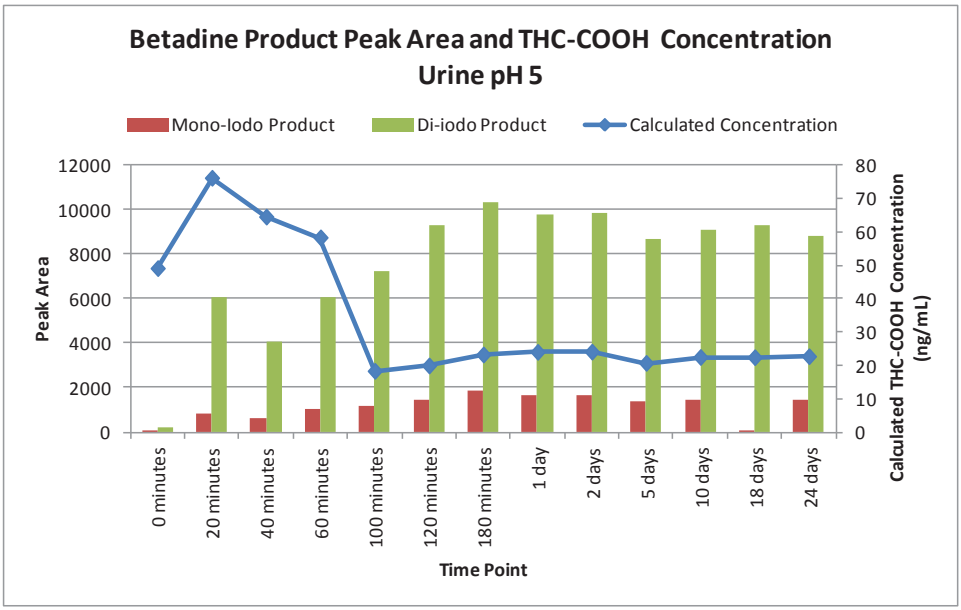


Figure 6.11 - Assessment of Betadine product formation in a spiked urine sample at pH 5 over 24 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-iodinated product is the sum of the recorded peak areas for both mono-iodinated products.

Results obtained from the analysis of the Betadine-adulterated urine sample under alkaline conditions are reported in **Figure 6.12**. Unlike the urine sample prepared under acidic conditions, the initial loss of THC-COOH is slowed, with a major decrease in estimated THC-COOH concentration observed 20 minutes after adulteration of the sample. In the 20 minute period following adulteration of the sample it can also be seen that initially product formation progresses quickly, with significant peak areas recorded for the targeted reaction products. It should be noted that in comparison with the equivalent study undertaken at pH 5, the mono-iodinated products form in a greater relative proportion. As with the sodium hypochlorite pH study, it is expected that under preferable reaction conditions, reaction of THC-COOH and the mono-iodinated products may continue to ultimately form the di-iodinated product.

As with the results obtained for the pH study of the hypochlorite-adulterated samples, occasional variations in peak area are observed. 20 minutes following adulteration there is a major decrease in these recorded peak areas, following which an increase is seen, with the extent of product formation stabilising approximately 100 minutes following adulteration. 120

minutes after adulteration a spike in the recorded peak area for the di-iodinated reaction product is seen, which subsequently decreases back to the previous level. For the mono-iodinated products a major decrease in peak area is recorded 2 days after adulteration of the sample, and it is interesting to note that this decrease in peak area is not reflected in the peak area of the di-iodinated product.

Despite these reported variations in peak area, the mono-iodinated and di-iodinated reaction products remain detected in the sample over the entirety of the 24 day period of study. As discussed previously for the pyridinium chlorochromate and sodium hypochlorite pH studies, detection of the reaction products over a total of 24 days represents a significant result, and suggests that the persistence of these compounds in spiked urine samples adulterated with Betadine may allow for substantial evidence of both cannabis use and adulteration of a sample submitted for drug testing analysis.

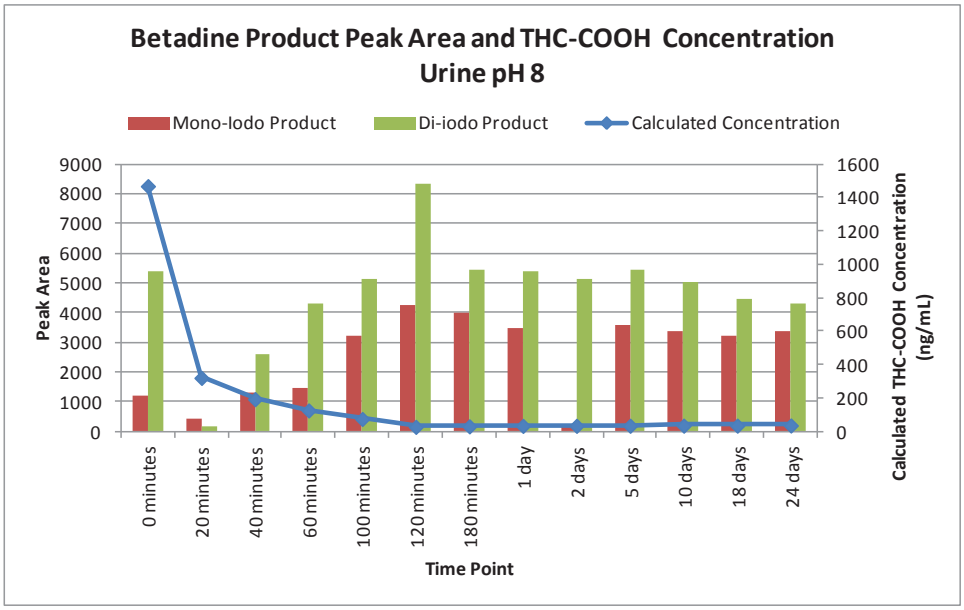


Figure 6.12 - Assessment of Betadine product formation in a spiked urine sample at pH 8 over 24 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-iodinated product is the sum of the recorded peak areas for both mono-iodinated products.

It is noted that from the data acquired through the pH studies for the selected adulterants, formation of the targeted reaction products does not appear to match the decrease in THC-COOH concentration over time. As mentioned previously for the sodium hypochlorite and Betadine reactions, sample pH appears to have a significant effect on the progression of the

reaction and the formation of specific reaction products. Under alkaline reaction conditions the di-chlorinated product forms in a higher proportion when compared to the sample prepared under acidic conditions. Similarly, Betadine antiseptic solution is reported to be slightly acidic (pH 4 – 5), and under acidic conditions, the di-iodinated product forms in a higher relative proportion when compared to the equivalent sample prepared under alkaline conditions.

A significant finding of these pH studies is that for all samples regardless of sample pH, the targeted reaction products remain detectable in solution at the end of the respective analysis periods. From the perspective of a drug testing laboratory, this means that if it is confirmed that these products are found to form in authentic cannabis-positive urine samples, then these compounds may be effectively used as markers of both cannabis use and urine adulteration.

6.2.4 – Sample Temperature and Storage

Further studies were also undertaken to assess the effects of sample storage temperature on the loss of THC-COOH and subsequent reaction product formation following adulteration of spiked urine samples (pH 6.5). To assess the effects of sample storage, two sets of samples were prepared for each adulterant. When not undergoing analysis, these samples were sealed and stored under two different conditions: one set was refrigerated at 4°C, whilst the other set was stored at room temperature away from any light sources. It is expected that storage of samples at lower temperatures will result in a general decrease in the loss of THC-COOH and subsequent product formation.

6.2.4.1 – Sample Temperature and Effect on THC-COOH Peak Area and Concentration

As with the pH studies undertaken with the selected adulterants, it is necessary to establish the effect of storage conditions on the in vitro reaction of THC-COOH and the formation of reaction products. As such, the peak areas and calculated THC-COOH concentrations were monitored for the three oxidants at both storage conditions over an extended period of time.

The results obtained for assessing the effect of storage conditions on the spiked urine samples adulterated with pyridinium chlorochromate are shown in **Figure 6.13**. It can be seen that for the refrigerated sample, loss of THC-COOH did not occur until approximately 20 minutes after

addition of the adulterant. In contrast, the sample stored at room temperature displayed a significant initial decrease in THC-COOH concentration, though the interpretation of this result is complicated with an apparent increase in THC-COOH concentration at the second monitoring time of 20 minutes. Despite this, an overall trend can be seen where the loss of THC-COOH in the sample occurs more quickly for the sample stored at room temperature, whilst for the refrigerated sample loss of THC-COOH occurs at a slower rate. Despite this, significant losses of THC-COOH are observed for both samples 120 minutes after adulteration of the sample.

At the end of the 24 day monitoring period a 96% reduction in THC-COOH is reported, revealing that under both sample storage conditions pyridinium chlorochromate represents an effective urine adulterant. The final reported concentration of THC-COOH for the refrigerated and room temperature samples is 70 ng/mL and 66 ng/mL, respectively. These concentrations would still potentially result in a positive test result from immunoassay-based screening techniques. At higher oxidant concentrations, or alternately with a lower initial THC-COOH concentration, it is likely that an apparent total loss of THC-COOH may be observed.

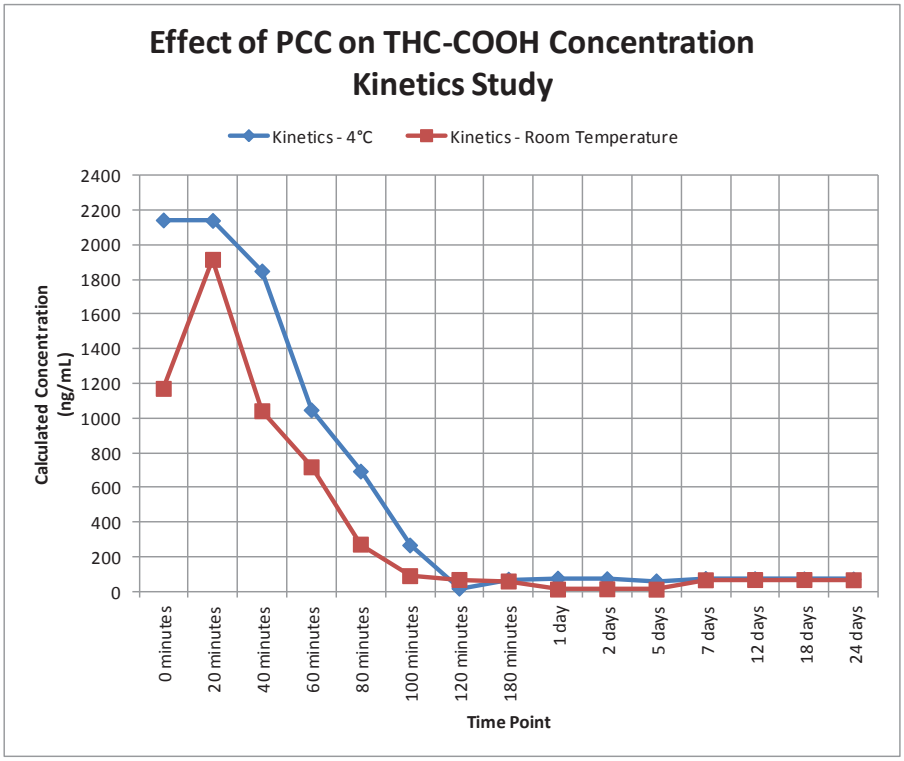


Figure 6.13 - Comparison of the PCC sample storage studies on the calculated THC-COOH concentration over 24 days.

For the study of sample storage condition on urine samples adulterated with sodium hypochlorite, results similar to those found in the pH study are observed (**Figure 6.14**). After adulteration of the samples, a major decrease in THC-COOH concentration is reported. For the refrigerated sample, the first data point reveals a 77% decrease in THC-COOH concentration, and for the sample stored at room temperature an 80% decrease in concentration is found. This indicates that despite changes in sample storage conditions, sodium hypochlorite undergoes a rapid reaction with THC-COOH in spiked urine samples. It is interesting to note that following the initial decrease in THC-COOH concentration in the hypochlorite-adulterated samples, the concentration of THC-COOH remains fairly steady over the remainder of the sampled time points, with slight variations in concentration that may be attributed to instrument response.

Analysis of the data for both storage conditions reveals that following the initial decrease in the concentration of THC-COOH, fluctuations in the calculated THC-COOH concentration are observed. Of particular note is that for the refrigerated sample, at the end of the period of study, the concentration of THC-COOH is slightly higher than at the beginning of the study. This is an interesting result, as it would be expected that the sample stored at room temperature would display such a trend due to possible degradation of the reaction products to regenerate THC-COOH in vitro.

At the end of the analysis period the final reported THC-COOH concentration for the refrigerated sample was found to be 513 ng/mL, corresponding to a 74% decrease in THC-COOH for a non-adulterated sample. For the sample stored at room temperature, the final reported concentration was 441 ng/mL. Though sodium hypochlorite represents a highly effective urine adulterant, under the tested sample storage conditions both sets of samples would be expected to return a positive test result following presumptive testing. As with pyridinium chlorochromate, an increase in sodium hypochlorite concentration or a decrease in the initial in vitro THC-COOH concentration could potentially result in the complete loss of THC-COOH, and would therefore lead to a false-negative test result for presumptive and confirmatory testing procedures.

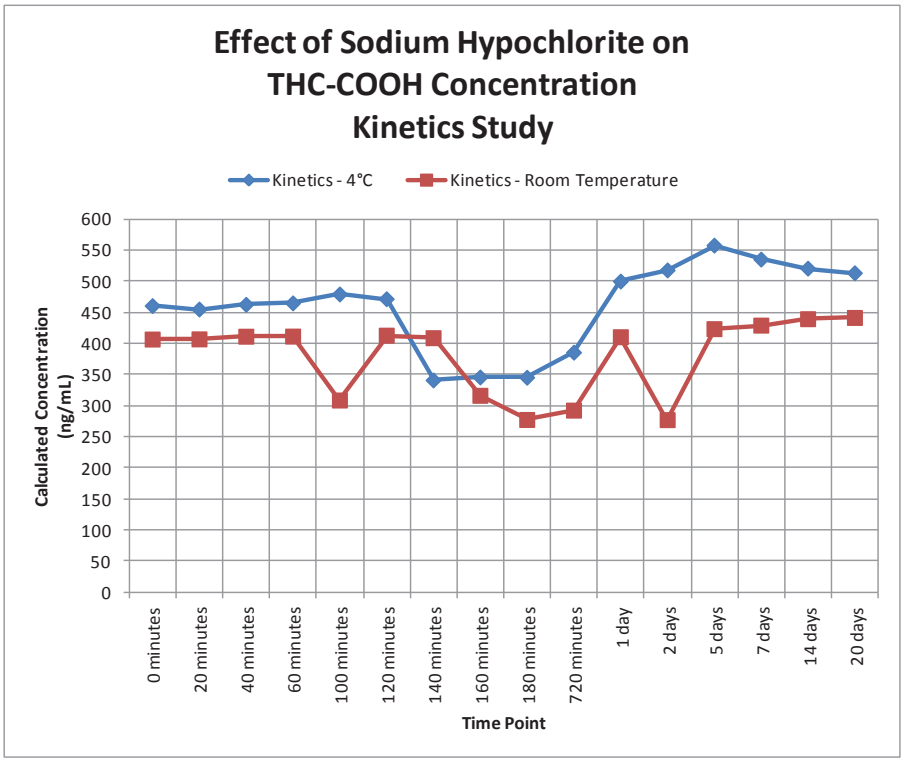


Figure 6.14 - Comparison of the sodium hypochlorite sample storage studies on the calculated THC-COOH concentration over 20 days.

The results obtained for the sample storage study on spiked urine samples adulterated with Betadine are shown in **Figure 6.15**. This study was undertaken over a period of 24 days. The effect of sample storage temperature on the loss of THC-COOH provides an unusual result. Unlike the sample storage study with pyridinium chlorochromate, the recorded loss of THC-COOH immediately after adulteration of the sample is highest with the refrigerated sample. In contrast, the recorded decrease in THC-COOH concentration for the sample stored at room temperature is comparatively slow. It would be expected that the results at this time point would be the opposite, with the reaction of THC-COOH with Betadine slowed when the sample is stored at 4°C.

Also of note is that for the refrigerated sample, a slight increase in THC-COOH concentration is seen 20 – 40 minutes after adulteration of the sample. After 40 minutes, the reported THC-COOH concentration begins to decrease. At the initial analysis point the concentration of THC-COOH for the refrigerated and room temperature samples are 69 ng/mL and 401 ng/mL, respectively. These reported concentrations correspond to a 67% and 80% decrease in THC-COOH concentration when compared to an unadulterated sample. Following storage of the samples for 24 days, the calculated concentration is found to be 53 ng/mL and 34 ng/mL for

the refrigerated and room temperature samples. Based on these results it is seen that, as with sodium hypochlorite and pyridinium chlorochromate, Betadine is effective at decreasing the concentration of THC-COOH in a urine sample.

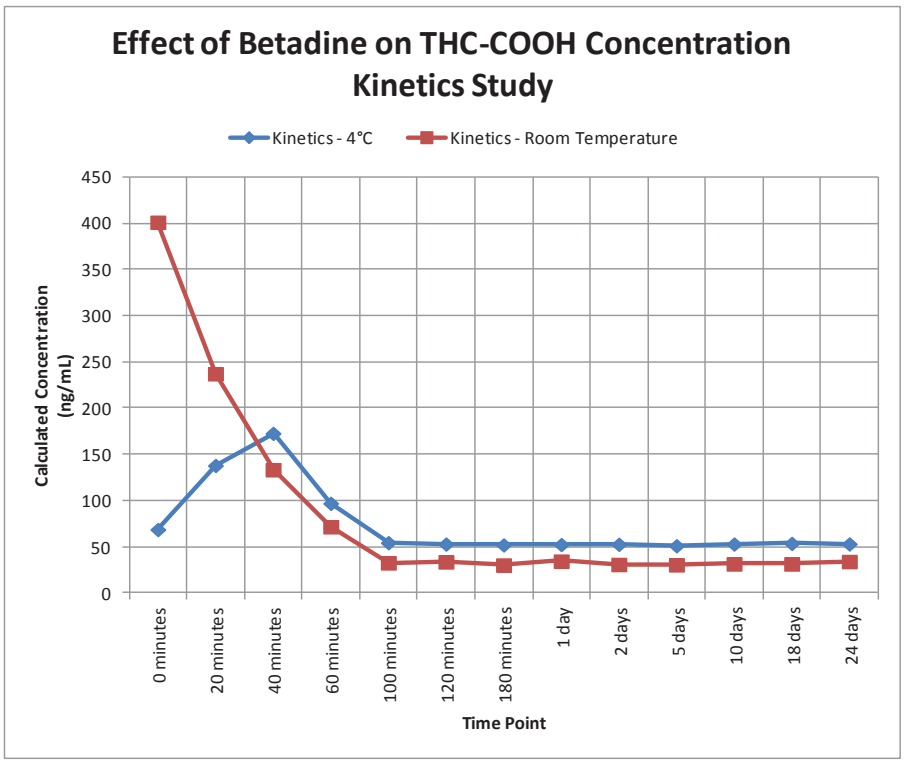


Figure 6.15 - Comparison of the Betadine sample storage studies on the calculated THC-COOH concentration over 20 days.

6.2.4.2 – Sample Temperature and Effect on Product Formation

The previous section demonstrates the relative effectiveness of the selected adulterants at masking the presence of THC-COOH in spiked urine samples at two sample storage conditions. In this section the effect of sample storage condition on the formation of the targeted reaction products will be studied. As the detection methods used for these analyses are quantitative only for THC-COOH, product formation is reported in terms of the product peak area.

The results obtained for the pyridinium chlorochromate reaction and subsequent sample storage at 4°C are shown in **Figure 6.16**. It can be seen that in terms of the reaction product, formation of this compound is similar to the results obtained in the acidified sample prepared in the pH study. 60 minutes after adulteration of the sample the first major peak areas are recorded for the reaction product, and remain stable over the next two time points. At 120

minutes after sample adulteration a major increase in reaction product peak area is recorded, and remains relatively stable over the remaining analysis period. As with the pH studies for pyridinium chlorochromate, it is interesting to note that the relative loss of THC-COOH in the sample does not correspond with reaction product formation.

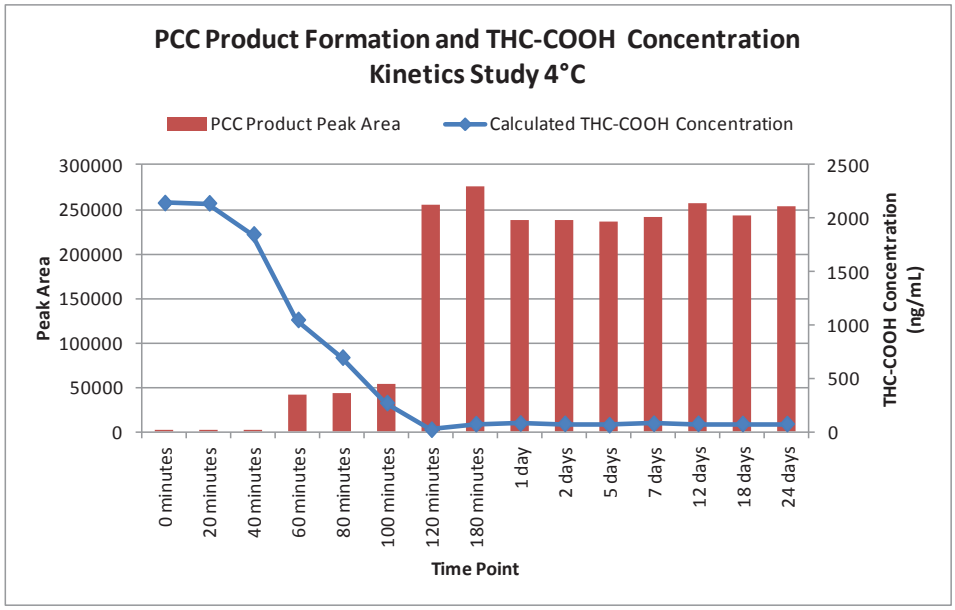


Figure 6.16 - Assessment of PCC product formation in a spiked urine sample stored at 4°C over 24 days. PCC product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period.

The results obtained for the pyridinium chlorochromate reaction stored at room temperature are shown in **Figure 6.17**. As with the refrigerated sample, the reaction is initially slow. Major peak areas are recorded approximately 120 minutes after sample adulteration, and remain relatively consistent over the remaining time points. One slight difference apparent between the refrigerated and room temperature samples is the highest peak area recorded following stabilisation of the peak areas. In the refrigerated sample the average product peak area is approximately 25,000. For the room temperature sample the average peak area value is slightly higher.

For both sample storage conditions it can be seen that pyridinium chlorochromate represents a highly effective urine adulterant. As with the pH study, major losses of THC-COOH are reported, and the product formed in the reaction between THC-COOH and pyridinium chlorochromate remains readily detectable for at least 24 days following sample adulteration.

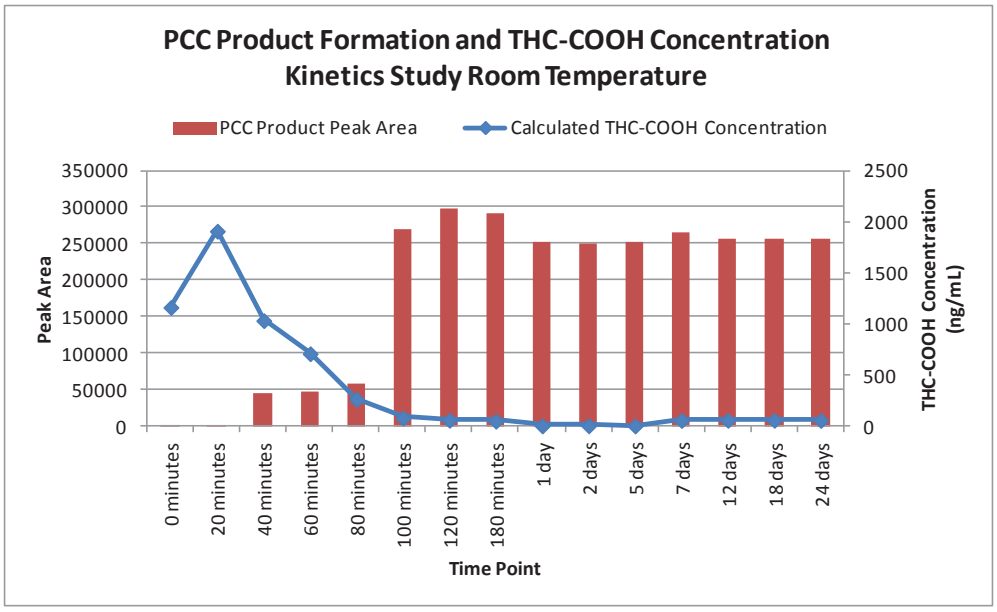


Figure 6.17 - Assessment of PCC product formation in a spiked urine sample stored at room temperature over 24 days. PCC product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period.

The results obtained for the sodium hypochlorite reaction and subsequent sample storage at 4°C are shown in **Figure 6.18**. Based on the peak areas recorded for the reaction products it appears that the reaction with sodium hypochlorite progresses relatively quickly despite storage of the sample at a lower temperature. Overall formation of the reaction products appears to stabilise over the first 160 minutes following adulteration of the sample. For the mono-chlorinated products, a decrease in peak area can be seen at approximately 180 minutes after adulteration, with further decreases seen after storage of the sample for 5 days. Over this same period the peak area of the di-chlorinated product increases, though after 7 days of storage a gradual decrease in the peak area for this compound is observed. During this period where the peak areas for the reaction products decreases, an increase in the reported THC-COOH concentration is seen, and potentially indicates that the reaction products may begin to degrade when stored for prolonged periods of time, resulting in a replenishment of THC-COOH.

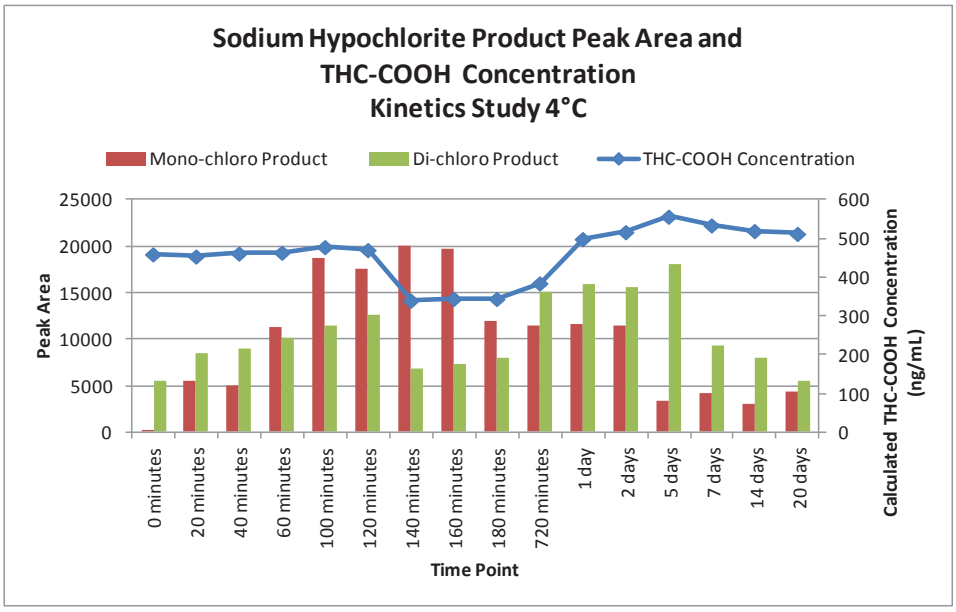


Figure 6.18 - Assessment of sodium hypochlorite product formation in a spiked urine sample stored at 4°C over 20 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-chlorinated product is the sum of the recorded peak areas for both mono-chlorinated products.

Figure 6.19 shows the recorded peak areas for the targeted reaction product for the sample stored at room temperature. At the higher storage temperature the sodium hypochlorite reaction displays several differences when compared with the sample stored at 4°C. The first difference noted is that the reaction progresses relatively quickly at room temperature, with significant peak areas recorded for the reaction products immediately after adulteration of the sample. In addition, it can be seen that unlike the refrigerated sample, the di-chlorinated product is formed in a higher proportion overall. This is likely due to the reaction between THC-COOH and sodium hypochlorite being more extensive at the higher sample storage temperature, resulting in further conversion of THC-COOH and the mono-chlorinated products to form the di-chlorinated product.

It is also interesting to note that the relative amount of the reaction products remaining in the sample at the end of the 20 day sampling period is significantly higher for the room temperature sample. Given that the refrigerated sample displayed gradual losses of the reaction products over the later stages of the sample storage study, this is somewhat unusual. If the loss of reaction products in the refrigerated samples is attributed to the degradation of the reaction products, it would be expected that this degradation would be more pronounced at higher temperatures. Due to this unusual result, it is not clear what mechanism/s are

involved in the increased degradation of the reaction products at lower temperatures in comparison to the sample stored at room temperature.

Despite the unusual loss of the reaction products in the refrigerated sample, the targeted reaction products remained readily detected over the entire 20 day period of analysis. As with the pyridinium chlorochromate pH and sample storage studies, this indicates that the products formed in the reaction between THC-COOH and sodium hypochlorite may represent viable markers of urine adulteration if their formation can be confirmed in authentic cannabis-positive urine samples.

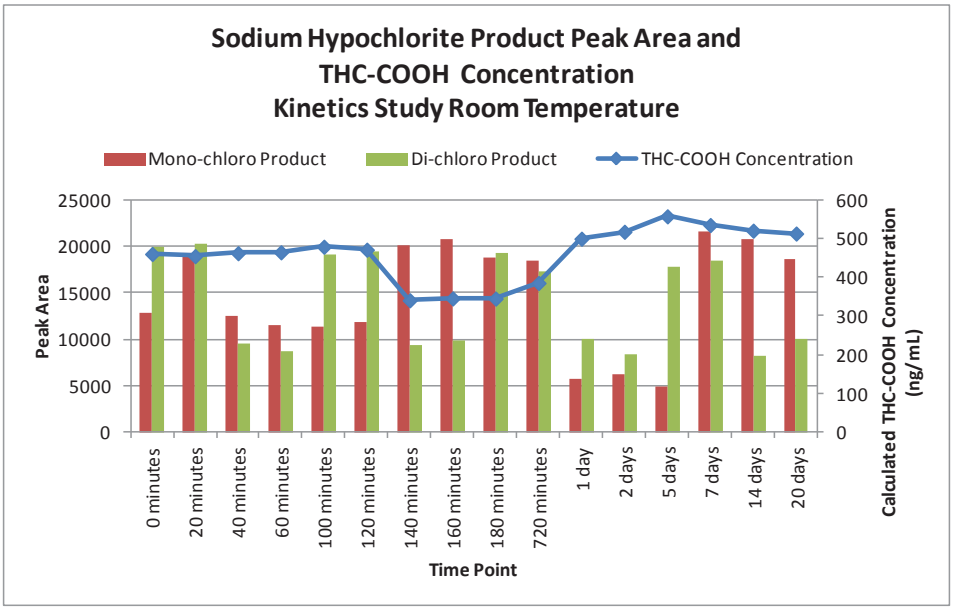


Figure 6.19 - Assessment of sodium hypochlorite product formation in a spiked urine sample stored at room temperature over 20 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-chlorinated product is the sum of the recorded peak areas for both mono-chlorinated products.

The results obtained for the Betadine reaction and subsequent sample storage at 4°C are shown in **Figure 6.20**. At this lower temperature the reaction of Betadine with THC-COOH is slowly initiated, with significant peak areas reported for the reaction products approximately 40 minutes following adulteration of the sample. Over this same period of time the apparent concentration of THC-COOH increases, and at 60 minutes after urine adulteration, quickly decreases and stabilises 100 minutes after sample adulteration.

Of particular note in this study of the refrigerated sample is that the formation of the di-iodinated reaction product is very low, with small peak areas recorded throughout the testing period. In contrast, it can be seen that the mono-iodinated products form extensively. It is expected that, as with the refrigerated sodium hypochlorite sample, that lower temperatures inhibit the reaction between THC-COOH and the adulterant, and thus preferentially forms the mono-iodinated products.

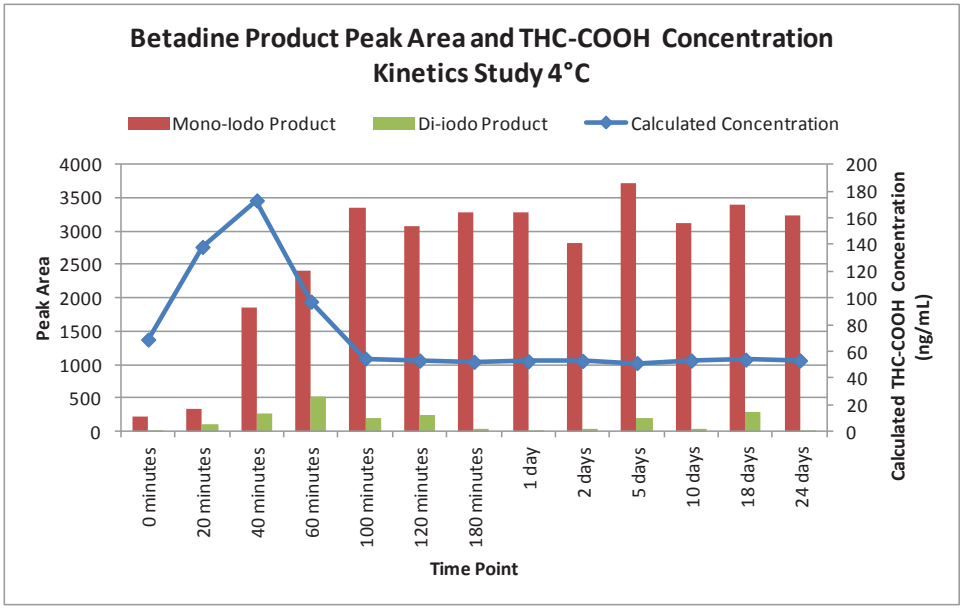


Figure 6.20 - Assessment of Betadine product formation in a spiked urine sample stored at 4°C over 24 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-iodinated product is the sum of the recorded peak areas for both mono-iodinated products.

The results obtained for the Betadine-adulterated urine sample stored at room temperature are shown in **Figure 6.21**. Unlike the refrigerated sample, product formation occurs more quickly, with the di-iodinated product produced in significant amounts. As with sodium hypochlorite, it is expected that storage of the sample at room temperature allows for the reaction to continually progress, through reaction with THC-COOH and the mono-iodinated products to form the di-iodinated product. The recorded peak areas for the products stabilise approximately 60 minutes after addition of the adulterant, and all targeted products remain readily detectable 24 days after adulteration. Also of note is that the decrease in THC-COOH concentration appears to coincide with the formation of the reaction products, and remains consistently low 100 minutes after sample adulteration.

As noted previously, for the refrigerated Betadine sample, an initial increase in THC-COOH concentration is noted in the 60 minute period following adulteration of the sample. As this effect was not observed with the sample stored at room temperature, it is proposed that this increase in concentration may be due to some manner of sequestration effect by either the polymer present in Betadine, PVP, or the other inactive ingredients present in this antiseptic solution. As the time following sample adulteration increases, it is possible that this interference between THC-COOH and the other compounds present in Betadine may slowly reverse; replenishing the amount of THC-COOH detected in the sample which subsequently undergoes reaction with the active component in Betadine to form the targeted reaction products, and thereby results in a gradual decrease in THC-COOH concentration. Indeed, as this effect is not observed at the higher temperature, this sequestration effect may only occur at low temperatures; alternately, the higher temperature of the unrefrigerated sample may provide a sufficient amount of energy to prevent or inhibit the possible sequestration of THC-COOH in vitro.

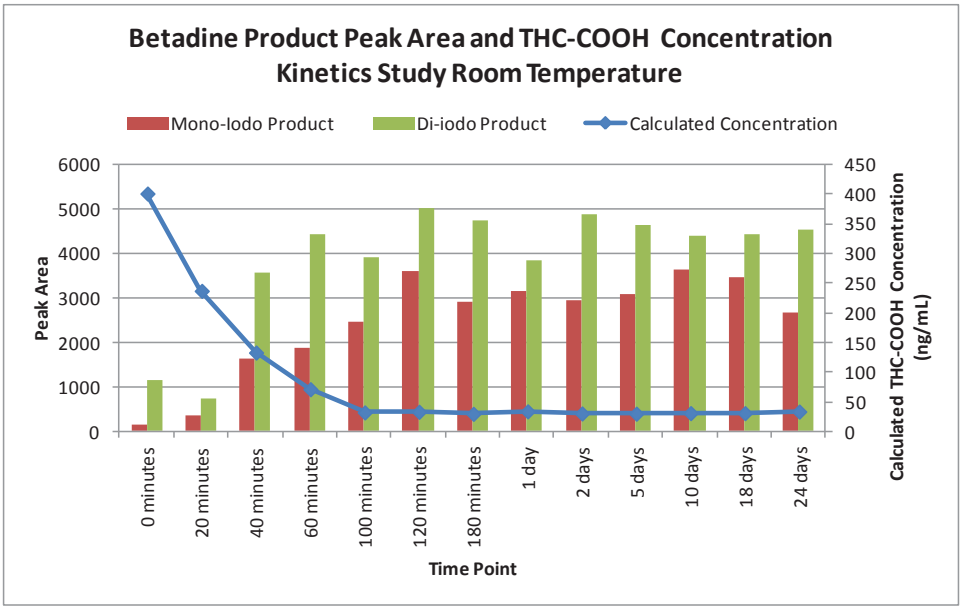


Figure 6.21 - Assessment of Betadine product formation in a spiked urine sample stored at room temperature over 24 days. Reaction product formation is reported in terms of peak area, and overlaid is the estimated THC-COOH concentration for the same period. Note that the recorded peak area for the mono-iodinated product is the sum of the recorded peak areas for both mono-iodinated products.

The study of storage conditions and their effect on adulteration of spiked urine samples with the selected adulterants provides further insight into urine adulteration and how sample

storage affects the loss of THC-COOH and formation of reaction products. For samples stored at 4°C, both the loss of THC-COOH and formation of the reaction products appear to be slowed. Regardless of the sample storage conditions, if a sample has been adulterated with a sufficient quantity of an oxidising adulterant, significant decreases in THC-COOH concentration will still be observed. Additionally, for all the reaction samples prepared for this study, all reactions appear to reach their endpoint between 60 – 120 minutes after sample adulteration. The reaction with pyridinium chlorochromate appears to take the longest amount of time to reach this reaction endpoint, taking approximately 120 minutes under both sample storage conditions. For sodium hypochlorite and Betadine, the reactions with THC-COOH appear to progress more quickly, and typically reach their endpoint approximately 60 minutes after adulteration.

From the perspective of a drug testing laboratory, it appears that refrigeration of a sample prior to analysis will not overtly effect the reaction of THC-COOH with the selected oxidising adulterants, with these reactions progressing under both storage conditions. Despite this, it will evidently remain best practice to refrigerate samples when not submitted for analysis. In addition, assuming that the adulterated sample is refrigerated immediately after collection, the main difference that a drug testing laboratory may expect is the preferential formation of specific reaction products. As seen in this study, samples adulterated with sodium hypochlorite and Betadine and subsequently refrigerated results in the preferential formation of the mono-halogenated products. The opposite was also found to be correct, in that storage of these samples at room temperature leads to the preferential formation of the di-halogenated reaction products.

It is also noted that for both the pH and sample storage studies, generally the targeted reaction products remained detectable in all samples at the final analysis point. The only exception to this was the Betadine reaction sample stored at 4°C, where only traces of the di-iodinated product were detected through the bulk of the time course study. As such, with regards to reaction product stability, these compounds may represent drug-specific markers of urine adulteration.

With regards to the possibility of incorporating these reaction products into current drug testing schemes to effectively identify cases of cannabis use and urine adulteration, further research is needed to ascertain whether these compounds are detected in authentic cannabis-positive urine samples. As THC-COOH is found as the glucuronide conjugate in urine, it is

possible that this may have an effect on reaction product formation, and will be discussed further in Chapter 7.

Chapter 7

Adulteration of Authentic Urine Specimens

The adulteration of spiked urine samples with Betadine, bleach and pyridinium chlorochromate has shown that these adulterants are highly effective at potentially masking a cannabis-positive urine result through their reaction with THC-COOH. In addition, it has been determined that through the use of these adulterants, decreases in THC-COOH concentration are concomitant with the formation of novel reaction products that may act as markers of adulteration of a cannabis-positive sample. However, as discussed in Chapter 1, urinary excretion of THC-COOH and related THC metabolites occurs primarily through the excretion of the glucuronidated conjugates of these molecules. It is possible that due to the different molecular structure of the glucuronidated molecules that the previously detected reaction products may no longer form following adulteration with the selected oxidants. As such, it is necessary to determine whether exposure of authentic cannabis-positive urine samples to the selected adulterants will still form with the glucuronidated conjugates, and furthermore, if the targeted reaction products can be detected following hydrolysis of the glucuronidated molecules.

An additional consideration is the effect of adulteration of authentic urine samples on immunoassay-based presumptive screening measures. As discussed previously, various adulterants are capable of interfering with immunoassay-based techniques. Furthermore, if it is determined that the desired reaction products do form in authentic urine samples, potential cross-reactivity of these products to a cannabinoid assay also needs to be assessed. Cross-reactivity of these compounds will potentially return a positive immunoassay test result; conversely, the interfering effects of the selected adulterants, or a lack of cross-reactivity for the targeted reaction products, may result in a false-negative test result being returned.

For the immunoassay-based presumptive testing of authentic urine samples, a batch of samples were prepared for analysis and submitted to the Drug Toxicology Unit for analysis. Prior to analysis aliquots of these samples were stored at 4°C for later confirmatory testing. Though such samples would normally undergo confirmatory testing by GC-MS, the high workload at the Drug Toxicology Unit meant that for the sake of convenience and expediency, confirmatory testing of the authentic urine samples prepared for this study would occur

through use of the validated LC-MS/MS methods involving sample hydrolysis generated and discussed in Chapter 5.

7.1 – Experimental

7.1.1 – Drug Standards and Reagents

Drug standards and reagents used in this chapter are the same as those found in Chapter 5. Due to storage of the sodium hypochlorite solution between experiments and possible degradation of this solution as a result, the hypochlorite concentration of this solution was determined spectrophotometrically, as per previous chapters. The hypochlorite concentration of the sodium hypochlorite solution was determined to be 0.240 M.

7.1.2 – Urine Specimens

Anonymised authentic urine specimens testing positive for THC-COOH and its glucuronide were supplied by the Drug Toxicology Unit, NSW Forensic & Analytical Science Service, Macquarie Hospital, NSW. Authentic urine specimens were stored in a freezer at -18°C prior to sample preparation. Two samples were available for this study, and were reported as having a THC-COOH concentration of 25 ng/mL and 825 ng/mL, respectively. A third authentic urine sample was prepared through a mixture of the two authentic urine specimens to form a pooled sample with an estimated THC-COOH concentration of 81 ng/mL. The pH of the three authentic urine specimens was recorded. For the low concentration authentic specimen sample pH was 7.5. For the remaining medium and high concentration authentic specimens urine pH was determined to be 6.5.

Blank pooled urine was prepared through the collection of urine specimens from healthy individuals (n= 10) and stored in polypropylene urine specimen containers at 4°C to create a representative blank urine matrix. Volunteers were selected randomly, with an age range from 18-50, and both males (n=5) and females (n=5) equally represented. The primary qualification factor for selected donors was that they had not used cannabis, nor had they been in contact with cannabis products, for the past three months. Individual samples were analysed by using LC-MS/MS methods developed in this research in order to ensure that it was negative for

cannabinoids prior to use. The combination of urine specimens to form pooled urine for research was not used for more than one experiment.

7.1.3 – Instrumentation

Presumptive screening of urine samples was undertaken on an Olympus AU 2700 analyser (Olympus America Inc., Melville, NY, USA) with a DRI™ Cannabinoid Assay (Microgenics Corporation, Fremont, CA, USA). The DRI™ Cannabinoid Assay provides a qualitative and semi-quantitative determination of cannabinoids in human urine. This technique uses mouse monoclonal anti-Δ^9-THC antibodies with a labelled Δ^9-THC enzyme conjugate. Calibration of assay response was achieved with 20 ng/mL, 50 ng/mL and 100 ng/mL calibrators.

LC-MS/MS analysis of the urine samples was as per the validated methods incorporating sample hydrolysis present in Chapter 5. **Table 7.1** provides the general instrument parameters used in the confirmatory testing of these samples. Chromatographic separation was undertaken on a Phenomenex Luna C_5 HPLC column (150 mm x 4.6 mm, 5 micron, Phenomenex Incorporated).

Table 7.1 – Instrument parameters for the analysis of authentic urine specimens.

LC-MS System Parameters		Setting
LC Parameters	Solvent Flow Rate	0.7 mL/min
	Injection Volume	2 µL
	Column Temperature	35°C
	Method Runtime	15 minutes
QQQ Parameters	Fragmentor Voltage	380 V
	Cell Accelerator Voltage	4 V
	Collision Energy	5 – 80 eV
	Ionisation Mode	Negative
	Sheath Gas Temperature	250°C
	Sheath Gas Flow Rate	11 L/min
	Scan Mode	Multiple Reaction Monitoring

7.1.4 – Sample Preparation

A total of 26 samples were prepared for presumptive screening by immunoassay and confirmatory testing by LC-MS/MS, with a final sample volume of 2 mL. **Table 6.2** lists the samples prepared for this study. Urine blanks, oxidant blanks and THC-COOH standards were prepared with the blank pooled urine. Oxidant reaction mixtures were prepared in the authentic urine specimens.

Working solutions of the selected adulterants were prepared for adulteration of the authentic urine specimens. The pyridinium chlorochromate working solution was prepared by dissolving pyridinium chlorochromate in water to a final concentration of 0.370 M. Betadine and sodium hypochlorite were used undiluted in these studies, with final stock solution concentrations of 1% w/v iodine and 0.240 M sodium hypochlorite. Final oxidant concentrations for the samples adulterated with pyridinium chlorochromate were 0.46 mM, 1.85 mM and 18.5 mM for the low, medium and high authentic urine samples respectively. For Betadine, the final oxidant concentration for the low, medium and high authentic urine specimens was 0.025‰, 0.1‰ and 1.0‰ available iodine. Finally, for the authentic urine specimens adulterated with bleach, the final oxidant concentrations were 0.3 mM, 1.2 mM and 12.0 mM.

Following sample preparation, urine specimens were left at room temperature for 12 hours to aid the reaction with THC-COOH after adulteration. Following this reaction period, samples were stored at room temperature overnight at 4°C. Prior to transportation of the samples to the Drug Toxicology Unit, a 1 mL aliquot of each sample was transferred to a glass GC vial, sealed and refrigerated at 4°C pending confirmatory testing. For the presumptive screening, samples were transported to the Drug Toxicology Unit in a polystyrene cooler on ice.

Table 7.2 – Samples prepared for testing of authentic urine specimens following in vitro adulteration with the selected oxidising agents.

Sample	THC-COOH Concentration (ng/mL)
Urine Blank 1	0
Urine Blank 2	0
THC-COOH Standard Low	25
THC-COOH Standard Medium	75
THC-COOH Standard High	750
Oxidant Blank Low	0
Oxidant Blank Medium	0
Oxidant Blank High	0
Oxidant Reaction Low	25[a,b]
Oxidant Reaction Medium	80 [a,b]
Oxidant Reaction High	825 [a,b]
Authentic Urine Low	25[b]
Authentic Urine Medium	80[b]
Authentic Urine High	825[b]

[a] Indicates initial THC-COOH concentration prior to adulteration
[b] Indicates reported concentration for authentic urine samples

7.1.5 – Alkaline Hydrolysis and Extraction

Alkaline hydrolysis of the urine samples was undertaken to recover unconjugated THC-COOH, and if they had formed from glucuronidated THC-COOH, the targeted reaction products. The tested samples had a volume of 1 mL, and were basified with 25 µL of 6 M sodium hydroxide, as per earlier research by Breindahl and Andreasen (Breindahl, T. & Andreasen, K., 1999). Following addition of sodium hydroxide, samples were heated at 50°C for 30 minutes, and upon cooling, were acidified with 0.5 M hydrochloric acid to pH 4.

After acidification of the samples, extraction of the unconjugated THC-COOH and desired reaction products was achieved through the use of liquid-liquid extraction. The samples were extracted with a 1:5 ethyl acetate/n-hexane solution in triplicate, with the organic layer transferred to a labelled vial. The organic fractions were then dried under a gentle stream of

nitrogen at 25°C, spiked with deuterated THC-COOH, and made up to a final volume of 1 mL with methanol. The concentration of the internal standard following reconstitution was 1000 ng/mL. Prior to sampling the samples were stored at 4°C.

7.1.6 – THC-COOH concentration

Unlike the pH and kinetics studies undertaken in Chapter 6, addition of the internal standard occurred after any potential reaction between the adulterants and the internal standard could occur. As a consequence, the proposed correction factors in Chapter 6 to aid in calculation of the concentration of THC-COOH in urine samples are not required.

7.2 – Results and Discussion

7.2.1 – Immunoassay Results

The batch of urine samples prepared for analysis by immunoassay were transferred to barcode-labelled vials and analysed. Results obtained from the immunoassay testing are shown in **Table 7.3**.

Table 7.3 - Immunoassay results from DRI™ Cannabinoid Assay for pooled and authentic urine samples.

Sample	Immunoassay Result (ng/mL)	Result
Urine Blank 1	-13	Negative
Urine Blank 2	-11	Negative
THC-COOH Standard Low	7	Negative
THC-COOH Standard Medium	52	**Positive**
THC-COOH Standard High	104	**Positive**
Betadine Blank Low	-14	Negative
Betadine Blank Medium	-11	Negative
Betadine Blank High	-11	Negative
Betadine Reaction Low	-10	Negative
Betadine Reaction Medium	-13	Negative
Betadine Reaction High	-11	Negative
Bleach Blank Low	-12	Negative
Bleach Blank Medium	-6	Negative

Table 7.3 (Continued) - Immunoassay results from DRI™ Cannabinoid Assay for pooled and authentic urine samples.

Sample	Immunoassay Result (ng/mL)	Result
Bleach Blank High	-6	Negative
Bleach Reaction Low	-8	Negative
Bleach Reaction Medium	-6	Negative
Bleach Reaction High	32	Negative
PCC Blank Low	-11	Negative
PCC Blank Medium	-9	Negative
PCC Blank High	-13	Negative
PCC Reaction Low	-9	Negative
PCC Reaction Medium	22	Negative
PCC Reaction High	109	**Positive**
Authentic Urine Low	19	Negative
Authentic Urine Medium	91	**Positive**
Authentic Urine High	106	**Positive**

The DRI™ Cannabinoid Immunoassay provides a linear range of 20 – 100 ng/mL. The 50 ng/mL calibration standard was used as the minimum cut-off concentration, with reported concentrations above this value considered positive. From this analysis a total of five samples returned a positive test result: the medium and high THC-COOH standard, the high PCC reaction mixture, and the medium and high authentic urine specimens. For the high THC-COOH standard and the high authentic urine sample, values above 100 were recorded. The values returned do not indicate that the THC-COOH concentration in these samples is around 100 ng/mL, rather it is an indication that the sample is strongly positive for the presence of cannabinoids. All other samples were found to be below the 50 ng/mL cut-off value.

All oxidant reagent blanks provided a negative cannabinoid concentration, as did the urine blanks. Negative values recorded for the immunoassay are considered as a strong indication that the sample is negative for cannabinoid compounds including THC-COOH. The Betadine reaction mixtures and the low and medium bleach reaction mixtures also recorded a negative response. This may be an indication that near-total loss of THC-COOH has occurred following reaction, or alternately, that the oxidants are causing an interference effect with the immunoassay, thereby skewing the recorded results.

Positive responses were found with the high bleach reaction and the medium and high PCC reaction mixtures. As with the other reaction mixtures, these results may be lower than the true THC-COOH concentration due to possible interference effects caused by the oxidants. As such, quantitative and validated methods for the detection of THC-COOH are required, and for the purposes of this study were achieved through LC-MS/MS. As discussed previously, GC-MS for the quantitative detection of drug metabolites has been well-established, and is routinely used by drug testing laboratories. Due to time constraints, and to have an effective means by which the targeted reaction products can be qualitatively detected, the validated LC-MS/MS methods from Chapter 5 were used. In addition, the qualitative detection of the targeted reaction products is critical in confirming whether these compounds do form after exposure to the glucuronide conjugate of THC-COOH present in the authentic urine samples. This would not be possible were the samples submitted for GC-MS analysis at the Drug Toxicology Unit, as these compounds are not targeted in their analyses.

7.2.2 – Analysis by LC-MS/MS methods

The authentic and spiked urine samples underwent alkaline hydrolysis, and following extraction and reconstitution in methanol, were analysed by LC-MS/MS in Multiple Reaction Monitoring mode. The calculated concentration of THC-COOH in these samples is reported in **Table 7.4**.

The results obtained for the calculated concentration of THC-COOH indicate that the concentration reported by the validated LC-MS/MS methods were generally in close agreement. For the THC-COOH standards prepared in blank pooled urine, it can be observed that reported concentrations were marginally lower than the expected concentration, and are attributed to losses during sample extraction after sample hydrolysis. This decrease in concentration is expected, based on the assessment of sample recovery undertaken prior to submitting these samples to the hydrolysis, extraction and reconstitution steps.

Differences are observed when comparing the data from **Table 7.4** to the results obtained from the DRI™ Cannabinoid Assay. For the THC-COOH standards, it can be seen that the results from the immunoassay are generally lower than the results obtained from confirmatory testing. However, it should be noted that the results obtained for the Authentic Urine Low sample (25 ng/mL) are in close agreement over the two analytical methods.

Significant differences between the results reported from the immunoassay analyses and confirmatory testing are readily apparent. It is expected that this is due to interference effects displayed by the oxidants, thereby providing an apparent lower concentration for THC-COOH as reported by the immunoassay. Another consideration is as to whether the targeted reaction products display any cross-reactivity in the DRI™ Cannabinoid Assay. As the reported THC-COOH concentrations from the immunoassay are generally lower than the concentrations reported by the LC-MS/MS methods, it may be the case that no significant cross-reactivity exists for the targeted reaction products. It is important to note, though, that due to the apparent interference effects caused by the oxidants at all tested concentrations, this apparent lack of cross-reactivity may be an artefact of the interference effect. As a consequence, further research into whether the targeted reaction products do display cross-reactivity is needed, and will require testing of the reaction products without the presence of the adulterants in the sample.

Table 7.4 – Reported THC-COOH concentrations for pooled and authentic urine samples by the validated LC-MS/MS methods incorporating sample hydrolysi.

Sample	Calculated THC-COOH Concentration (ng/mL)		
	Betadine Method	Bleach Method	PCC Method
Urine Blank 1	0.0	0.0	0.0
Urine Blank 2	0.0	0.0	0.0
THC-COOH Standard Low	22.4	23.2	20.8
THC-COOH Standard Medium	71.0	73.0	68.0
THC-COOH Standard High	734.1	726.1	724.3
Oxidant Blank Low	0.0	0.0	0.0
Oxidant Blank Medium	0.0	0.0	0.0
Oxidant Blank High	0.0	0.0	0.0
Oxidant Reaction Low	12.0	8.9	3.4
Oxidant Reaction Medium	22.7	23.4	11.8
Oxidant Reaction High	203.7	154.5	278.1
Authentic Urine Low	19.4	23.5	18.8
Authentic Urine Medium	68.4	73.1	79.1
Authentic Urine High	795.2	795.6	776.1

Based on the results obtained from the LC-MS/MS analysis, it is also possible to determine the effectiveness of the selected adulterants. **Table 7.5** provides a direct comparison of the initial concentration of THC-COOH in the authentic urine samples before and after adulteration. As can be seen, at the lowest THC-COOH concentration in an authentic urine sample, loss of THC-COOH due to reaction with the adulterants was highest for pyridinium chlorochromate, with Betadine and bleach also causing significant decreases in THC-COOH concentration. Similar results were found at the second-highest THC-COOH concentration in an authentic urine sample, whereby all three adulterants caused a considerable decrease in concentration. At the highest concentration, the effectiveness of pyridinium chlorochromate in reacting with THC-COOH appears lower than expected based on the previous results. This may be attributed to the reaction of this adulterant with the endogenous compounds present in the urine, or alternately, this adulterant may not readily react with the THC-COOH glucuronide. This latter possibility will be discussed later in this chapter.

Table 7.5 – Comparison of the recorded THC-COOH concentrations for the authentic urine specimens both before and after adulteration with the selected adulterants.

Sample	Pre-Adulteration THC-COOH Concentration (ng/mL)[a]	Post-Adulteration THC-COOH Concentration (ng/mL)		
		Betadine	Bleach	PCC
Oxidant Reaction Low	19.88	12.0	8.9	3.4
Oxidant Reaction Medium	73.52	22.7	23.4	11.8
Oxidant Reaction High	762.30	203.7	154.5	278.1

[a] Average THC-COOH concentration recorded for the three validated detection methods.

Further results obtained from the LC-MS/MS analyses of the prepared samples are shown in **Figure 7.1**. This reports the recorded peak areas detected for the targeted reaction products in their respective methods. As the reaction products will not be detected in the urine blanks, THC-COOH standards, authentic urine samples and oxidant reagent blanks, no peak areas were recorded for the reaction products in these samples, and are therefore excluded from **Figure 7.1**. These results are intended to confirm whether the targeted reaction products will form following exposure to the glucuronide conjugate of THC-COOH, and if these reactions do occur, whether they can be detected following sample hydrolysis.

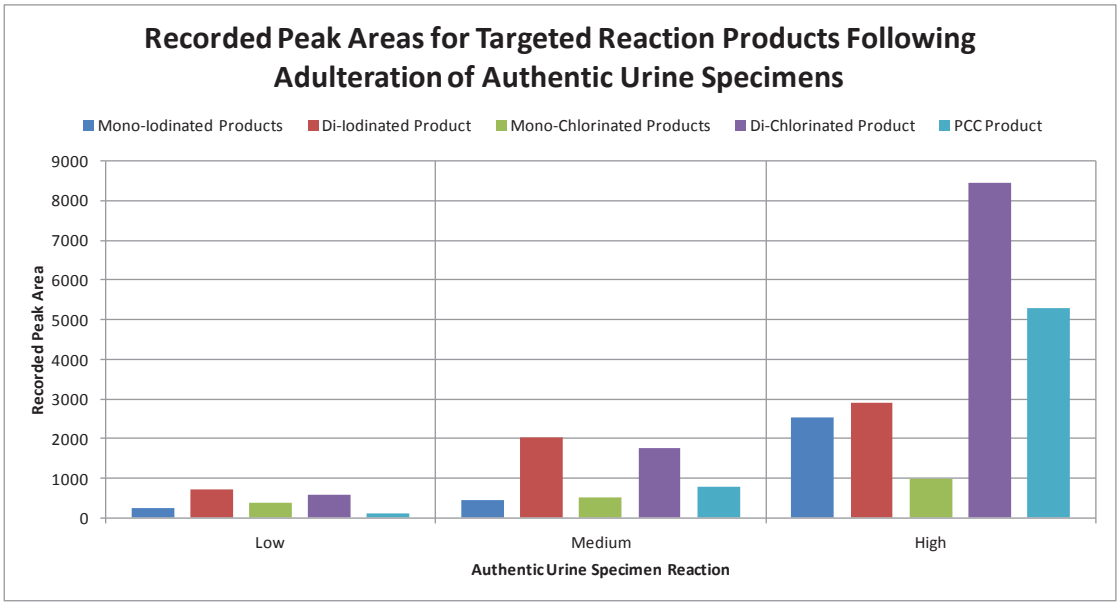


Figure 7.1 - Peak areas recorded for the targeted reaction products in the three reaction sets prepared in authentic cannabis-positive urine samples.

From **Figure 7.1** it can be seen that the targeted reaction products did not form to a significant extent at the lowest THC-COOH concentration in authentic urine. This result is expected due to the low quantity of THC-COOH available for reaction. When compared to **Table 7.4** and **Table 7.5**, it can be seen that the decrease in THC-COOH concentration post-adulteration was generally small, and is likely due to the reaction of the adulterants with the endogenous compounds present in urine. Also of note is that despite the limited formation of the reaction products, these compounds were successfully detected in an authentic urine sample, and suggests that these compounds may be effectively used by drug testing laboratories as markers of urine adulteration.

For the second authentic urine specimen, with an average THC-COOH concentration of 74 ng/mL across the three detection methods pre-adulteration, larger peak areas are recorded for all the targeted reaction products. Due to the increased amount of THC-COOH available for reaction, whether free or present as the glucuronide conjugate, this result is expected. For the reaction with Betadine and bleach in this authentic urine sample, preferential formation of the di-halogenated products is observed, with small peak areas reported for the corresponding mono-halogenated products.

Results obtained for the third authentic urine specimen, with an average THC-COOH concentration of 762 ng/mL pre-adulteration, initially appear similar to those obtained for the lower-concentration authentic urine specimens. It can be seen that for the reaction with bleach and pyridinium chlorochromate, the di-chlorinated product and sole PCC product readily form. In contrast, in the reaction with Betadine, this trend is not observed with only a marginal increase found for the di-iodinated product.

Based on the results obtained for the pH studies in Chapter 6, further information relating to the formation of the targeted reaction products in authentic urine may be obtained. As discussed previously, the reaction of THC-COOH with pyridinium chlorochromate and Betadine readily progresses under acidic conditions, whilst for sodium hypochlorite, alkaline reaction conditions are most beneficial for the formation of the chlorinated reaction products. The low-concentration authentic urine sample was slightly alkaline, with a reported pH of 7.5. In contrast, for the two other authentic urine specimens, a pH of 6.5 was reported.

Based on the results obtained in Chapter 6, we can expect that product formation for pyridinium chlorochromate and Betadine will be more favourable in the medium and high THC-COOH concentration authentic urine samples. This hypothesis appears to be correct, with

significant increases in peak area observed for the PCC reaction product in the two acidic urine samples. In addition, in the alkaline urine sample formation of the Betadine reaction products is poor. Through an increase in THC-COOH concentration, in addition to the slight acidity of the medium and high THC-COOH concentration authentic urine samples, a significant increase in peak area is recorded for the Betadine reaction products. In particular, it is observed that the formation of the mono-iodinated reaction products is markedly higher in the high-concentration authentic urine specimen.

For the reaction with bleach, the pH-based trends for pyridinium chlorochromate and Betadine are not as readily apparent. As the medium and high concentration authentic urine specimens are only slightly acidic, it appears that the reaction conditions encountered in these authentic specimens do not have a major negative effect on the formation of the bleach products. Indeed, formation of the di-chlorinated reaction product is quite good based on the peak areas recorded for this product.

Previously mentioned is the question of whether or not the targeted reaction products are capable of undergoing reaction with the glucuronide-bound form of THC-COOH. Superficially, the results obtained through the confirmatory testing of these samples appears to suggest that this is the case, and that the reaction products are capable of forming with both free and glucuronide-bound THC-COOH, considering that THC-COOH is primarily found as the glucuronide conjugate in urine. However, the interpretation of the results in this regard is complicated due to the ability of the THC-COOH glucuronide to undergo in vitro hydrolysis during sample storage.

Prior research by Skopp and Pötsch (2004) indicates that sample storage conditions and time may have a significant effect on the stability of THC-COOH-glucuronide. It was found that when stored at -20°C, the glucuronide conjugates of THC metabolites are relatively stable. However, storage temperatures above this result in a significant degree of auto-hydrolysis of the glucuronides, causing in the formation of free THC-COOH in vitro.

The authentic urine specimens used in this study were initially stored at -18°C, and immediately prior to sample preparation, were temporarily stored at 4°C. This has potentially significant implications on the interpretation of the data in this study, as it remains possible that the in vitro hydrolysis of the THC-COOH glucuronide may have made available sufficient quantities of free THC-COOH for reaction with the selected adulterants. As such, it is not possible to conclude whether the observed reaction products in the authentic urine specimens

were due to reaction of the adulterants with THC-COOH glucuronide, or if they were formed due to the presence of free THC-COOH in the specimen as a result of sample storage.

Conversely, it is important to point out that based on the characterisation of the PCC product, this adulterant appeared to be less effective in reacting with THC-COOH at higher concentrations in the authentic urine specimens relative to the other adulterants. This may suggest that if the authentic urine samples primarily contained THC-COOH-glucuronide, then this adulterant may be of limited effectiveness in authentic urine specimens and primarily reacted with the free THC-COOH present in the sample due to sample storage. As such, it may remain possible that both Betadine and bleach are capable of reacting with the glucuronide conjugate of THC-COOH, hence the greater reduction in THC-COOH concentration seen in these samples. An alternate proposal, however, is that for pyridinium chlorochromate, reactions with the endogenous compounds present in urine inhibited the reaction with THC-COOH, and in this scenario, extensive in vitro hydrolysis of the glucuronide conjugate may have occurred during sample storage.

Overall the results obtained in this study provide a further understanding of both the formation of the targeted reaction products in authentic urine specimens, as well as the potential effectiveness of these adulterants to potentially mask a cannabis-positive urine sample. The successful formation of all the targeted reaction products in the authentic urine specimens provides a proof of concept, whereby it can be seen that these compounds may represent viable markers of urine adulteration, and therefore may be targeted by drug testing laboratories to identify both cannabis use and urine adulteration.

Chapter 8

Conclusions

The issue of urine adulteration and urine manipulation is well known, and has been discussed extensively in literature. Methods for adulterating a drug-positive urine sample vary, and aim to decrease the concentration of a drug metabolite below the limit of detection, interfere with detection methods, or alternately, react with the drug metabolite in vitro. In these cases of urine adulteration, the primary goal is to return a false-negative drug test result, thereby avoiding detection of drug use. The reasons as to why an individual may wish to mask a positive drug test result are understandable, and the return of a positive test result may have significant ramifications for the individual where testing has been undertaken as a part of pre-employment screening, post-incident testing, routine screenings during an individual's employment, and sports drug testing. In addition, individuals discovered tampering with urine samples may also face legal penalties, especially in the above scenarios where a urine specimen may be requested by law enforcement or sports anti-doping officials.

Detection of urine adulteration and manipulation is well-established, with chemical and instrumental techniques available for urine integrity tests and identifying the presence of exogenous chemical substances that are known to mask a positive drug test result. Indeed, a large range of chemicals are available that have the potential to invalidate the results from drug testing. Some chemicals are widely available to the average consumer and may be freely purchased from supermarkets and pharmacies, and include bleach, Betadine and potassium permanganate. Others are available through online marketplaces, with these products both sold explicitly for the purposes of urine adulteration, or as detoxifying agents ostensibly used for improving a person's health.

As discussed in Chapter 1, one of the major problems faced by drug testing laboratories is the large range of adulterants that are available, and in addition, chemicals that have the potential to be used to invalidate drug test results. In the case of UrineLuck, previous formulations of this product were found to be pyridinium chlorochromate. However, as the use of this hexavalent chromium compound became well known, detection of hexavalent chromium compounds as a potential marker of urine adulteration was incorporated by drug testing laboratories. As a consequence, the formulation of UrineLuck has supposedly undergone

multiple revisions, with the current formulation alleged to be undetectable. In addition, the chemical composition of the latest formulation is not known, nor has been disclosed, at the time of writing, and does point out that the range of potential urine adulterants is extensive.

One method by which drug testing laboratories may detect cases of both drug use and urine adulteration is through the study of these adulterants and their effect on drug metabolites targeted during instrumental analysis of these samples. In cases where the adulterant reacts with the drug metabolite, it remains possible that novel compounds characteristic of both the targeted metabolite and its reaction with a selected adulterant may form. Detection of stable compounds following adulteration of a drug-positive urine sample may therefore act as markers signifying this act of adulteration. As discussed in Chapter 2, a range of novel compounds were found following adulteration of samples spiked with THC-COOH, and may prove useful to drug testing laboratories through incorporation into current testing measures.

In addition to the discovery of reaction products of THC-COOH post-adulteration, it is also important to note that though adulterants were found to be highly effective at masking the presence of THC-COOH in spiked urine samples, no reaction products were found during these experiments. For example, both potassium permanganate and sodium iodate resulted in significant decreases in the peak area of THC-COOH following adulteration, providing strong evidence of their potential as urine adulterants. However, the lack of any obvious reaction products limits the ability of a drug testing laboratory to conclusively identify both use of these adulterants, and in the case of this research, cannabis use.

Various hypotheses exist as to why certain adulterants do not form stable reaction products following exposure to THC-COOH. Some adulterants may react extensively with this metabolite, and ultimately lead to degradation of THC-COOH and any reaction products that may have formed in this reaction. Alternately, it is possible that the detection parameters utilised in these experiments were insufficient for the detection of these reaction products. As such, future research may seek to investigate different detection parameters, including the detected mass range, to explore whether these reactions are indeed capable of forming stable reaction products.

A major goal of the research undertaken for this thesis was the structural elucidation of selected reaction products. Aside from initially confirming their formation in spiked urine samples, this allowed for an examination of the potential reaction pathways that result in these products. For samples adulterated with Betadine and bleach, it is likely that these

reactions progress through the electrophilic aromatic substitution of the aromatic ring present in THC-COOH, with the results of the ^{1}H NMR experiment on the di-iodinated THC-COOH product providing strong evidence of this reaction pathway. Based on the results obtained for the di-iodinated THC-COOH product, it is expected that the proposed electrophilic aromatic substitution of THC-COOH is transferrable to both the adulteration of samples by bleach, and for the formation of the mono-iodinated products detected following adulteration by Betadine.

A notable limitation of this research was the production of sufficient quantities of the reaction products, with sufficient purity, for analysis by NMR. Though useful information relating to the chemical structures of the reaction products was obtained, acquired ^{13}C NMR data for the pyridinium chlorochromate reaction product revealed a poor signal-to-noise ratio. Similar issues were also encountered for the correlation spectra obtained for the reaction products analysed. These issues may be potentially attributed to insufficient quantities of the desired reaction products, insufficient purity, or in the case of the pyridinium chlorochromate reaction product, minor traces of chromium remaining present in the samples submitted for analysis. With regards to the NMR data acquired for the reaction products, future research may seek to improve upon the synthesis of these reaction products, and employing a more effective means by which purification of the reaction products may be achieved.

Determination of the reaction pathway that results in the detected pyridinium chlorochromate product is unclear. This oxidising adulterant typically reacts with primary and secondary alcohols, oxidising them to their respective aldehydes and ketones, and is also reported to selectively oxidise unsaturated alcohols and aldehydes. Based on the structure of THC-COOH, these reaction pathways are not possible, and complicates interpretation of how this reaction progresses. Data obtained from high-resolution accurate mass spectroscopy and NMR experiments ultimately provided evidence of possible regions of the THC-COOH molecule that are unlikely to have reacted with pyridinium chlorochromate. Specifically, the available data suggests that the aromatic ring and carboxylic acid functional groups, in addition to the alkyl sidechain present on the aromatic ring, have not undergone reaction. Though the information available is insufficient to conclusively propose a structure for this reaction product, a number of theorised candidates were found. As this compound was found to form in authentic urine specimens, as well as spiked urine specimens under different pH and storage conditions, future research may seek to elucidate the structure of this molecule, and thereby provide a stronger

understanding of the reaction pathway that results in this compound and the potential for other hexavalent chromium compounds to react with THC-COOH.

Synthesis of the reaction products, as discussed in Chapter 3, was ultimately successful, and was achieved through chromatographic separation of the compounds via liquid chromatography, and following dropwise collection of individual fractions, resulted in the collection of sufficiently pure samples of the desired reaction products. As noted in Chapter 3, careful monitoring of the dropwise collection of the desired fractions was required. Initial trials with small-scale reaction samples made light of potential contamination issues that were addressed. Following careful experimental design and ensuring cleanliness of instrumentation and glassware, this potential issue of contamination was not found following purification of the large-scale reaction samples. With regards to the small-scale reactions, it is expected that for the fractionation of both reactions, cross-contamination between fractions may have occurred post-column as the eluent exited the programmable absorbance detector. The solvent line exiting this detector module may have retained traces of the starting material and other products throughout fractionation. In the case of the Betadine/iodine reaction the differences in retention time of the reaction products combined with the timing of the drop-wise collection presents a likely source of contamination in the small-scale reaction tests.

Another primary consideration of this research was development of validated methods for both the quantitative detection of THC-COOH, and the qualitative detection of the reaction products formed following adulteration of authentic and spiked urine samples with pyridinium chlorochromate, Betadine and bleach. The research detailed in Chapter 6 was concerned with the study of the targeted reaction products and the effect of urine pH and sample storage conditions on the formation and stability of these reaction products, and thereby assess their potential suitability as markers of urine adulteration and cannabis use for drug testing laboratories. As the experiments detailed in Chapter 6 dealt exclusively with spiked urine samples, the first method that was validated did not incorporate a sample hydrolysis step. In contrast, as the adulterated urine specimens studied in Chapter 7 contain the glucuronide conjugate of THC-COOH, it was also necessary to validate a separate set of methods that incorporated an alkaline sample hydrolysis step. Overall these methods were found to be effective for the quantitative and qualitative detection of THC-COOH and the targeted reaction products, respectively.

Though the methods themselves were validated with regards to the quantitative detection of THC-COOH, and in addition, the qualitative detection of the targeted reaction products, the selection of the internal standard did limit the effectiveness of accurately determining the concentration of THC-COOH in adulterated urine samples. As the internal standard, THC-COOH-d_9, undergoes analogous reactions with the adulterants as THC-COOH also underwent. In the case of this research, the internal standard was selected due to its prevalence in previously validated methods.

As such, future research into the adulteration of THC-COOH positive urine samples may seek to assess alternate internal standards that are non-reactive towards common oxidising adulterants, in order to more fully explore both the formation of post-adulteration reaction products, and to accurately quantify THC-COOH concentration in adulterated samples. In addition, future research may also benefit from exploring the effect of when the internal standard is added at various post-adulteration time points. In the case of this research, addition of the internal standard was effectively concomitant with the addition of the selected adulterants. It is expected that, as greater periods of time pass between the addition of the adulterant, and the latter addition of the internal standard, a more accurate determination of THC-COOH concentration may be possible as the adulterants undergo extensive reactions with THC-COOH and the endogenous compounds found in urine. Indeed, ascertaining whether timing of the addition of the internal standard will have an effect on its final concentration pre-analysis may have implications for drug testing laboratories, whereby a small delay in the addition of the internal standard may limit possible reactions with supposed adulterated samples, and in turn, ensure that a sufficient concentration of the internal standard remains for quantitative analysis of the alleged adulterated sample.

Despite the success of these validated methods in detecting THC-COOH and the reaction products, it is limited by the lack of certified reference standards for the targeted reaction products. Synthesis and certification of the reaction products would be of significant benefit for future research concerning the effect of these adulterants, as it would allow for further development and validation of the detection methods, and potentially allow for the quantitative detection of compounds formed through the adulteration of drug-positive urine specimens. In addition, it would be of benefit in future studies to further explore additional time-points for the kinetics and stability studies undertaken in this research. Due to limits encountered in this research, it was necessary to select a sufficient number of time-points over which to collect data, in order to explore the formation of reaction products, reaction of THC-

COOH with the selected oxidising agents, and importantly, the stability of the reaction products. However, a more thorough analysis of the adulterated urine samples in the first twenty-four hours following initial adulteration may provide further insight into these reactions, and more importantly, allow for a greater understanding in relation to the formation of reaction products at different urine pH and storage temperature.

It is important to note that the research detailed in this thesis was designed to detect and further analyse potential oxidation products of THC-COOH through LC-MS/MS. As discussed previously, GC-MS is considered the gold standard for drug testing laboratories, offering high sensitivity and selectivity, and are critical for the confirmatory testing of suspected drug-positive urine samples. Though this instrumental method has been demonstrated to be highly effective for confirmatory screening, it was ultimately not incorporated into this research. Primarily, selection of LC-MS/MS minimised the number of steps and time required for sample preparation, as well as selection of a suitable derivatising agent to increase the volatility of THC-COOH and the detected reaction products. In the case of the pyridinium chlorochromate product, the lack of a conclusive chemical structure for this compound may complicate the selection of a derivatising agent.

Another consideration in the selection of LC-MS/MS over GC-MS in this research was sample throughput. By removing the time-consuming derivatisation and other sample preparation steps required for analysis of urine samples by GC-MS, additional information was able to be obtained regarding the formation and stability of the targeted reaction products through generation of additional data points during the time-based studies found in Chapter 6. Though such studies are able to be undertaken by GC-MS, simplification of sample preparation was ultimately a major consideration for the choice of LC-MS/MS for the detection and study of reaction products formed following adulteration of THC-COOH.

From the perspective of drug testing laboratories GC-MS is very likely to remain critical for analysis of suspected drug-positive urine samples in the future. It is stressed that the research presented in this thesis is not intended to undermine the utility and versatility of GC-MS for routine drug analysis. Indeed, selection of LC-MS/MS for this research was based on the versatility of this instrument to readily detect the potential markers of adulteration of cannabis-positive urine samples under a variety of conditions. Based on the detection of these novel reaction products for THC-COOH, future research may be concerned with application of

the LC-MS/MS detection parameters to GC-MS following selection of suitable derivatising agents.

Overall the research presented in this thesis provides a suitable starting point for the further exploration of the adulteration of drug-positive urine samples by a range of adulterants. In this research it was possible to detect a range of reaction products formed following adulteration of both water and urine samples containing THC-COOH. In addition, large-scale synthesis of selected products was explored, and though the yields of these products was lacking, further refinement of the synthesis of these compounds may allow for synthesis of larger quantities of these compounds in higher purity, and may lead to the production of certified reference standards for these compounds. Critically, it was also possible to develop validated detection methods to allow for the quantitative detection of THC-COOH, and as significant, the qualitative detection of the targeted reaction products.

Ultimately, further research is required to ascertain the effect of a range of both known and unknown adulterants on the validity of results obtained through drug testing analysis of drug-positive urine samples. As discussed previously, the detection of stable markers of both drug abuse and urine adulteration represents a potential benefit to drug testing laboratories. Previously, adulterated samples are classed as invalid, and an additional sample may be requested by the laboratory. The detection of a number of reaction products in authentic, cannabis-positive urine samples following adulteration in this research is therefore considered a proof-of-concept, and shows that the incorporation of novel markers of urine adulteration into current drug testing schemes is both possible and of significance for future research in this issue faced by drug testing laboratories.

Publications and Presentations

Publications

1. Charlton, N. & Fu, S. 2012, 'Effect of selected oxidising agents on the detection of 11-nor-9-carboxy-Δ-9-tetrahydrocannabinol (THC-COOH) in spiked urine', *TIAFT Bulletin*, vol. 42, no. 2, pp. 33-36.

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Presentations

- 2009 – Geneva, Switzerland: The International Association of Forensic Toxicologists (TIAFT)
- 2010 – Sydney, Australia: The Australian and New Zealand Forensic Science Society (ANZFSS)
- 2012 – Hamamatsu, Japan: The International Association of Forensic Toxicologists (TIAFT)
- 2013 – Sydney, Australia: The Forensic and Clinical Toxicology Association (FACTA)

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Appendix

This appendix contains additional data obtained during the experiments found in Chapter 4 and Chapter 5. This data provides raw data used in calculations, and data used in the interpretation of other results, as for the 2-dimensional NMR experiments. For further information regarding this data, please refer to the respective Chapter.

From Chapter 4 – Structural Elucidation

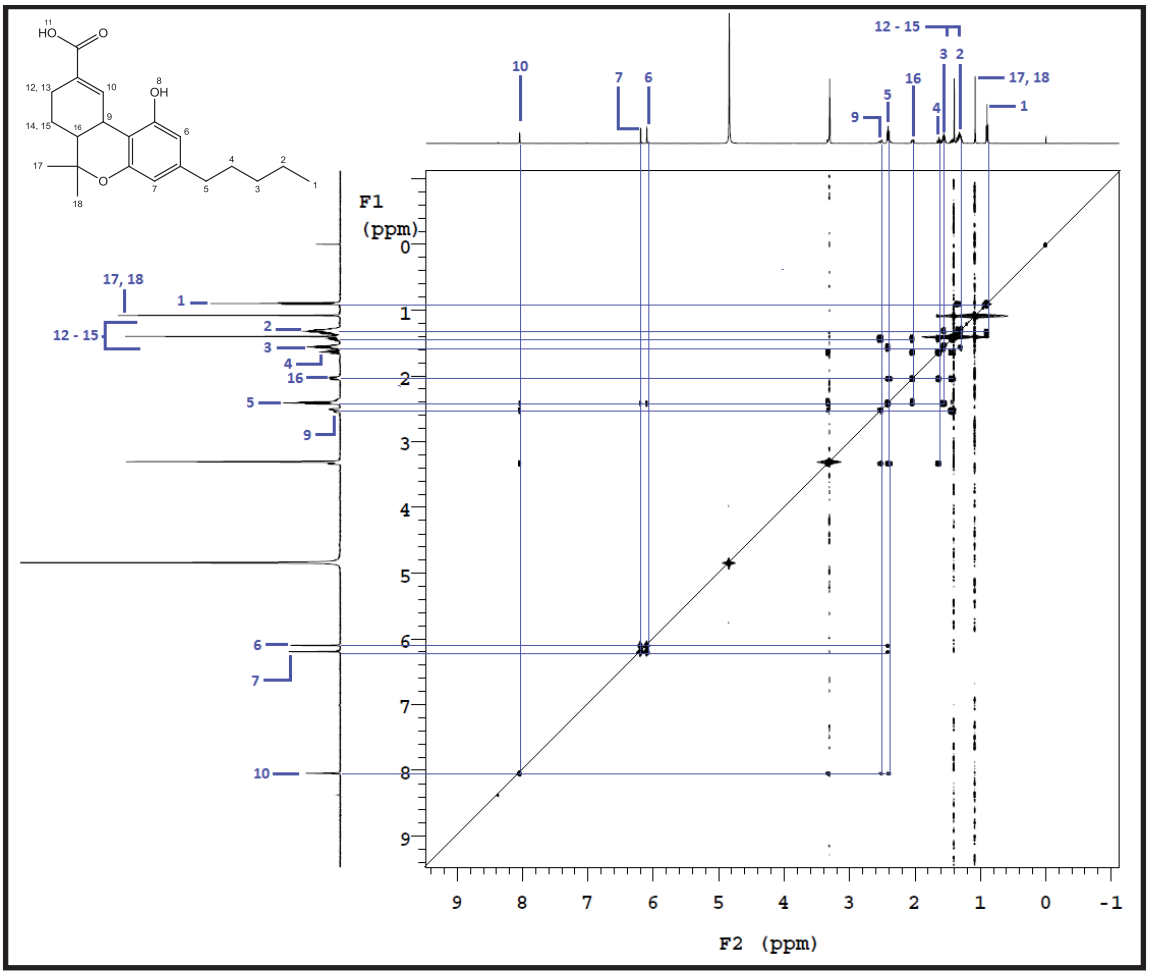

Figure A.1 – COSY spectra for THC-COOH. Correlation between protons has been indicated through blue lines. For further information regarding interpretation of this spectrum and a summary of results, please refer to Chapter 4, and for assignment of proton numbers, Figure 4.1.

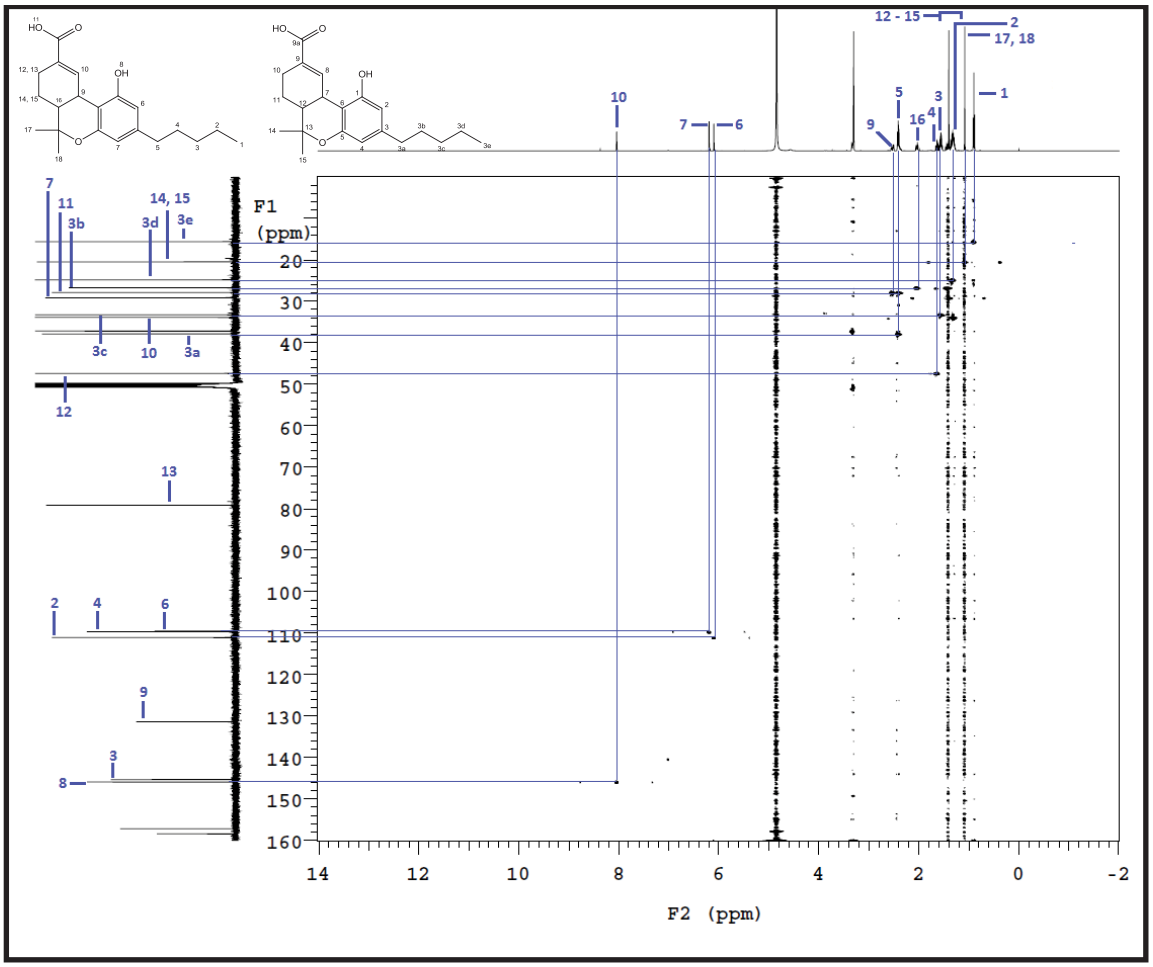

Figure A.2 – HSQC spectra for THC-COOH. Correlation between protons has been indicated through blue lines. For further information regarding interpretation of this spectrum and a summary of results, please refer to Chapter 4, and for assignment of proton and carbon numbers, Figure 4.1.

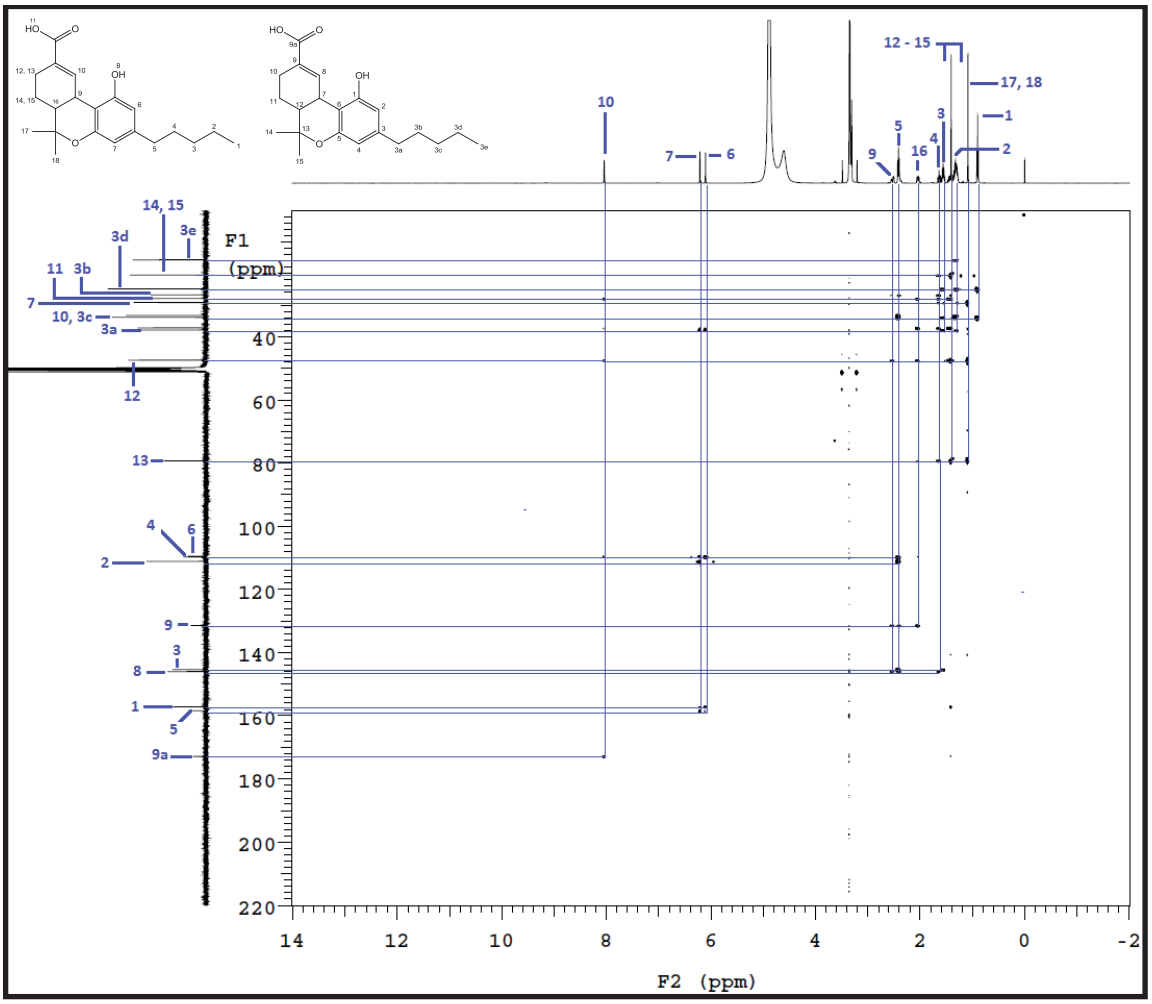

Figure A.3 - HMBC spectra for THC-COOH. Correlation between protons has been indicated through blue lines. For further information regarding interpretation of this spectrum and a summary of results, please refer to Chapter 4, and for assignment of proton and carbon numbers, Figure 4.1.

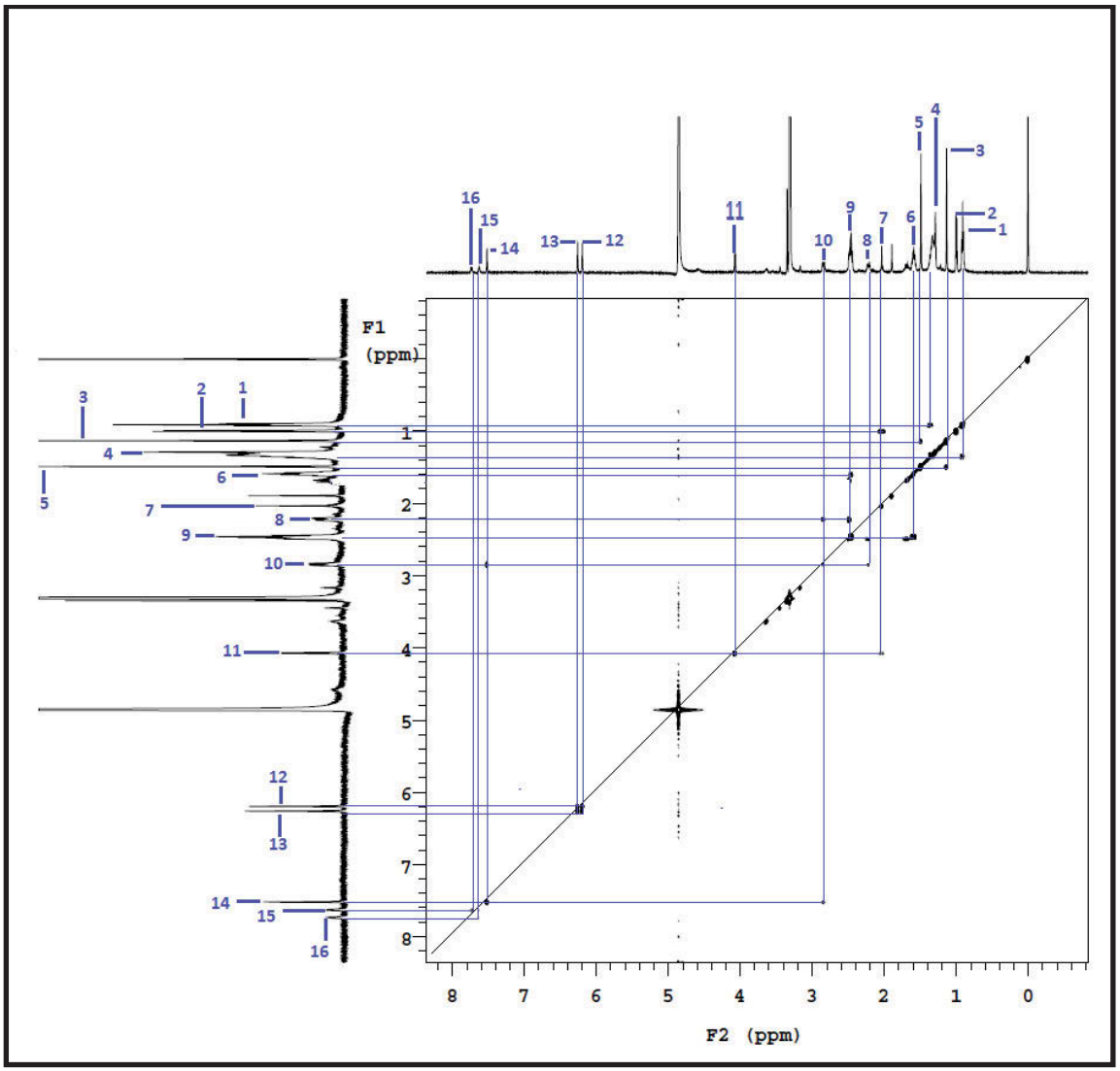

Figure A.4 - COSY spectra for the PCC Product. Correlation between protons has been indicated through blue lines. For further information regarding interpretation of this spectrum and a summary of results, please refer to Chapter 4.

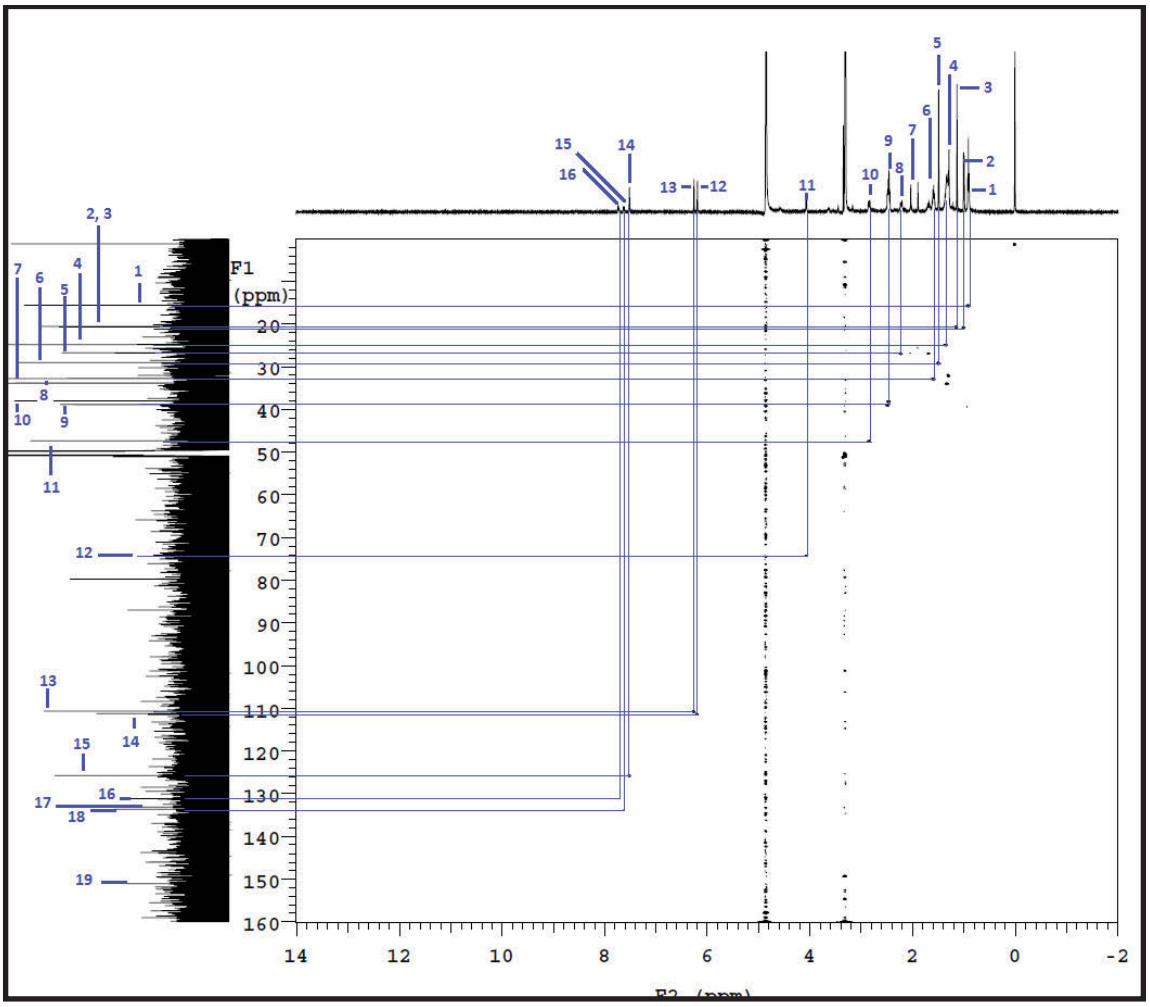

Figure A.5 - HSQC spectra for the PCC Product. Correlation between protons has been indicated through blue lines. For further information regarding interpretation of this spectrum and a summary of results, please refer to Chapter 4.

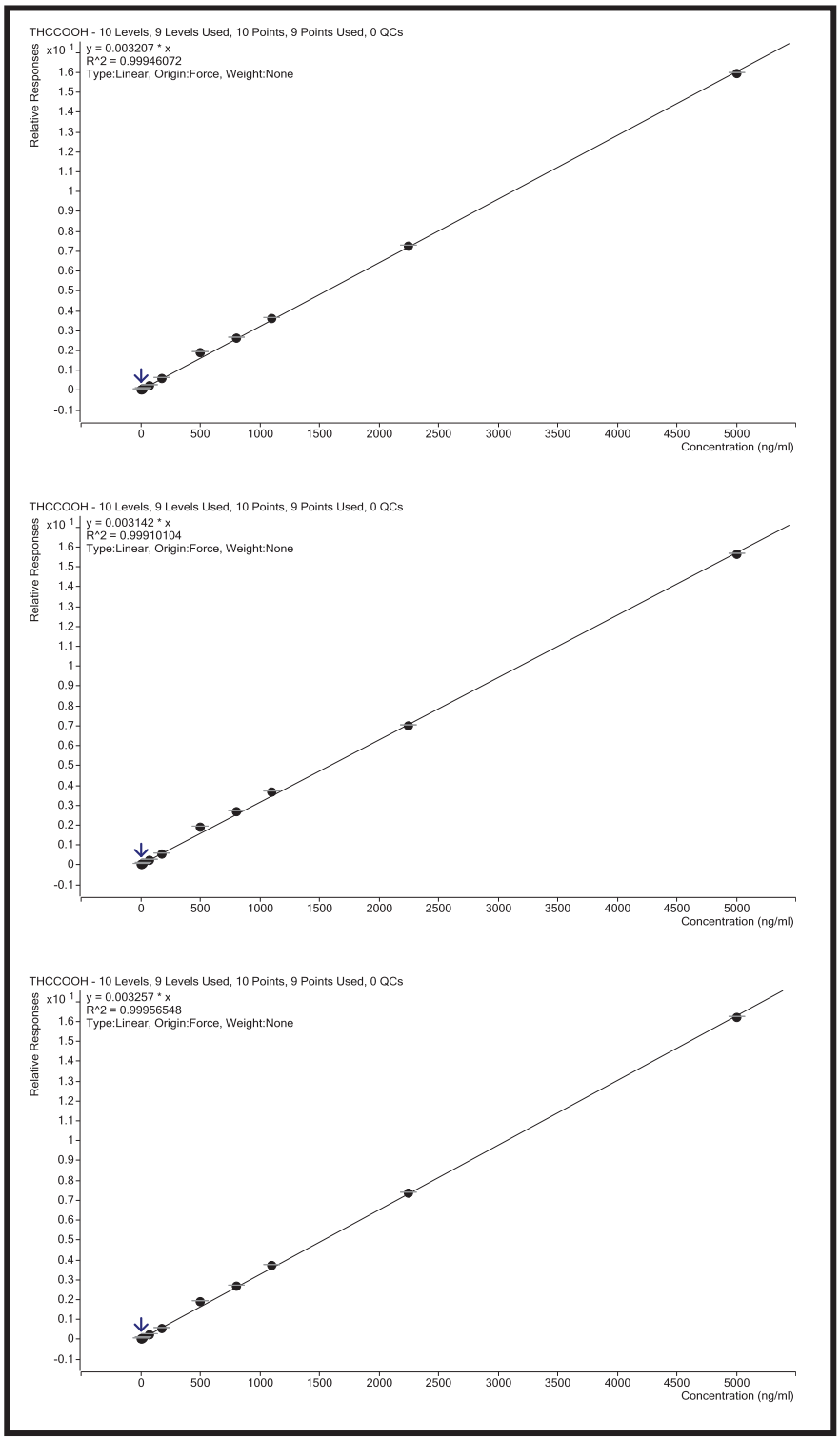

Figure A.6 – Calibration curves generated for the three validated methods involving the alkaline sample hydrolysis step: (Top) PCC method, (Middle) Betadine method, (Bottom) bleach method.

From Chapter 5 – Optimisation of Detection Parameters and Method Validation

Table A.1 – Intra-day precision calculations for the bleach method (without sample hydrolysis)

Intra-day Precision – Bleach			
Bleach	Injection	Calculated Concentration (ng/mL)	Standard Deviation (%RSD)
QC1	1	22.91	1.447
	2	19.10	
	3	21.20	
	4	21.40	
	5	22.30	
	6	19.81	
QC2	1	655.53	4.496
	2	650.31	
	3	648.29	
	4	653.65	
	5	648.15	
	6	659.52	
QC3	1	2512.26	4.841
	2	2503.96	
	3	2498.24	
	4	2506.58	
	5	2509.19	
	6	2504.16	

Table A.2 – Intra-day precision calculations for the bleach method (with sample hydrolysis)

Intra-day Precision – Bleach			
Bleach	Injection	Calculated Concentration (ng/mL)	Standard Deviation (%RSD)
QC1	1	22.78	1.439
	2	18.99	
	3	21.08	
	4	21.28	
	5	22.18	
	6	19.70	
QC2	1	651.79	4.470
	2	646.60	
	3	644.60	
	4	649.93	
	5	644.46	
	6	655.76	
QC3	1	2497.94	4.814
	2	2489.68	
	3	2484.00	
	4	2492.29	
	5	2494.89	
	6	2489.89	

Table A.3 – Intra-day precision calculations for the Betadine method (without sample hydrolysis)

Intra-day Precision - Betadine			
Betadine	Injection	Calculated Concentration (ng/mL)	Standard Deviation (%RSD)
QC1	1	21.66	2.757
	2	20.94	
	3	17.76	
	4	24.98	
	5	22.71	
	6	25.10	
QC2	1	648.19	3.513
	2	654.51	
	3	650.30	
	4	658.25	
	5	652.82	
	6	651.42	
QC3	1	2522.56	8.777
	2	2509.51	
	3	2498.90	
	4	2499.51	
	5	2507.15	
	6	2503.09	

Table A.4 – Intra-day precision calculations for the Betadine method (with sample hydrolysis)

Intra-day Precision - Betadine			
Betadine	Injection	Calculated Concentration (ng/mL)	Standard Deviation (%RSD)
QC1	1	21.54	2.742
	2	20.82	
	3	17.66	
	4	24.84	
	5	22.58	
	6	24.96	
QC2	1	644.50	3.493
	2	650.78	
	3	646.60	
	4	654.50	
	5	649.10	
	6	647.70	
QC3	1	2508.18	8.727
	2	2495.21	
	3	2484.66	
	4	2485.26	
	5	2492.86	
	6	2488.82	

Table A.5 – Intra-day precision calculations for the PCC method (without sample hydrolysis)

Intra-day Precision - PCC			
PCC	Injection	Calculated Concentration (ng/mL)	Standard Deviation (%RSD)
QC1	1	18.34	3.519
	2	19.00	
	3	24.90	
	4	26.17	
	5	21.43	
	6	25.99	
QC2	1	643.29	5.372
	2	658.60	
	3	652.03	
	4	656.08	
	5	649.10	
	6	652.06	
QC3	1	2518.32	10.064
	2	2502.74	
	3	2509.42	
	4	2497.01	
	5	2502.55	
	6	2489.05	

Table A.6 – Intra-day precision calculations for the PCC method (with sample hydrolysis)

Intra-day Precision - PCC			
PCC	Injection	Calculated Concentration (ng/ml)	Standard Deviation (%RSD)
QC1	1	18.24	3.499
	2	18.89	
	3	24.76	
	4	26.02	
	5	21.31	
	6	25.84	
QC2	1	639.63	5.342
	2	654.85	
	3	648.31	
	4	652.34	
	5	645.40	
	6	648.34	
QC3	1	2503.97	10.007
	2	2488.47	
	3	2495.12	
	4	2482.78	
	5	2488.29	
	6	2474.86	

Table A.7 – Inter-day precision calculations for the bleach method (without sample hydrolysis)

Inter-day Precision - Bleach			
Bleach	Injection	Calculated Concentration (ng/ml)	Standard Deviation (%RSD)
QC1	1	22.91	6.511
	2	20.93	
	3	10.25	
	4	5.81	
	5	24.86	
	6	20.07	
	7	19.44	
	8	19.44	
QC2	1	667.76	8.548
	2	656.75	
	3	652.24	
	4	648.29	
	5	655.45	
	6	672.74	
	7	667.48	
	8	657.80	
QC3	1	2509.09	10.300
	2	2493.26	
	3	2513.96	
	4	2521.26	
	5	2520.26	
	6	2498.55	
	7	2499.94	
	8	2511.10	

Table A.8 – Inter-day precision calculations for the bleach method (with sample hydrolysis)

Inter-day Precision - Bleach			
Bleach	Injection	Calculated Concentration (ng/ml)	Standard Deviation (%RSD)
QC1	1	22.80	6.481
	2	20.83	
	3	10.21	
	4	5.78	
	5	24.74	
	6	19.97	
	7	19.35	
	8	19.35	
QC2	1	664.70	8.509
	2	653.75	
	3	649.26	
	4	645.33	
	5	652.45	
	6	669.66	
	7	664.43	
	8	654.79	
QC3	1	2497.63	10.253
	2	2481.87	
	3	2502.47	
	4	2509.74	
	5	2508.75	
	6	2487.13	
	7	2488.51	
	8	2499.62	

Table A.9 – Inter-day precision calculations for the Betadine method (without sample hydrolysis)

Inter-day Precision - Betadine			
Betadine	Injection	Calculated Concentration (ng/ml)	Standard Deviation (%RSD)
QC1	1	23.51	4.266
	2	21.31	
	3	24.81	
	4	28.30	
	5	18.70	
	6	16.44	
	7	24.80	
	8	28.36	
QC2	1	654.31	10.259
	2	647.78	
	3	675.30	
	4	652.10	
	5	647.50	
	6	644.91	
	7	653.95	
	8	642.17	
QC3	1	2508.65	10.717
	2	2490.16	
	3	2515.17	
	4	2509.24	
	5	2511.91	
	6	2529.18	
	7	2510.11	
	8	2506.85	

Table A.10 – Inter-day precision calculations for the Betadine method (with sample hydrolysis)

Inter-day Precision - Betadine			
Betadine	Injection	Calculated Concentration (ng/ml)	Standard Deviation (%RSD)
QC1	1	23.40	4.246
	2	21.21	
	3	24.70	
	4	28.17	
	5	18.62	
	6	16.37	
	7	24.69	
	8	28.23	
QC2	1	651.31	10.212
	2	644.82	
	3	672.22	
	4	649.12	
	5	644.54	
	6	641.96	
	7	650.97	
	8	639.23	
QC3	1	2497.19	10.668
	2	2478.78	
	3	2503.67	
	4	2497.77	
	5	2500.43	
	6	2517.62	
	7	2498.64	
	8	2495.39	

Table A.11 – Inter-day precision calculations for the PCC method (without sample hydrolysis)

Inter-day Precision - PCC			
PCC	Injection	Calculated Concentration (ng/ml)	Standard Deviation (%RSD)
QC1	1	14.30	7.055
	2	28.71	
	3	24.65	
	4	12.81	
	5	14.60	
	6	27.31	
	7	11.09	
	8	22.61	
QC2	1	659.01	10.461
	2	672.51	
	3	635.99	
	4	653.50	
	5	660.51	
	6	652.81	
	7	648.93	
	8	657.81	
QC3	1	2520.65	12.968
	2	2479.11	
	3	2502.11	
	4	2494.32	
	5	2501.48	
	6	2489.17	
	7	2512.19	
	8	2503.17	

Table A.12 – Inter-day precision calculations for the PCC method (with sample hydrolysis)

Inter-day Precision - PCC			
PCC	Injection	Calculated Concentration (ng/ml)	Standard Deviation (%RSD)
QC1	1	14.24	7.023
	2	28.58	
	3	24.54	
	4	12.75	
	5	14.53	
	6	27.18	
	7	11.04	
	8	22.50	
QC2	1	656.00	10.414
	2	669.44	
	3	633.08	
	4	650.51	
	5	657.49	
	6	649.83	
	7	645.97	
	8	654.80	
QC3	1	2509.13	12.909
	2	2467.78	
	3	2490.68	
	4	2482.92	
	5	2490.05	
	6	2477.79	
	7	2500.71	
	8	2491.73	